The modulating effects of green rooibos (Aspalathus linearis) extract on vascular function and antioxidant status in obese Wistar rats

Zimvo Obasa, Mignon Albertha van Vuuren, Barbara Huisamen, Shantal Lynn Windvogel

Abstract

Purpose: Obesity is associated with the development of risk factors for cardiovascular disease (CVD) and polyphenols have been shown to possess ameliorative effects against obesity-induced CVD risk factors. Rooibos (Aspalathus linearis) is rich in polyphenols, therefore we investigated the cardio-protective effects of aspalathin-rich green rooibos (GRT) on obesity-induced CVD risk factors in obese Wistar rats.

Methods: Adult male Wistar rats (n = 20 per group) were fed a control or a high-fat diet (HFD) for 16 weeks and treated with GRT (60 mg/kg/day) for six weeks. Blood pressure was monitored throughout. Vascular reactivity was measured and Western blots of cell-signalling proteins (eNOS, AMPK and PKB) were performed in aortic tissues. Effects on oxidative stress were determined by measuring antioxidant enzyme activity and thiobarbituric reactive substance (TBARS) levels in the liver.

Results: HFD animals had (1) increased blood pressure, (2) impaired vasodilation, (3) attenuated PKB and AMPK expression, (4) decreased antioxidant enzyme activity, (5) increased malondialdehyde (MDA) levels, and (6) increased phosphorylated eNOS levels. Treatment with GRT extract significantly alleviated these obesity-induced CVD risk factors.

Conclusion: Supplementation with GRT extract alleviated cardiovascular risk factors in the HFD animals, suggesting a therapeutic potential for GRT in obesity-induced cardiovascular risk.

Keywords: obesity, blood pressure, vascular reactivity, oxidative stress, glucose homeostasis, Afriplex GRT™ extract

Cardiovascular disease (CVD), the leading cause of death globally, accounted for 17.7 million deaths in 2016, and is mostly prevalent in low- to middle-income countries. Behavioural risk factors for CVD include a sedentary lifestyle, tobacco smoking and alcohol abuse, which manifest as obesity, hypertension, type 2 diabetes, dyslipidaemia and raised blood glucose levels in individuals.

Obesity is a major global health problem and is on the rise, especially in developing countries where it is mostly prevalent in the adult population. It results when there is an energy imbalance between caloric intake and caloric expenditure, and is associated with development of the metabolic syndrome (MetS), a conglomerate of cardiometabolic risk factors that elevate CVD risk. The MetS is characterised by insulin resistance, elevated blood pressure, impaired glucose homeostasis, atherogenic dyslipidaemia and systemic inflammation. Obesity is also associated with the development of endothelial dysfunction and oxidative stress.

Rooibos (Aspalathus linearis), a leguminous shrub indigenous to the Cederberg Mountains of the Western Cape in South Africa, has numerous health-promoting properties, such as anti-hypertensive, anti-diabetic, anti-hyperglycaemic, anti-inflammatory, antioxidant, anti-cancer and anti-obesity effects. This is mainly attributed to its polyphenolic composition, particularly aspalathin, a unique major active flavonoid compound, and nothofagin, a 3-dehydroxydihydrochalcone glucoside.

When the rooibos plant is harvested, it is processed into fermented (oxidised) and unfermented (un-oxidised) products, which may be used to make herbal infusions and extracts. The unfermented ‘green’ rooibos is commonly used to prepare aspalathin-rich extracts due to the preservation of its polyphenolic content. Afriplex GRT™ (GRT) extract, a spray-dried powder with a high aspalathin content, used in this study, was prepared from unfermented rooibos.

To date, no studies have been performed investigating the relationship between the ameliorative effects of this GRT extract on obesity-induced CVD risk factors. Therefore, in view of the known health benefits of rooibos, we set out to determine whether GRT extract could improve obesity-induced CVD risk factors in an animal model.
Methods

Adult male Wistar rats weighing between 150 and 210 g and approximately seven to eight weeks old were obtained from the central animal facility of the Faculty of Medicine and Health Sciences at Stellenbosch University. They were housed in cages containing four rats per cage and maintained under a 12-hour day/night cycle at 24–25°C. Animals had ad libitum access to food and water.

Animals were randomly divided into five experimental groups (n = 8–10 per group) and fed either a control or high-fat diet (HFD) for a period of 16 weeks. The age-matched control group received standard Epol® [Epol (Pty) Ltd, Worcester, Western Cape, RSA] rat chow composed of: fat 4.8 g/100 g, protein 17.1%, carbohydrates 34.6%, sugar 6.6 g/100 g and energy 1 272 kJ/100 g. The HFD group however received a diet composed of: fat 27.9 g/100 g, cholesterol 6.4 mg/100 g, protein 14.6%, carbohydrates 29.5%, sugar 13.3 g/100 g, fructose 11 g/100 g and energy 1 823 kJ/100 g. The HFD diet was specifically adapted to induce high pressure effects and these rats were treated with captopril (50 mg/kg/day) as a positive control (n = 8). The additional HFD group was included, which served as a positive control (n = 8). The noradrenergic agonist phenylephrine (1 mM; 0.002g Phe in 10 ml 0.9% saline) was prepared. Acetylcholine (10 mM), labelled stock A, was serially diluted to make stock B (1 mM; 1 ml stock A in 9 ml 0.9% saline). Phenylephrine (1 mM; 0.002g Phe in 10 ml 0.9% saline), and finally stock C (100 µM; 1 ml stock B in 9 ml 0.9% saline) were prepared. Acetylcholine (10 mM), labelled stock A, was serially diluted to make stock B (1 mM; 1 ml stock A in 9 ml 0.9% saline). Phenylephrine (1 mM; 0.002g Phe in 10 ml 0.9% saline), and finally stock C (100 µM; 1 ml stock B in 9 ml 0.9% saline) were prepared.

At the time of sacrifice, the animals were weighed and euthanised with an overdose of sodium pentobarbital (Euthanize 160 mg/kg, intra-peritoneally). Following this, fasting or non-fasting blood was collected from all the animals, transferred to vacuum tubes (SGVac) and allowed to clot at room temperature (25°C). After 30 minutes, the blood was centrifuged at 1 200 × g for 10 minutes at 4°C. Thereafter, the serum was collected and stored at –80°C for biochemical analysis.

The blood pressure of each animal was measured using a CODA® non-invasive blood pressure acquisition system (Kent Scientific), which utilises a volume pressure-recording (VPR) tail-cuff system to measure the blood volume of the tail. Prior to the actual study, the animals were acclimatised to the apparatus for a period of two weeks. Blood pressure was then monitored on a weekly basis for 16 weeks, and baseline levels were determined for two weeks prior to treatment.

Glucose present in the urine was determined in weeks 10 and 16 of the study. Animals were individually placed in metabolic cages for a 24-hour period while having ad libitum access to food and water. Urine was collected in a plastic measuring cylinder attached to each cage. Glucose levels were determined using a Test-it™ 10 dipstick.

An oral sucrose tolerance test (OSTT) were performed in the week before commencement of treatment (week 10) and again a week before sacrifice (week 15) in both the controls and HFD groups. The animals were fasted overnight with free access to drinking water. Blood glucose levels were determined, using a handheld Glucopulse® glucometer, from a drop of blood collected after a tail prick with a lancet at the tail tip. Following measurement of baseline (0 minutes, fasting level) glucose levels, the animals were gavaged with 50% sucrose solution (1 g/kg) and the disappearance of glucose in the blood was monitored for two hours. After the OSTT procedure, the animals were left to recover for a week from this metabolic insult, prior to sacrifice.

Vascular contraction/relaxation of the aortic rings was performed to determine the endothelial function of the animals. A total of 10 aortae were used for each experimental group, except for the captopril group (n = 8). The noradrenergic agonist phenylephrine (1 mM; 0.002g Phe in 10 ml 0.9% saline) and the endothelium-dependant nitrogen oxide (NO)-releasing agent acetylcholine (10 mM; 0.0182 g in 10 ml 0.9% saline) stocks were prepared. Acetylcholine (10 mM), labelled stock A, was serially diluted to make stock B (1 mM; 1 ml stock A in 9 ml 0.9% saline), and finally stock C (100 µM; 1 ml stock B in 9 ml 0.9% saline).

The aortic ring (3–4 mm) was mounted onto two stainless steel hooks and slowly submerged in the organ bath (AD Instruments, Bella Vista, New South Wales, Australia) filled with Krebs-Henseleit buffer (KHB composition in mM: 119 NaCl, 25 NaHCO₃, 4.75 KCl, 1.2 KH₂PO₄, 0.6 MgSO₄·7H₂O, 0.6 Na₂SO₄, 1.25 CaCl₂, H₂O and 10 glucose) at 37°C and gassed with 95% O₂, 5% CO₂ at 100 mmHg.

Animal Welfare

All animal procedures were approved by the Ethics in Animal Research Committee of the Faculty of Medicine and Health Sciences, Stellenbosch University. The Good Manufacturing Practice (GMP)-certified GRT extract was prepared by Afriplex (Pty) Ltd (Paarl, South Africa) and it was kindly given as a donation. It was supplied with the total polyphenolic composition analysis (Table 1).

Table 1. High-performance liquid chromatography (HPLC) analysis of the GRT extract used in the study

<table>
<thead>
<tr>
<th>Compound (g compound/100 g soluble solids)</th>
<th>HPLC analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylpyruvic acid-2-O-glucoside (PPAG)</td>
<td>0.423265</td>
</tr>
<tr>
<td>Aspalathin</td>
<td>12.78348</td>
</tr>
<tr>
<td>Nothofagin</td>
<td>0.99418</td>
</tr>
<tr>
<td>Isoorientin</td>
<td>1.427281</td>
</tr>
<tr>
<td>Orientin</td>
<td>1.255839</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>nq</td>
</tr>
<tr>
<td>Vitexin</td>
<td>0.338513</td>
</tr>
<tr>
<td>Isovitexin</td>
<td>0.298022</td>
</tr>
<tr>
<td>Quercetin-3-robinobioside</td>
<td>1.040565</td>
</tr>
<tr>
<td>Hyperoside</td>
<td>0.398773</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.496034</td>
</tr>
<tr>
<td>Isoquercitin</td>
<td>0.572251852</td>
</tr>
<tr>
<td>Nq, not quantifiable.</td>
<td></td>
</tr>
</tbody>
</table>

An oral sucrose tolerance test (OSTT) were performed in the week before commencement of treatment (week 10) and again a week before sacrifice (week 15) in both the controls and HFD groups. The animals were fasted overnight with free access to drinking water. Blood glucose levels were determined, using a handheld Glucopulse® glucometer, from a drop of blood collected after a tail prick with a lancet at the tail tip. Following measurement of baseline (0 minutes, fasting level) glucose levels, the animals were gavaged with 50% sucrose solution (1 g/kg) and the disappearance of glucose in the blood was monitored for two hours. After the OSTT procedure, the animals were left to recover for a week from this metabolic insult, prior to sacrifice.
and 5% CO₂. The tension was slowly adjusted to 0.2 g and the preparation was initially stabilised for a period of 40 minutes, changing the buffer after every 10 minutes while gradually increasing the tension to 1.5 g. After 40 minutes, the KHB was changed and adjusted to exactly 25 ml.

Thereafter, 100 nM phenylephrine (2.5 μl of 1 mM stock) was added to induce maximal contraction, followed by 10 μM acetylcholine (25 μl of stock A) to induce at least 70% relaxation. The organ bath was rinsed three times with pre-warmed KHB to flush out the drugs and refilled to 25 ml. After another 30 minutes of stabilisation, cumulative concentrations of phenylephrine (to maximal vasocontraction), followed by titration with acetylcholine (to induce vasorelaxation) were added. Thereafter, the organ bath, string and steel hooks were thoroughly rinsed with boiled distilled water.

Western blot analysis was used to determine the expression and activation of selected proteins involved in endothelial function, such as AMPK, PKB and eNOS. Frozen sections of the aortic tissue (40–50 mg, n = 5 per group) were pulverised in a liquid nitrogen pre-cooled mortar and pestle and then transferred into a microcentrifuge tube [Scientific Group (Pty) Ltd, Milnerton, Western Cape, RSA] filled with 600 μl of lysis buffer [composition in mM: 20 Tris-HCl (pH 7.5), 1 EGTA, 1 EDTA, 150 NaCl, 1 β-glycerophosphate, 2.5 sodium pyrophosphate, 1 Na,VO₃, 50 mM NaF, 10 μg/μl leupeptin, 10 μg/ml aprotinin, 0.1% SDS, 1% Triton X-100 and 50 μg/ml PMSF, which was added last]. Samples were homogenised in a bullet blender® (Next Advance, Inc, New York) using 1.6-mm stainless steel beads at speed 8 for one minute at 4°C, for a total period of three minutes, with five-minute rests in-between cycles. Samples were allowed to stand on ice for 15 minutes and centrifuged at 15 000 rpm for 20 minutes at 4°C.

Protein concentration was determined using the Bradford method. The samples were diluted in Laemmli sample buffer, boiled for five minutes and stored at -80°C. Equal amounts of protein were separated using 26-well Bio-Rad TGX stain-free™ 5–20% gradient precast gels and transferred to polyvinylidene fluoride (PVDF) membranes using a Bio-Rad midi-transfer method. The samples were diluted in Laemmli sample buffer (composition in mM: 20 Tris-HCl (pH 7.5), 1% SDS, 1% Triton X100 and 50 μg/ml PMSF, which was added last). Samples were homogenised in a bullet blender® (Next Advance, Inc, New York) using 1.6-mm stainless steel beads at speed 8 for three minutes and speed 9 for four minutes, with one-minute rest periods in-between cycles. Samples were then allowed to stand on ice for 30 minutes and centrifuged at 15 000 rpm for 20 minutes at 4°C. Protein concentration was determined by means of a Bicinchoninic acid (BCA) protein assay kit (BCA1, Sigma Aldrich), using bovine serum albumin (BSA) (1 mg/ml) used as a standard, as supplied in the kit. The lysates were then frozen at -80°C and used for the downstream antioxidant enzyme assays and for the determination of lipid peroxidation.

Catalase (CAT) [enzyme commission number (EC) 1.11.1.6] catalyses the conversion of two H₂O₂ molecules into oxygen and two water molecules mostly in aerobic cells. Liver tissue homogenates were diluted to 0.1 μg/μl protein in assay buffer (50 mM potassium phosphate, 0.5% Triton X-100, pH 7.0). For the diluted tissue lysate, 5 μl was assayed in triplicate in a 96-well ultraviolet (UV) plate, followed by 170 μl of assay buffer. To initiate the reaction, 50 μl of H₂O₂ stock solution was added to all the wells and the absorbance was measured over five minutes to measure the linear decrease over time at 240 nm in a FLUOstar® Omega microplate reader. The molar extinction coefficient of H₂O₂ (43.6 M/cm), adjusted for the well path length, was used to determine catalase activity (μmol H₂O₂ consumed/min/μg protein).

Superoxide dismutase (SOD) (EC 1.15.1.1) catalyses the dismutation of the reactive superoxide radical (O₂•-) into H₂O₂ and oxygen. Activity was determined according to the method modified from Ellerby and Bredesen. Liver tissue homogenates were diluted to 0.1 μg/μl of protein in SOD assay buffer (50 mM sodium phosphate, pH 7.4), 6-hydroxydopamine (6-OHDOH) 1.6 mM was freshly prepared as follows: a volume of 50 μl of concentrated (70%) perchloric acid (HClO₄) was added to 10 ml deionised water (deiH₂O) and purged with nitrogen for 15 minutes to displace the oxygen. Thereafter, 4 mg of 6-OHDOH was added to this solution, wrapped in foil and kept on ice. The samples (10 μl) and the blank (15 μl SOD assay buffer) were assayed in triplicate and 170 μl of 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC), prepared in SOD assay buffer (1 mg in 25 ml), was added to all the wells. SOD assay buffer (5 μl) was added to all the sample wells, excluding the blank well and the reaction was initiated by adding 15 μl of 6-OHDOH to each well of the 96-well plate. The kinetics of the auto-oxidation of 6-OHDOH was monitored at 490 nm for five minutes at 25°C, using the FLUOstar® Omega microplate reader, and the results are expressed as units/mg protein.

Glutathione peroxidase (GPx) (EC 1.11.1.9) catalyses the dismutation of lipids and hydroperoxides, including H₂O₂, by reduced glutathione, and the activity was determined according to Ellerby and Bredesen. Liver tissue homogenates were diluted 2.5 × dilution (40 μl sample: 60 μl assay buffer) so that the...
protein concentration fell between the required range (5–10 mg/ml). A cocktail solution consisting of 210 µl assay buffer [50 mM potassium phosphate, 1 mM of ethylenediaminetetraacetic acid (EDTA), pH 7.0], 2.5 µl glutathione (GSH) solution (30.7 mg/ml in deH2O), 2.5 µl glutathione reductase (1.6 mg/ml, diluted in assay buffer), 2.5 µl of 0.1 M sodium azide to inhibit catalase and lastly, 2.5 µl of nicotinamide adenine dinucleotide phosphate (NADPH) [5 mg dissolved in 0.1% of sodium bicarbonate (NaHCO3)] was prepared.

Following this, 5 µl of the sample and blank (assay buffer: 50 mM potassium phosphate, 1 mM EDTA, pH 7.0) were assayed in triplicate, followed by the addition of 215 µl of the cocktail solution to the wells. The absorbance of NADPH oxidation in the absence of H2O2 was measured for three minutes with 30-second intervals, at 340 nm. To initiate the reaction, 25 µl of 1.5 mM H2O2 (3.4 µl of 30% stock solution in 20 ml assay buffer) was added immediately after the first absorbance measurement. The hydroperoxide-dependent linear NADPH oxidation was recorded for two to five minutes at 30-second intervals at the same wavelength. The GPx activity was expressed as µmol NADPH oxidised/min/mg protein.

### Thiobarbituric acid reactive substances (TBARS) assay

Measurement of TBARS is a widely used assay for the determination of lipid peroxidation in tissue homogenates and serves as an indicator of oxidative stress. The assay was performed according to the modified method of Esterbauer and Cheeseman. Liver tissue was homogenised in 0.01 mM sodium phosphate buffer (pH 7.5) containing 1.15% KCl in a Bullet Blender® 24 (Next Advance, NYC, USA) using 1.6-mm stainless steel beads, at speed 8 for three minutes and speed 9 for four minutes, with one-minute rest cycles in-between.

Protein concentrations of the samples were determined using a BCA assay kit (Sigma Aldrich). The MDA standard solution (500 µM) was prepared by diluting 1.23 µl of the MDA stock (125 µM) solution in 10 ml of deH2O and it was serially diluted in MDA diluent (nmol/ml MDA: 0, 0.322, 0.625, 1.25, 2.5, 5, 10, 25 and 30). Samples and standards (100 µl) were added to glass test tubes, followed by 100 µl of SDS solution (2%), 2 ml of trichloroacetic acid (TCA) reagent [composition: 10% TCA, BHT (12.5 mM butylated hydroxytoluene (BHT)/10 ml TCA solution)] and 2 ml thiobarbituric acid (TBA) (0.67% w/v) solution on the side of each tube, and the glass tubes were capped with marbles.

Test tubes were incubated in a water bath at 95°C for one hour and cooled at room temperature for 10 minutes. Thereafter, the samples were centrifuged at 3 000 rpm for 15 minutes and the supernatant was plated (150 µl) in triplicate. The absorbance was recorded for two to five minutes at 30-second intervals at the same wavelength. The GPx activity was expressed as µmol NADPH oxidised/min/mg protein.

### Statistical analysis

All the results were analysed using GraphPad Prism® 6. Statistical analysis was performed using one- or two-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test for comparison within the groups. The results are expressed as the mean ± standard error of mean (SEM). A probability of p < 0.05 was considered significant.

### Results

Food and water intake were measured three times weekly and body weight was measured once a week during the 10-week period (Table 2). The HFD group showed a significant increase in food intake and a significant decrease in mean water intake relative to the control group. Furthermore, the HFD group showed a significant increase in mean body weight when compared to the control group. Blood pressure was measured from week eight to 10 and the HFD group showed a significant increase in the mean systolic, diastolic and arterial blood pressure when compared to the control group. The mean arterial pressure (MAP) was calculated as follows:

\[
\text{MAP} = \text{mean DBP} + \frac{1}{3} (\text{mean SBP} – \text{mean DBP})
\]

where DBP is diastolic blood pressure and SBP is systolic blood pressure.

In week 10, quantitative blood glucose measurements were obtained in both the control and HFD group after an overnight fast. At baseline (Fig. 1A), the HFD group showed a significant increase in blood glucose levels compared to the control (5.56 ± 0.220 vs 4.66 ± 0.113 mmol/l; p < 0.01, n = 7–8 per group). After oral administration of the 50% sucrose solution, the HFD group showed a significant increase in plasma glucose levels at three (6.13 ± 0.219 vs 4.84 ± 0.341 mmol/l), five (6.59 ± 0.108 vs 5.06 ± 0.229 mmol/l), 10 (7.31 ± 0.437 vs 5.89 ± 0.245 mmol/l), 15 (7.63 ± 0.350 vs 5.51 ± 0.362 mmol/l), 20 (7.44 ± 0.270 vs 6.40 ± 0.229 mmol/l) and 25 minutes (7.04 ± 0.248 vs 6.31 ± 0.212 mmol/l; p < 0.05, n = 7–8 per group), compared to the control group (Fig. 1A). No significant differences were observed between the control and HFD groups from 30 to 120 minutes.

Additionally, according to the area under the curve (AUC, Fig. 1B) analysis, the HFD group presented with a significant increase in plasma glucose levels when compared to the control group (716 ± 15.4 vs 635 ± 31.2 arbitrary units, p < 0.05, n = 7–8 per group). After baseline measurement of plasma glucose levels (basal), the HFD group showed a significant increase in plasma glucose levels at three (6.13 ± 0.219 vs 4.84 ± 0.341 mmol/l), five (6.59 ± 0.108 vs 5.06 ± 0.229 mmol/l), 10 (7.31 ± 0.437 vs 5.89 ± 0.245 mmol/l), 15 (7.63 ± 0.350 vs 5.51 ± 0.362 mmol/l), 20 (7.44 ± 0.270 vs 6.40 ± 0.229 mmol/l) and 25 minutes (7.04 ± 0.248 vs 6.31 ± 0.212 mmol/l; p < 0.05, n = 7–8 per group), compared to the control group (Fig. 1A). No significant differences were observed between the control and HFD groups from 30 to 120 minutes.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>HFD</th>
<th>Sample size (n/group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean food intake (g)</td>
<td>17.18 ± 0.272</td>
<td>20.58 ± 0.429***</td>
<td>20–28</td>
</tr>
<tr>
<td>Mean water intake (ml)</td>
<td>20.48 ± 0.445</td>
<td>13.60 ± 0.229****</td>
<td>20–28</td>
</tr>
<tr>
<td>Mean body weight (g)</td>
<td>246.00 ± 4.404</td>
<td>274.10 ± 4.88***</td>
<td>20–28</td>
</tr>
<tr>
<td>Mean systolic blood pressure (mmHg)</td>
<td>125.00 ± 1.720</td>
<td>139.00 ± 2.460***</td>
<td>10</td>
</tr>
<tr>
<td>Mean diastolic blood pressure (mmHg)</td>
<td>84.48 ± 1.171</td>
<td>93.95 ± 1.226***</td>
<td>10</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>99.94 ± 0.518</td>
<td>111.70 ± 0.724***</td>
<td>10</td>
</tr>
<tr>
<td>Urinary glucose (mmol/l)</td>
<td>Normal</td>
<td>Normal</td>
<td>10</td>
</tr>
<tr>
<td>All data are expressed as mean ± SEM, ***p &lt; 0.001 HFD versus control. p &lt; 0.001 HFD versus control, n = 20–28 per group, except urinary glucose, mean systolic and diastolic blood pressure and mean arterial pressure, n = 10 per group.</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
The results in Table 3 represent biometric and blood pressure measurements during and after the 16-week period. The HFD animals showed a significant increase in food intake and a significant decrease in water intake compared to the control animals. Furthermore, the HFD animals showed a significant increase in leptin levels, and body weight when compared to the control animals. Additionally, the HFD group showed a significant increase in the mean systolic, diastolic and arterial blood pressure compared to the control group, measured over the last six weeks of the 16-week diet regime.

Treatment with the GRT extract showed no effect on food intake. However, control animals treated with the GRT extract showed a significant increase in water intake when compared to the untreated control animals. HFD animals treated with the GRT extract presented with a decrease in leptin levels, and IP fat, liver and absolute body weight when compared to the untreated HFD animals. Additionally, treatment with the GRT extract and captopril in the HFD animals significantly decreased the mean systolic, diastolic and arterial blood pressure compared to the untreated HFD group. Lastly, GRT treatment did not affect the blood pressure, leptin levels, and IP fat, liver and absolute body weight of the control animals.

In week 15, the blood glucose levels (Fig. 2A) of the HFD and control (GRT treated and untreated) animals were determined after an overnight fast. According to the AUC analysis (Fig. 2B), the HFD rats showed a significant increase in blood glucose levels when compared to the control animals (741.1 ± 16.20 vs 671.5 ± 23.93 arbitrary units; p < 0.05, n = 6–8 per group).

Table 3. Summary of the biometric and blood pressure measurements during and after the 16-week treatment period

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>HFD</th>
<th>Control + GRT extract</th>
<th>HFD + GRT extract</th>
<th>HFD + captopril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake (g)</td>
<td>17.11 ± 0.529</td>
<td>20.64 ± 0.631***</td>
<td>17.45 ± 0.468</td>
<td>21.11 ± 0.622</td>
<td>nd</td>
</tr>
<tr>
<td>Water intake (ml)</td>
<td>23.38 ± 0.442</td>
<td>17.01 ± 0.647****</td>
<td>26.66 ± 0.859***</td>
<td>14.76 ± 1.324</td>
<td>nd</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>339.50 ± 6.870</td>
<td>396.20 ± 13.660**</td>
<td>324.30 ± 7.460</td>
<td>344.50 ± 11.740@</td>
<td>nd</td>
</tr>
<tr>
<td>IP fat weight (g)</td>
<td>7.32 ± 0.995</td>
<td>23.79 ± 3.481***</td>
<td>8.27 ± 0.596</td>
<td>13.90 ± 1.315@</td>
<td>nd</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>10.30 ± 0.392</td>
<td>15.23 ± 0.803****</td>
<td>10.20 ± 0.411</td>
<td>11.70 ± 0.541</td>
<td>nd</td>
</tr>
<tr>
<td>Leptin assay (pg/ml)</td>
<td>2858 ± 210.80</td>
<td>5477 ± 791.50**</td>
<td>2948 ± 185.70</td>
<td>2431 ± 680.70</td>
<td>nd</td>
</tr>
<tr>
<td>Mean systolic blood pressure (mmHg)</td>
<td>122.30 ± 1.317</td>
<td>134.00 ± 1.770***</td>
<td>119.90 ± 1.252</td>
<td>120.60 ± 1.531****</td>
<td>115.40 ± 1.381****</td>
</tr>
<tr>
<td>Mean diastolic blood pressure (mmHg)</td>
<td>81.270 ± 1.645</td>
<td>91.64 ± 1.477****</td>
<td>79.22 ± 1.428</td>
<td>81.02 ± 1.482****</td>
<td>78.96 ± 0.739****</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>94.94 ± 1.417</td>
<td>105.80 ± 1.415***</td>
<td>92.80 ± 1.283</td>
<td>94.22 ± 1.431****</td>
<td>91.09 ± 0.843****</td>
</tr>
<tr>
<td>Urinary glucose (mmol/l)</td>
<td>Normal in 100% of animals</td>
<td>Glucose present in 50% of animals</td>
<td>Glucose present in 50% of animals</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SEM, two-way ANOVA was used for result analysis, nd, not determined.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 HFD versus control; *p < 0.001 control + GRT versus control; *p < 0.05, ***p < 0.0001 HFD + GRT versus HFD, ****p < 0.0001 HFD + captopril versus HFD, n = 10 per group.
with Western blot analysis. The data in Fig. 4 reflect the total (T) expression, phosphorylated (P) protein levels and phosphorylated:total (P:T) ratio of each protein. The HFD animals presented with a significantly lower T-AMPK expression and P-AMPK level, respectively, when compared to the control animals (Fig. 4A, B). Treatment of the HFD rats with the GRT extract upregulated the P:T AMPK ratio (Fig. 4C) but had no significant effect on T-AMPK and P-AMPK levels. Additionally, captopril upregulated P-AMPK levels and the P:T AMPK ratio. The HFD animals showed a significant decrease in the T-PKB expression and P-PKB levels when compared to the control group (Fig. 5A, B). Treatment with captopril significantly increased P-PKB levels and treatment with GRT extract showed no significant effect. Furthermore, no significant differences were observed in the PKB P:T ratio in all the groups assessed (Fig. 5C). HFD animals presented with an increase in P-eNOS (Fig. 6B) and P:T eNOS (Fig. 6C) ratio when compared to the control animals. The GRT extract significantly increased T-eNOS expression (Fig. 6A) with no significant effect on P-eNOS and P:T eNOS levels.

Activities of the primary antioxidant enzymes were determined in the liver (Table 4). The HFD animals had significantly lower SOD, CAT and GPx activity and increased MDA levels when compared to the control animals. Supplementation with GRT extract in the HFD animals significantly increased SOD and CAT activity and decreased MDA levels when compared to the untreated HFD animals. Additionally, the GRT extract in the treated control animals significantly increased GPx activity and decreased MDA levels when compared to the untreated control animals.

Discussion

Obesity, especially visceral obesity, results in enlargement of the adipose tissue, a major storage site for excess energy, which is
also considered a secretion site for pro- and anti-inflammatory cytokines. Therefore, in an obese state, there is upregulation in the release of pro-inflammatory cytokines, downregulation of anti-inflammatory cytokines and increased free fatty acid (FFA) release into the circulation. The released FFA and pro-inflammatory cytokines enter the liver and skeletal muscle cells and induce modifications in lipid and glucose homeostasis in these metabolic tissues, including modification in the inflammatory responses. As a result, this imbalance greatly contributes to the development of insulin resistance, hyperglycaemia, dyslipidaemia, hypertension, endothelial dysfunction (ED) and oxidative stress.

Leptin and angiotensinogen serve as examples of pro-inflammatory adipokines, which contribute to the dysregulation in adipocyte metabolism. A number of studies have shown the ameliorative effects of unfermented rooibos against the above obesity-induced CVD risk factors, resulting from adipocyte hypertrophy. Fermented rooibos has been shown to inhibit adipogenesis and intracellular lipid accumulation, and it attenuates leptin secretion. This was mainly attributed to its polyphenolic content, with aspalathin and nothofagin as active and the most abundant compounds.

The HFD used in this study successfully induced obesity in the Wistar rats, and excess consumption of a high-fat, high-sugar diet has been previously shown as a contributing factor to obesity. The increase in food intake by the HFD animals contributed to their higher body weight, leptin levels, and IP fat mass (Tables 2, 3), which has been previously shown in obese rats fed a HFD. Increased liver weight may be attributed to the increase in FFA release from the enlarged adipose tissue and increased lipid synthesis in the liver. Additionally, the HFD animals had impaired glucose homeostasis (Figs 1, 2), as was previously documented by studies that used a similar diet. Increased leptin levels and excessive accumulation of triglycerides in the liver have also been associated with dysregulation in glucose homeostasis. The impairment in glucose homeostasis observed in the HFD animals was also supported by downregulation in the expression of AMPK in the vascular system (Fig. 4), an insulin-independent signalling protein. This protein is responsible for glucose uptake and NO production via the phosphorylation of eNOS.

Furthermore, the HFD animals had increased blood pressure (Tables 2, 3), and decreased vasorelaxation (Fig. 3A) and vasocontraction (Fig. 3B). Increased blood pressure is as a result of an impairment in vasodilation, due to reduction
Fig. 5. PKB expression in the aortic rings of the HFD versus control groups (GRT treated and untreated), including the HFD animals treated with captopril. (A) T-PKB; ***p < 0.001, HFD versus control groups (n = 5 per group). According to two-way ANOVA, the HFD had a significant effect (p < 0.05) on T-PKB expression (n = 5 per group). (B) P-PKB; **p < 0.01, HFD versus control groups. According to two-way ANOVA, the HFD had a significant effect (p < 0.01) on P-PKB levels (n = 5 per group). (C) P:T PKB ratio; no significant differences between the groups.

Fig. 6. eNOS expression in the aortic rings of the HFD versus control groups (GRT treated and untreated), including the HFD animals treated with captopril. (A) T-eNOS; **p < 0.01, HFD + GRT versus HFD groups. According to two-way ANOVA, the GRT had a significant effect (p < 0.01) on T-eNOS expression (n = 5 per group). (B) P-eNOS; ***p < 0.001, HFD versus control groups; *p < 0.05, HFD + captopril versus HFD groups. According to two-way ANOVA, the HFD had a significant effect (p < 0.001) on the P-eNOS levels (n = 5 per group). (C) P:T eNOS; ***p < 0.001 HFD versus control groups; HFD + captopril versus HFD groups. According to two-way ANOVA, the HFD had a significant effect (p < 0.001) on P:T eNOS levels (n = 5 per group).
in NO availability or production in the endothelial cells, a condition defined as ED.\textsuperscript{3,5,6} Interestingly, the HFD animals presented with an upregulation in eNOS phosphorylation (Fig. 6B) despite the increased blood pressure and decrease in vasodilation. We speculate that this could be as a result of the decrease in SOD enzyme activity (Table 4), which contributes to a reduction in NO bioavailability via the eNOS uncoupling process. This process occurs when NO binds with superoxide radical-producing peroxynitrite, a highly reactive free radical, in the absence of the SOD enzyme.\textsuperscript{6}

The elevated blood pressure observed in the HFD animals could potentially be as a result of physical compression of the kidneys due to the accumulation of fat in and around the kidneys, and stimulation of the sympathetic nervous system as a result of increased leptin levels.\textsuperscript{17,18} Since leptin has been shown to be one of the factors that mediates increased blood pressure in obesity.\textsuperscript{19-21} The HFD animals also presented with an increase in oxidative stress, reflected by an increase in lipid peroxidation and downregulation of CAT, GPx and SOD activity in the liver (Table 4). HFD has previously been documented to induce oxidative stress in obese Wistar rats.\textsuperscript{21}

Treatment with GRT extract resulted in less weight gain, a decrease in IP fat mass, leptin levels, liver mass and blood pressure, as observed in the treated HFD animals. It also decreased vasocontraction (Fig. 3A) and improved vasorelaxation (Fig. 3B). Additionally, the GRT extract did not affect the body weight of the control animals. This corresponds with previous studies done in mice that used unfermented rooibos and green rooibos extract (GRE).\textsuperscript{22-24}

It is believed that these herbal substances bring about the anti-obesity effects by inhibiting adipocyte differentiation and downregulate mRNA expression of the transcription factors responsible for the adipocyte differentiation, such as peroxisome proliferator-activated receptor-gamma (\(\gamma\)) (PPAR-\(\gamma\)).\textsuperscript{25} This further results in reduction in leptin levels, AMPK activation, an increase in glucose uptake and a decrease in lipolysis and lipogenesis. Rooibos extracts have been shown to decrease the size and number of adipocytes.\textsuperscript{26} These changes may also have resulted in decreased liver weight, as fat accumulation inside the liver primarily contributes to an increase in liver weight.

The decrease in leptin levels may also have resulted in lowering of blood pressure, subsequently improving vascular function. However, further mechanisms need to be explored, such as measuring endothelial-derived vasorelaxation and vasoconstriction factors, as well as the renin–angiotensin system intermediates. The anti-hypertensive effects of unfermented rooibos have previously been documented.\textsuperscript{27-29} Effects on the expression and phosphorylation state of the main proteins involved in the activation of eNOS were affected by the ingestion of GRT observed in this study. This could be one of the mechanisms that potentially contributed to the reduction in blood pressure, as observed in the HFD animals treated with the GRT extract. Previously, treatment with rooibos and aspalathin have been shown to activate AMPK.\textsuperscript{30-32} Captoril possibly also resulted in upregulation of the production of NO by improved phosphorylation of AMPK, PKB and eNOS, decreasing blood pressure. Captoril is a well-known inhibitor of the angiotensin converting enzyme, thereby lowering the activity of the renin–angiotensin–aldosterone system.\textsuperscript{33,34}

Treatment with GRT extract improved glucose metabolism (Fig. 2) in the treated HFD animals. Previously, it was reported that GRE extract and aspalathin improved glucose uptake \textit{in vivo} via phosphorylation of AMPK and PKB signalling proteins and lipid metabolism.\textsuperscript{35,36} Furthermore, aspalathin decreased fasting blood glucose levels and improved glucose intolerance in a diabetic rat model, confirming the effects of aspalathin on glucose homeostasis.\textsuperscript{37} Interestingly, in the current study, the GRT extract showed no significant effect on phosphorylation of AMPK and PKB (Figs 4, 5), respectively, in the aorta. However, according to the P:T AMPK ratio, GRT extract increased the AMPK phosphorylation state in the treated HFD group (Fig. 4). This could mean that the GRT extract may have induced upregulation of glucose uptake in the tissues via the AMPK pathway.

In half of the control animals and in all the HFD animals, GRT interestingly increased urinary glucose levels (Table 3). The excretion of glucose in the urine is closely associated with inhibition of the sodium/glucose co-transporter 2 (SGLT2), thereby speculating that GRT might act as an SGLT2 inhibitor. Confirming this suggestion, a previous study reported that aspalathin can inhibit SGLT2.\textsuperscript{38} SGLT2 is predominantly expressed in the renal proximal tubules of the kidneys, and to a lesser extent in the liver, muscle, heart\textsuperscript{39-41} and pancreatic \(\beta\)-cells.\textsuperscript{42} Since glucose is mainly reabsorbed by the kidney via SGLT2, inhibition of SGLT2 offers an insulin-independent novel mechanism for the treatment of type 2 diabetes. We speculate that the improved glucose clearance in the HFD animals could be ascribed to the inhibition of SGLT2 by the GRT extract. Further studies are needed to explore the postulated mechanism at the level of this transporter. Lastly, the GRT extract restored the oxidant–antioxidant imbalance in the treated HFD animals, thus restoring the antioxidant defence system (Table 4).

**Conclusion**

There is a strong correlation between visceral obesity and the pathogenesis of CVD risk factors. The use of natural, safe and affordable therapeutics may be useful in alleviating these pathologies. As demonstrated in the HFD rats in this study, obesity was strongly associated with impaired glucose homeostasis, increased blood pressure, ED, dyslipidaemia and oxidative stress, synergistically increasing cardiovascular risk. These were, however, attenuated by treatment with GRT extract, but the mechanisms need to be explored to further elucidate some of the findings. Therefore, GRT extract may be a potential therapeutic agent against obesity-related vascular dysfunction, impaired glucose homeostasis, elevated blood pressure, oxidative stress, leptin resistance and weight gain.

We thank Airfplex (Pty) Ltd and the South African Medical Research Council (SAMRC) for provision of the Airfplex GRT\textsuperscript{43} extract and HPLC analysis, Harry Crosley Foundation for financial support and Mr LDM Bennie for performing the glutathione peroxidase and lipid peroxidation assays. We declare that the rats received humane care in compliance with the revised South African National Standard for the Animal Care and Use for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008) and the protocol was approved by Stellenbosch University Animal Ethics Committee, Tygerberg, South Africa (ethics number: SU-ACUM15-00102).
References


