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Supplementation with aspalathin and sulforaphane protects cultured cardiac cells against dyslipidemia-associated oxidative damage

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ABSTRACT

Dyslipidemia is a prominent pathological feature responsible for oxidative stress-induced cardiac damage. Due to their high antioxidant content, dietary compounds, such as aspalathin and sulforaphane, are increasingly explored for their cardioprotective effects against lipid-induced toxicity. Cultured H9c2 cardiomyoblasts, an in vitro model routinely used to assess the pharmacological effect of drugs, were pretreated with the dietary compounds, aspalathin (1 μM) and sulforaphane (10 μM) before exposure to palmitic acid (0.25 mM) to induce lipidemic-related complications. The results showed that both aspalathin and sulforaphane enhanced cellular metabolic activity and improved mitochondrial respiration correlating with improved mRNA expression of genes involved in mitochondrial function, including uncoupling protein 2, peroxisome proliferator-activated receptor, gamma coactivator 1-alpha, nuclear respiratory factor 1, and ubiquinol-cytochrome c reductase complex assembly factor 1. Beyond attenuating lipid peroxidation, the dietary compounds also suppressed intracellular reactive oxygen species and enhanced antioxidant responses, including the mRNA expression of nuclear factor erythroid 2-related factor 2. These envisaged benefits were associated with decreased cellular apoptosis. This preclinical study supports and warrants further investigation into the potential benefits of these dietary compounds or foods rich in aspalathin or sulforaphane in protecting against lipid-induced oxidative damage within the myocardium.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death globally [1]. This explains the growing interest in understanding the risk factors associated with the development of CVDs [2]. It's hypothesized that dyslipidemia, a pathophysiological condition that is associated with ectopic lipid accumulation may lead to the damage of various internal organs including the myocardium [3–5]. Indeed, available evidence suggests that dyslipidemia, specifically enhanced hypercholesterolemia, plays a significant role in causing lipid peroxidation, the pathological feature of oxidative stress that is linked to myocardial dysfunction [2]. Enhanced uptake or dysregulation of lipid products within the myocardium are being studied as prominent mechanisms for oxidative damage, the major characteristic feature of diabetic cardiomyopathy [6–8]. The latter describes undesired myocardial structural modifications that are likely facilitated by oxidative stress-related abnormalities, occurring independent of coronary artery disease and are considered one of the leading causes of CVD-related deaths [9,10]. As a result, beyond scrutinizing the total cholesterol content, determining the oxidative status, including the levels of lipid peroxidation products like

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malondialdehyde (MDA), has been increasingly used to assess the potential CVD risk in conditions of dyslipidemia or impaired metabolic function [11]. Pharmacological interventions with abundant antioxidant properties are emerging as a plausible strategy to attenuate oxidative stress and protect against dyslipidemia-associated cardiac damage [12,13].

Nutritional supplements containing strong antioxidant properties such as rooibos (*Aspalathus linearis*), including its major bioactive compound aspalathin, are actively screened for their cardioprotective effects, including attenuating oxidative stress-related complications [14]. Our group has already reviewed documented evidence indicating that the antioxidant properties of aspalathin, a dihydrochalcone *C*-glucoside of rooibos, can protect against oxidative damage in cultured cardiomyocytes or within the myocardium of diabetic animal models [15,16]. Similarly, another promising dietary compound is sulforaphane, an isothiocyanate rich in cruciferous vegetables of the genus Brassica like broccoli, which has also shown enhanced potential to protect against oxidative damage in preclinical models of diabetes [11, 17]. The antioxidant properties of aspalathin and sulforaphane have been linked with enhanced activation of nuclear factor erythroid 2-related factor 2 (Nrf2), which is proposed to be an important mechanism to promote cytoprotective responses against oxidative stress-induced damage within the myocardium [17,18]. Although such evidence is acknowledged, there is a need to further investigate the broader context implicating the cardioprotective effects of both aspalathin and sulforaphane against lipid-associated damage. In this study, we hypothesize that aspalathin and sulforaphane exert cardioprotective effects by enhancing mitochondrial health, through improved mitochondrial respiratory efficiency, reduced reactive oxygen species (ROS) production, and decreased lipid peroxidation. Additionally, we anticipate that these compounds will positively influence lipid homeostasis and mitigate cellular injury.

2. Reagents and methods

2.1. Reagents

The rat heart ventricular-derived H9c2 cardiomyoblasts were purchased from the American Type Culture Collection (Manassas, VA, USA; catalogue number: CRL-1446). Dulbecco's modified Eagle's medium (DMEM), (BE12-604F), Dulbecco's phosphate-buffered saline (DPBS, pH 7.4 with calcium and magnesium), (BE17-512F), and trypsin (CC-5012) were from Lonza BioWhittaker (Walkersville, MD, USA). Free fatty acid bovine serum albumin (BSA), (03117057001**)** was from Roche (Mannheim, Germany). The cell culture plates (3997) were from Corning (NY, USA); the Bradford kit (#5000201**)** was from Bio-Rad Laboratories (Hercules, CA, USA). Seahorse XF-96 microplate plates (103022-100), Seahorse XF assay media (103680-100), and Seahorse XF-cell Mito stress kit (103015-100) were all from Agilent (Santa Clara, CA, USA). QIAzol lysis reagent (79306) was from Qiagen (Hilden, Germany). Aspalathin (ca. 98 %, Batch SZI-356-54), synthesized following an already described method [19], was supplied by High Force Research LTD (Durham, UK). Gene expression probes, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), NADH: ubiquinone oxidoreductase core subunit s 3 (*Ndufs3*), nuclear factor erythroid 2-related factor 2 (*Nrf2*), nuclear respiratory factor 1 (*Nrf1*), peroxisome proliferator-activated receptor, gamma coactivator 1-alpha (*Pprgc1α)*, superoxide dismutase 2 (*Sod2*), ubiquinol-cytochrome c reductase complex assembly factor 1 (*Uqcc1*), and uncoupling protein 2 (*Ucp2*) as well as Fetal bovine serum (FBS) (10493106) were purchased from Thermo Fisher Scientific (Waltham, MA, USA), (Table). Whereas simvastatin (S6196**)** ≥97 % high-performance liquid chromatography (HPLC), sulforaphane (S6317) ≥95 % (HPLC), dimethyl sulfoxide (DMSO), (D8418**)**, palmitic acid (cell culture grade), (P5585**)**, cell culture tested water (W3500**)**. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Palmitic acid experimental model and preparation of treatment compounds, aspalathin and sulforaphane

Briefly, 204 mg of palmitic acid was dissolved in 2 ml absolute ethanol and heated in boiling water for 5 min to make a stock concentration of 400 mM. To prepare for the working solution, the appropriate volume of 400 mM palmitic acid was conjugated in 1 % BSA for 1 h in a sonicator bath. Stock solutions of aspalathin and sulforaphane were prepared by dissolving the compounds in 100 % DMSO to make stock solutions of 22.10 mM aspalathin and 28.20 mM sulforaphane, respectively. The final working solutions of these compounds were prepared by diluting the appropriate amounts of a stock solution with DMEM (supplemented with 8 mM glucose, 3.7 g/L $NaHCO_3$, and 0.1 % (w/v), 1 % BSA to yield a final working solution of DMSO <0.001 % DMSO, as previously described [20].

2.3. Cell culture conditions for H9c2 cardiomyoblasts

The H9c2 cardiomyoblasts are routinely used as a screening tool for novel therapeutic agents against cardiotoxicity [21]. Here, H9c2 cardiomyoblasts were cultured in DMEM supplemented with 10 % FBS at standard tissue culture conditions (37 °C, in humidified air and 5 % CO_2). Cells were regularly sub-cultured at a confluency of 80–90 % and seeded in 96-well or 6-well plates at a density of 5 x10^4 cells/ml for all the assays performed.

2.4. Determination of cellular metabolic activity using ATP production

To assess the potential toxicity of aspalathin, sulforaphane, and simvastatin on H9c2 cardiomyoblasts, a dose-response study was conducted. The concentrations tested were selected based on previous research: 1 and 10 μM for aspalathin (Dludla, Muller et al., 2017), 2.5 and 10 μM for sulforaphane [22], and 2.5 μM for simvastatin [23] which served as the experimental-comparative control. Additionally, a palmitic acid concentration of 0.25 mM was derived from our previous study study [3]. Subsequently, cellular metabolic activity was assessed. Briefly, cells were pretreated with aspalathin and sulforaphane, together with simvastatin as a comparator, for 24 h before exposure to 0.25 mM palmitic acid (together with treatment compounds) for an additional 24 h. Subsequently, cytoplasmic adenosine triphosphate (ATP), as a measure of cellular metabolic activity, was quantified using a CellTiter-Glo® Luminescent Cell Viability Assay Kit from Promega (MA, USA), as per the manufacturer's instructions. The SpectraMax i3x multi-mode microplate reader (Molecular Devices, CA, USA) was used to measure the luminescence. Protein concentrations quantified using Bradford assay (Bio-Rad Laboratories, Hercule, CA, USA), as per manufacturer instructions, were used for normalization.

2.5. Assessment of mitochondrial respiration status

To assess mitochondrial respiration in H9c2 cardiomyoblasts, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Mito Stress Kit and XF-96 Extracellular Flux Analyser from Seahorse Bioscience (MA, USA), following a method that has already been described [3]. Measuring oxygen consumption is crucial in this study as this can be used to explore how palmitic acid-induced metabolic shifts affect mitochondrial function and energy production in cardiomyoblasts. Briefly, the protocol involves injections of 10 μM oligomycin in port A (20 μl) to inhibit ATP synthase, 7.5 μM carbonyl cyanide 4 trifluoromethoxy-phenylhydrazone (FCCP) in port B (22 μl) for maximal respiration, as well as 5 μM of rotenone (complex I inhibitor) combined with antimycin A (complex III inhibitor) in port C (25 μl). After the assay protein concentrations were quantified using the Bradford for normalization, as previously explained [3].

2.6. mRNA expression analysis

To quantify the gene expression, RNA was extracted using QIAzol lysis reagent, cleaned, and reverse transcribed into complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription kit from Applied Biosystems (Thermo Scientific™, MA, USA). cDNA synthesis was carried out using Applied Biosystems® 2720 Thermal Cycler with the following conditions: 10 min at 25 °C, 120 min at 37 °C, 5 s at 85 °C and 4 °C for cooling the samples. The TaqMan gene expression probes used are included in Table 1 and gene expression was analyzed using a QuantStudio™ 7 Flex Real-Time PCR System (Thermo Scientific TM, MA, USA). The quantitative RT-PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Gene expression data was normalized to Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

2.7. Evaluation of mitochondrial mass and changes in mitochondrial membrane potential ($\Delta\psi m$)

The fluorescent dyes, MitoTracker Green (Thermo Fisher Scientific, MA, USA) and JC-10 (5,5',6,6'-tetrachloro-1,1',3,3-tetraethylbenzimidazolyl-carbocyanine iodide; Sigma-Aldrich, St Louis, MO, USA) were used to measure mitochondrial mass and determine changes in mitochondrial membrane potential, respectively. Both assays were performed using methods that have already been described [3]. Mitochondrial mass was measured using a BD Accuri® C6 flow cytometer (Becton Dickinson, NJ, USA). For the changes in mitochondrial membrane potential, the fluorescence intensity of JC-10 aggregates, orange fluorescence at ~590 nm (excited by 540 nm), and JC-10 monomers, green fluorescence at ~525 nm (excited by 490 nm) was measured using the SpectraMax i3x multi-mode microplate reader.

2.8. Measuring total cholesterol content and lipid peroxidation

Total cholesterol content was quantified using the Cholesterol/Cholesteryl Ester Assay Kit from Abcam (Cambridge, UK). Lipid peroxidation was assessed by measuring the MDA levels, using an OxiSelect™ Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit from Cell Biolabs (San Diego, USA). Both assays were performed using the manufacturer's protocol, and the relevant fluorescence was read on a SpectraMax i3x multi-mode microplate reader (Molecular Devices, CA, USA).

2.9. Evaluation of cytosolic and mitochondrial reactive oxygen species production (ROS)

Cytosolic and mitochondrial ROS production were detected using an OxiSelect Intracellular ROS Assay Kit from Cell Biolab (San Diego, CA, USA), and MitoSOX (mitochondrial superoxide indicators) Red kit (Thermo Fisher Scientific, MA, USA), respectively. Briefly, cytosolic and mitochondrial ROS were measured using fluorescent dyes, 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) and MitoSox red, respectively. For both assays, the fluorescence was measured using a BD Accuri® C6 flow cytometer (Becton Dickinson, NJ, USA).

Table 1
The list of TaqMan probes used in the study.

Probe	Gene	Assay ID
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	Rn01775763_g1
NADH: Ubiquinone Oxidoreductase Core Subunit S3	*Ndufs3*	Rn01484390_m1
Nuclear factor erythroid 2-related factor 2	*Nrf2*	Rn00582415_m1
Nuclear respiratory factor 1	*Nrf1*	Rn01455958_m1
Peroxisome proliferator-activated receptor Gamma coactivator 1-alpha	*Pprgc1α*	Rn00580241_m1
Superoxide dismutase 2	*Sod2*	Rn00690588_g1
Ubiquinol-Cytochrome C Reductase Complex Assembly Factor 1	*Uqcc1*	Rn01535673_m1
Uncoupling protein 2	*Ucp2*	Rn01754856_m1

2.10. Evaluation of intracellular antioxidants

The levels of intracellular antioxidants, total glutathione (GSH) content, and superoxide dismutase (SOD) activity were measured using the OxiSelect Total Glutathione Assay Kit from Cell Bio-lab (San Diego, USA), as well as the Superoxide Dismutase Activity Assay Kit from Abcam (Cambridge, UK), respectively. The assays were performed as per the manufacturer's instructions. The relative optical density was measured using a SpectraMax i3x multi-mode microplate reader at 405 nm for total GSH content and 440 nm for SOD activity.

2.11. Evaluation of cellular damage

Cellular apoptosis and necrosis in H9c2 cardiomyoblasts were assessed using Annexin V-FITC from Invitrogen (Carlsbad, CA, USA), and propidium iodide from Sigma-Aldrich (St Louis, MO, USA), respectively. Fluorescence measurements for both annexin V (apoptosis) and propidium iodide (necrosis) were acquired using a BD Accuri C6 flow cytometer following the method previously described [3]. The following channels were used, FITC signal detector FL1 (excitation = 488 nm; emission = 530 nm) for Annexin V positive (apoptotic) cells, and the FL3 detector (excitation = 488 nm; emission = 670/LP) for propidium positive (necrotic) cells.

2.12. Statistical analysis

Data was expressed as the mean ± standard error of the mean (SEM). Results for all experiments consist of three independent experimental repeats. Specifically, the ATP production assay and the Seahorse (Mito stress) analysis included six technical replicates per experiment (n = 6), while mRNA analysis and flow cytometry assays had three technical replicates each (n = 3). To ensure that repeated measurements were derived from biologically distinct samples, each well in the 96-well or 6-well plates was treated as an independent biological sample. To further guarantee the independence of the measurements, cells were seeded on different days and from different passages, across three separate plates. Statistical analysis was performed using GraphPad Prism software version 8.0.1 (GraphPad Software, Inc., La Jolla, CA, USA). To reduce the variability of data, seahorse and PCR mean values were log-transformed (Y = Log *Y). Prior to the statistical analysis both a normality test and an outlier test were performed using GraphPad Prism. After that, comparisons between groups were performed using one-way multivariate ANOVA, followed by a Tukey post-hoc test, this method is specifically designed to control for family-wise error rates across all pairwise comparisons, ensuring that the significance level remains valid despite multiple tests. Where appropriate, the student's *t*-test (and nonparametric test) was used, with $p < 0.05$ considered significant for all experiments performed.

3. Results

3.1. Aspalathin and sulforaphane enhanced the ATP production in cardiomyoblasts exposed to palmitic acid

ATP production served as a measure of cellular metabolic function (Fig. 1). After exposing cells to 0.25 mM palmitic acid for 24 h, there was a significant decrease in ATP production compared to the experimental control ($p < 0.001$). However, pretreatment with both doses of aspalathin and sulforaphane increased ATP production ($p < 0.01$) compared to the palmitate control (Fig. 1). The comparative control, simvastatin ($p < 0.01$) similarly demonstrated enhanced ATP production compared to the palmitic acid control (Fig. 1).

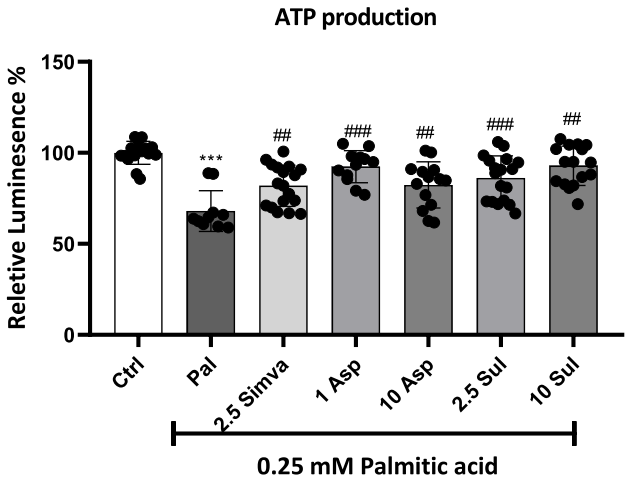


Fig. 1. Aspalathin and sulforaphane enhanced the metabolic activity in cardiomyoblasts exposed to palmitic acid. Briefly, H9c2 cardiomyoblasts were pretreated with aspalathin (Asp) and sulforaphane (Sul) followed by co-treatment with palmitic acid (Pal) for an additional 24 h. Simvastatin (Simva) was used as a comparative control. Subsequently, cellular metabolic activity was assessed using an ATP production kit. Results are presented as the mean ± standard error of the mean (SEM) from three independent experiments, each with at six technical replicates (n = 6), relative to the control (Ctrl). Comparisons between groups were performed using one-way multivariate ANOVA, followed by Tukey's multiple comparisons test. Statistical significance was represented by $^{***}p < 0.001$ compared to the experimental control, while $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ were compared to the palmitic acid control.

3.2. Aspalathin and sulforaphane enhanced mitochondrial respiration and glycolytic energy levels while reducing proton leak (H+) in cardiomyoblasts exposed to palmitic acid

Mitochondrial respiration was assessed by measuring OCR, and ECAR (Fig. 2AB), respectively. Exposure to palmitic acid significantly reduced basal respiration ($p < 0.001$), maximal respiration ($p < 0.001$), and non-mitochondrial respiration ($p < 0.01$), while increasing H+ (proton) leak ($p < 0.05$) (Fig. 2C–G). Aspalathin and sulforaphane demonstrated significant improvement in both basal ($p < 0.01$) and maximal respiration ($p < 0.05$) compared to the palmitic acid control. Remarkably, this improvement surpassed the effect observed with simvastatin, which was used as a comparative control, as depicted in Fig. 2CD. Spare capacity was also not affected by treatment compounds (Fig. 2E), however, elevated H+ (proton) leak was significantly reduced by aspalathin ($p < 0.05$), sulforaphane ($p < 0.001$), and simvastatin ($p < 0.05$) compared to the palmitic acid control (Fig. 2F). Notably, non-mitochondrial respiration was also improved by aspalathin ($p < 0.05$) and sulforaphane ($p < 0.05$), comparable to simvastatin relative to the palmitic acid control (Fig. 2G).

3.3. Aspalathin and sulforaphane increased the mRNA expression levels of some markers involved in mitochondrial function in cardiomyoblasts exposed to palmitic acid

We further explored the effects of aspalathin and sulforaphane on the mRNA expression of *Pprgc1α*, *Nrf1*, *Ucp2*, *Ndufs3*, and *Uqcc1* (Fig. 3A–E). Among the analyzed genes, results showed that palmitic acid significantly decreased the mRNA expression of *Pprgc1α* ($p < 0.01$), *Nrf1* ($p < 0.05$), and *Uqcc1* ($p < 0.001$) when compared to the experimental control (Fig. 3A–E). Aspalathin supplementation significantly increased the mRNA expression of *Pprgc1α* ($p < 0.05$), *Nrf1* ($p < 0.05$), and *Uqcc1* ($p < 0.05$) (Fig. 3A–E). Whereas sulforaphane supplementation significantly improved the mRNA expression of *Pprgc1α* ($p < 0.05$), *Nrf1* ($p < 0.05$), and *Uqcc1* ($p < 0.05$) (Fig. 3A–E). Simvastatin supplementation did not have much effect on regulating most genes, except for increasing the mRNA expression of *Ucp2* ($p < 0.05$) in comparison to the palmitic acid control (Fig. 3A–E).

3.4. Aspalathin and sulforaphane increased mitochondrial mass and improved mitochondrial membrane potential in cardiomyoblasts exposed to palmitic acid

Exposure to palmitic acid led to a significant decrease in mitochondrial mass ($p < 0.001$), accompanied by observable alterations in the morphology and structure of H9c2 cardiomyoblasts, as indicated by green fluorescence in the accompanying representative images (Fig. 4A). However, both aspalathin ($p < 0.001$) and sulforaphane ($p < 0.001$), along with the comparative control-simvastatin ($p < 0.001$) improved mitochondrial mass and improved cellular morphology/structure (as highlighted in accompanying images) compared to the palmitic acid control (Fig. 4A). Exposure to palmitic acid also induced undesirable changes in mitochondrial membrane potential ($p < 0.001$), as evidenced by increased intensity of green fluorescence intensity (highlighting JC-10 monomers) (Fig. 4B). Notably, treatment with both aspalathin ($p < 0.05$) and sulforaphane ($p < 0.05$) resulted in improved mitochondrial membrane potential, even more significantly compared to simvastatin (which did not show any significance) (Fig. 4B). Representative images further demonstrate improved mitochondrial membrane integrity, showing the increased intensity of orange fluorescence (highlighting JC-10 aggregates and orange color within accompanying images) (Fig. 4B).

3.5. Aspalathin and sulforaphane reduce cytosolic and mitochondrial production of reactive oxygen species (ROS) in cardiomyoblasts exposed to palmitic acid

Exposure to palmitic acid resulted in a significant increase in both cytosolic ($p < 0.001$) and mitochondrial ROS levels, respectively (Fig. 5AB). This effect was similar to the intracellular ROS control, H_2O_2, which significantly elevated cytosolic and mitochondrial ROS production ($p < 0.001$). Notably, treatment with aspalathin ($p < 0.001$) and sulforaphane ($p < 0.001$) effectively mitigated this effect by reducing cytosolic ROS production compared to the palmitic acid control (Fig. 5A). Both aspalathin ($p < 0.01$) and sulforaphane ($p < 0.001$) demonstrated comparable effects to simvastatin ($p < 0.01$) in reducing mitochondrial ROS production relevance to the palmitic acid control (Fig. 5B).

3.6. Aspalathin and sulforaphane did not affect cholesterol levels but protected against lipid peroxidation in cardiomyoblasts exposed to palmitic acid

We evaluated the impact of both aspalathin and sulforaphane on cholesterol levels (Fig. 6A) and lipid peroxidation (Fig. 6B). Exposure to palmitic acid caused an increase in total cholesterol content ($p < 0.001$) (Fig. 6A). However, only treatment with simvastatin, a known lipid-lowering agent, effectively reduced cholesterol content ($p < 0.001$) compared to the palmitic acid control (Fig. 6A). As expected, palmitic acid caused an elevation in MDA levels ($p < 0.05$), indicating enhanced lipid peroxidation compared to the experimental control (Fig. 6B). Treatment with aspalathin ($p < 0.001$) and sulforaphane ($p < 0.01$) demonstrated comparable efficacy in reducing MDA levels ($p < 0.001$) (Fig. 6B).

3.7. Aspalathin and sulforaphane enhance intracellular antioxidant response in H9c2 cardiomyoblasts exposed to palmitic acid

We further explored the protective effects of aspalathin and sulforaphane against oxidative damage by analyzing the mRNA expression of

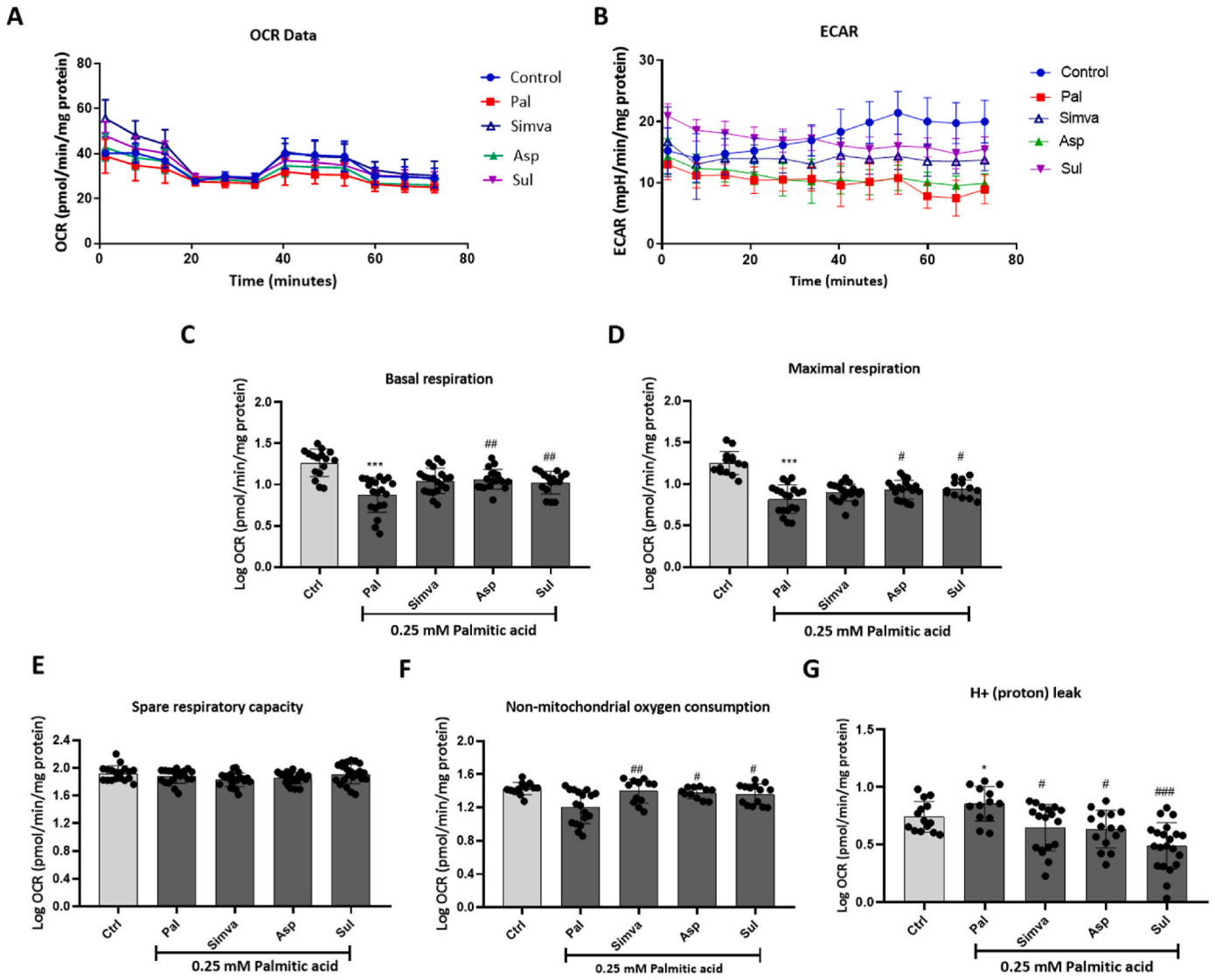


Fig. 2. Aspalathin and sulforaphane enhanced mitochondrial respiration and glycolytic energy levels while reducing proton leak (H+) in H9c2 cardiomyoblasts exposed to palmitic acid. Briefly, H9c2 cardiomyoblasts were pretreated with 1 μM aspalathin (Asp) and 10 μM sulforaphane (Sul) as well as 2.5 μM Simvastatin (Simva) which was used as a comparative control. Thereafter, cells were co-treated with 0.25 mM palmitic acid (Pal) for an additional 24 h. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured for all treatments (**A** and **B**). Parameters assessed included basal respiration (**C**), maximal respiration (**D**), spare respiratory capacity (**E**), proton leak (H+) (**F**), and non-mitochondrial respiration (**G**). Results are presented as Log values, representing the mean ± standard error of the mean (SEM) from three independent experiments, control consist of 6 technical repeats and all the treatment groups consists of 8 technical repeats per experiments. Comparisons between groups were performed using student's *t*-test (and nonparametric test). Statistical significance was represented by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the experimental control; and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the palmitic acid control.

intracellular antioxidants, specifically *Nrf2* and *Sod2*, together with SOD activity and total GSH content (Fig. 7AB). The mRNA expression of both *Nrf2* and *Sod2* was significantly reduced after exposure to palmitic acid ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 7AB). However, the mRNA expression of *Nrf2* and *Sod2* was significantly enhanced by the treatment of aspalathin and sulforaphane ($p < 0.01$), along with the comparative control, simvastatin ($p < 0.05$) in comparison to the palmitic acid control (Fig. 7AB). These results were collaborated by assessing the activity of SOD activity and total GSH content, which showed that both aspalathin ($p < 0.001$ and $p < 0.001$, respectively) and sulforaphane ($p < 0.05$ and $p < 0.001$, respectively) could significantly increase the levels of these antioxidants in comparison to the palmitic acid control (Fig. 7CD). This was similar to the effect of simvastatin on SOD activity ($p < 0.001$), whereas this comparative control did not affect the GSH content (Fig. 7CD).

3.8. Aspalathin and sulforaphane protect against cellular apoptosis induced by palmitic acid in cardiomyoblasts

In this study, we assessed the protective effects of aspalathin and sulforaphane against palmitate-induced cellular damage, by measuring the apoptotic rate and necrosis using annexin V and propidium iodide, respectively (Fig. 8). Measuring live cells showed that palmitic acid reduced cell viability ($p < 0.001$); however, both aspalathin ($p < 0.05$) and sulforaphane ($p < 0.01$) could reverse this effect (Fig. 8A). Palmitic exposure also significantly increased the rates of early ($p < 0.01$) and late ($p < 0.01$) apoptosis, including cell necrosis ($p < 0.01$) (Fig. 8B–D). Treatment with both aspalathin and sulforaphane decreased early ($p < 0.05$) and late ($p < 0.01$) apoptosis (Fig. 8BC). Simvastatin reduced early apoptosis ($p < 0.05$) but failed to affect the later stage of apoptosis or cell necrosis, while all treatment compounds did not protect against cell

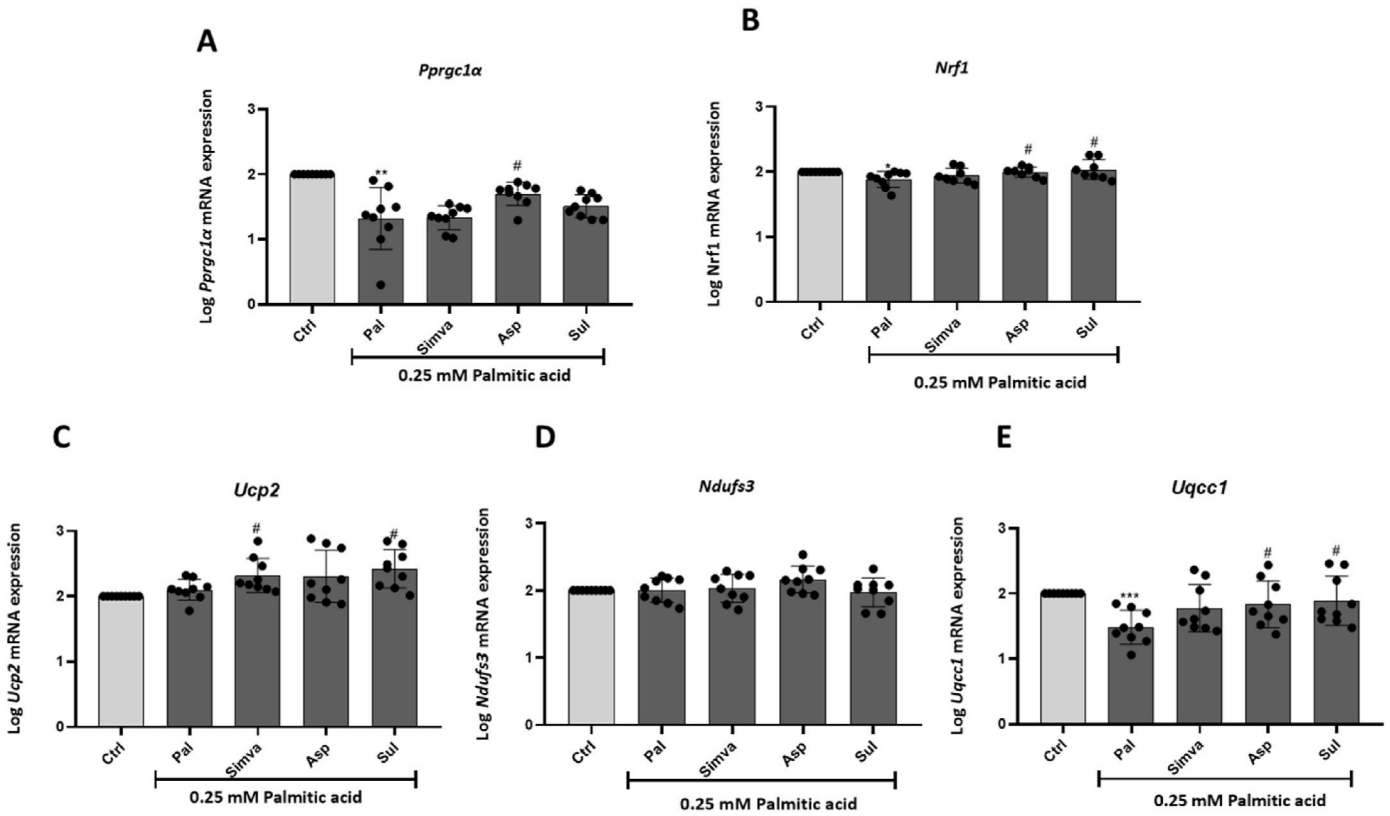


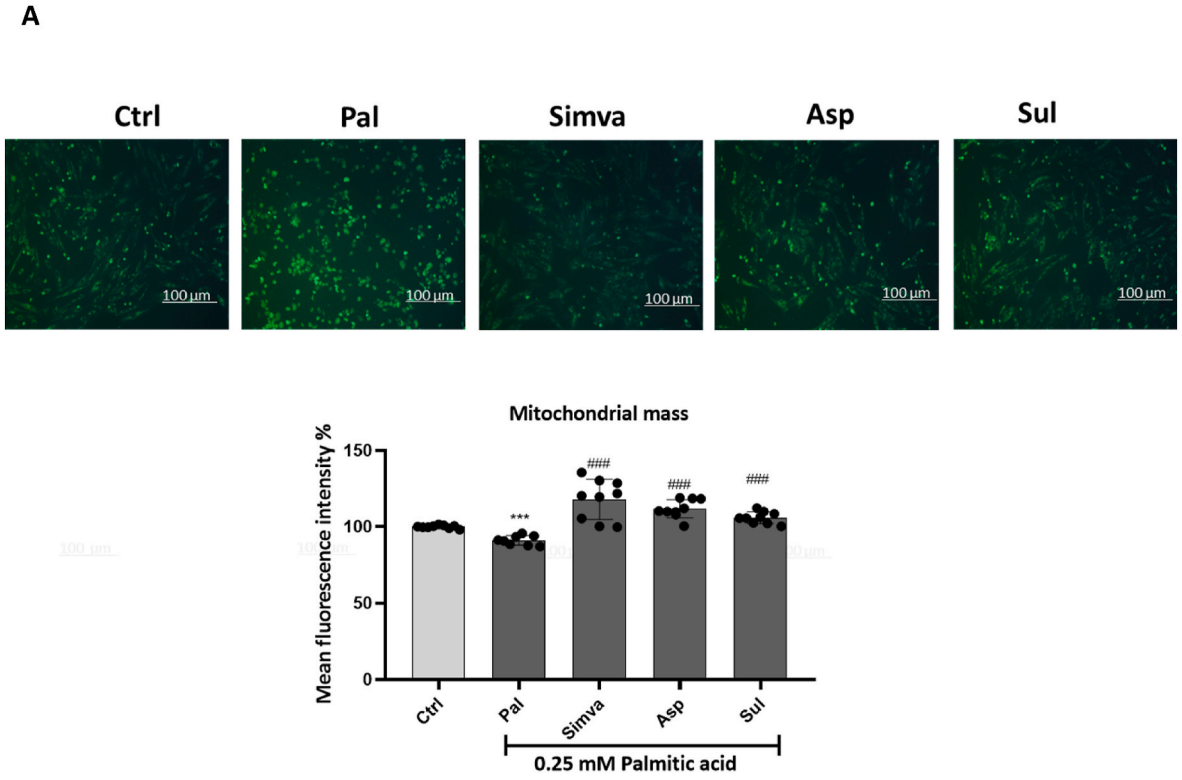
Fig. 3. Aspalathin and sulforaphane increased the mRNA expression of some markers involved in mitochondrial function in cardiomyoblasts exposed to palmitic acid. Briefly, H9c2 cardiomyoblasts were pretreated with 1 μM aspalathin (Asp) and 10 μM sulforaphane (Sul) as well as 2.5 μM Simvastatin (Simva) which was used as a comparative control. Thereafter, cells were co-treated with 0.25 mM palmitic acid (Pal) for an additional 24 h. The mRNA expression levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pprgc1α*), nuclear respiratory factor 1 (*Nrf1*), NADH: ubiquinone oxidoreductase core subunit S3 (*Ndufs3*), ubiquinol-cytochrome C reductase complex assembly factor 1 (*Uqcc1*), and uncoupling protein 2 (*Ucp2*) were quantified (**A, B, C, D,** and **E**, respectively). Results are presented as the mean ± standard error of the mean (SEM) from three independent experiments, each with three technical repeats (n = 3), relative to the experimental control (Ctrl). Comparisons between groups were performed using student's *t*-test (and nonparametric test). Statistical significance was represented by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the experimental control; and $^{\#}p < 0.05$ compared to the palmitic acid control.

necrosis (Fig. 8B–D).

4. Discussion

Myocardial-induced lipid overload, as a consequence of dyslipidemia, is considered a prominent pathological feature contributing to increased CVD risk [24]. As such, several experiments have made use of cultured heart cells, including H9c2 cardiomyoblasts, exposed to palmitic acid as an experimental model to study the detrimental effects of lipid overload on myocardial physiology [3,25–27]. In the current study, we evaluated whether the dietary compounds aspalathin and sulforaphane with reputable antioxidant properties can mitigate the detrimental effects associated with palmitic acid-induced cardiac toxicity. To assess cellular responses, an ATP production assay was utilized for its sensitivity, followed by real-time cell viability analysis through flow cytometry. This included the use of a reactive oxygen species (ROS) detection kit and fluorescent staining dyes (Annexin V and propidium iodide) to quantify apoptotic and necrotic cell populations, thereby offering detailed insights into the effects of palmitic acid-induced stress. Our results showed that exposing cardiomyoblasts to elevated levels of palmitic acid was associated with reduced metabolic activity which was measured by ATP production. The potentially toxic effects of palmitic acid could be reflected through reduced ATP production in collaboration with accelerated apoptosis in cultured H9c2 cardiomyoblasts [3]. The myocardium relies on a fine balance of respiratory production, and the availability of ATP levels, necessary for contractile function [28]. The current study showed that palmitic acid exposure interfered with the efficiency of the mitochondrial respiratory processes as seen with reduced basal and maximal respiration, which could have hindered the oxidative capacity of the cells. Measuring oxygen consumption is a highly informative experimental method that provides valuable insights [29]. It is particularly useful for identifying mitochondrial functionality parameters that could be influenced by pharmacological treatments and for characterizing energy metabolism within a physiological and pathological state [30]. For example, changes in oxygen consumption rates can indicate metabolic shifts, such as a transition from aerobic to anaerobic metabolism, triggered by exposure to palmitic acid [31,32]. This shift may disrupt ATP production and affect the overall cellular energy balance. Additionally, alterations in oxygen consumption can serve as a marker for oxidative stress, as increased proton leak and elevated production of ROS may lead to cellular damage [33,34].

Our results showed that palmitic acid exposure facilitated H+ (proton) leak, indicating an imbalance of membrane proton gradient, together with suppressing the mRNA expression of markers involved in mitochondrial function, including both biogenesis and bioenergetics (*Pprgc1α*, *Nrf1*, and *Uqcc1)*. In fact, beyond affecting mitochondrial oxidative capacity, palmitic acid exposure also interfered with mitochondrial biogenesis by reducing the expression of *Nrf1*, which is consistent with what has been previously reported ([35]; X. [36]). Furthermore, mitochondrial dysfunction has been linked with excessive accumulation of ROS [37]. Reviewed literature [38] suggests that exacerbated H+ (proton) leak may be associated with increased oxidative stress and subsequently cardiovascular complications. In this study, palmitic acid exposure enhanced the production of both cytosolic and



(caption on next page)

Fig. 4. Aspalathin and sulforaphane increased mitochondrial mass and membrane potential ($\Delta\psi m$) in cardiomyoblasts exposed to palmitic acid. Briefly, H9c2 cardiomyoblasts were pretreated with 1 μM aspalathin (Asp) and 10 μM sulforaphane (Sul) as well as 2.5 μM Simvastatin (Simva) which was used as a comparative control. Thereafter, cells were co-treated with 0.25 mM palmitic acid (Pal) for an additional 24 h. Mitochondrial mass was detected using Mito Tracker Green, while changes in mitochondrial membrane potential were probed through JC-10 fluorescent stain (**B**). The quantification of JC-10 aggregates, indicated by orange fluorescence at ~590 nm (excited by 540 nm), and JC-10 monomers, indicated by green fluorescence for mitochondrial membrane depolarization at ~525 nm (excited by 490 nm). Results are presented as the mean ± standard error of the mean (SEM) from three independent experiments, each with three technical replicates (n = 3) for mitochondrial mass and six technical replicates (n = 6) for mitochondrial membrane potential, relative to the control (Ctrl). Comparisons between groups were performed using one-way multivariate ANOVA, followed by Tukey's multiple comparisons test. Statistical significance was represented by ***$p < 0.001$ compared to the experimental control while $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the palmitic acid control. In **Figure A**, the images depict green fluorescence representing changes in cellular ultrastructure to represent intact mitochondrial mass. In **Figure B**, images depict intact mitochondria in orange colour, whereas mitochondrial membrane depolarization is stained green. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

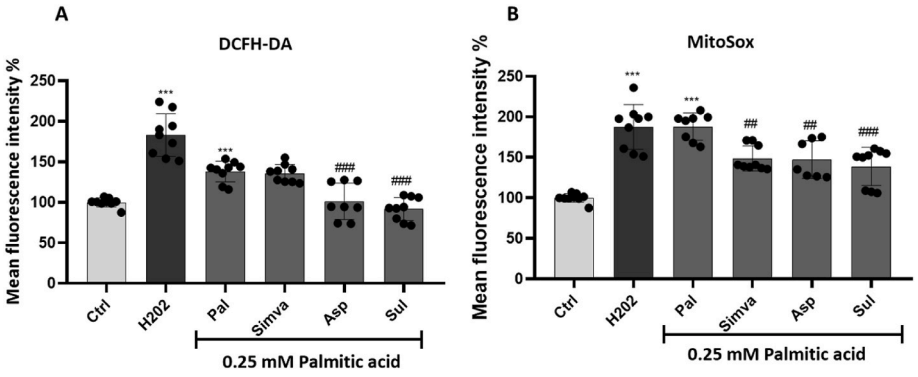


Fig. 5. Aspalathin and sulforaphane reduced cytosolic and mitochondrial production of reactive oxygen species (ROS) in cardiomyoblasts exposed to palmitic acid. Briefly, H9c2 cardiomyoblasts were pretreated with 1 μM aspalathin (Asp) and 10 μM sulforaphane (Sul) as well as 2.5 μM Simvastatin (Simva) which was used as a comparative control. Thereafter, cells were co-treated with 0.25 mM palmitic acid (Pal) for an additional 24 h. Simvastatin (Simva) was used as a comparative control. 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) (**A**) and MitoSox (mitochondrial superoxide indicators) fluorescent stains (**B**) were used for the detection of cytosolic and mitochondrial ROS production, respectively. Results are represented as the mean ± standard error of the mean (SEM) of three independent experiments, with three technical repeats (n = 3), per experiment relative to the control (Ctrl). Comparisons between groups were performed using one-way multivariate ANOVA, followed by Tukey's multiple comparisons test. Statistical significance was represented by ***$p < 0.001$ compared to the experimental control and $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the palmitic acid control.

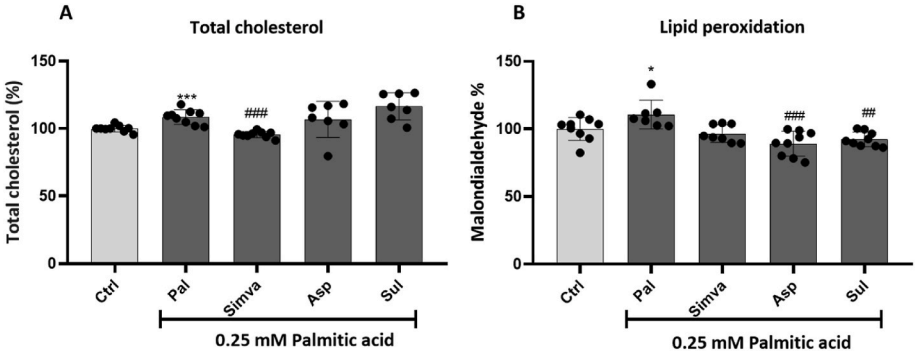


Fig. 6. Aspalathin and sulforaphane reduced cholesterol content and lipid peroxidation levels in H9c2 cardiomyoblasts. Briefly, H9c2 cardiomyoblasts were pretreated with 1 μM aspalathin (Asp) and 10 μM sulforaphane (Sul) as well as 2.5 μM Simvastatin (Simva) which was used as a comparative control. Thereafter, cells were co-treated with 0.25 mM palmitic acid (Pal) for an additional 24 h. Thereafter, the total cholesterol content (**A**) and malondialdehyde (MDA) levels were measured as indicators of lipid peroxidation (**B**). Results are represented as the mean ± standard error of the mean (SEM) of three independent experiments, with three technical repeats (n = 3), per experiment relative to the control (Ctrl). Comparisons between groups were performed using one-way multivariate ANOVA, followed by Tukey's multiple comparisons test. Statistical significance was represented by *$p < 0.05$, ***$p < 0.001$ compared to the experimental control; and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the palmitic acid control.

mitochondrial ROS consistent with impairments in mitochondrial respiration. In agreement, previous data [3,39,40] suggests that prolonged exposure to saturated fatty acids could cause impairments in mitochondrial respiration and enhance the generation of oxidative stress. As such, advances in treatments for CVD-related complications have increasingly focused on targeting alleviating mitochondrial abnormality with pharmacological compounds [41,42].

Furthermore, increased levels of ROS production are linked to decreased activities of intracellular antioxidants within a pathological state [43]. Endogenous antioxidants are vital for maintaining optimal cellular function and overall systemic health [44]. GSH is considered the most abundant endogenous antioxidant molecule in humans, found in relatively high concentrations enabling it to directly interact with superoxide radicals and other ROS molecules, while also indirectly supporting the function of other antioxidants [45]. Another crucial antioxidant is SOD also which also primarily neutralizes superoxide

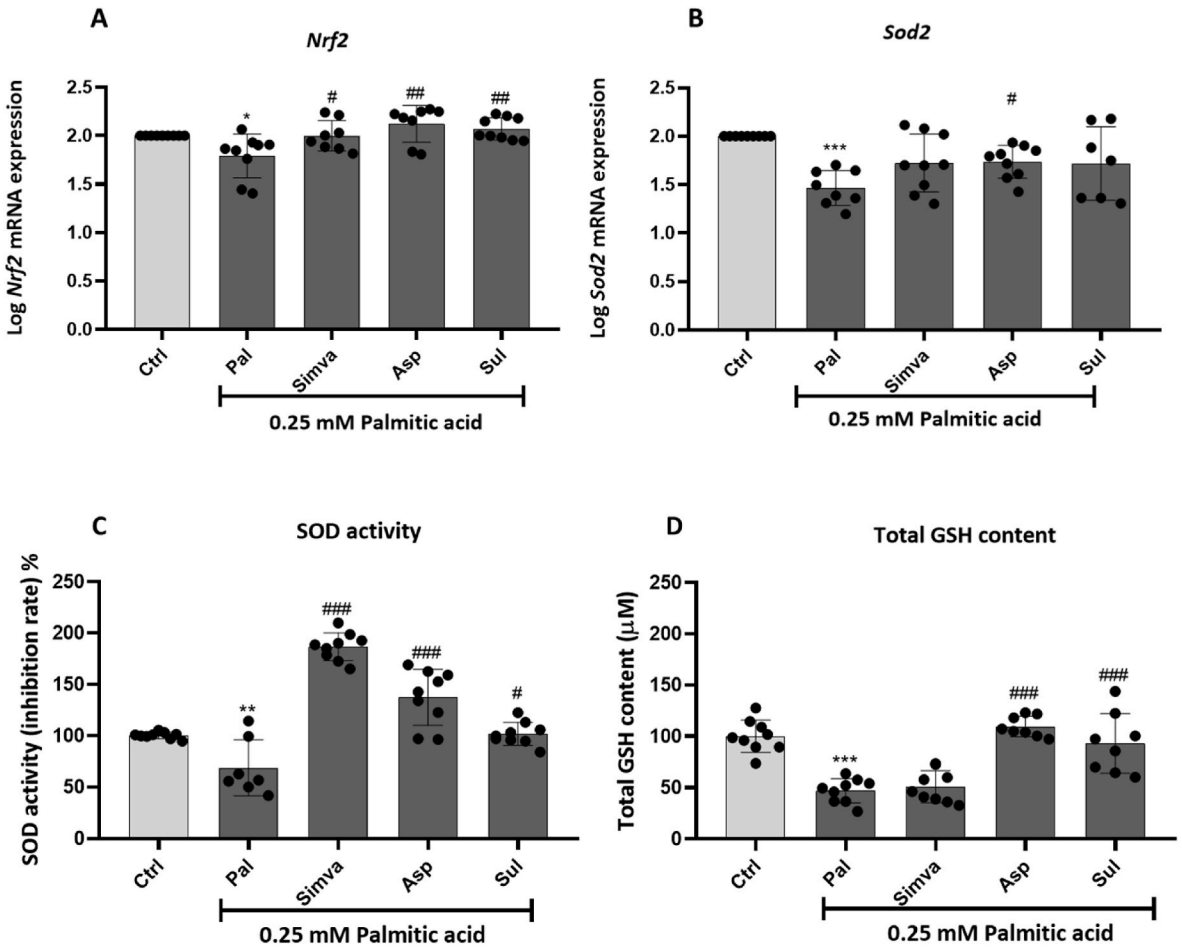


Fig. 7. Aspalathin and sulforaphane enhanced intracellular antioxidant responses in H9c2 cardiomyoblasts exposed to palmitic acid. Briefly, H9c2 cardiomyoblasts were pretreated with 1 μM aspalathin (Asp) and 10 μM sulforaphane (Sul) as well as 2.5 μM Simvastatin (Simva) which was used as a comparative control. Thereafter, cells were co-treated with 0.25 mM palmitic acid (Pal) for an additional 24 h. Subsequently, mRNA expression of nuclear factor erythroid 2-related factor 2 (*Nrf2*) and superoxide dismutase (*Sod2)* was quantified (**A** and **B,** respectively). This was in addition to the enzymatic capacity of superoxide dismutase (SOD) and glutathione (GSH) (**C** and **D**). Results are represented as the mean ± standard error of the mean (SEM) of three independent experiments, with three technical repeats (n = 3), per experiment relative to the control (Ctrl). Comparisons between groups were performed using student's *t*-test (and nonparametric test) for *Nrf2* and *Sod2* and one-way multivariate ANOVA, followed by Tukey's multiple comparisons test for SOD activity and GSH. Statistical significance was represented by *p < 0.05, **p < 0.01, ***p < 0.001 compared to the experimental control; and $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 compared to the palmitic acid control.

radicals as a protection against oxidative stress [46]. In this study, palmitic acid toxicity interfered with oxidative capacity and increased lipid peroxidation while diminishing the capacity of antioxidant enzymatic responses (SOD and GSH). Similar effects were observed with a reduction in the mRNA expression levels of *Nrf2,* the master regulator of antioxidant response, which is required for adequate responses for cytoprotective actions and attenuation of oxidative stress [47]. The reduced mRNA expression of *Nrf2*, together with other intracellular antioxidants, in response to palmitic acid exposure was associated with accelerated apoptosis and necrosis within our experimental model. These results support previous studies implying that cell death could occur as the consequence of impaired mitochondrial function under the condition of lipid-induced toxicity within the myocardium [48,49]. Furthermore, this study provides novel insights into the molecular mechanisms by which palmitic acid induces mitochondrial dysfunction in cardiac cells, specifically through the downregulation of key mitochondrial markers.

There is no doubt that oxidative stress is the sole driver of suppressed intracellular antioxidant response within many pathological conditions [50], necessitating the supplementation with dietary antioxidants to support cellular function [51]. The capacity of dietary compounds to enhance or conserve intracellular antioxidant responses could significantly contribute to cardiovascular protection [52]. Published preclinical studies indicate that plant extracts or bioactive compounds from plants, including those found in rooibos and broccoli, like aspalathin and sulforaphane, could potentially enhance the intracellular antioxidants to protect against diabetes-related complications [53,54]. These dietary compounds can protect the heart against oxidative damage by activating the antioxidant response element, *Nrf2,* within hyperglycemic conditions [55,56]. Clinical studies have demonstrated that consuming six cups of rooibos tea, which is rich in aspalathin, can help improve the oxidative status in individuals at risk of CVD [57]. Aspalathin has shown promising bioavailability, with evidence indicating its absorption in the gastrointestinal tract and detection in plasma following consumption [58]. It has been suggested that regular consumption of rooibos tea may further enhance its beneficial effects [59]. On the other hand, sulforaphane exhibits relatively low bioavailability due to rapid metabolism and instability [60]. This can limit its effectiveness in certain clinical contexts. However, consuming whole food sources or supplements, as well as utilizing advanced formulation strategies like myrosinase supplementation and encapsulation, have been shown to improve sulforaphane's absorption and stability[60–62]. Despite challenges in bioavailability, sulforaphane has shown therapeutic potential in clinical studies, particularly in cancer prevention,

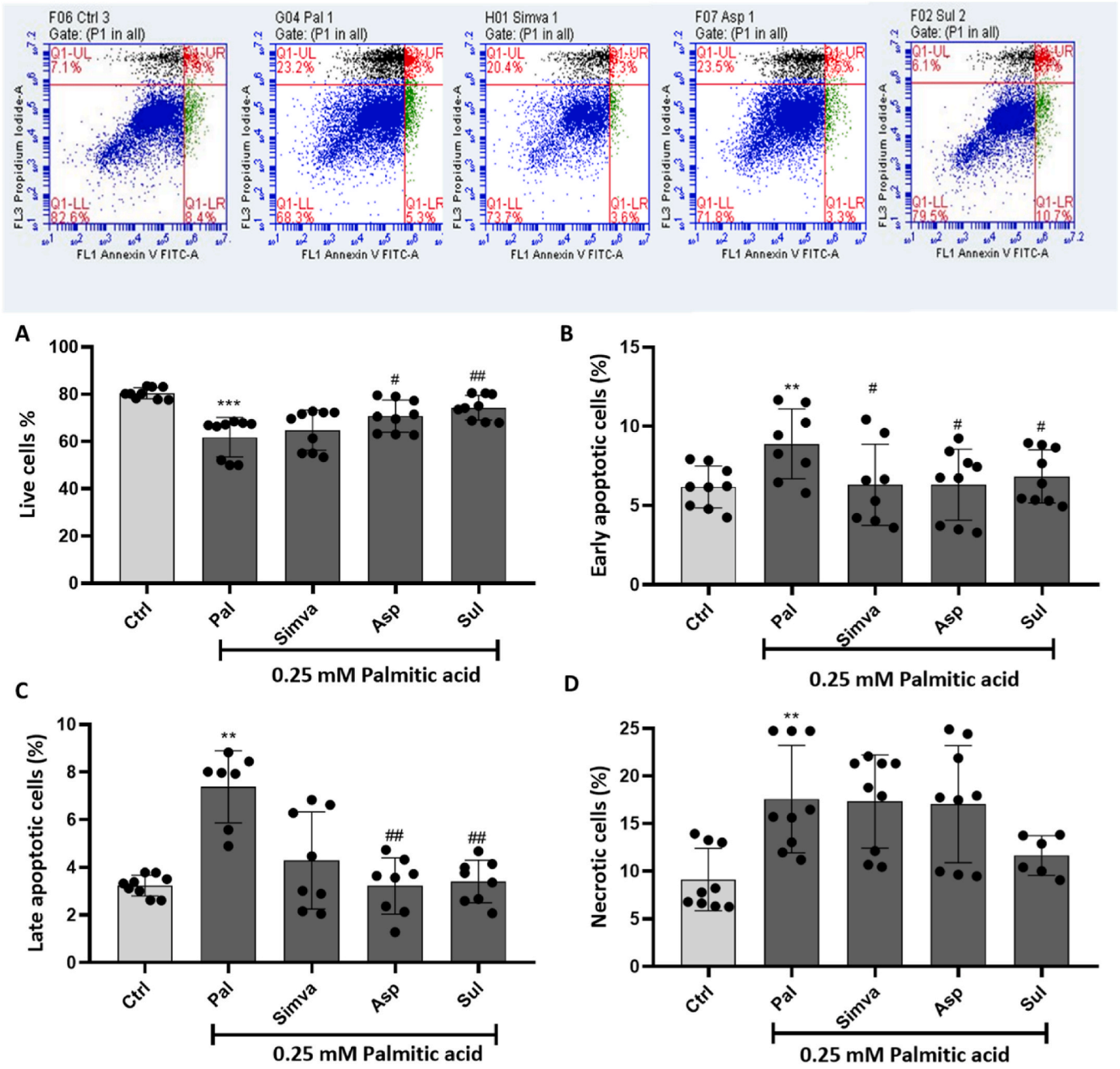


Fig. 8. Aspalathin and sulforaphane protected against cellular apoptosis induced by palmitic acid in cardiomyoblasts. Briefly, H9c2 cardiomyoblasts were pretreated with 1 μM aspalathin (Asp) and 10 μM sulforaphane (Sul) as well as 2.5 μM Simvastatin (Simva) which was used as a comparative control. Annexin V and propidium iodide staining were used to assess apoptotic and necrotic rates, respectively. The fluorescent images represent different stages of cell death, with viable cells in the lower left quadrant, early and late apoptotic cells in the lower and upper right quadrants respectively, and necrotic cells in the upper right quadrant. The graphs display the percentages of viable cells (**A**), as well as the rate of early apoptosis (**B**), and late apoptosis (**C**), including cell necrosis (**D**). Results are presented as the mean ± standard error of the mean (SEM) from three independent experiments, each with three technical repeats (n = 3), relative to the experimental control (Ctrl). Comparisons between groups were performed using student's *t*-test (and nonparametric test). Statistical significance was represented by $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the experimental control; and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the palmitic acid control.

neuroprotection, and inflammation, with promising effects even at lower concentrations [63]. Nonetheless, further research is required to understand its pharmacokinetics and optimize its clinical application fully.

The primary objective of this study was to evaluate the cardioprotective effects of aspalathin and sulforaphane, focusing on mitochondrial respiratory chain efficiency, reactive oxygen species (ROS) production, and lipid peroxidation, all of which serve as key indicators of mitochondrial health and oxidative stress. In addition to these primary outcomes, we also assessed secondary measures, including cholesterol levels and apoptosis/necrosis rates, to gain a more comprehensive understanding of the impact of these dietary compounds on lipid homeostasis and cellular injury. Our results showed that both aspalathin and sulforaphane could neutralize the harmful effects of palmitic acid by maintaining the metabolic activity of cells, while also positively affecting some parameters of mitochondrial respiration such as basal and maximal respiration, proton leak, and non-mitochondrial consumption. Spare capacity is a critical indicator of a cell's ability to

respond to increased energy demands and manage stress [64]. Notably, we observed no significant differences in spare capacity across the treatment groups. This lack of effect suggests that while aspalathin and sulforaphane enhance ATP production under baseline conditions, they do not significantly alter the cardiomyoblasts' capacity to respond to additional energy demands. This finding is noteworthy, as spare capacity is commonly used as an indicator of pathophysiological stress states [65]. The absence of an effect on spare capacity under these experimental conditions suggests that further exploration is needed to understand the specific mechanisms through which aspalathin and sulforaphane may influence cellular stress responses. The dietary compounds further improved mitochondrial membrane potential including the mRNA expression of key markers involved in this process, including *Pprgc1α, Nrf1*, and *Uqcc1*. Additionally, these dietary compounds could reduce H+ (proton) leaks while restoring the physiological state of cardiac cells by ameliorating the toxic effects of ROS. These results align with previously published studies from different experimental models, which demonstrated the protection of aspalathin by improving mitochondrial function in hepatic and skeletal muscle cells against palmitate insult [30,66]. Beyond aspalathin, other dietary compounds like curcumin and resveratrol have been shown to have enhanced therapeutic potential by improving mitochondrial function and detoxifying ROS production to improve cardiac function in conditions of metabolic stress [67,68]. Interestingly, these results corroborate previous findings supporting the potential therapeutic effects of sulforaphane improving the mitochondrial function and cellular function of cultured cardiomyocytes exposed to elevated palmitic acid levels [69]. Further, affirming the therapeutic potential of these dietary compounds, or foods rich in them, in protecting against diabetes-related complications.

We have also observed that conventional pharmacological approaches for both primary and secondary prevention of CVDs often focus on lowering circulating cholesterol levels [70,71]. Although our compounds of interest did not significantly affect the cholesterol levels, our results showed that they were able to reduce lipid peroxidation following the detrimental effect of palmitic acid. The strong antioxidant properties of these dietary compounds are aligned with their capability to up-regulate *Nrf2* expression as a strategy to protect against hyperglycemia-induced oxidative damage in cardiac cells [55,56]. Activation of *Nrf2*, including its downstream target genes, is one of the major mechanisms to protect against cardiac damage [72]. However, our results showed that both aspalathin and sulforaphane can inhibit apoptosis but fail to protect against cell necrosis. Perhaps inferring that these dietary compounds cannot reverse severe damage to the myocardium can serve as an appropriate intervention to protect against the development of the disease. However, this is a hypothesis that must be confirmed in well-designed in vivo studies, or clinical trials.

Overall, both these dietary compounds demonstrated improved beneficial effects in mitigating palmitic acid-induced cardiac abnormalities in our experimental model. The enhancement of intracellular antioxidants, possibly through the activation of *Nrf2*, appears to be the potential mechanism for blocking oxidative stress-induced cardiac apoptosis. Remarkably, both aspalathin and sulforaphane exhibit antioxidant effects that surpass simvastatin, a widely recognized lipid-lowering medication known to influence Nrf2/HO-1 signaling in diverse diseases ([73]; Q. [36]). These results indicate that both compounds not only have the capacity to protect against oxidative stress-induced cardiac damage but also potentially reduce lipid levels to prevent dyslipidemia and its associated complications. Our results offer promising insights into potential dietary strategies for mitigating cardiac oxidative damage, but we recognize that these findings are preliminary and largely based on in vitro models. The concentrations of compounds used in this study are higher than what is typically achievable through standard dietary intake. While dietary sources of these compounds may provide beneficial effects, their bioavailability and metabolism in humans could limit their ability to replicate the cellular responses observed in vitro [74]. To fully understand the practical applicability of these findings, further research is necessary to determine the optimal concentrations needed to achieve similar protective effects in human tissues, particularly in the context of lipid overload. Continued investigation will be crucial for refining dietary recommendations and assessing their real-world potential to improve cardiovascular health.

5. Conclusion

Our findings provide new insights into the potential therapeutic effects of aspalathin and sulforaphane in protecting against dyslipidemia-related cardiac toxicity. While we observed that these compounds enhanced ATP production under baseline conditions, there were no significant changes in spare capacity, suggesting that their protective effects may not extend to the ability of cardiomyoblasts to respond to additional energy demands under stress. These results highlight the need for further exploration into the mechanisms through which aspalathin and sulforaphane exert their cardioprotective effects.

This preclinical study supports the potential benefits of dietary compounds or foods rich in aspalathin or sulforaphane, such as rooibos and broccoli, in protecting against dyslipidemia-associated cardiac complications. However, it's important to recognize the limitations of using the H9c2 cardiomyoblasts model in vitro, which does not fully replicate human cardiac tissue complexities. Additionally, the concentrations of these dietary compounds used in the experiments may exceed those typically attainable through diet, posing challenges in applying these findings directly to dietary practices. Therefore, to comprehensively assess the cardioprotective properties of aspalathin and sulforaphane, further research using advanced models, such as primary cardiomyocytes, the AC16 human cardiac cell line, and animal studies, is essential to better understand their mechanisms and enhance the relevance of these findings to human health.

CRediT authorship contribution statement

Sinenhlanhla X.H. Mthembu: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Sithandiwe E. Mazibuko-Mbeje:** Writing – review & editing, Supervision, Funding acquisition. **Sonia Silvestri:** Writing – review & editing, Supervision. **Patrick Orlando:** Writing – review & editing, Conceptualization. **Bongani B. Nkambule:** Writing – review & editing, Supervision. **Christo J.F. Muller:** Writing – review & editing. **Luca Tiano:** Writing – review & editing. **Phiwayinkosi V. Dludla:** Writing – review & editing, Supervision, Funding acquisition.

Data availability statement

All data used to support the findings of this study are included in the article. Raw data can be available on request after publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

Δψm: changes in mitochondrial membrane potential; ATP: adenosine triphosphate; Asp: aspalathin; CVD: cardiovascular disease; Ctrl: control; DCFH-DA: 2', 7'-dichlorodihydrofluorescin diacetate; ECAR: glycolytic energy as measured by extracellular acidification rate; *Gapdh:* glyceraldehyde-3-phosphate dehydrogenase; GSH: glutathione; LDL: low-density lipoprotein; MDA: malondialdehyde; *Ndufs3:* NADH: ubiquinone oxidoreductase core subunit s 3; *Nrf1:* nuclear respiratory factor 1; *Nrf2:* nuclear factor erythroid 2-related factor 2; OCR: oxygen consumption rate; Pal: palmitic acid; *Pprgc1α:* peroxisome proliferator-activated receptor, gamma coactivator 1-alpha; ROS: reactive oxygen species; *Ucp2:* uncoupling protein 2; SEM: standard error mean; Sul: sulforaphane; SOD: superoxide dismutase; Simva: simvastatin; TBARS: thiobarbituric Acid Reactive Substances; *Uqcc1:* ubiquinol-cytochrome c reductase complex assembly factor 1.

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