

THE EFFECTS OF *Syzygium aromaticum*-DERIVED TRITERPENES ON GASTROINTESTINAL
GHRELIN EXPRESSION IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Abstract

Background: Diabetic polyphagia has been associated with elevated plasma ghrelin levels in experimental type 1 diabetes. This increase in food consumption contributes to chronic hyperglycaemia in diabetes thus contributing to the development of micro- and macrovascular complications. We have reported that plant-derived oleanolic acid (OA) and maslinic acid (MA) reduce blood glucose levels, in part, through the inhibition of intestinal carbohydrate hydrolyzing enzymes and glucose transporters. However, their effects on food intake and plasma ghrelin concentrations are unclear. Accordingly, we investigated the effects of these triterpenes on food intake and ghrelin expression in streptozotocin-induced diabetic rats.

Material: The effects of OA and MA on blood glucose concentration; food and water intake were monitored over five weeks after which plasma ghrelin concentrations were measured. Additionally, the expression of ghrelin in the various sections of the GIT was determined using Western blot analysis.

Results: Ghrelin concentrations in untreated STZ-induced diabetic rats were significantly higher in comparison to the non-diabetic control. Interestingly, the administration of OA and MA reduced food intake, blood glucose levels and plasma ghrelin levels in STZ-induced diabetic rats. This was further 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.

Conclusion: The findings of the study suggest that the control of food intake through the reduction of ghrelin expression by plant-derived OA and MA may constitute an avenue of glycaemic control in diabetes mellitus.

Keywords: diabetes mellitus, ghrelin, *Syzygium aromaticum*, triterpenes, oleanolic acid, maslinic acid.

Introduction

Diabetes mellitus is a metabolic disorder that is characterized by hyperglycaemia and glucose intolerance (Lin & Sun, 2010). Given the high prevalence of diabetes mellitus globally as well as the associated macro- and microvascular complications, identifying the factors that may affect its progress and cause the pathological changes in the body is important (Ceriello, 2006; Edmann et al., 2005). The secretion of ghrelin, an appetite-stimulating hormone in humans produced primarily in the gastrointestinal tract has been found to play a role in the progression of diabetes (Buss et al., 2014; Klok et al., 2007). Hypersecretion of ghrelin caused by a lack of insulin effects can lead to diabetic polyphagia. This in turn can lead to the development of several metabolic abnormalities that include obesity and cardiovascular complications (Duckworth, 2001; Ukkola, 2011). Therefore, increased food intake ascribed 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). Additionally, enhanced plasma ghrelin levels have also been observed in rats with streptozotocin-induced diabetes and are associated with diabetic polyphagia (Delhanty & van der Lely, 2011; Masaoka et al., 2003). The control of plasma ghrelin concentrations is of critical importance in the management of diabetes mellitus. Clinically, the use of intense glycaemic control via the subcutaneous administration of insulin has been shown to regulate plasma ghrelin levels (Tong et al., 2010). Metformin, a plant derived glycoside, has also been shown to reduce food intake in diabetes mellitus (Ariyasu et al., 2001; Tong et al., 2010). Various medicinal plant extracts have been reported to significantly reduce food intake possibly through the reduction of plasma ghrelin levels (Bast et al., 2002; Fong, 2002). Studies conducted in our laboratory have shown that *Syzygium aromaticum*-derived oleanolic acid (OA) and maslinic acid (MA) reduce blood glucose concentrations in STZ-induced diabetic rats in sub-chronic studies through a variety of mechanisms (Khathi et al., 2013; Mkhwanazi et al., 2014; Musabayane et al., 2010; Ngubane et al., 2011). Furthermore, these triterpenes have previously been reported to reduce food intake through unknown mechanisms (Khathi et al., 2013; Mkhwanazi et al., 2014). This study was therefore designed to evaluate the effects of these triterpenes on plasma ghrelin concentrations as well as the gastrointestinal expression of this hormone.

Methods

Drugs

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

Extraction of OA and MA

OA and MA were isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] cloves using a standard protocol that has been previously validated in our laboratory by (Khathi et al., 2013; Madlala et al., 2012; Mkhwanazi et al., 2014).

The structures of these plant-derived triterpenes were confirmed by spectroscopic analysis using 1D and 2D, ¹H and ¹³C 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 protocols involving animals were reviewed and approved by the University of KwaZulu-Natal animal ethics committee.

Induction of diabetes

Experimental type 1 diabetes mellitus was induced in male Sprague-Dawley rats using a previously described protocol (Mapanga et al., 2009; Ngubane et al., 2011). Briefly, each animal was administered a single intraperitoneal injection of 60 mg/kg streptozotocin in freshly prepared 0.1M citrate buffer (pH 6.3). The control group were injected with citrate buffer. Animals that presented with glucosuria after tested using urine strips (Rapidmed Diagnostics, Sandton, South Africa) and had blood glucose concentrations exceeding 18 mmol/L in STZ-induced diabetic rats after seven days post-induction were considered as stable diabetics.

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 weight 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 (stomach, small intestine and large intestine) was determined using Western Blot analysis.

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-week period (n = 6 in each group).

In the animals where the effects of the triterpenes 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. At the end of the 5 week experimental period, all animals were sacrificed by exposure to Isofor (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 tissue samples were removed, snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -70°C for Western blot analysis of ghrelin expression. 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 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.

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 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 (1 mg/mL). The proteins were then denatured by boiling in Laemmli sample buffer 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 running buffer. Following electrophoresis, the resolved proteins were electro-transferred to a nitrocellulose membrane for 30 min in transfer buffer. After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% 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

All data is expressed as means ± Standard Error of Means. For statistical analysis, one-way analysis of variance (ANOVA) was employed followed by using the Tukey-Kramer multiple comparison test. Statistical significance was calculated using GraphPadInStat Software (version 5.00, GraphPad Software, San Diego, California, USA).

Results**Structure elucidation**

Table 1 compares the resonance frequencies of all the carbon atoms in the *S. aromaticum*-derived OA and MA with previously reported data (Júlio et al., 2003; Martinez et al., 2013). The carbon signals at 68.3 ppm, 121.9 ppm and 143.7 ppm correspond to carbon-OH (C-2) and carbon-carbon double bond (C-12 and C-13), respectively and confirm the chemical structure of OA and MA (See Figure 2). The purity of the *S. aromaticum*-derived triterpenes was 98% and the percentage yields varied from 0.79% to 1.72%

Table 1: Comparison of ^{13}C Bruker NMR spectra of *S. aromaticum*-derived OA and MA with that reported by Martinez et al., 2013 and Juli et al., 2003 respectively.

Carbon number	OA		MA	
	Reported (Martinez et al., 2013)	<i>Syzygium aromaticum</i> derived	Reported (Júlio et al., 2003)	<i>Syzygium aromaticum</i> derived
1	39.9	39.9	46.2	46.4
2	27.9	27.5	68.3	68.8
3	79.7	79.5	83.3	83.7
4	39.3	39.3	39.1	39.1
5	56.8	56.8	55.0	55.5
6	19.5	19.5	18.1	18.5
7	33.8	33.8	32.7	32.5
8	40.6	40.5	39.0	39.1
9	49.1	49.1	47.4	47.5
10	38.2	38.2	38.0	38.1
11	24.5	24.5	23.2	23.2
12	123.7	123.5	121.9	121.9
13	145.2	145.2	143.7	143.7
14	42.9	42.8	41.6	41.8
15	28.9	28.8	27.4	27.5
16	24.1	24.1	23.0	23.3
17	47.6	47.4	46.2	46.6
18	42.8	42.8	41.0	41.3
19	47.3	47.1	45.7	45.8
20	31.6	31.6	30.4	30.7
21	34.9	34.6	33.6	33.9
22	34	34.1	32.3	32.3
23	28.8	28.6	28.3	28.8
24	16.3	16.3	16.6	16.9
25	15.9	15.9	16.5	16.9
26	17.7	17.3	16.4	16.9
27	26.4	26.4	23.2	23.5

28	180.8	180.1	178.5	178.1
29	33.6	33.4	32.2	33.2
30	24	24	23.2	23.5

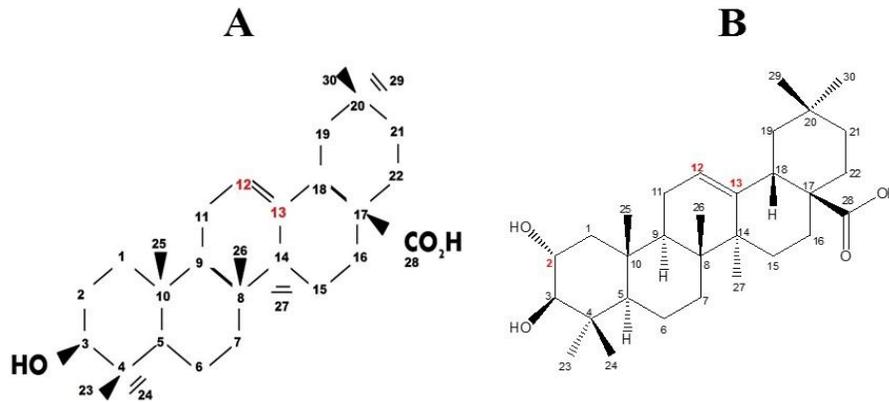


Figure 1: Chemical structure and IUPAC numbering of (A) OA and (B) MA as determined through ¹H and ¹³C NMR spectroscopy

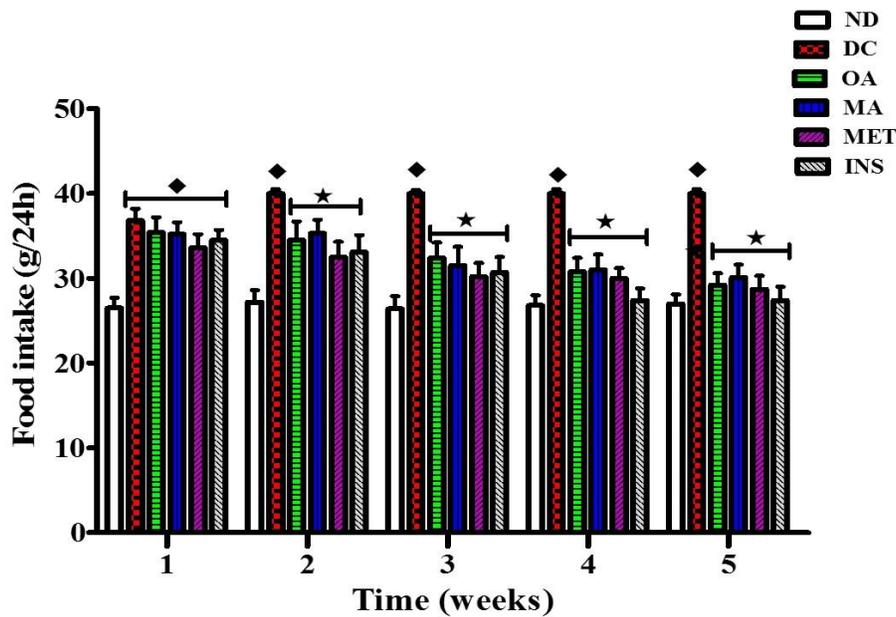


Figure 3

Figure 2: Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on food intake 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 2: 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).

	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 [*]

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

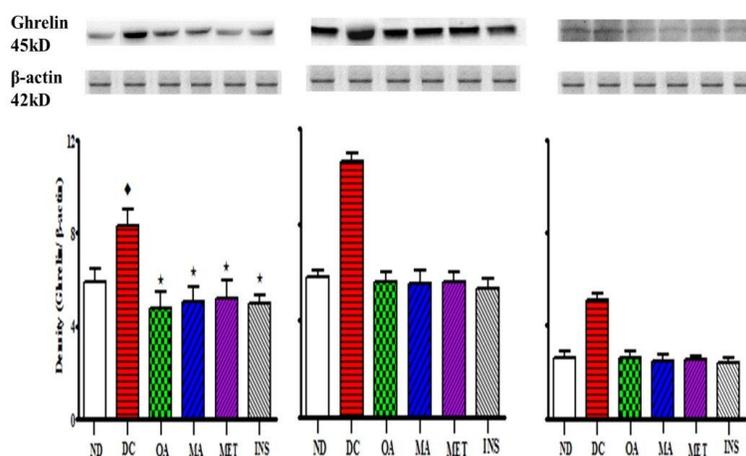


Figure 3: Comparison of the effects of OA and MA administered in STZ-diabetic rats twice every third day for 5 weeks on gastrointestinal ghrelin expression in the gastric fundus of the stomach (A), small intestine (B) and large intestine (C) with untreated STZ-diabetic rats and those treated with the 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 anti-hyperglycaemic mechanisms of these triterpenes. 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., 2001; Dezaki et al., 2004; Egido et al., 2002; Reimer et al., 2003). Ghrelin suppresses glucose-induced insulin release via Kv channel-mediated attenuation of Ca²⁺ 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. (Flanagan et al., 2003; Foster-Schubert et al., 2008; McCowen et al., 2002). Indeed, enhanced plasma ghrelin levels have been observed in individuals with diabetes mellitus and these are associated with diabetic polyphagia (Ariyasu et al., 2001; Broglia et al., 2001; Delhanty & van der Lely, 2011). Plant bioactive compounds such as triterpenes have been reported to exert their anti-hyperglycaemic effects through a variety of mechanisms (Abdul-Ghani & Defronzo, 2014; Ali et al., 2002; Bhat et al., 2008; Dzubak et al., 2006; Grover et al., 2002). 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. This study was therefore partly aimed at investigating plant bioactive compounds such as OA and MA on food intake in STZ-diabetic animals. The stereostructure of *S. aromaticum*-derived OA and MA was elucidated using ¹H- and ¹³C-NMR and were comparable to the previously reported data (García-Granados et al., 2000; Julio et al., 2003; Mahato & Kundu, 1994; Martinez et al., 2013). The administration of OA and MA was found to significantly reduce food intake in the STZ-diabetic animals. Furthermore, the administration of these triterpenes 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; Mkhwanazi et al., 2014; Musabayane et al., 2010; Ngubane et al., 2011).

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. 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 (Khathi et al., 2013). The results of this 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 (Buss et al., 2014). These results were accompanied by increases in the gastrointestinal expression of ghrelin. Interestingly, 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. 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 these 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 & Morley, 2012). Previous studies have shown that OA and MA possess anti-hyperglycaemic properties in STZ-induced diabetic animals (Khathi et al., 2013; Mapanga et al., 2009; Mkhwanazi et al., 2014; Musabayane et al., 2010; Ngubane et al., 2011). These studies have also shown that these triterpenes exert their effects through various mechanisms. This study has, for the first time, shown that these triterpenes exert their effects, in part, through the reduction of food intake through the reduction of gastrointestinal ghrelin expression in STZ-induced diabetic animals. Taken together, the findings of the study suggest that the control of food intake through the reduction of gastrointestinal 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 supplement for managing eating patterns and thus preventing chronic hyperglycaemia and thereby averting diabetic complications.

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Disclosure

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

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