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Morin protects against palmitic acid-induced diabetic cardiomyopathy via SIRT6 activation and mitochondrial preservation

K. P Divya[1], P. V Anuranjana[1], Krupa Thankam George[1], Krishnaprasad Baby[1], Fathima Beegum[1], Navjot Kanwar[2], Nitesh Kumar[3], Krishnadas Nandakumar[1*], Abhinav Kanwal[4]

[1]Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal, India.
[2]Department of Pharmaceutical Sciences and Technology, Maharaja Ranjit Singh Technical University, Bathinda, India.
[3]Department of Pharmacology, National Institute of Pharmaceutical Education and Research, Hajipur, India.
[4]Department of Pharmacology, All India Institute of Medical Sciences, Bathinda, India.

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ABSTRACT

Diabetic cardiomyopathy (DCM) is a serious diabetes complication marked by cardiac dysfunction and structural abnormalities. Mitochondrial dysfunction, driven by lipotoxicity and glucotoxicity, is central to DCM pathogenesis. SIRT6, a sirtuin family protein, is a potential therapeutic target due to its role in mitochondrial protection. This study explored morin, a natural flavonoid, as a SIRT6 activator to protect against palmitic acid-induced cardiomyopathy in H9c2 cells. *In silico* molecular docking identified morin as a potential SIRT6 activator with strong binding affinity, especially at residues ASP116 and PHE82. *In vitro* studies in H9c2 cardiomyoblasts confirmed morin's protective effects against palmitic acid-induced toxicity. Morin pretreatment improved cell viability and reduced cytotoxicity. It enhanced glucose uptake, preserved mitochondrial membrane potential, and protected against mitochondrial damage. Morin also decreased reactive oxygen species (ROS), and lowered early and late apoptosis rates, showing strong anti-apoptotic effects. Additionally, morin upregulated SIRT6 and OPA1 expression, key genes for mitochondrial function and protection. These results suggest morin's cardioprotective effects involve SIRT6 activation, mitochondrial protection, antioxidant activity, and anti-apoptotic mechanisms. By upregulating SIRT6 and OPA1, morin may counteract mitochondrial dysfunction in DCM. This study highlights morin's therapeutic potential as a natural compound for DCM treatment, warranting further preclinical and clinical research.

1. INTRODUCTION

Diabetic cardiomyopathy (DCM) is a distinct clinical condition marked by structural and functional myocardial abnormalities, independent of coronary artery disease, valvular defects, or hypertension. It is primarily driven by hyperglycemia, dyslipidemia, and insulin resistance, which collectively induce mitochondrial dysfunction, oxidative stress, cardiomyocyte apoptosis, and fibrosis. These metabolic derangements disrupt glucose uptake and utilization, exacerbate mitochondrial dysfunction, and lead to reduced contractility and myocardial remodeling [1,2].

Excess reactive oxygen species (ROS) generation causes oxidative damage to lipids, proteins, and DNA, activating pro-apoptotic pathways and compromising mitochondrial membrane potential (MMP), creating a vicious cycle of oxidative stress and cell loss [3–6]. Mitochondrial dynamics, especially the role of OPA1, a dynamin-like GTPase essential for mitochondrial fusion and cristae maintenance, are critically impaired in DCM. The downregulation of OPA1 leads to mitochondrial fragmentation, bioenergetic failure, and increased apoptosis [7–13].

SIRT6 downregulation in diabetic hearts is associated with impaired antioxidant gene expression, heightened oxidative stress, and increased apoptosis. Beyond oxidative defense, SIRT6 regulates insulin signaling, lipid metabolism,

*Corresponding Author
Krishnadas Nandakumar, Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal, India.
E-mail: nandakumar.k @ manipal.edu

chromatin remodeling, and genomic stability. It also modulates apoptotic pathways through GATA4 and enhances antioxidant defenses via the Nrf2/ARE axis [14–31].

Cardiomyocytes are essential for cardiac contractility and electrical conductivity. Their high metabolic demand makes them particularly vulnerable to oxidative stress, leading to mitochondrial dysfunction and apoptosis, hallmark features of DCM [32–36]. H9c2 cardiomyoblasts, derived from rat embryonic heart tissue, offer a suitable *in vitro* model for studying DCM-related mitochondrial dysfunction due to their structural and metabolic resemblance to cardiomyocytes and sensitivity to oxidative insults [37,38].

Palmitic acid (PA), a major dietary saturated fatty acid, induces cardiomyocyte apoptosis and mitochondrial dysfunction by triggering oxidative stress, making it a well-established model for lipid-induced DCM [39–46]. Despite its role as an energy source, high PA intake contributes to cardiovascular pathologies due to its lipotoxic effects.

Morin, a plant-derived flavonoid, exhibits potent antioxidant and anti-apoptotic properties. It enhances enzymatic antioxidants (e.g., superoxide dismutase, catalase, glutathione peroxidase) and reduces oxidative stress markers (e.g., malondialdehyde). Morin also regulates apoptotic pathways by increasing Bcl-2 and Bcl-XL while suppressing Bax and caspase-3/8 [47–52]. It activates the TrkB/Akt pathway and improves antioxidant status in diabetic rat models, suggesting protective potential for cardiomyocytes under lipotoxic conditions.

Although several agents have shown protective effects against PA-induced injury, morin remains understudied in this context. This study investigates the protective role of morin in H9c2 cardiomyocytes exposed to PA, focusing on its effects on cell viability, ROS levels, apoptosis, mitochondrial integrity, and on the modulation of SIRT6 and OPA1.

Importantly, this study is the first to demonstrate morin's ability to modulate SIRT6 and OPA1 expression in the context of PA-induced oxidative injury. Our findings highlight morin's potential as a therapeutic agent against lipid-induced mitochondrial dysfunction and apoptotic damage in DCM. By examining the interconnections among SIRT6, OPA1, ROS, apoptosis, and glucose metabolism, we aim to deepen the understanding of DCM pathogenesis and identify novel therapeutic strategies.

2. METHODOLOGY

2.1. H9c2 cell culture

The H9c2 cell line (CRL-1446, ATCC), derived from embryonic rat heart tissue, served as the model system in this study due to its similarities with primary rat cardiac myocytes. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Cat. No: D5648, Sigma-Aldrich) with 10% inactivated fetal bovine serum (Cat. No: F9665, Sigma-Aldrich) and penicillin and streptomycin (Cat. No: P0781, Sigma-Aldrich) under a humidified 5% CO_2 atmosphere [53]. After thawing in a 37°C water bath, the cells were cultured through a multi-step process involving centrifugation to form a pellet, washing with phosphate-buffered saline (PBS) (Cat. No: P2272, Sigma-Aldrich), and detachment using a cell dissociation solution (Trypsin-EDTA [Cat. No: 25300054, Thermo Fisher Scientific]: 0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The cells were regularly monitored for growth, and upon reaching approximately 80% confluency, they were passaged and resuspended in fresh DMEM for future use. Cells were routinely tested and found negative for mycoplasma contamination. STR profiling was not applicable, as H9c2 is a rat-derived cell line.

2.2. MTT assay

The MTT assay was used to independently assess morin (Cat. No: M4008, Sigma Aldrich) and palmitic acid (PA) (Cat. No: TC386, HiMedia) on cell proliferation. The H9c2 cells (5,000 cells/well) were seeded into 96-well culture plates and allowed to adhere for 24 hours. After the incubation period, 100 µl of different concentrations of palmitic acid (disease control; 0–800 µM/0–200 µg/ml) and morin (test drug; 0–2666 µM or 0–800 µg/ml). Then, 100 µl of MTT (Cat. No: M6494, Thermo Fisher Scientific) solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 hours at 37°C in the dark. During this time, viable cells metabolized MTT to form insoluble formazan crystals. After the incubation, the formazan crystals were solubilized by adding 100 µl of dimethyl sulfoxide (DMSO) to each well. The plates were gently shaken for 10 minutes to ensure complete dissolution of the formazan. The absorbance of each well was then measured at a wavelength of 590 nm using a microplate reader (Spectramax i3X plate reader). The absorbance values were used to assess cell viability, with higher absorbance corresponding to more viable cells [54].

2.3. Cardioprotection study against palmitic acid treatment

Palmitic acid was used at a selected concentration of 433.1 µM to induce cardiomyopathy in the cell culture model. For further studies, a concentration of 450 µM was used instead of 433.1 µM for practical feasibility and ease of experimental execution, as the slight difference is unlikely to affect the overall biological response. The cardioprotective effects of morin were assessed by pre-incubating cardiomyocytes with various concentrations of morin (0–666.5 µM or 0–200 µg/ml) and a fixed concentration of PA (450 µM). The viability of H9c2 cells was determined as described above; the percentage growth inhibition was calculated, and the concentration of drug needed to inhibit cell growth by 50% (IC_{50}) was computed from the dose-response curve.

2.4. Effect of morin on glucose uptake in H9c2 cells

A non-radioactive assay was used to assess Glucose uptake using the Glucose Uptake-Glo™ Assay (Promega, Cat. No: J1341) in H9c2 cardiomyoblasts. Initially, the cultured H9c2 cells were seeded at 5,000 cells/well density and placed in a 96-well plate. The growth medium was replaced with a differentiation medium (DMEM supplemented with 2% horse serum) for 3 days to promote differentiation. A day before the assay, the cells were serum-starved for 18 hours, with 100 µl DMEM to minimize the influence of serum factors on glucose uptake. On the day of the assay, the medium was replaced with

100 µl of DMEM ± 1 µM insulin or, in control conditions, incubated in serum- and glucose-free DMEM for 24 or 48 hours to establish baseline glucose uptake levels. Subsequently, glucose uptake was initiated by removing this medium and incubating the cells with 50 µl of 0.1 mM 2-deoxy-D-glucose (2DG) for 30 minutes at 25°C. Following incubation, the glucose uptake was quantified according to the manufacturer's protocol, with luminescence recorded using a luminometer [55,56]. The integration time for luminescence detection was set between 0.3 and 1 second.

Luminescence values were converted to absolute concentrations of 2DG6P (µM) using a standard curve generated with known 2DG6P concentrations supplied in the kit. These values were then converted to femtomoles (fmol) by multiplying by the sample volume. Background luminescence (no-cell controls) was subtracted before conversion. Uptake rates were normalized per cell number and per minute and are expressed as fmol·cell^{-1}·min^{-1}. The glucose uptake was calculated using the formula: Rate of glucose uptake = [(2DG6P) × (volume of sample)) ÷ ((number of cells) × (time of uptake)].

2.5. Determination of mitochondrial membrane potential

To assess the impact of morin on the mitochondrial membrane potential (H9c2) in PA-treated H9c2 cells, Rhodamine-123 (R-123, Cat. No: R8004, Sigma Aldrich), a lipophilic cationic fluorescent dye, was employed. The H9c2 cells were seeded at a density of 1 × 10^5 cells per well in 12-well plates and incubated for 24 hours to allow for attachment [57]. Upon reaching approximately 80% confluency, the cells were treated with morin (333.25 µM and 666.5 µM) for 24 hours. The cells were subjected to PA (450 µM) treatment for an additional 24 hours to induce cellular stress, mimicking pathological conditions related to metabolic dysfunction. After the treatments and incubation, the cells were washed twice with 1X-PBS to remove any residual culture medium. Subsequently, cells were incubated with 500 µl of 10 µM Rhodamine-123 (R-123) in PBS at 37°C for 10 minutes in the dark, allowing the dye to accumulate in healthy mitochondria in a membrane potential-dependent manner. Following incubation, cells were centrifuged at 2,000 rpm for 5 minutes, and the supernatant was discarded. The cell pellet was then washed twice with 1X-PBS to remove excess dye, and the cells were resuspended in 500 µl PBS for fluorescence analysis. The fluorescence intensity was measured using flow cytometry (BD-FACS Calibur) allowing determination of mitochondrial membrane potential in response to morin treatment in the presence of palmitic acid. The data collected were analyzed to assess any potential protective effects of morin on mitochondrial integrity, as indicated by changes in Rhodamine-123 fluorescence.

2.6. Assessment of ROS generation using DCFDA fluorescence of morin in H9c2 cells

To evaluate intracellular ROS production, 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA; Cat. No: D6883, Sigma Aldrich) was utilized as a fluorescent indicator. The H9c2 cells were seeded into 96-well plates at a density of 5 × 10^4 cells/well and incubated for 24 hours [58]. Subsequently, cells were treated with varying concentrations of morin, prepared in DMSO at stock concentrations of 100 mg/ml and 10 mM, and serially diluted in plain DMEM to achieve final concentrations of 20.83µM, 41.66µM, 83.31µM, 166.63µM, 333.25µM, 666.5µM, and 450 µM PA, respectively. After 24-hour treatment with morin, the cells were further exposed to 450 µM PA for 24 hours to induce oxidative stress. Post-treatment, the cells were washed once with 1X PBS, and 100 µl of a 25 µM DC-FDA solution was added to each well. The cells were then incubated for 30 minutes at 37°C, in a 5% CO_2 atmosphere to allow for DCFDA loading and ROS generation. The fluorescence was measured using a SpectraMax i3X plate reader, with an excitation wavelength of 485 nm and an emission wavelength of 535 nm as per the methodology laid by Souza *et al.* [59] and Tungalag et al [60].

2.7. Detection of the anti-apoptotic potential of morin in H9c2 cells using flow cytometry

To evaluate the anti-apoptotic potential of morin in H9c2 cells, the cells were cultured in 6-well plates and allowed to adhere overnight. After an 18-hour pretreatment with morin, the induced apoptosis and cell death were quantified using Annexin V (Cat. No: A13199, Thermo Fisher Scientific)-PI (Cat. No: P-4864, Sigma Aldrich) staining and flow cytometry [61]. The cells were harvested and washed twice with cold PBS before resuspending in 1 ml of binding buffer. Ten microliters of Annexin V and 5 µl of propidium iodide were added to the cell suspension, and the cells were incubated for 15 minutes at room temperature in the dark. The flow cytometric analysis was performed within 1 hour of staining to determine apoptotic cell populations.

2.8. Quantitative real-time PCR (qPCR)

To investigate the anti-apoptotic effects of morin in H9c2 cells, total RNA was extracted using TRIzol reagent (Cat. No: 15596026, Life Technologies) following standard protocols. The RNA yield was quantified using a SpectraDrop (SpectraMax i3x, Molecular Devices, USA) to ensure sufficient input for downstream analyses. Complementary DNA synthesis was performed using 500 ng of total RNA with the PrimeScript RT reagent kit (Cat. No. RR037A, Takara and an oligo(dT) primer, per the guidelines laid by the manufacturer. Subsequently, qPCR was conducted on an Applied Biosystems Veriti thermal cycler with SYBR Green I (Cat. No. PB20.15, PCR Biosystems) as the fluorescent dye. Gene-specific primers were designed using Primer-BLAST (NCBI/PRIMER 3 Software). Expression levels of the target genes were normalized to a reference housekeeping gene, and fold changes were computed using the 2−ΔΔCT method [62]. These measurements were integral to quantifying the effects of morin on apoptotic signaling pathways in H9c2 cells. All qPCR experiments were performed in two biological replicates (n = 2), which provided preliminary insights but may limit statistical power.

2.9. Computational study

Molecular docking and simulation studies were conducted using the Maestro Molecular platform (version

12.1) from Schrödinger on an HP desktop with Linux Ubuntu 18.04.1 LTS, featuring an Intel Core i3-4160 processor and 8 GB RAM. The optimization of the morin structure was achieved through the LigPrep tool, creating low-energy 3D structures at pH 7.2 ± 2.0 under the OPLS3e force field. The X-ray structure of human SIRT6, referenced as PDB ID: 5MF6 and resolved at 1.87 Å, was sourced from the RCSB Protein Data Bank for preparing the protein structure. The "Protein Preparation Wizard" workflow enhanced the protein structure, filling missing residues, modifying, and minimizing its conformation while retaining critical active site residues. A receptor grid around the ligand-binding site of the prepared SIRT6 structure facilitated subsequent molecular modeling The receptor grid for docking morin was established using the Receptor Grid Generation Tool. Molecular docking studies were conducted with the Glide module at extra precision mode. The binding free energy of morin to SIRT6 was calculated via the Molecular mechanics/generalized born and surface area (MM/GBSA) method in the Prime module utilizing the VSGB solvation model and OPLS3e force field. Recognizing the limitations of rigid docking, a 20 ns molecular dynamics (MD) simulation was subsequently performed to explore the interaction's stability and dynamics using a three-step process, namely system setup with the docked complex in a water-filled box, energy minimization to rectify unfavorable interactions, followed by a production run [63–65].

2.10. Statistical analysis

Experimental results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism software (versions 8 and 8.4.2; Dotmatics). One-way ANOVA followed by Tukey's multiple comparison test was used to assess significance between groups, unless stated otherwise. A p-value < 0.05 was considered statistically significant. All assays were performed in three independent experiments (n = 3), except for qPCR, which was conducted in two independent experiments (n = 2) for gene expression analysis.

3. RESULT

3.1. Cytotoxicity assessment of palmitic acid and morin

Before assessing morin's efficacy, we determined the cytotoxic effects of palmitic acid on H9c2 cells using the MTT assay. Palmitic acid exhibited dose-dependent toxicity, with an IC_{50} value of 433.1 µM Fig. 1B. This concentration was selected for subsequent experiments to replicate lipotoxic stress in H9c2 cells, simulating the pathophysiological environment of diabetic cardiomyopathy. We next investigated the cytotoxicity of morin across a wide concentration range. At 2666 µM, morin induced approximately 33% cell death, indicating potential toxicity at higher doses. However, morin concentrations at or below 666.5 µM maintained cell viability above 75%, remaining below the accepted cytotoxicity threshold of 25% (Fig. 1A). Based on these observations, we selected 333.25 µM and 666.5 µM as the working concentrations for subsequent cardioprotective studies. These doses allowed the evaluation of therapeutic potential while minimizing any direct cytotoxic effects.

3.2. Cardioprotection of Morin against palmitic acid treatment

To assess the cardioprotective potential of morin, H9c2 cells were pretreated with increasing concentrations of morin for 24 hours, followed by exposure to 450 µM palmitic acid for another 24 hours. Cell viability was then measured using the MTT assay to determine the extent of protection afforded by morin.

As illustrated in Figure 1C, lower concentrations of morin (5.21 µM–41.7 µM) produced minimal protective effects, with cell viability remaining close to that of PA-treated cells alone. However, a dose-dependent increase in cell survival was observed at higher concentrations. The most pronounced effect was seen at 666.5 µM, where morin pretreatment increased cell viability by approximately 31.36% compared to PA-only treated cells, indicating a substantial protective benefit.

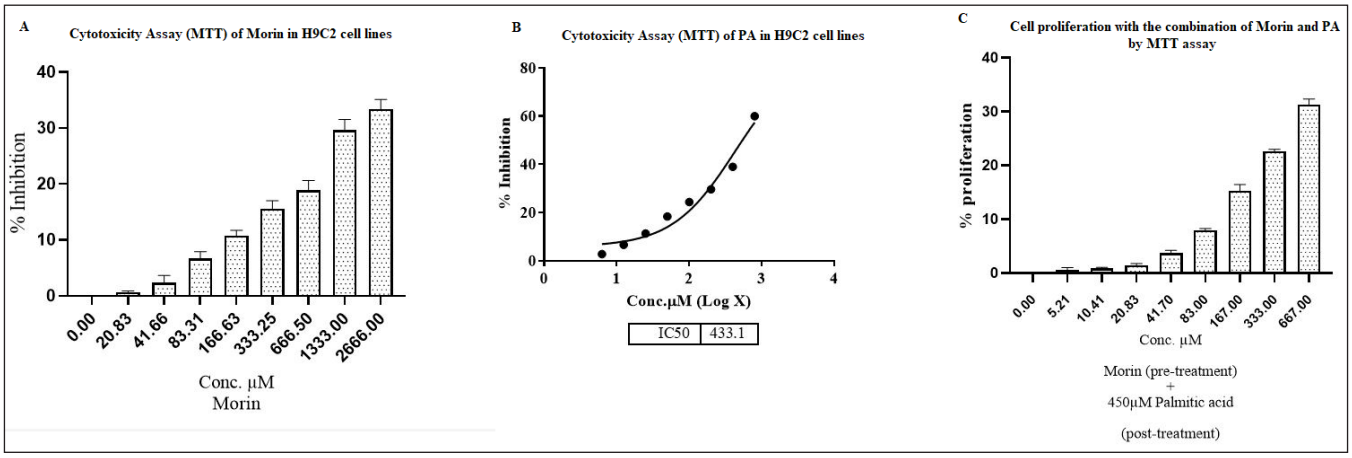

Figure 1. (A) Bar diagram showing cytotoxic effects of morin in H9c2 cells. (B) Log concentration versus % inhibition curve for palmitic acid in H9c2 cells, showing an IC_{50} of 433.1 µM. (C) Bar diagram depicting the dose-dependent protective effects of morin in combination with PA on H9c2 cells. Cell viability was assessed by MTT assay, and values are expressed as mean ± SEM (n = 3). The highest proliferation (~33%) was observed at 667 µM morin, confirming its protective role against PA-induced toxicity.

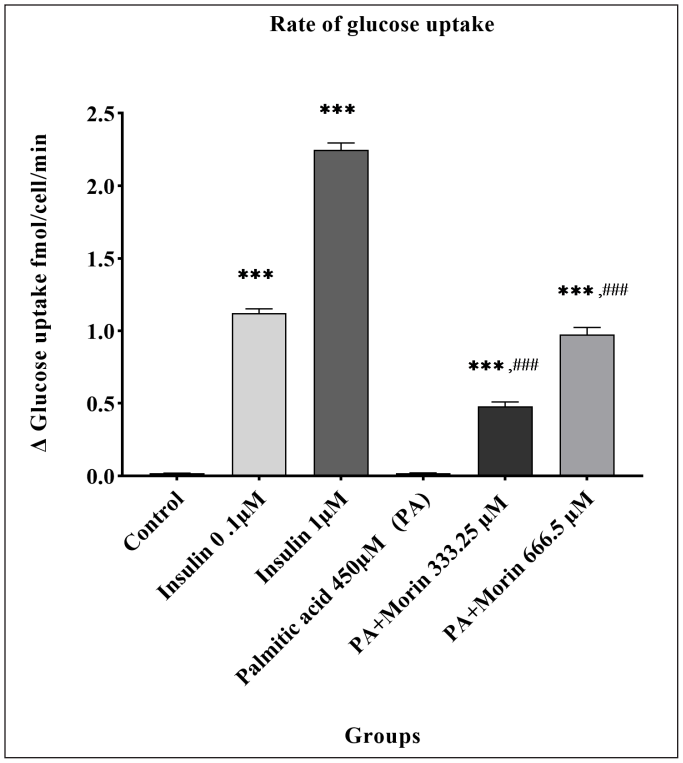

Figure 2. Effect of morin on insulin-stimulated glucose uptake in H9c2 cardiomyoblasts exposed to palmitic acid (PA). Cells were treated with insulin (0.1 or 1 μM), PA (450 μM, 24 hours), or PA in combination with morin (333.25 or 666.5 μM). Insulin alone significantly increased glucose uptake compared with untreated controls. PA impaired insulin-mediated glucose uptake, consistent with insulin resistance. Morin co-treatment significantly restored insulin-stimulated glucose uptake in a dose-dependent manner. Data are presented as mean ± SEM (n = 3). Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test. ***$p < 0.001$ versus untreated control; $^{###}p < 0.001$ versus insulin alone.

Based on these findings, 333.25 μM (100 μg/ml) and 666.5 μM (200 μg/ml) morin concentrations were selected for subsequent molecular and mechanistic studies. These doses were chosen as they demonstrated significant efficacy while remaining below cytotoxic thresholds established in earlier experiments.

3.3. Effect of Morin on glucose uptake in palmitic acid-treated cardiomyocyte

This study assessed the effects of morin on insulin-stimulated glucose uptake in H9c2 cardiomyoblasts. Insulin alone significantly increased glucose uptake in a dose-dependent manner: 0.1 μM insulin enhanced uptake by 1.10-fold versus untreated cells ($p < 0.001$), while 1 μM insulin produced a 2.23-fold increase ($p < 0.001$) (Fig. 2).

Exposure to 450 μM palmitic acid (PA, 24 hours) impaired insulin-mediated glucose uptake, consistent with its known role in inducing insulin resistance. Morin co-treatment significantly counteracted this inhibitory effect of PA in a dose-dependent manner: 333.25 μM morin restored uptake by 0.46-fold, and 666.5 μM morin by 0.96-fold compared with PA + insulin (both $p < 0.001$).

These results indicate that morin protects cardiomyocytes from PA-induced insulin resistance by restoring insulin-stimulated glucose uptake.

3.4. Determination of mitochondrial membrane potential

Palmitic acid exposure led to a significant decrease in mitochondrial membrane potential in H9c2 cells, as evidenced by reduced cell fluorescence ($p < 0.001$ compared to untreated control cells). Specifically, treatment with 450 μM palmitic acid decreased MMP by approximately 67% compared to control cells.

Pretreatment with morin demonstrated a significant protective effect against palmitic acid-induced mitochondrial damage. Morin at both 333.25 μM and 666.5 μM significantly attenuated the palmitic acid-induced decrease in MMP, increasing MMP to 0.61 and 0.83 folds of the control, respectively ($p < 0.001$ for both compared to palmitic acid alone) (Fig. 3).

These findings suggest that morin can protect cardiomyocytes from palmitic acid-induced mitochondrial dysfunction, potentially by preserving MMP. This protective effect may be attributed to morin's antioxidant properties, which could mitigate palmitic acid-induced oxidative stress and subsequent mitochondrial damage.

3.5. Morin modulates palmitic acid-induced ROS generation in cardiomyocytes

This study investigated the protective effects of morin against palmitic acid-induced oxidative stress in the H9c2 cell line. As anticipated, treatment with 450 μM palmitic acid significantly increased ROS levels compared to the control group (***$p < 0.001$), confirming its role as an inducer of oxidative stress.

Interestingly, co-treatment with morin significantly attenuated this PA-induced ROS increase in a dose-dependent manner. While 333.25 μM morin partially reduced ROS levels, 666.5 μM morin effectively restored ROS levels to near-control levels (***$p < 0.001$ *vs.* PA control). This finding suggests that morin's antioxidant properties can effectively counteract the oxidative stress induced by palmitic acid, which is illustrated in Figure 4.

Furthermore, the ROS-reducing effect of morin was comparable to that of N-acetylcysteine, a well-established antioxidant, which served as a positive control in this study. NAC treatment resulted in the most significant decrease in ROS levels (###p < 0.001 *vs.* PA control), even surpassing the control group, highlighting its potent antioxidant capacity

3.6. Morin attenuates palmitic acid-induced apoptosis in H9c2 cardiomyocytes

This study's findings indicate that palmitic acid treatment significantly reduced H9c2 cell viability, reflecting a potent apoptotic effect ($p < 0.001$). However, pretreatment with morin, at concentrations of both 333.25 μM and 666.5 μM, significantly mitigated this palmitic acid-induced reduction in cell viability ($p < 0.01$ and $p < 0.001$, respectively). Notably, a dose–response relationship was evident, with the higher morin concentration, 666.5 μM, conferring significantly

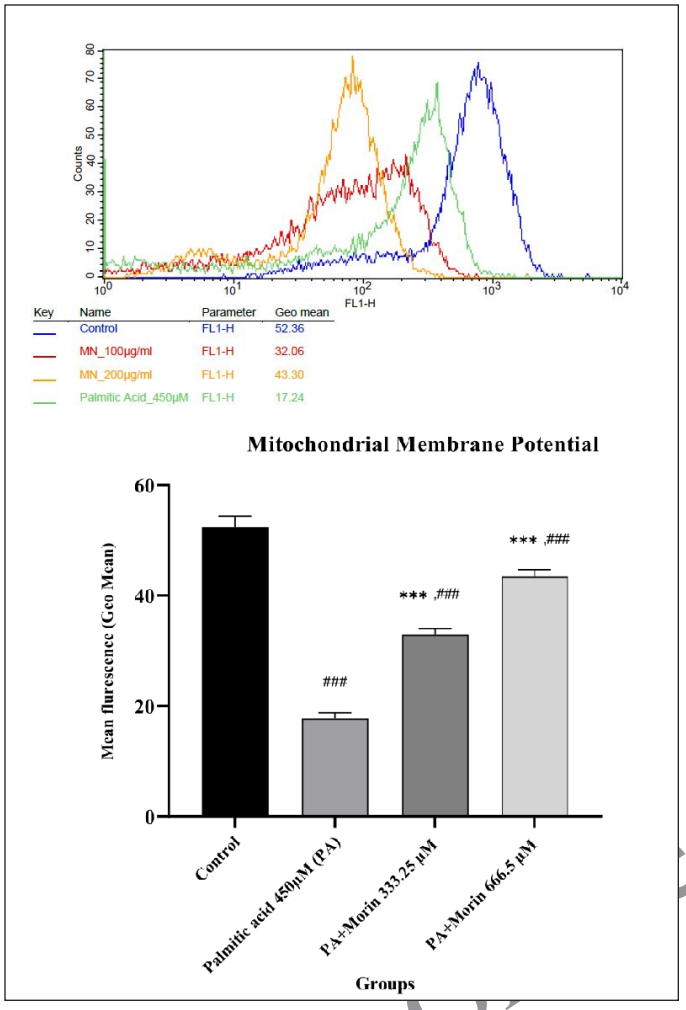

Figure 3. The mitochondrial membrane potential analysis of H9c2 cells treated with morin and PA. PA treatment has produced a decreased MMP whereas the combination of morin with PA has improved the MMP of the cells. All values are expressed in mean ± SEM (n = 3). Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test. where, ***p < 0.001, when compared to PA control, $^{###}p$ < 0.001, when compared to non-treated control.

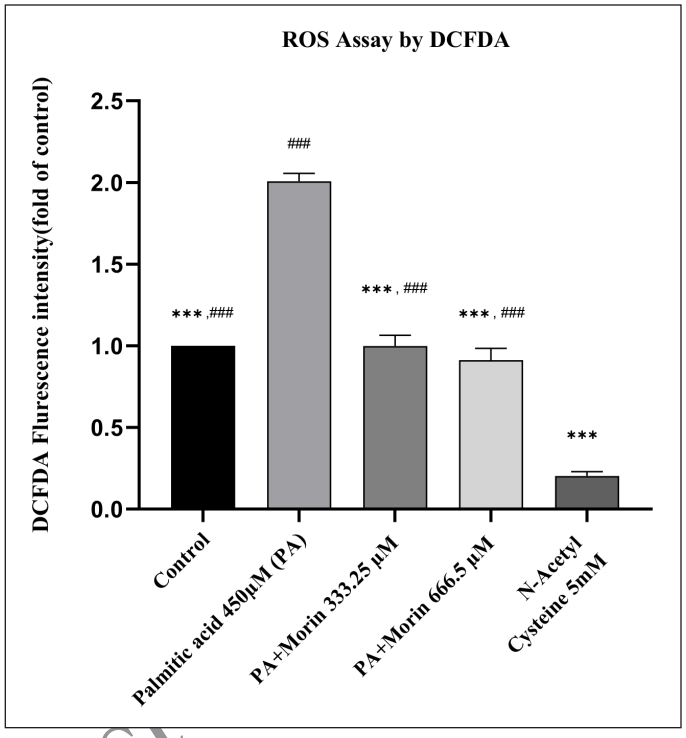

Figure 4. The ROS analysis of H9C2 cells treated with morin and PA. PA treatment has produced increased levels of ROS, whereas the combination of morin with PA has attenuated the ROS levels of the cells. All values are expressed in mean ± SEM (n = 3). Statistical analysis was performed by One-way ANOVA followed by Tukey's multiple comparison test, where***p < 0.001 when compared to PA control and $^{###}p$ < 0.001 when compared to N-acetyl cysteine.

greater protection compared to the lower concentration (p < 0.001). These results suggest that morin exerts a protective effect against palmitic acid-induced apoptosis in H9c2 cells, and this cytoprotective effect appears to be dose-dependent. Figure 5A and 5B illustrates that morin pretreatment improved cardiomyocyte viability and reduced PA-induced apoptosis in a dose-dependent manner. Cell viability was assessed using Annexin V/PI staining, and statistical analysis confirmed significant protective effects of morin. Data are presented as Mean ± SEM (n = 3), with ***p < 0.001, **p < 0.01 versus PA control, and ###p < 0.001 versus untreated control.

3.7. Effects of morin on the expression of SIRT6 and OPA1

3.7.1. Morin upregulates SIRT6 expression, counteracting potential palmitic acid-induced suppression

Palmitic acid exposure modestly suppressed SIRT6 gene expression in H9c2 cells, indicating a potential disruption in mitochondrial regulation. However, morin treatment counteracted this effect in a dose-dependent manner. At 333.25 µM, morin significantly increased SIRT6 expression compared to the PA-treated group (p < 0.001), and this upregulation was also statistically significant versus the untreated control group (#p < 0.05). The effect was more pronounced at 666.5 µM, where SIRT6 expression was elevated beyond both PA and control groups (p < 0.001), demonstrating morin's ability to restore or enhance mitochondrial regulatory signaling impaired by lipotoxic stress.

These findings confirm that morin significantly mitigates PA-induced suppression of SIRT6, particularly at higher concentrations, which may underlie its protective mechanism in diabetic cardiomyopathy models (Fig. 6).

3.7.2. Morin upregulates OPA1 expression, counteracting potential palmitic acid-induced suppression

While palmitic acid treatment alone did not cause significant changes in OPA1 expression (p = 0.925), morin induced a dose-dependent increase. Pretreatment with 333.25 µM morin led to a 1.93-fold increase in OPA1 expression compared to control (p = 0.078), and the effect was statistically significant when compared to PA alone (p = 0.027). A more substantial upregulation was observed at 666.5 µM, where

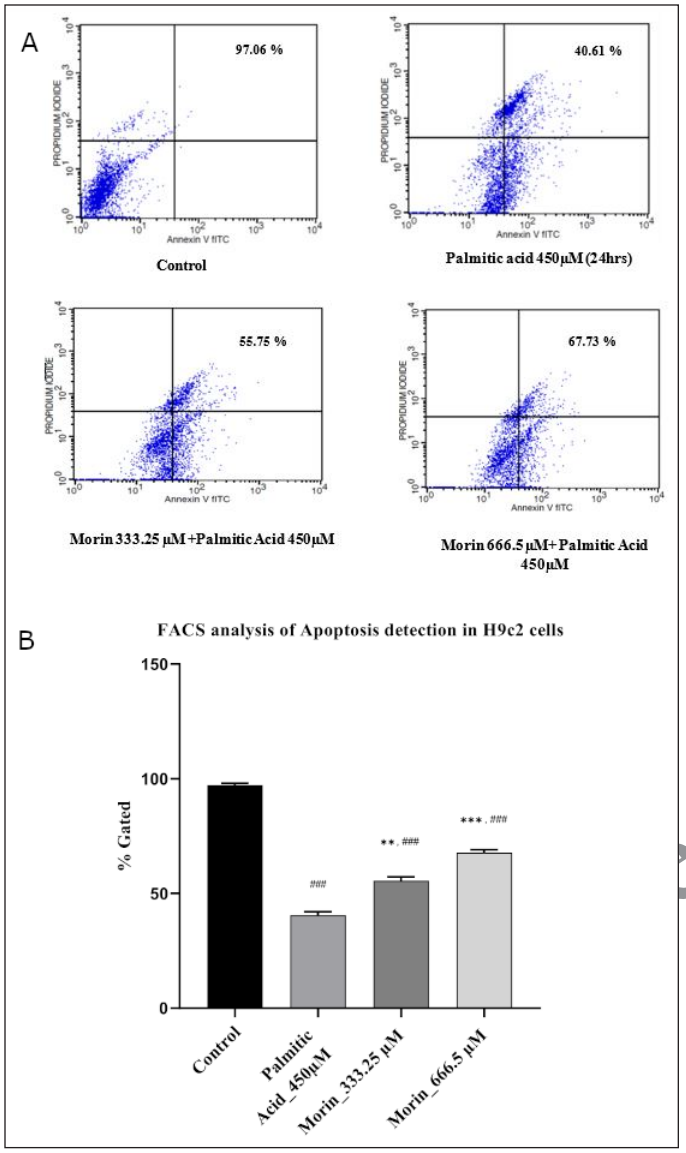

Figure 5. A Effect of morin treatment on cardiomyocyte viability with palmitic acid for 24 hours. The cells were preincubated with 333.25 µM and 666.5 µM morin for 24 hours before palmitic acid 450µM. Cell viability was measured using Annexin V/PI double staining. Representative dot plots of a cardiomyocyte sample are shown, with numbers indicating the percentage of viable cells (Annexin V/PI double negative). B The apoptotic assay of H9C2 cells indicates that PA treatment significantly reduced the cell viability, reflecting a potent apoptotic effect. However, morin pretreatment produced a dose-dependent reduction in PA-induced apoptosis in the cells revealing the protective effect of morin. All values are expressed in mean ± SEM (n = 3). Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison tests where ***$p < 0.001$, **$p < 0.01$ when compared to PA control, and $^{###}p < 0.001$ when compared to control.

OPA1 levels rose 4.06-fold over control and significantly surpassed both the PA-only and 333.25 µM groups ($p < 0.001$ in all comparisons).

These data highlight a strong dose-dependent enhancement of OPA1 by morin, reinforcing its role in promoting mitochondrial fusion and integrity under lipotoxic conditions (Fig. 7).

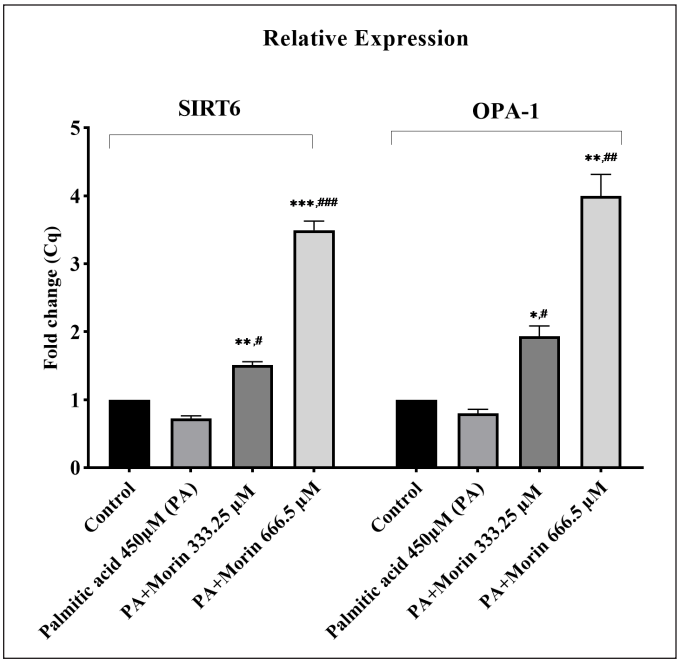

Figure 6. Relative fold change in mRNA expression of SIRT6 and OPA1 genes in H9c2 cells following morin treatment in the presence of PA. PA treatment alone reduced SIRT6 expression compared to the untreated control, while morin co-treatment significantly mitigated this reduction in a dose-dependent manner. Similarly, OPA1 expression increased in a dose-dependent manner with morin co-treatment. Data are presented as mean ± SEM from $n = 2$ independent experiments (biological replicates), providing preliminary insights. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Significance levels: ***$p < 0.001$, **$p < 0.01$ compared to the PA control; $^{#}p < 0.05$, $^{###}p < 0.001$ compared to the untreated control.

3.8. *In silico* prediction of morin's binding affinity to SIRT6

3.8.1. Molecular docking

Molecular docking simulations were performed to assess the binding affinity of morin to the active site of SIRT6. Morin exhibited a docking score of –6.81, indicating a favorable binding interaction. Analysis of the binding pose revealed key interactions with amino acid residues within the active site, including a hydrogen bond with PRO62 and a π–π interaction with PHE86. Hydrophobic interactions were observed with ALA53, VAL115, PHE86, ILE185, MET157, ILE63, PHE82, and VAL70. Additionally, charged (negative) interactions with ASP116 and ASP63, as well as polar interactions with SER56 and ASN114, contributed to the stable binding of morin to SIRT6. MM/GBSA calculations were then performed to further evaluate the binding energetics. MMGBSA ΔG bind is calculated to be –52.56.

3.8.2. Molecular dynamics simulation study

Molecular dynamics simulations were performed to investigate the stability of the morin–SIRT6 complex and to further characterize the interactions between morin and the SIRT6 active site. Analysis of the root-mean-square deviation of the protein and ligand throughout the simulation revealed stable binding of morin to SIRT6. The protein backbone RMSD

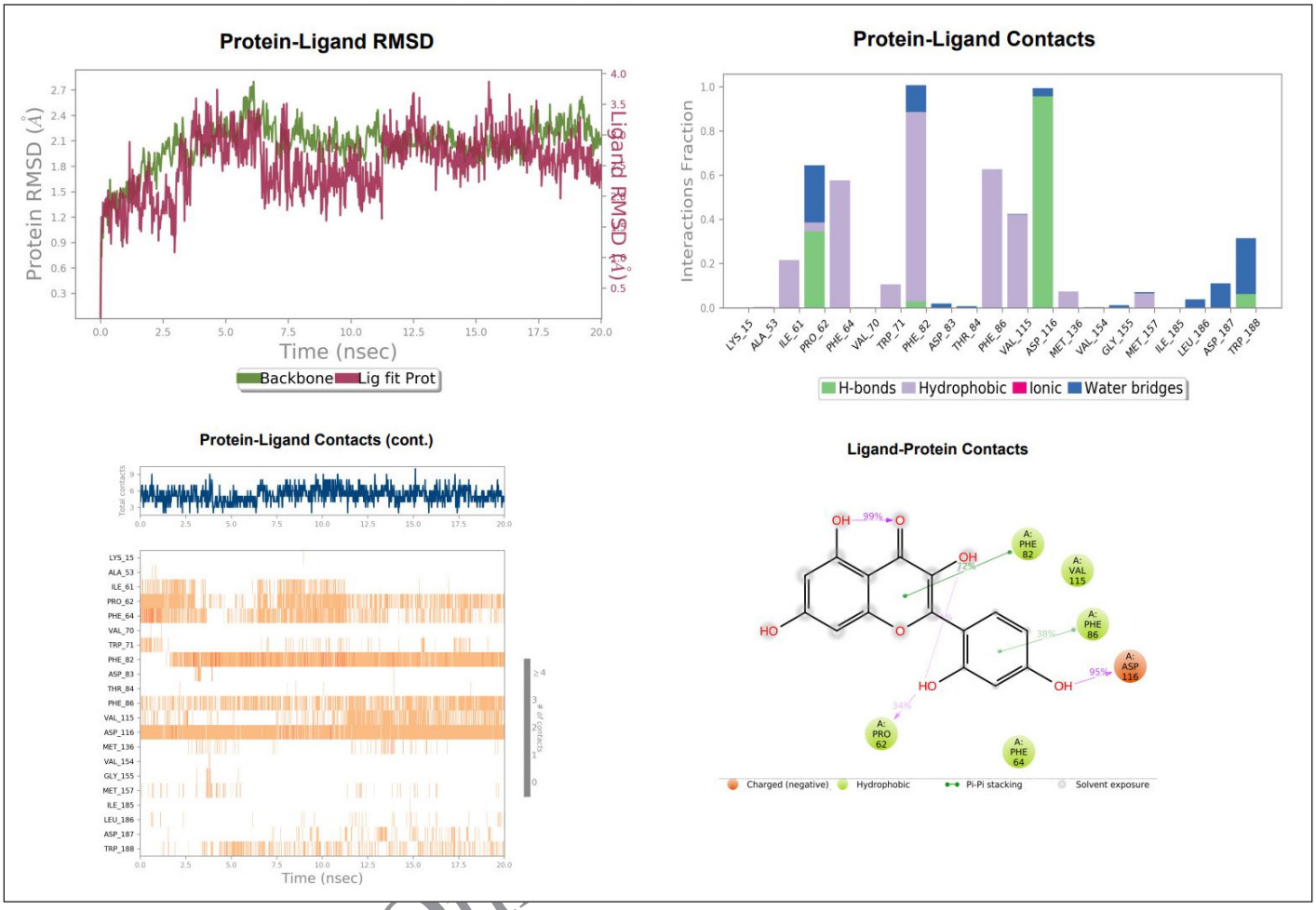

Figure 7. RMSD and protein–ligand contact plot of morin with SIRT6 and A timeline representation of the interactions and contacts (H-bonds, hydrophobic, ionic, water bridges) observed during MD simulation.

fluctuated between 1.0 and 2.1 Å, indicating minimal structural changes during the simulation. Similarly, the ligand RMSD remained within a range of 2.5–2.9 Å, further supporting the stability of the morin–SIRT6 complex.

Detailed analysis of protein–ligand interactions throughout the MD simulation revealed consistent interactions between morin and key residues within the SIRT6 active site. Notably, ASP116 exhibited a strong hydrogen bonding interaction with morin, with a high occupancy rate of 95%, suggesting a crucial role in stabilizing morin within the binding pocket. PHE82 also formed a significant hydrogen bond with morin (72% occupancy), further contributing to binding stability. Hydrophobic interactions with VAL115 and PHE86 provided additional stability to the complex. PRO62 was observed to interact with morin for 34% of the simulation, while PHE64 contributed to hydrophobic interactions. The presence of high-occupancy water bridges further suggests stabilization of morin within the active site via water-mediated interactions.

The observed interactions, particularly those with ASP116 and PHE82, suggest that morin has the potential to activate SIRT6. These findings support the selection of morin as a candidate SIRT6 activator for further investigation. Figure 8 illustrates the root mean square deviation (RMSD) and protein–ligand contact plot of morin with SIRT6, along with a timeline representation of the interactions and contacts (H-bonds, hydrophobic, ionic, and water bridges) observed throughout the MD simulation. RMSD and protein–ligand contact plot of morin with SIRT6 and interactions observed during MD simulation is given in Figure 8. As this analysis was based on a 20 ns simulation, the results should be regarded as preliminary structural insights, with longer trajectories needed for definitive stability assessment.

4. DISCUSSION

DCM, a debilitating complication of diabetes, is characterized by myocardial dysfunction and structural alterations in the heart [66]. The pathophysiology of DCM is closely linked to mitochondrial dysfunction, which is aggravated by lipotoxicity and glucotoxicity in diabetic conditions [6,67,68]. Mitochondria, vital for cellular energy production, become impaired in DCM, resulting in increased oxidative stress, apoptosis, and compromised cardiac function [69,70]. Recent studies have highlighted the potential of sirtuins, particularly SIRT6, as therapeutic targets due to their pivotal

role in mitochondrial integrity and cellular stress responses [30,71–73]. In the present study, we investigated the potential of morin, a natural flavonoid, as an SIRT6 activator to mitigate mitochondrial dysfunction and protect against palmitic acid-induced cardiotoxicity using *in vitro* and *silico* approaches. Our *in vitro* findings demonstrated that morin mitigates palmitic acid-induced damage in H9c2 cardiomyocytes, highlighting its potential as a therapeutic agent. To further strengthen these findings, computational studies were performed to evaluate morin's binding affinity and interactions with SIRT6, a critical protein in mitochondrial regulation and cardiac health.

In the *in vitro* experiments, we investigated morin's effects on H9c2 cardiomyocytes, a well-established model for studying lipotoxicity and diabetic cardiomyopathy. H9c2 cardiomyocytes were exposed to palmitic acid, a well-established inducer of lipotoxicity and oxidative stress, to mimic diabetic cardiomyopathy. Previous studies have established PA as a reliable *in vitro* model to induce lipotoxicity and cardiomyocyte dysfunction, mimicking diabetic cardiomyopathy conditions. PA exposure in H9c2 cells leads to increased ROS generation, mitochondrial membrane potential loss, and apoptosis, highlighting the involvement of oxidative stress in PA-induced cardiotoxicity [74]. Similarly, another study reported that PA downregulates key mitochondrial regulators such as SIRT3 PGC-1α, and SIRT6, contributing to impaired mitochondrial function and cell death [75,76].

We demonstrated that morin pretreatment significantly attenuated palmitic acid-induced cytotoxicity, which suggests a cardioprotective role for morin in this context. This observation is consistent with previous research highlighting the protective effects of polyphenols, particularly flavonoids such as morin, against cardiomyocyte injury [77]. The ability of morin to reduce palmitic acid-induced apoptosis further strengthens its potential as a therapeutic agent. We observed a significant reduction in apoptotic markers in morin-treated H9c2 cells, suggesting that its anti-apoptotic properties contribute to the preservation of cardiomyocyte viability. This aligns with earlier studies demonstrating the anti-apoptotic effects of flavonoids in various cellular models of cardiovascular disease [78,79].

A deeper exploration of the mechanisms underlying morin's cardioprotective effects revealed several critical pathways. First, morin significantly enhanced glucose uptake in palmitic acid-treated cardiomyocytes, which may help ameliorate the metabolic dysfunction characteristic of diabetic cardiomyopathy. Morin effectively improves glucose uptake in insulin-resistant H9c2 cardiomyocytes, suggesting its potential to enhance glucose metabolism and mitigate insulin resistance, a key pathological feature in diabetic cardiomyopathy. This finding is particularly relevant in light of the growing body of literature indicating the importance of metabolic regulation in the pathogenesis of DCM [80,81].

PA alone did not significantly alter basal glucose uptake ($p > 0.05$). This finding is consistent with earlier reports showing that PA-induced insulin resistance primarily impairs insulin-stimulated rather than basal glucose transport. Mechanistically, PA does not directly trigger GLUT4 translocation, and its inhibitory effects on glucose uptake generally require either insulin co-stimulation or prolonged exposure. Thus, the impaired uptake observed in our model reflects insulin-dependent resistance rather than a reduction in basal glucose transport. These results also clarify that while PA-induced cytotoxicity in cardiomyocytes is mediated via mitochondrial dysfunction and oxidative stress, its impact on glucose uptake emerges predominantly under insulin-stimulated conditions [82–84].

Moreover, morin's ability to reduce ROS generation suggests that it possesses strong antioxidant properties, which are crucial in combating oxidative stress, a hallmark of diabetic cardiomyopathy. Our findings are in line with studies reporting the antioxidant effects of flavonoids in various models of oxidative stress and cardiovascular dysfunction [78,85]. Morin significantly protects against palmitic acid-induced MMP loss, indicating its potential to preserve mitochondrial function and integrity, which is crucial for maintaining cardiomyocyte viability in conditions of oxidative stress and lipotoxicity.

Although morin effectively reduced PA-induced ROS generation to near-control levels, restoration of MMP remained partial. This discrepancy indicates that oxidative stress is not the sole driver of PA-induced mitochondrial dysfunction. Previous reports have shown that PA can impair mitochondrial function through additional mechanisms, including direct disruption of respiratory chain complexes (Complexes I and III), induction of mitochondrial permeability transition pore (mPTP) opening, and cardiolipin remodeling within the inner mitochondrial membrane. Such alterations may persist even after ROS neutralization, accounting for an incomplete recovery of MMP in our study. These findings suggest that morin's antioxidant action alleviates a major component of lipotoxic injury but may not fully prevent structural or bioenergetic impairments. Future studies evaluating respiratory chain activity and mPTP regulation will help clarify these additional pathways [86–90].

While Annexin V/PI staining provided clear evidence of reduced apoptosis with morin treatment, additional markers such as Bax/Bcl-2 ratio and cleaved caspase-3 were not assessed in the present study. Future work will include these analyses to provide deeper mechanistic insight.

Additionally, we observed that morin modulated the expression of SIRT6 and OPA1, two proteins central to mitochondrial function and dynamics. SIRT6 activation has been shown to protect against mitochondrial dysfunction and improve mitochondrial stability [17,91]. By upregulating SIRT6 expression and influencing the levels of OPA1, morin may enhance mitochondrial fusion and function, counteracting the mitochondrial damage observed in DCM. This is particularly significant as mitochondrial dysfunction is a key feature of diabetic cardiomyopathy [5]. Our results suggest that morin's cardioprotective effects may be mediated, at least in part, through SIRT6 activation, which supports mitochondrial integrity and function. Morin enhanced OPA1 expression in PA-treated cells, consistent with its reported ability to protect mitochondrial integrity and function under oxidative and lipotoxic stress in previous studies [92–96]. These results highlight the multifaceted effects of morin in countering lipotoxicity-induced cellular damage, including its ability

to improve metabolic function, reduce oxidative stress, and preserve mitochondrial health.

To complement and support our *in vitro* findings, *in silico* studies were performed to explore morin's potential interaction with SIRT6, a protein critical for mitochondrial function and cellular metabolism. Molecular docking simulations revealed that morin binds favorably to the active site of SIRT6, forming stable interactions with key residues such as ASP116 and PHE82. This was further substantiated by molecular dynamics simulations, which demonstrated the stability of the morin-SIRT6 complex. These computational results suggest that morin may act as a direct modulator of SIRT6 activity. Previous studies have highlighted SIRT6's role in mitigating mitochondrial dysfunction, inflammation, and oxidative stress, key pathological features of DCM [97,98]. The observed interactions with SIRT6 align with the reported ability of flavonoids to modulate sirtuins, further supporting morin's therapeutic potential [85].

The combination of *in vitro* and *in silico* approaches provides a comprehensive understanding of morin's mechanisms of action. *In vitro* findings demonstrated its cardioprotective effects through enhanced glucose uptake, reduced ROS generation, and preserved mitochondrial function. These effects are further supported by computational evidence of morin's strong binding affinity and potential activation of SIRT6, which may contribute to its protective effects. By upregulating SIRT6 expression and influencing mitochondrial regulatory proteins such as OPA1, morin addresses multiple pathological pathways in DCM.

Our 20 ns MD simulations revealed that the morin–SIRT6 complex attained equilibrium with stable RMSD and acceptable RMSF fluctuations, supporting the plausibility of the docking interactions. However, we recognize that 20 ns trajectories provide only preliminary structural insights. Longer MD runs (≥100 ns) are generally recommended for robust assessments of conformational convergence and dynamic stability and thus should be undertaken in future work to fully validate these observations.

Furthermore, our results are consistent with the protective effects observed with other natural antioxidants such as quercetin, resveratrol, and kaempferol, which have been shown to mitigate PA-induced injury via antioxidant, anti-apoptotic, and mitochondrial regulatory pathways. Compared to these compounds, morin exhibits comparable effects in restoring mitochondrial function and reducing ROS, reinforcing its therapeutic potential [99–103].

In comparison to synthetic SIRT6 activators such as MDL-800, morin exhibits distinct features in terms of potency, binding mechanism, and broader bioactivity. MDL-800 is a well-characterized allosteric activator that enhances SIRT6 catalytic activity by up to ~22-fold, with an EC_{50} around 10–11 μM, and functions via binding to an N-terminal hydrophobic pocket, leading to downstream deacetylation of histone targets and demonstrating *in vivo* efficacy in tumor xenograft models. While morin activates SIRT6 *in vitro*, it does so at higher, micromolar concentrations, and its mechanism involves general upregulation of SIRT6 expression and mitochondrial regulatory signaling rather than direct allosteric modulation. As a naturally occurring flavonoid, morin may offer complementary antioxidant and metabolic regulatory benefits beyond potency, positioning it as a potentially safer, multi-target candidate—albeit one that requires more comprehensive *in vivo* validation to substantiate therapeutic claims [104,105].

Although morin treatment significantly upregulated SIRT6 and OPA1 gene expression in H9c2 cardiomyocytes, we acknowledge that transcriptional or expression data do not directly establish catalytic activation of SIRT6. SIRT6 is an NAD^+-dependent histone deacetylase, and its enzymatic activity is typically assessed through functional assays such as fluorometric deacetylase activity assays or by measuring acetylation levels of canonical histone substrates (e.g., H3K9Ac, H3K56Ac). While our phenotypic findings improved mitochondrial membrane potential, reduced ROS, enhanced glucose uptake, and reduced apoptosis, which are consistent with enhanced SIRT6 activity, future studies incorporating direct enzymatic assays will be essential to confirm morin as a definitive activator of SIRT6 at the catalytic level.

A limitation of the present study is the absence of genetic validation experiments (e.g., SIRT6 or OPA1 knockdown/overexpression) and the use of a cardiomyocyte-validated SIRT6 activator for direct comparison. While our findings consistently support morin's cardioprotective effects and its modulatory influence on SIRT6–OPA1 signaling, definitive proof of mechanistic necessity will require future studies incorporating these approaches. Nevertheless, the convergence of expression analyses, functional readouts, and *in silico* modeling provides a strong preliminary rationale for morin as a modulator of SIRT6 activity.

A key constraint of this study is that the gene expression analysis was conducted with $n = 2$ biological replicates, which may not fully capture biological variability. While the results provide preliminary support for the observed transcriptional changes, future validation with ≥3 replicates will be required to confirm these findings with greater statistical robustness.

In conclusion, our study highlights morin as a promising candidate for the treatment of diabetic cardiomyopathy. Its ability to improve glucose metabolism, combat oxidative stress, and preserve mitochondrial function underscores its therapeutic potential. While the computational findings strongly suggest that SIRT6 activation contributes to morin's effects, further *in vivo* studies and clinical investigations are warranted to validate these outcomes. Additionally, optimizing morin's pharmacokinetic properties will be essential for its clinical application. This study paves the way for future research into the potential of morin as a natural therapeutic agent for diabetic cardiomyopathy, potentially offering a safer, more accessible alternative to current treatments.

5. CONCLUSION

This study comprehensively establishes morin as a promising therapeutic candidate for DCM, acting through SIRT6 activation and mitochondrial preservation. By integrating *in vitro* and *in silico* approaches, we demonstrated that morin significantly protects H9c2 cardiomyocytes from palmitic acid-induced injury. It enhances glucose uptake, reduces oxidative stress, and maintains mitochondrial membrane potential, thereby counteracting key pathological processes such as insulin

resistance, lipotoxicity, and mitochondrial dysfunction. These protective effects underscore morin's capacity to counteract key pathological processes in DCM, such as insulin resistance, lipotoxicity, and mitochondrial dysfunction.

Complementing the experimental findings, *in silico* studies highlighted morin's strong binding affinity to the SIRT6 active site, supported by stable interactions with critical residues, such as ASP116 and PHE82. Molecular dynamics simulations further validated the stability of the morin-SIRT6 complex, suggesting its potential as a direct modulator of SIRT6 activity. SIRT6 plays a pivotal role in regulating glucose and lipid metabolism, mitochondrial function, and oxidative stress, making it a promising target for DCM therapy.

Collectively, these findings establish morin as a novel and potent SIRT6 activator with multiple mechanisms of action. By bridging *in vitro* evidence with computational predictions, this study provides a robust foundation for further exploration of morin's therapeutic potential. Future research should focus on *in vivo* validation and clinical studies to translate these findings into therapeutic applications. Additionally, optimization of morin's pharmacokinetic profile will be crucial to enhance its clinical utility.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. AVAILABILITY OF DATA AND MATERIALS

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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