Phenylpyruvic Acid-2-O-β-D-Glucoside Attenuates High Glucose-Induced Apoptosis in H9c2 Cardiomyocytes

Abstract

Chronic hyperglycemia is closely associated with impaired substrate metabolism, dysregulated mitochondrial membrane potential, and apoptosis in the diabetic heart. As adult cardiomyocytes display a limited capacity to regenerate following an insult, it is essential to protect the myocardium against the detrimental effects of chronic hyperglycemia. This study therefore investigated whether phenylpyruvic acid-2-O-β-D-glucoside, present in Aspalathus linearis (rooibos), is able to attenuate hyperglycemia-induced damage in H9c2 cardiomyocytes. H9c2 cardiomyocytes were exposed to a high glucose concentration (33 mM) prior to treatment with phenylpyruvic acid-2-O-β-D-glucoside (1 µM), metformin (1 µM), or a combination of phenylpyruvic acid-2-O-β-D-glucoside and metformin (both at 1 µM). Our data revealed that high glucose exposure increased cardiac free fatty acid uptake and oxidation, mitochondrial membrane potential, and apoptosis (caspase 3/7 activity and TUNEL), and decreased the Bcl2/Bax protein expression ratio. Phenylpyruvic acid-2-O-β-D-glucoside treatment, alone or in combination with metformin, attenuated these glucose-induced perturbations, confirming its protective effect in H9c2 cardiomyocytes exposed to chronic hyperglycemia.

Abbreviations

CVD: cardiovascular disease
DCFH-DA: 2′,7′-dichlorofluorescein diacetate
DM: diabetes mellitus
DPBS: Dulbecco’s phosphate buffered saline
FAO: fatty acid oxidation
FAU: fatty acid uptake
FFAs: free fatty acids
JC-1: 5,5′,6′,6′-tetrachloro-1′,1′,3′,3′-tetraethylbenzimidazolyl-carboxyanine iodide
p53: tumor protein p53
PPAG: phenylpyruvic acid-2-O-β-D-glucoside
ROS: reactive oxygen species

Introduction

CVDs are a leading cause of death worldwide [1]. Moreover, the continuous rise in noncommunicable diseases such as DM and obesity remains a major burden contributing to increased CVD onset [1]. There are currently ~415 million individuals burdened with DM and this is estimated to reach ~642 million by the year 2040 [2]. Patients with DM are at an increased risk of developing CVD [2,3]. In addition, the chronic hyperglycemia of DM contributes to the development of diabetic cardiomyopathy [4,5]. Cardiomyopathies are a class of heart diseases that distinctly affect the structure of the heart muscle independently of coronary artery disease and hypertension [4,5]. Although mainly nonischemic in nature, cardiomyopathies play a predominant role to induce heart failure and are one of the major causes of death in southern Africa [5,6]. The mechanisms by which chronic hyperglycemia induces myocardial injury are varied and have not been fully elucidated [7]; however, enhanced intracellular flux of FFAs is implicated, as it can result in mitochondrial deficiencies and subsequently trigger myocardial apoptosis [8–10]. FFAs are the preferred substrate for energy generation within the normal mammalian heart, while glucose provides the majority of the remaining proportion [11]. The diabetic heart utilizes a high

Supporting information available online at http://www.thieme-connect.de/products
proportion of FFAs as an energy source that can elicit detrimental effects, e.g., the deterioration of heart function and a state of energy deficiency [11–13]. Furthermore, increased FFA uptake within cardiomyocytes subjected to elevated concentrations of glucose is accompanied by aberrant mitochondrial membrane potential, preceding myocardial apoptosis [14, 15]. Phytochemicals, especially those present in plant foods, are gaining increasing popularity due to potential health-promoting properties. Such compounds are therefore actively screened for novel antidiabetic and cardioprotective properties [16–19]. For example, Aspalathus linearis (Brum.f) Dahlg. (Fabaceae), used to brew a popular herbal tea known as rooibos, contains polyphenols such as the novel dihydrochalcone, aspalathin, with robust antioxidant properties [17]. A precursor in the flavonoid biosynthesis pathway and a non-phenolic compound, PPAG (Fig. 1) [20], is not unique to rooibos, but it occurs rarely in nature and only a few studies have reported on its presence in plants [21]. Of note, in vitro and in vivo models of diabetes previously demonstrated that PPAG improved metabolic parameters such as glucose intolerance, and it also protected pancreatic β-cells against endoplasmic reticulum stress and palmitate-induced apoptosis [19, 22]. However, as its effects within the mammalian heart are unknown, this study aimed to establish whether PPAG could protect heart cells exposed to chronic hyperglycemia.

Results

Compared to the experimental control (cells exposed to media only) (100.0 ± 8.2%), PPAG induced a concentration-dependent response with the highest ATP concentrations at 1 µM (119.4 ± 0.5%, \(p = 0.0001\)) (Fig. 2). We therefore employed this dosage for the remainder of experiments conducted in this study. High glucose (33 mM) induced a robust increase in FAU (135.2 ± 4.9%, \(p = 0.0001\)) and FAO (140.0 ± 7.0%, \(p = 0.0002\)) in H9c2 cardiomyocytes when compared to the normal glucose control (100.0 ± 3.9% and 100.0 ± 4.2%, respectively) (Fig. 3A, B). Post-treatment with metformin (123.2 ± 6.1%, \(p = 0.05\) and 120.1 ± 8.2%, \(p = 0.05\)), PPAG (115.0 ± 4.7%, \(p = 0.009\) and 111.2 ± 3.2%, \(p = 0.002\)), as well as a combination of metformin + PPAG (114.0 ± 2.4%, \(p = 0.011\) and 113.2 ± 3.9%, \(p = 0.004\)) significantly reduced FAU and FAO, respectively (Fig. 3A, B).

DCFH-DA fluorescence intensity as a representative of ROS formation was significantly increased (141.3 ± 4.9%, \(p = 0.0001\)) after chronically exposing H9c2 cells to 33 mM of glucose when compared to the normal glucose control (100.1 ± 3.1%) (Fig. 1S, Supporting Information). Enhanced ROS in high glucose-exposed cells occurred concomitant to the reduction of glutathione (GSH) content (CellTracker fluorescent intensity) (62.4 ± 1.6%, \(p = 0.0001\)) and superoxide dismutase (SOD) activity (59.4 ± 3.9%, \(p = 0.0001\)) (Figs. 2S and 3S, Supporting Information). Although PPAG was able to reduce enhanced ROS production (127.3 ± 5.0%, \(p = 0.05\)), it failed to increase the decreased GSH content (68.4 ± 4.0%) and SOD activity (61.4 ± 4.8%). Interestingly, metformin and its combination with PPAG showed more potency in ameliorating ROS generation (123.4 ± 2.9%, \(p = 0.001\) and 110.4 ± 5.0%, \(p = 0.0001\)) and improving GSH content (81.2 ± 4.2%, \(p = 0.0001\) and 88.4 ± 4.4%, \(p = 0.0001\)) and SOD activity (86.0 ± 4.5%, \(p = 0.0001\) and 75.2 ± 4.9%, \(p = 0.0001\)) when compared to PPAG monotherapy (Figs. 1S–3S, Supporting Information).

Exposure of H9c2 cardiomyocytes to high glucose increased mitochondrial membrane potential in cells stained with JC-1 fluorescence stain (Fig. 4A). Treatment with metformin, PPAG, or metformin + PPAG ameliorated this effect (Fig. 4A). Quantitative fluorescent analysis revealed that high glucose increased the mitochondrial membrane potential by 138.0 ± 4.1% (\(p = 0.0001\)) when compared to the normal glucose-exposed cells (100.0 ± 3.6%) (Fig. 4B). PPAG, metformin, and metformin + PPAG treatment attenuated this effect (110.4 ± 5.3%, \(p = 0.0003\); 121.2 ± 4.1%, \(p = 0.006\); and 115.3 ± 7.3%, \(p = 0.001\), respectively) (Fig. 4A, B).

High glucose-induced cells displayed increased levels of fluorescence intensity associated with late apoptosis (propidium iodide and TUNEL staining) when compared to the normal glucose controls (Fig. 5A). In support, H9c2 cardiomyocytes exposed to high glucose exhibited increased caspase 3/7 activity (132.2 ± 6.1%, \(p = 0.01\)), with a concomitant upregulation in TUNEL positive cells (8.4 ± 1.1, \(p = 0.001\)) when compared to normal glucose-treated cells (100.0 ± 3.7% and 2.6 ± 0.7, respectively) (Fig. 5A, B). Treatment with PPAG and metformin + PPAG decreased both the activity of caspase 3/7 and TUNEL positive cells ([109.3 ± 5.3%, \(p = 0.01\) and 4.6 ± 0.4, \(p = 0.01\), and (108.2 ± 3.7%, \(p = 0.007\) and 4.1 ± 1.0, \(p = 0.01\)]) (Fig. 5A–C). The Bcl2/Bax protein expression ratio was robustly diminished in H9c2 cells exposed to high glucose (80.1 ± 8.8%, \(p = 0.03\)) when compared to normal glucose-treated cells (100.0 ± 1.7%) (Fig. 6). Treatment with PPAG (107.4 ± 4.8%, \(p = 0.009\)) and metformin (104.4 ± 3.6%, \(p = 0.01\)) was able to ameliorate this ef-
fect (Fig. 6). However, treatment with metformin + PPAG (95.2 ± 2.5%) was ineffective (Fig. 6).

Discussion

Previous studies found that rooibos can trigger a peripheral hypoglycemic effect, improve glucose uptake, and relieve endoplasmic reticulum stress-induced cell apoptosis in muscle, liver, and pancreatic β-cells of diabetic animals [19,22]. Focusing on the heart, selective rooibos compounds can attenuate myocardial dysfunction by improving endothelial function and limiting ischemia/reperfusion injury [23,24]. However, it remains unknown whether PPAG is able to protect the myocardium against hyperglycemia-mediated apoptosis. The current study, therefore, focussed specifically on this phenylpropenoic acid glucoside, present in rooibos, by employing an in vitro cardiac model of chronic hyperglycemia. Our findings demonstrate that relatively low PPAG doses attenuated high glucose-induced apoptosis in H9c2 cardiomyoblasts, thus offering early promise as a novel cardioprotective agent.

The initial experiments revealed that low PPAG doses increased metabolic activity of H9c2 cardiomyocytes. However, higher PPAG concentrations significantly reduced cell viability. These data are in agreement with an earlier work demonstrating that a lower PPAG dose stimulated glucose uptake in Chang cells [19], unlike the high dose that resulted in a reduced uptake.

Chronic hyperglycemia can result in a number of pathophysiological disturbances that contribute to myocardial injury [7,9]. Such perturbations include derangements in both glucose and fatty acid metabolic pathways, with increased ROS generation emerging as a central, damaging outcome in this process. This study found that high glucose availability resulted in altered cardiac FAU and FAO concomitant to increased ROS and reduced intracellular antioxidant capacity of the cells. In parallel, mitochondrial membrane potential was higher, together with increased myocardial apoptosis. This is consistent with the findings of others [15,25], where high glucose exposure induced mitochondrial membrane hyperpolarization and apoptosis in undifferentiated H9c2 cells compared to controls. It is likely that in our model, elevated fuel substrate availability (e.g., fatty acids) results in an increased flux through the mitochondrial electron transport chain leading to enhanced proton extrusion into the inter-mitochondrial membrane space [11,12,14]. Higher intracellular glucose levels will also lead to increased mitochondrial membrane potential due to a greater availability of reducing equivalents for the mitochondrial electron transport chain [7]. Subsequently, this will generate increased mitochondrial ROS levels (due to an impaired electron flow at the electron transport complexes) and contribute to myocardial apoptosis [15,26]. For example, others found that chronic hyperglycemia and associated mitochondrial deficiency are key factors that trigger myocardial apoptosis in a diabetic state [10,11]. Increased mitochondrial membrane potential can also cause the release of proapoptotic proteins into the cytosol [27]. In agreement, our data show lower Bcl2/Bax protein expression together with increased caspase activity and higher propidium iodide and TUNEL staining. These observations are consistent with enhanced propidium iodide and TUNEL staining found in isolated heart cells of diabetic mice [28,29]. Higher intracellular glucose and fatty acid levels can also trigger multiple, non-mitochondrial effects that can also contribute to the onset of myocardial cell death [30,31].

Our data revealed that 1 µM PPAG treatment for 12 h attenuated several of the high glucose-induced perturbations earlier discussed. For example, PPAG treatment limited cardiac FAU and FAO, decreased mitochondrial membrane potential, and lowered cardiac apoptosis. These findings are in agreement with the other results showing that PPAG ameliorated apoptosis by increasing Bcl2 expression and inhibiting Bax translocation to the mitochondrion. Similar findings were also reported in pancreatic β-cells of mice fed a high-fat diet [22]. In agreement with this, fermented rooibos extract with abundant levels of PPAG protected primary rat cardiomyocytes against oxidative stress-induced myocardial injury [16]. We have previously demonstrated that aspalathin, the major phenolic compound in rooibos, protected primary rat cardiomyocytes against oxidative stress-induced myocardial injury [16]. We have previously demonstrated that aspalathin, the major phenolic compound in rooibos, protected primary rat cardiomyocytes against oxidative stress-induced myocardial injury [16]. We have previously demonstrated that aspalathin, the major phenolic compound in rooibos, protected primary rat cardiomyocytes against oxidative stress-induced myocardial injury [16]. We have previously demonstrated that aspalathin, the major phenolic compound in rooibos, protected primary rat cardiomyocytes against oxidative stress-induced myocardial injury [16].
The potential mechanism by which PPAG inhibits hyperglycemia-induced myocardial injury remains to be fully elucidated. However, recent research is highlighting a strong connection between the activation of tumor protein p53 and the increased expression of Bax or inhibition of Bcl2 in various disease models [33–35]. In the hearts of diabetic mice, upregulated expression of p53 is associated with enhanced lipid accumulation and mitochondrial generated ROS, leading to apoptosis [35]. Thus, we propose that PPAG prevents hyperglycemic-induced cardiac apoptosis through modulating increased FFA substrate flux into mitochondrial respiration complexes with a concomitant amelioration of the Bcl2/Bax ratio. However, further studies are required to assess the involvement of the p53 tumor suppressor gene in our model.

In summary, this study reveals the cardioprotective potential of PPAG against hyperglycemia-induced cell injury and provides im-

![Fig. 4](image-url)

**Fig. 4** Effect of PPAG, metformin (MET), and MET + PPAG on mitochondrial transmembrane potential (ΔΨm) measured by JC-1 stain. A Representative images of H9c2 cardiomyocytes exposed to 33 mM of glucose for 48 h before treatment with either PPAG, MET, or MET + PPAG; cells with non-depolarized mitochondria exhibited an orange fluorescence, while depolarized cell mitochondria displayed green fluorescence. Mannitol (MAN) did not have an effect on mitochondrial membrane potential and was comparable to the normal glucose (NG) control. B Quantitative analysis of JC-1 fluorescence staining. Results are the mean ± SEM of three independent biological experiments relative to the NG control, each done in triplicate. ***P < 0.0001 versus NG; *P < 0.05, **P < 0.001, and ###P < 0.0001 versus high glucose (HG). (Color figure available online only.)

![Fig. 5](image-url)

**Fig. 5** Effect of PPAG, metformin (MET), and MET + PPAG on high glucose-induced cell apoptosis in H9c2 cardiomyocytes. A Representative images of H9c2 cardiomyocytes exposed to 33 mM of glucose for 48 h before treatment with either PPAG, MET, or MET + PPAG; cells positive for propidium iodide nuclear stain exhibited red fluorescence, while those positive for TUNEL displayed green fluorescence. B The antiapoptotic potential of PPAG to reduce caspase 3/7 activity (C) and TUNEL fluorescent staining was comparable to an additive effect of MET and PPAG. Results are the mean ± SEM of three independent biological experiments, each done in triplicate. *P < 0.05 versus normal glucose (NG); *P < 0.05 and **P < 0.001 versus high glucose (HG). (Color figure available online only.)
petus for future investigations into its long-term effects on CVD in diabetic individuals.

Materials and Methods

Materials

The ViaLight plus ATP kit, cell culture tested water, Dulbecco’s modified Eagle’s medium, Hank’s balanced salt solution, and DPBS were obtained from Lonza. 14C-palmitate was from American Radiolabelled Chemicals, fetal bovine serum and horse serum from Biochrom, Bcl2 antibody from Cell Signaling Technology, secondary antibodies, Bax, and β-actin were from Santa Cruz Biotechnology, and the Caspase-Glo 3/7 kit was from Promega. PPAG (99% purity; batch: MC1(2)-248–91D) was synthesized by High Force Research. H9c2 rat-derived cardiomyoblasts (ECACC No. 8809294) were purchased from the European Collection of Cell Cultures. All consumables and reagents, including metformin (99% purity) and mannitol (99% purity), were purchased from Sigma-Aldrich Corp., unless otherwise specified.

Preparation of PPAG solution

PPAG (MW: 326) at 10 mg was dissolved in 1 mL of cell culture-tested water to give a final concentration of 30.67 mM stock solution which was stored at – 80 °C. Working solutions of 1 mM were made up from the initial stock using Dulbecco’s modified Eagle’s medium to give dilutions ranging from 0.01 to 1000 µM.

H9c2 cell culture and treatment with PPAG

Embryonic heart-derived H9c2 cardiomyoblasts were cultured in supplemented Dulbecco’s modified Eagle’s medium (10% fetal bovine serum) for 48 h under standard tissue culture conditions (37 °C in humidified air and 5% CO2). Depending on the assay performed, 60–80% confluent cells were seeded at a density of 2 x 10⁴ or 0.8 x 10⁴ cells/mL in 6-well or 96-well multiplates, respectively. After 48 h of culture, H9c2 cardiomyoblasts were differentiated into cardiomyocytes by substituting the growth media with differentiation media consisting of Dulbecco’s modified Eagle’s medium supplemented with 1% horse serum and 10 nM all-trans retinoic acid for 6 days [36]. All experiments were initiated on day 7. H9c2 cells were exposed to a range of PPAG log concentrations (0.01 to 1000 µM) for 3, 6, 12, 24, and 48 h, respectively. Thereafter, the effect of PPAG on hyperglycemia-induced cell apoptosis was tested. Here, H9c2 cells were exposed to 33 mM of glucose for 48 h prior to treatment with either PPAG (1 µM), metformin (1 µM), or metformin + PPAG (both at 1 µM) for an additional 12 h. Cells exposed to either 5.5 mM glucose, 33 mM glucose, or 33 mM mannitol served as controls for normal glucose, high glucose, and osmotic stress, respectively [25].

Measurement of metabolic activity

ATP production as a measurement of metabolic activity in H9c2 cells was determined using the ViaLight plus ATP kit, according to the manufacturer’s instructions. Briefly, treated cells were lysed and incubated for 10 min at room temperature. Thereafter, ATP monitoring reagent was added and the luminescence determined after 2 min using a BioTek FLx800 plate reader with data acquisition using Gen 5 software (Bio-Tek Instruments, Inc.).

Fatty acid uptake and oxidation

FAU and FAO were determined as previously described [32, 37]. Following treatment, H9c2 cells were washed twice with warm DPBS and recultured in high glucose Dulbecco’s modified Eagle’s medium (without phenol red) containing 0.5 µCi/mL of the final concentration of palmitate-D-[14C] in clear 6-well multiplates. Each well was covered with filter paper wetted with 0.1 M NaOH for collection of 14CO2 released from 14C-palmitate. The filter paper was replaced every 3 h and at the end of the experiment, 14C activity was measured by liquid scintillation (2220 CA, Packard Tri-Carb series, PerkinElmer) to calculate FAO. The filter paper was replaced every 3 h and at the end of the experiment. 14C activity was measured by liquid scintillation (2220 CA, Packard Tri-Carb series, PerkinElmer) to calculate FAO. The remaining cells were rinsed in ice-cold DPBS to stop metabolism, then lysed with 0.3 M NaOH + 1% sodium dodecyl sulfate buffer. The cell lysate was used to assess 14C-palmitate by liquid scintillation as well as protein content using Bradford protein assay [38]. The counts per minute (CPM) over specific activity (pre-determined using GraphPad radioactivity calculator: http://www.graphpad.com/quickcalcs/radcalcform.cfm) was used to determine fmol/mg protein.

Measurement of oxidative stress markers

Intracellular production of ROS was detected using DCFH-DA fluorescent dye (Cell Biolabs Inc.) as previously described [32]. Briefly, 100 µL of a 1-µM DCFH-DA final solution prepared in...
Hank’s balanced salt solution was added to H9c2 cells in a multiwell plate and incubated at 37 °C for 30 min in humidified air with 5% CO₂. After 30 min, the dye was aspirated and the cells were rinsed in Hank’s balanced salt solution. DCFH-DA fluorescence intensity (Ex 485 ± 20 nm; Em 528 ± 20 nm) was measured using a BioTek FLx800 plate reader.

GSH content was determined using 7-amino-4-chloromethylcoumarin (CellTracker Blue CMAC) (Invitrogen). Briefly, 100 µL of 2.5 µM CellTracker solution were added to H9c2 cells and incubated at 37 °C for 30 min. Thereafter, media containing CellTracker solution was removed and the cells were rinsed in DPBS before fluorescence intensity (Ex 360 ± 20 nm; Em 460 ± 40 nm) was measured using a BioTek FLx800 plate reader. SOD activity was quantified using a Biovision kit. Briefly, following treatment, H9c2 cells were lysed by the addition of 100 µL lysis buffer. Ten-microliters of cell lysate were then transferred to a new 96-well plate to which 200 µL of tetratozolium working solution was added. SOD activity was measured at an absorbance of 450 nm using a BioTek ELx800 plate reader.

**Determination of mitochondrial membrane potential (ΔΨm)**

Membrane depolarization was assessed by staining H9c2 cardiomyocytes with JC-1, according to a previously described method [15]. Briefly, treated H9c2 cells were washed twice with warm DPBS, 100 µL of 2 µM JC-1 solution (made up in Dulbecco’s modified Eagle’s medium without phenol red) were added, and the cells were then incubated at 37°C in humidified air and 5% CO₂ for 30 min in the dark. After JC-1 exposure, cells were rinsed in DPBS before fluorescence intensity (Ex 485 ± 20 nm; Em 530 ± 25 nm and 590 ± 35 nm) was measured using a BioTek FLx800 plate reader and Gen 5 software. In addition, fluorescent photomicrographs were taken at 10× magnification using a Nikon Eclipse Ti inverted microscope and NIS-Elements imaging software.

**Propidium iodide fluorescent stain**

A propidium iodide staining assay was performed following a published protocol [39]. Briefly, treated cells were exposed to 1 µg/mL propidium iodide solution for 30 min at 37°C in humidified air with 5% CO₂. After 30 min, cells were rinsed in DPBS and analyzed as described for determination of membrane depolarization. Fluorescent photomicrographs were taken at 10× magnification using a Nikon Eclipse Ti inverted microscope and NIS-Elements imaging software.

**Caspase 3/7 activity assay**

In this assay, Caspase-Glo 3/7 reagent was cleaved by caspases producing a luminescent signal proportional to caspase activity. Briefly, treated cells were washed twice with warm DPBS before being lysed. Caspase-Glo 3/7 reagent was mixed with cell lysates and the mixture was incubated in the dark at 37 °C in humidified air with 5% CO₂ for 30 min. Luminescence was measured using an integration time of 1 s and equipment described for measurement of metabolic activity. Results were normalized to the protein content determined according to the method of Bradford [38].

**TUNEL assay**

DNA fragmentation, as a measure of apoptosis in H9c2 cells, was detected using a DeadEnd Fluorometric TUNEL kit, according to the manufacturer’s instructions. Briefly, treated cells were washed twice in DPBS and permeabilized with 0.2% Triton X-100. Thereafter, the equilibration buffer and TdT reaction mix (supplied with the kit) were added before incubation at 37°C in humidified air with a 5% CO₂ incubator for 60 min. The apoptotic rate was calculated as the average number of condensed TUNEL-positive cells in nonoverlapping fields of 1 mm² under a 10× magnification field (average of at least five fields per well) using a Nikon Eclipse Ti inverted fluorescent microscope and NIS-Elements imaging software.

**Western blot analysis**

Protein extracts were isolated from different treatment groups and Western blot analysis was performed according to an already described method [40]. Membranes were immunoblotted for 16 h at 4°C with the following primary antibodies: anti-Bax (1:250 dilution), anti-Bcl2 (1:1,000 dilution), and anti-β-actin (1:1,000 dilution) antibody, included as a loading control. The relevant horseradish peroxidase conjugated secondary antibodies were applied the following day for 90 min at room temperature. The protein signal was detected using a chemiluminescence solution and quantified using a Chemidoc-XRS imager (Bio-Rad).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc.). Comparisons between treatment groups were performed using one-way ANOVA followed by a Tukey post hoc test or unpaired Student t-test where appropriate. A p value of ≤ 0.05 was deemed as statistically significant.

**Supporting information**

Effect of PPAG on oxidative stress markers.

**Acknowledgements**

This research was funded in part by the National Research Foundation (NRF) Thuthuka Programme Grant 87836 and the South Africa Medical Research Council’s Biomedical Research and Innovation Platform. The grant holders acknowledge that opinions, findings, and conclusions or recommendations expressed in any publication generated by the NRF supported research are those of the authors, and that the NRF accepts no liability whatsoever in this regard. Funding from Stellenbosch University and Ernst Ethel Erikson Trust is also acknowledged.

**Conflict of Interest**

Coauthors Christo John Frederick Muller, Elizabeth Joubert, and Johan Louw declare their interest as inventors in PCT patent applications, dealing with the antidiabetic activity of RX-1 and analogues (WO2011/120576 A1, WO2012/045363 A1). RX-1 is the code used for PPAG. The other authors declare no conflict of interest.
Affiliations
1 Biomedical Research and Innovation Platform (BRIIP), Medical Research Council (MRC), Tygerberg, South Africa
2 Division of Medical Physiology, Faculty of Health Sciences, Stellenbosch University, Tygerberg, South Africa
3 Post-Harvest and Wine Technology Division, Agricultural Research Council (ARC) Infruct-Nietvoortij, Stellenbosch, South Africa
4 Department of Food Science, Stellenbosch University, Stellenbosch, South Africa
5 Cardio-Metabolic Research Group (CMRG), Department of Physiological Sciences, Stellenbosch University, Stellenbosch, South Africa

References
10 Opie LH. Metabolic management of acute myocardial infarction comes to the fore and extends beyond control of hyperglycemia. Circulation 2008; 117: 2172–2177
11 Dnytar D. Diabetic cardiomyopathy: effects of fatty acids and glucose on adult rat cardiomyocytes [dissertation]. Zürich: Naturwissenschaften ETH Zürich; 2003; Nr. 15175
12 Carlsson M, Wessman Y, Aldercreutz P, Group L. High levels of nonesterified fatty acids are associated with increased familial risk of cardiovascular disease. Arterioscler Thromb Vasc Biol 2003; 20: 1588–1594
23 Kwik S, Han MS, Bae JS. Aspalathin and nothofergin from rooibos (Aspalathus linearis) inhibit endothelial protein C receptor shedding in vitro and in vivo. Fitoferapia 2015; 100: 179–186
39 Chellan N. The effect of Cyclophia malacca extract on β-cell function, protection against oxidative stress and cell survival [dissertation]. Stellenbosch University; 2014