

UNIVERSITY OF KWAZULU-NATAL

***Syzygium aromaticum*-derived triterpenes modulate intestinal
glucose handling in streptozotocin-induced diabetic rats**

Andile Khathi

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***Syzygium aromaticum*-derived triterpenes modulate intestinal glucose handling in streptozotocin-induced diabetic rats**

By

Andile Khathi (207515130)

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

Supervisor: Professor C.T. Musabayane

Discipline of Human Physiology

School of Laboratory Science and Medical Sciences

College of Health Sciences



DECLARATION

I, **Andile Khathi** hereby declare that the dissertation entitled:

“Syzgium aromaticum-derived triterpenes modulate intestinal glucose handling in streptozotocin-induced diabetic rats”

is the result of my own investigation and research and that this work 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, there is due acknowledgment in the text.

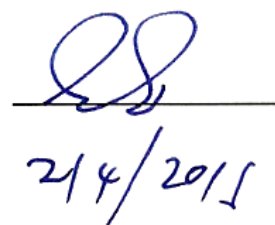
Student : Mr Andile Khathi

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Supervisor: Professor C. T. Musabayane

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21/4/2015

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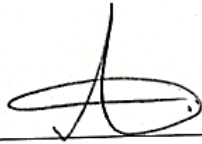
PLAGIARISM DECLARATION

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PhD IN HUMAN PHYSIOLOGY 2011-2014

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3. This thesis is my own work.
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Signature _____

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List of abbreviations

α	Alpha
ACE	Angiotensin converting enzyme
Al^{3+}	Aluminium
AGE	Advanced glycation end products
ANOVA	Analysis of variance
ARB's	Angiotensin receptor blockers
ATP	Adenosine triphosphate
AUC	Area Under Curve
β	Beta
BBM	Brush-border membrane
BHT	Butylatedhydroxytoluene
BCA	Bicinchoninic acid
$\text{C}_2\text{H}_5\text{OH}$	Ethanol
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CO_2	Carbon dioxide
CVD	Cardiovascular disease
DAG	Diacylglycerol
DCM	Dichloromethane
DCMS	Dichloromethane solubles
DMSO	Dimethylsulphoxide
EA	Ethyl acetate
EAS	Ethyl acetate soluble
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

eNOS	Endothelium nitric oxide synthase
Fe ²⁺	Iron
GFAT	Glutamine fructose-6-phosphate amidotransferase
GFR	Glomerular filtration rate
GIT	Gastrointestinal tract
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GP	Glycogen phosphorylase
GPCR's	G-protein coupled receptors
GPx	Glutathione Peroxidase
H ₂ SO ₄	Sulphuric acid
HBP	Hexosamine biosynthesis pathway
HDL	High density lipoproteins
HES	Hexane solubles
Hg ²⁺	Mercury
HIV	Human immunodeficiency virus
HRP	Horse-radish peroxidase
IRS	Insulin receptor substrate
KCl	Potassium chloride
K ₂ HPO ₄	Dipotassium phosphate
KOH	Potassium hydroxide
LDL	Low density lipoprotein
μmol	Micromole
MA	Maslinic acid

MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MES	Methanol solubles
MI	Myocardial infarction
mmol/l	Millimoles per litre
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
Na ₂ EDTA	Sodium ethylene diaminetetraacetic acid
NAD ⁺	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
OGT	Oral glucose tolerance
OA	Oleanolic acid
PKC	Protein kinase C
PI3K	Phosphoinositol 3 kinase
PMSF	Phenyl methyl sulphonyl fluoride
PPA	Porcine pancreatic amylase
PPAR γ	Peroxisome proliferator gamma
PVDF	Polyvinylidenedifluoride
RAGE	Receptor for advanced glycation end product
RT	Room temperature
SA-HRP	Streptavidin-horse radish peroxidase
SDS	Sodium docedylsulphate
SEM	Standard error of means

SGLT1	Sodium dependent glucose transporter 1
SGLT2	Sodium dependent glucose transporter 2
SOD	Superoxide dismutase
STZ	Streptozotocin
SU	Sulphonylurea
SUR1	Sulphonylurea receptor 1
SUR2	Sulphonylurea receptor 2
SUR2A	Sulphonylurea receptor 2A
SUR2B	Sulphonylurea receptor 2B
T2D	Type 2 diabetes
TBA	Thiobarbituric acid
TLC	Thin layer chromatography
TGF- β	Transforming growth factor beta
TMB	3,3',5,5'-tetramethylbenzidine
TZD	Thiazolidinedione
UA	Ursolic acid
UDP-GlcNAc	Uridinediphosphate-acetylglucosamine
UKZN	University of KwaZulu Natal
VEGF	Vascular endothelial growth factor

ABSTRACT

Background

Polyphagia in diabetes mellitus ascribed to elevated plasma ghrelin concentrations is associated with prolonged postprandial hyperglycaemia due to increased activities of intestinal carbohydrate hydrolyzing enzymes and glucose transporters. Postprandial hyperglycaemia is a major risk factor in the development of diabetic complications, and as such, should be managed to prevent chronic vascular complications. Previous studies in our laboratory have shown that *Syzygium aromaticum*-derived oleanolic acid (OA) and maslinic acid (MA) use various mechanisms to lower blood glucose concentrations in experimental diabetes. The effects of these triterpenes, however, on intestinal glucose handling remain unknown. Accordingly, this study was designed to investigate the effects of these triterpenes on intestinal glucose handling in STZ-induced diabetic rats.

Materials and methods

OA and MA were extracted from *Syzygium aromaticum* cloves using a previously validated protocol. Briefly, *S. aromaticum*-derived OA and MA were sequentially extracted with dichloromethane and ethyl acetate to obtain ethyl acetate solubles which contained mixtures of OA/ursolic acid (UA) and methyl maslinate/methyl corosolate. These solubles were purified by silica gel 60 column chromatography with hexane: ethyl acetate solvent systems to produce OA and MA. The structures of these triterpenes were confirmed by using ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy and were comparable with those previously reported in literature. The *in vitro* studies investigated the inhibitory effects of OA and MA against enzymes such as α -amylase, α -glucosidase and sucrase. Additionally, the effects of various concentrations of OA and MA (0.82 - 6.56 mmol/L) on intestinal glucose transport were investigated using the everted intestinal sacs protocol. The *in vivo* studies investigated the effects of OA and MA on intestinal carbohydrate handling in separate groups of non-diabetic and STZ-diabetic male Sprague Dawley rats. These studies were subdivided into oral glucose tolerance (OGT) responses which were carried out over two hours following loading with various carbohydrates as well as sub-chronic studies that were carried out over 5-weeks where the rats

were kept on standard rat chow. OGT responses were monitored in separate groups of non-diabetic and STZ-induced diabetic animals the rats treated with OA and MA (80 mg/kg, p.o.). The rats were loaded with monosaccharides, disaccharides and polysaccharides after an 18-hour fast. The sub-chronic studies investigated the effects of the triterpenes on blood glucose concentrations over 5-weeks in groups of non-diabetic and STZ-induced diabetic male Sprague-Dawley rats. In those animals in which the effects of OA/MA were investigated, the rats were administered with OA/MA (80 mg/kg, p.o.) twice daily. Blood glucose, body weights as well as food and water intake were assessed every third day for the duration of the experimental period. At the end of the experimental period, the rats were killed and blood was collected for plasma insulin and ghrelin measurements. Furthermore, mid portions of the small intestine were snap frozen in liquid nitrogen and stored in a BioUltra freezer at -70 °C for Western blot analysis of glucose transporters, carbohydrate hydrolyzing enzymes and ghrelin expression. Additionally, the effects of OA and MA on intestinal oxidative stress were evaluated through malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) measurements.

Results

The *in vitro* studies revealed that OA and MA possess inhibitory effects on the activity of α -amylase, α -glucosidase and sucrase comparable with those of the standard drug acarbose. In addition, OA and MA significantly ($p<0.05$) inhibited intestinal glucose transport in the everted intestinal sacs in a dose-independent manner. The OGT studies showed that OA and MA had no significant effects on blood glucose concentrations in non-diabetic rats loaded with the various carbohydrates by comparison with the non-diabetic control. However, the triterpene-treated STZ-diabetic rats loaded with the various carbohydrate showed significant ($p<0.05$) reductions in blood glucose concentrations by comparison with untreated STZ-diabetic rats. OA and MA progressively reduced blood glucose concentration as well as food and water intake over the 5-week study in STZ-induced diabetic rats by comparison with untreated STZ-diabetic rats. Treatment with OA and MA had no effects on plasma insulin concentration in STZ-induced diabetic rats. However, these triterpenes significantly ($p<0.05$) reduced plasma ghrelin concentrations by comparison with untreated STZ-induced diabetic rats. Furthermore, rats treated with OA and MA showed significant ($p<0.05$) decreases in ghrelin, SGLT1, GLUT2, α -

amylase and α -glucosidase expression in the gastrointestinal tract by comparison with untreated STZ-diabetic rats. This was accompanied by improvements in their intestinal antioxidant status as there were significant ($p<0.05$) reductions in MDA concentrations with significant ($p<0.05$) increases in SOD and GPx by comparison with the STZ-diabetic control. Additionally, OA and MA-treated rats showed significant ($p<0.05$) increases in intestinal glycogen concentrations with concomitant significant ($p<0.05$) increases in the intestinal expression of glycogen synthase by comparison with untreated STZ-diabetic animals.

Discussion

The results of the present study indicate that the blood glucose lowering effects of OA and MA in STZ-induced diabetic rats are mediated, in part, via modulating postprandial hyperglycaemia. These findings suggest that this is achieved through the ghrelin-mediated reduction in food intake leading to decreased expression of intestinal carbohydrate hydrolyzing enzymes as well as intestinal glucose transporters. This was followed by significant improvements in the antioxidant status in the rats suggesting that these triterpenes could, by preventing chronic postprandial hyperglycaemia, prevent the onset of the development of diabetic complications. The results of this study are of considerable importance as they suggest another mechanism for the anti-diabetic properties of the triterpenes and further explain the role of the gastrointestinal tract in the management of diabetes mellitus.

Conclusion

The results of the present study suggest that the *S. aromaticum*-derived triterpenes possess anti-diabetic properties that arise, in part, through the modulation of intestinal glucose handling.

Keywords: Postprandial hyperglycaemia, diabetes mellitus, *Syzygium aromaticum*; oleanolic acid, maslinic acid

Table of contents

	Topic	Page number
	Declaration	ii
	Acknowledgements	iii
	Plagiarism declaration	v
	List of abbreviations	vi
	Abstract	viii
	List of figures	x
	List of tables	xi
	List of appendices	xii
 CHAPTER 1		
1.0	Background	1
1.1	Introduction/Literature review	2
1.1.1	Glucose homeostasis	2
1.1.2	Fasting state	3
1.1.3	Postprandial state	4
1.1.4	Ghrelin	4
1.2	The small intestine	7
1.2.1	Intestinal carbohydrate metabolism	8
1.2.2	Amylases	8
1.2.3	α-glucosidases	9

1.3	Glucose transport	10
1.3.1	Mode of action of SGLT	12
1.4	Diabetes mellitus	13
1.4.1	Aetiology of diabetic complications	15
1.4.1.1	Polyol pathway	15
1.4.1.2	Advanced glycation end products (AGEs)	16
1.4.1.3	Protein kinase C (PKC)	17
1.4.1.4	The hexosamine pathway	18
1.5	Macrovascular complications of diabetes	19
1.5.1	Atherosclerosis	19
1.5.2	Microvascular complications of diabetes	20
1.5.2.1	Diabetic retinopathy	20
1.5.2.2	Diabetic nephropathy	21
1.5.2.3	Diabetic neuropathy	21
1.6	Conventional management of diabetes mellitus	22
1.6.1	Insulin	24
1.6.2	Thiazolidinediones	25
1.6.3	Sulphonylureas	25
1.6.4	Biguanides	26
1.6.5	Intestinal enzyme inhibitors	26
1.6.5.1	α -amylase inhibitors	27
1.6.5.2	α -glucosidase inhibitors	29
1.6.5.3	Intestinal glucose transport inhibitors	30
1.7	Traditional plant extracts	32

1.7.1	<i>Syzygium aromaticum</i>	33
1.8	OA	34
1.9	MA	35
1.10	Experimental techniques used to study intestinal glucose handling	35
1.11	Justification of the study	37

CHAPTER 2

2.0	Drugs and chemicals	38
2.1	Isolation of OA and MA	38
2.2	Animals	39
2.3	Ethical consideration	39
2.4	Induction of diabetes mellitus	40
2.5	Experimental design	40
	Series I	43
2.6	<i>In vitro</i> assays	43
2.6.1	Inhibitory enzyme assay studies	43
2.6.2	α-amylase	43
2.6.3	α-glucosidase	43
2.6.4	Sucrase	44
2.7	Glucose transport in the small intestine	45
	Series II	48
2.8	Oral glucose tolerance (OGT) responses	48
2.9	Intestinal glucose handling	49
2.10	Tissue sample harvesting	49
2.11	Laboratory analyses	50

2.11.1	Intestinal glycogen concentration	50
2.11.2	Hormone measurements	51
2.11.2.1	Insulin	51
2.11.2.2	Ghrelin	52
2.11.3	Western blot analysis	53
2.12	Oxidative stress	54
2.12.1	MDA	54
2.12.2	SOD	55
2.12.3	Glutathione peroxidase (GPx)	56
2.12.4	Histological studies	57
2.13	Analysis of data	57
CHAPTER 3		58
	Results	58
3.0	Structure elucidation	58
3.1	OA	58
3.2	MA	61
	Series I	63
3.3	Carbohydrate hydrolyzing enzyme activity	63
3.4	Glucose transport in the everted gut	64
	Series II	68
3.5	Acute effects	68
3.5.1	OGT responses	68
3.6	Sub-chronic effects	75
3.6.1	Physicochemical changes	75
3.6.1.1	Body weight changes, food and water intake	75

3.6.1.2	Blood glucose concentrations	77
3.7	Intestinal glycogen concentrations	79
3.8	Hormone concentrations	81
3.9	Protein expression	84
3.9.1	Glycogen synthase	84
3.9.2	α-amylase and α-glucosidase	86
3.9.3	SGLT1 and GLUT2	89
3.9.4	Ghrelin	92
3.10	Oxidative stress assessment	96
3.11	Histological studies	99
 CHAPTER 4		
4.0	Discussion	102
 CHAPTER 5		
5.0	Conclusions	110
5.1	Limitations and directions for future studies	110
 CHAPTER 6		
6.0	References	112
 CHAPTER 7		
7.0	Appendices	129

List of figures

	Legend	Page no.
Figure 1	Sequential carbohydrate hydrolysis by intestinal carbohydrate hydrolyzing enzymes yielding monosaccharides that are subsequently absorbed in the small intestine.	10
Figure 2	The various classes of compounds used in the management of diabetes mellitus. These classes use various mechanisms to maintain glucose homeostasis.	23
Figure 3	<i>Syzygium aromaticum</i> leaves adapted from Raina <i>et al.</i> , 2001.	34
Figure 4	Flow diagram detailing acute <i>in vivo</i> studies carried out over a 2-hour period. Non-diabetic and STZ-induced diabetic rats were loaded with mono-, di- and polysaccharides to observe the effects of triterpene treatments on blood glucose levels. This was in an effort to investigate the effects of the triterpenes on the intestinal carbohydrate hydrolyzing enzymes and the resultant postprandial blood glucose concentrations.	41
Figure 5	Flow diagram detailing sub-chronic <i>in vivo</i> studies carried out over a 5-week period. Non-diabetic and STZ-induced diabetic rats were maintained on a starch-based diet to observe the effects of triterpene treatments on blood glucose levels, food and water intake. Following the 5-week study various biochemical parameters were investigated to elucidate the effects of the triterpenes on the gastrointestinal tract.	42
Figure 6	The everted intestinal sac protocol developed by Wilson and Wiseman (Wilson and Wiseman, 1954). This technique measures the influence of drugs on intestinal glucose uptake by	47

measuring glucose concentrations in the mucosal and serosal fluid before and after the 30-minute incubation period.

Figure 7	Chemical structure and IUPAC numbering of OA as determined through ^1H and ^{13}C NMR spectroscopy.	59
Figure 8	<i>Syzygium aromaticum</i> -derived OA ^1H (A) and ^{13}C NMR (B) spectroscopic data. Pure OA was obtained following recrystallization of EAS with methanol and elucidated using ^1H and ^{13}C NMR spectroscopy.	60
Figure 9	Chemical structure and IUPAC numbering of MA as determined through ^1H and ^{13}C NMR spectroscopy	61
Figure 10	<i>Syzygium aromaticum</i> -derived MA ^1H (A) and ^{13}C NMR (B) spectroscopic data. Pure OA was obtained following recrystallisation of EAS with methanol and elucidated using ^1H and ^{13}C NMR spectroscopy.	62
Figure 11	Effects of graded concentrations of OA (0.82 - 6.56 mmol/l) on mucosal glucose disappearance and serosa glucose appearance in rat everted intestinal sacs. The initial amount of glucose in the mucosal fluid was 500 μmol indicated by the horizontal line. Values are presented as means, and vertical bars indicate SEM of means (n=6 for each concentration). \blacklozenge =p<0.05 by comparison to the initial mucosal glucose concentration. \star = p <0.05 by comparison with control.	65
Figure 12	Effects of graded concentrations of MA (0.82 - 6.56 mmol/l) on mucosal glucose disappearance and serosa glucose appearance in rat everted intestinal sacs. The initial amount of glucose in the mucosal fluid was 500 μmol indicated by the horizontal line.	66

Values are presented as means, and vertical bars indicate SEM of means (n=6 for each concentration). ♦=p<0.05 by comparison to the initial mucosal glucose concentration. ★ = p <0.05 by comparison with control.

- Figure 13** Effects of graded concentrations of phlorizin on mucosal glucose disappearance and serosa glucose appearance in rat everted intestinal sacs. The initial amount of glucose in the mucosal fluid was 500 µmol indicated by the horizontal line. Values are presented as means, and vertical bars indicate SEM of means (n=6 for each concentration). ♦ = p<0.05 by comparison to the initial mucosal glucose concentration. ★ = p <0.05 by comparison with control. **67**
- Figure 14** Comparison of OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug phlorizin following a glucose load. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the respective control. **69**
- Figure 15** Comparison of the AUC_{glucose} values obtained from OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug phlorizin following a glucose load. The AUC_{glucose} was calculated by incremental method. Values are presented as means, and vertical bars indicate SEM (n = 6 rats in each group). ★p< 0.05 by comparison with control animals. **70**

Figure 16	Comparison of OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug phlorizin following a sucrose load. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the respective control.	71
Figure 17	Comparison of the AUC _{glucose} values obtained from OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug acarbose following a sucrose load. The AUC _{glucose} was calculated by incremental method. Values are presented as means, and vertical bars indicate SEM (n = 6 rats in each group). ★p< 0.05 by comparison with control animals.	72
Figure 18	Comparison of OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug phlorizin following a starch load. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the respective control.	73
Figure 19	Comparison of the AUC _{glucose} values obtained from OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug acarbose following a starch load. The AUC _{glucose} was calculated by incremental method. Values	74

are presented as means, and vertical bars indicate SEM (n = 6 rats in each group). ★p< 0.05 by comparison with control animals.

- | | | |
|------------------|---|-----------|
| Figure 20 | Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on blood glucose concentration with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦ =p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control. | 78 |
| Figure 21 | Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine glycogen concentration with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦ =p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control. | 80 |
| Figure 22 | Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on plasma insulin concentrations with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦ =p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control | 82 |
| Figure 23 | Comparison of the effects of OA and MA administered in STZ- | 83 |

diabetic rats twice every third day for 5 weeks on plasma ghrelin concentrations with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control

- | | | |
|------------------|--|-----------|
| Figure 24 | Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine glycogen synthase expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control. | 85 |
| Figure 25 | Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine α -amylase expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control. | 87 |
| Figure 26 | Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine α -glucosidase expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦=p<0.05 by comparison to the non- | 88 |

diabetic control. ★ = $p<0.05$ by comparison to the STZ-induced diabetic control.

- Figure 27** Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine SGLT1 expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group).◆= $p<0.05$ by comparison to the non-diabetic control. ★ = $p<0.05$ by comparison to the STZ-induced diabetic control. **90**
- Figure 28** Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine GLUT2 expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group).◆= $p<0.05$ by comparison to the non-diabetic control. ★ = $p<0.05$ by comparison to the STZ-induced diabetic control. **91**
- Figure 29** Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on gastric fundus ghrelin expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group).◆= $p<0.05$ by comparison to the non-diabetic control. ★ = $p<0.05$ by comparison to the STZ-induced diabetic control. **93**
- Figure 30** Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine **94**

ghrelin expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control.

- | | | |
|------------------|--|-----------|
| Figure 31 | Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on large intestine ghrelin expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control. | 95 |
| Figure 32 | Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on MDA concentration with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control. | 97 |
| Figure 33 | Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine SOD activity with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic | 98 |

control

Figure 34 Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine GPx activity with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ♦ = p < 0.05 by comparison to the non-diabetic control. ★ = p < 0.05 by comparison to the STZ-induced diabetic control **99**

Figure 35 H & E stains illustrating the effects of OA and MA on the morphology of the small intestine in STZ-induced diabetic rats. Picture A represents an intact longitudinal layer (blue arrow), an intact mucous membrane (yellow arrow), goblet cells in the circular layer (orange arrow) and normal length villi (black arrow) in non-diabetic control animals. Picture B represents a disrupted longitudinal layer (, an intact mucous membrane, goblet cells in the circular layer and villi with reduced length in untreated STZ-diabetic control animals. Pictures C, D, E and F represent an intact longitudinal layer, an intact mucous membrane, goblet cells in the circular layer and normal length villi in OA-, MA-, metformin- and insulin-treated STZ-diabetic animals respectively. **101**

List of tables

	Legend	Page no.
Table 1	Various medicinal plant extracts that have been reported to possess α -amylase inhibitory activity. They are given in order of increasing IC_{50} values as well as with their maximum percentage inhibition values.	28
Table 2	Medicinal plant extracts that have been reported to have α -glucosidase inhibitory activity. They are given in order of increasing IC_{50} values as well as with their maximum percentage inhibition values.	30
Table 3	IC_{50} values of OA, MA and acarbose on the activities of sucrase, α -glucosidase and α -amylase <i>in vitro</i> . Values are presented as means \pm SEM (n=4 in each group).	63
Table 4	Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on body weight changes, food and water intake with control non-diabetic, untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin (n=6 in each group). Values are presented as means \pm SEM	76

List of appendices

		Page no.
Appendix I	Ethical clearance 2011	129
Appendix II	Ethical clearance 2012	130
Appendix III	Ethical clearance 2013	131
Appendix IV	Ethical clearance 2014	132
Appendix V	Published article Khathi A, Masola B, Musabayane CT (2013). Effects of <i>Syzygium aromaticum</i> -derived oleanolic acid on glucose transport and glycogen synthesis in the rat small intestine. Journal of Diabetes 5(1):80-87	133
Appendix VI	Published article Khathi A, Serumula M, Myburgh R, Musabayane CT (2013). Effects of <i>Syzygium aromaticum</i> -derived triterpenes on postprandial blood glucose in streptozotocin-induced diabetic rats following carbohydrate challenge. PLOS One 87:1-7	141
Appendix VII	Draft article Khathi A, Mbongwa HP and Musabayane CT (2014). The effects of <i>Syzygium aromaticum</i> -derived triterpenes on gastrointestinal ghrelin expression in streptozotocin-induced diabetic rats.	149

Conference proceedings

Appendix VIII	Khathi A, Mbongwa HP and Musabayane CT (2014). The effects of <i>Syzygium aromaticum</i> -derived triterpenes on gastrointestinal ghrelin expression in streptozotocin-induced diabetic rats.	166
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42nd Conference of the Physiological Society of Southern Africa. 14-17 September 2014. University of KwaZulu-

Natal. South Africa

- Appendix IX** Khathi A, Luvuno M, Mbongwa HP and Musabayane CT (2014). The effects of *Syzygium aromaticum*-derived triterpenes on gastrointestinal ghrelin expression in streptozotocin-induced diabetic rats. **167**

College of Health Sciences Symposium. 11-12 September 2014 University of KwaZulu Natal. South Africa

- Appendix X** Dube SC, Khathi A, Serumula MR, Myburg R and Musabayane CT (2013). Effects of *Syzygium aromaticum*-derived oleanolic acid administration on postprandial glucose concentration and key intestinal carbohydrate hydrolyzing enzymes in streptozotocin-induced diabetic rats. **168**

The Annual Society for Endocrinology conference. 15-19 March 2013. Harrogate International Conference Centre. United Kingdom

- Appendix XI** Khathi A, Serumula MR, Myburg R and Musabayane CT (2013). Effects of *Syzygium aromaticum*-derived triterpenes on postprandial blood glucose in streptozotocin-induced diabetic rats following carbohydrate challenge. **169**

41st Conference of the Physiological Society of Southern Africa. 15-18 September 2013. University of the Limpopo (Medunsa campus). South Africa

- Appendix XII** Khathi A, Mkhwanazi BN, van Heerden FR and Musabayane CT (2012). Effects of *Syzygium aromaticum*-derived maslinic acid on blood glucose of streptozotocin induced-diabetic rats. **170**

The Annual Society for Endocrinology conference. 19-22 March 2012. Harrogate International Conference Centre. United Kingdom

Appendix XIII	Khathi A, Ngubane PS, Masola B and Musabayane CT (2011). Effects of <i>Syzygium aromaticum</i> -derived oleanolic acid on glucose transport and glycogen synthesis in the rat small intestine.	171
	Physiological Society. 18-22 July 2011 University of Oxford. United Kingdom	
Appendix XIV	Khathi A, Masola B and Musabayane CT (2011). Effects of <i>Syzygium aromaticum</i> -derived oleanolic acid on glucose transport and glycogen synthesis in the rat small intestine.	172
	39 th Conference of the Physiological Society of Southern Africa. 29-31 August 2011. University of the Western Cape. South Africa	

CHAPTER 1

Introduction/Literature review

1.0 Background

Intestinal glucose absorption resulting from dietary carbohydrates increases postprandial blood glucose to levels which are returned to within the homeostatic levels by insulin. However, in diabetes mellitus where there is impaired cellular glucose transport, blood glucose levels continually rise resulting in postprandial hyperglycaemia which is associated with the onset of microvascular and macrovascular complications. Therefore, controlling postprandial glucose levels is a critical component in the management of diabetes mellitus. Current conventional diabetes therapy involves the use of intense glycaemic control via insulin as well as various classes of oral hypoglycaemic drugs such as α -glucosidase inhibitors. These drugs prevent postprandial hyperglycaemia by either retarding intestinal carbohydrate hydrolysis or inhibiting intestinal glucose absorption. The drugs are, however, expensive and not readily available to populations in developing countries. Interestingly, these drugs were first isolated from medicinal plant products. Metformin, a standard drug used in the management of diabetes, was isolated from the plant *Galega officinalis*. Furthermore, there are a variety of plants with α -glucosidase inhibitory activity. While medicinal plants have drawn increasing attention as a potential viable source of anti-diabetic drugs for researchers across the world, the use of medicinal plant products in developing countries remains unabated. Of interest in this study, are the bioactive compounds isolated from the plant *Syzygium aromaticum*, oleanolic acid (3 β -hydroxy-olean-12-en-28-oic acid, OA) and maslinic acid (2 α , 3 β -dihydroxy-olean-12-en-oic acid, MA).

Previous studies conducted in our laboratory demonstrated that OA and MA reduce blood glucose concentrations of streptozotocin (STZ)-induced diabetic rats. The mechanism(s) by which these triterpenes reduce blood glucose are yet to be fully established although studies suggest that the effects involve many organs. Indeed, both OA and MA have been reported to increase glycogen synthesis as well as increase glycogenic enzyme activity in both the liver and skeletal muscle. Furthermore, some studies suggest that OA increases insulin secretion in isolated rat pancreatic β -cells. Guided by these fundamental observations and that the blood

glucose lowering effects of anti-diabetic drugs are mediated via a variety of mechanisms of action, we hypothesized that the hypoglycaemic effects of OA and MA may involve the inhibition of glucose transport or the delay of carbohydrate hydrolysis in the small intestine. Indeed, some plant extracts lower blood glucose in experimental animals by reducing food intake and interfering with the effects of ghrelin in the gastrointestinal tract. The current study was, therefore, designed to investigate whether the blood glucose lowering properties of OA and MA could arise, in part, through effects on intestinal carbohydrate hydrolysis, intestinal glucose absorption as well as on the gastrointestinal expression of ghrelin in STZ-induced diabetic rats.

Chapter 1 describes glucose homeostasis, intestinal carbohydrate metabolism, diabetes mellitus as well as the conventional anti-diabetic management strategies. This chapter also describes the medicinal properties of OA and MA as well as justification of the study.

1.1 Introduction/Literature review

1.1.1 Glucose homeostasis

In healthy individuals, plasma glucose levels are maintained within a relatively narrow range throughout the day despite large and frequent fluctuations in the delivery and removal of glucose from the circulation (Gerich, 2000). The diet plays a large role in the determination of blood glucose levels as well as ensuring that the brain receives enough energy. The brain cannot produce nor store glucose and is therefore primarily dependent on plasma glucose for survival. A fall of blood glucose concentrations to levels between 1.2 and 3.8 mM can decrease brain glucose uptake and trigger the release of glucagon, catecholamines, cortisol as well as growth hormone (Cummings *et al.*, 2001). These hormones increase blood glucose concentrations through various mechanisms. A decrease in glucose concentration also triggers the secretion of ghrelin which results in the suppression of insulin release to prevent further decreases in blood glucose concentrations (Gerich, 2000; Broglio *et al.*, 2001). An increase in glucose concentration, however, triggers the secretion of insulin to facilitate cellular glucose uptake. This results in a decrease in plasma ghrelin concentrations as well as inhibition of the processes of gluconeogenesis and glycogenolysis. In pathological states such as diabetes mellitus, these physiological processes are exploited as therapeutic targets in reducing hyperglycaemia (Beckman *et al.*, 2002).

1.1.2 Fasting state

Following a 14-16h overnight fast, plasma glucose concentrations average 5 mM and are relatively stable (Bolli *et al.*, 1984; Gerich, 2000). Consequently, the rates of release of glucose into the blood must closely approximate the rates of glucose removal. During this time, there is no net storage of glucose, therefore glucose taken up by tissues is either completely oxidized to CO₂ or released back into the circulation as lactate, alanine and glutamine for reincorporation into glucose via gluconeogenesis (Heydari *et al.*, 2010). Prolonged fasting causes the release of glucose into the circulation and this is regulated by both hormonal and non-hormonal mechanisms. Many tissues contain enzymes that break down glycogen to glucose-6-phosphate (glycogenesis) or synthesize glucose-6-phosphate from glycerol (gluconeogenesis). However, the liver and kidney are the only organs that mainly contain enough glucose-6-phosphatase that makes significant amounts of glucose (Marshall, 1991; Juhász *et al.*, 2007). The liver is responsible for approximately 80% of the glucose released into circulation in this state. Of this amount, 50% is attributed to glycogenesis while the rest is attributed to gluconeogenesis. The kidney, however, normally contains little glycogen as the majority of renal cells lack glucose-6-phosphatase. Consequently, a significant amount of the glucose released by the kidney is the result of gluconeogenesis (Gerich, 2000).

Interestingly, the small intestine has also been found to express the enzyme glucose-6-phosphatase (Rajas *et al.*, 1999), although the extent to which this organ can store glycogen has not yet been determined. Considering the role of the small intestine in glucose absorption, the conversion of dietary glucose to glycogen could be a mechanism that prevents postprandial hyperglycaemia in the diabetic state.

1.1.3 Postprandial state

Postprandial glucose concentrations are defined as the glucose levels that occur after a meal. The total duration for complete assimilation of a meal containing carbohydrates, proteins and fats and the return to normal blood glucose levels takes at least 6 hours. However, the complete assimilation of a pure carbohydrate meal is complete within 5 hours (Gerich, 2000; Ceriello, 2005). Humans generally consume 3 meals a day and therefore spend the majority of the day in

the postprandial state. Various factors play a role in the extent and the duration of the postprandial state. These include exogenous factors such as the amount of physical activity following the meal and the composition and form of the meal. These can also include factors such as the rate of gastric emptying, carbohydrate hydrolysis within the intestine, the rate of absorption of glucose across the small intestine into the hepatic portal vein, the release of glucose into the circulation as well as glucose uptake by the post-hepatic tissues. Once glucose uptake into the cells has occurred, the glucose is stored as glycogen (Hirsch, 2005; Juhász *et al.*, 2007). Therefore, the small intestine plays a major role in the determination of postprandial glucose concentrations. As previously mentioned, the diet is a major component influencing blood glucose concentrations. Feeding patterns are regulated by a hunger/satiety cycle that is regulated by plasma concentrations of leptin, ghrelin and insulin. However, a study showed that STZ-induced diabetic rats exhibit polyphagia that is associated with increases in both ghrelin gene expression and plasma ghrelin concentrations (Masaoka *et al.*, 2003). This abnormality consequently leads to chronic hyperglycaemia and therefore accelerates the progression of diabetic complications. Previous studies have shown the triterpenes, OA and MA, to possess insulin-mimetic effects (Musabayane *et al.*, 2010; Ngubane *et al.*, 2011). Therefore, this study investigated the effects of these triterpenes on ghrelin plasma ghrelin concentrations. The following section briefly describes the role of ghrelin in the maintenance of glucose homeostasis.

1.1.4 Ghrelin

Ghrelin belongs to a group of conserved humoral factors characterized by strong interspecies homology in the composition and proportions of their amino acid constituents (Broglia *et al.*, 2001; Smith *et al.*, 2005). Human ghrelin has 82.9% similarity with the rat protein and is derived from a 117-amino-acid preprohormone (Gul *et al.*, 2011). Convertase cleaves a signal sequence from preproghrelin, and the remaining 94-amino-acid prohormone undergoes post-translational acylation in a serine in position 3 of the protein through the action of n-capric acid. This reaction is catalyzed by the enzyme ghrelin O-acyltransferase (GOAT). Proghrelin undergoes further

proteolytic processing by convertasePC1/3, a member of the prohormone convertase (PC) family, which transforms proghrelin into a 28-amino acid peptide (Smith *et al.*, 2005). Many tissues express preproghrelin mRNA, but the gastric fundus has the highest levels of expression (Kirsz *et al.*, 2011). A-like cells, which are mainly present in the gastric fundus have been reported to be the main source of ghrelin (Ariyasu *et al.*, 2001; Smith *et al.*, 2005). Ghrelin is the only known circulating hormone that acts on peripheral and central targets to increase food intake and promote adiposity. Approximately 55% of circulating ghrelin is produced in the stomach while substantially lower amounts are detected in the small intestine (25%), large intestine (15%), pancreas, kidney, placenta, testis, pituitary, lung, and hypothalamus (Takaya *et al.*, 2000; Ariyasu *et al.*, 2001; Masaoka *et al.*, 2003). Ghrelin plays a major role in appetite regulation as studies show that this hormone stimulates feeding (Nakazato *et al.*, 2001; Wren *et al.*, 2001). In humans, ghrelin peaks before meals, suggesting the role of ghrelin as a hunger signal. Studies show that the biological effects of ghrelin are mediated through the specific receptor GHS-R, which belongs to the family of G protein-coupled receptors (GPCRs). The primary transcript of the GHRS gene consists of two exons and one intron. As a result of alternative splicing, two isomeric forms of the receptor exist: GHS-R1a and GHS-R1b. Human GHSR1a consists of 366 amino acids and shares 96% identity with the rat receptor. GHS-R1b contains 289 amino acids with five domains and does not have any biological activity (Smith *et al.*, 2005). The strongest expression of GHS-R1a is in the hypothalamic cell populations that regulate feeding and body weight homeostasis (Zigman *et al.*, 2006). Ghrelin binding to the GHS-R1a receptor results in the activation of two calcium-signaling cascades which lead to various metabolic processes such as changes in appetite (Broglia *et al.*, 2001; Egido *et al.*, 2002). Circulating levels of ghrelin are regulated by both short and long-term changes in nutritional status. Plasma levels of this peptide increase on fasting and decrease after habitual feeding, thus showing a pattern reciprocal to that of insulin.

Studies reveal that when ghrelin is injected subcutaneously in mice, rats and humans, there is inhibition of insulin release (Broglia *et al.*, 2001; Egido *et al.*, 2002; Reimer *et al.*, 2003; Dezaki *et al.*, 2004). Low plasma ghrelin levels are associated with elevated fasting insulin levels and insulin resistance in humans (Poykko *et al.*, 2003). These findings suggest both physiological and pathophysiological roles for ghrelin in the regulation of insulin release. Furthermore, the

findings raised the possibility that endogenous ghrelin has a role in physiological insulin secretion, and that ghrelin antagonists could improve pancreatic β -cell function (Tong *et al.*, 2010). Plasma ghrelin concentrations were decreased in the healthy offspring of type 2 diabetes subjects and the compensatory hyperinsulinaemia due to insulin resistance was associated with significantly reduced ghrelin concentrations (Østergård *et al.*, 2003). A study has shown that fasting plasma concentrations of total ghrelin to be lower in type 2 diabetic subjects compared other diabetic patients (Poykko *et al.*, 2003). These findings suggest that low blood ghrelin levels affect the growth hormone/insulin like growth factor-1-axis which in turn might increase insulin resistance and lead eventually to the development of type 2 diabetes mellitus (Barazzoni *et al.*, 2007). Enhanced plasma ghrelin levels have also been observed in rats with streptozotocin-induced diabetes and are associated with diabetic polyphagia (Ariyasu *et al.*, 2001). This increase in food consumption causes the body to spend more time in the postprandial state and in diabetes mellitus this could lead chronic hyperglycaemia. Consequently, this contributes to the development of diabetic complications which are discussed at a later stage. Medicinal plant extracts such as those from *Aloe vera*, *Ocimum sanctum*, *Azadirachta indica*, *Solanum variable*, *Urtica salicifolia*, *Asparagus racemosus* and *Araucaria araucana* have all been reported to significantly reduce food intake through the reduction of plasma ghrelin levels (Bast *et al.*, 2002; Fong, 2002; Dzubak *et al.*, 2006 ; Gao *et al.*, 2009). *Syzygium aromaticum*-derived OA and MA have been previously shown to possess insulin-mimetic properties as they reduce blood glucose concentrations in STZ-induced diabetic rats in sub-chronic studies through a variety of mechanisms. In this study, we speculated that these triterpenes reduce blood glucose levels, in part, through the reduction of food intake and intestinal ghrelin secretion. The following section further discusses the physiological role of the small intestine in maintaining glucose homeostasis.

1.2 The small intestine

The small intestine forms an integral part of the gastrointestinal tract. The primary function of this organ is the absorption of nutrients and minerals obtained in the diet. The small intestine is made up of three structural parts: the duodenum, jejunum and ileum. The length of the small intestine in an adult human male varies from 4.6 m to 9.8 m with glucose absorption occurring throughout the tract. Various studies have shown, however, that the absorption of carbohydrates across the small intestine is not uniform (Drozdowski and Thompson, 2006). The upper duodenum has been found to mostly absorb free glucose sub-units directly from the stomach while the lower duodenum together with the jejunum has been found to absorb the majority of the ingested glucose (Ferraris, 2001; Drozdowski and Thompson, 2006). This has been found to be due to the presence of the carbohydrate hydrolyzing enzymes that break down the carbohydrates into glucose sub-units thus allowing for maximal absorption. By the time the food reaches the upper part of the ileum, approximately 95% of glucose has been absorbed leaving the ileum to do the remainder of the absorption.

Large proteins are hydrolyzed into small peptides and amino acids by pancreatic enzymes such as trypsin and chymotrypsin while the intestinal brush-border enzyme carboxypeptidase cleaves one enzyme at a time prior to absorption. Lipids are broken down into fatty acids and glycerol by the enzyme pancreatic lipase. Studies have shown, however, that this enzyme is water soluble while the fatty triglycerides are hydrophobic and tend to orient towards each other and away from the watery environment of the small intestine. The bile salts have been identified to be largely responsible for the emulsification of these fats to allow the pancreatic lipase to break down the triglycerides into free fatty acids and monoglycerides and thus allowing them to enter the villi of the intestine for absorption.

1.2.1 Intestinal carbohydrate metabolism

The breakdown of starch begins in the mouth under the action of salivary amylase. Once swallowed, the carbohydrates are propelled through the oesophagus into the stomach. The food from the stomach enters the duodenum of the small intestine through the pylorus mediated by the pyloric sphincter. Once food enters the small intestine, the secretion of the hormone cholecystokinin is triggered in the small intestine. The entry of food in the small intestine also triggers the release of the hormone secretin that allows for the entry of bicarbonate from the pancreas into the small intestine for the neutralization of gastric acid. This results in the release of digestive enzymes from the small intestine and pancreas.

1.2.2 Amylases

Amylases are enzymes that hydrolyze starch molecules to give diverse products including dextrans and progressively smaller polymers (oligosaccharides) composed of glucose units (Gupta *et al.*, 2003). Amylases are widely distributed in microorganisms, plants and animals. In animals, amylases work together with α -glucosidases to digest complex carbohydrates into monosaccharides that are then absorbed (see Figure 1). They are classified into three major groups on the basis of the mechanism of action. Endoamylases, also known as α -amylases, hydrolyze internal α -(1,4)-glycosidic bonds in amylose amylopectin and related polysaccharides such as glycogen to produce oligosaccharides of varying chain length. In animals, these exist in two major isoforms, namely salivary and pancreatic amylase (Smith *et al.*, 2005). Exoamylases, also known as β -amylases cleave α -1,4-glycosidic bonds in amylose, amylopectin and glycogen from the non-reducing end by successive removal of maltose or glucose in a stepwise manner (Drozdowski and Thompson, 2006). De-branching amylases, also known as γ -amylases, hydrolyze mainly α -1,6-glycosidic bonds in branched starches such as amylopectin and glycogen (Muralikrishna and Nirmala, 2005).

1.2.3 α -glucosidases

α -glucosidases (glycosidases) are enzymes that catalyze the hydrolysis of $\alpha(1,4)$ glycosidic linkages in starch and related compounds. These enzymes are found in the intestinal brush-border and comprise of sucrase, maltase and iso-maltase. The major function of these enzymes is to hydrolyze disaccharides ingested or produced by the action of α -amylases on starch to yield monosaccharides which are then absorbed (see Figure 1)(Ali *et al.*, 2002). A deficiency in these enzymes may lead to hypoglycaemia while hyperactivity of these enzymes can lead to rapid increases in postprandial glucose concentrations. Inhibition of this enzyme prevents a pronounced postprandial increase in blood glucose concentration due to delayed carbohydrate absorption (Ajjan and Grant, 2006).

In diabetes mellitus the expression of enzymes such α -amylase and α -glucosidase is greatly elevated leading to increased rates of carbohydrate hydrolysis (Floris *et al.*, 2005). This along with the lack of insulin effects leads to prolonged postprandial hyperglycaemia and subsequent development of diabetic complications. Some anti-diabetic drugs target the expression of these enzymes to slow down intestinal carbohydrate hydrolysis as a management strategy. In this study, we investigated the effect of OA and MA on these enzymes to not only retard intestinal carbohydrate hydrolysis, but to also slow down intestinal glucose transport.

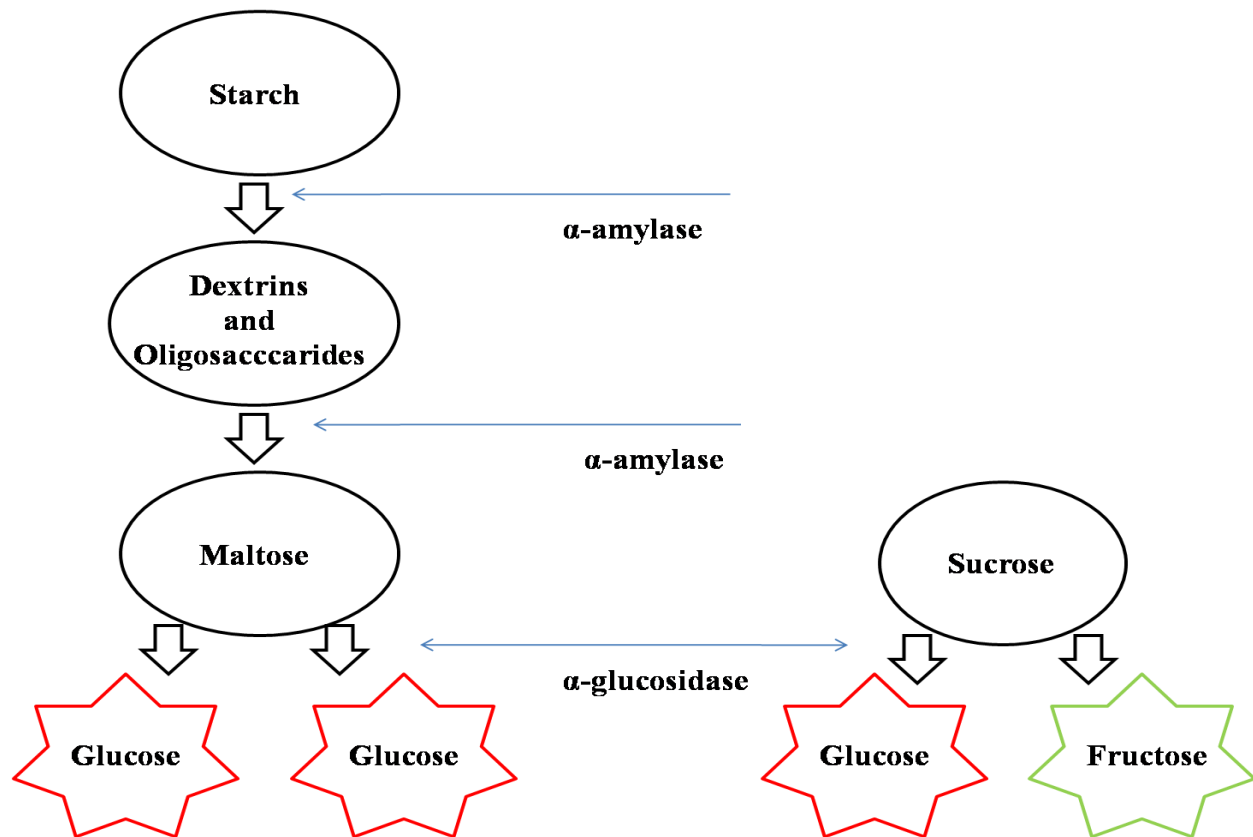


Figure 1. Sequential carbohydrate hydrolysis by intestinal carbohydrate hydrolyzing enzymes yielding monosaccharides that are subsequently absorbed in the small intestine

1.3 Glucose transport

Insulin is largely responsible for the maintenance of blood glucose homeostasis through the promotion of glucose uptake as well as glucose utilization by cells. This leads to the storage of glucose in the form of glycogen in the liver and skeletal muscle. Other organs such as the kidney and the small intestine have been postulated to have the ability to store glycogen because of the presence of the enzyme glucose-6-phosphatase (Aiston *et al.*, 2003). Insulin is synthesized by the β -cells of the pancreas in the islets of Langerhans in the form of pro-insulin which is further modified to insulin. This hormone has also been found to also stimulate protein and lipid anabolism as a deficiency of insulin results in weight loss through severe muscle wasting and lipid breakdown. This is largely attributed to the activation of hormone sensitive lipase and inhibition of lipoprotein lipase (Shen *et al.*, 2007). The secretion of insulin is stimulated by

elevated blood glucose and fatty acid concentrations. As the concentration of glucose increases in the blood stream, more glucose enters the β cell through the GLUT2 transporter. This glucose is phosphorylated by glucokinase and subsequent reactions leading to the formation of ATP. The ATP: ADP ratio rises due to the oxidative and glycolytic metabolism of glucose causing ATP to bind and inactivate the ATP-dependent K^+ channel. Inactivation of this channel leads to depolarization and an influx of Ca^{2+} leading to insulin secretion (Scheepers *et al.*, 2004). Low levels of blood glucose or starvation inhibit secretion of insulin.

Insulin receptors are present in most tissues which rely on insulin for glucose uptake. Insulin receptors have two components, alpha (α) components, which are found in the cell membrane surface and β components which are within the cell (Saltiel and Pessin, 2002). Insulin binds to α - components and simultaneously activates β components. The activation of β components causes phosphorylation of tyrosine kinase. Tyrosine kinase activation causes phosphorylation of phosphoinositol 3 kinase (PI3K) which causes insertion of GLUT4 across the membrane of tissues such as skeletal muscle (Annandale *et al.*, 2004). GLUT4 transporters then transport glucose into the cell. Cells using this transport system are said to be insulin dependent (Annandale *et al.*, 2004). After insulin binding has been terminated, insulin is released and circulates in the blood. Finally insulin is cleared mainly due to degradation by the liver. In addition to insulin dependent transporters, there are also various glucose transporters which are insulin independent such as sodium glucose transporters (SGLTs) as well as GLUT1 and GLUT2 (Tahrani *et al.*, 2013). The small intestine and kidneys use SGLTs which are sodium-mediated.

The transport of glucose in the small intestine is determined by the amount of sodium in the intestinal lumen. A high concentration of sodium on the mucosal surface of cells facilitates the movement of glucose into the epithelial cells. A low concentration inhibits glucose influx into the cells. This is because glucose and sodium share a sodium dependent glucose transporter (SGLT)(Furtado *et al.*, 2002). The two isoforms of this type of transporter, SGLT1 and SGLT2, resemble the glucose transporters responsible for facilitated diffusion in that they contain 12 transmembrane-spanning domains and have their carboxyl and amino terminals on the cytoplasmic side of the membrane (Liu *et al.*, 2012; Tahrani *et al.*, 2013). There is however, no homology with the GLUT series of transporters. These SGLT transporters are also responsible for glucose transport out of the renal tubules.

1.3.1 Mode of action of SGLT

SGLT1 expression in the small intestine is responsive to the presence of carbohydrates in the gut lumen. Glucose and Na^+ bind to the common carrier SGLT1 in the luminal membrane and glucose is carried into the cell as the Na^+ moves down the electrical and chemical gradient. The Na^+ is then pumped out of the cell into the lateral intercellular spaces. Glucose is transported by GLUT2 into the interstitial fluid. The energy for glucose transport is provided indirectly by the active transport of sodium out of the cell. This maintains the concentration gradient across the luminal border of the cell (Asano *et al.*, 2004; Wright *et al.*, 2011; Tahrani *et al.*, 2013). Enterocytes lining intestinal villi express very large numbers of SGLT1 transporters in the apical (brush border) membrane. Transfer of glucose (or galactose), along with sodium from the lumen, into the enterocyte is driven by active extrusion of sodium at the basolateral membrane via the Na^+/K^+ -ATPase pump. The increasing concentration of glucose within the cytosol of the enterocyte enables the passive transfer of glucose out of the cell across the basolateral membrane via the sodium-independent facilitative transporter GLUT2. Glucose uptake via SGLT1 by intestinal K cells and L cells contributes to the secretion of gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP1), respectively and up-regulates GLUT2 production (Wright *et al.*, 2011). In patients with diabetes, expression of SGLT1 and other monosaccharide transporters in the duodenum increases by 3-4 fold, suggesting that patients with diabetes have an increased capacity for glucose absorption (Asano *et al.*, 2004; Wright *et al.*, 2011). Therefore, aberrations in glucose transporter function can result in diabetes mellitus and the associated complications. The following section briefly describes diabetes mellitus.

1.4 Diabetes mellitus

Diabetes mellitus is a metabolic disorder of multiple aetiologies characterized by chronic hyperglycaemia, disturbances of carbohydrate, fat and protein metabolism resulting from defects in either insulin secretion, insulin action or both (Zimmet *et al.*, 1997; WHO, 2006a). Diabetes mellitus can be categorized into three main types: type 1, type 2 and gestational diabetes.

Type 1 diabetes is usually a progressive autoimmune disease, in which the pancreatic β -cells that secrete insulin are slowly destroyed by the body's own immune system. The exact mechanism that describes what first triggers this cascade of immune events is unclear, but there is evidence which suggests that both a genetic predisposition and environmental factors such as a viral infection are involved (Brownlee, 2001; Bonadonna, 2004).

Rare genetic disorders such as Klinefelters syndrome and Huntington's chorea, as well as hormonal disorders in acromegaly, Cushing syndrome, pheochromocytoma and hyperthyroidism have been associated with an increased risk for the development of type 1 diabetes, but these account for less than 5% of reported cases (Almdal *et al.*, 2004). Researchers have found at least 18 genetic locations, labeled IDDM1 - IDDM18 that are related to type 1 diabetes. The IDDM1 region contains the HLA genes that encode major histocompatibility complex proteins (Bonadonna, 2004). The genes in this region affect the immune response. New advances in genetic research are identifying other genetic components of type 1 diabetes (American Diabetes Association, 2011). Ironically; most people who develop type 1 diabetes do not have a family history of the disease. The odds of inheriting the disease are only 10% if a first-degree relative has diabetes, and even in identical twins, one twin has only a 33% chance of having type 1 diabetes if the other has diabetes (Almdal *et al.*, 2004). Children are more likely to inherit the disease from a father with type 1 diabetes (Beckman *et al.*, 2002). This then implies that genetic factors alone cannot fully explain the development of type 1 diabetes. Some researchers suggest that viral infections may trigger the disease in genetically susceptible individuals (WHO, 2006b; American Diabetes Association, 2011). Among the viruses that are under scrutiny are enteric viruses as these viruses attack the intestinal tract. The family of Cocksackie viruses are of particular interest in these studies (Brownlee, 2001; Beckman *et al.*, 2002). This is because epidemics of the Cocksackie virus, mumps and congenital rubella, have

been associated with incidence of type 1 diabetes. There are other conditions that damage or destroy the pancreas, such as pancreatitis, pancreatic surgery or the entry of certain industrial chemicals. Over the past 40 years, a major increase in the incidence of type 1 diabetes has been reported in some European and African countries (WHO, 2006b; American Diabetes Association, 2011).

In type 2 diabetes (T2D), the pancreas still has the ability to secrete insulin, but the cells are partially or completely unable to utilize the insulin (Tiwari and Rao, 2002). This is referred to as insulin resistance and there are various theories as to why this occurs. One view is that type 2 diabetes occurs due to genetic defects. These include defects in genes coding for glucokinase, the insulin molecule, the insulin receptor, GLUT 4 or the insulin receptor substrate IRS-1 (Bethel and Feinglos, 2002). Another view is that insulin resistance occurs due to obesity. This view postulates that as body weight increases, the ability of insulin to access insulin receptors in fat tissue and muscle is greatly diminished (American Diabetes Association, 2011). This increases insulin resistance and is thus associated with type 2 diabetes. The pancreas tries to overcome this resistance by secreting more insulin which is why type 2 diabetes patients often exhibit hyperinsulinaemia (Marshall, 1991; Tiwari and Rao, 2002). T2D is typically diagnosed in adulthood, usually after age 45 years. At least 90% of adult individuals with diabetes have type 2 diabetes (WHO, 2006a). T2D is usually controlled with diet, weight management, exercise, and oral medications. Some of these oral medications exert their effects by either slowing down the hydrolysis of carbohydrates in the small intestine or inhibiting glucose transport from the small intestine to the systemic circulation. The small intestine thus plays a large role in the management of diabetes and therefore justifies why this study investigated the effects of OA and MA on intestinal glucose handling.

Gestational diabetes arises when there is insulin resistance which occurs during the sixth and seventh month of pregnancy. During pregnancy, increased levels of progesterone and oestrogen secreted in the placenta assist in transporting nutrients from the mother to the developing foetus. Other hormones like human placental lactogen and human placental growth hormone are produced by the placenta to help prevent the mother from developing hypoglycaemia. They work by resisting the actions of insulin. Over the course of the pregnancy, these hormones lead to progressive increases of blood glucose concentration. Usually, the mother's pancreas is able to

produce more insulin to overcome the effect of the pregnancy hormones on blood glucose levels. If, however, the pancreas cannot produce enough insulin to overcome the effect of the increased hormones during pregnancy, blood glucose levels will rise, resulting in gestational diabetes.

The various types of diabetes mellitus discussed in the previous sections are all characterized by chronic hyperglycaemia. If untreated, they result in the development of diabetic complications which account for the majority of diabetes related deaths. The following section briefly describes the pathophysiology of the various diabetic complications.

1.4.1 Aetiology of diabetic complications

1.4.1.1 Polyol pathway

The polyol pathway is activated in diabetes mellitus in non-insulin sensitive tissues such as the lenses, peripheral nerve and the glomerulus. In the pathway, the enzyme aldose reductase is involved in the catalysis of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of glucose to sorbitol. The sorbitol is oxidized to fructose by sorbitol dehydrogenase, an enzyme that uses NAD^+ as a cofactor. Studies suggest that sorbitol causes osmotic vascular damage and cataracts in the eyes (Buse, 2006). The development of diabetic complications also involves the decrease in activity of enzymes that use NADPH/NADH as a cofactor. These include enzymes such as glutathione reductase, which would ultimately cause redox imbalances due to the depletion of glutathione levels (Brownlee, 2001; Sheetz and King, 2002; Lorenzi, 2007). The generation of nitric oxide by the endothelium is also impaired by the decreased NADPH levels. There is also the additional inhibition of glyceraldehydes-3-phosphate dehydrogenase which leads to an increase in triose phosphate. The accumulation of this phosphate increases the formation of methylglyoxal which is a precursor of advanced glycation end products as well as diacylglycerol (DAG). This increase in DAG activates protein kinase C (PKC). The inhibition of aldose reductase by various synthetic drugs such as zenarestat and sorbinil has been effective in alleviating and preventing diabetic complications (Neves *et al.*, 2009). There are also anti-diabetic plants which have proven their success in this area. For example the glycoside derivatives of the plant extracts of *Stelechocarpus cauliflorous*

[Annonaceae], engeletin and astilbin, have been reported to inhibit aldose reductase (Brownlee, 2001). There are also plant-derived bioactive compounds that inhibit aldose reductase activity which include quercetin, silymarin, flavonoids and puerarin (Matsuda *et al.*, 2001). OA and MA have been shown to be anti-hyperglycaemic bioactive compounds that reduce the amount of reactive oxygen species (Madlala *et al.*, 2012; Mkhwanazi *et al.*, 2014). This study investigated the effects of these triterpenes on both the amount of reactive oxygen species as well as antioxidants in the small intestine.

1.4.1.2 Advanced glycation end products (AGEs)

In diabetes mellitus, the levels of dicarbonyl compounds are elevated leading to the formation and accumulation of AGEs. The glycation reaction requires carbonyl groups obtained from glucose and carbonyl compounds such as glyceraldehyde. The formation and accumulation of AGEs from dicarbonyl compounds is accelerated in diabetes. AGEs have also been shown to reduce matrix protein flexibility through modification of extra-cellular matrix proteins leading to abnormal interactions with other matrix components and the receptors for matrix components on cells (Brownlee, 2001). The activation of these events leads to development of diabetic complications like retinopathy, nephropathy and atherosclerosis.

There are several strategies that can be used to prevent the effects of AGEs accumulation. One strategy may be the use of antioxidants that inhibit the final Maillard step catalyzed by oxidative stress. In diabetic atherogenesis, blocking or genetically deleting receptors for AGE (RAGE) in experimental animals reverses atherosclerosis (Ihara and Virella, 2007). Synthetic amino guanidine and pyridoxamine, which are known AGEs formation inhibitors, have been found to have reno-protective effects in diabetic animals (Hartog *et al.*, 2007). Additionally, the inhibition of AGEs effects can be achieved through breaking of the AGEs cross links by synthetic drugs such as alagebrium and also by inhibiting AGE signal transduction with drugs like cerivastatin (Hartog *et al.*, 2007). Another strategy may be to involve glycaemic control and therefore prevent the glucose-dependent first step in the Maillard reaction. A well-documented strategy of glycaemic control involves the inhibition of glucose transport across the small intestine (Dyer *et al.*, 1997b; Kwon *et al.*, 2007a), a focus of the current study.

There are several medicinal plants with their associated derivatives that have been shown to prevent the complications in diabetes mellitus through the inhibition of the AGEs or RAGE formation (Brownlee, 2001). The extract of the plant *Panaxquin quefolium* (Linnaeus) [Araliaceae] has been shown to inhibit the formation of AGEs or RAGE (Beckman *et al.*, 2002; Kudzuma, 2002). Additionally, bioactive compounds such as resveratrol and curcumin which are derived from the plants *Vitis vinifera* (Linnaeus) [Vitaceae] and *Curcuma longa* (Linnaeus) [Zingiberaceae] respectively, have also been found to inhibit the formation of AGEs or RAGE (Sheetz and King, 2002; Rahbar and Figarola, 2003; Kim *et al.*, 2007; Wirasathien *et al.*, 2007). There are also other plant extracts that have been shown to reduce glucose transport across the small intestine (Mahomoodally *et al.*, 2004). By using this mechanism, this significantly reduces the amount of glucose available for the formation of AGEs. Furthermore, the triterpenes of interest in this study have been found to improve kidney function in non-diabetic and STZ-induced diabetes (Madlala *et al.*, 2012; Mkhwanazi *et al.*, 2014). Accordingly, this study investigated *Syzygium aromaticum*-derived OA on intestinal glucose transport.

1.4.1.3 Protein kinase C (PKC)

In diabetes mellitus, diacyl-glycerol(DAG) levels are increased both in vascular tissues, including those of the aorta, heart and renal glomeruli and also in non-vascular tissues such as liver and skeletal muscles (Brownlee, 2001). DAG is a second messenger that plays a critical role in signaling and regulates many vascular functions such as endothelial activation, vasodilator release as well as growth factor signaling (Brownlee, 2001). There have been several pathways that have been shown to increase DAG levels in the diabetic state. One of these involves the hydrolysis of phosphatidylcholine by phospholipase C. The generation of the reactive oxygen species, like hydrogen peroxide in the polyol pathway also activates PKC. The activation of PKC is associated with changes in enzymatic activity, extra cellular matrix expansion, vascular permeability, angiogenesis, cell growth, basement membrane thickening and blood flow (Sheetz and King, 2002).

In early diabetes, the activation of PKC has been implicated in the impairment of retinal and renal blood flow possibly by increasing endothelin-1 levels. The effects of PKC activation on nitric oxide remain unclear though there is evidence of reduced production of nitric oxide.

Studies show that the activation of PKC directly increases the permeability of macromolecules across endothelial or epithelial barriers by phosphorylating cytoskeletal proteins or indirectly by regulating expression of various growth factors such as vascular endothelial growth factor (VEGF) (Keenan *et al.*, 2007). The majority of the diabetic manifestations due to PKC activation are reversed with the use of PKC inhibitors. One of these inhibitors is ruboxistaurin. This is a PKC- β inhibitor that has been shown to reverse haemodynamic changes in retinopathy, nephropathy and neuropathy (Sheetz and King, 2002; Evcimen and King, 2007). A variety of hydrolysable tannins purified from the plant *Phyllanthus amarus* have been found to inhibit PKC- β . However, if blood glucose levels in the diabetic state are kept within the homeostatic range via the inhibition of glucose uptake in the small intestine, the PKC would be inhibited resulting in an alleviation of diabetic complications.

1.4.1.4 The hexosamine pathway

The hexosamine biosynthesis pathway (HBP) involves conversion of fructose-6-phosphate to glucosamine-6-phosphate by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT). The major end-product of this pathway is uridinediphosphate-acetylglucosamine (UDP-GlcNAc). This compound along with the other amino-carbohydrates generated by hexosamine biosynthetic pathway (HBP) provides essential building blocks for glycosyl side chains, of proteins and lipids (Buse, 2006). Shunting of excess intracellular glucose into the HBP may account for several manifestations of diabetic renal and vascular complications (Buse, 2006). Therefore, there are increased amounts of fructose-6-phosphate under hyperglycaemic conditions diverted from glycolysis that provide substrates for reactions which require UDP-vV-acetylglucosamine such as those of proteoglycan synthesis and the formation of O-linked glycoproteins (Brownlee, 2001). The altered protein function due to O-linked GlcNAcylation results in diminished expression of sarcoplasmic reticulum Ca^{2+} ATPase in cardiomyocytes and induction of TGF-P and plasminogen activator inhibitor-1 in mesangial cells, vascular smooth muscle cells and aortic endothelial cells (Buse, 2006). Activation of the HBP is associated with reduced insulin mediated translocation of GLUT-4 transporters (Furtado *et al.*, 2002; Li *et al.*, 2007). Some of the effects of the HBP in diabetes include inhibition of endothelium nitric oxide synthase (eNOS) through hyperglycaemia-induced O-acetyl-glucosaminylation. In essence the

excess intracellular glucose leads to the diabetic manifestations associated with HBP (Brownlee, 2001).

There are various interventions that have been tried to alleviate the diabetic complications. These involve the use of hypoglycaemic drugs to inhibit the polyol and hexosamine pathways. Medicinal plant extracts from plants such as *Phyllanthus amarus*, *Momordica charantia*, *Ocimum sanctum* and *Pterocarpus marsupium* have also been investigated (Katz *et al.*, 2007). The primary target in the management of diabetes mellitus is the removal of excess glucose in the bloodstream, which in this case is associated with complications of HBP. The control of glucose that enters the bloodstream is, therefore, a crucial factor and thus many conventional drugs such as acarbose and miglitol inhibit the absorption of glucose in the small intestine. In a recent study, Musabayane and colleagues showed that orally administered herbal extracts decrease both food consumption and blood glucose concentrations in normal and STZ-induced diabetic rats (Musabayane *et al.*, 2006). Since OA and MA, which are plant derived triterpenoids, have been found to be anti-hyperglycaemic this study seeks to investigate the effects of both OA and MA on carbohydrate breakdown and absorption in the small intestine.

1.5 Macrovascular complications of diabetes

The central pathological mechanism in macrovascular disease is the process of atherosclerosis, which leads to narrowing of arterial walls throughout the body.

1.5.1 Atherosclerosis

Atherosclerosis is thought to result from chronic inflammation and injury of arterial walls of peripheral or coronary vascular vessels (Ajjan and Grant, 2006). Diabetes increases the risk of cardiovascular disease (CVD). CVD is the primary cause of death in people with either type 1 or type 2 diabetes (Laing *et al.*, 2003; Paterson *et al.*, 2007). Recent studies have shown that the risk of myocardial infarction (MI) in diabetes is equivalent to the risk in non-diabetic patients with a history of previous MI (Duckworth, 2001; Ajjan and Grant, 2006). Type 2 diabetes typically occurs in the setting of the metabolic syndrome which also includes abdominal obesity, hypertension and hyperlipidaemia. These other factors can also promote CVD. Even in this

setting of multiple risk factors, type 2 diabetes acts as an independent risk factor for the development of ischaemic disease, stroke, and death (Almdal *et al.*, 2004). Among people with type 2 diabetes, women may be at higher risk for coronary heart disease than men. The presence of microvascular disease is also a predictor of coronary heart events (Avogaro *et al.*, 2000). The increased risk of CVD has led to more aggressive treatment of these conditions to achieve primary or secondary prevention of coronary heart disease before it occurs. Studies in type 1 diabetes have shown that intensive diabetes control is associated with a lower resting heart rate and that patients with higher degrees of hyperglycemia tend to have a higher heart rate, which is associated with higher risk of CVD (Paterson *et al.*, 2007). Since hyperglycaemia is the generally the focal point of these complications, treatment and management of these complications involve glycaemic control which can be brought about in many ways, including the inhibition of glucose in the small intestine.

1.5.2 Microvascular complications of diabetes

1.5.2.1 Diabetic retinopathy

Diabetic retinopathy is the most common microvascular complication of diabetes (Lindholm *et al.*, 2002). The risk of developing diabetic complications of diabetes depends on both the duration and the severity of hyperglycaemia. Aldose reductase is the initial enzyme in the intracellular polyol pathway that participates in the development of diabetes complications. This pathway involves the conversion of glucose to sorbitol. High glucose levels increase the flux of glucose molecules through the polyol pathway, which causes sorbitol accumulation in cells. Cells are also thought to be injured by glycoproteins. High glucose concentrations can promote the non-enzymatic formation of AGEs. In animal models, these substances have also been associated with formation of microaneurysms and pericyte loss. Evaluations of AGE inhibitors are underway (Lindholm *et al.*, 2002). Oxidative stress may also play an important role in cellular injury from hyperglycemia. High glucose levels can stimulate free radical production and reactive oxygen species formation. Animal studies have suggested that treatment with antioxidants, such as vitamin E, may attenuate some vascular dysfunction associated with diabetes, but treatment with antioxidants has not yet been shown to alter the development or progression of retinopathy or other microvascular complications of diabetes (Gabbay, 2004).

This study was designed to investigate the antioxidant effects of OA and MA in the small intestine.

1.5.2.2 Diabetic nephropathy

Diabetic nephropathy is the leading cause of renal failure worldwide. The pathological changes in the kidney that occur in diabetic nephropathy include increased glomerular basement membrane thickness, microaneurysm formation and mesangial nodule formation (Kimmelsteil-Wilson bodies). The underlying mechanism of injury may also involve some or all of the same mechanisms as diabetic retinopathy. As with other complications of diabetes, prevention of the onset of diabetic nephropathy is part of the initial treatments. Like other microvascular complications of diabetes, there are strong associations between glucose control (as measured by haemoglobinA_{1c} [A1C]) and the risk of developing diabetic nephropathy. Patients should be treated to generate the lowest safe glucose level that can be obtained to prevent or control diabetic nephropathy (Watkins, 2003). Treatment with angiotensin-converting enzyme (ACE) inhibitors has not been shown to prevent the development of microalbuminuria in patients with type 1 diabetes, but has been shown to decrease the risk of developing nephropathy and cardiovascular events in patients with type 2 diabetes (Watkins, 2003; Gross *et al.*, 2005). There are several literature reports that implicate the use of plant extracts in the treatment and prevention of diabetic nephropathy. For example, garlic (*Allium sativum*) and ginger (*Zingiber officinale*) ameliorate structural nephropathy progression in streptozotocin-induced diabetic rats through attenuation of the SGLT1 transporters (Ali *et al.*, 2002). Interestingly, these transporters are also found in the small intestine and play a major role in facilitating intestinal glucose absorption. Therefore, this study sought to investigate the effects of OA and MA, on intestinal glucose handling.

1.5.3.3 Diabetic neuropathy

The precise nature of the inflammation caused by hyperglycaemia to the peripheral nerves is not known, but the likelihood is that this is related to mechanisms such as polyol product accumulation, injury from AGEs, and oxidative stress. Peripheral neuropathy in diabetes may

manifest in several different forms, including sensory, focal/multifocal, and autonomic neuropathies. More than 80% of amputations occur after foot ulceration or injury which can result from diabetic neuropathy (Keenan *et al.*, 2007). Diabetic neuropathy is, therefore, a direct consequence of sustained hyperglycaemia.

There is no specific treatment of diabetic neuropathy, although many drugs are available to treat the symptoms. The primary goal of therapy is to control symptoms and prevent worsening of neuropathy through improved glycaemic control. Some studies have suggested that control of hyperglycaemia and avoidance of glycaemic excursions may improve symptoms of peripheral neuropathy (Gabbay, 2004). Amitriptyline, imipramine, paroxetine, citalopram, gabapentin, pregablin, carbamazepine, topiramate, duloxetine, tramadol, and oxycodone have all been used to treat painful symptoms, but only duloxetine and pregablin possess official indications for the treatment of painful peripheral diabetic neuropathy (Laing *et al.*, 2003). Treatment with some of these medications may be limited by side effects of the medication, and no single drug is universally effective. Treatment of autonomic neuropathy is targeted toward the organ system that is affected, but also includes optimization of glycaemic control. This is mostly achieved through dietary interventions in combination with drugs that alter intestinal glucose handling. These drugs either inhibit intestinal glucose absorption or inhibit the hydrolysis of carbohydrates to their monomers.

1.6 Conventional management of diabetes mellitus

Evidence from the preceding paragraphs shows that various complications can arise in diabetes mellitus due to prolonged postprandial hyperglycaemia. The therapies that are currently available do not always achieve glycaemic goals. With intensive treatment, patients may still face spikes in blood glucose levels after meals, weight gain and a loss of effectiveness of their treatments over time (Musi *et al.*, 2002). The present therapies for T2DM mainly rely on the approaches to reduce hyperglycaemia by means of oral hypoglycaemic agents such as sulphonylureas, which increase the release of insulin from pancreatic islets; metformin, which acts to suppress gluconeogenesis and thus reduce hepatic glucose production; PPAR γ agonists

(thiazolidinediones), which enhance insulin action primarily through indirect effects on lipid metabolism; α -glucosidase inhibitors, which interfere with gut glucose absorption and exogenous insulin injections, which suppress glucose production and augment glucose utilization (Kellett and Helliwell, 2000; Stumvoll *et al.*, 2000; Dhindsa *et al.*, 2002). The following sections briefly discuss the various classes of compounds, which include α -glucosidase inhibitors that are used in the management of diabetes mellitus as shown in Figure 3 (Stümpel *et al.*, 1996).

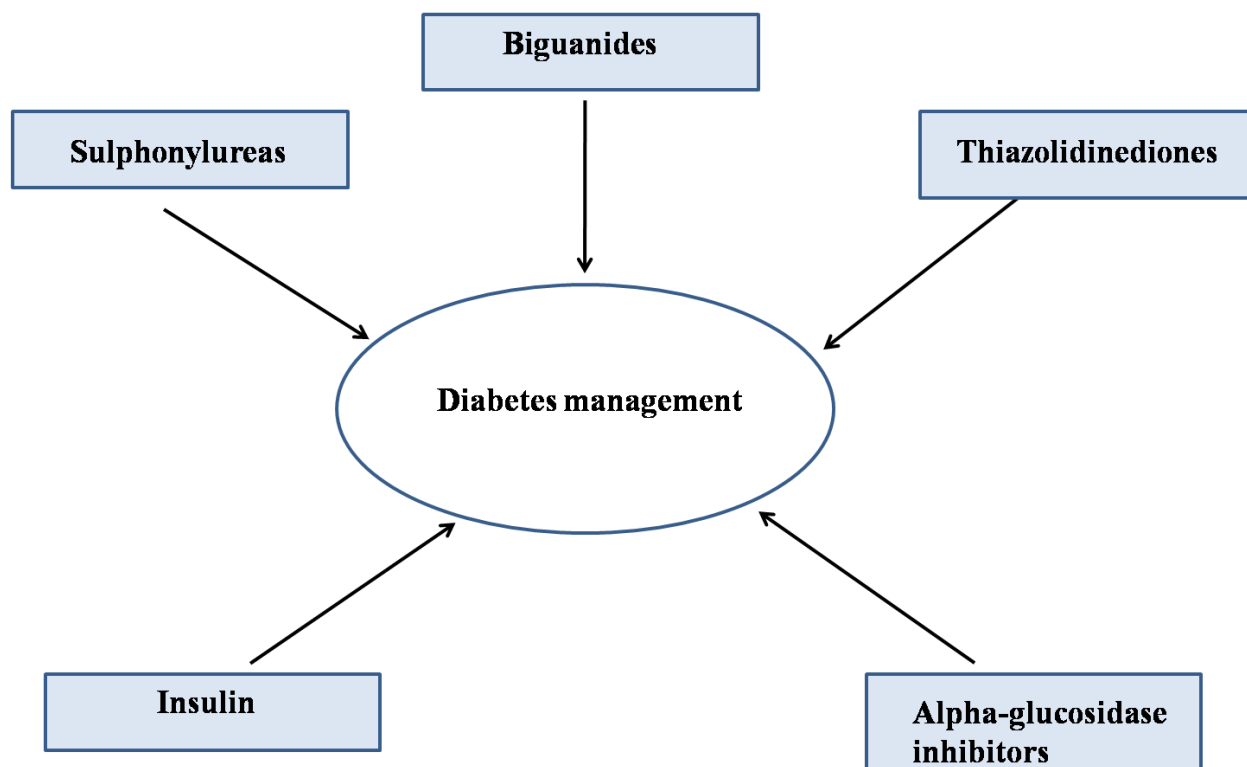


Figure 2: The various classes of compounds used in the management of diabetes mellitus. These classes use various mechanisms to maintain glucose homeostasis.

1.6.1 Insulin

The various insulin formulations are classified based on the duration of action. and there are three main types; short-acting, intermediate-acting and long-acting (Bethel and Feinglos, 2002;

Vazquez-Carrera and Silvestre, 2004). The short-acting class is designed to mimic bolus insulin secretion which is used in emergency situations, while intermediate or long acting insulin classes are designed to mimic basal glycaemic control and are more suitable for long term management of diabetes (Vazquez-Carrera and Silvestre, 2004). A disadvantage of subcutaneously delivered insulin is that this insulin is delivered in a bolus. With this method of delivery, the diabetic patient runs a risk of developing hyperinsulinaemia which can result in severe hypoglycaemia. At extremely low blood glucose levels, patients can exhibit lethargy which can lead to coma, convulsions and eventual death.

Due to the short half-life of insulin, there is a need for multiple insulin injections daily in order to maintain homeostatic blood glucose levels. This results in decreased patient compliance as the process is not only cumbersome, but also painful as this is an invasive method. This method however, has been found to be efficient in managing postprandial hyperglycaemia as the injections are administered with every meal. Recent studies have shown the small intestine to be an insulin-sensitive organ as the small intestine expresses the enzyme glucose-6-phosphatase which is involved in both gluconeogenesis as well as glycolysis (Aiston *et al.*, 2003). This suggests that the small intestine could be a site for glucose storage. Furthermore, insulin has been found to inhibit the intestinal absorption of glucose *in vitro* (Stümpel *et al.*, 1996 ; Lidia *et al.*, 2010). This suggests that the hypoglycaemic effects of insulin observed in diabetes mellitus could arise, in part, via the effects on the small intestine.

The subcutaneous administration of insulin, however, is more suitable to the management of type 1 diabetes (Aiston *et al.*, 2003; Hirsch, 2005). Type 2 diabetes management, however, requires a different approach as insulin is still present. There are various classes of compounds that are used to manage diabetes. The following paragraphs briefly discuss these.

1.6.2 Thiazolidinediones

Thiazolidinediones (TZD's) are orally administered hypoglycaemic agents that require the presence of insulin to exert their effects. They are mainly used in the management of type 2 diabetes mellitus. These compounds are known to exert their hypoglycaemic effects by increasing insulin sensitivity in tissues such as skeletal muscle and adipose tissue to enhance insulin effects (Bailey, 2011). TZD's are selective and potent agonists for the peroxisome proliferator-activated receptors (PPAR γ). These receptors are found on insulin-sensitive tissues and not only act as lipid sensors, but also as regulators of carbohydrate and lipid metabolism. Activation of PPAR γ improves insulin sensitivity resulting in increased glucose uptake in the skeletal muscles and reduced hepatic glucose output (Ajjan and Grant, 2006). Commercially available TZD's include troglitazone, pioglitazone and rosiglitazone. The drugs have been found to modulate most of the risk factors associated with cardiovascular diseases (Kudzuma, 2002). In addition, TZD's modify lipid profiles and prevent inflammation and atherosclerosis in vascular tissues as well as lower blood pressure in diabetic patients. These drugs, have not been found to influence intestinal glucose handling as yet, but have been associated with increased glycogen synthesis in the liver and skeletal muscle (Sun, 2006).

1.6.3 Sulphonylureas

Sulphonylureas (SU), also known as insulin secretagogues, are orally administered hypoglycaemic agents which exert their hypoglycaemic effects primarily by increasing the release of insulin from residual functioning pancreatic β cells. Drugs such as tolbutamide and gliclazide exert their effects by blocking the SUR1 subunit, whereas drugs like glibenclamide and glimepiride have high affinity for both SUR1 and SUR2. The class of drugs has additional effects because the SUR2 receptors are also present on the KATP channels of cardiac (SUR2A) and vascular smooth muscles (SUR2B) and are thus able to assist the heart adapt during ischaemic conditions through dilatation of coronary vessels (Ajjan and Grant, 2006). SU's are used with extreme caution as their effects cannot be regulated. These drugs cause the release of insulin irrespective of the blood glucose concentration and this can result in severe hypoglycaemia (Ajjan and Grant, 2006). While studies reveal that insulin may play a role in

intestinal glucose handling, these drugs have not been found to exert any effects on the small intestine.

1.6.4 Biguanides

Biguanides are a class of orally administered anti-diabetic drugs that lower blood glucose by mainly reducing hepatic gluconeogenesis and increasing skeletal muscle glucose uptake and utilization (Musi *et al.*, 2002). Most of these drugs exert their effects by activating AMP-activated protein kinase (AMPK). This is an enzyme which plays a major role in the stimulation of glucose uptake in skeletal muscle (Musi *et al.*, 2002), but also by inhibiting liver gluconeogenesis (Grover *et al.*, 2002; Lidia *et al.*, 2010). The AMPK pathway is usually activated in response to the depletion of cellular energy stores observed in diabetes mellitus. Metformin has also been reported to increase muscle hexokinase (HK) activity in STZ-induced diabetic rats (Dyer *et al.*, 2002). HK plays an important role in the maintenance of glucose homeostasis in all cells that are metabolized by ATP to produce G-6-P (Bonadonna, 2004; Ndong *et al.*, 2007). Metformin has been reported to delay the digestion of complex carbohydrates in the small intestine while some studies suggest that biguanides may shunt some glucose towards glycogen synthesis within the small intestine (Dhindsa *et al.*, 2002; Ali *et al.*, 2002). However, there is paucity of studies that have investigated the effects of these biguanides on intestinal glucose handling. This study therefore investigated the effects of OA and MA on intestinal glucose handling as well as glycogen synthesis in the small intestine.

1.6.5 Intestinal enzyme inhibitors

Studies during the last few decades have identified a number of carbohydrate hydrolyzing enzymes as possible targets for diabetes management. These include enzymes such as α -glucosidase and α -amylase which are briefly discussed in the sections below.

1.6.5.1 α -amylase inhibitors

The activity of α -amylases is reported to be inhibited by metal chelators, organic acids, heavy inorganic metal ions, pure natural products as well as crude plant extracts (Muralikrishna and Nirmala, 2005). Metal chelators are strong inhibitors of amylases as they are metalloenzymes (Matsuda *et al.*, 2001; Yong and Robyt, 2003). Organic acids such as citric acid and oxalic acid have been reported to be potent inhibitors of amylase while heavy metal ions such as Fe^{2+} , Hg^{2+} and Al^{3+} have been known to inhibit amylases at high concentrations (Muralikrishna and Nirmala, 2005). Acarbose, a synthetic pseudotetrasaccharide originally isolated from actinoplane species of microorganisms is used in the management of diabetes mellitus. Acarbose has the ability to inhibit both α -amylase and α -glucosidase (Yong and Robyt, 2003). Interestingly, various studies have confirmed the α -amylase inhibitory activity of medicinal plant extracts. A majority of plant extracts that have been investigated have showed strong α -amylase inhibitory effects that compare with those of the standard drug acarbose. Table 1 shows a few of the plants that have been identified as possessing α -amylase inhibitory effects.

Table 1. Various medicinal plant extracts that have been reported to possess α -amylase inhibitory activity. They are given in order of increasing IC₅₀ values together with their maximum percentage inhibition values.

Plant species	Type of extract	Max inhibition (%)	IC ₅₀ (mg/ml)	Reference
<i>Phyllanthus amarus</i>	Hexane	100	0.003	(Patel <i>et al.</i> , 2011)
<i>Ocinum tenuiflorum</i>	Aqueous	76	1.6	(Bhat <i>et al.</i> , 2008)
<i>Murraya koenigii</i>	Aqueous	84	1.9	(Bhor <i>et al.</i> , 2004)
<i>Morus alba</i>	Ethanolic	76	18	(Abdul-Ghani and Defronzo, 2014)
<i>Salvia veticilata</i>	Ethanolic	100	18	Nickavar <i>et al.</i> , 2008
<i>Salvia virgate</i>	Ethanolic	77	20	Nickavar <i>et al.</i> , 2008
<i>Andrographis paniculata</i>	Ethanolic	80	51	Subramanian <i>et al.</i> , 2008
<i>Bergenia ciliata</i>	Methanolic	94	401	(Bhandari <i>et al.</i> , 2008)

1.6.5.2 α -glucosidase inhibitors

α -glucosidase inhibitors such acarbose (precose) and miglitol (glyset) are orally administered anti-diabetic drugs that exert their hypoglycaemic effects by either inhibiting glucose absorption in the small intestine or slowing down the hydrolysis of carbohydrates (Tiwari and Rao, 2002). These agents can also exert their effects via either inhibition of other digestive enzymes such as α -amylase (Kim *et al.*, 2008). α -glucosidase inhibitors can also exert their effects via the inhibition of active transport of glucose across the intestinal brush border membrane or by decreasing gastric emptying (Mahomoodally *et al.*, 2005). The inhibition of glucose transport mechanism relies heavily on the interaction with the SGLTs in the small intestine. These transporters aid in the transfer of glucose from the mucosa to the serosa. Although there are a few commercially available α -glucosidase inhibitors, various medicinal plant extracts have been shown to lower blood glucose concentrations through inhibition of α -glucosidase.

Pharmacologically, the potency (inhibitory strength) of a medicinal enzyme inhibitor is reported in terms of the compounds IC_{50} value. This is the concentration at which the inhibitory compound reduces the enzyme activity by 50%. The IC_{50} value depends on concentrations of the enzyme (or target molecule), the type of inhibitor used as well as the substrate along with other experimental conditions. Inhibitors that require a smaller concentration to reduce the enzyme activity by 50% have smaller IC_{50} values suggesting that the lower the IC_{50} value, the greater the inhibitory potency of the compound. Studies have reported that medicinal plants extracts like those derived from *Momordica charantia* not only exert their hypoglycaemic effects by inhibiting α -glucosidase activity but that they also have IC_{50} values comparable to those of drugs such as acarbose and miglitol (Mahomoodally *et al.*, 2004). Recent studies that have identified various plant extracts to have effects on α -glucosidase activity are summarized in Table 2.

Table 2. Medicinal plant extracts that have been reported to have α -glucosidase inhibitory activity. They are given in order of increasing IC₅₀ values together with their maximum percentage inhibition values.

Plant species	Type of extract	Max inhibition (%)	IC ₅₀ (mg/ml)	Reference
<i>Alstonia scholaris</i>	Methanolic	74 51	1.7 (maltose) 1.9 (sucrose)	(Jong-Anurakkhun <i>et al.</i> , 2007)
<i>Grateloupia elliptica</i>	Ethyl acetate	64	4.8	Kim <i>et al.</i> , 2008
<i>Morus alba</i>	Tea infusion	67	7.7 (maltose)	(Hansawasdi and Kawabata, 2006)
<i>Andrographis paniculata</i>	Ethanolic	80	17.2	(Tandon <i>et al.</i> , 1993)
<i>Bergenia ciliate</i>	Methanolic	69	33.3	(Bhandari <i>et al.</i> , 2008)
<i>Euclea undulate</i>	Ethanolic	62	49.5	(Deutschlander <i>et al.</i> , 2009)
<i>Momordica charantia</i>	Ethanolic	72	56.1	(Mahomoodally <i>et al.</i> , 2006)

1.6.5.3 Intestinal glucose transport inhibitors

In the 1980s, Rossetti and colleagues introduced the concept of normalization of glucose concentrations by increasing urinary glucose excretion (Rossetti *et al.*, 1980). The researchers showed that phlorizin, a naturally occurring phenolicglycoside first isolated from apple tree bark in 1835 increased urinary glucose and lowered blood glucose in partly pancreatectomised rats. In

the 1990s, phlorizin was identified as an inhibitor of the SGLT1 and SGLT2 transporters. However, phlorizin offered little oral bioavailability because of degradation at an *O*-glucoside linkage by intestinal glucosidases. In addition, the poor selectivity for blockade of SGLT2 compared with SGLT1 in the intestine caused sufficient gastrointestinal side-effects to exclude this drug as a treatment for patients with type 2 diabetes. Although several potent selective SGLT2 inhibitors such as T1095, sergliflozin, and remogliflozin were developed, they are degraded by intestinal glucosidases at their *O*-glucoside linkage and have not progressed in clinical development. Oral SGLT inhibitors that are either approved or in advanced clinical development have circumvented glucosidase degradation by replacement of the *O*-glucoside linkage with a C-aryl linkage. Inhibitors of SGLT2 eliminate 60–80 g of glucose per day and sustain this for 2 years in clinical trials. This elimination represents inhibition of reabsorption of about a third of the filtered glucose load. The extent to which SGLT2 can be inhibited varies with the dose, binding affinity, and retention time of the inhibitor at the transporter. Exposure of the inhibitor to the transporter also shows the rate at which the inhibitor is filtered, secreted or reabsorbed by the proximal tubule. Inhibition of glucose reabsorption by SGLT2 will be partly off-set by the uptake of glucose by SGLT1. Because inhibition of SGLT2 and SGLT1 is not insulin-dependent, and is not altered by deteriorating β -cell function or insulin resistance, these inhibitors should in principle be operative at any stage in the natural history of diabetic states. However, although these inhibitors can reduce hyperglycaemia, they do not directly address the fundamental underlying endocrinopathies. Thus, although a sustained reduction in glucotoxicity improves the metabolic environment and reduces hyperglycaemia-related complications, insulin is always necessary to meet other physiological requirements. Many studies have reported the efficacy of various medicinal plant extracts in inhibiting intestinal glucose absorption (Mahomoodally *et al.*, 2005; Mahomoodally *et al.*, 2006; Gao *et al.*, 2008). Plants extracts of *Neurolena lobata* and *Luthrum salicaria* have all been shown to be able to completely inhibit intestinal glucose absorption *in vitro* while the extracts of plants such as *Fraxinus excelsior* and *Momordica charantia* have been shown to avert hyperglycaemia *in vivo* through this mechanism (Liu *et al.*, 2012; Tahrani *et al.*, 2013).

1.7 Traditional plant extracts

As mentioned previously, current conventional diabetes therapy using blood glucose-lowering medications such as insulin or oral hypoglycaemic agents has limitations. Alternative methods of lowering blood glucose are therefore needed. The World Health Organisation (WHO) estimated that 80 % of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs (Matsuda *et al.*, 2001; Raptis and Dimitriadis, 2001). Scientific investigations of the plant extracts that possess hypoglycaemic effects not only assists in validating indigenous knowledge systems, but could also provide a more affordable alternative source of anti-diabetic drugs. Extracts of the plants *Erythroxylum macrocarpum* (Erythroxylaceae), *Punica granatum*, *Rosa damascena* and *Vaccinium arctostaphylos* have all been found to possess anti-diabetic properties (Grover *et al.*, 2002). Further investigations reveal that these plants exhibit their anti-diabetic properties using different mechanisms such as reducing food intake, stimulating insulin secretion, increasing glycogen synthesis and delaying carbohydrate digestion. This is testament that the various plants used around the world to manage diabetes are a rich source of anti-diabetic drugs and possess the potential to finding the ideal drug to manage diabetes. For instance, the use of herbs and medicinal plant products has become a mainstream phenomenon over the past two decades in the United States where herbs and phytomedicines have become one of the fastest growing segments in retail pharmacies and supermarkets (Antignac *et al.*, 2011; Delhanty and van der Lely, 2011). In Asian regions such as India and China, western scientists have rediscovered many of these traditional medicines as cheap sources of complex bioactive compounds (Ndong *et al.*, 2007). A significant number of many potent drugs used today trace their origins to plants (Al-Qattan *et al.*, 2008). For instance, the anti-diabetic drug metformin was isolated from the plant *Galega officinalis* (Linnaeus) [Fabaceae]. Another anti-diabetic drug that is used to delay the digestion of carbohydrates in the small intestine, acarbose, is derived from various plants such as *Phaseolus vulgaris* and *Irvingia gabonensis* (Al-Qattan *et al.*, 2008). Currently, several kinds of plant extracts from various exotic, endemic and indigenous plants are sold as decoctions in several markets globally to treat diabetes mellitus, but very few of these have been validated scientifically for their therapeutic efficacy. Some of these plants extracts, while hypoglycaemic, have other negative side-effects

such as kidney failure, increase blood pressure and lead to inflammatory responses due to possessing antigenic properties (Katz *et al.*, 2007). These extracts contain many compounds, some of which are toxic. Some of the compounds found in these extracts are not naturally toxic, but because they are given in excessively high amounts they become toxic. This has led to the identification and isolation of the bioactive compounds responsible for the hypoglycaemic effects to investigate their individual effects.

Some of the work in our laboratory thus far has investigated the blood glucose lowering effects of crude plant extracts. These include the extracts derived from plants such as *Persea americana* (Miller) [Lauraceae], *Sclerocarya birrea* [(A. Rich) Hochst.] *Ficus thonningii* (Blume) [Moraceae] and *Psidium guajava*. More recently, the focus has been on plants of the *Syzygium* species, these include plants such as *Syzygium cordatum* as well as *Syzygium aromaticum* (Ngubane *et al.*, 2011; Madlala *et al.*, 2012; Mkhwanazi *et al.*, 2014). Other studies also showed that these extracts contained pentacyclic triterpenoids (Chen *et al.*, 2006).

1.7.1 *Syzygium aromaticum*

Syzygium aromaticum is a clove tree that belongs to *Myrtaceae* family (See Figure 3). This plant is an evergreen tree that grows to a height of 15 to 30 meters and is native in North Moluccas (Indonesia). This plant is also grown in India, Jamaica, Brazil, and other tropical areas (Agbaje *et al.*, 2009). *S. aromaticum* has opposite; ovate leaves which are more than 5 inches long. When fully grown, the flowers are red and white, bell-shaped, and grow in terminal clusters. *S. aromaticum* also has brown, dried, unopened flower buds that are called cloves. These cloves are familiarly used in cooking and the fruit is a one- or two-seeded berry (Agbaje *et al.*, 2009). The leaves of the plant are glabrous, with numerous oil glands on the lower surface. *S. aromaticum* contains bioactive chemical compounds which include tannins, glycosides, saponins, polyphenols and triterpenes. An essential oil is produced from the cloves of this plant and this oil is used to add aroma to food. The main constituents of the oil are eugenol (70-85%), eugenol acetate (15%), β -caryophyllene (5-12%). The triterpenes, ursolic acid (UA), oleanolic acid (OA) and maslinic acid (MA) make up the remainder of the constituents of this oil. However, the UA has been found, in previous studies conducted in our laboratory, to be toxic to tissues resulting in the

investigations being discontinued (Musabayane *et al.*, 2007). This study, therefore seeks to investigate the effects of OA and MA on intestinal glucose handling.



Figure 3: *Syzygium aromaticum* plant. Picture adapted from (Raina *et al.*, 2001)

1.8 OA

Oleanolic acid (3 β -hydroxy-olea-12-en-28-oic acid, OA is a plant pentacyclic triterpene found as free acid or aglycones for triterpenoid saponins (Liu, 1995). This compound appears abundantly as a major constituent in many African medicinal plants (Al-Qattan *et al.*, 2008; Liu *et al.*, 2012). OA has therapeutic importance due to the triterpenes low systemic toxicity as well as diverse pharmacological properties (Liu, 1995; Braga *et al.*, 2007; Patil *et al.*, 2010). OA possesses anti-inflammatory and anti-oxidant properties (Teodoro *et al.*, 2008). In our laboratory, this triterpene has been shown to possess hypoglycaemic, hypotensive, reno- and cardio-protective effects in experimental animals (Musabayane *et al.*, 2005; Mapanga *et al.*, 2009; Musabayane *et al.*, 2010). The effects of this triterpene on postprandial glucose concentrations however, have not yet been established. This study therefore investigated the effects of this triterpene on intestinal carbohydrate hydrolyzing enzymes and intestinal glucose transport.

1.9 MA

The pentacyclic triterpene, maslinic acid (MA) (2a, 3b-2,3-dihydroxy-olean-12-en-28-oic acid), has wide-spread occurrence throughout the plant kingdom. This compound possesses a broad spectrum of pharmacological properties which include anti-inflammatory, anti-humoral, hepatoprotective, anti-oxidation, antibacterial and anti-HIV effects (Aiston *et al.*, 2003; Dzubak *et al.*, 2006 ; Juhász *et al.*, 2007). In addition this triterpene has previously been shown to have hypoglycaemic effects via antagonism of hepatic glucose production (Liu, 2008; Tarling *et al.*, 2008). MA has been previously reported to present a new class of glycogen phosphorylase (GP) inhibitor (Aiston *et al.*, 2003; Juhász *et al.*, 2007). This bioactive compound causes pharmacological inhibition of this key enzyme responsible for glycogen breakdown thus providing a therapeutic approach for treating diseases associated with abnormalities in glycogen metabolism. The increase in glycogen synthesis caused by MA enhances glucose utilisation thus decreasing blood glucose (García-Granados *et al.*, 2000; Reyes-Zurita *et al.*, 2009). In addition, MA has been reported to possess hypoglycaemic properties in adrenaline-induced diabetic mice (Al-Qattan *et al.*, 2008). Furthermore, studies have shown that this triterpene ameliorates kidney function through increased glomerular filtration rate and increased urinary sodium output in STZ-induced diabetic rats. The effects of this triterpene on postprandial glucose concentrations however, have not yet been established. This study therefore investigated the effects of this triterpene on intestinal carbohydrate hydrolyzing enzymes and intestinal glucose transport.

1.10 Experimental techniques used to study intestinal glucose handling

There are various methods available for studying glucose handling in the gastro-intestinal tract, but they all have certain limitations. The serial measurement of blood glucose concentrations after the oral administration of carbohydrates is an indirect method influenced by many factors besides intestinal absorption. These studies, however, do not account for the disappearance of glucose which may be influenced by such factors as bacterial decomposition in the large intestine (Zakeri-Milania *et al.*, 2007). One technique that was used for measuring the rate of absorption of sugars in the intact animal was by giving a sugar by stomach tube and then killing the animal

and measuring the amount of sugar recovered from the gastro-intestinal tract (Reynell and Spray, 1956). This method is open to the objections that variations in gastric emptying and intestinal transit cannot be taken into account and the site of absorption cannot be determined. Another method of evaluating intestinal glucose handling is the *in situ* jejunal perfusion technique. This technique is a well-established technique to investigate the intestinal absorption behavior of both drugs and carbohydrates in which the glucose is monitored in perfusate. This technique offers the advantage of being able maintain high intestinal enzyme activity, however, organ integrity as well as enzyme activity may become fragile and compromised during long-term perfusions (Liu and Liu, 2013). Another *in vitro* technique used to assess intestinal function is the use of Caco-2 cells. These cells are derived from human colorectal adenocarcinomas and form monolayer (like human intestinal epithelium) under conventional culture conditions. They have been widely used as a potent *in vitro* model to predict drug absorption in humans, to explore mechanisms of drug absorption, and to identify substrates or inhibitors of transporters (Sjoberg *et al.*, 2013). These cells exhibit remarkable morphologic and biochemical similarity to the small intestinal columnar epithelium; they are extremely useful for mechanistic studies of drug absorption and are widely used by pharmaceutical companies in absorption screening assays for preclinical drug selection. Although Caco-2 cells system has many advantages, there also include some limitations such as the long (21 day) cell culturing time, heterogeneity, and non-specificity due to the expression of multiple transporters.

The everted intestinal sac technique is a widely used *in vitro* model to evaluate the absorption of drugs by transporters (Wilson and Wiseman, 1954). This system offers the advantage of being able to monitor glucose transport across the small intestine through the quantification of glucose (Mahomoodally *et al.*, 2004). Furthermore, the cells of the small intestinal tissue receive sufficient amounts of oxygen throughout the experimental period thus keeping them alive for the duration of the experiment. Additionally, this technique requires a low number of animals for experiments as many intestinal segments can be obtained from each animal. This technique was employed in this study to evaluate the effects of triterpenes on intestinal glucose absorption. This is discussed in more detail in the next chapter.

1.11 Justification of the study

Postprandial hyperglycemia ascribed to hydrolysis of carbohydrates by digestive enzymes in the small intestine is a major risk factor for complications in diabetic patients. Small intestine α -glucosidase and pancreatic α -amylase are the key enzymes of dietary carbohydrate digestion in humans. Glucose released upon carbohydrate digestion is absorbed from the intestinal lumen into the blood mainly via sodium-dependent glucose co-transporter (SGLT1), localized to the enterocyte apical or brush-border membrane (BBM), and the basolateral facilitative glucose transporter 2 (GLUT2). Evidence obtained from experimental diabetes indicates that the capacity of the small intestine to absorb glucose in diabetes increases mainly due to enhanced activity and abundance of SGLT1 and GLUT2. Thus, controlling postprandial glucose concentrations is critical during early treatment of diabetes mellitus to avert postprandial glucose excursions thereby reducing chronic vascular complications. Commercially available synthetic inhibitors of carbohydrate hydrolyzing enzymes are effective in retarding carbohydrate hydrolysis and glucose absorption to suppress postprandial hyperglycaemia. Furthermore, dietary interventions are incorporated into many diabetes management strategies so as to reduce food intake without compromising the nutritional status of the individual. α -glucosidase inhibitors with increased potency and lesser adverse effects than the existing drugs have also been isolated from medicinal plants. Interestingly, various medicinal plant extracts have been found to influence food intake through the modulation of plasma ghrelin concentrations as well as through the attenuation of intestinal carbohydrate hydrolyzing enzymes. We have reported that the hypoglycaemic properties of plant-derived oleanolic acid (OA) and maslinic acid (MA) are mediated via other mechanisms such as increased glycogenesis. However the effects on key carbohydrate hydrolyzing enzymes remain unanswered. Therefore, the present project was designed to evaluate the effects of *Syzygium spp*-derived triterpenes oleanolic acid (OA) and maslinic acid (MA) on intestinal glucose handling in normal and STZ-induced diabetic rats. This was done through the evaluation of the triterpene effects on postprandial hyperglycaemia associated with various carbohydrates and their hydrolyzing enzymes; intestinal glucose transport and the expression of the respective glucose transporters as well as evaluating the effects of the triterpenes on food intake by evaluating the expression of ghrelin as well as plasma ghrelin levels.

CHAPTER 2

MATERIALS AND METHODS

2.0 Drugs and chemicals

Drugs and chemicals were sourced as indicated:

streptozotocin (STZ), Tris-HCl, dimethyl sulphoxide (DMSO), acarbose, porcine pancreatic amylase (PPA), α -glucosidase, sodium dodecyl sulphate (SDS), glycerol, phenyl methyl sulphonyl fluoride (PMSF), bicinchoninic acid (BCA) reagent, nitrocellulose membrane, Trizma base (tris), triton X-100, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), sodium bicarbonate (NaHCO_3), sodium chloride (NaCl), potassium chloride (KCl), dipotassium phosphate (K_2HPO_4) and sodium hydroxide (NaOH) (Sigma-Aldrich, St Louis, Missouri, United States of America);

acetic acid, citric acid, sodium citrate, hexane, dichloromethane (DCM), ethyl acetate (EA), methanol, calcium chloride, potassium hydroxide, potassium chloride, 95% ethanol ($\text{C}_2\text{H}_5\text{OH}$), disodium hydrogen orthophosphate dehydrate, sodium dihydrogen orthophosphate dihydrate, sucrose, maltose, starch, silica gel, methanol (CH_3OH), chloroform (CHCl_3), sulphuric acid (H_2SO_4) (Merck chemicals (PTY) LTD, Wadeville, Gauteng, South Africa) and

halothane (Isofor, Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa).

All chemical reagents were of analytical grade.

2.1 Isolation of OA and MA

The extraction of OA and MA was performed in the Chemistry laboratory at UKZN Pietermaritzburg campus. These triterpenes were isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] flower buds using a previously validated standard protocol that has been reported from our laboratories (Mapanga *et al.*, 2009; Musabayane *et al.*, 2010; Ngubane *et al.*, 2011; Madlala *et al.*, 2012; Mkhwanazi *et al.*, 2014). Briefly, air-dried flower buds of *S. aromaticum* (500 g) were sequentially extracted twice at 24h intervals with 1 L of dichloromethane (DCM) and ethyl acetate (720 mL) on each occasion. The resulting filtrate was filtered using a 30 cm filter paper (Whatman, Maidstone, England). The filtrates were

concentrated *in vacuo* $60 \pm 1^{\circ}\text{C}$ using a laboratory 4000 efficient rotary evaporator (Laboratory Consumables and Chemical Supplies, Johannesburg, South Africa) to obtain DCM solubles (63 g) and ethyl acetate solubles (EAS, 85 g). Crude EAS were subjected to further purification since previous studies indicated that they contain mixtures of OA/UA and methyl maslinate/methyl corosolate (Somova *et al.*, 2003; Musabayane *et al.*, 2005). The EAS containing mixtures of oleanolic/ursolic acid and methyl maslinate/methyl corosolate were purified by silica gel 60 column chromatography with hexane: ethyl acetate solvent systems, 7:3 for OA and 6:4 for MA. This yielded OA and MA, respectively which were further purified by recrystallization from chloroform-methanol (1:1 v/v). The structures of these triterpenes were confirmed by spectroscopic analysis using 1D and 2D, ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) techniques.

Preliminary studies indicated that the properties of *S. aromaticum*-isolated OA and MA such as hypoglycemic effects were similar to respective commercial triterpenes and hence the plant-extracted compounds were used in the experiments as they were less costly.

2.2 Animals

Male Sprague-Dawley rats weighing 250-300g bred and housed in the Biomedical Research Unit (BRU) of University of KwaZulu-Natal were used in this study. The animals were maintained under standard laboratory conditions of constant temperature ($22 \pm 2^{\circ}\text{C}$), CO_2 content of <5000 p.p.m., relative humidity of $55 \pm 5\%$, and illumination (12 h light/dark cycles) and the noise levels of <65 decibels and had free access to water and standard rat chow (Meadow Feeds, Pietermaritzburg, South Africa). Procedures involving animals and their care were conducted in conformity with institutional guidelines of the University of KwaZulu-Natal.

2.3 Ethical consideration

Ethical clearance was obtained from the University of KwaZulu-Natal Ethics committee (references: 082/11/Animal, 026/12/Animal, 056/13/Animal and 031/14/Animal see Appendices I, II, III and IV).

2.4 Induction of diabetes mellitus

Experimental type 1 diabetes mellitus was induced in male Sprague-Dawley rats with the administration of a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ) in freshly prepared 0.1 M citrate buffer (pH 6.3). The control animals were injected with the vehicle. Animals that exhibited glucosuria after 24 h, tested by urine strips (Rapidmed Diagnostics, Sandton, South Africa) were considered diabetic. Animals that showed blood glucose concentrations of 20 mmol/L or above, measured after one week were considered as having a stable diabetic state before experimental procedures.

2.5 Experimental design

The studies were divided into series I (*in vitro*) and series II (*in vivo*) experiments. The inhibitory activities of OA and MA against carbohydrate hydrolyzing enzymes were studied *in vitro*. The *in vitro* studies investigated the effects of OA and MA on glucose transport using the isolated small intestines. *In vivo* studies investigated the effects of OA/MA on postprandial blood glucose concentration, intestinal carbohydrate-hydrolyzing enzymes and glucose transporters (see Figure 4 and Figure 5).

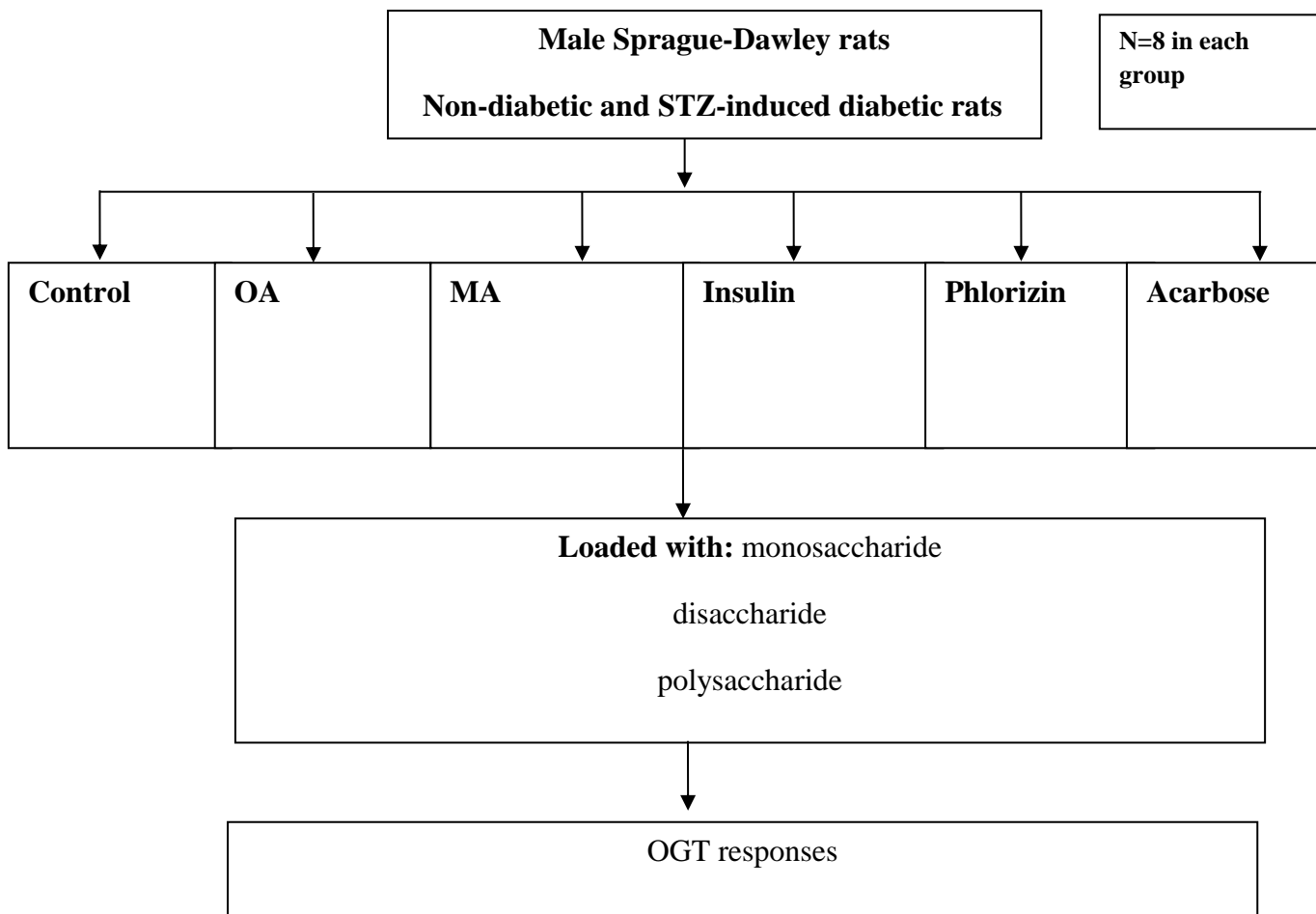


Figure 4: Flow diagram detailing acute *in vivo* studies carried out over a 2-hour period. Non-diabetic and STZ-induced diabetic rats were loaded with mono-, di- and polysaccharides to observe the effects of triterpene treatments on blood glucose levels. This was in an effort to investigate the effects of the triterpenes on the intestinal carbohydrate hydrolyzing enzymes and the resultant postprandial blood glucose concentrations.

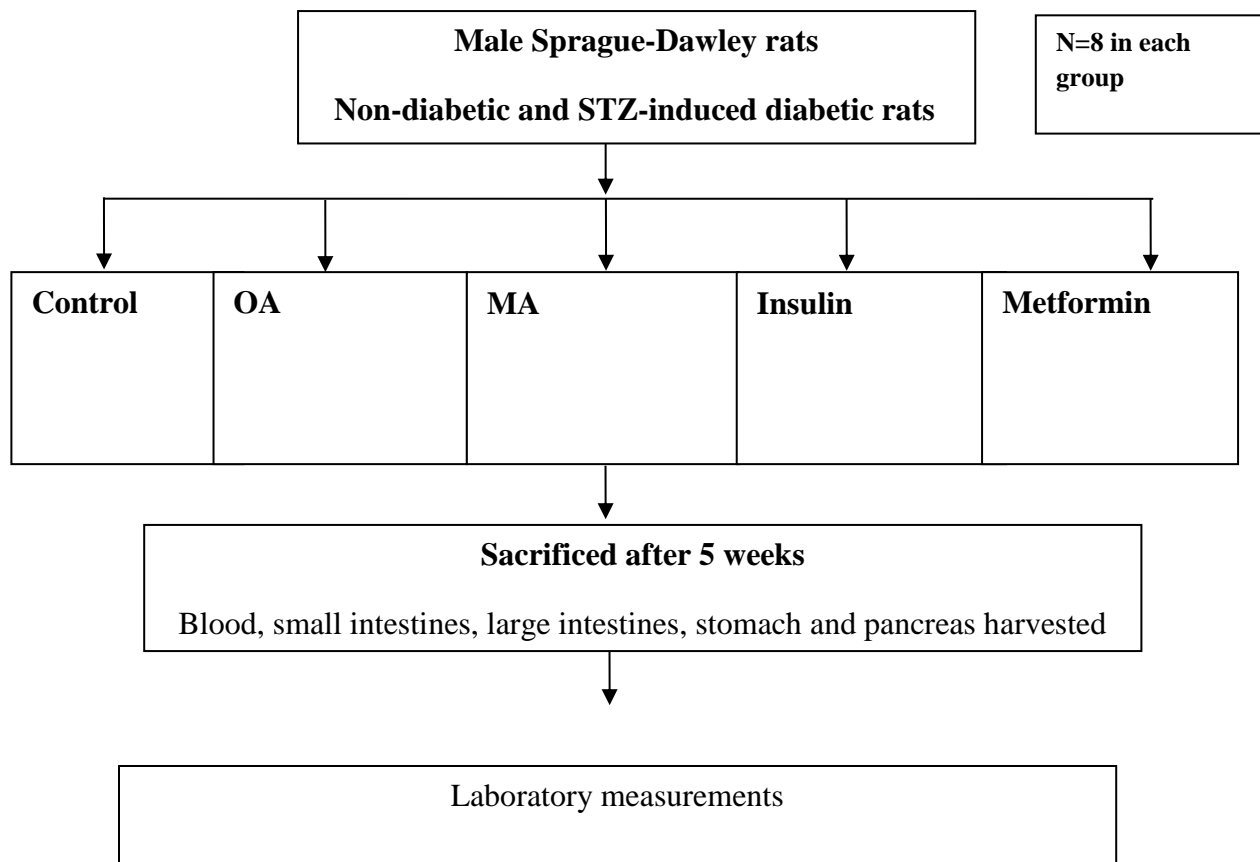


Figure 5: Flow diagram detailing sub-chronic *in vivo* studies carried out over a 5-week period. Non-diabetic and STZ-induced diabetic rats were maintained on a starch-based diet to observe the effects of triterpene treatments on blood glucose levels, food and water intake. Following the 5-week study various biochemical parameters were investigated to elucidate the effects of the triterpenes on the gastrointestinal tract.

Series I

These studies were carried out to investigate the effects of the triterpenes on carbohydrate hydrolyzing enzymes and intestinal glucose transport *in vitro*.

2.6 *In vitro* assays

2.6.1 Inhibitory enzyme assay studies

To investigate the effects of OA and MA against enzymes such as α -amylase, α -glucosidase and sucrase, the inhibitory activities of these triterpenes were investigated *in vitro*. The inhibitory activities of OA and MA on α -amylase and α -glucosidase were studied using an α -amylase/ α -glucosidase-starch model system while inhibitory effects on sucrose utilized the dextran sucrase-dextran sucrose reaction mixture.

2.6.2 α -amylase

The assessment of the inhibitory effects of OA/MA against α -amylase activity *in vitro* was based on the modified method previously described by (Bhandari *et al.*, 2008; Gao *et al.*, 2009).. Briefly, soluble maize starch (1 mg) was boiled for 5 min in 0.5 mL of 0.5 M Tris-HCl buffer (pH 6.9) containing 0.01 M CaCl₂. After cooling, deionized water was added to a final volume of 100 mL. The solution was kept in the refrigerator and was used within 2-3 days. A reaction mixture of 500 μ L containing 200 μ L starch, 100 μ L of OA/MA at various concentrations (4.37-21.90 μ mol/L) to which 200 μ L of α -amylase (porcine pancreas, 2.60 mmol/L) was added to initiate the reaction and incubated at 37 °C for 37 min. The reaction was terminated by addition of 100 μ L of 50% acetic acid.

2.6.3 α -glucosidase

The assessment of the inhibitory effects of OA and MA against α -glucosidase *in vitro* utilized a similar method described above for α -amylase except that the 0.1 M potassium phosphate buffer

(pH 6.9) was used. The assay mixture (500 μ L) comprising of 200 μ L of α -glucosidase (Type 1, Bakers yeast, 1.30 mmol/L) was premixed with OA/MA (100 μ L) at various concentrations (4.37-21.90 μ mol/L). The mixture was incubated at 37 °C for 30 min after adding starch in phosphate buffer and stopped by adding 1.5 mL of 2M Tris-HCl buffer (pH 6.9).

2.6.4 Sucrase

The *in vitro* sucrase inhibitory effects of OA/MA were performed as described above for α -amylase except for that the assay mixture (700 μ L) comprised of 200 μ L dextran sucrose (56mmol/L) in the potassium phosphate buffer, 100 μ L of OA/MA at various concentrations (4.37-21.90 μ mol/L) and 400 μ L of dextran sucrase (*Leuconostoc mesenteroides*, 2.60 mmol/L). In all cases, the liberated glucose was measured by the glucose oxidase method and the absorbance was recorded at 595nm using Varian Cary 1E UV-visible spectrophotometer (Varian Australia Pty Ltd, Victoria, Australia). Results expressed as the percentage inhibition of the corresponding control were calculated using the formula:

$$\% \text{ Inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}} \times 100}{\text{Abs}_{\text{control}}}$$

Where $\text{Abs}_{\text{control}}$ was the absorbance without sample and $\text{Abs}_{\text{samples}}$ was the absorbance of sample extract.

The IC_{50} values were calculated from plots of log concentration of inhibitor concentration versus percentage inhibition curves.

2.7 Glucose transport in the small intestine

To establish the effects of OA/MA on intestinal glucose transport, the everted intestinal sac technique described by Mahomoodally *et al*, was used in this study (Mahomoodally *et al.*, 2006). Briefly, non-diabetic animals were rendered unconscious by a severe blow on the head against a hard surface after an 18-h fast. The whole small intestine was removed and rinsed with cold normal saline solution. Mid portions of the small intestine were removed by cutting across the upper end of the duodenum and the lower end of the ileum from the pyloric sphincter to the ileocecal junction and rinsed with cold normal saline solution (0.9%, w.v, NaCl). These intestinal segments (10 ± 2 cm) were then everted according to the method previously described by Wilson and Wiseman (Wilson and Wiseman, 1954; Mahomoodally *et al.*, 2005; Mahomoodally *et al.*, 2006). After weighing, the empty sac was filled with 1 ml of Krebs–Henseleit bicarbonate buffer (KHB composition in mmol/L: NaHCO_3 , 25; NaCl, 118; KCl, 4.7; MgSO_4 , 1.2; NaH_2PO_4 , 1.2; CaCl_2 , 1.2; and Na_2EDTA , 9.7 (serosal fluid), and tying the other end with a similar thread. The distended sac was immersed in an organ bath containing 50 mL of KHB buffer incubation medium (mucosal fluid) which was continuously being bubbled with a mixture of 95% O_2 and 5% CO_2 . The organ bath was surrounded by a water jacket maintained at 37 ± 1 °C (see Figure 6).

In separate preparations, 50 μL of 10mM d-glucose (500 μmol) was added to the medium just before the start of the experiments and incubated with graded concentrations OA/MA (0.82 - 6.56 mmol/L) in the mucosal solution as shown in Figure 5. To establish whether OA or MA possess activities comparable to synthetic drugs already in use, studies were conducted in separate everted gut preparations incubated with phlorizin (a positive control for glucose uptake inhibition). Everted preparations to which an equal volume of the vehicle or phlorizin (10^{-3} - 10^{-6} mmol/L) acted as untreated and treated positive controls, respectively. At the end of the incubation period (30 min), the sacs were removed from the organ bath, blotted and weighed again. The serosal fluid was drained through a small incision into a test tube. The emptied sac was shaken gently to remove the adhered fluid and the tissue was reweighed. The weight of the empty sac before and after the incubation did not significantly differ. The transport of d-glucose across the everted gut was evaluated by measuring the change in concentration of d-glucose inside and outside the intestinal sacs after 30 minutes of incubation. Mucosal glucose transfer is

the amount of glucose that disappeared from the mucosal fluid while serosal glucose uptake is the amount of glucose that entered the serosal fluid. Glucose concentration was measured using a commercial glucose oxidase kit (Boehringer Mannheim, Germany). In this protocol, glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to form a coloured product. The oxidized o-dianisidine reacts with sulphuric acid to form a more stable pink-coloured product. The intensity of this colour is measured at 540nm and this is proportional to the glucose concentration. All samples were read in a Novaspec II spectrophotometer against a blank. A standard curve was obtained after measuring the absorbance of standards of glucose which were prepared in the range of 5.5-22.2 mmol/L. Using absorbances obtained from the spectrophotometer, glucose concentration was then extrapolated from this standard curve and the results obtained were standardized by converting them to μmol .

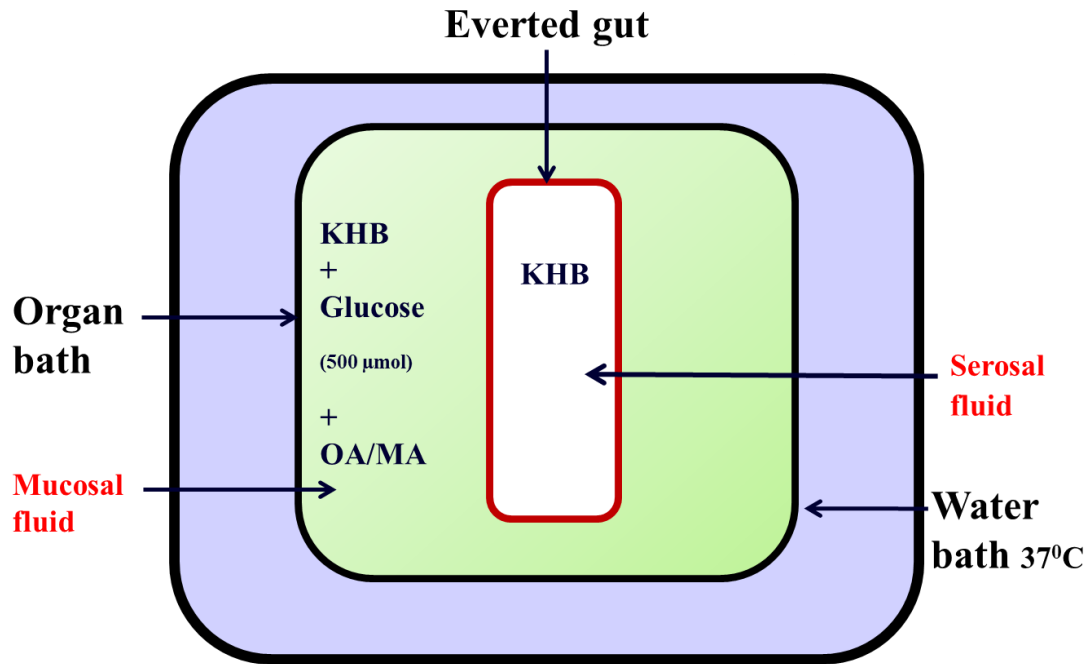


Figure 6. The everted intestinal sac protocol developed by Wilson and Wiseman (Wilson and Wiseman, 1954). This technique measures the influence of drugs on intestinal glucose uptake by measuring glucose concentrations in the mucosal and serosal fluid before and after the 30-minute incubation period.

Series II

These studies were carried out to investigate the effects of the triterpenes on intestinal carbohydrate handling *in vivo* in non-diabetic and STZ-diabetic animals. These were subdivided into oral glucose tolerance (OGT) responses and sub-chronic studies.

2.8 Oral glucose tolerance (OGT) responses

In order to determine how the small intestine handles different types of carbohydrates, non-diabetic and STZ-induced diabetic animals were loaded with monosaccharides, disaccharides and polysaccharides. Oral glucose tolerance responses were monitored in these animals as previously described by our laboratory with slight modifications (Mapanga *et al.*, 2009; Musabayane *et al.*, 2010). The rats were divided into the following groups: control and treated non-diabetic rats as well as control and treated STZ-induced diabetic rats (n = 6 in each group). After an 18 h fasting period, glucose was measured (time 0) followed by loading with monosaccharide (glucose; 0.86 g/kg, p.o.), disaccharide (sucrose; 1.72 g/kg, p.o.) or polysaccharide (starch; 0.086 g/kg, p.o.) delivered into the stomach by a gavage needle (18-gauge, 38 mm long curved, with a 21/4 mm ball end (Able Scientific, Canning Vale, Australia). The selection of these doses was based on previous studies conducted in our laboratory (Musabayane *et al.*, 2006; Gondwe *et al.*, 2008; Musabayane *et al.*, 2010). In order to determine the effects of triterpenes on postprandial glucose, separate groups of non-diabetic and STZ-induced diabetic rats were administered OA and MA (80 mg/kg, p.o.) dissolved in dimethyl sulphoxide and deionized water. Rats treated with DMSO/water (3 mL/kg, p.o.) and standard drugs acarbose (100mg/kg, p.o.), insulin (175µg/kg, sc) and phlorizin (100mg/kg, p.o.) served as untreated and positive controls, respectively (Mapanga *et al.*, 2009; Musabayane *et al.*, 2010; Ngubane *et al.*, 2011). Blood glucose concentrations were measured using the tail-prick method with the use of a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom) were made at 15, 30, 60, and 120 minutes after carbohydrate loading (see Figure 3). The area under the curve (AUC) for increase in glucose over baseline was calculated during OGT responses by the incremental method that calculates changes in blood glucose concentrations relative to the beginning of the experiment.

2.9 Intestinal glucose handling

To assess the influence of OA and MA on intestinal glucose handling, the activity of intestinal carbohydrate hydrolyzing enzymes and glucose transporters, groups of non-diabetic and STZ-induced diabetic male Sprague-Dawley rats were housed individually in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa) for a 5-week period (n=6 in each group).

In those animals in which the effects of OA/MA were investigated, the rats were administered with OA/MA (80 mg/kg, p.o.) twice daily at 09h00 and 15h00 by means of an oral gavage needle (18-gauge, 38 mm long curved, with a 21/4 mm ball end (Able Scientific, Canning Vale, Australia). Rats treated with DMSO/saline (3 mL/kg, p.o.) served as untreated controls while those treated with standard anti-diabetic drugs metformin (500 mg/kg, p.o.) and insulin (175 µg/kg sc) served as treated positive controls (see Figure 4). Body weight changes as well as food and water intake were assessed every third day at 09h00 for the duration of the experimental period. At the end of the 5 week experimental period, the animals were killed by exposing to halothane (100 mg/kg, for 3 min) (Isofor, Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) via a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa).

2.10 Tissue sample harvesting

Blood was collected by cardiac puncture from groups of untreated non-diabetic and STZ-induced diabetic as well as treated STZ-induced diabetic rats into individual pre-cooled heparinized containers at the end of the 5-week experimental period. The blood was centrifuged (Eppendorf centrifuge 5403, Germany) at 4 °C and a speed of 503 g for 15 minutes and separated plasma was stored at -70 °C in a Bio Ultra freezer (Snijers Scientific, Holland) for hormonal assays that are described in section 2.11. Thereafter, the whole of the small intestine was removed by cutting across the upper end of the duodenum and the lower end of the ileum from the pyloric sphincter to the ileocecal junction and rinsed with cold normal saline solution. Mid portions of the small intestine were snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -70 °C for Western blot analysis of glucose transporters (SGLT1 and GLUT2), carbohydrate hydrolyzing enzymes (α -amylase and α -glucosidase) and ghrelin expression. The stomach and large intestine were also removed, snap frozen in liquid nitrogen

and stored at -70°C for Western blot analysis for ghrelin. Other mid portions of the small intestine were placed in formalin and stored at room temperature (22°C) for histological analysis. These procedures will be discussed in more detail in later sections.

2.11 Laboratory analyses

2.11.1 Intestinal glycogen concentration

The glycogen concentration was measured in mid portion segments of the small intestine of starved and non-starved non-diabetic and STZ-induced diabetic rats with access to water 18-h after treatment with OA and MA (80 mg/kg p.o.) while those obtained from the metformin-treated (500 mg/kg p.o.) and insulin-treated (175 $\mu\text{g/kg}$ sc) served as positive controls. The animals were rendered unconscious by a severe blow to the head against a hard surface. The glycogen concentration was determined using a previously validated protocol with modifications (Ong and Khoo, 2000; Ngubane *et al.*, 2011). Briefly, the tissue samples (0.5- 0.7 g) were homogenized in 2 mL of 30% KOH solution and digested at 100°C for 30 min and then cooled in ice saturated sodium sulphate. The glycogen was precipitated with ethanol, and then pelleted, washed, and redissolved in deionized water. Glycogen standards (10-2000 mg/L) were also prepared using glycogen powder. The glycogen concentration was determined by its reaction with the anthrone reagent [2 g anthrone/ 1 of 95% (v:v) H_2SO_4] after which absorbance was measured at 620 nm using a Novaspec II spectrophotometer (Biochrom Ltd, Cambridge, England).

2.11.2 Hormone measurements

2.11.2.1 Insulin

Plasma insulin concentrations were measured using an ultra-sensitive rat insulin ELISA kit (DRG diagnostics EIA-2746 GmbH, Marburg, Germany) in untreated non-diabetic and STZ-induced diabetic controls as well as the STZ-diabetic treated groups. The kit contained a 96 well plate coated with mouse monoclonal anti-insulin, standards, enzyme conjugate, enzyme conjugate buffer, wash buffer, substrate 3,3',5,5'-tetramethylbenzidine (TMB) and a stop solution. The principle of the assay is a solid phase two-site enzyme immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed towards separate antigenic determinants antigenic epitopes of insulin. During the incubation period, insulin in the sample reacts with the peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration well. The washing step removes all unbound enzyme labeled antibody, leaving only the bound conjugate which reacts with TMB. This reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically. Each determination was performed in duplicate for standards and samples. The insulin assay procedure was as follows: 50µL of each insulin standard (20.0, 50.0, 150.0, 400.0 and 1000.0 pmol/L) were added to anti-insulin wells. 50µL of the plasma samples were then added to the remaining wells followed by 50µL enzyme conjugate to all wells of standards and plasma samples. The plates were incubated at room temperature for 2 hours on a plate shaker (VelpScientifica, Milano, Italy) followed by aspirating the reaction volume, adding wash buffer (350 µL) and aspirating 6 times. After the final wash the plates were inverted firmly against absorbent paper to absorb all the liquid in the plates. 200 µL substrate TMB was added to all the wells and incubated for 30 minutes. The reaction was stopped by adding 50µL of stop solution to all wells and mixing on a shaker for 5minutes. The absorbance was measured at 450 nm using a Biotekmicroplate reader (Winooski, Vermont, USA). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in GraphPad InStat software (version 5.00). The respective insulin concentrations of the unknown samples were then extrapolated from the standard curve. The lower and upper limits of detection were 1.39 pmol/L and 960 pmol/L, respectively. The intra-assay analytical coefficient of

variation ranged from 4.4 to 5.5% while the inter-assay coefficient variation ranged from 4.7 to 8.9%.

2.11.2.2 Ghrelin

In order to evaluate the effects of treatment with OA and MA on ghrelin secretion, plasma ghrelin was measured using ultra-sensitive rat ghrelin ELISA kit (DRG diagnostics EIA-3706 GmbH, Marburg, Germany) in untreated non-diabetic and STZ-induced diabetic controls as well as the STZ-diabetic treated groups. The kit contained a 96 well plate pre-coated with secondary antibody and the nonspecific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the ghrelin antibody whose Fab fragment will be competitively bound by biotinylated ghrelin in sample. The biotinylated peptide is able to interact with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution composed of 3,3', 5,5'-tetramethylbenzidine (TMB) and hydrogen peroxidase to produce a blue colored solution. The enzyme-substrate reaction is stopped by hydrogen chloride (HCl) and the solution turns to yellow. The intensity of the yellow is directly proportional to the amount of biotinylated peptide-SA-HRP complex but inversely proportional to the amount of the peptide in standard solutions or samples. This is due to the competitive binding of the biotinylated ghrelin and the ghrelin in standard solutions or in the samples to the ghrelin antibody. A standard curve of ghrelin with known concentrations was established accordingly. The concentration of ghrelin in samples was determined by extrapolation from the standard curve. The lower and upper limits of detection were 0.1 pmol/L and 960 pmol/L, respectively. The intra-assay analytical coefficient of variation ranged from 4,2% to 6,8% while the inter-assay coefficient variation ranged from 5.9% to 9.2%.

2.11.3 Western blot analysis

In order to further elucidate the mechanism of the triterpenes, we investigated the gastrointestinal expression of ghrelin, carbohydrate hydrolyzing enzymes and glucose transporters using Western blot analysis. Small intestine tissues obtained from untreated non-diabetic and STZ-induced diabetic controls as well as the STZ-diabetic treated groups (0.1 g) were homogenized on ice in isolation buffer (0.5 mM Na₂EDTA, 0.1 M KH₂PO₄, 0.1 mM dithiothreitol, 0.25 M sucrose) and then centrifuged at 400 x g for 10 min (4 °C). The protein content was quantified using the Lowry method (Lowry *et al.*, 1951). The Lowry method involves reaction of protein with cupric sulphate and tartrate in an alkaline solution, resulting in formation of tetradentate copper-protein complexes. When the Folin-Ciocalteu reagent is added, this reagent is effectively reduced in proportion to these chelated copper complexes, producing a water-soluble product whose blue color can be measured at 750 nm. Upon completion of protein quantification, all the samples were standardized to one concentration (1 mg/mL). The proteins were then denatured by boiling in Laemmli sample buffer (0.5 M Tris-HCl, glycerol, 10% sodium dodecyl sulphate (SDS), 2-mercaptoethanol, 1% bromophenol blue for 5 min. The denatured proteins were loaded (25 µL) on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with molecular weight marker (5 µL). The gel was electrophoresed for 1 h at 150 V in electrode (running) buffer (Trisbase, glycine, SDS), pH 8.3). Following electrophoresis, the resolved proteins were electro-transferred to an equilibrated polyvinylidenedifluoride (PVDF)/ membrane for 1 h in transfer buffer (192 mM glycine, 25 mM Tris, 10% methanol). After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (20 mM Tris, 150 mM NaCl, KCl, 0.05% Tween-20). The membrane was then immuno-probed with antibodies- glycogen synthase (1:1 000 in 1% BSA, Neogen, USA) for 1 h at room temperature (RT); α -amylase and α -glucosidase (1:500 in 1% BSA, Neogen, USA); SGLT1 and GLUT2 (1:1 000 in 1% BSA, Neogen, USA) and ghrelin (1:1000 in 1% BSA, Neogen, USA) for 1 h at room temperature (RT). The PVDF membrane was then subjected to 5 washes (10 min each with gentle agitation) with TTBS. Following which, the membrane was incubated in horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:1000; Bio-Rad) for 1 h at RT. After further washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system

and analysed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands.

2.12 Oxidative stress

To determine the effects of OA and MA on oxidative stress, we compared levels of malondealdehyde (MDA), a known marker of oxidative stress and of antioxidant defense enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) in non-diabetic, untreated STZ-diabetic and STZ-diabetic treated groups following the sub-chronic study.

2.12.1 MDA

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

$$\text{Concentration of MDA (mM)} = \frac{\text{Average absorbance}}{\text{Absorption coefficient (156mmol/L)}}$$

2.12.2 SOD

The antioxidant effects of OA and MA were determined in small intestine samples collected after the 5-week study by assessing superoxide dismutase (SOD) activity. SOD activity was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine in a convenient 96 well format. SOD activity was measured using the Biovision SOD Assay Kit according to manufacturers' instructions (BioVision Research Products, Mountain View, CA). Small intestine tissues (50 mg) were homogenized in ice cold 0.1 M Tris/HCl (pH 7.4) containing 0.5% Triton X-100, 5mM β -mercaptoethanol (ME) and 0.1 mg/mL 1 phenyl methane sulphonylonyl fluoride (PMSF). The tissue homogenate was centrifuged at 14,000g for 5 min at 4 °C. The supernatant obtained was added to each sample (20 mL) and blank 2 (20 mL) well, while blank 1 and blank 3 wells received 20 mL of H₂O. Thereafter, 200 mL of working solution was added to each well. Subsequently, dilution buffer (20 mL) was added to each blank 2 and blank 3 well, while each sample and blank 1 well received enzyme working solution (20 mL). The solutions were mixed thoroughly before reading the plate. Inhibition activity of SOD was colorimetrically measured on an Anthos Venytech-200 Spectrophotometer (Biochrom limited, Cambridge, United Kingdom) after a reaction period of 20 min at 37 °C. SOD activity was calculated as percentage inhibition using the equation:

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

2.12.3 Glutathione peroxidase (GPx)

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

$$B - B^0 \times \text{Sample dilution}$$

GPx activity = _____

$$(T2 - T1) \times V$$

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

2.12.4 Histological studies

In order to determine if the treatment of rats with OA and MA induced any morphological changes in the small intestine, histological analysis was used. Following the sub-chronic study, small intestine tissues were stored in 10% formalin immediately after killing the non-diabetic control, STZ-induced diabetic control, as well as the STZ-diabetic animals treated with OA, MA, metformin as well as insulin. The tissues were cut in the longitudinal as well as cross-section manner using a sterile blade. The tissues were then rinsed with water to remove excess formalin and then placed in 70%, 80%, 90%, as well as 100% ethanol for 2, 2, 3 and 2 minutes, respectively. During this process, the water within the tissue is replaced with alcohols of varying strengths to facilitate dehydration. The tissue was then placed in xylene that clears the alcohol and leaves the tissue translucent. The tissues were embedded in wax blocks which are then sectioned at a thickness of 4microns using a Microm rotary microtome (Robert-Bosch-Strabe, Walldorf, Baden-Wurttemberg, Germany). The tissue sections were mounted onto clean slides and placed onto a Ransom warming plate to allow for drying. The sections were then de-paraffinized with xylene and stained with the haematoxylin and eosin stain (Sigma-Aldrich, St Louis, Missouri, USA). The section was then permanently mounted with a drop of mounting medium and covered with a coverslip. The slides were then scanned with a Leica scanner SCN 400 (Leica Microsystem CMS, GmbH, Ernst-Leitz-Strasse, Wetzlar, Germany).

2.13 Analysis of data

All data were expressed as means \pm S.E.M. Statistical comparisons between control and treated groups were performed with GraphPadInStat Software (version 5.00, GraphPad Software, Inc., San Diego, California, USA) using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

3.0 Structure elucidation

This section gives the structures of OA and MA as determined by ^1H and ^{13}C NMR techniques.

3.1 OA

The purity of the *S. aromaticum*-derived OA (see Figure 7) determined by ^1H NMR and ^{13}C NMR (1D and 2D) was approximately 98% and the percentage yields varied from 0.79% to 1.72%. The ^1H NMR and ^{13}C NMR (1D and 2D) given below was obtained.

^1H NMR CDCl_3 δ , 0.73, 0.75, 0.88, 0.89, 0.90, 0.96, 1.11 (each 3H, s), 2.84 (^1H , dd, $J = 10.36$), 3.22 (^1H , dd, $J = 4.56$), 5.26 (^1H , t, $J = 3.76$);

^{13}C NMR (CDCl_3) : δ 183.5, 143.8, 122.7, 79.2, 55.4, 47.8, 46.8, 46.1, 41.8, 41.2, 39.5, 38.9, 38.6, 38.3, 34.0, 33.3, 32.8, 32.7, 31.6, 28.4, 27.9, 27.4, 26.2, 23.8, 23.7, 23.1, 18.5, 17.4, 15.8, 15.5.

Spectra in Figure 8 are given as supporting information.

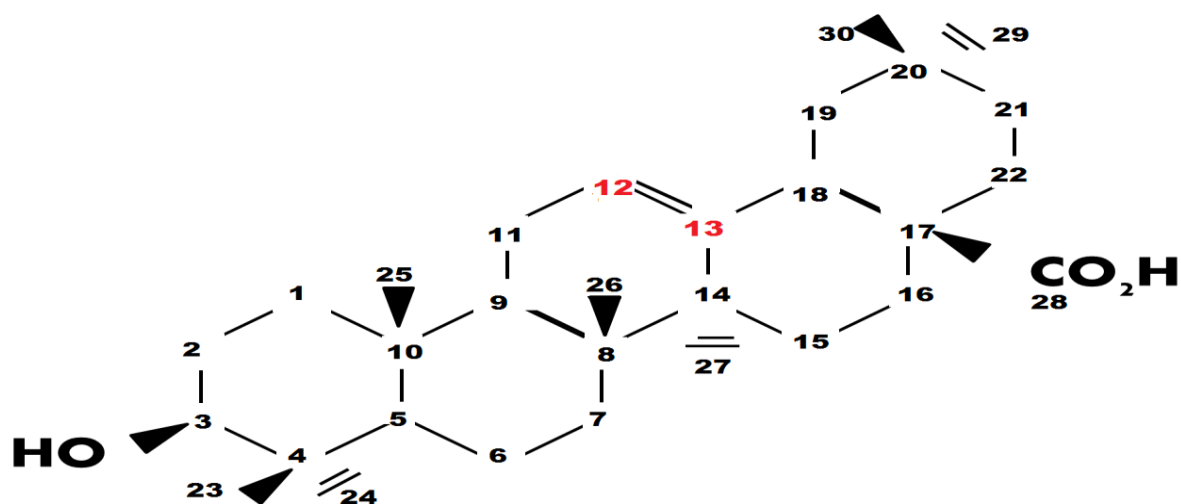


Figure 7. Chemical structure and IUPAC numbering of OA as determined through ¹H and ¹³C NMR spectroscopy

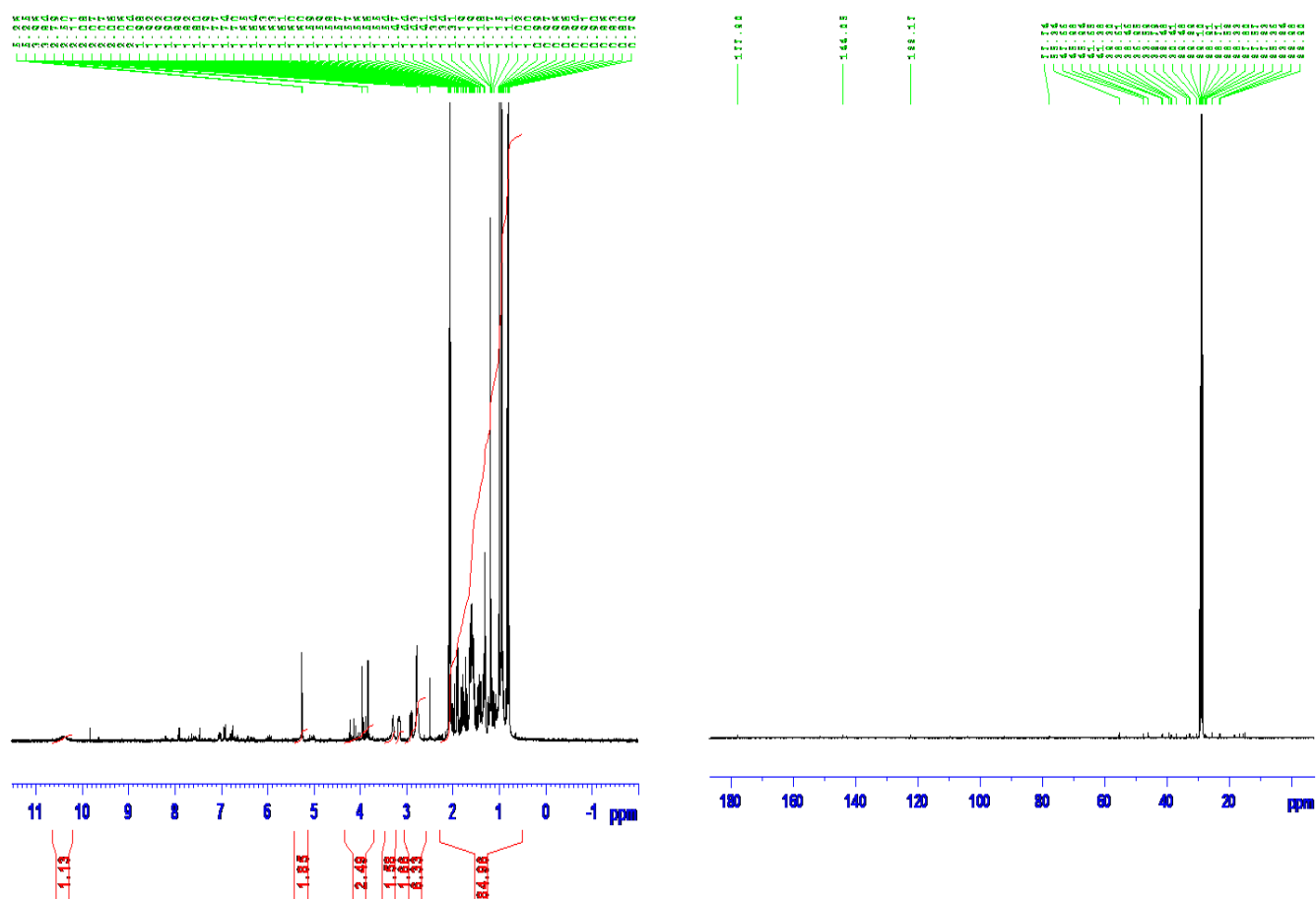


Figure 8. *Syzygium aromaticum*-derived OA ^1H (A) and ^{13}C NMR (B) spectroscopic data. Pure OA was obtained following recrystallization of EAS with methanol and elucidated using ^1H and ^{13}C NMR spectroscopy.

3.2 MA

The purity of the *S. aromaticum*-derived MA (see Figure 9) determined by ^1H and ^{13}C NMR (1D and 2D) was approximately 98% and the percentage yields varied from 0.02% to 0.03%. The ^1H and ^{13}C NMR (1D and 2D) given below was obtained.

^{13}C NMR: δ_{H} (^{13}C NMR (400 MHz, CD_3OD): 46.2 (C-1), 68.3 (C-2), 83.3 (C-3), 39.1 (C-4), 55.0 (C-5), 18.1 (C-6), 32.7 (C-7), 39.0 (C-8), C-9(47.4), C-10 (38.0), C-11 (23.2), C-12 (121.9), C-13 (143.7), C-14 (41.6), C-15 (27.4), C-16 (23.0), C-17 (46.2), C-18(41.0), C-19 (45.7), C-20 (30.4), C-21 (33.6), C-22 (32.3), C-23 (28.3), C-24 (16.6), C-25 (16.5), C-26 (16.4), C-27(23.2), C-28 (178.5), (C-29) 32.2, C-30 (23.2).

Spectra in Figure 10 are given as supporting information.

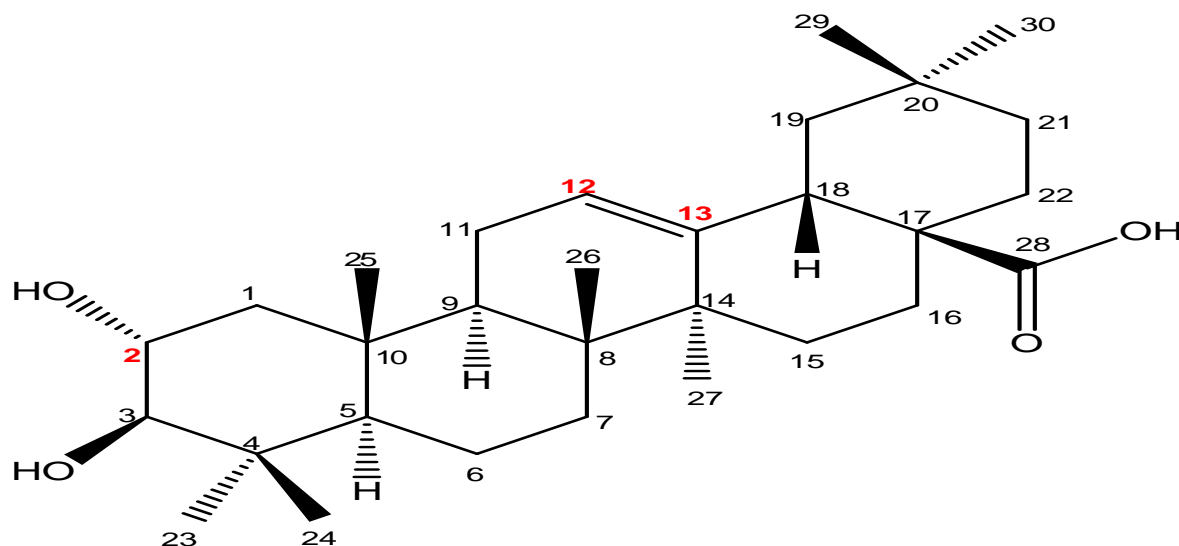


Figure 9. Chemical structure and IUPAC numbering of MA as determined through ^1H and ^{13}C NMR spectroscopy

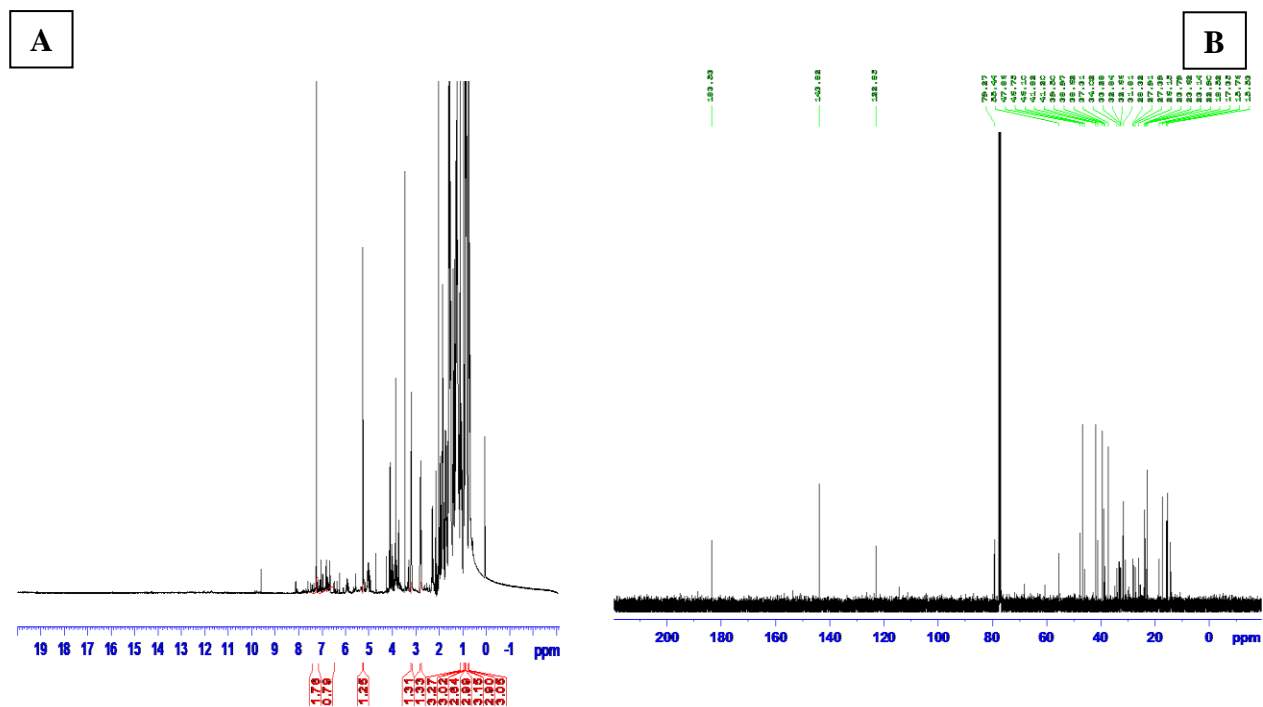


Figure 10. *Syzygium aromaticum*-derived MA ^1H (A) and ^{13}C NMR (B) spectroscopic data. Pure OA was obtained following recrystallisation of EAS with methanol and elucidated using ^1H and ^{13}C NMR spectroscopy.

Series I

Series I studies were carried out to investigate the effects of OA and MA on carbohydrate hydrolyzing enzymes and intestinal glucose transport *in vitro* using the enzyme-carbohydrate model system and the everted intestinal sacs technique, respectively.

3.3 Carbohydrate hydrolyzing enzyme activity

The effects of OA and MA on key intestinal carbohydrate hydrolyzing enzymes were performed using *in vitro* assays to assess the inhibitory activities of the triterpenes against these enzymes. The inhibitory activities of OA and MA on intestinal α -glucosidase/ α -amylase were studied using an α -glucosidase/ α -amylase starch model system while inhibitory effects on sucrose utilized the dextran sucrase- dextran sucrose reaction mixture. The IC₅₀ values of the triterpenes were calculated from plots of log concentration of inhibitor concentration versus percentage inhibition curves. Table 3 shows that OA and MA displayed inhibitory effects on the activity of sucrase, α -glucosidase and α -amylase as these triterpenes were found to possess IC₅₀ values comparable with those of the standard drug acarbose.

Table 3: IC₅₀ values of OA, MA and acarbose on the activities of sucrase, α -glucosidase and α -amylase *in vitro*. Values are presented as means \pm SEM (n=4 in each group).

IC ₅₀ (μ g/mL)			
	Sucrase	α - glucosidase	α -amylase
OA	59.74 \pm 1.76	55.45 \pm 1.75	62.11 \pm 1.80
MA	60.91 \pm 4.91	57.61 \pm 3.08	71.01 \pm 1.85
Acarbose	43.65 \pm 1.84	47.97 \pm 1.68	80.25 \pm 1.90

3.4 Glucose transport in the everted gut

The effects of OA and MA on intestinal d-glucose absorption (mucosal glucose transfer) and transport across the everted gut (serosa glucose uptake) across rat everted small intestinal sacs are shown in Figures 11 and 12.

The amount of glucose in the mucosal fluid was significantly ($p<0.05$) reduced throughout the 30-minute incubation period in the control everted intestinal sacs by comparison to the initial amount of glucose in the mucosa. This was accompanied by a simultaneous steady appearance of glucose in the serosa. Both OA and MA at a low concentration (0.82 mmol/L) had no significant effect on mucosal glucose disappearance and serosa glucose disappearance. However, OA and MA at concentrations ranging from 1.64 - 6.56 mmol/L significantly ($p<0.05$) suppressed mucosal glucose disappearance and serosa glucose appearance in a dose-independent manner. A similar trend was observed in the intestinal sacs treated with the standard drug phlorizin (see Figure 13).

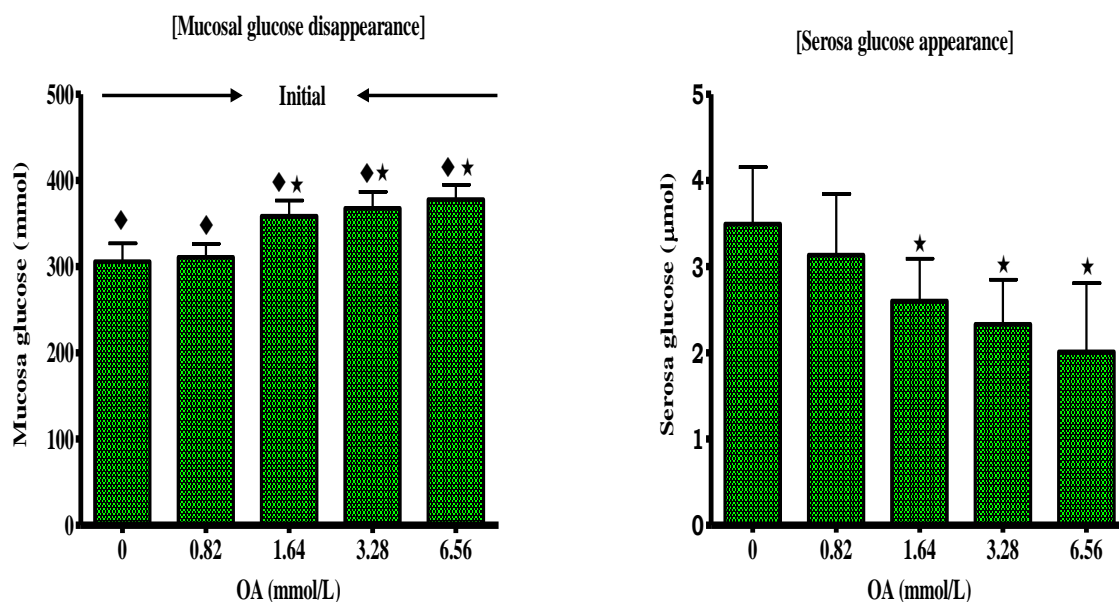


Figure 11. Effects of graded concentrations of OA (0.82 - 6.56 mmol/l) on mucosal glucose disappearance and serosa glucose appearance in rat everted intestinal sacs. The initial amount of glucose in the mucosal fluid was 500 μmol indicated by the horizontal line. Values are presented as means, and vertical bars indicate SEM of means ($n=6$ for each concentration). ◆= $p<0.05$ by comparison to the initial mucosal glucose concentration. ★ = $p < 0.05$ by comparison with control.

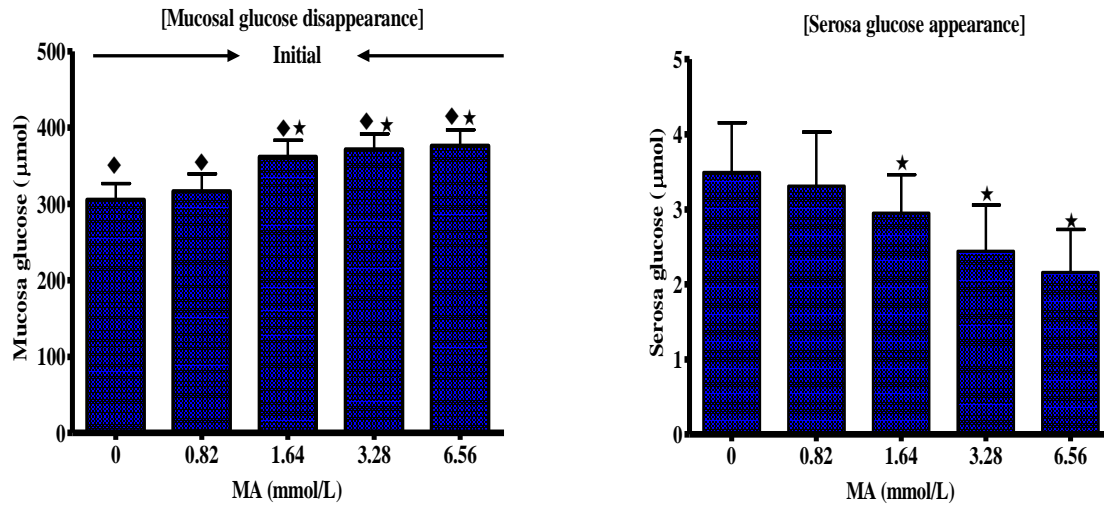


Figure 12. Effects of graded concentrations of MA (0.82 - 6.56 mmol/l) on mucosal glucose disappearance and serosa glucose appearance in rat everted intestinal sacs. The initial amount of glucose in the mucosal fluid was 500 μmol indicated by the horizontal line. Values are presented as means, and vertical bars indicate SEM of means ($n=6$ for each concentration). ◆= $p<0.05$ by comparison to the initial mucosal glucose concentration. ★ = $p < 0.05$ by comparison with control.

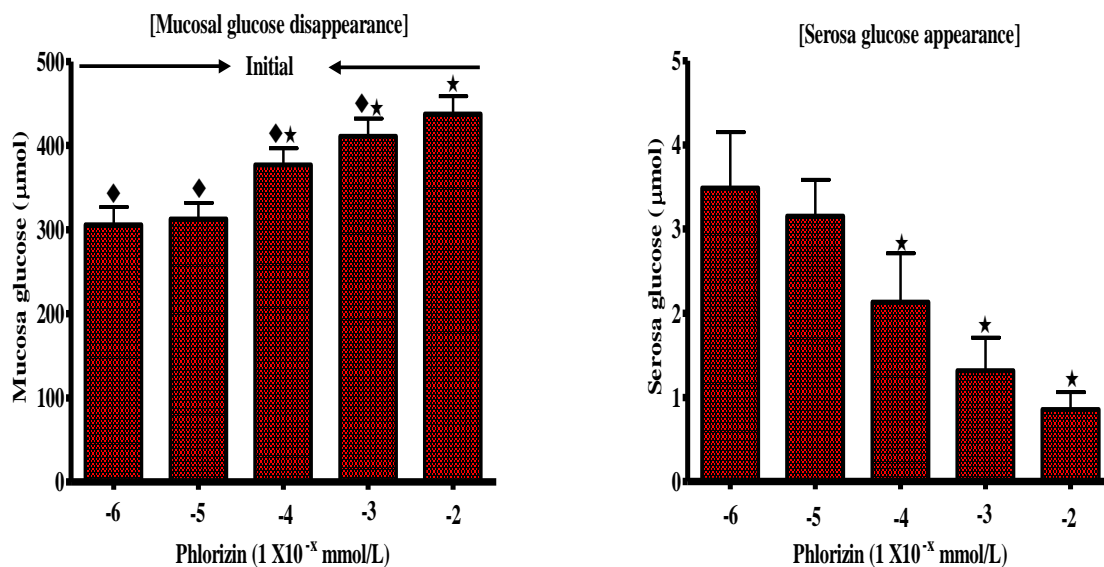


Figure 13. Effects of graded concentrations of phlorizin on mucosal glucose disappearance and serosa glucose appearance in rat everted intestinal sacs. The initial amount of glucose in the mucosal fluid was 500 μmol indicated by the horizontal line. Values are presented as means, and vertical bars indicate SEM of means ($n=6$ for each concentration). ♦ = $p < 0.05$ by comparison to the initial mucosal glucose concentration. ★ = $p < 0.05$ by comparison with control.

Series II

Series II studies were subdivided into influences on postprandial blood glucose as assessed by oral glucose tolerance (OGT) responses over two hours (acute) following loading with various carbohydrates and sub-chronic studies carried out over 5-weeks where the rats were kept on standard rat chow.

3.5 Acute effects

3.5.1 OGT responses

OGT responses to OA and MA (80 mg/kg) were monitored in separate groups of non-diabetic and STZ-induced diabetic rats following glucose (0.86 g/kg, p.o.), sucrose (1.72 g/kg, p.o.) and starch (0.086 g/kg, p.o.) loading after an 18-h fast.

Blood glucose concentrations were significantly ($p < 0.05$) higher in untreated STZ-induced diabetic rats loaded with glucose, sucrose and starch at time 0 during the OGT response tests compared to the control non-diabetic rats. Following the loading of glucose, sucrose and starch, the blood glucose concentration of control non-diabetic and untreated STZ-induced diabetic rats increased for 60 minutes before declining to blood glucose concentrations comparable with those at time 0 by the end of the 2 h experimental period. Administration of OA and MA had no significant effects on the blood glucose concentration in glucose, sucrose and starch-loaded non-diabetic rats. This was further shown by a lack of significant changes in the AUC values in the OA and MA-treated rats by comparison with the non-diabetic control. However, the blood glucose concentrations in OA and MA-treated STZ-diabetic rats persisted declining below the preloading values by the end of the 2 h experimental period. This was accompanied by significant ($p < 0.05$) reductions in the AUC values of the STZ-diabetic OA and MA-treated rats loaded with the various carbohydrates (see Figures 14, 15 and 16). A similar response pattern of results was observed in glucose-loaded STZ-diabetic rats treated with phlorizin (100 mg/kg, p.o.) and sucrose or starch-loaded STZ-diabetic rats treated with acarbose (100 mg/kg, p.o.) (see Figures 17, 18 and 19).

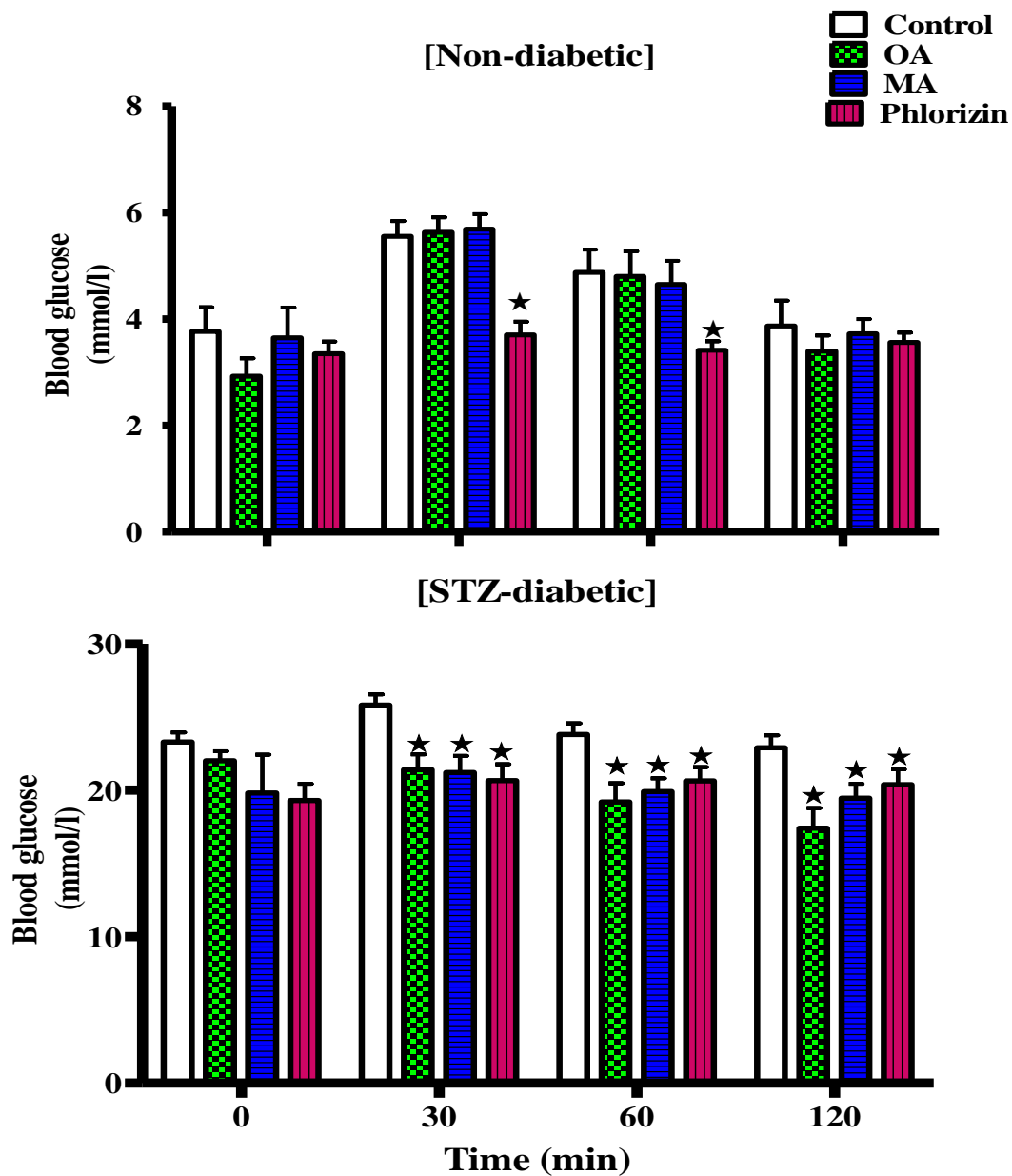


Figure 14. Comparison of OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug phlorizin following a glucose load. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ◆=p<0.05 by comparison to the non-diabetic control. ★=p<0.05 by comparison to the respective control.

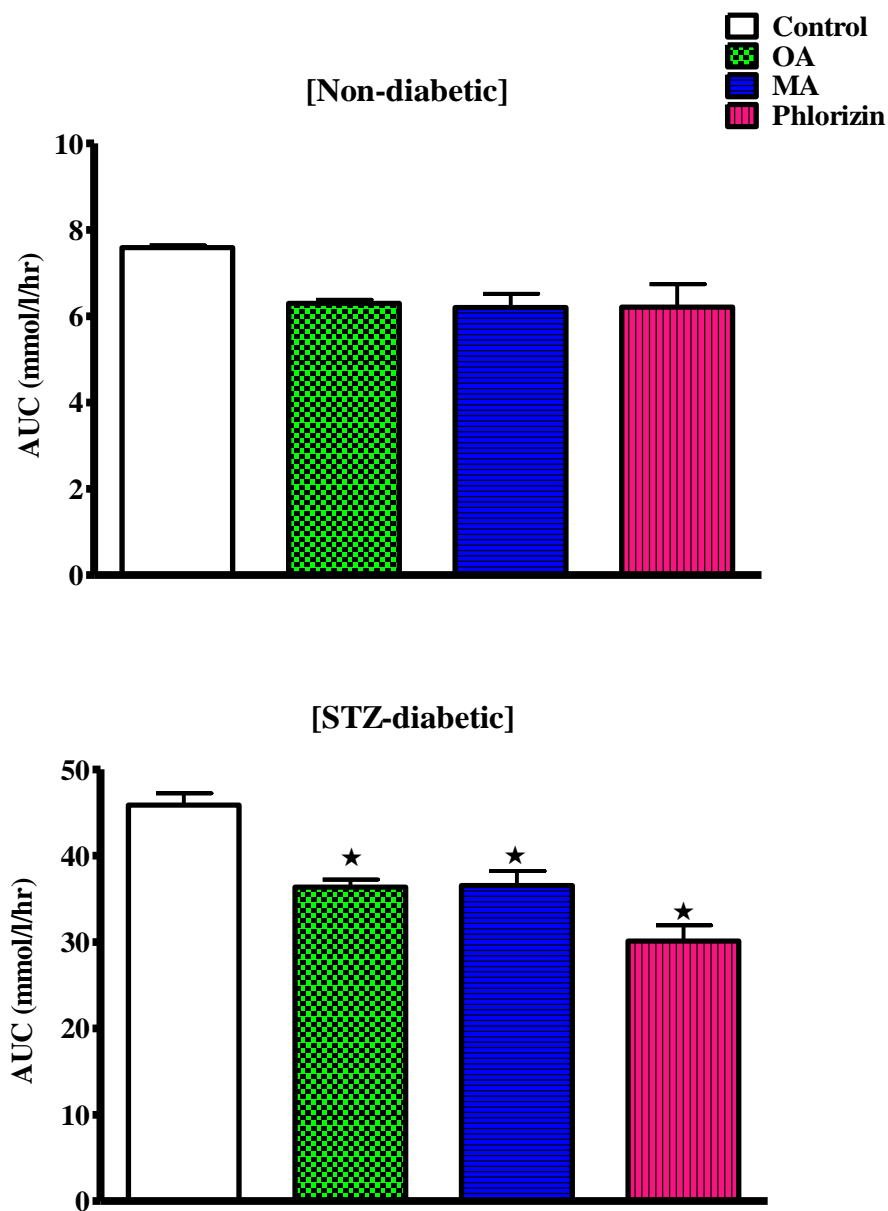


Figure 15. Comparison of the AUC_{glucose} values obtained from OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug phlorizin following a glucose load. The AUC_{glucose} was calculated by incremental method. Values are presented as means, and vertical bars indicate SEM ($n = 6$ rats in each group). ★ $p < 0.05$ by comparison with control animals.

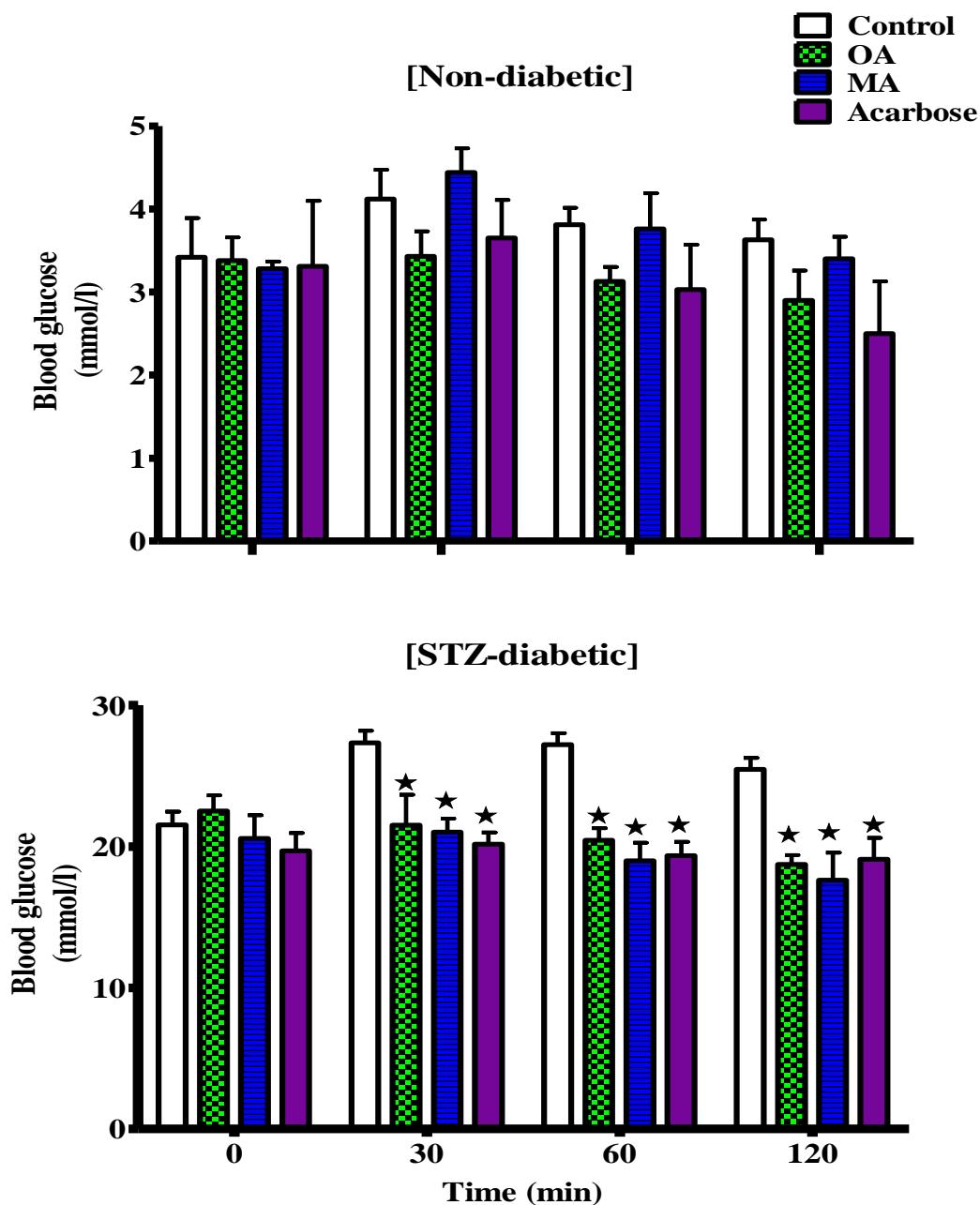


Figure 16. Comparison of OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug phlorizin following a sucrose load. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the respective control.

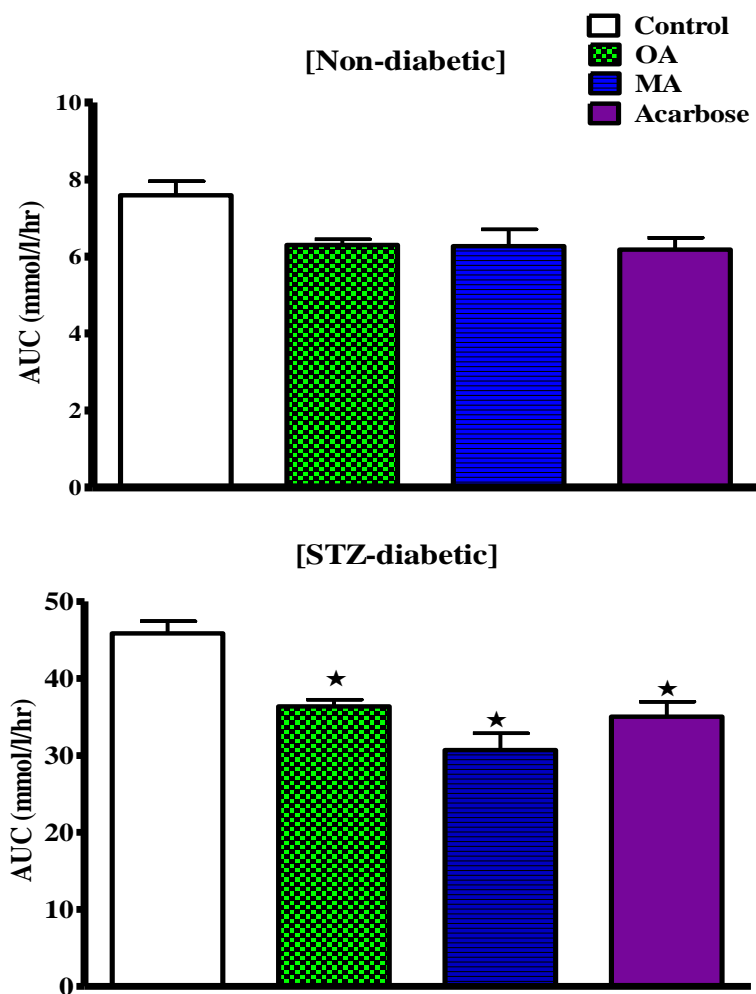


Figure 17. Comparison of the AUC_{glucose} values obtained from OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug acarbose following a sucrose load. The AUC_{glucose} was calculated by incremental method. Values are presented as means, and vertical bars indicate SEM (n = 6 rats in each group). ★p < 0.05 by comparison with control animals.

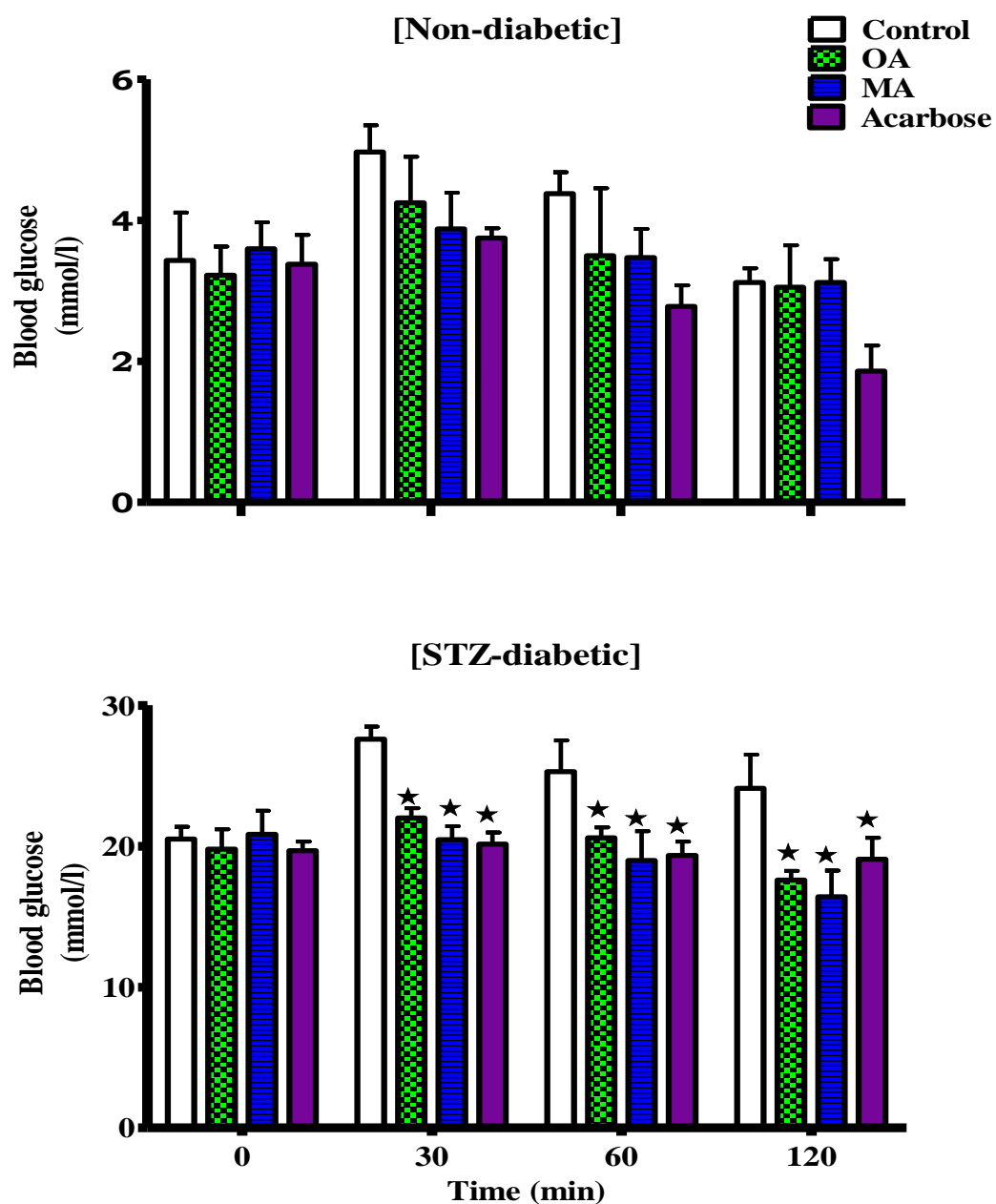


Figure 18. Comparison of OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug phlorizin following a starch load. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the respective control.

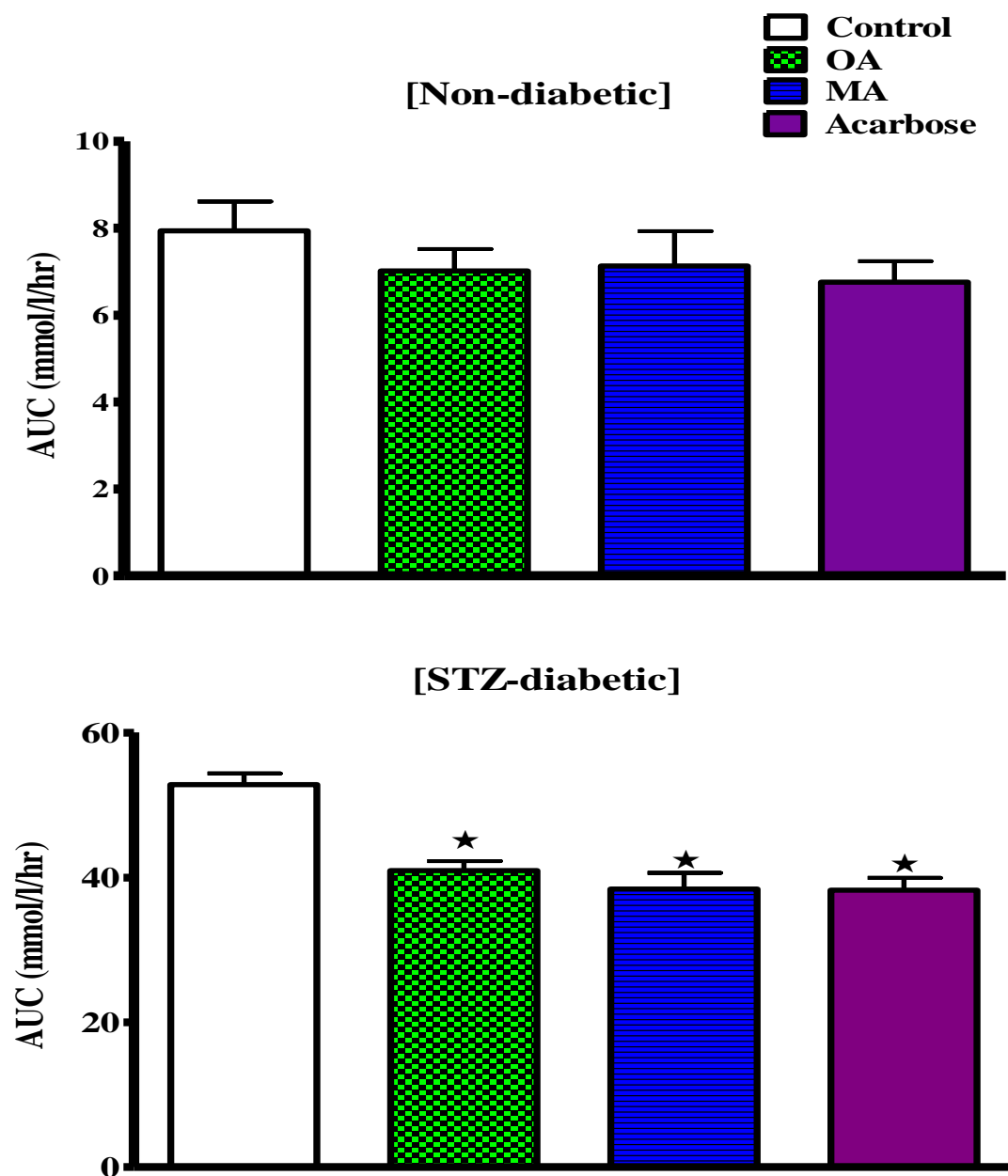


Figure 19. Comparison of the AUC_{glucose} values obtained from OGT responses to OA and MA in non-diabetic and STZ-diabetic rats with control non-diabetic, untreated STZ-diabetic and those treated with the standard drug acarbose following a starch load. The AUC_{glucose} was calculated by incremental method. Values are presented as means, and vertical bars indicate SEM ($n = 6$ rats in each group). ★ $p < 0.05$ by comparison with control animals.

3.6 Sub-chronic effects

3.6.1 Physicochemical changes

3.6.1.1 Body weight changes, food and water intake

Non-diabetic control rats progressively gained weight while STZ-induced diabetic rats exhibited severe weight loss throughout the experimental period. Treatment of the STZ-diabetic rats with OA and MA (80 mg/kg, p.o.) significantly ($p<0.05$) increased the body weights of STZ-induced diabetic rats from the third week to the fifth week by comparison with the STZ-diabetic control rats. A similar trend was observed in those rats treated with the standard drugs metformin (500 mg/kg, p.o.) and insulin (175 μ g/kg, p.o.).

The daily food and water intake of STZ-induced diabetic control rats significantly ($p<0.05$) increased by comparison to the non-diabetic control over the 5-week period. On the contrary, STZ-diabetic rats treated with OA and MA (80 mg/kg, p.o.) showed significant ($p<0.05$) decreases in food intake by comparison to the STZ-induced diabetic controls from the third week onwards. A similar response pattern of results was observed in those rats treated with the standard drugs metformin (500 mg/kg, p.o.) and insulin (175 μ g/kg, p.o.) (see Table 6).

Table 4: Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on body weight changes, food and water intake with control non-diabetic, untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin (n=6 in each group). Values are presented as means \pm SEM.

Parameter	Group	Week 1	Week 2	Week 3	Week 4	Week 5
Food intake (g/100 g)	Non-diabetic	10.6 \pm 1.2	10.9 \pm 1.4	10.5 \pm 1.0	10.7 \pm 1.2	11.0 \pm 1.1
	STZ-diabetic	15.4 \pm 1.0 [♦]	16.0 \pm 0.5 [♦]	16.0 \pm 0.4 [♦]	16.0 \pm 0.5 [♦]	16.0 \pm 0.5 [♦]
	STZ-OA	14.1 \pm 1.8 [♦]	13.9 \pm 2.2 [♦]	12.4 \pm 1.8*	11.3 \pm 1.6*	11.2 \pm 1.4*
	STZ-MA	14.0 \pm 1.4 [♦]	14.1 \pm 1.6 [♦]	12.6 \pm 2.0*	11.4 \pm 1.8*	11.2 \pm 1.5*
	STZ-Met	13.5 \pm 1.6 [♦]	13.0 \pm 1.8 ^{♦*}	12.1 \pm 1.6*	12 \pm 1.2*	11.5 \pm 1.8*
	STZ-Ins	13.8 \pm 1.2 [♦]	13.2 \pm 2.0 ^{♦*}	12.3 \pm 1.8*	11.0 \pm 1.4*	11.0 \pm 1.6*
Water intake (ml/100 g)	Non-diabetic	14.0 \pm 1.0	12.0 \pm 1.5	12.0 \pm 2.0	15.2 \pm 1.4	14.0 \pm 1.9
	STZ-diabetic	40.0 \pm 2.5 [♦]	40.0 \pm 3.0 [♦]	48.0 \pm 2.0 [♦]	48.0 \pm 3.0 [♦]	48.0 \pm 3.4 [♦]
	STZ-OA	36.0 \pm 1.7 [♦]	36.0 \pm 2.4 [♦]	35.0 \pm 1.4 ^{♦*}	34.0 \pm 1.9 ^{♦*}	32.0 \pm 1.6 ^{♦*}
	STZ-MA	38.0 \pm 2.1 [♦]	36.0 \pm 3.0 [♦]	36.0 \pm 1.9 ^{♦*}	34.0 \pm 2.7 ^{♦*}	32.0 \pm 1.9 ^{♦*}
	STZ-Met	36.0 \pm 1.9 [♦]	34.0 \pm 1.8 ^{♦*}	32.0 \pm 3.1 ^{♦*}	32.0 \pm 2.5 ^{♦*}	30.0 \pm 2.0 ^{♦*}
	STZ-Ins	36.0 \pm 2.0 [♦]	34.0 \pm 1.7 ^{♦*}	32.0 \pm 2.0 ^{♦*}	30.0 \pm 3.2 ^{♦*}	28.0 \pm 2.3 ^{♦*}
% b.wt change /week	Non-diabetic	2.8 \pm 0.7	4.8 \pm 0.9	6.0 \pm 0.5	8.4 \pm 0.6	9.6 \pm 0.8
	STZ-diabetic	1.0 \pm 0.5 [♦]	-1.4 \pm 0.2 [♦]	-2.4 \pm 0.5 [♦]	-4.0 \pm 0.3 [♦]	-6.4 \pm 0.7 [♦]
	STZ-OA	1.1 \pm 0.3 [♦]	1.0 \pm 0.4 ^{♦*}	1.9 \pm 5.7 ^{♦*}	3.2 \pm 0.5 ^{♦*}	3.4 \pm 0.6 ^{♦*}
	STZ-MA	1.2 \pm 0.4 [♦]	1.0 \pm 0.3 [♦]	1.6 \pm 0.5 ^{♦*}	1.6 \pm 0.2 ^{♦*}	1.7 \pm 0.7 ^{♦*}
	STZ-Met	1.0 \pm 0.3 [♦]	1.4 \pm 0.4 [♦]	2.4 \pm 0.1 ^{♦*}	2.6 \pm 0.8 ^{♦*}	4.2 \pm 0.2 ^{♦*}
	STZ-Ins	1.6 \pm 0.7 [♦]	1.6 \pm 0.4 [♦]	2.4 \pm 0.7 ^{♦*}	3.2 \pm 0.4 ^{♦*}	3.2 \pm 0.3 ^{♦*}

♦=p<0.05 by comparison to the non-diabetic control. *=p<0.05 by comparison to the STZ-diabetic control.

3.6.1.2 Blood glucose concentrations

Figure 20 shows the sub-chronic effects of treatment of STZ-diabetic rats with OA and MA (80 mg/kg, p.o.) on blood glucose concentrations measured throughout the 5-week experimental period. Untreated STZ-diabetic rats showed significantly ($p<0.05$) increased blood glucose concentrations by comparison with non-diabetic rats for the duration of the 5-week experimental period. On the other hand, STZ-diabetic rats treated with OA and MA (80 mg/kg, p.o.) showed significant ($p<0.05$) reductions in blood glucose concentrations from the first week by comparison to the STZ-induced diabetic control rats. These concentrations gradually decreased throughout the experimental period to levels comparable with those of the non-diabetic control animals by the fifth week of the study. STZ-diabetic rats treated with insulin (175 $\mu\text{g/kg}$, sc) or metformin (500 mg/kg) showed a similar response pattern of results (see Figure 20).

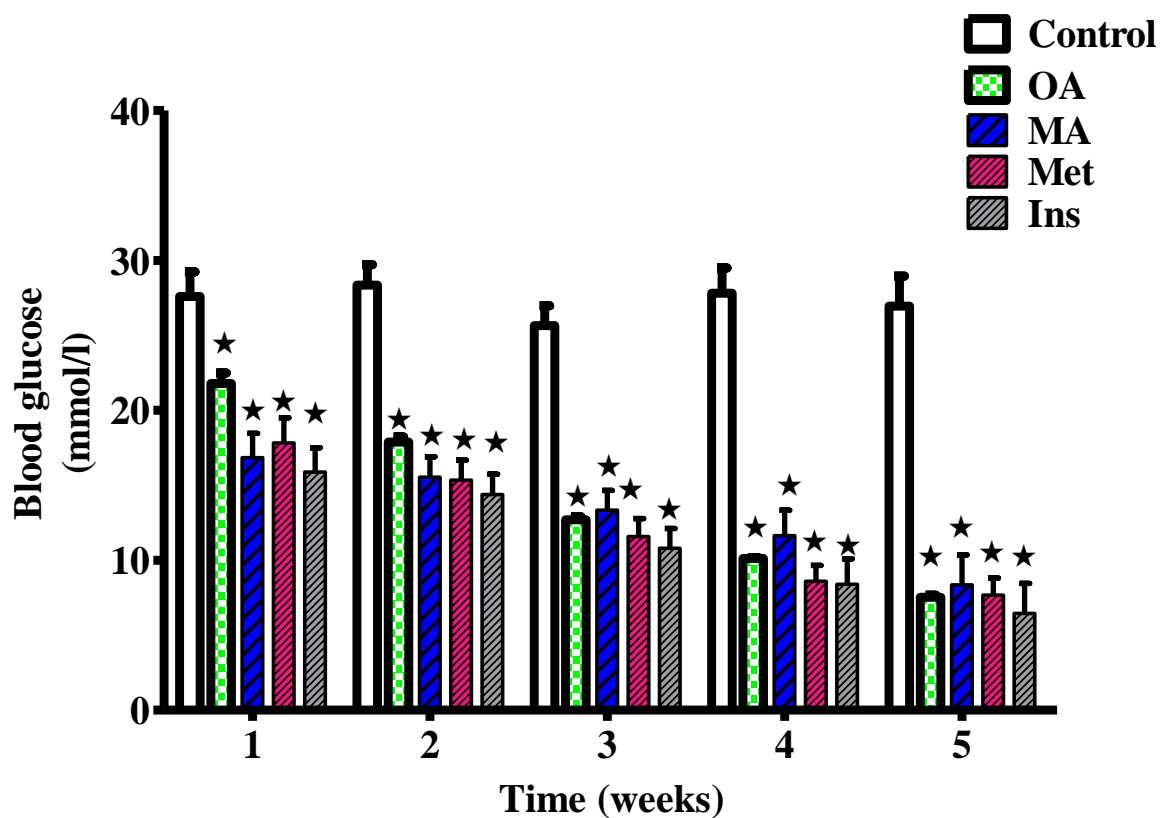


Figure 20. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on blood glucose concentration with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). ♦ = $p < 0.05$ by comparison to the non-diabetic control. ★ = $p < 0.05$ by comparison to the STZ-induced diabetic control.

3.7 Intestinal glycogen concentrations

To establish the effects of OA and MA on intestinal glycogen synthesis, intestinal glycogen concentrations were measured in separate groups of non-diabetic control, untreated STZ-diabetic and STZ-diabetic treated rats following the 5-week study.

The small intestine glycogen concentrations of STZ-induced diabetic rats were significantly ($p<0.05$) decreased by comparison with those of the non-diabetic rats after the 5-week experimental period. On the other hand, treatment of STZ-induced diabetic rats with OA and MA significantly ($p<0.05$) increased the intestinal glycogen concentrations. Similarly, rats treated with the standard anti-diabetic drugs, metformin and insulin showed comparable significant ($p<0.05$) increases in intestinal glycogen concentrations after the 5-week period (see Figure 21).

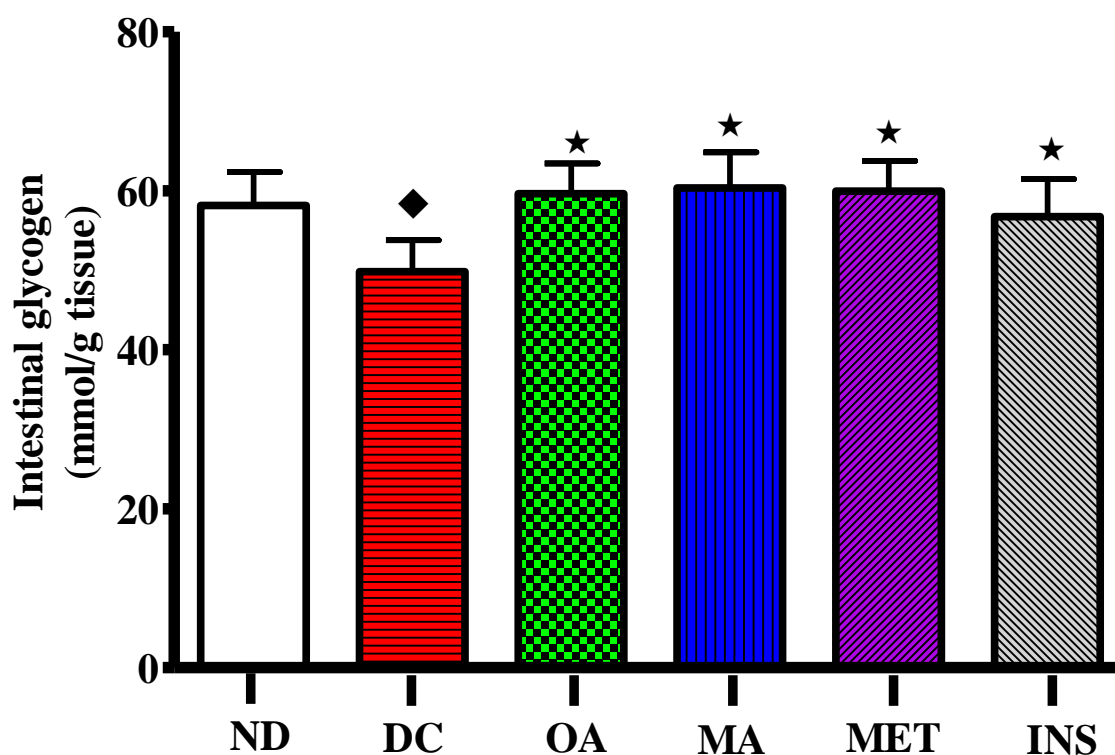


Figure 21. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine glycogen concentration with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).◆=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control.

3.8 Hormone concentrations

In order to determine the effects of OA and MA on insulin and ghrelin secretion, plasma insulin and ghrelin concentrations of STZ-induced diabetic rats treated with OA and MA every third day for 5 weeks were determined using ELISA. The plasma insulin concentrations of STZ-induced diabetic rats which were significantly ($p<0.05$) low by comparison with those of the non-diabetic rats were not altered by any of the treatments (see Figure 22). On the other hand the plasma ghrelin concentrations of STZ-induced diabetic rats were significantly ($p<0.05$) increased by comparison with those of the non-diabetic rats after the 5-week experimental period. However, treatment of STZ-induced diabetic rats with OA and MA significantly ($p<0.05$) decreased the plasma ghrelin concentrations. Similarly, rats treated with the standard anti-diabetic drugs, metformin and insulin showed significant ($p<0.05$) decreases in plasma ghrelin concentrations after the 5-week period (see Figure 23).

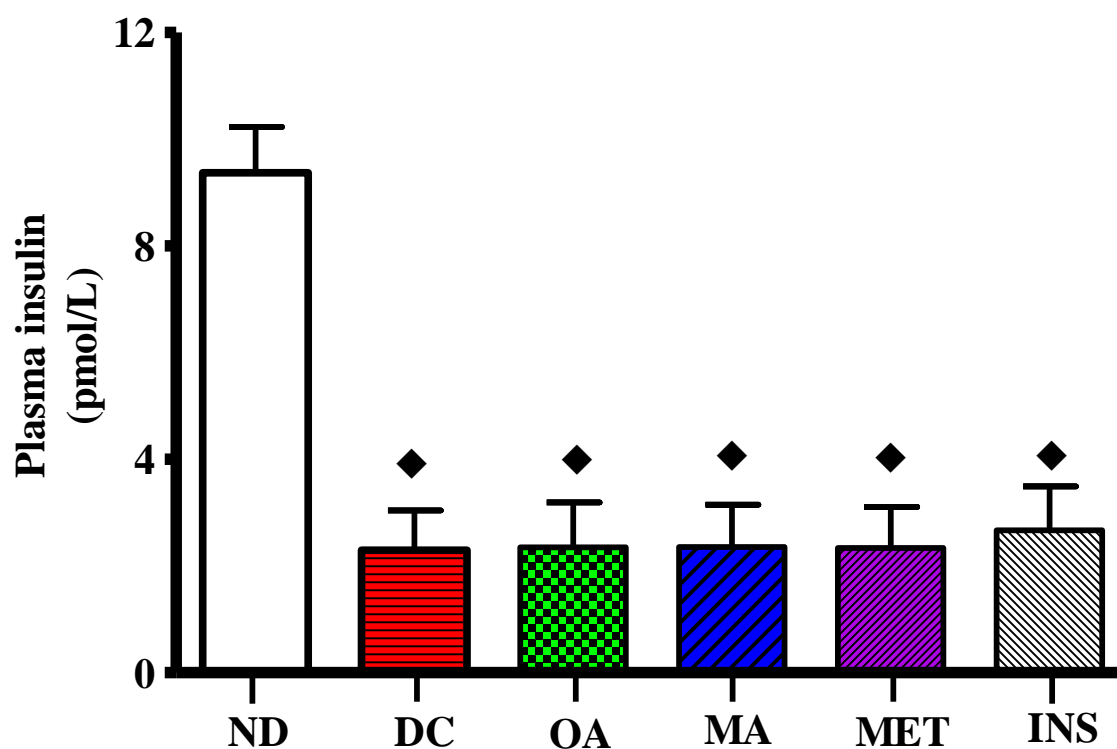


Figure 22. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on plasma insulin concentrations with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). $\blacklozenge = p < 0.05$ by comparison to the non-diabetic control. $\star = p < 0.05$ by comparison to the STZ-induced diabetic control.

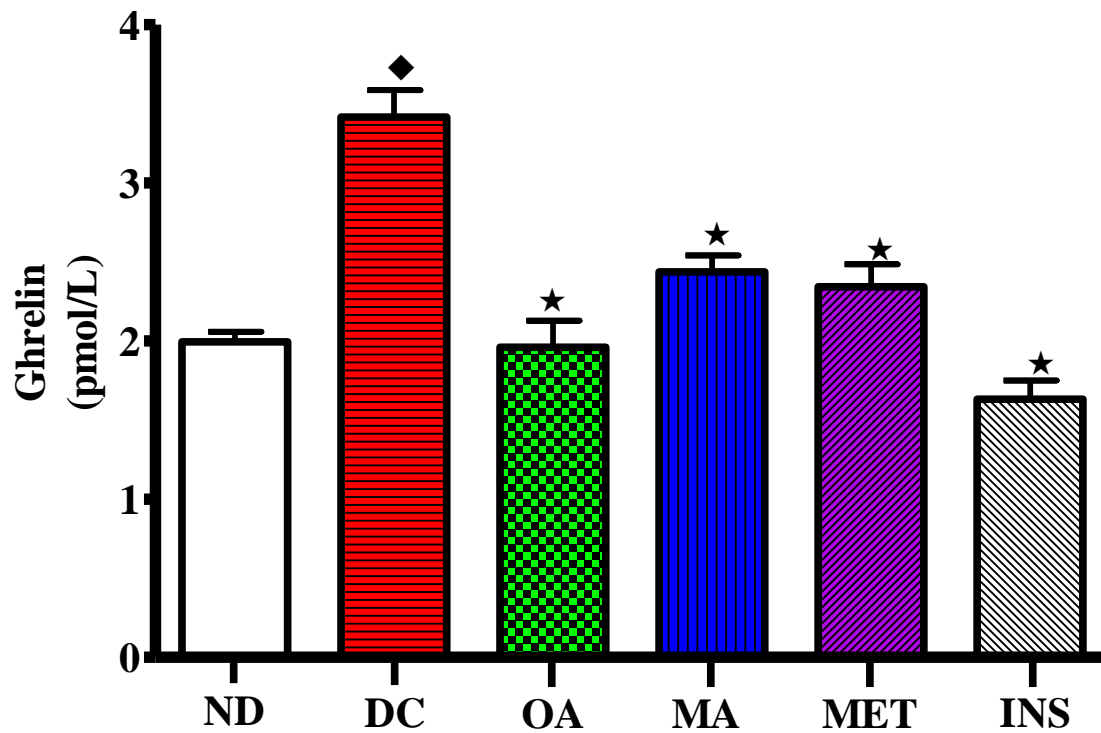


Figure 23. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on plasma ghrelin concentrations with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). ♦= $p < 0.05$ by comparison to the non-diabetic control. ★= $p < 0.05$ by comparison to the STZ-induced diabetic control

3.9 Protein expression

In order to determine the effects of OA and MA on glycogen synthase, SGLT1, GLUT2, α -glucosidase, α -amylase and ghrelin expression, Western blot analysis of these proteins was conducted in various sections of the gastrointestinal tract in separate groups of non-diabetic, untreated STZ-diabetic rats and STZ-diabetic rats treated with OA and MA following the 5 week study.

3.9.1 Glycogen synthase

The expression of glycogen synthase in the small intestine of untreated STZ-induced diabetic rats was significantly ($p<0.05$) decreased by comparison with those of the non-diabetic control rats after the 5-week experimental period. Interestingly, treatment of STZ-induced diabetic rats with OA and MA significantly ($p<0.05$) increased the expression of glycogen synthase in the small intestine. Similarly, rats treated with the standard anti-diabetic drugs, metformin and insulin showed significant ($p<0.05$) increases in the intestinal expression of glycogen synthase after the 5-week period (see Figure 24).

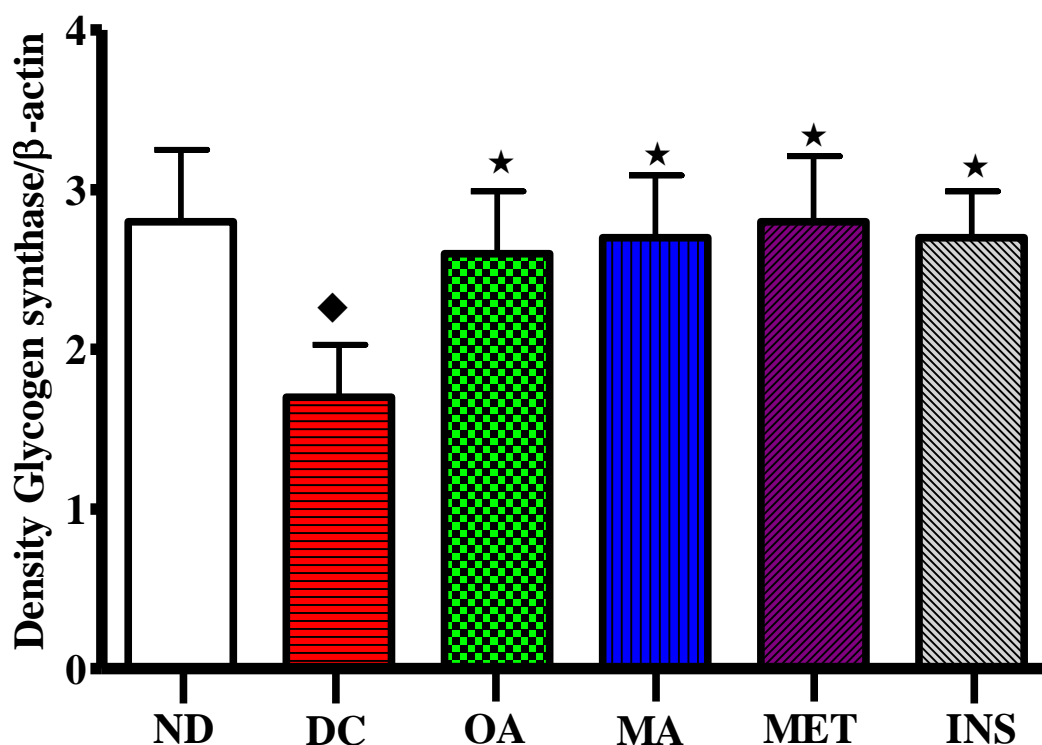


Figure 24. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine glycogen synthase expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). \blacklozenge = $p < 0.05$ by comparison to the non-diabetic control. \star = $p < 0.05$ by comparison to the STZ-induced diabetic control.

3.9.2 α -amylase and α -glucosidase

The small intestine α -amylase and α -glucosidase expression of untreated STZ-induced diabetic rats were significantly ($p < 0.05$) increased by comparison with those of the non-diabetic control rats after the 5-week experimental period. However, treatment of STZ-induced diabetic rats with OA and MA significantly ($p < 0.05$) decreased the intestinal expression these carbohydrate hydrolyzing enzymes. STZ-diabetic rats treated with standard drugs metformin and insulin showed a similar response pattern of results (see Figure 25 and Figure 26).

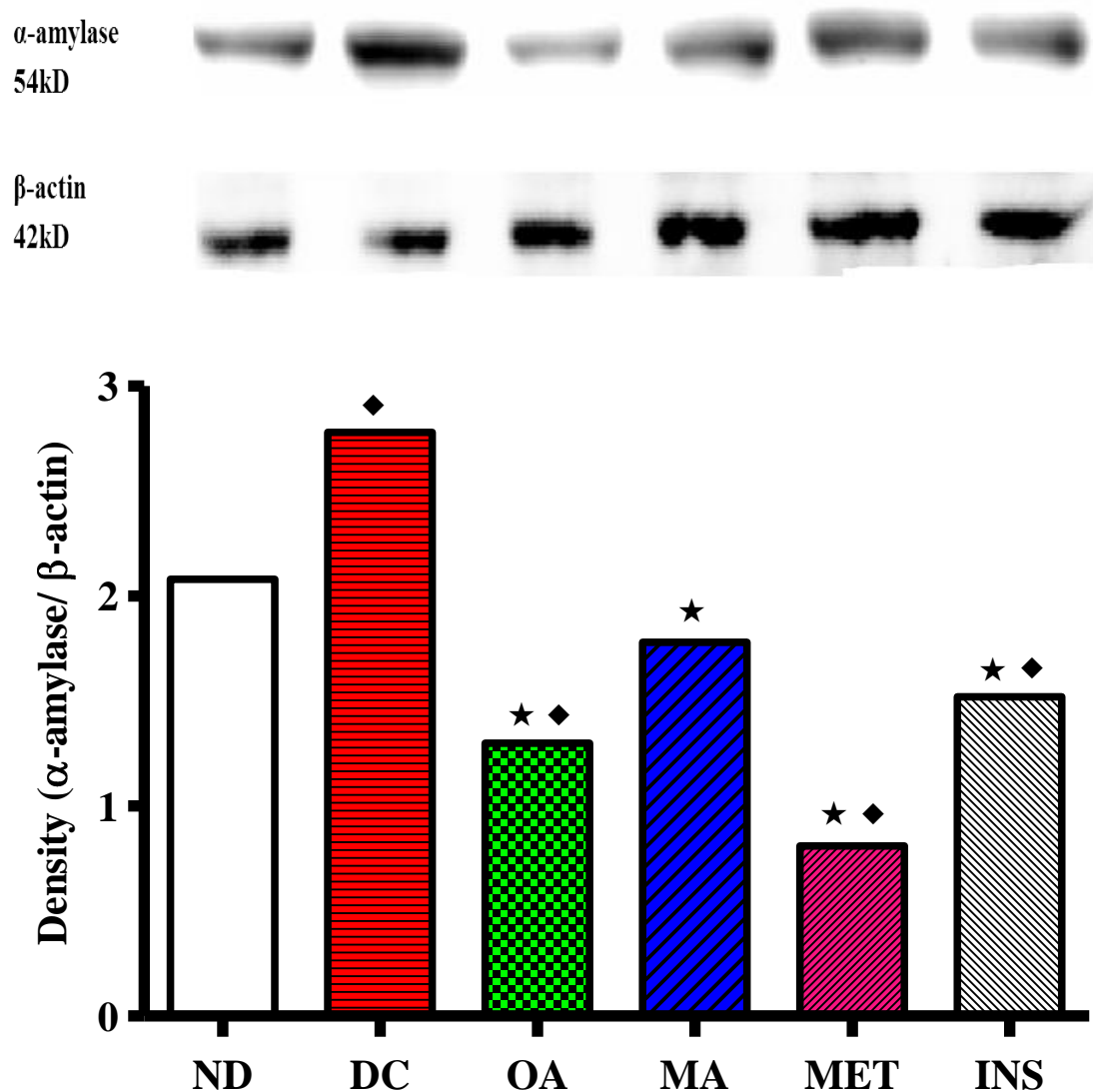


Figure 25. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine α -amylase expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ◆ = p < 0.05 by comparison to the non-diabetic control. ★ = p < 0.05 by comparison to the STZ-induced diabetic control.

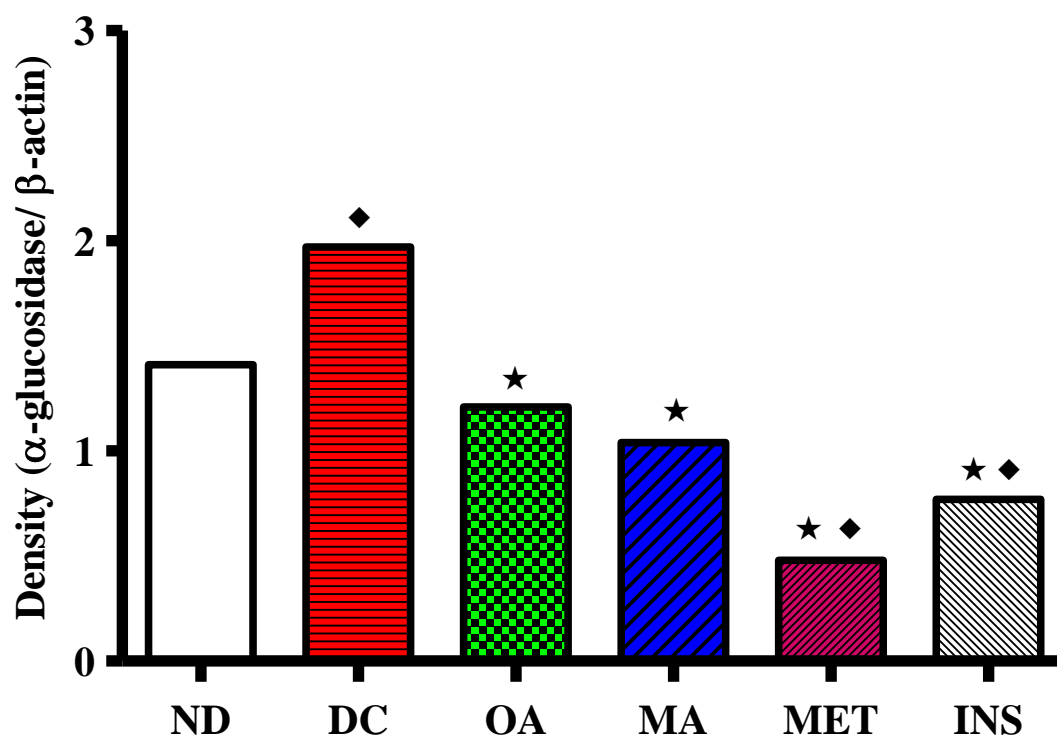
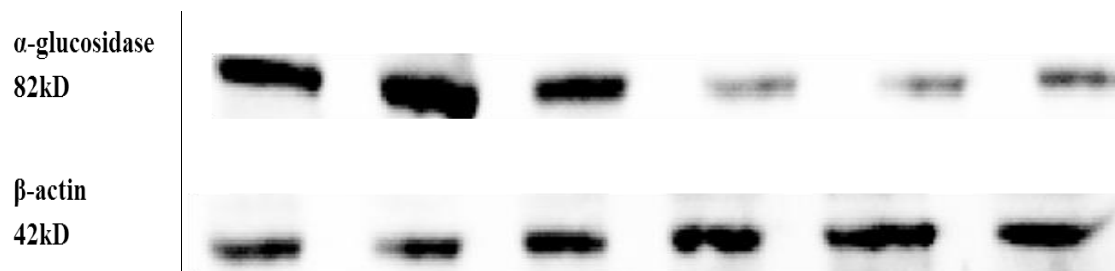


Figure 26. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine α -glucosidase expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). ♦= $p < 0.05$ by comparison to the non-diabetic control. ★= $p < 0.05$ by comparison to the STZ-induced diabetic control.

3.9.3 SGLT1 and GLUT2

The small intestine expression of SGLT1 and GLUT2 in STZ-induced diabetic rats were significantly ($p < 0.05$) increased by comparison with those of the non-diabetic rats after the 5-week experimental period. On the other hand, treatment of STZ-induced diabetic rats with OA and MA significantly ($p < 0.05$) decreased the intestinal expressions of SGLT1 and GLUT2. STZ-diabetic rats treated with standard drugs metformin and insulin showed a similar response pattern of results (see Figure 27 and Figure 28).

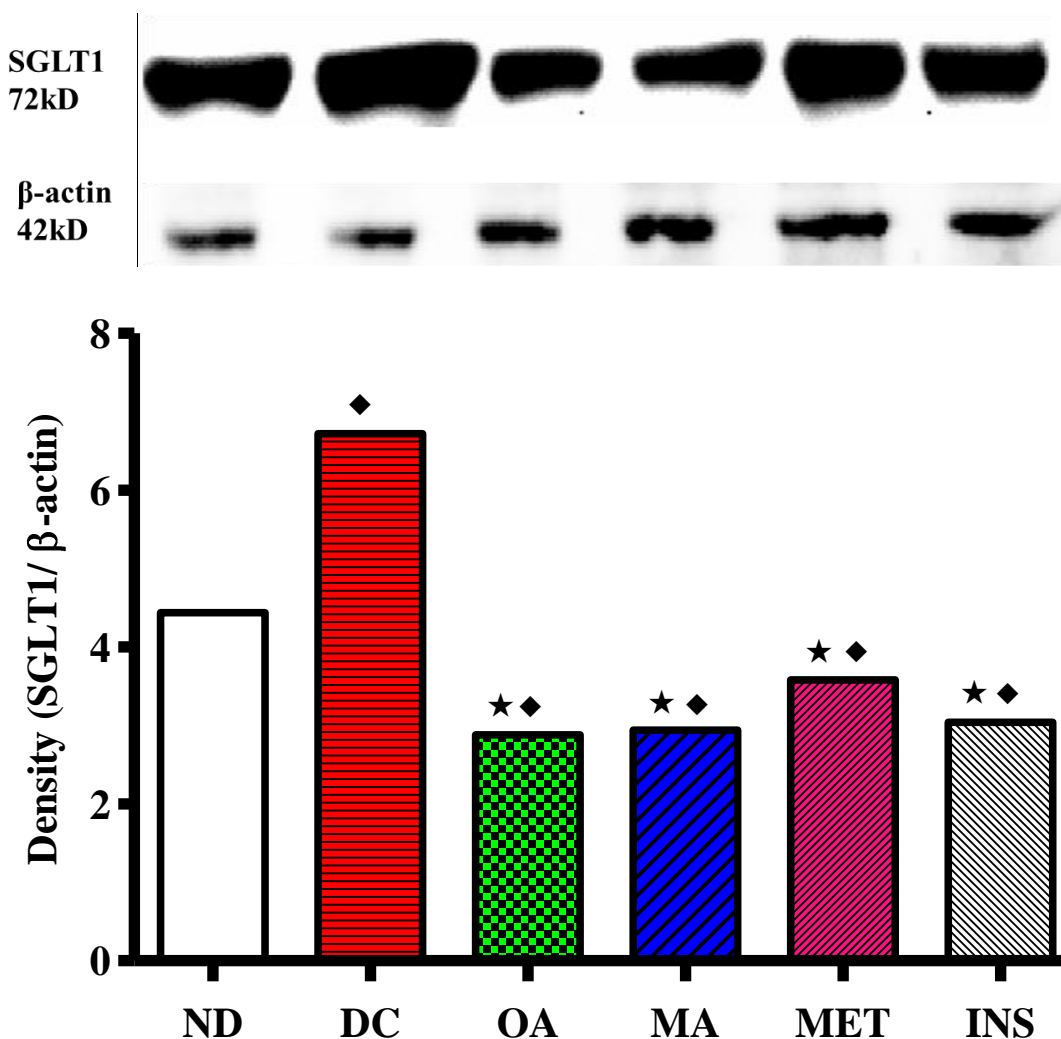


Figure 27. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine SGLT1 expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). \blacklozenge =p<0.05 by comparison to the non-diabetic control. \star =p<0.05 by comparison to the STZ-induced diabetic control.

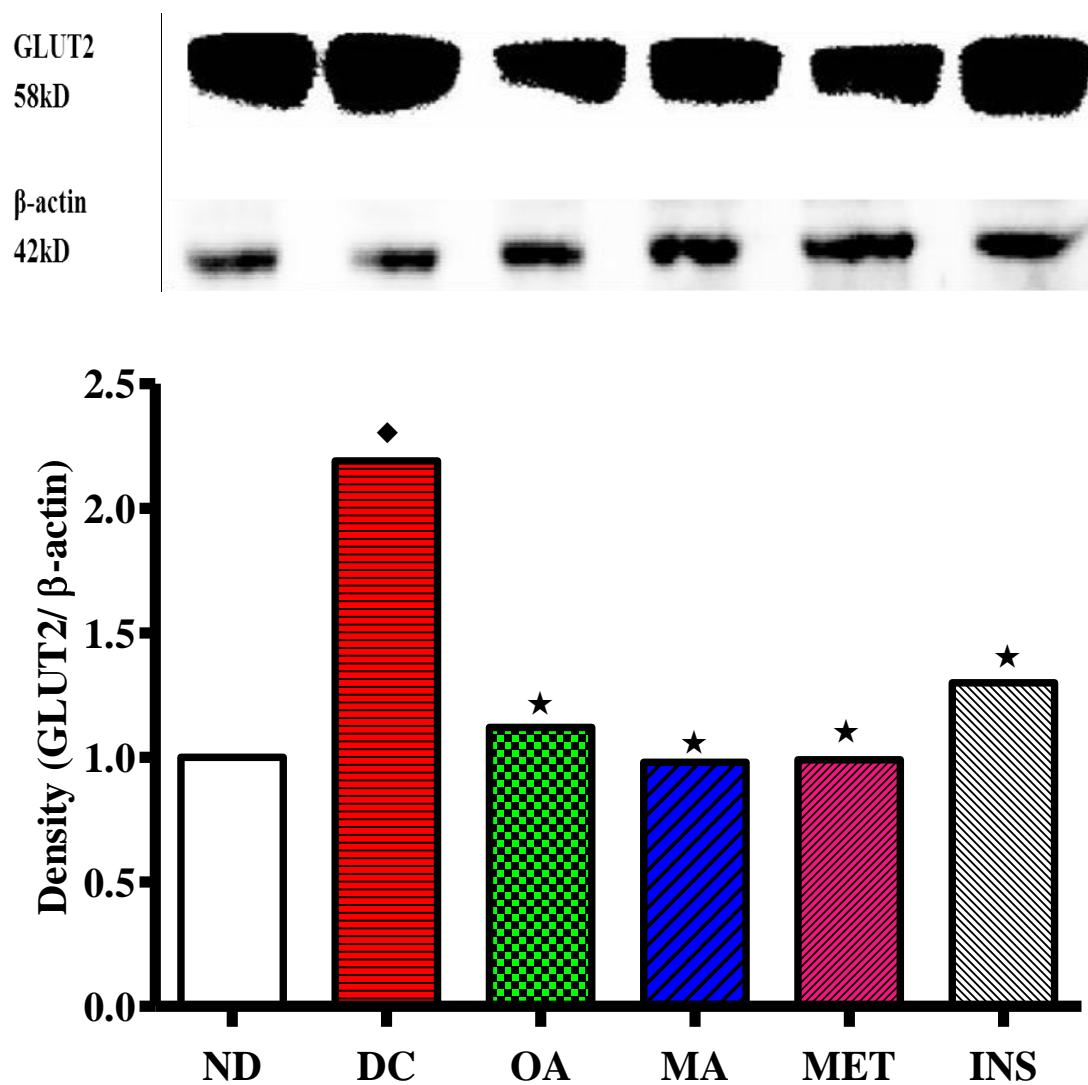


Figure 28. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine GLUT2 expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).◆=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control.

3.9.4 Ghrelin

The expression of ghrelin in the gastric fundus, small and large intestine of STZ-induced diabetic rats was significantly ($p<0.05$) increased by comparison with those of the non-diabetic rats after the 5-week experimental period. On the other hand, treatment of STZ-induced diabetic rats with OA and MA significantly ($p<0.05$) decreased the expression of ghrelin in the gastric fundus as well as in the small and large intestine. Additionally, STZ-diabetic rats treated with standard drugs metformin and insulin showed a similar response pattern of results (see Figure 29, Figure 30 and Figure 31).

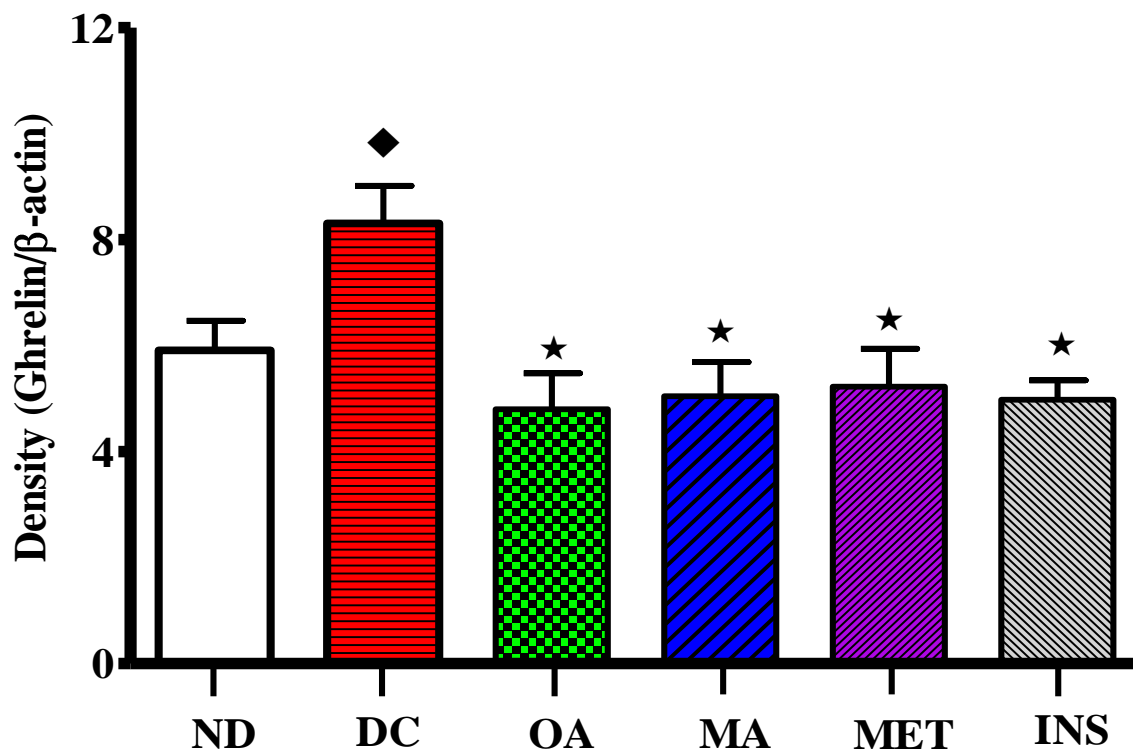
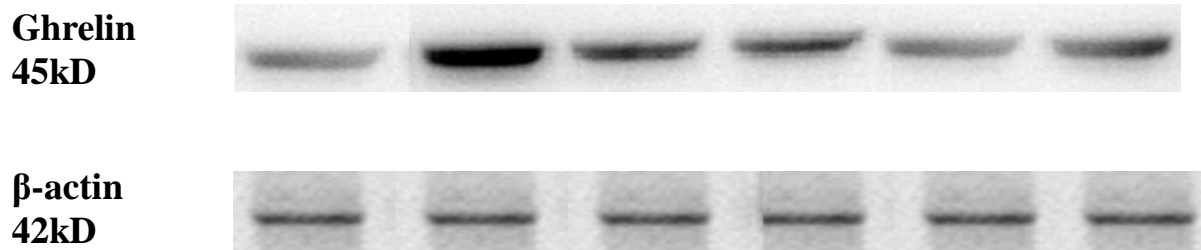


Figure 29. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on gastric fundus ghrelin expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). \blacklozenge = $p < 0.05$ by comparison to the non-diabetic control. \star = $p < 0.05$ by comparison to the STZ-induced diabetic control.

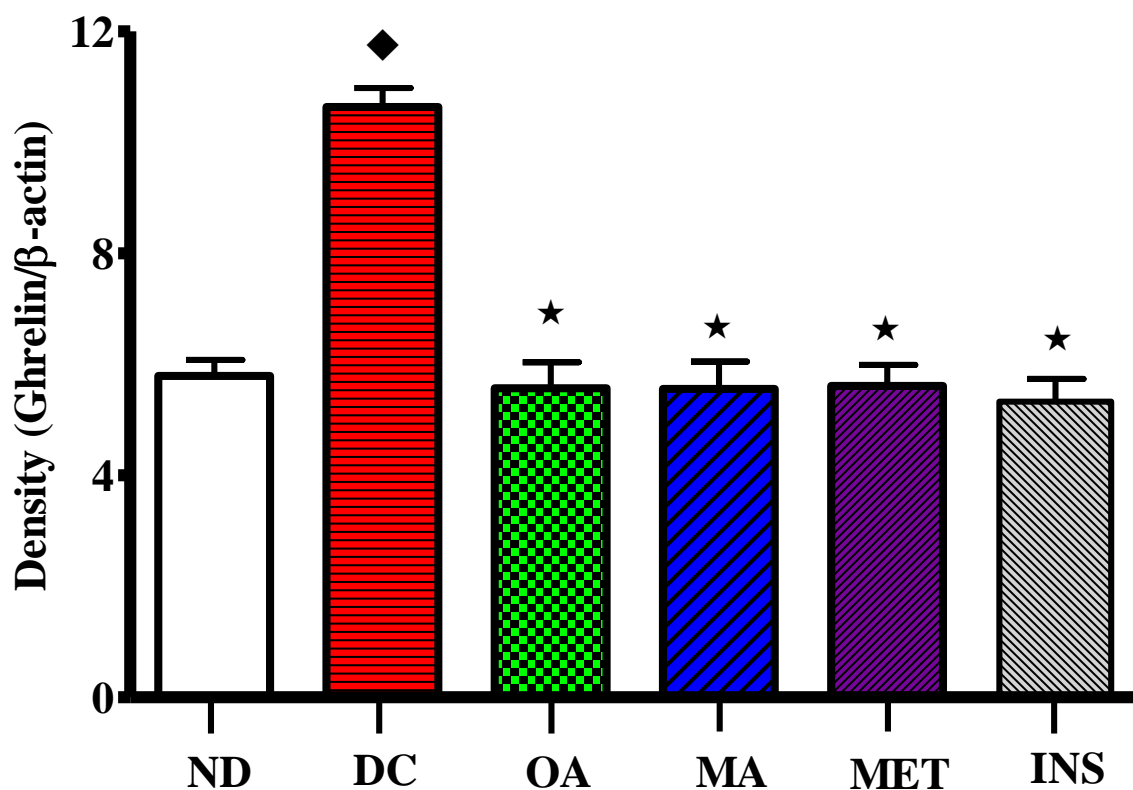
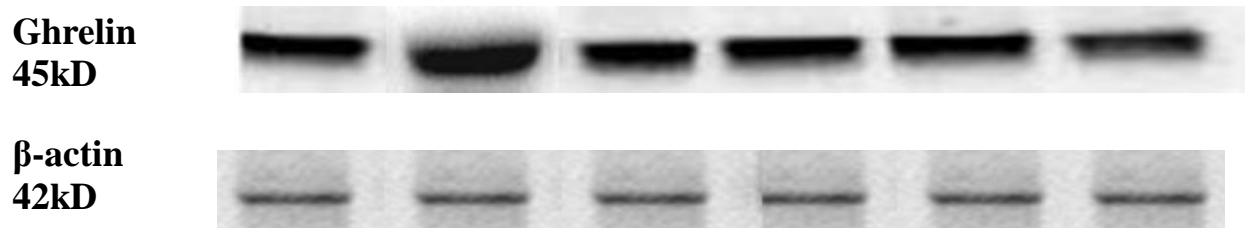


Figure 30. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine ghrelin expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). ♦= $p < 0.05$ by comparison to the non-diabetic control. ★= $p < 0.05$ by comparison to the STZ-induced diabetic control.

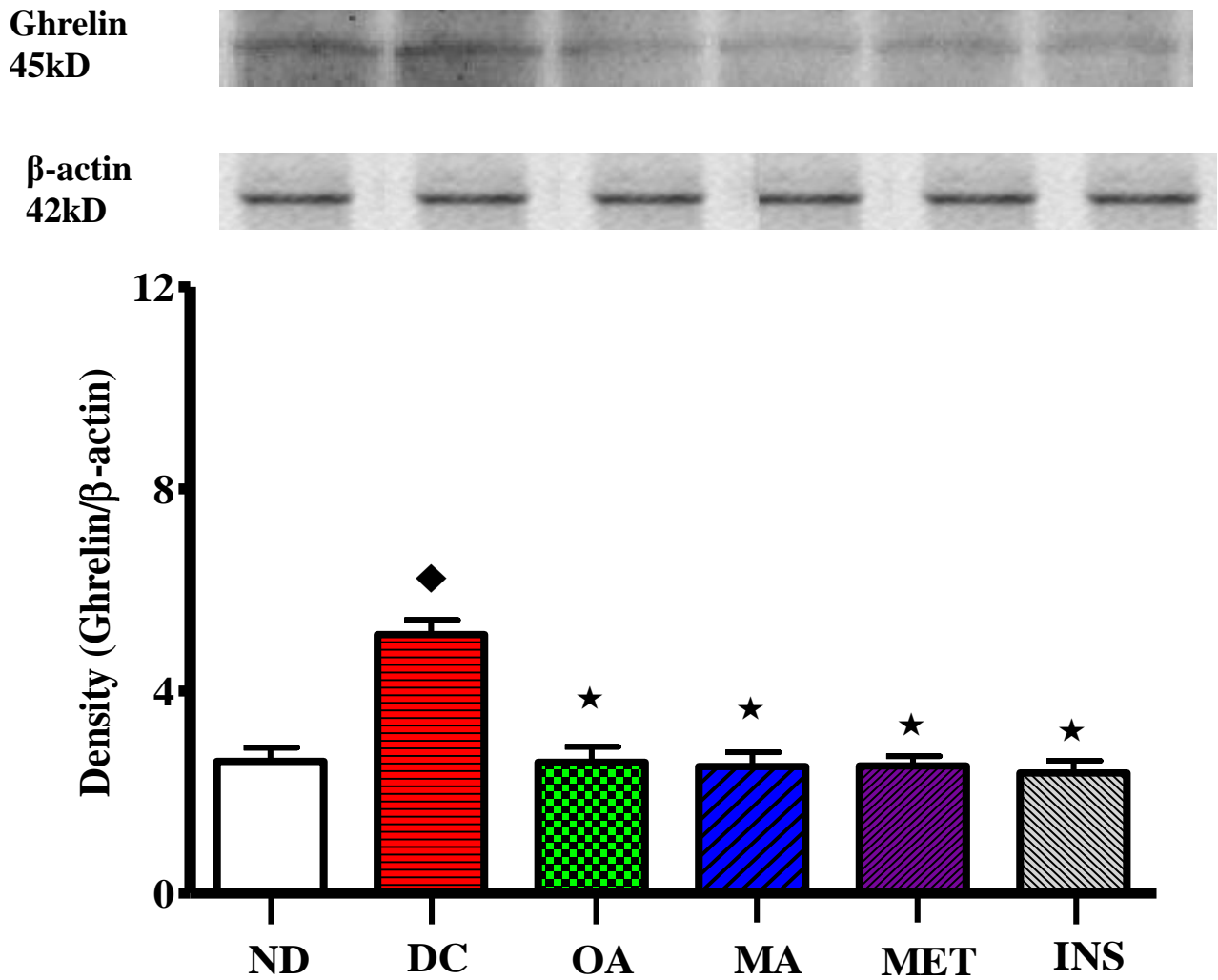


Figure 31. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on large intestine ghrelin expression with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ◆=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control.

3.10 Oxidative stress

In order to determine the effects of OA and MA on oxidative stress, we measured levels of MDA, a commonly known marker of oxidative stress and levels of antioxidant defence enzymes SOD and GPx in the small intestines of non-diabetic control, untreated STZ-diabetic and STZ-diabetic rats treated with OA and MA following the 5 week study. The concentrations of MDA and antioxidant enzymes (SOD and GPx) in non-diabetic control rats represent baseline/ normal activity levels found in the small intestine. There was a significant ($p<0.05$) increase in intestinal MDA concentrations and a simultaneous significant ($p<0.05$) decrease of SOD and GPx activity in the untreated STZ-diabetic rats by comparison with the non-diabetic control rats. Treatment of the STZ-diabetic rats with OA and MA significantly ($p<0.05$) reduced the MDA concentrations while significantly ($p<0.05$) increasing the activities of SOD and GPx in the small intestine thereby reversing the changes seen in the untreated STZ-diabetic rats. Similar effects were observed in STZ-diabetic rats treated with the standard drugs metformin and insulin (see Figure 32, Figure 33 and Figure 34).

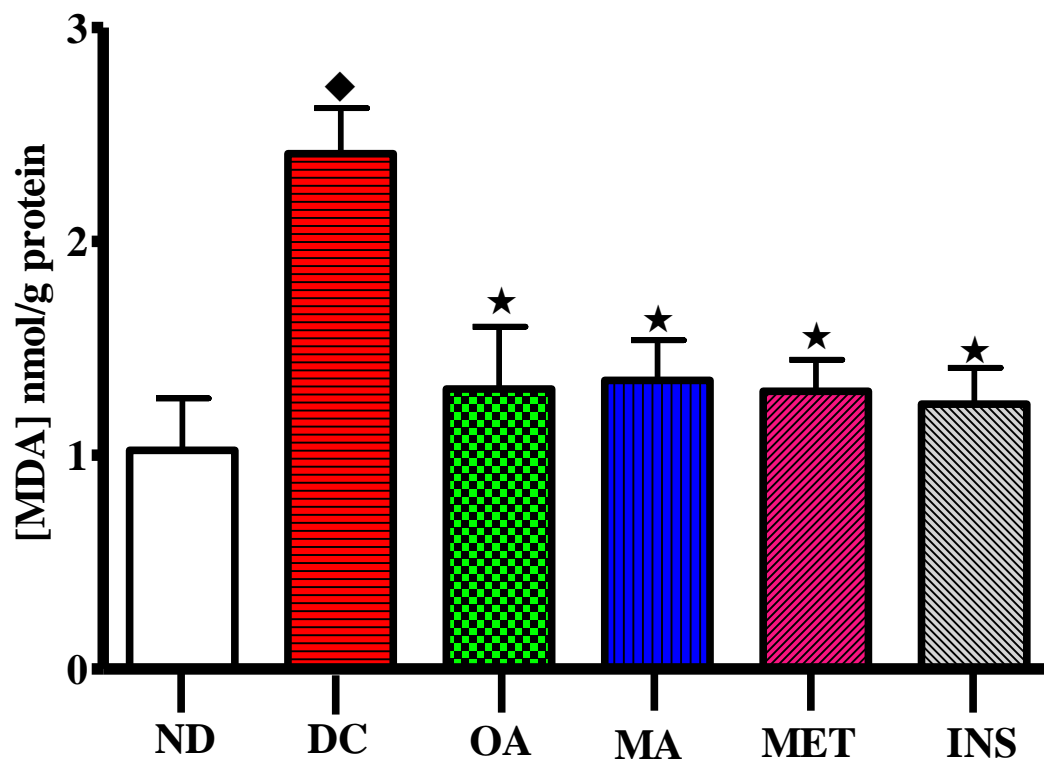


Figure 32. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on MDA concentration with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).◆=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control.

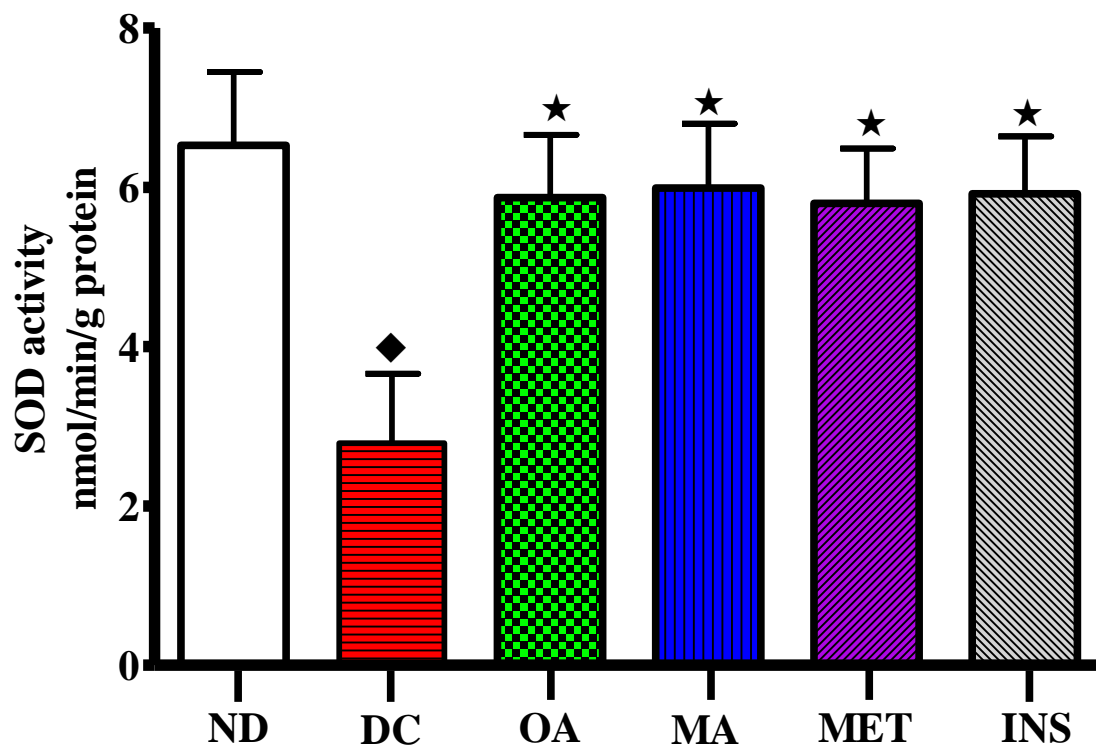


Figure 33. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine SOD activity with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ♦=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control

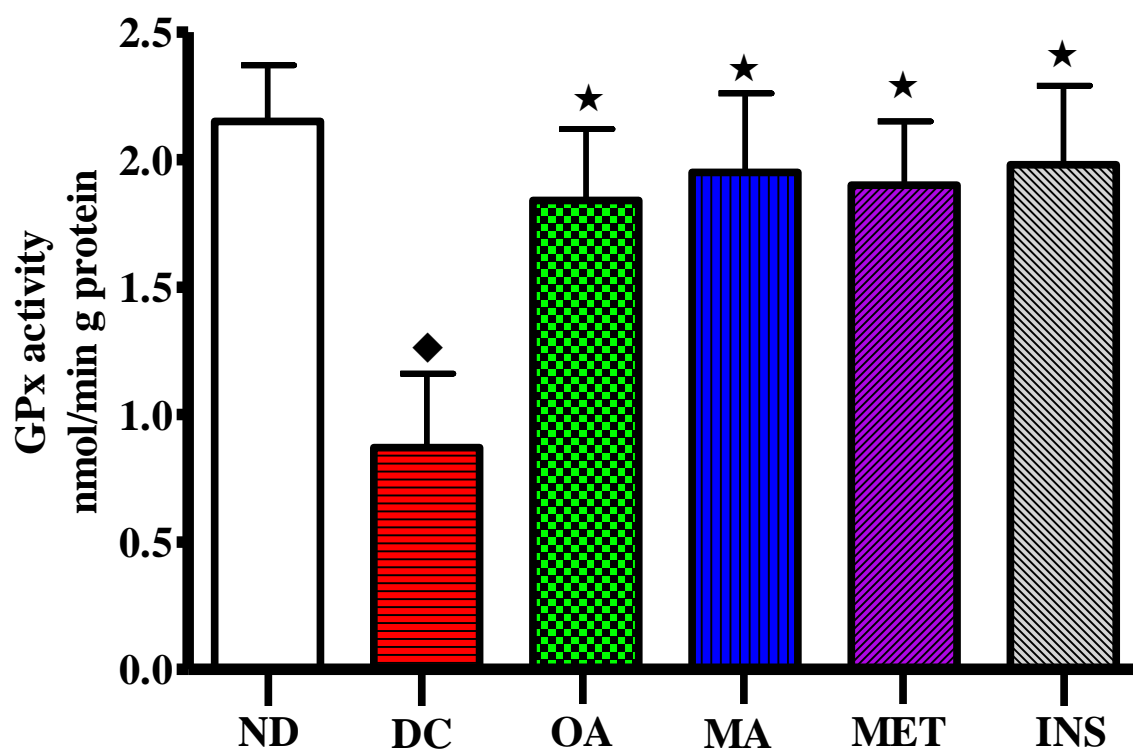


Figure 34. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on small intestine GPx activity with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).◆=p<0.05 by comparison to the non-diabetic control. ★ =p<0.05 by comparison to the STZ-induced diabetic control

3.11 Histological studies

To investigate the effects of OA and MA on the structural integrity of the small intestine, haematoxylin and eosin staining was performed in the small intestines of non-diabetic control, untreated STZ-diabetic and STZ-diabetic rats treated with OA and MA following the 5 week study. H and E-stained sections of untreated STZ-diabetic rats showed disruptions in the longitudinal layer of the small intestine by comparison with those of the non-diabetic controls observed under a light microscope. On the other hand, the intestinal mucous membrane in the circular layer of the small intestines of the untreated STZ-diabetic rats showed observable reductions in the length of the villi and disruption in the brush-border membrane by comparison with the non-diabetic rats. However, those animals treated with OA and MA showed no disruptions in the longitudinal layer by comparison to those of the untreated STZ-diabetic rats. Additionally, intact brush-border membranes and increases in the height of the villi were observed in those animals treated with OA and MA when compared to the untreated STZ-diabetic groups. Similar results were observed in animals treated with the standard drugs metformin and insulin (see Figure 35).

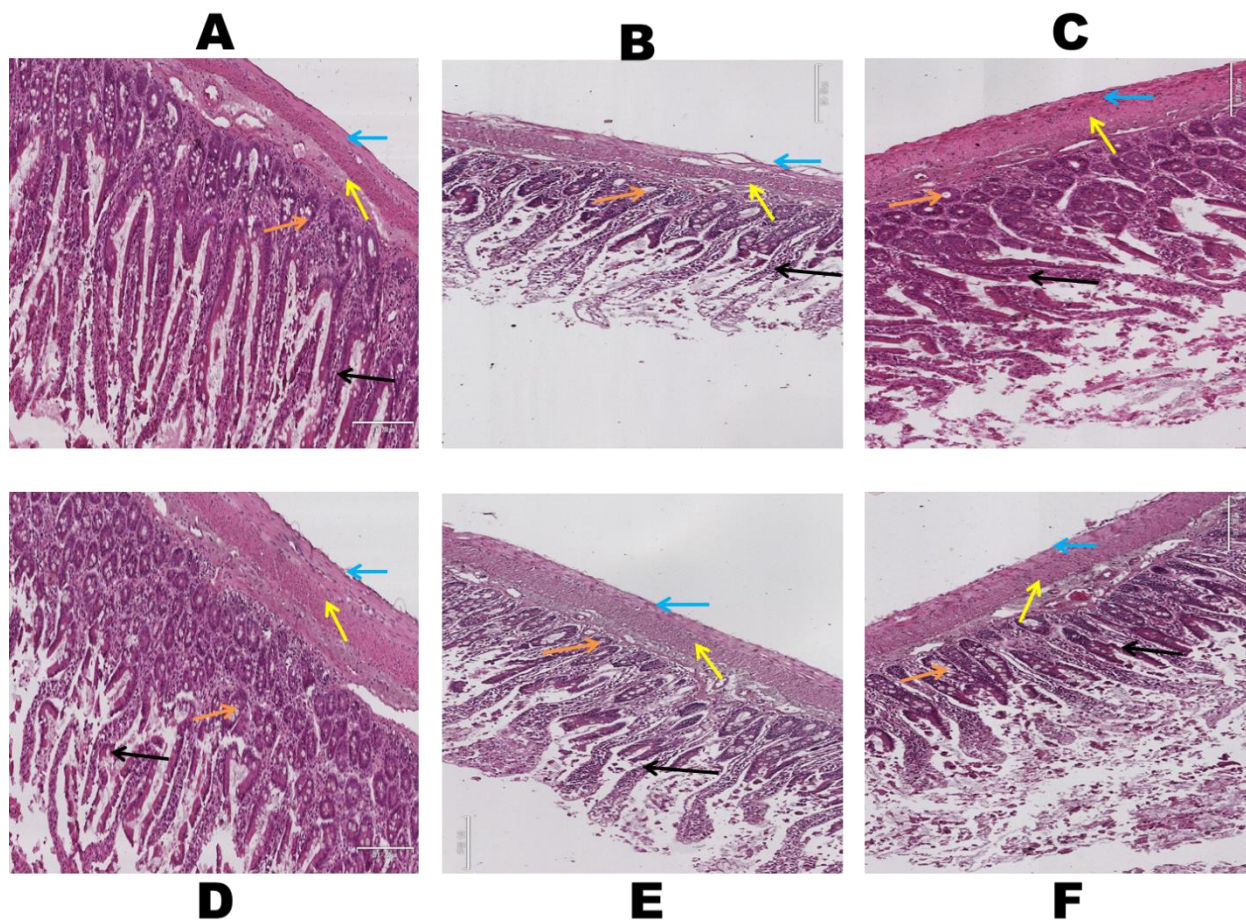


Figure 35. H & E stains illustrating the effects of OA and MA on the morphology of the small intestine in STZ-induced diabetic rats. Picture A represents an intact longitudinal layer (blue arrow), an intact mucous membrane (yellow arrow), goblet cells in the circular layer (orange arrow) and normal length villi (black arrow) in non-diabetic control animals. Picture B represents a disrupted longitudinal layer, an intact mucous membrane, goblet cells in the circular layer and villi with reduced length in untreated STZ-diabetic control animals. Pictures C, D, E and F represent an intact longitudinal layer, an intact mucous membrane, goblet cells in the circular layer and normal length villi in OA-, MA-, metformin- and insulin-treated STZ-diabetic animals respectively.

CHAPTER 4

DISCUSSION

The aim of the present study was to investigate the effects of *Syzygium-aromaticum* derived triterpenes, OA and MA on intestinal glucose handling in STZ-induced diabetic rats in an effort to further elucidate the hypoglycaemic mechanisms of the triterpenes. The results suggest that *Syzygium aromaticum*-derived OA and MA prevents of postprandial hyperglycaemia in streptozotocin-induced diabetic rats primarily through the modulation of intestinal glucose handling. . The findings of the current study are of considerable importance as they not only further explain the hypoglycaemic effects of OA and MA but they also justify the use of the small intestine as a therapeutic target in the management of diabetes mellitus.

The absolute stereostructure of *S. aromaticum*-derived OA and MA elucidated from the spectra using ^1H - and ^{13}C -NMR were comparable to the previously reported data (Mahato and Kundu, 1994; Mapanga *et al.*, 2009; Uddin *et al.*, 2011). The presence of the olefinic bond between C-12 and C-13 bond served as confirmation that the two compounds were indeed triterpenes as this is a bond unique to triterpenes (García-Granados *et al.*, 2000; Ju'lio *et al.*, 2003). Additionally, the identification of MA was confirmed through the C-2 which represents a hydroxyl group that is absent in other triterpenes like oleanolic acid (OA) and ursolic acid (UA) (Ju'lio *et al.*, 2003).

The induction of experimental diabetes using streptozotocin significantly increased plasma ghrelin concentrations in male Sprague-Dawley rats. Ghrelin acts on peripheral and central targets to stimulate food intake (Nakazato *et al.*, 2001; Wren *et al.*, 2001). Plasma levels of this peptide increase on fasting and decrease after habitual feeding, thus showing a pattern reciprocal to that of insulin (Broglia *et al.*, 2001; Egido *et al.*, 2002; Reimer *et al.*, 2003; Dezaki *et al.*, 2004). Enhanced plasma ghrelin levels have been observed in diabetic patients are associated with diabetic polyphagia (Ariyasu *et al.*, 2001). This increase in food consumption causes the body to spend more time in the postprandial state leading to chronic hyperglycaemia (Ariyasu *et al.*, 2001; Broglia *et al.*, 2001; Delhanty and van der Lely, 2011). This study served to advance the current knowledge on how OA and MA achieve and maintain glucose homeostasis in experimental type 1 diabetes mellitus in Sprague-Dawley rats.

In order to ensure that there was no residual insulin secretion in the rats, pancreatic β -cells were obliterated using a single intraperitoneal injection of STZ (60 mg/kg in citrate buffer, pH 6.3). STZ is an antibiotic first isolated from *Streptomyces achromogenes* selectively destroys pancreatic β -cells by inducing cytotoxicity effects in these cells (Weiss, 1982; Pinent *et al.*, 2004). The glucose moiety of STZ is important for recognition by the β -cells and thus selectively facilitates the entry of STZ into these cells through the GLUT2 transporter (Coskun *et al.*, 2005). Studies show that STZ induces cytotoxicity of the pancreatic B-cells through the alkylation of DNA as well as through the generation of reactive oxygen species. Furthermore, STZ has been reported to inhibit the Krebs cycle to decrease oxygen consumption which leads to the reduction of ATP levels. Finally, the pro-apoptotic genes are expressed thus causing β -cell death (Sztudelski, 2001). Additionally, previous studies have shown that the toxic effects of streptozotocin are short-lived and that this compound is quickly removed from circulation therefore suggesting that the observed effects in the animals are due to derangements in insulin secretion (Papaccio *et al.*, 1991; Kwon *et al.*, 1994; Sztudelski, 2001; Bhor *et al.*, 2004).

Indeed, the plasma insulin concentrations were significantly lower in the untreated STZ-induced diabetic rats by comparison with the non-diabetic rats. Furthermore, there were increases in plasma ghrelin levels that were possibly a result of the increased gastrointestinal expression of this hormone. This was further accompanied by significant increases in food intake as well as fasting and postprandial blood glucose concentrations in the untreated STZ-induced diabetic rats by comparison with the non-diabetic control rats. Taking this information into consideration, we were able to investigate the effects of OA and MA intestinal glucose handling without the influence of pancreatic insulin secretion thus mimicking an experimental type 1 diabetes model.

However, administration of OA and MA significantly reduced the food intake in the STZ-diabetic rats. This was possibly due to reduced plasma ghrelin levels that were associated with reduced gastrointestinal expression of this hormone after treatment with OA and MA. This reduction in food intake possibly decreased the amount of glucose available for absorption into circulation. Furthermore, the administration of these triterpenes to STZ-diabetic rats significantly reduced the blood glucose concentrations in both the acute and sub-chronic studies. These results are in agreement with previous observations from studies conducted in our laboratory (Mapanga *et al.*, 2009; Musabayane *et al.*, 2010; Ngubane *et al.*, 2011; Mkhwanazi *et*

al., 2014). Interestingly, both OA and MA had no significant effect on plasma insulin concentrations in the STZ-diabetic rats suggesting that the blood glucose lowering effects may be exerted via extra-pancreatic mechanisms. In addition, previous studies have indeed shown that while triterpenes such as OA enhance insulin secretion in isolated pancreatic islets, they have no effects on plasma insulin concentrations in whole animal studies (Teodoro *et al.*, 2008; Musabayane *et al.*, 2010). Indeed, OA and MA administration did not have significant effects on blood glucose concentrations in non-diabetic rats following a carbohydrate challenge. This could perhaps be due to the masking effects of insulin arising from the functional β -cells.

Plant bioactive compounds such as triterpenes have been reported to exert their anti-hypoglycaemic effects through a variety of mechanisms (Grover *et al.*, 2002; Ali *et al.*, 2002 ; Dzubak *et al.*, 2006 ; Bhat *et al.*, 2008; Abdul-Ghani and Defronzo, 2014). One of these mechanisms is slowing down the absorption of glucose in the small intestine to prevent postprandial hyperglycaemia (Ferraris, 2001; Kim *et al.*, 2008). This can be mediated through the modulation of key carbohydrate hydrolyzing enzymes such as α -amylase and α -glucosidase as well as intestinal glucose transporters such as SGLT1 and GLUT2 (Floris *et al.*, 2005; Jong-Anurakkhun *et al.*, 2007; Bhandari *et al.*, 2008). Enzyme inhibitor drugs such as acarbose, miglitol and voglibose prevent postprandial hyperglycaemia through the inhibition of the carbohydrate hydrolyzing enzymes to delay digestion and absorption of dietary carbohydrates (Dhindsa *et al.*, 2002). These drugs are characterized by their potency and mode of inhibition. The potency of an enzyme inhibitor is usually determined and reported in terms of an IC_{50} value which is the concentration of the inhibitor corresponding to 50% inhibition (DeFronzo *et al.*, 2014). In the current study, the potency of OA and MA as inhibitors of sucrase, and α -glucosidase and α -amylase were assessed and reported in terms of IC_{50} values. Interestingly, the *in vitro* studies indicated that the IC_{50} values of OA and MA against sucrase, α -amylase and α -glucosidase at various concentrations were less than those shown of acarbose suggesting that these triterpenes are highly potent and have fewer side effects.

Acarbose is associated with gastrointestinal side effects due to excessive inhibition of pancreatic α -amylase, resulting in the abnormal bacterial fermentation of undigested carbohydrates in the large intestine (Hanefeld, 1998; Kwon *et al.*, 2007b). Furthermore, acarbose has been reported to increase the incidence of renal tumors, serious hepatic injury, and acute hepatitis (Diaz-

Gutierrez *et al.*, 1998; Charpentier *et al.*, 2000). Against this background are reports that several α -glucosidase inhibitors isolated from medicinal plants possess lesser adverse effects than the existing drugs (Matsuda *et al.*, 2002; Gowri *et al.*, 2007; Tarling *et al.*, 2008; Kim *et al.*, 2009).

Indeed, the *in vivo* results also suggest that OA and MA inhibit the activity of key carbohydrate hydrolyzing enzymes. This was shown through the suppression of postprandial hyperglycaemia and significant reductions in AUC_{glucose} in the OGT studies. This was further evidenced through the down-regulation in the intestinal expression of both α -amylase and α -glucosidase following the sub-chronic study. The down-regulation of these enzymes in the small intestines by the OA and MA, in a manner similar to the standard drug metformin possibly contributed to the prevention of postprandial hyperglycaemia (Brayer *et al.*, 2000; Raptis and Dimitriadis, 2001).

Metformin is an anti-diabetic drug that was first isolated from the plant *Galega officinalis*. This drug prevents postprandial hyperglycaemia, in part, through the inhibition of gastric emptying and intestinal glucose transport (Dhindsa *et al.*, 2002; Musi *et al.*, 2002; Mahomoodally *et al.*, 2006; Mahrouf *et al.*, 2006). Phlorizin is another plant-derived (*Malus domestica*) drug that is used to prevent postprandial hyperglycaemia (Eddouks and Maghrani, 2004; Ehrenkranz *et al.*, 2005). The structure of phlorizin consists of a glucose moiety and two aromatic rings joined by an alkyl spacer thus allowing this drug to be a specific and competitive inhibitor of the SGLT transporters located within the small intestine (Panayotova-Heiermann *et al.*, 1995). Glucose is actively taken up from the lumen of the small intestine into the cells across the brush-border membrane by a Na⁺-dependent glucose co-transporter (SGLT1) and then exits the cell across the basolateral membrane by a Na⁺-independent glucose transporter (GLUT2) (Mizuma, 1993; Hediger and Rhoads, 1994; Strugala *et al.*, 1995; Meinild *et al.*, 1998). The inhibition of intestinal glucose transporters has been considered as a potential target for drug development for glycaemic control in diabetes mellitus (Coady *et al.*, 1990; Lee *et al.*, 1994; Asano *et al.*, 2004).

This study accordingly also investigated the influence of OA and MA on glucose transport across the small intestine using the rat everted gut sacs *in vitro*. The everted gut sac technique developed by Wilson and Wiseman, (1954) is a standard method for studying the absorption of substances in the mammalian intestine *in vitro* (Wilson and Wiseman, 1954; Porteous *et al.*, 1984; Burgmann *et al.*, 1992; Tandon *et al.*, 1993; Obatomi *et al.*, 1994; Mahomoodally *et al.*,

2005; Mahomoodally *et al.*, 2006). The results show that both OA and MA inhibited glucose absorption in the small intestine. OA and MA, like phlorizin, significantly inhibited the intestinal mucosal glucose absorption and simultaneously inhibited glucose release into serosal fluid.

Indeed, the administration of OA and MA in STZ-diabetic glucose-loaded rats significantly reduced postprandial glucose concentrations. Furthermore, Western blot analysis showed that untreated STZ-induced diabetic rats exhibited increased SGLT1 and GLUT2 expression in the small intestine over the 5-week study in comparison to non-diabetic control animals. Studies have shown in rats with experimentally induced diabetes that the capacity of the small intestine to absorb glucose increases at least in part, due to enhanced activity and abundance of brush border SGLT1 and basolateral GLUT2 (Dyer *et al.*, 1997a; Dyer *et al.*, 1997b; Liu *et al.*, 2012; Tahrani *et al.*, 2013). However, the results also indicate that administration of OA and MA significantly reduced the expression of these transporters in STZ-diabetic rats suggesting that the blood glucose lowering effects of these triterpenes could arise, in part through the inhibition of intestinal glucose transporters. The down regulation of these transporters by OA and MA reduces the total glucose absorption capacity in the small intestine in diabetes and therefore significantly reduces the magnitude of postprandial hyperglycaemia.

Other studies have noted that when intestinal glucose inhibitors such as phlorizin are taken for extended periods, they lead to adverse side-effects such as polyuria, glycosuria and extreme weight loss (Charpentier *et al.*, 2000; Beckman *et al.*, 2002; Kwon *et al.*, 2007b; Tahrani *et al.*, 2013). These are classical symptoms of diabetes mellitus and thus, the administration of these drugs may worsen the diabetic condition. Indeed, the results of the present study revealed that there was marked reduction in the total body weights of the untreated STZ-diabetic group compared to that of the non-diabetic control group. On the contrary, treatment with OA and MA stabilized the body weights of the STZ-induced diabetic rats suggesting that there may be a compensatory mechanism. Additionally, we have previously shown that OA and MA reduce blood glucose concentrations of STZ-induced diabetic rats through a variety of mechanisms (Mapanga *et al.*, 2009; Musabayane *et al.*, 2010).

We suggest that OA and MA, apart from inhibiting glucose transport from the mucosa to the serosa, these triterpenes could possibly shunt the glucose accumulated within the gut wall towards glycogen synthesis. This is consistent with our observations of increased glycogen concentrations in the small intestine as well increased expression of the glycogenic enzyme glycogen synthase. In keeping with this hypothesis, there are several reports of the presence of glycogen stores and insulin sensitive enzymes, glucose-6-phosphatase (Glc6Pase), glycogen phosphorylase and glycogen synthase in both rodent and human small intestines (Anderson and Jones, 1974 ; Rousset *et al.*, 1984 ; Croset *et al.*, 2001). We have previously reported that the hypoglycaemic effects of OA and MA are exerted in part via increased glycogen synthesis in the liver and skeletal muscle (Ngubane et al., 2011; Mkhwanazi et al., 2014). In addition, MA has been reported to reduce hepatic glucose production at least in part, through inhibiting glycogen phosphorylase activity (Singh *et al.*, 1992). Inhibition or inactivation of glycogen phosphorylase has been regarded as the remedial strategy for the control of blood glucose in diabetes (Chen *et al.*, 2006). Glycogen inhibition activates glycogen synthase, a critical component of the mechanism of glycogen synthesis (Aiston *et al.*, 2003). Indeed, the current study suggests that the observed increases in intestinal glycogen concentration of STZ-diabetic rats treated with OA and MA could be mediated through an increase in the expression of glycogen synthase as determined through Western blot analysis.

This conversion of glucose to glycogen in the small intestine reduces the amount of glucose available for the generation of reactive oxygen species. The continued generation of reactive oxygen species has been shown to contribute to the progression of diabetic complications, consequently, there are various drugs used to inhibit the generation of reactive oxygen species (Pazdro and Burgess, 2010). Indeed, OA and MA have been shown in the current study to suppress reactive oxygen species formation as seen through significant reductions in MDA levels which is a marker of lipid peroxidation. These triterpenes have also been shown to increase the activity of antioxidant defence enzymes such as glutathione peroxidase and superoxide dismutase (Mkhwanazi *et al.*, 2014). Current anti-diabetic drugs such as captopril, allopurinol, melatonin and metformin are used to suppress the elevation of reactive oxygen species in diabetes mellitus (Dhindsa *et al.*, 2002; Musi *et al.*, 2002; Mahrouf *et al.*, 2006). Captopril has been reported to increase superoxide dismutase levels and decrease MDA concentration in the

plasma (Shirpoor *et al.*, 2009). Allopurinol inhibits xanthine oxidase which is involved in the production of hydrogen peroxide and superoxide. Metformin is also used in the management of reactive oxygen species in diabetes mellitus. The antioxidant effects of metformin are exerted through the inhibition of NADPH oxidase which contributes to the generation of reactive oxygen species particularly in the kidneys and small intestines. Metformin also lowers the mitochondrial respiration chain thus reducing the formation of superoxide iron (Mahrouf *et al.*, 2006). This drug therefore inhibits lipid peroxidation and elevates glutathione levels (Gumieniczek, 2005). The elevation of reactive oxygen species results in structural changes in the small intestine that further lead to the development of diabetic complications. Indeed, haematoxylin and eosin staining showed that the intestinal mucous membrane of the STZ-diabetic rats showed significant reductions in the length of the villi by comparison with the non-diabetic rats. These results can be related the functional activity of L-glutamine which is considered the main energy source for the enterocytes to promote proliferation and cellular migration of the intestinal mucosa (Bhor *et al.*, 2004; Katz *et al.*, 2007). L-glutamine is a precursor of glutathione which is significantly reduced in diabetes mellitus (Bhor *et al.*, 2004; Coskun *et al.*, 2005; Gao *et al.*, 2009; Kim *et al.*, 2009). However, those animals treated with OA and MA had significant increases in the height of the villi when compared to the untreated STZ-diabetic groups. Indeed, this could be related to the significant increases in superoxide dismutase and glutathione peroxidase activity in the small intestine. Indeed, several studies have shown that various medicinal plant extracts such as those of *Allium sativum*, *Emblica officinalis* and *Momordica charantia* possess beneficial effects on the oxidative status of rats with experimental diabetes (Khanna *et al.*, 1981; Bhor *et al.*, 2004; Coskun *et al.*, 2005; Uebanso *et al.*, 2007). These effects have been observed with simultaneous improvements in postprandial blood glucose concentrations. OA and MA have also been shown to possess antioxidant properties in experimental diabetes that arise through the alleviation of reactive oxygen species due to reduced blood glucose levels.

This study identifies the small intestine as a glycogen synthesizing organ which may be involved in the pathophysiology of diabetes. OA and MA have been found to possibly play a role in the reduction in gastrointestinal expression of ghrelin leading to reduced food intake. Western blot analysis not only confirmed the inhibitory effects of OA and MA against α -glucosidase and

pancreatic α -amylase, but additionally showed that the triterpenes decrease the levels of SGLT and GLUT2 in the small intestines of STZ-induced diabetic rats to perhaps reduce glucose reabsorption (Hediger and Rhoads, 1994; Meinild *et al.*, 1998). The results, therefore, extend our *in vitro* observations by showing that the hypoglycaemic effects of OA and MA are in part mediated via inhibition of carbohydrate hydrolyzing enzymes as in the small intestines of diabetic rats. Taken together, the *in vitro* and *in vivo* observations of the study suggest that the control of the intestinal absorption of glucose by OA and MA may constitute an avenue of glycaemic control in diabetes. Additionally, the data suggests that OA and MA could be used as a potential supplements for treating postprandial hyperglycemia. We believe that these results will certainly enable a better understanding of the mechanisms involved in the pathophysiology of human diabetes and further justify the use of the small intestine as a target in the management of diabetes mellitus.

CHAPTER 5

5.0 Conclusions

The results presented in the current study show that *S. aromaticum*-derived OA and MA exert their blood glucose lowering effects in part, through the reduction of food intake mediated through a decrease in the gastrointestinal expression of ghrelin. Furthermore, this study shows that the hypoglycaemic effects of OA and MA could be due to increased glycogen synthesis in the small intestine. The results also show that OA and MA prevent postprandial hyperglycaemia in STZ-induced diabetic rats. This is mediated in part, through reductions in the intestinal expression of carbohydrate hydrolyzing enzymes such as α -amylase and α -glucosidase as well intestinal glucose transporters such as SGLT1 and GLUT2.

Additionally, OA and MA alleviate oxidative stress in the small intestine of STZ-diabetic rats. Taken together, the results suggest that OA and MA modulate intestinal glucose homeostasis in diabetes mellitus through the prevention of postprandial hyperglycaemia and can therefore prevent the development of diabetic complications. The results of this study are of considerable importance as they add further knowledge in the bid to fully elucidate the mechanism of the hypoglycaemic effects of these triterpenes and further explain the role of the gastrointestinal tract in the management of diabetes mellitus.

5.1 Limitations and directions for future studies

One of the limitations of the current study is that plasma leptin levels were not measured after the 5-week study. This hormone plays a role in the regulation of the hunger-satiety cycle. Another limitation of the study is that the effects of OA and MA on the enzyme glucose-6-phosphatase were not determined. This is a gluconeogenic enzyme that has been implicated to play a role in intestinal glucose homeostasis. Due to time constraints, we were not able to determine the effects of OA and MA on other glycogenic enzymes. This study therefore also opens up further investigations on the effects of these triterpenes on the expression of other glycogenic enzymes such as hexokinase and glucokinase. Additionally, the effects of OA and MA on the measured parameters were investigated in isolation. In order to further elucidate the anti-diabetic mechanisms of these triterpenes, they should be investigated in combination with

standard anti-diabetic drugs. Further studies should also look at the expression of the various transporters, enzymes and hormones at the gene level using techniques such as polymerase chain reaction (PCR) and microarrays to fully establish the mechanisms used by OA and MA to exert their anti-diabetic effects.

CHAPTER 6

6.0 References

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CHAPTER 7

APPENDICES

APPENDIX I



UNIVERSITY OF
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

Research office
Animal Ethics Research Committee

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

27 July 2011

Reference: 082/11/Animal

Mr. A Khathi
Human physiology
UKZN
WESTVILLE CAMPUS

Dear Mr. Khathi

Ethical Approval of Research Projects on Animals

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

The effects of novel transdermally delivered hypoglycaemic agents on intestinal glucose handling

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee


Cc Registrar, Prof. J Meyerowitz
Research Office, Mr N Moodley
Head of School, Prof. W Daniels
Supervisor, Prof. CT Musabayane
Co-Supervisor, Dr. B Masola
Biomedical Resource Unit, Dr S Singh



Founding Campuses:

Edgewood
Howard College
Medical School
Pietermaritzburg
Westville

APPENDIX II



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

1 December 2011

Reference: 026/12/Animal

Mr. Andile Khathi
Department of Physiology F2-413
School of Medical Science
University of KwaZulu- Natal
Private Bag X54001
Durban 4000


Dear Mr. Khathi

Renewal: 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:

"The effects of transdermally delivered hypoglycaemic agents on intestinal glucose handling.."

Yours sincerely



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

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



Founding Campuses:

- Edgerwood
- Howard College
- Medical School
- Pietermaritzburg
- Westville

APPENDIX III



UNIVERSITY OF
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

Research Office
Animal Ethics Research Committee

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

20 December 2012

Reference: 056/13/Animal

Mr A Khathi
School of Laboratory Medicine
and Medical Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Mr Khathi

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:

"The effects of transdermally delivered hypoglycaemic agents on intestinal glucose handling."

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



Founding Campuses:

- Edgewood
- Howard College
- Medical School
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APPENDIX IV



23 December 2013

Reference: 031/14/Animal

Mr A Khuthi
Discipline of Physiology
School of Laboratory Medicine &
Medical Sciences
WESTVILLE Campus

Dear Mr Khuthi

RENEWAL: Ethical Approval of Research Projects on Animals

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

"The effects of *Syzygium aromaticum*-derived triterpenes on intestinal glucose handling."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

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

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag 3031, Pietermaritzburg, 3201, South Africa
Telephone: +27 (0)33 260 5463/35 Facsimile: +27 (0)33 260 5105 Email: animalethics@ukzn.ac.za Website: www.ukzn.ac.za
Reading Campus: ■ Edgewood ■ Howard College ■ Medical School ■ Pietermaritzburg ■ Westville

INSPIRING GREATNESS



APPENDIX V



ORIGINAL ARTICLE

Effects of *Syzygium aromaticum*-derived oleanolic acid on glucose transport and glycogen synthesis in the rat small intestine

Andile KHATHI,¹ Bubuya MASOLA² and Cephas T. MUSABAYANE¹

¹Faculty of Health Sciences, Discipline of Human Physiology, and ²Faculty of Science and Agriculture, Department of Biochemistry, University of KwaZulu-Natal, Durban, South Africa

Correspondence

Cephas T. Musabayane, Department of Human Physiology, School of Medical Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa.
Tel: +27 31 260 7975
Fax: +27 31 260 7132
Email: musabayane@ukzn.ac.za

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Abstract

Background: In the present study, we investigated the effects of oleanolic acid (OA), which has hypoglycemic properties, on glucose transport and glycogen synthesis in the small intestine, an organ that secretes enzymes involved in carbohydrate metabolism.

Methods: The OA was isolated from *Syzygium aromaticum* ethyl acetate-soluble fractions followed by recrystallization with ethanol. It was diluted to required concentrations in freshly prepared dimethyl sulfoxide (2 mL) and normal saline (19 mL) before being administered to rats (p.o.). Glycogen concentrations were determined in isolated small intestines from fasted and non-fasted non-diabetic and streptozotocin-diabetic rats after 18 h treatment with 80 mg/kg, p.o., OA or standard hypoglycemic drugs (i.e. 100 µg/kg, s.c., insulin; 500 mg/kg, p.o., metformin). In a separate series of experiments, the effects of 30-min incubation with graded concentrations of OA (0.82–6.56 mmol/L) on D-glucose were evaluated by monitoring changes in glucose concentrations inside and outside of intestinal sacs isolated from fasted, non-diabetic rats and mounted in an organ bath containing Krebs–Henseleit bicarbonate buffer.

Results: All *in vivo* treatments increased the glycogen concentration in rat small intestine, although the effects of metformin treatment in non-fasted diabetic rats failed to reach statistical significance. *In vitro*, both OA (1.64–6.56 mmol/L) and phlorizin (10^{-5} – 10^{-3} mol/L) decreased glucose transport from the mucosa to the serosa.

Conclusion: The data suggest that OA may be a potential alternative drug treatment for postprandial hyperglycemia because of its inhibition of glucose uptake across the small intestine and its concomitant conversion of glucose to glycogen in the intestinal wall.

Keywords: glucose transport, glycogenesis, oleanolic acid, small intestine, *Syzygium aromaticum*.

Significant findings of the study: Targeting of the small intestine may be a novel therapeutic strategy for diabetes treatment because OA-elicited glycogenesis in the small intestine in the present study was associated with a concomitant decrease in blood glucose levels in diabetic rats.

What this study adds: In addition to the liver and skeletal muscle, the small intestine appears to be another organ involved in the pathophysiology of diabetes.

Introduction

Traditional herbal medicine is used by many African communities to treat a range of diseases, including diabetes. Anecdotal evidence suggests that this approach is effective, because diabetic complications are less common in rural populations. Indeed, more than 200 compounds from plant sources have been reported to exhibit blood glucose-lowering activity.^{1–3} We have also demonstrated that oleanolic acid (OA), an active ingredient isolated from *Syzygium* spp., exerts hypoglycemic effects in streptozotocin (STZ)-induced diabetic rats, in part by increasing glycogen synthesis in the liver and skeletal muscle.^{4,5} On the basis of these observations and the fact that the blood glucose-lowering effects of many antidiabetic drugs are mediated via a variety of mechanisms of action, we hypothesized that the hypoglycemic effects of OA may involve inhibition of glucose transport in the small intestine. Indeed, some plant extracts lower blood glucose in experimental animals by interfering with food intake and by decreasing the intestinal absorption of glucose, as does metformin, a plant-derived antidiabetic drug.^{6–8} Similarly, antidiabetic drugs such as acarbose, miglitol, and voglibose delay glucose absorption in the small intestine.^{9,10} Thus, the main aim of the present study was to investigate the effects of OA on glucose uptake in the small intestine *in vitro* using the everted gut sac model to further elucidate the mechanism(s) underlying the hypoglycemic effects of this triterpene. Because insulin maintains blood glucose homeostasis by controlling hepatic and peripheral glycogen metabolism,¹¹ we further investigated the effects of OA on glycogen synthesis in the small intestine. The small intestine is an insulin-sensitive organ¹² that expresses glucose-6 phosphatase (Glc6Pase),¹³ the last enzyme in the gluconeogenesis and glycogenolysis pathways. If OA inhibits glucose absorption and transport in the small intestine and increases glycogen synthesis in this tissue, it may be useful in decreasing blood glucose levels in diabetes mellitus.

Methods

Isolation of OA

The OA used in the present study was isolated from the air-dried powdered flower buds of *Syzygium aromaticum* (Linnaeus) Merrill & Perry (Myrtaceae; cloves) using a standard protocol that has been validated in our laboratory.¹⁴ The air-dried powdered flower buds (1.74 kg) were extracted three times at room temperature with hexane (3 × 3 L), dichloromethane (3 × 3 L), ethyl acetate (3 × 3 L), and methanol

(3 × 3 L), yielding mixtures of OA/ursolic acid and methyl maslinate/methyl corosolate. Pure OA (109 mg) was obtained by recrystallization of the ethyl acetate-soluble (EAS) fraction (25.5 g) with ethanol. Its structure was confirmed by spectroscopic analysis using one- and two-dimensional (1D and 2D, respectively) ¹H and ¹³C nuclear magnetic resonance (NMR) techniques. Preliminary studies indicated that OA isolated from *S. aromaticum* and commercially available OA exhibited similar hypoglycemic effects and so the plant extract was used in subsequent experiments because it is less expensive.

Animals

Male Wistar rats, weighing 250–300 g, were used in the study. Rats had free access to standard rat chow (Meadows, Pietermaritzburg, South Africa) and water and were kept under standard environmental conditions under a 12-h light–dark cycle. The procedures involving animals and their care were conducted in accordance with the guidelines of the University of KwaZulu-Natal.

Diabetes was induced in rats by a single intraperitoneal injection of STZ (60 mg/kg). Seven days after STZ injection, diabetic rats were used in the experiments. Note, fasted rats were allowed free access to water at all times.

Experimental design

The effects of OA on glucose transport across the small intestine *in vitro* were investigated using the rat everted small intestine. Conversely, the effect of the triterpene on glycogen synthesis was evaluated in small intestines harvested from non-fasted and fasted non-diabetic and STZ-diabetic rats 18 h after treatment with 80 mg/kg, p.o., OA or standard hypoglycemic drugs (100 µg/kg, s.c., insulin; 500 mg/kg, p.o., metformin). Preliminary studies indicated that there were no significant differences in fasting blood glucose levels of non-diabetic and STZ-diabetic rats after 18 or 24 h fasting, so 18 h was chosen as the sampling period in the present study.

Everted intestinal sac preparation

The everted intestinal sac technique described by Wilson and Wiseman¹⁵ and modified by Mahomoodally et al.¹⁶ was used in the present study. Briefly, rats were killed after an overnight fast. The whole of the small intestine was then removed by cutting across the upper end of the duodenum and the lower end of

the ileum from the pyloric sphincter to the ileocecal junction, and it was then rinsed with cold normal saline solution. Mid-portions of the small intestine (mean [\pm SEM] 10 ± 2 cm in length) were everted according to the method of Wilson and Wiseman.¹⁵ The sac was made by tightly tying the distal end of the segment with a dry thread and everting the sac. The empty sac was weighed before being filled with 1 mL Krebs–Henseleit bicarbonate (KHB) buffer (composition [in mmol/L]: NaHCO_3 25; NaCl 118; KCl 4.7; MgSO_4 1.2; NaH_2PO_4 1.2; CaCl_2 1.2; plus Na_2EDTA 9.7 mg/L), referred to as the “serosal fluid”. Once the sac had been filled, its open end was tied off with another thread. The distended sac was immersed in a water-jacketed organ bath containing 50 mL KHB (mucosal fluid), which was bubbled continuously with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Prior to the beginning of each experiment, the everted intestinal sac was checked for potential leaks at the ligated ends.

Effects of OA on glucose transport

In a separate series of experiments, D-glucose (10 mmol/L) was added to the KHB just prior to the start of the experiments and the everted intestinal sac preparations were incubated with graded concentrations of OA (0.82–6.56 mmol/L) in the mucosal solution. To establish whether the activity of OA is comparable to that of synthetic drugs already in use, in some experiments the preparations were incubated in the presence of 10^{-6} – 10^{-3} mol/L phlorizin as a positive control for the inhibition of glucose uptake. In other experiments, an equal volume of KHB was added to the organ baths as a control. At the end of the incubation period (30 min), the sacs were removed from the organ bath, blotted, and weighed. The serosal fluid was drained through a small incision into a test tube. The emptied sac was shaken gently to remove any remaining fluid and the empty sac was weighed again. There was no significant difference in the weight of the empty sacs before and after incubation. The transport of D-glucose across the everted gut was evaluated by measuring changes in D-glucose concentrations inside and outside the intestinal sacs after 30 min incubation. Mucosal glucose transfer was defined in the present study as the amount of glucose that disappeared from the mucosal fluid, whereas serosal glucose uptake was defined as the amount of glucose that entered the serosal fluid. Glucose concentrations were measured using a commercially available glucose oxidase kit (Sigma-Aldrich, St Louis, MO, USA).

Effects of OA on intestinal glycogen concentrations

Glycogen concentrations were measured in mid-portion segments of the small intestine of non-fasted and starved non-diabetic and STZ-diabetic rats 18 h after treatment with 80 mg/kg, p.o., OA. The effects of OA on glycogen synthesis in the small intestine were compared with those of 500 mg/kg, p. o., metformin and 100 $\mu\text{g/kg}$, s.c., insulin. The control group was injected with 3 mL/kg deionized water 18 h before the study.

Glycogen concentrations were determined as described previously,¹⁷ with some modification.¹⁸ Briefly, tissue samples (0.5–0.7 g) were homogenized in 2 mL of 30% KOH solution, digested at 100°C for 30 min, and then cooled in ice-cold saturated sodium sulfate. Glycogen was precipitated with ethanol and then pelleted, washed, and resolubilized in deionized water. Glycogen standards (10–2000 mg/L) were prepared using glycogen powder. The glycogen concentration was determined by allowing it to react with anthrone reagent (2 g anthrone/L of 95% [v/v] H_2SO_4) and then measuring absorbance at 620 nm using a Novaspec II spectrophotometer (Biochrom, Cambridge, UK).

Statistical analysis

All data are expressed as the mean \pm SEM. The significance of differences between the control and experimental groups was determined by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparison test using GraphPad InStat Software (version 4.00; GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

Results

Structural elucidation of compounds

Spectral data obtained for OA using ^1H - and ^{13}C -NMR (1D and 2D) were compared with the literature.¹⁹ The purity of OA was determined to be approximately 99% on the basis of chemical and physicochemical evidence.

Effect of OA on glucose transport *in vitro*

The effects of OA on D-glucose absorption in the rat small intestine (mucosal disappearance) and transport across the everted gut sac (serosal appearance) are shown in Fig. 1. At concentrations ranging from 1.64 to 6.56 mmol/L, OA significantly suppressed the mucosal disappearance and serosal appearance of D-glucose in a dose-independent manner. However,

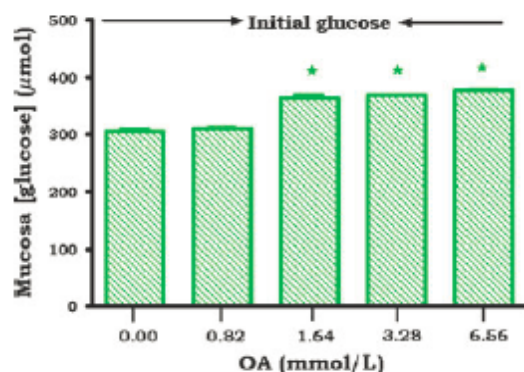


Figure 1 Effects of increasing concentrations of oleic acid (OA; 0.82–6.56 mmol/L) on the disappearance of glucose from the mucosa in the rat everted intestinal sac preparation. The initial amount of glucose in the mucosal fluid was 500 μ mol, indicated by the horizontal line. Data are the mean \pm SEM ($n = 6$ rats in each group). * $P < 0.05$ compared with control.

0.82 mmol/L OA had no significant effect on these parameters. Similarly, phlorizin at concentrations between 10^{-5} and 10^{-3} mol/L inhibited both mucosal glucose disappearance in everted intestinal sacs bathed in KHB buffer containing 10 mmol/L glucose (Fig. 2) and serosal glucose appearance (data not shown). The total amount of glucose accumulated or metabolized by the wall of the everted small intestinal sac decreased with increasing concentrations of OA (Table 1). The amount of glucose accumulated by the wall of the

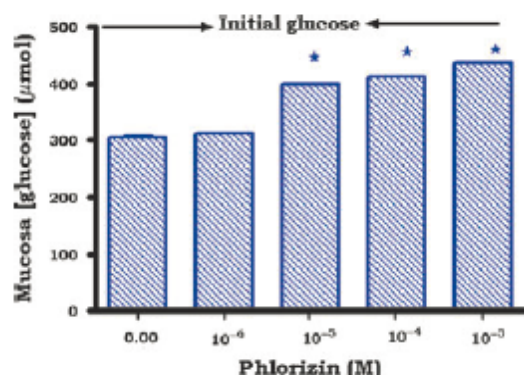


Figure 2 Effects of increasing concentrations of phlorizin (10^{-5} – 10^{-3} mol/L) on the disappearance of glucose from the mucosa in the rat everted intestinal sac preparation. The initial amount of glucose in the mucosal fluid was 500 μ mol, indicated by the horizontal line. Data are the mean \pm SEM ($n = 6$ rats in each group). * $P < 0.05$ compared with control.

Table 1 Effects of increasing concentrations of oleic acid on the transport of D-glucose across rat everted intestinal sacs

OA (mmol/L)	Mucosal disappearance of D-glucose (μ mol/g tissue)	Serosal appearance of D-glucose (μ mol/g tissue)	Intestinal accumulation of D-glucose (μ mol/g tissue)
0	252.64 \pm 0.08	2.88 \pm 0.08	176.70
0.82	256.81 \pm 0.12	2.58 \pm 0.12	167.87
1.64	296.40 \pm 0.06*	2.14 \pm 0.06*	131.82*
3.28	303.63 \pm 0.05†	1.93 \pm 0.05†	125.42†
6.56	312.11 \pm 0.09‡	1.66 \pm 0.09‡	108.54‡

Where appropriate, data are given as the mean \pm SEM ($n = 6$ rats in each group). * $P < 0.05$ compared with control; † $P < 0.05$ compared with lower concentrations of oleic acid (OA); ‡ $P < 0.05$ compared with all other groups.

Intestinal accumulation of D-glucose was calculated as the difference between the amount of glucose lost from the mucosal fluid and the amount of glucose in the serosal fluid after the 30-min incubation period.

small intestine sac was calculated as the difference between the amount of glucose lost from the mucosal fluid and the amount of glucose in the serosal fluid after incubation.

Effects of OA on small intestine glycogen concentrations

To assess whether the glucose that accumulated in the intestinal walls stimulated glycogenesis, glycogen was measured in isolated small intestines obtained from non-fasted or starved non-diabetic and STZ-diabetic rats 18 h after treatment with OA or standard hypoglycemic drugs (metformin or insulin). The mean blood glucose concentration of STZ-diabetic rats was significantly than that of control non-diabetic rats (see Fig. 3). Relative to the respective control groups, OA treatment did not significantly alter plasma glucose levels of non-diabetic rats, but it did significantly decrease plasma glucose concentrations in STZ-diabetic rats. There was no significant difference in the glycogen concentration in the small intestine of control non-fasted non-diabetic and diabetic rats. However, the glycogen concentration in the isolated small intestine was significantly reduced in non-fasted and starved STZ-diabetic rats. Treatment with OA significantly increased glycogen concentrations in the isolated small intestines of rats. More notable, however, were the marked effects of OA (80 mg/kg, p.o.) in non-fasted non-diabetic rats, in which the magnitude of the increase in glycogen content was almost twofold that seen in starved non-diabetic animals. Treatment of rats with insulin and metformin resulted in significant

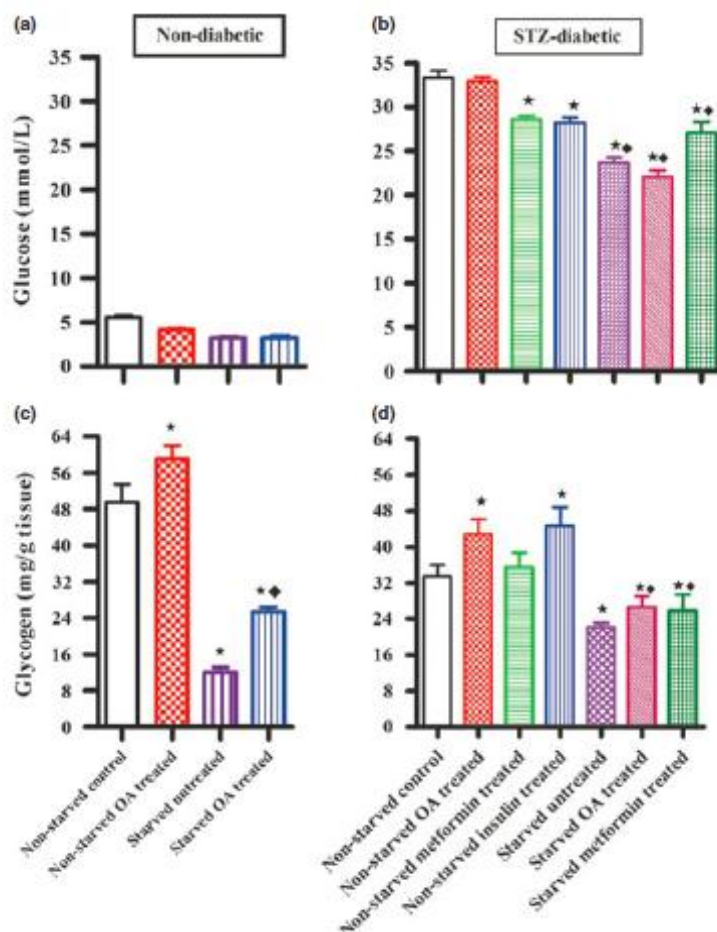


Figure 3 (a,b) Blood glucose and (c,d) small intestine glycogen concentrations in non-fasted or fasted non-diabetic (a,c) and streptozotocin-diabetic (b,d) rats 18 h after treatment with oleic acid (OA; 80 mg/kg, p.o.), 500 mg/kg, p.o., metformin (Met) and 100 µg/kg, s.c., insulin (Ins). Data are the mean ± SEM ($n = 6$ rats in each group). * $P < 0.05$ compared with control; † $P < 0.05$ compared with non-fasted untreated control rats.

increases in the glycogen content in the small intestine of fasted STZ-diabetic rats. The results in fasted insulin-treated STZ-diabetic rats (data not shown) were similar to those in non-fasted diabetic rats.

Discussion

The present study investigated the *in vitro* effects of OA on glucose transport across the small intestine using rat everted gut sacs, as well as the *in vivo* effects of the triterpene on small intestine glycogen synthesis, in an effort to further elucidate the mechanism(s) underlying the hypoglycemic effects of OA. Previously, we reported that OA increases glycogen synthesis in the liver and skeletal muscle.^{4,5} In the present study we used the everted gut sac technique developed by Wilson and Wiseman,¹⁵ a standard method for investigating the absorption of substances in mammalian

intestines *in vitro*.^{20–24} Using this model, we found that OA inhibited glucose absorption and increased glycogen synthesis in the small intestine. *In vitro* studies showed that not all the glucose that was in the mucosa was transferred into the serosa after the 30-min incubation period (Table I). We suggest that, in addition to inhibiting glucose transport from the mucosal to serosal side of the small intestine, OA also enhances glycogen synthesis in the small intestine, using glucose that has accumulated in the gut wall. A marked lowering of glycogen concentrations was seen in the small intestines of fasted non-diabetic and diabetic rats, suggesting that glucose uptake accounts for glycogenesis in this organ. Indeed, food deprivation reduced glycogen concentrations in the liver and skeletal muscle of rats, which was reversed by the consumption of carbohydrates.^{25,26} Thus, the results of the present study identify the small intestine as a glycogen-synthesizing

organ that may be involved in glucose homeostasis in diabetes. This is at variance with the current view that only the liver and skeletal muscles are important glycogen-storing organs that play a pivotal role in buffering hyperglycemia in mammals.

Like phlorizin, OA significantly inhibited intestinal mucosal glucose absorption, as well as its release into the serosal fluid, possibly by binding to glucose transporters. Glucose is actively taken up from the lumen of the small intestine into cells across the brush border membrane by the Na⁺-dependent glucose cotransporter SGLT1.²⁷ It then exits the cell across the basolateral membrane via the Na⁺-independent glucose transporter GLUT2.^{28–30} Phlorizin blocks intestinal glucose absorption by inhibiting SGLT1 and GLUT2.^{27–31} Potential effects of OA on intestinal SGLT1 and GLUT2 cannot be inferred from the results of the present study. Further studies are required to establish the effect of OA on intestinal SGLT1 and GLUT2 to determine whether the triterpene decreases intestinal glucose uptake in the rat everted gut by regulating these glucose transporters. However, the fate of glucose within the intestinal wall is difficult to quantify because glucose is rapidly metabolized by enterocytes.³² Another interesting finding in the present study is that OA lowered the blood glucose of fasted diabetic rats, but not fed diabetic rats. The lack of effect of OA on blood glucose levels in the latter group may be attributed to continuous intestinal carbohydrate absorption arising from uninterrupted food intake. Indeed, we have reported previously that food intake of STZ-diabetic rats is significantly greater than that of non-diabetic rats.³³

It is of note that OA reduced both the mucosal disappearance and serosal appearance of glucose, which may reflect inhibition of glucose transfer at both the mucosal and serosal boundaries. Accordingly, it is possible that OA increases small intestine glucose conversion to glycogen to reduce glucose transport into the serosal solution. In keeping with this hypothesis are reports of the presence of glycogen stores and the insulin-sensitive enzymes Glc6Pase, glycogen phosphorylase (GP), and glycogen synthase (GS) in both the rodent and human small intestine.^{12,34,35} Indeed, the results of whole-animal studies demonstrated that OA induces increases in small intestine glycogen synthesis in non-diabetic and diabetic rats (Figs 3b and 4d). Interestingly, OA has also been reported to reduce hepatic glucose production by, at least in part, inhibiting GP activity.³⁶ Inhibition or inactivation of GP has been regarded as the remedial strategy for the control of blood glucose in diabetes.³⁷ Inhibition of GP activates GS by removing GP-induced inhibition of

glycogen synthase phosphatase.¹ This is a critical component of the mechanism for stimulating glycogen synthesis. The role of the small intestine in lowering blood glucose in diabetes relative to the liver and skeletal muscle cannot be established based on the results of the present study. However, reports indicate that the capacity of the small intestine to influence glucose homeostasis increases in experimentally induced diabetes.³⁸ Together, the *in vivo* and *in vitro* observations in the present study suggest that the control of the intestinal absorption of glucose by OA may constitute an avenue of glycemic control in diabetes.

We believe that the results of the present study contribute to a better understanding of the mechanisms involved in the pathophysiology of human diabetes because regulation of SGLT1 can be mediated by insulin.³⁹ Both SGLT1 and GLUT2 in intestinal epithelial cells, as well as in renal epithelial cells, have been considered potential targets of drug development for glycemic control in diabetes.^{40–42} The results of the present study suggest that the hypoglycemic properties of OA may arise, in part, from the inhibition of glucose transport across the small intestine with a concomitant conversion of glucose to glycogen. Although the limitations of the present study include the lack of assessment of the effects of OA on the activity of key glycogenic enzymes and SGLT1 and GLUT2 in the small intestine, further studies are expected to provide this information. Such data would establish the direct effects of OA on small intestine glucose transport and glycogen synthesis.

Acknowledgments

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Disclosure

The authors have no conflicts of interest to declare.

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Effects of *Syzygium aromaticum*-Derived Triterpenes on Postprandial Blood Glucose in Streptozotocin-Induced Diabetic Rats Following Carbohydrate Challenge

Andile Khathi¹, Metse R. Serumula¹, Rene B. Myburg¹, Fanie R. Van Heerden², Cephas T. Musabayane^{1*}

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

Abstract

Purpose: Recent reports suggest that the hypoglycaemic effects of the triterpenes involve inhibition of glucose transport in the small intestine. Therefore, the effects of *Syzygium spp*-derived triterpenes oleanolic acid (OA) and maslinic acid (MA) were evaluated on carbohydrate hydrolyzing enzymes in STZ-induced diabetic rats and consequences on postprandial hyperglycaemia after carbohydrate loading.

Methods: We determined using Western blot analysis the expressions of α -amylase and α -glucosidase and glucose transporters SGLT1 and GLUT2 in the small intestine intestines isolated from diabetic rats treated with OA/MA for 5 weeks. *In vitro* assays were used to assess the inhibitory activities of OA and MA against α -amylase, α -glucosidase and sucrase.

Results: OA and MA ameliorated postprandial hyperglycemia in carbohydrate loaded diabetic rats as indicated by the significantly small glucose area under the curve (AUC) in treated diabetic animals compared with that in untreated diabetic rats. Western blotting showed that OA and MA treatment not only down-regulated the increase of SGLT1 and GLUT2 expressions in the small intestine of STZ-induced diabetic rats, but also inhibited small intestine α -amylase, sucrase and α -glucosidase activity. IC₅₀ values of OA against α -amylase (3.60 ± 0.18 mmol/L), α -glucosidase (12.40 ± 0.11 mmol/L) and sucrase (11.50 ± 0.13 mmol/L) did not significantly differ from those of OA and acarbose.

Conclusions: The results of suggest that OA and MA may be used as potential supplements for treating postprandial hyperglycemia.

Novelty of the Work: The present observations indicate that besides improving glucose homeostasis in diabetes, OA and MA suppress postprandial hyperglycaemia mediated in part via inhibition of carbohydrate hydrolysis and reduction of glucose transporters in the gastrointestinal tract. Inhibition of α -glucosidase and α -amylase can significantly decrease the postprandial hyperglycaemia after a mixed carbohydrate diet and therefore can be an important strategy in the management of postprandial blood glucose levels in NIDDM patients.

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* E-mail: musabayane@ukzn.ac.za

Introduction

Postprandial hyperglycemia ascribed to hydrolysis of carbohydrates by digestive enzymes in the small intestine is a major risk factor for complications in diabetic patients [1–3]. Small intestine α -glucosidase and pancreatic α -amylase are the key enzymes of dietary carbohydrate digestion in humans. Glucose released upon carbohydrate digestion is absorbed

from the intestinal lumen into the blood mainly via sodium-dependent glucose co-transporter (SGLT1), localized to the enterocyte apical or brush-border membrane (BBM), and the basolateral facilitative glucose transporter 2 (GLUT2) [4]. Evidence obtained from experimental diabetes indicates that the capacity of the small intestine to absorb glucose in diabetes increases mainly due to enhanced activity and abundance of SGLT1 and GLUT2 [5]. Thus, controlling postprandial glucose

level is critical during early treatment of diabetes mellitus to avert postprandial glucose excursions thereby reducing chronic vascular complications [6]. The duration of postprandial hyperglycaemia and magnitude of glucose concentration trigger oxidative stress-linked diabetic complications [7].

Commercially available synthetic inhibitors of carbohydrate hydrolyzing enzymes are effective in retarding carbohydrate hydrolysis and glucose absorption to suppress postprandial hyperglycaemia [8]. Quercetin, a flavonoid antioxidant, isolated from medicinal plants blunts postprandial hyperglycaemic spike via inhibition of carbohydrate hydrolysis [9,10]. Furthermore, α -glucosidase inhibitors with increased potency and lesser adverse effects than the existing drugs have also been isolated from medicinal plants [11]. We have reported that the hypoglycaemic properties of plant-derived oleanolic acid (OA) are in part mediated via inhibition of glucose transport in the small intestine [12], but effects on key carbohydrate hydrolyzing enzymes remain unanswered. Therefore, the present project was designed to evaluate the effects of *Syzygium* spp.-derived triterpenes oleanolic acid (OA) and maslinic acid (MA) on carbohydrate hydrolyzing enzymes in normal and diabetic rats and consequences on postprandial hyperglycaemia associated with polysaccharide, disaccharide and monosaccharide challenge. We also evaluated the effects of OA and MA on the expression of α -amylase and α -glucosidase as well as on glucose transporters SGLT1 and GLUT2 in the small intestine to establish whether these triterpenes had direct effects on carbohydrate digestion and glucose transport in the small intestine.

Materials and Methods

Drugs and chemicals

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

Extraction methods

OA and MA were isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] (cloves) using a standard protocol that has been validated in our laboratory [13,14]. Air-dried *S. aromaticum* flower buds (500 g) were milled and sequentially extracted twice at 24 h intervals at room temperature using 1 L dichloromethane (DCM), and ethyl acetate (720 ml) on each occasion. Subsequently, the extract was concentrated under reduced pressure at $55 \pm 1^\circ\text{C}$ using a rotary evaporator to yield dichloromethane solubles (DCMS) and ethyl acetate solubles (EAS). The EAS containing mixtures of oleanolic/ursolic acid and methyl maslinic/methyl corosolate were purified by silica gel 60 column chromatography with hexane: ethyl acetate solvent systems, 7:3 for OA and 6:4 for MA. This yielded OA and MA, respectively which were further purified by recrystallization from chloroform-methanol (1:1, v/v). The structures of OA and MA were confirmed by spectroscopic analysis using 1D and 2D, ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopic experiments.

Animals

Male Sprague-Dawley rats (250–300 g) maintained on free access to standard rat chow (Meadows, Pietermaritzburg, South Africa) and water *ad libitum* were used throughout the study. They were maintained in standard environmental conditions with 12h light/12h dark cycle. All animal protocols were reviewed and approved by the University of KwaZulu-Natal animal ethics committee.

Induction of diabetes mellitus

Experimental type 1 diabetes mellitus was induced in male Sprague-Dawley rats using a previously described protocol [15]. Briefly, the animals were administered a single intraperitoneal injection of 60 mg/kg STZ in freshly prepared 0.1 M citrate buffer (pH 6.3). Control group received the vehicle, citrate buffer through the same route. Animals that exhibited glucosuria after 24 h, tested by urine strips (Rapidmed Diagnostics, Sandton, South Africa) were considered diabetic. Seven days later, the blood glucose concentration of STZ-induced diabetic rats greater than 20 mmol L⁻¹ was considered as stable diabetes.

Experimental design

The effects of OA/MA on postprandial blood glucose concentration and intestinal carbohydrate-hydrolyzing enzymes and glucose transporters were examined in non-diabetic and STZ-induced diabetic male Sprague-Dawley rats. The inhibitory activities of OA and MA against carbohydrate hydrolyzing enzymes were studied *in vitro*.

Oral glucose tolerance (OGT) responses

The rats were divided into the following groups: control and treated non-diabetic rats and control and treated STZ-induced diabetic rats ($n = 6$ in each group). After an 18 h fasting period, glucose was measured (time 0) followed by loading with monosaccharide (glucose; 0.86 g/kg, p.o.), disaccharide (sucrose; 1.72 g/kg, p.o.) or polysaccharide (starch; 0.086 g/kg, p.o.) delivered into the stomach by a gavage needle (18-gauge, 38 mm long curved, with a 21/4 mm ball end). To determine the effects of triterpenes on postprandial glucose, separate groups of animals were administered OA and MA (80 mg/kg) dissolved in dimethyl sulfoxide and deionized water. The selection of these doses was based on the posology from previous studies in our standard laboratory [16]. Rats treated with DMSO/water (3 ml/kg, p.o.) and standard drugs acarbose (100 mg/kg, p.o.) and phlorizin (100 mg/kg, p.o.) served as untreated and positive controls, respectively. Blood glucose measurements were made at 15, 30, 60, and 120 min after carbohydrate loading. The area under the curve (AUC) for increase in glucose over baseline was calculated during OGT responses by incremental method.

Short-term studies

To assess the influence of OA and MA on the activity intestinal carbohydrate hydrolyzing enzymes and glucose transporters, groups of non-diabetic and STZ-induced diabetic male Sprague-Dawley rats were housed individually in

Makrolon polycarbonate metabolic cages (Techniplats, Labotec, South Africa) for a 5-week period ($n = 6$ in each group). In those animals in which the effects of OA/MA were investigated, the rats were administered with OA/MA (80 mg/kg) twice daily at 09h00 and 15h00 by means of a bulb-dipped steel tube. Rats similarly treated with DMSO/saline (3 mL/kg, p.o.) and standard anti-diabetic drugs (acarbose, 100 mg/kg, p.o.) acted as untreated and treated positive controls, respectively.

Tissue sample harvesting

At the end of the 5 week experimental period, all animals were sacrificed by exposing to halothane via a gas anaesthetic chamber (100 mg/kg, for 3 min). The tail part of the pancreas was quickly removed from each rat through the abdominal incision. Thereafter, the whole of the small intestine was removed by cutting across the upper end of the duodenum and the lower end of the ileum from the pyloric sphincter to the ileocecal junction and rinsed with cold normal saline solution. Mid portions of the small intestine (10 ± 2 cm) were removed, snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijders Scientific, Tilburg, Netherlands) at -70°C for Western blot analysis of glucose transporters, SGLT1 and GLUT 2 and carbohydrate hydrolyzing enzymes, α -amylase, α -glucosidase.

Western Blot Analysis

Small intestine tissues harvested from untreated OA and MA treated STZ-induced diabetic rats at the end of 5 week were analyzed for SGLT1, GLUT 2, α -amylase and α -glucosidase using Western blotting. Pancreatic tissues were only analyzed for α -amylase. Small intestine and pancreatic tissues (0.1 g) were homogenized on ice in isolation buffer (0.5 mM Na_2EDTA , 0.1 M KH_2PO_4 , 0.1 mM dithiothreitol, 0.25 M sucrose) and then centrifuged at $400 \times g$ for 10 min (4°C). The protein content was quantified using the Lowry method [17]. All the samples were standardized to one concentration (1 mg/mL). The proteins were then denatured by boiling in Laemmli sample buffer (0.5 M Tris-HCl, glycerol, 10% sodium dodecyl sulphate (SDS), 2-mercaptoethanol, 1% bromophenol blue) for 5 min. The denatured proteins were loaded (25 μL) on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with molecular weight marker (5 μL). The gel was electrophoresed for 1 h at 150 V in electrode (running) buffer (Trisbase, glycine, SDS), pH 8.3). Following electrophoresis, the resolved proteins were electro-transferred to an equilibrated polyvinylidene difluoride (PVDF) membrane for 1 h in transfer buffer (192 mM glycine, 25 mM Tris, 10% methanol). After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (20 mM Tris, 150 mM NaCl, KCl, 0.05% Tween-20).

The intestinal membranes were then immuno-probed with antibodies- SGLT1, GLUT 2, α -amylase and α -glucosidase (1:1000 in 1% BSA, Neogen, USA) while the pancreatic membranes were immuno-probed with α -amylase (1:500 in 1% BSA, Neogen, USA) for 1 h at room temperature (RT). The PVDF membrane was then subjected to 5 washes (10 min each with gentle agitation) with TTBS. The membranes were then incubated in horse radish peroxidase (HRP)-conjugated

secondary antibody (rabbit anti-mouse 1:10 000; Bio-Rad) for 1 h at RT. After further washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analysed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands.

In-vitro inhibitory enzyme assay studies

The inhibitory activities of OA and MA on α -amylase and α -glucosidase were studied using an α -amylase/ α -glucosidase-starch model system while inhibitory effects on sucrose utilized the dextran sucrose- dextran sucrose reaction mixture.

α -amylase

The assessment of the inhibitory effects of OA/MA against α -amylase activity *in vitro* was based on the modified method previously described Bhandari et al., 2008 and Gao et al., 2008 [18,19]. Briefly, soluble maize starch (1 mg) was boiled for 5 min in 0.5 mL of 0.5M Tris-HCl buffer (pH 6.9) containing 0.01M CaCl_2 . After cooling, deionized water was added to a final volume of 100 mL. The solution was kept in the refrigerator and was used within 2-3 days. A reaction mixture, 500 μL containing 200 μL starch, 100 μL of OA at various concentrations (4.37-21.90 $\mu\text{mol/L}$) to which 200 μL of α -amylase (porcine pancreas, 2.60 mmol/L) was added to initiate the reaction and incubated at 37°C for 37 min. The reaction was terminated by addition of 100 μL of 50% acetic acid.

α -glucosidase

The assessment of the inhibitory effects of OA and MA against α -glucosidase *in vitro* utilized a similar method described above for α -amylase except that the 0.1M potassium phosphate buffer (pH 6.9) was used. The assay mixture (500 μL) comprising of 200 μL of α -glucosidase (Type 1, Bakers yeast, 1.30 mmol/L) was premixed with OA (100 μL) at various concentrations (4.37-21.90 $\mu\text{mol/L}$). The mixture was incubated at 37°C for 30 min after adding starch in phosphate buffer and stopped by adding 1.5 mL of 2M Tris-HCl buffer (pH 6.9).

Sucrase

The *in vitro* sucrase inhibitory effects of OA/MA were performed as described above for α -amylase except for that the assay mixture (700 μL) comprised of 200 μL dextran sucrose (56 mmol/L) in the potassium phosphate buffer, 100 μL of OA/MA at various concentrations (4.37-21.90 $\mu\text{mol/L}$) and 400 μL of dextran sucrase (*Leuconostoc mesenteroides*, 2.60 mmol/L).

In all cases, the liberated glucose was measured by the glucose oxidase method and the absorbance was recorded at 595 nm using Varian Cary 1E UV-visible spectrophotometer (Varian Australia Pty Ltd, Mulgrave, Victoria, Australia). Results expressed as the percentage inhibition of the corresponding control were calculated using the formula:

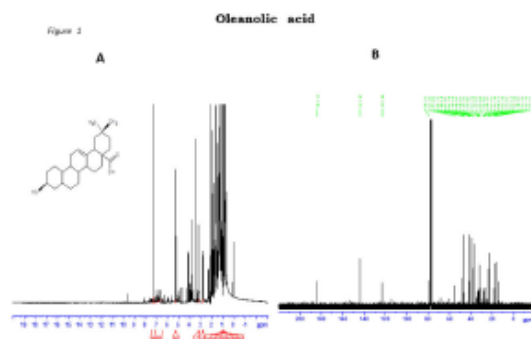


Figure 1. ^1H (A) and ^{13}C (B) NMR of oleanolic acid in dissolved in chloroform.

doi: 10.1371/journal.pone.0081632.g001

$$\% \text{ Inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

Where $\text{Abs}_{\text{Control}}$ was the absorbance without sample, $\text{Abs}_{\text{Sample}}$ was the absorbance of sample extract.

The IC_{50} values were calculated from plots of log concentration of inhibitor concentration versus percentage inhibition curves.

Statistical analysis

Data are expressed as means \pm SEM. Statistical significance was performed with GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA), for analysis of one-way variance (ANOVA), followed by Tukey-Kramer multiple comparison test.

Results

Structure elucidation

The ^1H - and ^{13}C -NMR (1D and 2D) spectroscopic data for OA and MA obtained from *S. aromaticum* powder EAS white powder following recrystallization with chloroform-methanol were given as follows:

OA

^1H NMR CDCl_3 δ , 0.73, 0.75, 0.88, 0.89, 0.90, 0.96, 1.11 (each 3H, s), 2.84 (1H, dd, $J = 10.36$), 3.22 (1H, dd, $J = 4.56$), 5.26 (1H, t, $J = 3.76$); ^{13}C NMR (CDCl_3) : δ 183.5, 143.8, 122.7, 79.2, 55.4, 47.8, 46.8, 46.1, 41.8, 41.2, 39.5, 38.9, 38.6, 38.3, 34.0, 33.3, 32.8, 32.7, 31.6, 28.4, 27.9, 27.4, 26.2, 23.8, 23.7, 23.1, 18.5, 17.4, 15.8, 15.5.

These values compared with those reported in literature [20]. Spectra in Figure 1 are given in the supporting information. The purity of the plant-derived OA was approximately 98% and the percentage yield varied from 0.79% to 1.72%.

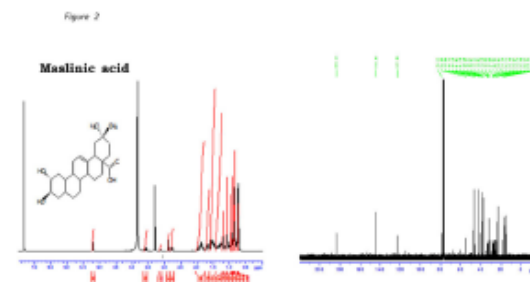


Figure 2. ^1H (A) and ^{13}C (B) NMR spectroscopic analysis of MA dissolved in deuterated methanol.

doi: 10.1371/journal.pone.0081632.g002

MA

^{13}C NMR: δ , (^{13}C NMR (400MHz, CD_3OD): 46.2 (C-1), 68.3 (C-2), 83.3 (C-3), 39.1 (C-4), 55.0 (C-5), 18.1 (C-6), 32.7 (C-7), 39.0 (C-8), C-9 (47.4), C-10 (38.0), C-11 (23.2), C-12 (121.9), C-13 (143.7), C-14 (41.6), C-15 (27.4), C-16 (23.0), C-17 (46.2), C-18 (41.0), C-19 (45.7), C-20 (30.4), C-21 (33.6), C-22 (32.3), C-23 (28.3), C-24 (16.6), C-25 (16.5), C-26 (16.4), C-27 (23.2), C-28 (178.5), (C-29) 32.2, C-30 (23.2).

These data compared with those reported in literature [3]. Spectra in Figure 2 are given in the supporting information. The purity of the plant-derived MA was approximately 98% and the percentage yield varied from 0.02% to 0.03%.

Postprandial glucose concentrations

Blood glucose concentrations were significantly higher in the STZ-induced diabetic rats groups loaded with sucrose and at all time-points during the OGT response tests compared to non-diabetic rats (Figure 3). The glucose area under the curve (AUC) also increased significantly compared to non-diabetic rats (Figure 4). Treating STZ-induced diabetic rats with OA significantly reduced blood glucose levels during the OGT protocol. In addition, the $\text{AUC}_{\text{glucose}}$ was smaller in treated diabetic animals compared with that in untreated diabetic rats. In the MA-treated diabetic rats, the OGT responses and $\text{AUC}_{\text{glucose}}$ were not significantly different from those observed with OA. Similar kind of suppression effect was observed in the diabetic groups that received acarbose as the positive control along with the carbohydrates. Considering the whole 120 min experiment, the glycaemic responses of STZ-induced diabetic rats to sucrose or starch co-administration with OA/MA were not significantly different.

The blood glucose concentrations of control non-diabetic rats increased slightly after carbohydrate load, but co-administration with OA and MA decreased the blood glucose levels to values that did not achieve statistical significance (Figure 3). This was reflected the $\text{AUC}_{\text{glucose}}$ of groups of animals administered with the triterpenes that did not achieve statistical difference when compared to control non diabetic rats (Figure 3).

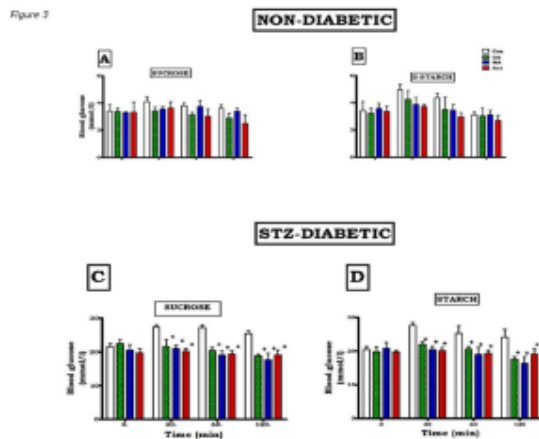


Figure 3. Comparison of OGT responses to OA and MA in non-diabetic (A and B) and STZ-induced diabetic (C and D) rats after sucrose and starch loading. Values are presented as means, and vertical bars indicate SEM (n=6 rats in each group). ★ p<0.05 by comparison with control animals. doi: 10.1371/journal.pone.0081632.g003

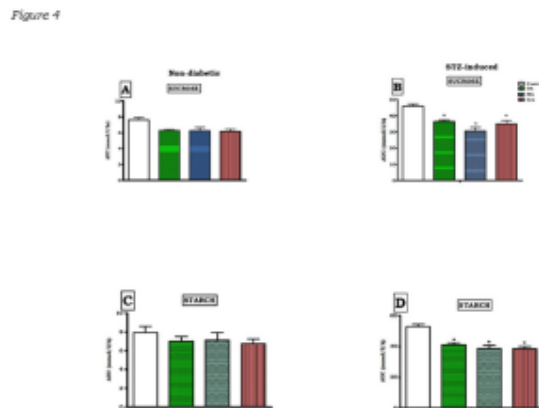


Figure 4. Inhibitory effects of OD and MA on blood glucose increases in non-diabetic (A and B) and STZ-induced diabetic (C and D) rats after sucrose and starch loading for 2h. The area under the curve for glucose (AUC_{glucose}) was calculated by incremental method. Values are presented as means, and vertical bars indicate SEM (n = 6 rats in each group). ★ p < 0.05 by comparison with control animals. doi: 10.1371/journal.pone.0081632.g004

Western blots

Western blot analysis of glucose transporters SGLT1 and GLUT2 and carbohydrate hydrolyzing enzymes, α -amylase, α -glucosidase proteins in small intestines isolated from control and treated non-diabetic and STZ-induced diabetic rats were

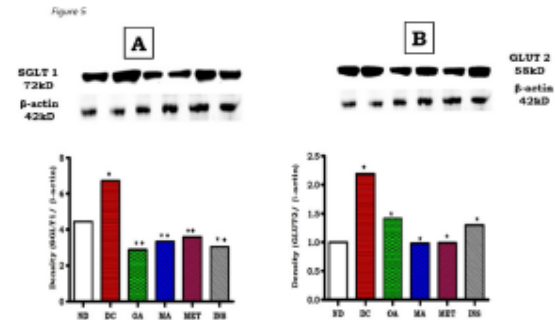


Figure 5. Effects of OA and MA on expression of SGLT1 (A) and GLUT2 (B) as determined by Western blotting of control, OA, MA, metformin and insulin-treated small intestine tissues of non-diabetic and STZ-induced diabetic rats. Values are expressed as mean \pm S.E.M. Values were obtained from Western blots for six preparations. ★ p < 0.05 by comparison with respective control animals.

◆ p < 0.05 by comparison with respective non-diabetic animals. doi: 10.1371/journal.pone.0081632.g005

assessed to explore the inhibitory mechanism of OA and MA on glucose absorption. The small intestines revealed that STZ-induced diabetes increased the expression of SGLT1 and GLUT2 proteins in the intestines of diabetic rats by 43% and 23%, respectively compared to non-diabetic control rats (Figure 5). The increases of SGLT1 and GLUT2 induced in diabetes were significantly abrogated by OA and MA indicating that the triterpenes reduced small intestine glucose absorption in part via inhibition of glucose transporters. Interestingly, treatment with OA and MA reduced the SGLT1 expression in small intestines isolated from diabetic rats to values significantly lower than those of untreated diabetic and non-diabetic control rats, but comparable to the standard hypoglycaemic drugs (insulin and metformin).

To determine whether these effects also involved intestinal carbohydrate hydrolyzing enzymes, the levels of α -amylase and α -glucosidase were determined using Western blot analysis in small intestines isolated from control and treated non-diabetic and STZ-induced diabetic rats. As assessed by densitometric analyses, α -amylase and α -glucosidase levels in untreated diabetic rats were elevated in comparison with non-diabetic control rats. OA significantly reduced the expression of both α -amylase and α -glucosidase of STZ-induced rats, but effects were more potent on the former enzyme (Figure 6). MA like OA inhibited the expression of both enzymes with no significant difference in potency.

To further explore these findings, we used *in vitro* assays to quantify the inhibitory activities of OA and MA against α -amylase, α -glucosidase and sucrase. Both OA and MA dose dependently inhibited α -amylase, α -glucosidase and sucrase activities (Figure 7). By comparison with the standard anti-amylase drug acarbose, IC₅₀ values of OA against α -amylase, α -glucosidase and sucrase of 13.60 ± 0.18 vs 17.50 ± 0.17 , 10.40 ± 0.11 vs 15.50 ± 0.14 and 10.50 ± 0.13 vs 13.10 ± 0.17

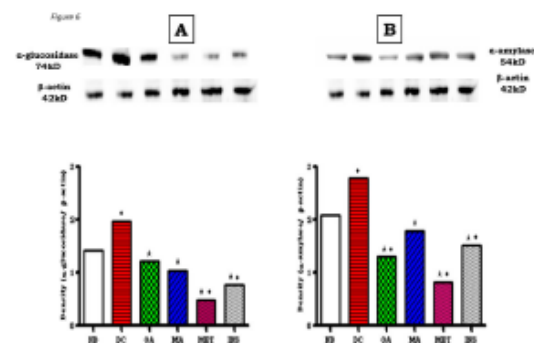


Figure 6. Effects of OA and MA on expression of α -glucosidase (A) and α -amylase (B) as determined by Western blotting of control, OA, MA, metformin and insulin-treated small intestine tissues of non-diabetic and STZ-induced diabetic rats. Values are expressed as mean \pm S.E.M. Values were obtained from Western blots for six preparations.

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

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

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mmol/L, respectively, were significantly low. The MA IC_{50} values against these key carbohydrate hydrolyzing enzymes did not significantly differ from those of OA. The effects of diabetes and subsequent OA and MA treatments on α -amylase expression in the pancreas were also evaluated in STZ-induced diabetic rats. As evaluated by Western blot analysis, pancreatic tissues from untreated STZ-induced diabetic rats exhibited significant increases ($p < 0.05$) in pancreatic amylase expression compared to non-diabetic control animals (Figure 8) which was significantly ($p < 0.05$) towards control values in non-diabetic rats after OA and MA treatment. The reversal of these changes by the triterpenes was similar to those changes that occurred following standard drug treatments.

Discussion

The present study was designed to evaluate whether *Syzygium aromaticum*-derived triterpenes, OA and MA reduce postprandial glucose in STZ-induced diabetic rats loaded with carbohydrates via inhibition of carbohydrate hydrolysis and reduction of glucose transporters in the gastrointestinal tract. The structures of OA and MA were elucidated by 1H and ^{13}C NMR spectral data compared with previously reported values [3,20]. We have previously reported that the hypoglycaemic properties may arise, in part, from the inhibition of glucose transport across the small intestine [12]. The results suggest that OA and MA inhibit the activity of key carbohydrate hydrolyzing enzymes and down regulate the activity of glucose transporters in the small intestines to decrease postprandial hyperglycaemia.

OA and MA improved glycaemic control of STZ-induced diabetic rats as evidenced by the suppression of postprandial

Figure 7

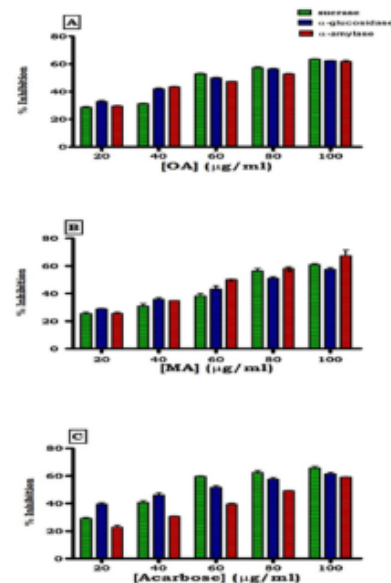


Figure 7. Percent inhibition by OA (A), MA (B) and acarbose (C) sucrase on dextran sucrose and of α -glucosidase and α -amylase on starch. Values presented are means \pm SEM ($n = 8$ for each concentration).

doi: 10.1371/journal.pone.0081632.g007

glucose concentration and reduction in the AUC_{glucose} . The inhibition of the α -amylase and α -glucosidase as well as down regulating of glucose transporters in the small intestines by the triterpenes possibly contributed to the decrease in postprandial glucose [21,22]. Interestingly, intestinal α -glucosidase enzyme inhibitors also improve postprandial hyperglycaemia by delaying digestion of polysaccharides, disaccharides and monosaccharides [21,23,24]. Pancreatic α -amylase and intestinal α -glucosidase hydrolyze the α -1, 4-glucoside linkages carbohydrates to glucose which is then transported through the mucosa of the bowel [25]. On the other hand, SGLT1 in the apical membranes of the intestinal mucosa reabsorbs glucose from the lumen across the brush border membrane which is released into the bloodstream via basolaterally expressed GLUT2 [26,27]. Therefore, the extend our previous observations by showing that the hypoglycaemic effects of triterpenes are in part mediated via inhibition of carbohydrate hydrolyzing enzymes as in the small intestines of diabetic rats. We have previously shown that triterpenes reduce blood glucose concentrations of STZ-induced diabetic rats via a variety of mechanisms [12–14].

Western blot analysis not only confirmed the inhibitory effects of OA and MA against α -glucosidase and pancreatic α -amylase, but additionally showed that the triterpenes may potentially reduce glucose absorption by decreasing the levels of SGLT and GLUT2 in the small intestines of STZ-induced

Figure 8

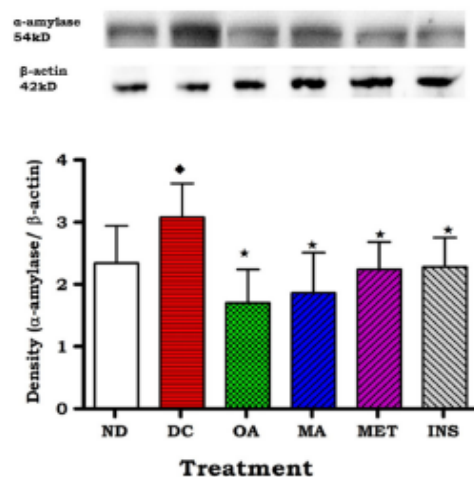


Figure 8. Effects of OA and MA on expression of α -amylase as determined by Western blotting of control, OA, MA, metformin and insulin-treated pancreatic tissues of non-diabetic and STZ-induced diabetic rats. Values are expressed as mean \pm S.E.M. Values were obtained from Western blots for six preparations.

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

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

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diabetic rats. It has been shown in rats with experimentally induced diabetes that the capacity of the small intestine to absorb glucose increases at least in part, due to enhanced activity and abundance of brush border SGLT1 and basolateral GLUT2 [5]. Therefore, it is possible that down regulation of these transporters by OA and MA reduces the total glucose

absorption capacity in the small intestine in diabetes. Indeed, untreated STZ-induced diabetic rats exhibited increased SGLT1 and GLUT2 expression in the small intestine in comparison to non-diabetic control animals.

Interestingly, *in vitro* studies indicated that the IC_{50} values of OA and MA against sucrase, α -amylase and α -glucosidase at various concentrations were less than those shown of acarbose suggesting that the triterpenes have fewer side effects. Acarbose is associated with gastrointestinal side effects due to excessive inhibition of pancreatic α -amylase, resulting in the abnormal bacterial fermentation of undigested carbohydrates in the large intestine [28,29]. Additionally, acarbose has been reported to increase the incidence of renal tumors, serious hepatic injury and acute hepatitis [30,31]. Against this background are reports that several α -glucosidase inhibitors isolated from medicinal plants possess lesser adverse effects than the existing drugs [11,32–34].

Altogether, it can be suggested that the OA and MA modulates the activity of intestinal glucose transporters and carbohydrate hydrolyzing enzymes to reduce postprandial hyperglycaemia in diabetes. The reduction by OA and MA of postprandial hyperglycaemia and the AUC_{glucose} are characteristics of effective compounds which control diabetes [35,36]. The data suggest that OA and MA could be used as a potential supplements for treating postprandial hyperglycemia.

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Author Contributions

Conceived and designed the experiments: AK MRS RBM FRVH CTM. Performed the experiments: AK MRS RBM FRVH CTM. Analyzed the data: AK MRS RBM FRVH CTM. Contributed reagents/materials/analysis tools: AK MRS RBM FRVH CTM. Wrote the manuscript: AK MRS RBM FRVH CTM.

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APPENDIX VII

The effects of *Syzygium aromaticum*-derived triterpenes on gastrointestinal ghrelin expression in streptozotocin-induced diabetic rats

Khathi A, Mbongwa HP and Musabayane CT

School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa,

Corresponding author: Professor C.T. Musabayane
Department of Human Physiology
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban 4000
South Africa
Phone: (27) (31) 260 7975
Fax: (27) (31) 260 7132
E-mail: musabayane@ukzn.ac.za

Abstract

Previous studies have shown that ghrelin regulates homeostatic food intake and hedonic eating. Elevated plasma ghrelin levels associated with diabetic polyphagia have been observed in experimental diabetes. This increase in food consumption prolongs postprandial hyperglycaemia in diabetes contributing to the development of micro- and macrovascular complications. In our laboratory we have reported that plant-derived oleanolic acid (OA) and maslinic acid (MA) reduce postprandial hyperglycaemia, in part, through the inhibition of intestinal carbohydrate hydrolyzing enzymes. However, their effects on the plasma ghrelin concentration are unclear. Accordingly, we investigated whether the effects of these triterpenes on glucose handling in various sections of the gastrointestinal tract in streptozotocin-induced diabetic rats. The effects of OA and MA on blood glucose concentration; food and water intake; as well as body weight changes were monitored over a five weeks period after which plasma ghrelin and insulin concentrations were measured. Additionally, the expression of ghrelin in the various sections of the GIT was determined using Western blot analysis. Ghrelin concentration in STZ-induced diabetic rats (3.42 ± 0.17 nmol/L) was significantly higher in comparison to the non-diabetic control (1.99 ± 0.07 nmol/L). Interestingly, the administration of these triterpenes (like the standard drugs) reduced food intake, blood glucose levels and plasma ghrelin levels in STZ-induced diabetic rats. This was complemented by significant reductions in the gastrointestinal expression of ghrelin suggesting that the anti-diabetic properties of these triterpenes are mediated in part through the reduction of food intake and the modulation of ghrelin expression.

Introduction

Ghrelin, the appetite-stimulating hormone in humans, is produced principally in the stomach (Klok *et al.*, 2007; Buss *et al.*, 2014). Hypersecretion of ghrelin can lead to several metabolic abnormalities, including obesity; hypertension; and impaired glucose tolerance in conditions such as diabetes mellitus (DM) (Ukkola, 2011). Diabetes mellitus is a heterogeneous disease that is characterized by hyperglycemia and glucose intolerance (Lin and Sun, 2010). Given the high prevalence of DM, identifying the factors that may affect its process and cause the pathological changes in the body is important (Edmann *et al.*, 2005; Ceriello, 2006). A number of obesity-related peptide hormones have been identified and have been suggested to play a role in the pathogenesis of metabolic syndrome (MS). One such molecule is ghrelin, a blood-borne signal from gut to brain, with multiple hormonal; metabolic; and cardiovascular activities (Duckworth, 2001; Poykko *et al.*, 2003). Low ghrelin concentrations have been associated with the components of the MS, like obesity, insulin resistance, and blood pressure (Ukkola, 2011). The secretion of ghrelin by the stomach depends largely on the

nutritional state (Broglia *et al.*, 2001a; Klok *et al.*, 2007). Ghrelin levels show preprandial increases and postprandial decreases (Ariyasu *et al.*, 2001a; Cummings *et al.*, 2001; Ceriello, 2005).

Increased food intake attributed to elevated plasma ghrelin concentrations is a major contributor to the development of macro- and microvascular complications of diabetes (Shintani *et al.*, 2001; Ukkola, 2011). Enhanced plasma ghrelin levels have also been observed in rats with streptozotocin-induced diabetes and are associated with diabetic polyphagia (Masaoka *et al.*, 2003; Delhanty and van der Lely, 2011). This increase in food consumption causes the body to spend more time in the postprandial state, and in diabetes mellitus this could lead chronic hyperglycaemia. Therefore, the control of plasma ghrelin levels is a critical component in the reduction of vascular diabetic complications. Clinically, the use of intense glycaemic control using the subcutaneous administration of insulin has been shown to regulate plasma ghrelin levels while metformin, a plant derived glycoside, has been shown to reduce food intake in diabetes mellitus (Ariyasu *et al.*, 2001a; Tong *et al.*, 2010). Various medicinal plant extracts have been reported to significantly reduce food intake through the reduction of plasma ghrelin levels (Bast *et al.*, 2002; Fong, 2002). *Syzygium aromaticum*-derived OA and MA have been previously shown to reduce blood glucose concentrations in STZ-induced diabetic rats in sub-chronic studies through a variety of mechanisms. Furthermore these triterpenes have been previously reported to reduce food intake (Mapanga *et al.*, 2009; Mkhwanazi *et al.*, 2014) but the effects on plasma ghrelin concentrations are not yet fully established. This study was therefore designed to evaluate the effects of these triterpenes on plasma ghrelin concentrations. We also evaluated the effects of these triterpenes on the gastrointestinal expression of ghrelin to establish whether these triterpenes had direct effects on food intake.

Materials and Methods

Drugs and chemicals

Drugs were sourced from standard pharmaceutical suppliers. All chemicals and reagents were of analytical grade and were purchased from standard commercial suppliers.

Extraction methods

OA and MA were isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] cloves using a standard protocol that has been validated in our laboratory by (Madlala *et al.*, 2012; Mkhwanazi *et al.*, 2014). Air-dried *S. aromaticum* flower buds (500 g) were milled and sequentially extracted twice at 24 h

intervals at room temperature using 1 L dichloromethane (DCM), and ethyl acetate (720 mL) on each occasion. Subsequently, the individual extract was concentrated under reduced pressure at 55 ± 1 °C using a rotary evaporator to yield dichloromethane solubles (DCMS) and ethyl acetate solubles (EAS). The EAS fraction containing mixtures of oleanolic/ursolic acid and methyl maslinic/methyl corosolic was purified by silica gel 60 column chromatography with hexane: ethyl acetate solvent systems, 7:3 for OA and 6:4 for MA. This yielded OA and MA, respectively which were further purified by recrystallization from chloroform: methanol (1:1, v/v). The structures of OA and MA were confirmed by spectroscopic analysis using 1D and 2D, ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopic experiments.

Animals

Male Sprague-Dawley rats (250-300 g) maintained on free access to standard rat chow (Meadows, Pietermaritzburg, South Africa) and water *ad libitum* were used throughout the study. These animals were maintained in standard environmental conditions with 12h light/12h dark cycle. All animal protocols were reviewed and approved by the University of KwaZulu-Natal animal ethics committee.

Induction of diabetes mellitus

Experimental type 1 diabetes mellitus was induced in male Sprague-Dawley rats using a previously described protocol (Ngubane *et al.*, 2011; Khathi *et al.*, 2013). Briefly, each animal was administered a single intraperitoneal injection of 60 mg/kg STZ in freshly prepared 0.1M citrate buffer (pH 6.3). The control group received the citrate buffer through the same route. Animals that exhibited glucosuria after 24 h, tested using urine strips (Rapidmed Diagnostics, Sandton, South Africa) were considered diabetic. A blood glucose concentration greater than 20 mmol/L in STZ-induced diabetic rats after seven days was considered as stable diabetes.

Experimental design

Non-diabetic and STZ-induced diabetic rats were divided into separate groups to study the sub-chronic effects of OA and MA treatment on blood glucose; food and water intake; as well as changes in body after a period of 5 weeks, after which plasma ghrelin and insulin concentrations were measured ($n = 6$ in each group). Furthermore, the expression of ghrelin in the various sections of the gastrointestinal tract was determined using Western Blot analysis.

Sub-chronic studies

To assess the influence of OA and MA on blood glucose, food intake, water intake as well as changes in body weight, groups of non-diabetic and STZ-induced diabetic male Sprague-Dawley rats were housed individually in Makrolon polycarbonate metabolic cages (Techniplats, Labotec, South Africa) for a 5-weeks period (n = 6 in each group). In the animals where the effects of OA/MA were investigated, the rats were administered with OA/MA (80 mg/kg) twice daily at 09h00 and 15h00 by means of a bulbed steel tube. Rats treated with DMSO/saline (3 mL/kg, p.o.) served as untreated controls while those treated with standard anti-diabetic drugs (metformin, 500 mg/kg, p.o. and insulin, 175µg/kg, sc) served as treated positive controls.

Tissue sample harvesting

At the end of the 5 weeks experimental period, all animals were sacrificed by exposure to halothane (100 mg/kg, for 3 min) via a gas anaesthetic chamber. Blood was collected from separate parallel groups of non-diabetic and STZ-induced diabetic rats prepared as for the sub-chronic study for plasma ghrelin and insulin determination. Thereafter, stomach, small intestine and large intestine samples tissues were removed, snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijders Scientific, Tilburg, Netherlands) at -70°C for Western blot analysis of ghrelin expression.

Hormone measurements

The plasma ghrelin concentrations were measured by using ultra-sensitive rat ghrelin ELISA kit (DRG diagnostics EIA-3706 GmbH, Marburg, Germany). This immunoassay allows for accurate quantification due to the competitive binding of the biotinylated ghrelin and the ghrelin in samples to the ghrelin antibody. The lower and upper limits of detection were 0.1pmol/L and 960pmol/L respectively. The intra-assay analytical coefficient of variation ranged from 4.2% to 6.8% while the inter-assay coefficient variation ranged from 5.9% to 9.2%. The plasma insulin concentrations were measured by ultrasensitive rat insulin ELISA kit (DRG Instruments GmbH, Marburg, Germany). The immunoassay is a quantitative method utilizing two monoclonal antibodies which together are specific for insulin. The lower limit of detection was 1.74pmol/L. The intra- and inter-assay analytical coefficients of variation ranged from 4.4 to 5.5% and from 4.7 to 8.9%, respectively.

Western Blot Analysis

Stomach, small intestine and large intestine tissues harvested from the untreated and treated controls as well as from the triterpene treated STZ-induced diabetic rats at the end of 5 weeks were analyzed for ghrelin expression using Western blotting. These tissues (0.1 g) were homogenized on ice in isolation buffer (0.5 mM Na₂EDTA,

Page | 5

0.1 M KH_2PO_4 , 0.1 mM dithiothreitol, 0.25 M sucrose) and then centrifuged at 400 x g for 10 min (4°C). The protein content was quantified using the Lowry method (Lowry *et al.*, 1951). All the samples were standardized to one concentration (1mg/mL). The proteins were then denatured by boiling in Laemmli sample buffer (0.5 M Tris-HCl, glycerol, 10% sodium dodecyl sulphate (SDS), 2-mercaptoethanol, 1% bromophenol blue) for 5min. The denatured proteins were loaded (25µL) on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with molecular weight marker (5µL). The gel was electrophoresed for 1h at 150 V in electrode (running) buffer (Trisbase, glycine, SDS), pH 8.3). Following electrophoresis, the resolved proteins were electro-transferred to a nitrocellulose membrane for 30 min in transfer buffer (192 mM glycine, 25 mM Tris, 10% methanol). After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (20 mM Tris, 150 mM NaCl, KCl, 0.05% Tween-20).

The stomach and intestinal membranes were then immuno-probed with the antibody for ghrelin (1:500 in 1% BSA, Neogen, USA) for 1 h at room temperature (RT). The nitrocellulose membrane was then subjected to 5 washes (10min each with gentle agitation) with TTBS. The membranes were then incubated in horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:1000; Bio-Rad) for 1 h at RT. After further washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analysed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was done on the resultant bands.

Statistical analysis

Data are expressed as means \pm SEM. Statistical significance was performed with GraphPadInStat Software (version 5.00, GraphPad Software, San Diego, California, USA), for analysis of one-way variance (ANOVA), followed by Tukey-Kramer multiple comparison test.

Results

Structure elucidation

OA

The purity of the *S. aromaticum*-derived OA (see Figure 6) determined by ^1H NMR and ^{13}C NMR (1D and 2D) was approximately 98% and the percentage yields varied from 0.79% to 1.72%. The ^1H NMR and ^{13}C NMR (1D and 2D) given below was obtained.

^1H NMR CDCl_3 δ , 0.73, 0.75, 0.88, 0.89, 0.90, 0.96, 1.11 (each 3H, s), 2.84 (^1H , dd, $J = 10.36$), 3.22 (^1H , dd, $J = 4.56$, 5.26 (^1H , t, $J = 3.76$);

^{13}C NMR (CDCl_3) : δ 183.5, 143.8, 122.7, 79.2, 55.4, 47.8, 46.8, 46.1, 41.8, 41.2, 39.5, 38.9, 38.6, 38.3, 34.0, 33.3, 32.8, 32.7, 31.6, 28.4, 27.9, 27.4, 26.2, 23.8, 23.7, 23.1, 18.5, 17.4, 15.8, 15.5.

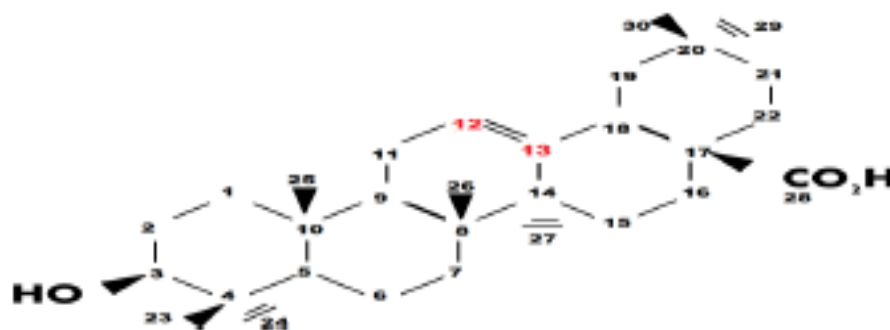


Figure 1. Chemical structure and IUPAC numbering of OA as determined through ^1H and ^{13}C NMR spectroscopy

MA

The purity of the *S. aromaticum*-derived MA (see Figure 8) determined by ^1H and ^{13}C NMR (1D and 2D) was approximately 98% and the percentage yields varied from 0.02% to 0.03%. The ^1H and ^{13}C NMR (1D and 2D) given below was obtained.

^{13}C NMR: δ_{H} (^{13}C NMR (400 MHz, CD_3OD): 46.2 (C-1), 68.3 (C-2), 83.3 (C-3), 39.1 (C-4), 55.0 (C-5), 18.1 (C-6), 32.7 (C-7), 39.0 (C-8), C-9(47.4), C-10 (38.0), C-11 (23.2), C-12 (121.9), C-13 (143.7), C-14 (41.6), C-15 (27.4), C-16 (23.0), C-17 (46.2), C-18(41.0), C-19 (45.7), C-20 (30.4), C-21 (33.6), C-22 (32.3), C-23 (28.3), C-24 (16.6), C-25 (16.5), C-26 (16.4), C-27(23.2), C-28 (178.5), (C-29) 32.2, C-30 (23.2).

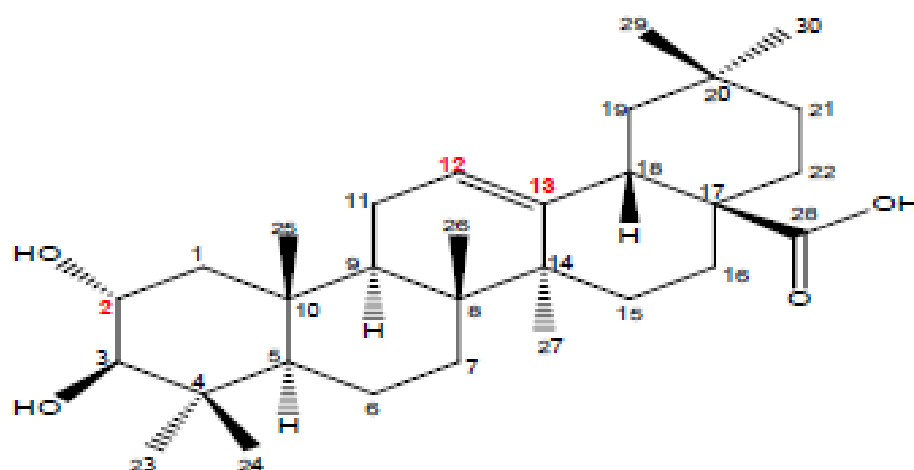


Figure 2. Chemical structure and IUPAC numbering of MA as determined through ^1H and ^{13}C NMR spectroscopy

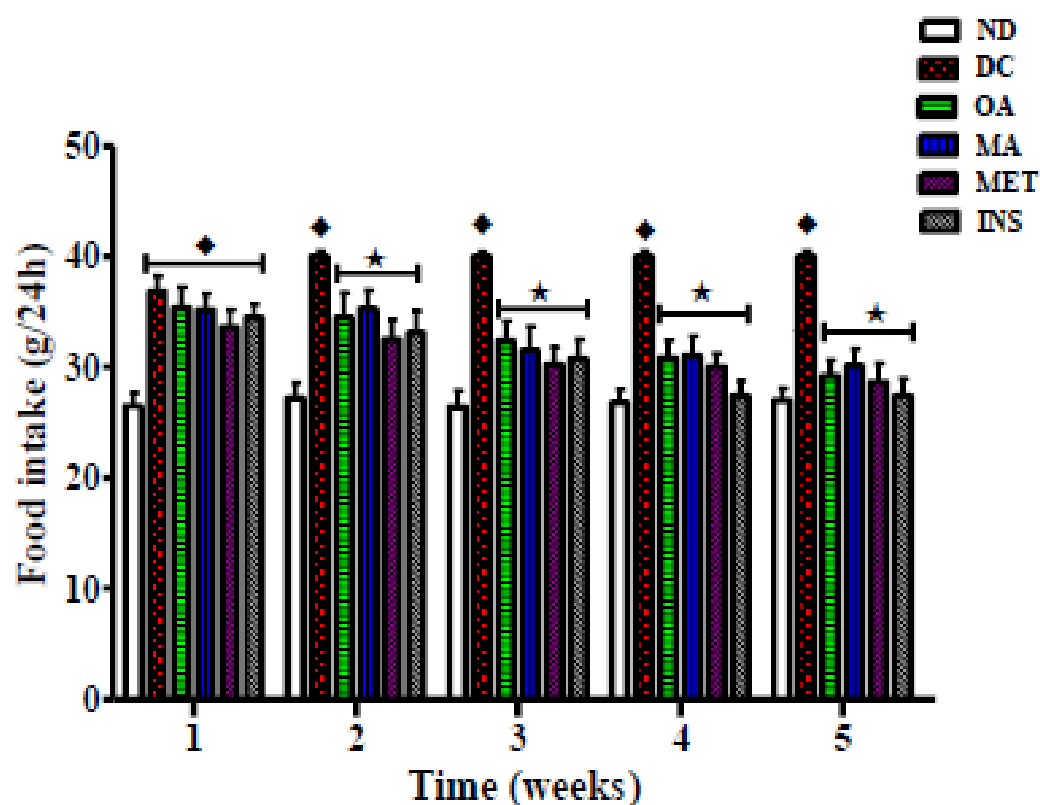


Figure 3. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on plasma ghrelin concentrations with untreated STZ-diabetic rats. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). * = $p < 0.05$ by comparison to the non-diabetic control. ★ = $p < 0.05$ by comparison to the STZ-induced diabetic control

Table 1. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on terminal blood glucose concentrations, plasma insulin and ghrelin concentrations with untreated STZ-diabetic rats. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). * = p<0.05 by comparison to the non-diabetic control. ★ = p<0.05 by comparison to the STZ-induced diabetic control

	Terminal blood glucose (mmol/L)	Plasma insulin (pmol/L)	Plasma ghrelin (pmol/L)
Non-diabetic	5.46 ± 0.83	9.37 ± 0.86	1.99 ± 0.07
STZ-diabetic	26.95 ± 2.00*	2.30 ± 0.74*	3.42 ± 0.17*
STZ-OA treated	7.54 ± 1.10*	2.35 ± 0.84*	1.96 ± 0.11*
STZ-MA treated	8.35 ± 2.00*	2.36 ± 0.79*	2.44 ± 0.10*
STZ-Metformin treated	7.68 ± 1.15*	2.34 ± 0.77*	2.34 ± 0.14*
STZ-Insulin treated	6.45 ± 1.27*	2.37 ± 0.43*	1.91 ± 0.15*

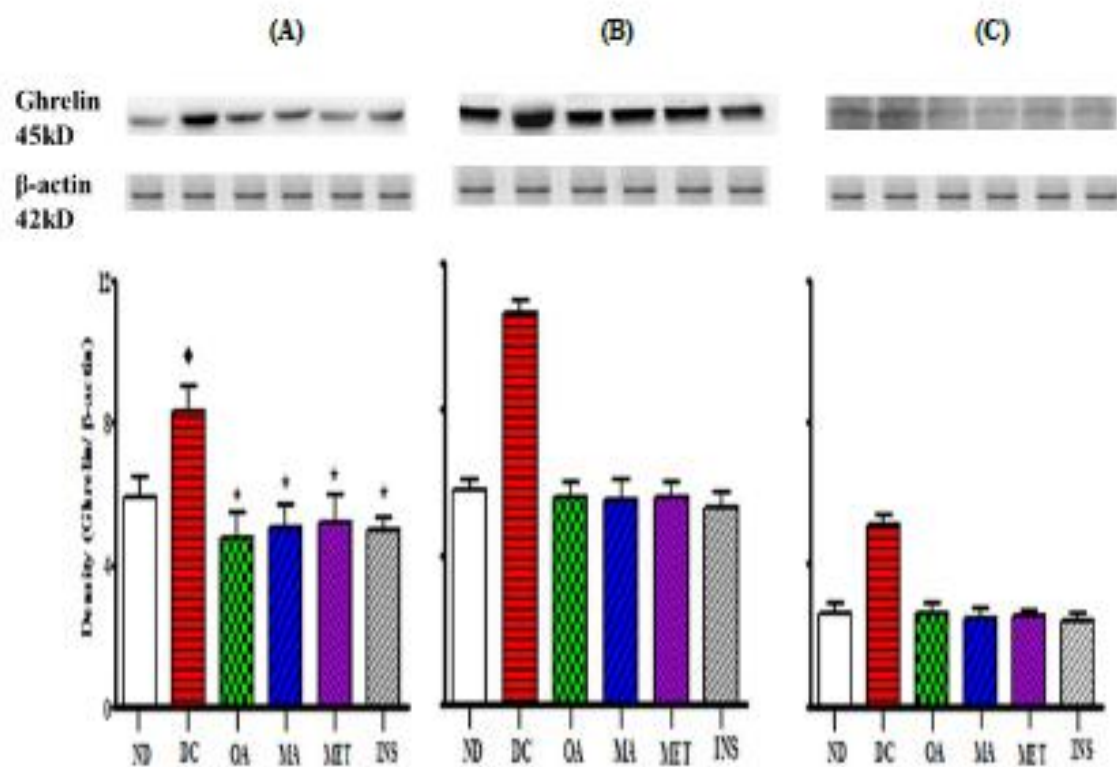


Figure 4. Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on gastric fundus ghrelin expression in the gastric fundus of the stomach (A), small intestine (B) and large intestine (C) with untreated STZ-diabetic rats and those treated with the standard drugs metformin and insulin. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). + = $p < 0.05$ by comparison to the non-diabetic control. * = $p < 0.05$ by comparison to the STZ-induced diabetic control.

Discussion

The aim of the present study was to investigate the effects of *Syzygium-aromaticum* derived OA and MA on plasma ghrelin concentrations in STZ-induced diabetic rats in an effort to further elucidate the hypoglycaemic mechanisms of these triterpenes. The stereostructure of *S. aromaticum*-derived OA and MA was elucidated using ^1H - and ^{13}C -NMR and were comparable to the previously reported data (Mahato and Kundu, 1994; García-Granados *et al.*, 2000; Julio *et al.*, 2003). We have previously reported that OA and MA could prevent postprandial hyperglycaemia through the down-regulation of key intestinal carbohydrate hydrolyzing enzymes such as α -amylase and α -glucosidase as well as intestinal glucose transporters such as SGLT1 and GLUT2. The results of the present study suggest that OA and MA down-regulate the gastrointestinal expression of ghrelin to reduce food intake and thereby prevent postprandial hyperglycaemia.

The administration of OA and MA to STZ-diabetic rats significantly reduced the blood glucose concentrations in the sub-chronic studies. These results were in agreement with previous observations from studies conducted in our laboratory (Mapanga *et al.*, 2009; Musabayane *et al.*, 2010; Ngubane *et al.*, 2011; Mkhwanazi *et al.*, 2014). However, both OA and MA had no significant effect on plasma insulin concentrations in the STZ-diabetic rats suggesting that the blood glucose lowering effects may be exerted via extra-pancreatic mechanisms.

Plant bioactive compounds such as triterpenes have been reported to exert their anti-hypoglycaemic effects through a variety of mechanisms (Grover *et al.*, 2002; Ali *et al.*, 2002; Dzubak *et al.*, 2006; Bhat *et al.*, 2008; Abdul-Ghani and DeFronzo, 2014). One of these mechanisms is slowing down the absorption of glucose in the small intestine to prevent postprandial hyperglycaemia (Ferraris, 2001; Kim *et al.*, 2008). Another mechanism is through the reduction of food intake to decrease the amount of glucose available for absorption in the gastrointestinal tract. The administration of OA and MA was found to significantly reduce food intake in the STZ-diabetic animals.

Ghrelin is a circulating hormone that acts on peripheral and central targets to stimulate food intake (Nakazato *et al.*, 2001; Wren *et al.*, 2001). Plasma levels of this peptide increase on fasting and decrease after habitual feeding, thus showing a pattern reciprocal to that of insulin (Broglia *et al.*, 2001b; Egido *et al.*, 2002; Reimer *et al.*, 2003; Dezaki *et al.*, 2004). Ghrelin suppresses glucose-induced insulin release via Kv channel-mediated attenuation of Ca^{2+} signalling in the pancreatic β -cells (Dezaki *et al.*, 2007). Previous *in vivo* studies in humans also support the notion that insulin can regulate ghrelin secretion suggesting that the absence of insulin for the homeostatic control of blood glucose levels impedes the suppression of ghrelin secretion from the gastrointestinal tract (McCowen *et al.*, 2002; Flanagan *et al.*, 2003; Foster-Schubert *et al.*, 2008). Indeed, enhanced plasma ghrelin levels have been observed in individuals with diabetes mellitus and these are

associated with diabetic polyphagia (Ariyasu *et al.*, 2001b; Broglio *et al.*, 2001b; Delhanty and van der Lely, 2011). Similarly, the results of the present study showed that the plasma ghrelin levels were significantly higher in the STZ-diabetic animals possibly as a result of the decreased plasma insulin levels.

Interestingly, the administration of OA and MA reduced food intake with concomitant decreases in plasma ghrelin concentrations. The administration of other standard anti-diabetic drugs such as insulin and metformin were also found to significantly reduce the magnitude of the parameters. In one study, the infusion of insulin with purposeful maintenance of normoglycaemia led to a rapid fall in ghrelin levels, suggesting that insulin suppresses ghrelin secretion independently of the degree of glycaemia (Flanagan *et al.*, 2003). In another study, metformin was found to decrease food intake in obese individuals with type 2 non-insulin dependent diabetes mellitus (Lee and Morley, 2012). Western blot analysis further confirmed our results as the STZ-diabetic rats treated with OA and MA showed significant reductions in the expression of ghrelin possibly leading to the reduction in food intake. These results further support our previous observations that the administration of these triterpenes prevents postprandial hyperglycaemia through a reduction of the activity of both the carbohydrate hydrolyzing enzymes as well as the intestinal glucose transporters as this would be a direct consequence of a reduction in food intake.

Taken together, the findings of the study suggest that the control of food intake through the reduction of ghrelin expression by OA and MA may constitute an avenue of glycaemic control in diabetes mellitus. Additionally, the data suggests that OA and MA could be used as a potential supplements for treating postprandial hyperglycaemia.

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Declaration of Interest

The authors declare that there is no interest that could be perceived as prejudicing the impartiality of the research reported.

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APPENDIX VIII

Society for Endocrinology BES 2013, Harrogate, UK



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EFFECTS OF SYZYGIUM AROMATICUM-DERIVED OLEANOLIC ACID ADMINISTRATION ON POSTPRANDIAL GLUCOSE CONCENTRATION AND KEY INTESTINAL CARBOHYDRATE HYDROLYZING ENZYMES IN STREPTOZOTOCIN-INDUCED DIABETIC RATS.

Sinenkosi C Dube, Andile Khathi, Metse Serumula, Rene Myburg & Cephas T Musabayane

School of Laboratory Medicine & Medical Sciences, University of KwaZulu-Natal,
Private Bag X54001, Durban 4000, South Africa.

INTRODUCTION

The magnitude and duration of postprandial hyperglycaemia due to hydrolysis of carbohydrates in the small intestine are major risk factors of macro- and microvascular complications in diabetes¹. Indeed, diabetes management strategies may involve several α-glucosidase inhibitors (acarbose, voglibose, miglitol) that suppress postprandial glucose peaks². Reports from our laboratory indicate that *Syzygium aromaticum*-derived oleanolic acid (OA) inhibits the absorption of glucose across the small intestine³. The influence of this terpene on postprandial blood glucose concentrations is not yet established.

OBJECTIVES

- The objectives of this study were to:
 - I. evaluate the influence of OA on postprandial hyperglycaemia after mono-, di- and polysaccharide loading in normal and STZ-induced diabetic rats
 - II. examine the effects of OA on the hydrolysis of di- and polysaccharides *in vitro*.

MATERIALS AND METHODS

Isolation of oleanolic acid
OA was isolated from *Syzygium aromaticum* (Linnæus) Merril & Perry (Myrtaceae) (leaves) flower buds using a standard protocol that has been validated in our laboratory⁴. 2 g aromatic flowers were sequentially extracted twice of 24 h intervals with 1 L on each occasion of dichloromethane (DCM) and ethyl acetate yielding dichloromethane soluble (DCMS) and ethyl acetate soluble (EAS). Recrystallisation of EAS with ethanol yielded pure OA whose structure was confirmed by spectroscopic analysis using 1D and 2D, ¹H and ¹³C NMR techniques.

EXPERIMENTAL DESIGN

The effects of OA on postprandial blood glucose changes were assessed in non-diabetic and STZ-induced diabetic male Sprague Dawley rats while the effects on intestinal carbohydrate-hydrolyzing enzymes were investigated *in vitro*.

IN VIVO STUDIES

Oral glucose tolerance (OGT) responses
OGT responses were monitored in non-diabetic and STZ-induced diabetic rats loaded with monosaccharide (glucose; 0.66 g/kg, p.o.), disaccharide (sucrose; 1.72 g/kg, p.o.) and polysaccharide (starch; 0.66 g/kg, p.o.) after an 18 h fast (n = 4 in each group). Rats treated with deionised water (3 ml/kg, p.o.), or acarbose (100 mg/kg, p.o.) acted as negative and positive controls, respectively. Blood glucose was monitored at 15 min intervals for the first 60 minutes and once at 120 minutes.

IN VITRO STUDIES

Effects of OA on carbohydrate hydrolysing enzymes
Various concentrations of OA (25-100 µg/ml) were used to determine the IC₅₀ value for OA on sucrose, dextrane and α-glucosidase with sucrose (2 mg/ml) and starch (2 mg/ml) serving as substrates.

STATISTICAL ANALYSIS

All data are expressed as means ± S.E.M. The AUC₀₋₁₂₀ values were calculated using blood glucose concentrations following the 2-hour loading with mono-, di- or polysaccharide. The IC₅₀ values of OA against carbohydrate hydrolysing enzymes were calculated using the inhibitory activity of OA at various concentrations. Overall statistical comparisons between the control means and experimental group were performed with GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. A value of p < 0.05 was considered significant.

RESULTS AND DISCUSSION

By comparison with animals pre-loaded with carbohydrates alone, co-administration of OA with glucose, sucrose and starch significantly reduced the peak blood glucose spikes of separate groups of non-diabetic and STZ-induced diabetic rats (Figure 1 and 2) with a concomitant reduction in the C_{max} values (Table 1). The suppression of the postprandial glucose spikes response by OA to carbohydrate loads was associated with the reduction of the area under the blood glucose-time curve (AUC₀₋₁₂₀) of non-diabetic and diabetic animals (Table 1). The *in vitro* hydrolytic inhibitory concentrations (IC₅₀) of OA on sucrose, α-amylase and α-glucosidase compared with that of acarbose, the standard drug (Table 2).

CONCLUSION

These results support that OA suppresses postprandial hyperglycaemia perhaps via the inhibition of the carbohydrate hydrolysing enzymes in the small intestine.

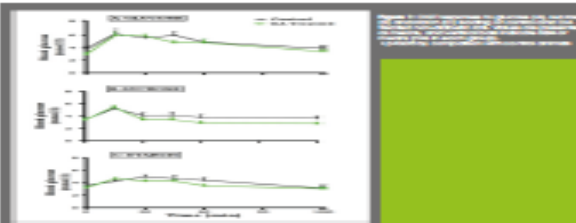


Table 1: Postprandial blood glucose levels (mg/dL) and AUC₀₋₁₂₀ (mg/dL·min) for non-diabetic and STZ-induced diabetic rats.

Group	Substrate	Peak Blood Glucose (mg/dL)	AUC ₀₋₁₂₀ (mg/dL·min)
Non-diabetic	Glucose	~100	~1000
	Sucrose	~120	~1200
	Starch	~110	~1100
STZ-induced diabetic	Glucose	~150	~1500
	Sucrose	~180	~1800
	Starch	~160	~1600

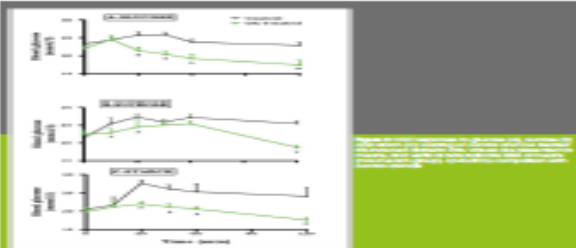


Table 2: IC₅₀ values (µg/ml) of OA and acarbose against sucrose, dextrane, and α-glucosidase.

Enzyme	OA IC ₅₀ (µg/ml)	Acarbose IC ₅₀ (µg/ml)
Sucrose	~25	~10
Dextrane	~50	~20
α-glucosidase	~100	~40

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APPENDIX IX

Endocrine Abstracts

endocrine-abstracts.org/ea/0028/ea0028p207.htm

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

Blessing Mkhwanazi¹, Andile Khathi¹, Masola Bubuya², Fanie van Heerden³ & Cephas Musabayane¹

Author affiliations

¹Human Physiology, University of KwaZulu-Natal, Durban, South Africa; ²Biochemistry, University of KwaZulu-Natal, Durban, South Africa; ³Chemistry, University of KwaZulu-Natal, Durban, South Africa.

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

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

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APPENDIX X

ORAL PRESENTATION 15

THE EFFECTS OF *SYZYGIUM AROMATICUM*-DERIVED TRITERPENES ON GASTROINTESTINAL GHRELIN EXPRESSION IN STREPTOZOTOCIN –INDUCED DIABETIC RATS

AUTHOR/S	INSTITUTE	EMAIL
¹ A Khathi ¹ H Mbongwa ² FR Van Heerden ¹ CT Musabayane	Schools of ¹ Laboratory Medicine and Medical Sciences ² Chemistry and Physics, University of KwaZulu-Natal	akhathi@gmail.com

ABSTRACT

Enhanced plasma ghrelin levels have been observed in rats with streptozotocin-induced diabetes and are associated with diabetic polyphagia. This increase in food consumption causes the body to spend more time in the postprandial state and in diabetes mellitus this could lead to chronic hyperglycaemia. Consequently, this contributes to the development of micro- and macrovascular complications of diabetes. The subcutaneous administration of insulin has been shown to reduce plasma ghrelin concentrations; however, this has been associated with the development of hyperinsulinaemia. Recent reports from our laboratory indicate that *Syzygium aromaticum* derived oleanolic acid (OA) and maslinic acid (MA) reduce postprandial hyperglycaemia, in part, through the inhibition of intestinal carbohydrate hydrolyzing enzymes, however, their effects on the gastrointestinal expression of ghrelin are not yet known. Accordingly, this study investigated the effects of these triterpenes on gastrointestinal ghrelin expression in streptozotocin induced diabetic rats. The effects of OA and MA on blood glucose concentration, food and water intake as well as body weight changes were evaluated in STZ-induced diabetic rats kept on a standard rat diet for 5-weeks. Plasma ghrelin and insulin concentrations were investigated after the study. Furthermore the expression of ghrelin was analyzed by Western blot analysis in the gastrointestinal tract. The induction of diabetes significantly increased plasma ghrelin concentrations in comparison to the non-diabetic control. Interestingly, the administration of the triterpenes, like insulin, reduced food intake, blood glucose levels and plasma ghrelin levels in STZ-induced diabetic rats. This was accompanied by significant reductions in the gastrointestinal expression of ghrelin.

APPENDIX XI

University of Oxford (2011) **Proc Physiol Soc 23, C29**

Oral Communications

The effects of *Syzygium aromaticum* derived oleanolic acid on glucose transport across rat-everted intestinal sacs *in vitro*

A. Khathi¹, P. S. Ngubane¹, B. Masola² and C. T. Musabayane¹

1. Human Physiology, University of KwaZulu-Natal, Durban, KwaZulu-Natal, South Africa. 2. Biochemistry, University of KwaZulu-Natal, Durban, KwaZulu-Natal, South Africa.

The hypoglycaemic effects of *Syzygium aromaticum* derived triterpene, oleanolic acid (OA) in streptozotocin (STZ)-induced diabetic rats are mediated in part via increased hepatic glycogen synthesis [1]. The wide range of available anti-diabetic suggests that a variety of mechanisms of action are involved in the blood glucose lowering effects. The present study was designed to investigate the effects of OA on glucose transport *in vitro* using the everted rat intestinal protocol which has been previously described by Mahomoodally *et al.*, 2005. [2] Everted intestinal sacs from rats filled with 1 ml of Krebs-Henseleit bicarbonate buffer (KHB) were mounted in an organ bath containing 50 ml of the same incubation medium. D-glucose (10 mM) was added to the medium just before the start of the appropriate experiments. In separate preparations graded concentrations of either OA (0.375-3.00 mM) or the standard drug, phlorizin (10^{-6} - 10^{-3} M) were incubated for 30 min in the mucosal bathing fluid containing glucose (10 mM) to investigate effects on glucose transport across the intestine gut wall. The external incubation medium will be continuously bubbled with gas mixture of 95% oxygen) and 5% carbon dioxide during the whole incubation period. The organ bath was surrounded by a water jacket maintained at 37-40 °C. The transport of D-glucose was evaluated by measuring the increase in glucose concentration inside the intestinal sacs after 30 min of incubation. The change in glycogen concentration in the gut wall was interpreted as assessed glucose metabolized. Graph Pad InStat software (version 4) using one way analysis of variance (ANOVA) followed by Tukey- Kramer multiple comparison test was used. P values < 0.05 were considered significant. Lower concentration (0.375 and 0.750 mM) of OA significantly inhibited ($p < 0.05$) D-glucose transport across the rat everted intestinal sac in a dose-dependent manner whereas the inhibitory effects of higher concentrations of 0.750, 1.500 and 3.000 mM could not be statistically separated. Phlorizin, however, exhibited dose-dependent inhibition of glucose uptake across the everted intestinal sac. The accumulation of glycogen concentration in the gut wall increased significantly in the presence of OA. The fate of glucose retained within intestinal wall is difficult to quantify since glucose is rapidly metabolized by enterocytes. We hypothesize that OA inhibits the active transport of d-glucose suggesting that the triterpene can be a potential alternative drug therapy of postprandial hyperglycaemia via inhibition of glucose uptake across the small intestine.

APPENDIX XII

College of Health Science Research Symposium 2014

THE EFFECTS OF PLANT DERIVED TRITERPENES ON PLASMA GHRELIN CONCENTRATIONS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

Luvuno, M; **Khathi A**; Mbongwa, H.P and Musabayane, C.T

School of Laboratory Medicine and Medical Sciences

Recent studies in our laboratory suggest that the hypoglycaemic effects of plant-derived triterpenes oleanolic acid (OA) and maslinic acid (MA) arise, in part, through the reduction of food intake. Conventional treatments reduce food intake through the suppression of ghrelin secretion and therefore prevent sustained hyperglycaemia.

Accordingly, we investigated the effects of OA and MA on plasma ghrelin concentrations as well as gastrointestinal ghrelin expression in non-diabetic and STZ-induced diabetic rats treated with OA and MA for 5 weeks. We determined the plasma ghrelin concentrations of non-diabetic and STZ-induced diabetic rats treated with OA and MA for 5 weeks using ELISA. The results have shown that the plasma ghrelin concentrations of untreated diabetic rats were significantly increased compared to non-diabetic rats (3.42 ± 0.17 vs. 1.99 ± 0.06 mmol/L) respectively. Interestingly, the plasma ghrelin concentrations of triterpene-treated diabetic rats were significantly decreased to levels comparable with the non-diabetic control (OA: 1.96 ± 0.17 ; MA: 2.43 ± 0.11 mmol/L).

Furthermore, we also determined the ghrelin gene expression in the gastrointestinal tract using Western blot analysis. The results show that the ghrelin expression of untreated diabetic rats was greatly increased in comparison to the non-diabetic rats. However, the gastric and intestinal expressions of triterpene-treated rats were significantly reduced in comparison to the STZ-diabetic control.

We conclude that the anti-hyperglycaemic properties of OA and MA arise, in part, through the reduction of food intake mediated by decreases in plasma ghrelin concentrations. Therefore, OA and MA can be utilised as alternative conventional drugs to prevent sustained hyperglycaemia.

APPENDIX XIII

PSSA 2013; Wyndham entry 24

EFFECTS OF *SYZYGIUM AROMATICUM*-DERIVED TRITERPENES ON POSTPRANDIAL BLOOD GLUCOSE IN STREPTOZOTOCIN-INDUCED DIABETIC RATS FOLLOWING CARBOHYDRATE CHALLENGE

¹Andile Khathi, ²Metse Serumula, ²Rene Myburg & ¹Cephas T Musabayane

School of Laboratory Medicine and Medical Sciences, Disciplines of ¹Human Physiology & ²Biochemistry, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa.

ABSTRACT

Dietary carbohydrate hydrolysis in the small intestine increases the magnitude and duration of postprandial hyperglycaemia in diabetes leading to the development of macro- and microvascular complications. Indeed, drugs such as acarbose and miglitol which inhibit key carbohydrate hydrolyzing enzymes in the small intestine are used to manage diabetes. Reports indicate that *Syzygium spp*-derived oleanolic acid (OA) and maslinic acid (MA) possess hypoglycaemic properties, but their effects on postprandial hyperglycaemia and intestinal carbohydrate metabolism remain unclear. Accordingly, this study was designed to investigate the effects of OA and MA on intestinal carbohydrate hydrolyzing enzymes and postprandial blood glucose concentrations in non-diabetic and STZ-induced diabetic rats associated with polysaccharide, disaccharide and monosaccharide challenge after an 18h fast. To assess the influence of OA and MA on intestinal glucose transport, the expression of SGLT1 and GLUT2 were assessed in small intestines isolated from rats treated with OA and MA twice daily for 5 weeks using Western blot analysis.

OA and MA ameliorated postprandial hyperglycemia in carbohydrate loaded diabetic rats as indicated by the significantly small glucose area under the curve (AUC_{0-2h}) in treated diabetic animals compared with untreated diabetic rats. Western blotting showed significantly elevated SGLT1 and GLUT2 expressions in the small intestine tissues of untreated STZ-induced diabetic rats compared with control non-diabetic animals. OA and MA treatment not only down-regulated the diabetes-induced increase of these transporters, but also inhibited intestinal α -amylase and α -glucosidase activity. These results suggest that OA and MA could be used as potential supplements for treating postprandial hyperglycaemia.

Keywords

: Postprandial glucose; α -glucosidase; α -amylase; oleanolic acid; maslinic acid

APPENDIX XIV

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Effects of *Syzygium aromaticum*-derived oleanolic acid on glucose transport and glycogen synthesis in the rat small intestine.

Khathi A¹, Masola B², Musabayane CT¹

¹Faculty of Health Sciences, Discipline of Human Physiology Faculty of Science and Agriculture, ²Department of Biochemistry, University of KwaZulu-Natal, Durban, South Africa.

Abstract

In the present study, we investigated the effects of oleanolic acid (OA), which has hypoglycemic properties, on glucose transport and glycogen synthesis in the small intestine, an organ that secretes enzymes involved in carbohydrate metabolism. The OA was isolated from *Syzygium aromaticum* ethyl acetate-soluble fractions followed by recrystallization with ethanol. It was diluted to required concentrations in freshly prepared dimethyl sulphoxide (2 mL) and normal saline (19 mL) before being administered to rats (p.o.). Glycogen concentrations were determined in isolated small intestines from fasted and non-fasted non-diabetic and streptozotocin-diabetic rats after 18 h treatment with 80 mg/kg, p.o., OA or standard hypoglycemic drugs (i.e. 100 µg/kg, s.c., insulin; 500 mg/kg, p.o., metformin). In a separate series of experiments, the effects of 30-min incubation with graded concentrations of OA (0.82-6.56 mmol/L) on d-glucose were evaluated by monitoring changes in glucose concentrations inside and outside of intestinal sacs isolated from fasted, non-diabetic rats and mounted in an organ bath containing Krebs-Henseleit bicarbonate buffer. All in vivo treatments increased the glycogen concentration in rat small intestine, although the effects of metformin treatment in non-fasted diabetic rats failed to reach statistical significance. In vitro, both OA (1.64-6.56 mmol/L) and phlorizin (10^{-5} - 10^{-3} mol/L) decreased glucose transport from the mucosa to the serosa. The data suggest that OA may be a potential alternative drug treatment for postprandial hyperglycemia because of its inhibition of glucose uptake across the small intestine and its concomitant conversion of glucose to glycogen in the intestinal wall.