International Scholars Journals

African Journal of Botany ISSN: 2314-9825, Vol. 12 (1), pp. 001-014, December, 2024. Available online at www.internationalscholarsjournals.org © International Scholars Journals

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Full Length Research Paper

Rooibos (Aspalathus linearis) has cardioprotective properties against isoproterenol-induced hypertrophy in H9c2 cardiomyoblasts. Improving mitochondrial and antioxidant activity

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Accepted 21 December, 2024

Systemic hypertension is one of the risk factors for cardiovascular disease (CVD), a global health concern. The left ventricle experiences severe stress from prolonged high blood pressure, which can cause left ventricular hypertrophy and eventually lead to left ventricular failure. The majority of pharmaceutical therapies are expensive and have long-term negative effects, particularly for patients who live in low-resource environments. In order to help manage CVD, efforts are being made to find more natural and reasonably priced adjuvant medicines. The purpose of this study was to assess Aspalathus linearis (Rooibos, RB)'s ability to prevent isoproterenol-induced hypertrophy in H9c2 cardiomyoblasts. Isoproterenol (50 mM, 24 h), RB (100 mg/mL, 24 h), co-treatment with isoproterenol (50 mM, 24 h), and control (2 % FBS media, 24 h) were the treatments administered to cardiomyoblasts. We performed the following tests: Western blots, high-resolution respirometry, lipid peroxidation assay (TBARS), antioxidant assays (SOD and CAT), cell viability assay (MTT), cell size (light microscopy), and an ATP assay. Isoproterenol decreased mitochondrial routine respiration, decreased SOD and CAT activities, increased oxidative stress through an increase in lipid peroxidation, decreased cell size and viability, complex-I linked OXPHOS and complex-II's contribution to the ETS via the S-pathway, and increased complex-IV activity without adversely affecting ATP levels. When RB and isoproterenol were administered together, cell size decreased, antioxidant enzyme activity improved, rotundine respiration increased, and complex-I increased the expression of total Akt and decreased the expression of total NFAT, while also linking OXPHOS and decreasing the cytochrome-c response. According to our research, RB might be used as an adjunct therapy to treat left ventricular hypertrophy. In this situation, RB's protective impact is attributed to its strong antioxidant properties and ability to rescue mitochondrial dysfunction.

Keywords: Left ventricular hypertrophy, Rooibos, Isoproterenol, Oxidative stress, Mitochondrial function.

INTRODUCTION

The Up to 32% of all deaths worldwide are caused by cardiovascular disease (CVD), making it the main cause of death worldwide (WHO, 2021). Low-to-middle-income countries account for 80% of deaths from CVD (World Heart Federation, 2017; Agyemang and van den Born, 2018; WHO, 2021; HSFSA, 2022). Nearly 225 people die from

CVD per day in South Africa, making it a major cause of death (HSFSA, 2022). Coronary artery disease, ischemic heart disease, rheumatic heart disease, and hypertensive heart disease are among the conditions that make up the spectrum of cardiovascular disease (CVD) (WHO, 2021; HSFSA, 2022).

More than 75% of the underlying risk factors for the onset and progression of CVD can be changed, according to the WHO (WHO, 2021). These include smoking, diabetes, high blood pressure, poor diet, and a sedentary lifestyle (WHO, 2021; Ghodeshwar et al., 2023). Around 1.3 billion individuals worldwide suffer from hypertension, and in South Africa, the prevalence has significantly increased from 25% in 2012 to over 40% in recent years (Kohli-Lynch et al., 2022; WHO, 2023). Increased cardiomyocyte lipid peroxidation, altered mitochondrial bioenergetics, and excessive reactive oxygen species (ROS) production are all linked to left ventricular hypertrophy, which is caused by increased pressure overload from hypertension (Gonz´alez et al., 2018; D'Oria et al., 2020). Effective management of left ventricular hypertrophy is crucial since, in the end, it causes left ventricular failure and death (Saheera and Krishna-murthy, 2020) (Fuchs and Whelton, 2020; WHO, 2023).

In contrast to industrialized nations, a significant number of people in low-resource nations cannot afford or obtain conventional pharmaceutical medications, which have been utilized to treat CVD (Tarride et al., 2009; Bangalee and Suleman, 2016; Dzudie et al., 2020). Moreover, bradycardia, tachycardia, hypotension, fainting, oedema, stroke, and angina are among the negative effects of prolonged use (Naganathan, 2013; Julian and Pocock, 2015; Rossello et al., 2015; Mabhida et al., 2021). As a result, more "natural," efficient, and thus less expensive adjuvant cardioprotective treatments are required (Smith and Swart, 2018). As adjuvant cardioprotective therapeutic agents, medicinal plants and herbs have received attention (Maarman, 2019; Jacobs et al., 2024). Their strong antioxidant properties and high polyphenolic content are responsible for these cardioprotective effects (Song et al., 2010). Compared to conventional pharmaceutical medications, medicinal herbs and plants are more accessible, less expensive, and generally safe (Mashour et al., 1998; Chen et al., 2016). Aspalathus linearis (Rooibos, RB), a native plant species of South Africa, is cultivated in the Western Cape's Cedarberg Mountains and is frequently consumed as herbal tisane (Smith and Swart, 2018). Additionally, RB has drawn a lot of interest due to its many health advantages, including as its anti-inflammatory, anti-apoptotic, and antioxidant properties, which protect the heart and vascular system. Its high polyphenol content, which has been demonstrated to lessen oxidative stress, lower blood pressure, and enhance endothelial function, is primarily responsible for these benefits. Furthermore, Rooibos has shown promise in regulating important cellular pathways, including those related to inflammation and apoptosis, which can protect the cardiovascular system and lessen tissue damage in diseases like heart failure and hypertension (Tse et al., 2013; Windvogel, 2020; Maarman and Lecour, 2022). RB has never been examined in relation to left ventricular hypertrophy, though. Thus, the purpose of this work is to examine how RB prevents isoproterenol-induced hypertrophy in H9c2 cardiomyoblasts and the underlying processes that underlie this impact.

2. Methods

2.1. Preparation of extract and experimental drugs

A gift from Rooibos Limited® (Clan William, South Africa) was a cold-water soluble RB extract (fermented batch number: E1CCJ/23/PP/A). A stock solution with a concentration of 10 mg/mL was created by dissolving the powdered extract in 1 mL of cold distilled water after it had been weighed to 10 mg. After filtering, this stock solution was kept at —80 °C. No previous studies had examined the effects of Rooibos in a H9c2 cardiomyoblast hypertrophy model at the time of the investigation. Thus, we used this special Rooibos extract in a dose-response test. Following the dose-response test, the stock solution was further diluted to a final concentration of 100 mg/mL in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 2% fetal bovine serum (FBS). Isoproterenol (Sigma Aldrich, South Africa) was created by diluting the stock solution of 0.1 M in newly manufactured DMEM supplemented with 2% FBS to a working concentration of 50 mM (Tsai et al., 2017). Our initial dose-response experiments serve as the basis for all concentrations. For twenty-four hours, cells were exposed to RB and isoproterenol.

2.2. Quantification of individual flavonoids

The Agricultural Research Council used a previously reported method of high-performance liquid chromatography (HPLC) analysis to identify the specific components in our RB extract (Walters et al., 2017). To put it briefly, we used an Agilent 1200 system (Agilent, Santa Clara, CA, USA) with HPLC and diode-array detection. A ZORBAX SB C18 guard column (12.5 £ 4.6 mm, 5 mm) and a Poroshell 120 C18 column (150 £ 4.6 mm, 2.7 mm) were utilized in the HPLC study. The column temperature was kept at 44.5 °C, and the flow rate was fixed at 1 mL/min. Since different substances have different spectra with different wavelengths for maximum absorbance, it is impossible to directly compare the peak height or area for different peaks. As a result, we utilized 288 nm for compounds like PPAG and Aspalathin that had a maximum absorbance close to 288 nm, and 350 nm for compounds like orientin and isoorientin that had a maximum absorbance close to 350 nm. Furthermore, various compounds may have different extinction coefficients—that is, different quantities of the compound are required to produce the same peak area—even though their maximum absorbance occurs at a comparable wavelength. As a result, we created calibration curves against which each compound is measured using genuine reference standards. The final results are given as g compound/100 g of soluble solids.

2.3. Cell culturing and maintenance

The Division of Medical Physics donated the H9c2 rat cell line. The cells were cultured in DMEM (Capricorn Scientific, GmbH, Germany) supplemented with 10% FBS (Capricorn Scientific, GmbH, Germany) and 1% penicillin/streptomycin solution (Capricorn Scientific, GmbH, Germany). The cells were kept in a monolayer at 37 °C and in a humidified air atmosphere with 95% oxygen and 5% carbon dioxide. The growth medium was replaced every 48 hours while the cells were allowed to multiply in 100 mm cell culture plates (NEST, New Jersey, USA). Cultures were separated after they achieved 70% confluency by washing the cell monolayer with phosphate-buffered saline (PBS) that had been preheated to 37 °C. This was followed by trypsinization with 0.25 percent trypsin-ethylenediami-netetraacetic acid (EDTA) (Capricorn Scientific, GmbH, Germany). Following their collection and separation, the cells were resuspended in new growth media and seeded at the appropriate seeding density for the tests that followed. For all tests, cells from passages 14–18 were utilized.

2.4. Cell culture treatments

In preparation for the experiment, cells were cultivated at a seeding density of 3.5 £ 105 in 100 mm plates (NEST, New Jersey, USA). Prior to the addition of therapy, cells were rinsed with warm PBS once they had reached 60% confluency. RB (100 mg/mL, 24 h, following a dose-response test), isoproterenol (50 mM, 24 h), control (DMEM supplemented with 2% FBS, 24 h), and a co-treatment group that received RB (100 mg/mL, 24 h) and isoproterenol (50 mM, 24 h) concurrently for 24 h were the various experimental groups. Following treatment, the cells underwent two PBS washes before being trypsinized to extract the cells from the plate's surface. Three biological repeats (n=three) made up each treatment group in all trials, while three technical repeats made up one biological repeat.

2.5. Cell viability

A 3-(4, 5-dimethylthiazolyl-2)—2, 5-diphenyltetrazolium bromide (MTT) cell viability assay was used to evaluate cell viability. H9c2 cells were planted in fresh medium supplemented with 10% FBS at a density of 5.0 $ 104 cells per well in a 24-well plate (NEST, New Jersey, USA). After being cultivated to 60% confluency, the cells received a 24-hour treatment. The positive control group's media was taken out 30 minutes prior to the completion of the experiment, and 10% dimethyl sulfoxide (DMSO) (Merck, South Africa) was added to 2% FBS-supplemented treatment media. Following treatment, all wells were cleaned with PBS and incubated for 2.5 hours at 37 °C without CO2 using an incu-shaker (Benchmark Scientific, Sayreville, USA) with MTT solution (2 mg/mL) (Invi-trogen, Massachusetts, USA). After being treated with DMSO and Sorenson's buffer, the cells were incubated for 30 minutes on the incu shaker. A FLUOstar Omega® (BMG LABTECH, Germany) microplate reader was used to read the plate at an absorbance of 570 nm after the incubation period. The quantity of live cells directly correlates with the intensity and concentration of the purple color produced. The final data is presented as a percentage of viability.

2.6. Cell size determination

Prior to treatment, cells were allowed to reach 50% confluency for 24 hours after being seeded at a density of 2.8 $ 104 cells per 35 mm cell culture dish (NEST, New Jersey, USA). The DMEM was taken out after 24 hours, and the cells were then rinsed with warm PBS before being cultured in treatment media for another 24 hours. Following treatment, cells were viewed at a magnification of 20 pounds using a Nikon inverted microscope with a Polaroid digital camera. For every dish, ten random photos were obtained, and using ImageJ software (NIH), at least ten measurements of the surface area of each individual cell were acquired from each picture. mm2 (area) is the final data expression.

2.7. Assay lysate preparation

Following treatment, the samples underwent a 5-minute, 92-g centrifugation to pellet the cells. After discarding the supernatant, the pellet was mixed with lysis buffer (0.5 mM potassium phosphate buffer containing 10% Triton X-100 (Merck, South Africa)). After adding 0.5 mm zirconium beads, the mixture was bullet-blended (Next Advance, USA) at 8 rpm for three 1-minute cycles, with one-minute cold rests in between. The samples were centrifuged at 5000 g for 5 minutes after being treated in lysis buffer on ice for 30 minutes. The cytosolic fraction, or supernatant, was separated into aliquots and kept at -80 °C until it was required. Langdon (2008) provided the optimal lysate preparation method.

2.8. Protein determination

The Qubit protein assay kit (Thermo Fischer Scientific, Massachusetts, USA, Cat: Q33211) was used to measure the protein content on the Qubit fluorometer (Thermo Fischer Scientific, Massachusetts, USA). The Qubit protein reagent is first diluted 1:200 into the Qubit protein buffer to create the Qubit working solution. To get a final amount of 200 mL, 190 mL of working solution is added to 10 mL of standard and samples in specialized Qubit Eppendorf tubes (0.5 mL). After two to three seconds of low-speed vortexing, the standards and samples were allowed to sit at room temperature for fifteen minutes. The standard curve was then calculated by reading each standard on the Qubit fluorometer. To calculate the final concentration, each sample was read and compared to the standard curve. The results were expressed in milligrams per milliliter.

2.9. Antioxidant determination

An antioxidant enzyme called superoxide dismutase (SOD) is essential for converting superoxide into hydrogen

peroxide and molecular oxygen (Zheng et al., 2023). The autooxidation of 6-hydroxydopamine (6-OHD) is measured to determine the activity of SOD (Sigma Aldrich, South Africa). As previously stated, cell lysates were made. Ellerby and Bredesen's (2000) approach was used to determine the SOD activity assay. In a 96-well flat bottom plate (Merck, South Africa), the samples and blank (SOD assay buffer, 0.5 mM potassium phosphate buffer pH = 7.4) were tested in triplicate. The SOD test buffer was used to make 0.1 mM diethylenetriamine penta-acetic acid (DETAPAK) (Merck, South Africa) and add it to each well. SOD assay buffer was then added to the wells until 185 mL was filled. Prior to reading the plate, freshly made 1.6 mM 6-OHD was added to the wells. Using the FLUOstar Omega® (BMG LABTECH, Germany) microplate reader, the auto-oxidation of 6-OHD was observed at 490 nm for five minutes at 25 °C. The data are reported as units/mg protein/min.
The antioxidant enzyme catalase (CAT) is in charge of converting hydrogen peroxide into oxygen and water (Nandi et al., 2019). The reduction in hydrogen peroxide molecules is used to measure CAT activity. Cell lysates were made using the previously mentioned procedure. A 96-well UV flat bottom plate (Merck, South Africa) was used for the triplicate analysis of all samples and blanks. The CAT activity assay was altered from Ellerby and Bredesen's (2000) previously established technique. Each sample and blank received 170 mL of CAT assay buffer (0.5 mM potassium phosphate buffer pH = 7.0). Before reading, a 30% hydrogen peroxide solution (Merck, South Africa) was made in distilled water and added to each well. A FLUOstar Omega® (BMG LABTECH, Germany) microplate reader was used to measure the absorbance over a period of five minutes in order to determine the linear decrease over time at 240 nm. CAT activity was quantified using the molar extinction coefficient of hydrogen peroxide (43.6 M/cm) and mmole H2O2 consumed/min/mg protein.

2.10. Lipid peroxidation

Malondialdehyde (MDA) levels are measured using the Thiobarbituric Acid Reactive Substances (TBARS) assay, which is frequently used to gauge oxidative stress (Aguilar Diaz De Leon and Borges, 2020; Esterbauer and Cheeseman, 1990). With the exception of utilizing a lysis solution made of 0.01 potassium phosphate buffer with a pH of 7.4 and 1.15 percent potassium chloride (KCl) (Merck, South Africa), lysates were produced as previously reported. The MDA standards (0, 0.322, 0.625, 1.25, 2.5, 5, 10, 25, and 30 nmol/mL MDA) were produced by further diluting a 500 mM MDA (Merck, South Africa) standard solution with distilled water. One milliliter of a 2% sodium lauryl sulphate (SDS) (Merck, South Africa) solution, two milliliters of a 10% trichloroacetic acid (TCA)—8% butylated hydroxytoluene (BHT) (Merck, South Africa) reagent, and two milliliters of a 0.67 percent w/v tertiary butyl alcohol (TBA) (Merck, South Africa) solution were added to each glass test tube for the assay. Marbles were then used to cap the tubes in order to stop evaporation. The test tubes were then submerged for an hour at 95 °C in a water bath. The tubes were let to cool for ten minutes to reach room temperature when the incubation period was over. 200 mL of the supernatant of all standards and samples was plated in triplicate in a 96-well flat bottom plate (Merck, South Africa) after the samples were centrifuged at 121 £ g for 15 minutes. The FLUOstar Omega® microplate reader (BMG LABTECH, Germany) was used to measure the absorbance at 530 nm.
The mmoles of MDA per milligram of protein were used to express the consequences of lipid peroxidation.

2.11. Western blotting

In 100 mm cell culture dishes, cells were planted at a density of 3.5 £ 105, allowed to reach 60% confluency, and then treated for 24 hours. Five milliliters of PBS were added for harvesting after the cells had been cleaned with prewarmed PBS. After being scraped, the cells were put into 15 mL containers and centrifuged for five minutes at 4 °C at 92 x g. After removing the supernatant, 400 mL of lysis buffer (20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris—HCl), 1 mM ethylene glycol tetra-acetic acid (EGTA), pH 7.4, 1 mM EDTA, 150 mM sodium chloride (NaCl), 1 mM b-glycerophosphate, 2.5 mM tetra-Na-phosphate, 1 mM sodium orthova-nadate (Na3VO4), 50 mg/mL phenylmethylsulfonyl fluoride (PMSF), 0.5% protease inhibitor cocktail, and 1% Triton X-100) (Merck, South Africa) were added to the pellet and kept at —80 °C for western blotting. The Bradford protein test (Bradford, 1976) was used. In order to see the proteins in the gel, 14 mg of protein were loaded and separated using 10% hand-cast polyacrylamide gels with 2, 2, 2 tri-chloro-ethanol added to the gel. The foundation of this approach is the UV alteration of naturally occurring tryptophan residues in proteins by trichloro chemicals. Once the proteins in the gel have separated, they can be seen in a ChemiDoc without causing the gel to become stained, resulting in a "stain-free gel." The stain-free gels were then photographed using the ChemiDocTM XRS+ System (Bio-Rad) to activate the gel and visualize the total proteins in the gels. Proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were subsequently moved using the Transfer system (Bio-Rad) to a polyvinylidene fluoride (PVDF) membrane (Merck, South Africa). 5% fat-free milk in Tris-buffered saline solution with 0.1% Tween (pH 7.4) was used to prevent nonspecific binding sites for two hours at 24 °C. Phospho-specific and total protein expression antibodies, such as Total Akt (1:1000, ab81283, Abcam), Total mTOR (1:1000, ab134903, Abcam), p-GSK 3b (1:1000, CST#9323, Cell signaling), Total GSK 3b (1:1000, CST#12456, Cell signaling), Pan Calcineurin (1:1000, CST#2614, Cell signaling), NFAT 1 (1:1000, ab283649, Abcam), and ERK 1/2 (1:1000, ab136926, Abcam) were incubated on membranes for an entire night at 4 °C.
All antibody dilutions were made in a Tris-buffered saline solution with 0.1% Tween and 5% fat-free milk. Horseradish peroxidase-linked secondary antibody (1:4000) was then used to incubate the membranes. A chemiluminescent agent (ECL) from Bio-Rad was then used to enhance the

signals, and ChemiDoc from Bio-Rad was used to visualize the results using Image Lab 6.1. Image Lab software was used to analyze the results, and the stain-free membrane pictures were used to normalize the data to the total protein loaded. All values were presented as a fold change from the untreated control samples for each gel.

2.12. High-Resolution respirometry

Employing the previously published protocols, mitochondrial respiration was measured in an Oroboros O2K-FluoRespirometer employing a polarographic oxygen sensor (Gnaiger et al., 2020). DatLab acquisition computer software (Oroboros Instruments®, Innsbruck, Austria) was used to capture the oxygen sensor's amplified signal at two-second intervals. Before the experiment began, the respirometer was calibrated at 37 °C and air saturation. Following treatment schedules and counting, the H9c2 cell pellets were put in the 2 mL chambers of the Oroboros, resuspended in 2.5 ml of MiRO5 respiration media, and rinsed with PBS. Following the stabilization of the oxygen flux, normal respiration was documented. To permeabilize the cell membranes, digitonin (10 mg/mL) was then introduced to the chambers. Adenosine diphos- phate (ADP) (2.5 mM) was then added to measure complex-I linked oxidative phosphorylation (OXPHOS) after pyruvate (5 mM) and malate (2 mM) were titrated to stimulate beta-oxidation-linked leak respiration via the electron-transferring flavoprotein dehydrogenase (ETF). The integrity of the outer mitochondrial membrane was next evaluated by titrating cytochrome c (10 mM). The maximum electron transfer (ETS) capacity was then measured by titrating a carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (0.5 mM) uncoupler in increments of 1 mL until it reached the maximal responsive level. To quantify complex-I's contribution to the ETS through the N-pathway, glutamate (10 mM) was titrated. Then, to increase the S-pathway's contribution to the ETS, suc-cinate (10 mM) was added. To evaluate the contribution of the F-pathway, or beta-oxidation's contribution to the ETS, octanoylcarnitine (0.5 mM) was introduced. Rotenone (0.5 mM), a complex-I inhibitor, was then titrated to determine how much complex-II contributed to the ETS capacity. In order to stimulate residual oxygen consumption (ROX), which is oxygen consumption used for redox processes outside of the mitochondria and not ATP synthesis, antimycin A (2.5 mM), a complex-III inhibitor, was employed. In order to evaluate complex-IV activity, ascorbate (2 mM) and TMPD (0.5 mM) were titrated last, followed by sodium azide (100 mM) (Gnaiger, 2014). The final results and all of the reagents (Merck, South Africa) were adjusted for ROX and normalized to cell number (per million). The final results are shown as pmolO2/s/mL.

2.13. ATP assay

In order to achieve 60% confluency, H9c2 cells were seeded at 5.0 $ 104 in 35 mm cell culture dishes (NEST, New Jersey, USA) and incubated for 24 hours. We employed the cell viability luciferase assay (Merck, South Africa, cat: # SCT149) to measure the amount of ATP. As directed by the protocol, standards were prepared as a serial dilution in PBS with concentrations of 100 mM, 10 mM, 1 mM, 100 nM, 10 nM, and 0 (blank). Following treatment, cells underwent washing, trypsinization, and centrifugation at 92 g for five minutes at 4 °C. In a 96-well white/clear flat-bottom cell culture dish (NEST, New Jersey, USA), the standards and samples were plated in triplicate after the pellet was resuspended in 300ul of PBS for each treatment group. Prior to reading the plate, firefly lucif- erase was added to the ATP assay solution in a ratio of 1 mL to 100 mL after an ATP detection assay cocktail had been pipetted. Each well (apart from the blank) received 100 m L of the ATP detection assay cocktail, and the plate reader was set up to read the sample for 10 s. The plate was read using the FLUOstar Omega® (BMG LABTECH, Germany) micro-plate reader's luminescence program function. The luciferase enzyme used in this assay oxidizes D-luciferin using ATP, producing light in the process. Consequently, relative light units (RLU) are used to assess ATP.

2.14. Statistical analysis

Utilizing GraphPad Prism 10.2 (GraphPad Software, Inc., Boston, MA, USA), statistical analysis was carried out. The Shapiro-Wilk and D'Agostino-Pearson omnibus normality tests were used to determine whether the data was normal. A one-way ANOVA analysis was used to compare the treatment groups, and a Bonferroni multiple comparisons as a post hoc test or, if appropriate, a student t-test were used afterward. The significance level was set at $p < 0.05$, and all results are shown as the mean § (standard error of the mean) SEM.

3. Results

3.1. HPLC analysis of rooibos extract

High amounts of S/R Eriodictyol-6-glc (0.313 % and 0.290 %), Phenylpyruvic acid-2-O-glucoside (PPAG) (0.436 %), and flavones such as isoorientin (0.410 %), orientin (0.610 %), and vitexin (0.112 %) were found in the extract, according to the HPLC analysis. Bioquercetin (0.139%) was the quercetin glycoside identified in the extract. Additionally present were S/R-Eriodictyol-8-glc (0.138 % and 0.145 %). The extract also included dihydrochalcones such nothofagin (0.035%) and aspalathin (0.164%). The cold-water extract also included the flavone luteoloside (0.248%) and the compounds hyperoside and ferulic acid (0.029%). Rutin and isovitexin, the other chemicals, were detected in trace amounts (0.090% and 0.023%). Isoquercetin, however, was below the assay's detection limit (Table 1).

3.2. Dose-response tests for isoproterenol and RB

The viability of 100 mM isoproterenol was lower than that of the control (72.86 § 4.872 % versus 100 %, $p = 0.0051$) (Fig. 1). Cell viability was lower with RB at 80 mg/mL than with control (83.11 § 2.765% vs. 100%, $p = 0.0036$) (Fig. 1).

3.3. The effect of treatments on cell viability and cell size

While RB had no effect, isoproterenol reduced cell viability in comparison to the control group (80.55 § 6.19 % vs 100 § 0.00 %, p = 0.02) (Fig. 2). RB and isoproterenol co-treatment decreased cell size compared to the isoproterenol group (3324 § 124.9 mm2 vs 5884 § 142.1 mm2, p < 0,0001), whereas isoproterenol increased cell size compared to the control (5884 § 142.1 mm2 vs 3727 § 79.07 mm2, p < 0.0001) (Figs. 3 and 4).

3.4. Effect of treatments on antioxidant activity and lipid peroxidation

The use of RB and isoproterenol together increased SOD activity in comparison to the isoproterenol group (1400 § 165.2 U/mg/min vs 853.9 § 118.3 U/mg/min, p = 0.02), while isoproterenol reduced SOD activity in comparison to controls (853.9 § 118.3 U/mg/min vs 1218 § 171.7 U/mg/min, p < 0.024) (Fig. 5). Additionally, RB and isoproterenol co-treatment increased the CAT activity compared with the isoproterenol group (129.5 § 26.63 mmol H2O2/min/mg vs 76.15 § 11.4 mmol H2O2/min/mg, p = 0.01), while isoproterenol decreased the CAT activity compared to controls (75.15 § 11.4 mmol H2O2/min/mg vs 185.6 § 29.25 mmol H2O2/min/mg, p = 0.0033) (Fig. 5).

Fig. 3. The effect of RB on cell size in an isoproterenol-induced H9c2 hypertrophy model. H9c2 rat cardiomyoblasts were treated with 50 mM isoproterenol for 24 h, fol- lowed by exposure to 100 mg/mL RB alone or a combination of 50 mM ISO and 100 mg/ mL RB. Control cells were exposed to 2 % FBS media only. The graph represents cell size presented as a measurement in mm compared to the control. Results are pre- sented as the mean § standard error of the mean (SEM) from three independent
experiments, each with three technical replicates. Comparisons between groups were performed using one-way ANOVA, followed by Bonferroni post hoc test. *p < 0.05 (vs control), #p < 0.05 (vs isoproterenol).

While co-treatment had no impact, isoproterenol increased lipid peroxidation in comparison to the control group (36.96 § 1.549 mmol MDA/mg vs 19.47 § 4.69 mmol MDA/mg, p = 0.033) (Fig. 6). In comparison to the isoproterenol group, the group that received only RB showed higher SOD activity (1186 § 80.65 U/mg/min vs 853.9 § 118.3 U/mg/min, p = 0.036) and higher CAT activity (145.9 § 21.63 mmol H2O2/min/mg vs 76.15 § 11.4 mmol H2O2/min/mg, p = 0.0122) (Fig. 5). Furthermore, lipid peroxidation was lower in the group that only got RB than in the group that received isoproterenol (20.05 § 2.46 mmol MDA/mg vs. 36.96 § 1.549 mmol MDA/mg, p < 0.0001) (Fig. 6).

3.5. The effect of treatments on protein expression

None of the signaling proteins' expression was impacted by isoproterenol. In contrast to the isoproterenol groups, co-treatment with RB and isoproterenol decreased overall Akt expression (0.7308 § 0.082 AU vs. 1.188 § 0.076 AU, p = 0.0149) (Fig. 7).

Furthermore, compared to the isoproterenol group, the co-treatment decreased the expression of total NFAT (0.4858 § 0.159 AU vs 0.9109 § 0.093 AU, p = 0.04) (Fig. 7). In comparison to the control group, the group that received only RB showed lower levels of total GSK 3b (0.4408 § 0.123 AU vs 1.0 § 0.087 AU, p = 0.0207), calcineurin (0.2959 § 0.028 AU vs 1.178 § 0.115 AU, p = 0.0017), and total NFAT (0.4203 § 0.119 AU vs 0.9109 § 0.093 AU, p = 0.0313). In comparison to the control group, the data also showed decreased expression of total mTOR (0.3704 § 0.063 AU vs 1.0 § 0.098 AU, p = 0.0401). Furthermore, the ERK 1/2 ratio was lower in the group that only received RB than in the control group (0.7831 § 0.034 AU vs. 1.0 § 0.029 AU, p = 0.0076) (Fig. 7).

3.6. The effect of treatments on mitochondrial respiration

In comparison to the control group, isoproterenol decreased routine respiration (27.58 § 2.338 pmolO2/s/mL vs 75.23 § 3.935 pmolO2/s/mL, p < 0.0001), and co-treatment with RB and isoproterenol decreased routine respiration (27.58 § 6.177 pmolO2/s/mL vs 55.78 § 2.338 pmolO2/s/mL, p = 0.0023) (Fig. 8). RB and isoproterenol co-treatment increased complex-1 linked OXPHOS compared to the isoproterenol group (69.15 § 10.73 pmolO2/s/mL vs 27.44 § 4.256 pmolO2/s/mL, p = 0.0056), while isoproterenol decreased it in comparison to the control (27.44 § 4.256 pmolO2/s/mL vs 100.1 § 13.5 pmolO2/s/mL, p = 0.0011) (Fig. 8). In comparison to the control, the co-treatment decreased the cytochrome c response (15.78 § 4.181 pmolO2/s/mL vs 97.47 § 13.04 pmolO2/s/mL, p = 0.0012).

In addition, the N-pathway (23.73 § 1.49 pmolO2/s/mL vs 92.75 § 16.87 pmolO2/s/mL, p = 0.0114), S-pathway (39.73 § 10.63 pmolO2/s/mL vs 164.1 § 23.1 pmolO2/s/mL, p = 0.0033), and F-pathways (24.9 § 12.88 pmolO2/s/mL vs 128.3 § 30.69 pmolO2/s/mL, p = 0.0314) were all decreased in comparison to the control.. Furthermore, compared to the control group, isoproterenol increased complex-IV activity (481.1 § 69.42 pmolO2/s/mL vs 296.8 § 28.57 pmolO2/s/mL, p = 0.0270) and decreased complex-II linked respiration via the S-pathway (67.54 § 30.71 pmolO2/s/mL vs 164.1 § 23.1 pmolO2/s/mL, p = 0.0306) (Fig. 8). ATP levels were unaffected by either RB or isoproterenol (Fig. 9).

4. Discussion

Our study's key conclusions were that isoproterenol caused H9c2 hypertrophy by increasing cell size, decreasing cell viability, decreasing SOD and CAT activity, increasing lipid peroxidation, decreasing mitochondrial routine respiration, decreasing complex-I linked OXPHOS, decreasing complex-II linked respiration via the S-pathway, and increasing complex-IV activity without having an adverse effect on ATP levels. We also showed that co-treatment with RB and

isoproterenol enhanced mitochondrial respiration, reduced total Akt and NFAT expression, enhanced antioxidant enzyme activity, and reduced cell size.
By simulating the effects of excessive adrenaline and noradrenaline exposure on the heart, isoproterenol, a catecholamine and beta-adrenergic receptor agonist, is frequently used to cause cardiac hypertrophy in both in vivo and in vitro models (Leenen et al., 2001; Chowdhury et al., 2013). When the beta-adrenergic receptor is stimulated, oxidative stress rises and major changes in gene expression occur, which leads to a hypertrophic response that resembles human left ventricular hypertrophy (Zhao et al., 2017; Velusamy et al., 2020). At a concentration of 50 mM, isoproterenol in our study increased cell surface area while also producing more dead cells. This is consistent with our viability data, which indicate that isoproterenol had cytotoxic effects at this dose (supplementary material: Figure 10 and 11). These results are in line with the research of Tsai et al. (2017), who demonstrated that giving H9c2 cells 50 mM isoproterenol for 24 hours was able to effectively trigger hypertrophic response. Through an increase in cell size, they were also able to validate hypertrophy.
Because oxidative stress increases the generation of reactive oxygen species (ROS), which damage cellular components and interfere with essential signaling pathways, it plays a critical role in the development of cardiac hypertrophy. Free radicals harm the carbon-carbon double bonds in lipids, particularly the polyunsaturated fatty acids in the cell membrane, a process known as lipid peroxidation, which is a type of oxidative stress (Aguilar Diaz De Leon and Borges, 2020). MDA is one of the several products produced by the lipid peroxidation process (Ayala et al., 2014). Lipid peroxidation is therefore a significant indicator of oxidative stress (Ito et al., 2019).

According to our findings, the group treated with isoproterenol had higher MDA levels. Attalla et al. (2018) reported similar findings, showing a rise in cardiac oxidative stress indicators such TBARS. Shen and Qian (2006) supported this by demonstrating that lipid peroxidation rose during the generation of ventricular hypertrophy. By causing oxidative stress through increased ROS generation, isoproterenol raises lipid peroxidation and causes oxidative damage to cell membrane lipids (Khalil et al., 2015; Khan et al., 2018). These findings imply that increased lipid peroxidation leads to increased oxidative damage to the cell membrane, which accelerates the hypertrophic response. According to Zehiroglu and Ozturk Sarikaya (2019), antioxidant systems like SOD and CAT are essential for controlling the amount of ROS in cells. These enzymes shield cells from damage caused by ROS, making them the first line of defense against oxidative stress (Karthikeyan et al., 2010; Zehiro-glu and Ozturk Sarikaya, 2019). According to our findings, the isoproterenol-treated group's SOD and CAT activity decreased. Similar findings have been reported in other studies. For instance, Mohammed et al. (2020) observed that in a rat model of cardiac hypertrophy, isoproterenol reduced SOD and CAT. De Lacerda Alexandre et al. (2021) provided additional support for this by showing that administering isoproterenol to mice resulted in a decrease in endogenous SOD and CAT activities and that the oxidative effect mediated the induction of hypertrophy within the tissue. The enhanced catecholamine signaling via beta-adrenergic receptors is probably the cause of the decline in antioxidant enzyme activity. Superoxide ions are produced in excess as a result of enhanced adrenergic receptor signaling, which inhibits SOD function (Rathore et al., 1998; Fekry et al., 2014). Additionally, since SOD converts superoxide to hydrogen peroxide, which CAT then breaks down into water and oxygen, the decrease in CAT activity may be the consequence of either reduced SOD activity or overwhelmed antioxidant defenses brought on by increased oxidative stress (Murtaza et al., 2008; Qin et al., 2010; Lee and Park, 2021).
Numerous intracellular signaling pathways that control the growth and function of cardiac cells are among the intricate processes involved in the formation and evolution of left ventricular hypertrophy (Takano et al., 2020). We employed western blot analysis to examine the expression levels and activation state of hypertrophic protein markers, such as Akt, GSK 3b, calcineurin, NFAT, mTOR, and ERK 1/2, in order to comprehend the molecular signaling pathways and how they are connected to the development of left ventricular hypertrophy (Antos et al., 2002; Zhang et al., 2011; Takano et al., 2020). Since we used an MTT dose-response test to determine the drug's concentration at which enhanced cell growth occurs, it was surprising that isoproterenol had no effect on the signaling pathways. Since the 50 mM concentration increased cell size more than the other concentrations, we decided to use it. Prior research with a comparable concentration served as our guide (Tsai et al., 2017). Although we lack a logical explanation for this event, another investigation found that the medicine did alter proteins, suggesting that either the dosage or the duration of therapy was insufficient (Fan et al., 2019). This data should be regarded cautiously because we were also unable to blot for the phosphorylated forms of these proteins.
Heart enlargement is largely caused by mitochondrial dysfunction, which raises oxidative stress and ROS production. Excessive ROS produced by damaged mitochondria promote hypertrophic development (Nollet et al., 2023). We conducted a thorough investigation of mitochondrial respiratory pathways using high-resolution respirometry. The term "routine respiration" describes the baseline amount of oxygen that cells' mitochondria use when they are engaged in normal cellular functions. The amount of ATP required by the cell, its efficiency in producing ATP, and any disturbances in OXPHOS all affect this respiratory state (Gnaiger, 2023). Because they contain leftover substrates from the culturing process that may affect energy generation, it also shows the general health of the cells. Thus, decreased routine respiration in cells may be a sign of serious injury or a cell type's vulnerability to damage. Because reduced oxygen consumption under basal conditions may indicate compromised OXPHOS capacity resulting from ETS dysfunction, isoproterenol therapy decreased mitochondrial routine respiration, indicating a deleterious influence on mitochondrial function. Rats given

isoproterenol injections showed a comparable decrease in intrinsic respiration in a prior study (Krestinin et al., 2020). This observation, when combined with our data, indicates that isoproterenol increases the likelihood of cell damage, which leads to the cells using their remaining substrates for energy rather than the ETS through various mitochondrial complexes.
In order to do this, complex-I-linked OXPHOS—the oxygen consumption rate fueled by NADH-linked substrata like pyruvate and malate in the presence of ADP—was decreased by isoproterenol (Gnaiger et al., 2020; Gnaiger, 2023). Given that complex I connects glucose oxidation, the TCA cycle, and the ETS to produce ATP, isoproterenol's action suggests decreased mitochondrial activity. This is in line with research that indicates hypertrophic hearts have decreased complex-I activity. Chouchani et al. (2014) discovered a link between the onset of hypertrophic cardiomyopathy and a reduction in complex-I activity, which is enough to cause hypertrophic cardiomyopathy without the need for pathways linked to oxidative stress. This decrease in complex-I activity may possibly result from isoproterenol's propensity to raise ROS, which could further oxidatively damage complex-I in the mitochondria. Several of complex-I's cysteine residues undergo oxidative thiol modification as a result of these ROS, which lowers activity (Okoye et al., 2023).
Isoproterenol decreased complex-II's contribution to the ETS, or the S-pathway. Through the transport of electrons from succinate into complex II, this route calculates the amount of oxygen consumed. The TCA cycle produces succinate, which feeds electrons to ubiquinone through complex II and functions as a substrate (Gnaiger et al., 2020; Gnaiger, 2023). The S-pathway was decreased by isoproterenol, which would indicate compromised complex II activity. According to our data, complex-II also experienced oxidative damage from isoproterenol, which would indicate a decreased capacity to oxidize succinate and, consequently, a decreased oxidation of glucose. This is consistent with earlier research showing that decreased glucose oxidation is linked to pathological ventricular hypertrophy (Tran and Wang, 2019).

We also measured the activity of cytochrome-c oxidase, or complex-IV. The last complex of the mitochondrial ETS, complex-IV oxidase, converts oxygen to water by using cytochrome c as a substrate. Ascorbate and TMPD are introduced to complex-IV to donate electrons in order to evaluate complex-IV activity in the SUIT technique. In line with earlier research that demonstrated the effect of isoproterenol in H9c2 cells, where complex-IV activity is elevated during compensated ventricular hypertrophy, our investigation found that isoproterenol boosted complex-IV activity (Rosca et al., 2013; Signorile et al., 2022).
There is increasing interest in employing natural products as adjuvant therapy for CVD because of the role that mitochondrial function and oxidative stress play in heart health. Due in large part to its abundant antioxidant qualities, RB, a well-known tisane from South Africa, has become well-known for its possible cardioprotective benefits (Marnewick et al., 2010; Ajuwon et al., 2015). RB has long been used to treat conditions like allergies, eczema, asthma, and colic (Mckay and Blumberg, 2007). It has well-established anti-inflammatory and antioxidant qualities, is free of caffeine, and contains little tannins (Joubert et al., 2008; Marnewick et al., 2010). The South African fynbos plant Aspalathus linearis, which is used to make RB, has a high polyphenolic content and offers a number of health advantages (Afrifa et al., 2023).
The extract included high concentrations of PPAG, orientin, iso-orientin, luteoloside, and S/R-Eriodictyol-6-glc, according to our HPLC investigation. Numerous studies have demonstrated that these phenolic components of RB have antioxidant properties and lessen the metabolic consequences of CVD (Dlu-dla et al., 2014; Windvogel, 2020). According to reports, PPAG protects overall cellular integrity by lowering the mitochondrial membrane potential through oxidative stress reduction and mitochondrial hyperpolarization prevention (Dludla et al., 2017a). By lowering oxidative stress and inflammation, orientin can lessen cardiac remodeling (Li et al., 2017). By preventing migration and proliferation, luteoloside can lessen vascular remodeling (Su et al., 2015). By drastically lowering cell size in comparison to the isoproterenol group, a study evaluating the effects of vitexin, a common component of RB and several other natural products, was able to lessen isoproterenol-induced cardiac hypertrophy in rat cardiomyoblasts (Lu et al., 2013). Although aspalathin has been demonstrated to have numerous health benefits, our HPLC analysis revealed that it was lower than the other component, which is specific to the extract (Muller et al., 2012; Johnson et al., 2016; Dludla et al., 2017b).
In our investigation, RB (as a stand-alone treatment) decreased lipid peroxidation and raised the activity of antioxidant enzymes SOD and CAT, suggesting an improved antioxidant response. The decrease in lipid peroxidation is probably caused by the high polyphenolic content of RB, which shields cardiomyoblasts from oxidative damage. Several research have shown similar results, showing that RB has cardioprotective effects on the heart by increasing antioxidant capacity and reducing ROS (Marnewick et al., 2003; Waisundara and Hoon, 2015; Dludla et al., 2020). Through a variety of signaling pathways, especially those related to cell signaling and stress response, like Akt and ERK, RB is known to have cardioprotective effects. For example, a study examining the impact of RB on vascular function in the aortas of diet-induced obese rats discovered that RB therapy significantly reduced PKB/Akt phosphorylation (Eldieb, 2017). It has been demonstrated that vitexin, a component of RB, modulates ERK1/2 signaling, lowering calcium excess during ischemia-reperfusion injury, even though ERK signaling in RB treatment is less well-established (Che et al., 2016). Additionally, there was a decrease in the expression of GSK-3b, calcineurin, total NFAT, total mTOR, and total ERK1/2 in the group that received only RB. However, some of these positive effects were diminished when RB and isoproterenol were combined. This is probably because isoproterenol causes severe oxidative damage, which may be too much for RB's antioxidant capacity to handle. The toxicity of isoproterenol seems to restrict its

protective potential, even though RB's antioxidant qualities can somewhat reduce oxidative stress. However, RB may offer cardioprotection beyond merely lowering oxidative stress by influencing pathways like Akt and ERK. These mechanisms could be further explored in future studies to have a better understanding of RB's entire spectrum of cardioprotective effects.

The dosage utilized in this study was carefully chosen with consideration for human intake in light of our results about the cardioprotective benefits of RB and its impact on important signaling pathways. Joubert et al. (2012) state that the average concentration of soluble solids in a cup of RB tea is roughly 1172 mg/mL, with a range of 843 to 1666 mg/mL. Understanding the true exposure, however, depends critically on the bioavailability of these substances after ingestion. Only 0.26% of RB is bioavailable after consumption, according to earlier studies (Breiter et al., 2011).

This means that 304.72 mg/mL of RB would be bioavailable after drinking one cup of RB tea. Therefore, our study's dose is roughly three times lower than what one cup of RB tea would provide in terms of bioavailability. This makes our results more applicable and interpretable since it implies that the dosage used in our experimental model is well within a reasonable and attainable range for human intake.

Although it had no effect on lipid peroxidation, co-treatment of RB and isoproterenol was able to reverse the isoproterenol-induced increase in cell size without affecting cell viability. Our results are in contrast to earlier studies by Chipofya (2024), in which RB decreased indicators of lipid peroxidation in a cell type subjected to oxidative stress. The lack of lipid peroxidation reduction in the RB co-treatment group in our investigation raises the possibility that RB's protective benefits work by directly restricting cell development as opposed to lowering lipid peroxidation. Nonetheless, as previously noted, the co-treatment did raise SOD and CAT activities, most likely as a result of RB's high polyphenolic flavonoid content or its stimulatory influence on antioxidant synthesis (Dludla et al., 2017a). Our findings support those of Fekry et al. (2014), who demonstrated a considerable rise in the activity of several antioxidant enzymes, such as SOD and CAT. Furthermore, Hong et al. (2014) showed that RB reduces the inhibitory effects on the activity of SOD and CAT, hence modulating their activity. Curiously, we did not see a decrease in MDA levels in spite of the smaller cells. This could suggest that the RB concentration in our model was insufficient to counteract lipid peroxidation in response to the high dose of isoprotere- nol. The strong effects of isoproterenol and the severity of the used hypertrophy model are probably to blame for this result. On the other hand, RB might have affected additional oxidative stress indicators that were not measured in this investigation, like iso- prostanes, protein carbonyls, oxidative DNA damage, or nitrotyro- sine. The strong protective effects RB shown in decreasing cell size and increasing antioxidant enzyme activity are noteworthy, even though it had no effect on lipid peroxidation. These results demonstrate the potential of RB's distinct polyphenol profile and mix of bioactive components to provide notable cardioprotective benefits, especially given that it has not been well investigated in the setting of hypertrophy. To fully grasp RB's potential in cardiovascular health, future studies could investigate these pathways in more detail.

RB decreased the overall expression of Akt and NFAT in the co-treatment. This result is consistent with another work by Eldieb (2017) that found that a diet-induced model of heart hypertrophy similarly showed a decrease in Akt. The transcription and translation of hypertrophic genes are further promoted by Akt's regulation of several downstream targets, including GSK 3b and mTOR (Le´ger et al., 2006; Shioi et al., 2002; Shiojima et al., 2005). By blocking the calcineurin/NFAT pathway, GSK 3b, which is active at rest, acts as a negative regulator of cardiac hypertrophy. Its activity is regulated by Akt, which phosphorylates GSK 3b at a particular serine 9 location, making it inactive, when PI3 K activates it (Morisco et al., 2000). Additionally, the hypertrophic response has been linked to calcineurin, a crucial calcium-dependent phosphatase that links modifications in cardiac cells' calcium handling to hypertrophic growth (Wilkins and Molkentin, 2002).

A crucial transcription factor called NFAT can be dephosphorylated and activated by activated calcineurin, which causes it to go into the nucleus and transcribe the hypertrophic genes needed for the development of cardiac cells (Sugden, 1999). However, except from lowering the expression of NFAT and total Akt, RB had no effect on these proteins in the co-treatment in our investigation. Since we were unable to blot for the phosphorylated versions, we cannot be positive, but the decreased total Akt and total NFAT may indicate that RB provides protection in our model through this anti-hypertrophic pathway. Notably, phosphorylated NFAT causes the transcription factor to stay in the cell's cytoplasm and stops hypertrophic genes from being transcribed (Antos et al., 2001; Sugden et al., 2008). However, because of budgetary limitations, we were only able to blot for total NFAT expression in our investigation. Determining activity levels is difficult because the total expression encompasses both NFAT's active and inactive stages. The results should therefore be regarded cautiously.

Co-treatment of RB with isoproterenol improved complex-I-linked OXPHOS and routine respiration in terms of mitochondrial respiration. In the setting of antimycin A-induced dysfunction, Mthembu et al. (2021) examined the impact of RB flavonoids, particularly Aspalathin, isoor-ientin, and orientin, on mitochondrial function. According to their research, these flavonoids enhanced complex-I expression and mitochondrial respiration. Furthermore, complex-IV activity was unaffected by RB, which is consistent with prior research that examined the impact of flavonoids on complex-IV activity and found none (Lagoa et al., 2011). Additionally, the combination-treated group showed a decrease in the cytochrome c response, indicating that RB offers protection against possible harm to the outer mitochondrial membrane. When combined with our data, these results show that RB improved routine respiration and complex-I-linked OXPHOS while preventing the negative effects of isoproterenol on mitochondrial function. Accordingly, RB increases the capacity of the mitochondrial

phosphorylation system and triggers a compensatory process that enables cells to use their natural substrates sparingly in order to avert further harm.

5. Conclusion

By improving antioxidant enzyme (SOD, CAT) activities, decreasing the expression of total Akt and total NFAT, and improving mitochondrial activity through a complex-I route, Rooibos reversed the isoproterenol-induced increase in H9c2 cell size. This is the first study to examine RB's effects against isoproterenol-induced H9c2 hypertrophy, and it shows promise as an adjuvant cardioprotective treatment, particularly for patients with heart disease who develop cardiac hypertrophy. More research is needed, though.

Declaration of competing interest

The authors affirm that none of the work described in this publication may have been influenced by any known conflicting financial interests or personal ties.

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