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Cytoprotective and Antioxidant Effects of Silver Nanoparticles Synthesised from the *Pisonia grandis* L. Extracts Against Hydrogen Peroxide-induced Toxicity in Human Cardiomyocytes

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Abstract

Background: Myocardial Infarction (MI) is a major cause of global mortality, with oxidative stress being a central mechanism underlying cardiomyocyte injury. Hydrogen peroxide (H_2O_2) induced oxidative damage is widely used as an *in vitro* model to mimic myocardial stress. Plant-mediated nanomaterials have emerged as promising cardioprotective agents due to their antioxidant potential. **Aim:** This study evaluates the protective effect of Polyvinyl Alcohol (PVA)-encapsulated silver nanoparticles synthesised using the ethanolic extract of *Pisonia grandis* leaves (PVA AgNPs Pg) against H_2O_2-induced oxidative stress in Human Cardiomyocytes (HCM). **Methods:** The synthesised nanoparticles were characterised using Ultraviolet (UV)–visible spectroscopy, Transmission Electron Microscopy (TEM), X-ray Powder Diffraction (XRD), Fourier Transform Infrared (FTIR) spectroscopy, Energy-Dispersive X-ray (EDX) analysis, and Scanning Electron Microscopy (SEM). HCM cells were pretreated with PVA AgNPs Pg followed by H_2O_2 exposure. Cell viability, intracellular Reactive Oxygen Species (ROS), antioxidant enzyme levels (SOD, CAT, GSH, and GPX), and mitochondrial integrity using Mitotracker Green (MTG) staining were evaluated. Digoxin was used as the reference drug. **Results:** H_2O_2 exposure significantly decreased cell viability and induced morphological alterations. Pretreatment with PVA AgNPs Pg markedly improved cell survival and significantly enhanced antioxidant enzyme levels. ROS generation was effectively suppressed, demonstrating protection comparable to digoxin. Confocal microscopy revealed modulation of mitochondrial function, indicated by altered MTG expression in treated cells. **Conclusion:** PVA-encapsulated silver nanoparticles derived from *P. grandis* exert significant cardioprotective effects against oxidative stress by enhancing antioxidant defence, reducing ROS, and preserving mitochondrial integrity, suggesting their potential in myocardial infarction management.

Major Findings: PVA-encapsulated silver nanoparticles from *P. grandis* leaves showed cardioprotective effects against H_2O_2-induced oxidative stress by enhancing antioxidant enzymes, reducing ROS generation, preserving mitochondrial function, and demonstrating efficacy comparable to digoxin.

Keywords: Antioxidants, Cardiomyocyte, Digoxin, Mitotracker Green, Myocardial Infarction

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1. Introduction

Antioxidants derived from herbs are being used more frequently as preventive measures against various cardiovascular disorders. By scavenging the production of free radicals, bioactive compounds derived from natural sources have become essential components of the contemporary medical system, lowering the risk of heart conditions. Although medicinal plants are acclimated to make numerous pharmaceuticals, the phytochemical compounds existing in the plant's original substance are extra potent and have fewer side effects than their pharmaceutical equivalents. It is known that many plants and their bioactive phytoconstituents have few adverse effects, which makes them a viable alternative treatment option for cardiac conditions. Plants possess cardioprotective substances such as saponin, flavonoids, steroids, alkaloids, glycosides, tannins, phenols, terpenoids and anthraquinones[1]. Cardiovascular Diseases (CVD) are a prominent category of non-communicable diseases and have become widespread, in consonance with the World Health Organisation. Roughly 17.5 million people worldwide lose their lives due to CVD yearly. Low- and middle-income nations have recorded more than 75% of the mortality rate. According to the World Health Organisation (WHO) data, heart attacks and strokes accounted for 85% of all CVD deaths[2]. Cardiotoxicity has been linked to a range of cardiac conditions, including heart failure. The immune and inflammatory responses to infection appear as sepsis, which is still a significant cause of death and can progress to multiorgan failure. Reactive oxygen species (ROS) are the principal root of oxidative stress, present at relatively low concentrations under healthy settings. This chemical is crucial for signal transduction and has the ability to control the body's synthesis of inflammatory and immunological factors. Hydrogen peroxide, a crucial bridge and a metastable oxygen species, is produced by almost every source of oxygen radicals. Surprisingly, organic compounds hardly ever react with hydrogen peroxide. Apoptosis and necrosis can be brought on by H_2O_2. When H_2O_2 oxidises lipids, proteins, or DNA, oxidised chemicals are produced that have been connected to organ damage[3].

Redox equilibrium of ROS and free radicals is maintained by cells through two kinds of antioxidant modes in the body. Alpha-lipoic acid, glutathione, vitamin C, vitamin E, coenzyme Q10 and other antioxidants are part of the non-enzymatic antioxidant system. The enzymatic antioxidant network comprises Superoxide Dismutase (SOD), Catalysis (CAT) and Glutathione Peroxidase (GSH-Px). These antioxidants can avert oxidative damage to cells and tissues, deactivate free radicals and ROS and lessen the impairment that these substances cause to cells. The body employs a range of antioxidants to defend against the harm brought by free radicals and ROS. Endogenous antioxidant expression controls the production of these potentially dangerous ROS and free radicals. When antioxidants are utilised, the body's antioxidant capability is increased. Research conducted *in vitro* verifies that different plant active ingredients can reduce oxidative stress[4].

The fundamental goal of this research is to determine how plant-derived nanomedicine protects cardiomyocytes from oxidative stress. This study looked at the oxidative stress generated by hydrogen peroxide in HCM using PVA-encapsulated silver nanoparticles derived from an ethanolic extract of *P. grandis* leaves.

2. Materials and Methods

2.1 Collection of Plant Material and Extraction

Pisonia grandis leaves (Figure 1) were gathered from Coimbatore, Tamil Nadu, cleaned, and then shade-dried for three weeks before being ground into a fine powder. The plant was duly authenticated by the Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore, and assigned the authentication number BSI/SRC/5/23/2022/Tech/406.

To extract it, 10 grams of this powder were combined with 100ml of ethanol and cold macerated. Unextractable elements were removed from the sample by filtering it. Silver nanoparticles (AgNPs) were synthesised by dissolving 200mg of the above sample in 100ml of water. 90ml of a 1mM silver nitrate solution was mixed with 10ml of this solution (0.017g $AgNO_3$ in 100ml water). A 1% PVA solution that had been heated to 80°C for five hours was used to encapsulate the nanoparticles. To create PVA-encapsulated silver nanoparticles, the leaf extract, $AgNO_3$ and PVA solution were combined in a 1:10:3 ratio and magnetically swirled for two hours at 24°C[5].



Figure 1. *Pisonia grandis* R.

2.2 Chemicals and Reagents

Human Cardio Myocytes (Cat No: C12881), Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Phosphate Buffer Saline (DPBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT), Dimethyl Sulfoxide (DMSO), Fetal Bovine Serum (FBS), Digoxin, Sigma Chemicals Co. provided H_2O_2. Superoxide Dismutase Kit (#KBH0918, KrishGen Biosystems, India), Human Glutathione, GSH Assay Kit (#KBH1462, KrishGen Biosystems, India), Human Glutathione Peroxidase-1 (GPx1) assay kit (Cat No: E-EL-H5410, Elab Science), Human Catalase ELISA kit (Cat No: KBH3053, KrishGen Biosystems, India.

2.3 Characterisation of Silver Nanoparticles

UV-visible absorption spectroscopy (Shimadzu BioSpec-nano, Japan) was used to study the optical characteristics of AgNPs, with spectra obtained between 200 and 800nm. The surface configuration and chemical constituents of the biological components were determined using Fourier Transform Infrared (FTIR) spectroscopy. X-Ray powder Diffraction (XRD) patterns were acquired by means of 2-hour scans spanning a 2θ range of 30 to 80° to evaluate crystallinity. The structural features and chemical composition of the AgNPs were analysed using SEM and energy-dispersive X-Ray Spectroscopy (EDX). The size of the nanoparticles was determined using Transmission Electron Microscopy (TEM). The colloidal behaviour and electrostatic properties were determined through zeta potential analysis.

2.4 Maintenance of Cell Lines

The HCM cells (Cat No: C12881) were purchased from NCCS, Pune, India. This research was authorised by the Institutional Ethics Committee of Avinashilingam University under the number (AUW/IHEC/BC-22-23/XPD-03). DMEM with excess glucose augmented with 5% Fetal Bovine Serum was used to maintain the cells, together with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2 at 37^0C temperature in the CO_2 incubator and sub-cultured every 2-3 days.

2.5 Cytotoxicity Assay of Human Cardiomyocytes (HCM) Using H_2O_2

Cell viability was examined by MTT assay[6,7]. Cells were allowed to develop for approximately a twenty-four-hour period. Cardio-toxicity was induced in the cells with 100uM of H_2O_2 for 2 hours, after which the appropriate concentrations of PVA AgNPs (6.25, 12.5, 25,50 and 100ug/ml) were added to compare the protective ability. For twenty-four hours, the plates were incubated at 37°C with 5% CO_2 and left to incubate for three hours following the addition of the MTT reagent. Note that incubation durations differ depending on the cell line. It is important to have a consistent incubation period within a single experiment when doing comparisons. 100µl of DMSO was added once the MTT reagent had been separated. In a gyratory shaker, gentle spinning helped dissolve.

2.6 Antioxidants Estimation Assay

A 6-well plate was used to cultivate HCMs (0.5 x 10^6 cells/ml), and they were incubated for 24 hours to achieve the necessary cell count. Induce the cardiotoxicity to the HCM cells with 100uM of H_2O_2 for 2hours and further treat the cells with 25ug/ml PVA AgNPs Pg and 10uM of reference std, Digoxin, followed by 24 hours of incubation. H_2O_2-exposed cells were maintained as the positive (disease) control, untreated cells served as the normal control, and medium itself was used as the blank in all ELISA studies. Various enzymic antioxidant levels were measured using a specific assay kit from Krish Gen Biosystems, India and Elabscience, Texas, USA. Monoclonal antibodies were pre-coated onto microwells, where the added samples and standards bound specific human antioxidants. A biotin-labelled antibody and Streptavidin-HRP were incorporated to form a detection complex. The addition of TMB substrate led to a colour change relative to antioxidant levels. Absorbance was measured at 450nm after stopping the reaction

2.7 Determination of Intracellular ROS

The fluorescent probe 2',7'dichlorodihydrofluorescein diacetate (H2DCFDA) (#D-399, Life Technologies,

Invitrogen) was employed to judge intracellular ROS levels in HCM cells. After seeding HCM cells (0.5 x 106 cells/2 ml), mixed with 100uM H_2O_2 for two hours, followed by 25 ug/ml of the test chemical, PVA AgNPs, Pg and controls in 1 ml of culture media to induce cardiotoxicity. The mixture was rinsed with 500µl of PBS solution in a polystyrene tube and incubated at 37°C for three to four minutes. Cells were dispersed in dichlorodihydrofluorescein diacetate working solution, then the supernatant was removed by centrifugation, and the cells were mixed in 400µl warmed DPBS. A flow cytometer was employed to quantify the results. It was recommended to excite and detect at 535nm using the 488nm laser[8].

2.8 Mito Tracker Green FM Expression (Mitochondrial Damage) Study on H_2O_2 Induced HCM Cell Lines by Confocal Microscopy

A mitochondrial-selective fluorescent marker called MitoTracker Green (MTG) is frequently employed in flow cytometry and confocal imaging. By interacting with the free thiol groups of cysteine residues, this dye is predicted to selectively concentrate in the mitochondrial matrix and covalently attach to mitochondrial proteins. Its emission and excitation peaks are located at 512 and 490nm, respectively.

Culture cells in a 35mm glass-bottom dish at a density of 50,000cells/1000ul were incubated for twenty hours. Incubation continued for twenty hours after mixing with 100uM H_2O_2. Untreated cells were well considered as a negative control, and H2O2-treated cells alone were considered as a toxic control. After washing in PBS, resuspend the cells with 400nM of mitotracker green Fei Mao (FM) dye, followed by incubation in the absence of light and washing the cells with PBS to remove excess dye. The cells were counterstained with 1000µL of Hoechst 33342 solution (1ug/ml) without the presence of light. Cells were examined using confocal Microscopy applying a 488nm laser and a filter cube for excitation and detection at 490/512nm nm for green fluorescence (MTG) and Excitation 341nm and emission 452nm for Hoechst stain for the blue channel to perceive the nucleus. Images were evaluated by ZEN Blue Software and verified; The expression of Dichlorofluorescein (DCF) intensity is calculated using ImageJ software.

3. Results and Discussion

Numerous cardiac diseases, including heart failure, cardiac hypertrophy, abnormalities in calcium management, energetics, and substrate metabolism, as well as anomalies in contractile properties and electrical activity, have been linked to oxidative stress, which causes macromolecular damage. All aerobic cells maintain oxidative stress equilibrium through the regulation of free radicals by endogenous antioxidant enzymes[9].

ROS levels are quite low under physiological conditions. This signal transducer is essential because it can regulate the body's production of immunological inflammatory substances. Additionally, peroxides can cause apoptosis[10,11].

ROS attacks can cause proteins to oxidise, change structure, and lose physiological function. It has the ability to oxidise lipids on cell membrane surfaces, hence changing their composition and functionality. It also has the power to change bases and disturb the way genetic material works[12]. When Fe^{2+} is present, H_2O_2 forms hydroxyl radicals (OH), which have a high oxidising power and cause additional reactive oxygen species to break down organic molecules, resulting in oxidative damage to the body[13].

The findings of this investigation demonstrate that *P. grandis* extract may have a cardioprotective effect by lowering oxidative stress caused by H_2O_2 in HCM cardiomyocytes. Results demonstrate that by lowering reactive oxygen species, the leaf extract significantly decreased cellular damage. Both the cellular reaction to endogenous toxic substances and mitochondrial oxidative metabolism lead to reactive oxygen species (ROS) formation. Cellular functions imbalance brought on by an additional of reactive oxygen species and a slower antioxidant defence system.

3.1 Characterisation Studies

Various characterisation analyses validated the effective formation of silver nanoparticles synthesised from *P. grandis* leaf extract. The extract's colour change confirmed the Ag ion reduction to metallic silver within 30 minutes, which was the initial sign of the effective production of PVA-encapsulated silver nanoparticles (PVA AgNPsPg). No additional intensification followed, indicating complete reduction. The PVA-encapsulated

nanoparticles stayed stable at ambient temperature for three months.

UV-visible spectroscopy showed a noticeable Surface Plasmon Resonance (SPR) peak at 430nm (Figure 2), which is suggestive of nanoparticle production. The polymer matrix is responsible for the band broadness. The greatest absorption peaks at 400nm in solutions were obtained by silver nanoparticles made with Diospyros maritima Blume leaf extract, indicating the Localised Surface Plasmon Resonance (LSPR) of the particles[14].

FTIR spectra disclosed functional groups responsible for AgNP reduction and stabilisation, such as -OH stretching (3302cm^{-1}), -CH stretching (1635cm^{-1}), phenols/tertiary alcohols, cyclic ethers, aliphatic bromo compounds, and aryl disulfides (Figure 3). These findings suggest the involvement of amino acids, triterpenoids, alkaloids, phytosterols, and flavonoids in nanoparticle formation. The functional groups in nanoparticles synthesised from *Citrus reticulata* Blanco (Kinnow mandarin hybrid) peel extract. were identified via FTIR analysis. Peaks were identified in the 525-3251cm^{-}1 range. The signal at 1641.8cm^{-}1 shows stretching of C-N and C-C bonds[15].

Based on the crystallographic lattice planes, the indexed and Miller Indices (hkl) are (111), (200), (220), and (311) (Ref: Joint Committee on Powder Diffraction Standards, Joint Committee on Powder Diffraction Standards (JCPDS) file No. 04-0783). The Miller indices, which indicate a face-centred cubic structure, were correlated with diffraction peaks at 2θ values of 38.1°, 44.2°, 64.4°, and 77.3° in the XRD analysis, confirming the crystalline character of AgNPs (Figure 4). The average crystalline size was calculated using the Scherrer equation and matched previous reports. The XRD motif of Pathor Kuchi Leaf (PKL) extract showed diffraction peaks at 38.070, 44.290, 64.440, and 77.340 locations. The extremely strong peak indicates a polycrystalline phase configuration of generated silver nanoparticles, validating the formation of Face-Centred Cubic (FCC) crystalline silver nanoparticles[16].

SEM of the investigation showed primarily spherical nanoparticles with different sizes in the plant sample (Figure 5). SEM image generated for silver nanoparticles of *Gamphrena serrata* clearly explained the formation of polydispersed nanoparticles of size 2 micrometer[17].

Transmission Electron Microscopy (TEM) offered a more accurate view, displaying spherical particles with an average size of 10.2nm (Figure 6). The average AgNP particle size, according to TEM images taken for collective plant leaf extracts such as *Azadirachta indica* and *Mangifera indica* L., is 10.14nm, and a small percentage of them are 20nm, which suggests AgNP aggregation[18]. Energy-Dispersive X-ray (EDX) spectroscopy of the PVA AgNPs Pg verified the occurrence of silver with a distinctive peak at 3 keV, consistent with metallic silver nanocrystals (Figure 7). This technique was utilised to conduct qualitative assessments of micro and nanoparticle materials, while it can also produce semi-quantitative results. The spectrum of Silver nanoparticles (AgNPs) brought from strawberry pomace extracts revealed that silver, carbon and oxygen account for 53.29%, 33.5% and 13.21%, respectively, indicating AgNPs creation[19].

PVA AgNPs Pg showed greater colloidal stability, with a mean zeta potential of −7.4mV compared to −20 mV for pure silver nanoparticles (Figure 8). This suggests improved electrostatic repulsion and resistance to aggregation. These combined analyses indicate the effective synthesis, structural integrity, and stability of PVA-encapsulated silver nanoparticles made from *P. grandis* leaf extract. Zeta potential study results for silver nanoparticles (AgNPs) made from plant leaf extracts of *Ocimum gratissimum* (Og) and *Vernonia amygdalina* (Va) vary from -15.03mV to -20.3mV and from 23.41mV to -27.23mV, respectively. This range of zeta potentials suggests some instability and demonstrates that the nanoparticles were in the aggregation threshold. While they might aid in stabilising, they might not promote drug protein absorption[20].

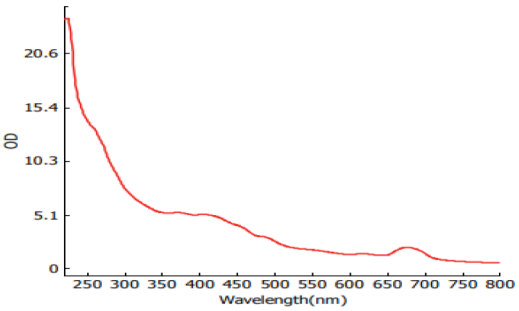


Figure 2. UV-visible absorption spectra of the PVA AgNPs Pg.

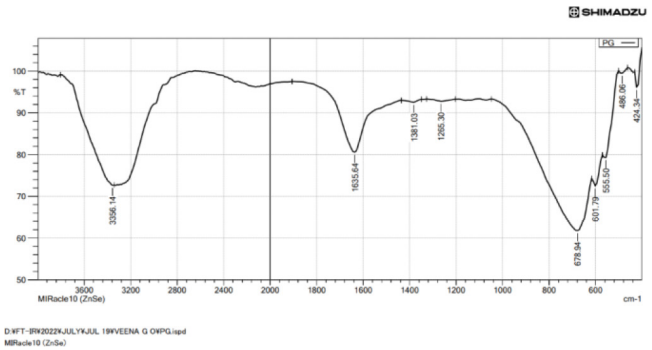


Figure 3. Fourier transform infrared spectroscopy (FTIR) of PVA AgNPs Pg.

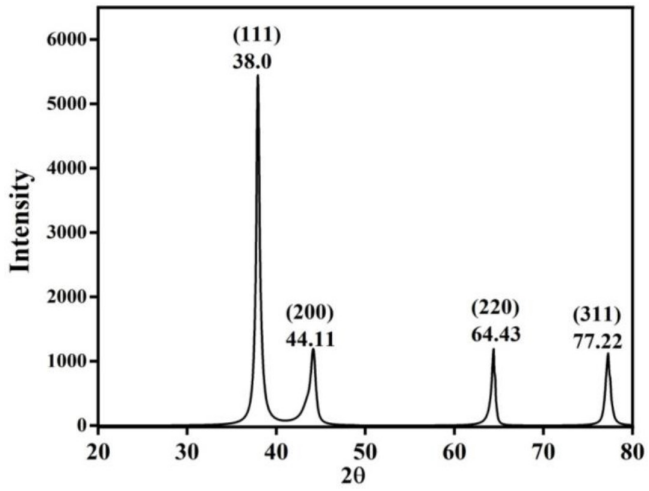


Figure 4. XRD spectra analysis of PVA AgNPs Pg.

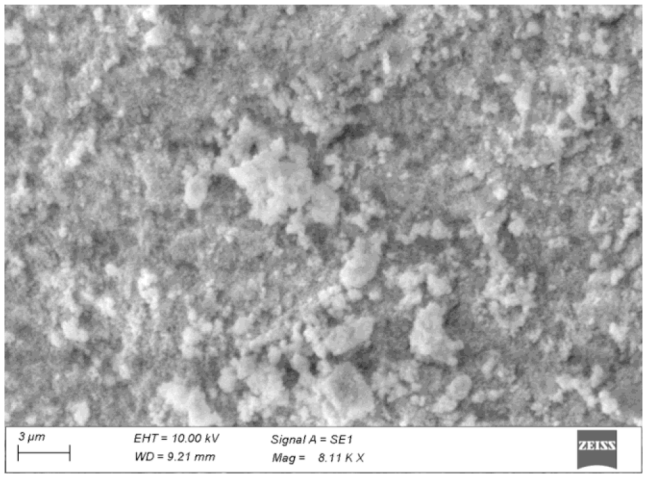


Figure 5. Scanning electron microscopy (SEM) of PVA AgNPs Pg.

3.2 MTT Assay

The outcomes of the MTT assay suggest the cytotoxic nature of the 100uM H_2O_2, with 25% cell viability and

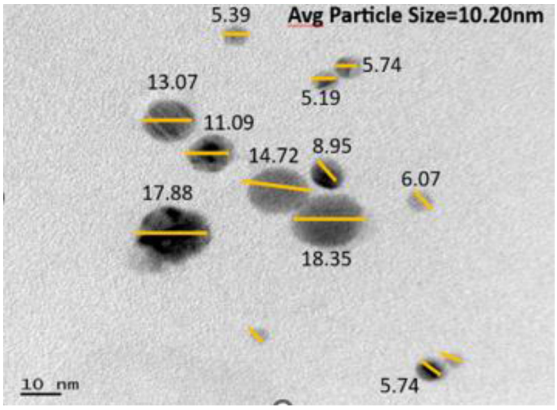


Figure 6. Transmission electron microscopy (TEM) of PVA AgNPs Pg.

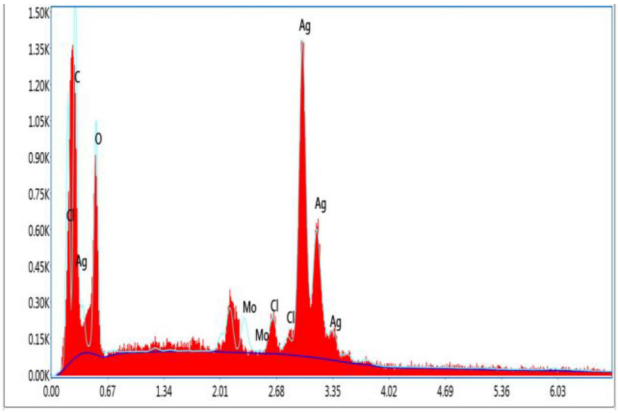


Figure 7. Energy-dispersive X-ray spectroscopy (EDX) of the PVA AgNPs Pg.

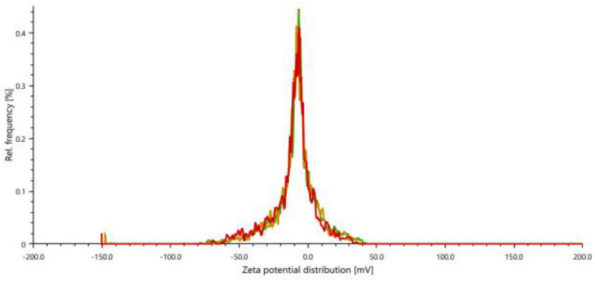


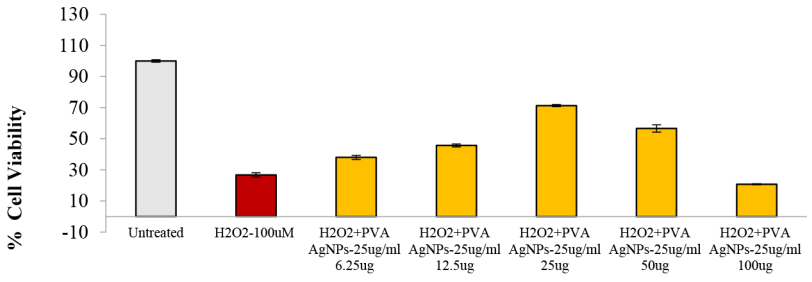
Figure 8. Zeta potential analysis of PVA AgNPs Pg.

25ug/ml PVA AgNPs. Pg was non-cytotoxic in nature on HCM, with the % cell viability of 93% after a 24-hour treatment period. Other higher concentrations caused moderate toxicity in HCM cells. In the H_2O_2 induced model against HCM cells, PVA AgNPs showed protective ability with increased cell viability values till the maximum dose of 25ug/ml. The maximum protective dose observed was 25ug/ml, which can

protect the cells from H_2O_2 damage with 71% cell viability (Figure 9). Similar studies carried out by researchers on cardioprotective effects of Pomegranate Seed Oil (PSO) of diverse concentration (25, 50, 100 and 200 µg/ml) on H_2O_2-induced toxicity revealed a substantial rise in viability of HCM cells to 60% to 80% respectively[21]. Treatment with H_2O_2 alone showed decreased viability of 47% compared to the control. Antioxidants put off the oxidation process by averting the instigation or propagation of oxidising chain reactions.

3.3 Endogenous Antioxidant Activity in Human Cardiomyocytes

Antioxidants are indispensable homeostatic agents; when cells are experiencing oxidative stress, they comprise enzymatic and non-enzymatic antioxidants. From the cell viability assays, it could be inferred that 25ug/ml of PVA AgNPs Pg showed similar cell viability as analogous to the control. Antioxidants, including SOD, CAT, GSH, and GPX activity, were measured to verify that cardiomyocytes treated with *P. grandis* extract were protected against oxidative stress caused by H_2O_2. PVA Ag NP concentrations of 25 ug/ml were used in the antioxidant tests. Following initial treatment with extract concentrations of 25 ug/ml, cells' antioxidant parameters were discovered to have dramatically increased with 3.51±0.06 ng/ml, 3.94±0.10 ng/ml, 10.94±0.14 ng/ml and 592.51±12.08 pg/ml, respectively, for SOD and CAT, GSH and GPX on exposure to H_2O_2 (Figures 10-13). However, these antioxidant parameters were found to be very low in H_2O_2 treated cells. It signifies that the treatment with *P. grandis* extract improved the antioxidants SOD and CAT, GSH and GPX, respectively, by mitigating H_2O_2 induced toxicity. Similar studies using *Malus doumeri* Leaf Extract (MDLE) also revealed a significant improvement in the CAT, SOD, GSH, and GPX expression of H_2O_2-applied oxidative damage in H9C2 rat cardiomyocytes[4]. After rat H9c2 cardiomyocytes were exposed to H_2O_2, the study on *Nelumbo nucifera* leaf extract demonstrated the capacity of enzymatic antioxidants such as SOD and CAT. Two different concentrations of *N. nucifera* leaf extract were used for the study (10.7 and 47.3 units/mg of protein for SOD and CAT, respectively)[9]. The ethyl acetate fraction of *Dracocephalum moldavica* L. is found to assess the expression of total SOD and catalase activity. In comparison to the control group, the ethyl acetate (EtOAc) fraction significantly increased the CAT activity. Additionally, EtOAc fraction treatment restored the H_2O_2 induced total SOD levels[22]. Research using hydrogen peroxide to treat the red algae *Alsidium corallinum* (*A. corallinum*) showed that 30 mg/ml had harmful effects on rat cardiomyocytes. Through preventing ROS generation and influencing the activity of antioxidants like SOD, CAT, GPX, and GSH, *A. corallinum* may shield the cardiomyocytes from H_2O_2 induced damage[3]. The cellular antioxidant assay reports suggest that the given test compound, PVA AgNPs Pg, showed significant cardio-protective activity by enhancing the cellular oxidant enzymes viz., SOD, CAT, GSH and GPX in H_2O_2 induced model, whereas



The results are expressed as mean ± SD. Statistical analysis was performed using Student's *t*-test. $p < 0.05$, $p < 0.01$, $p < 0.001$ compared with H_2O_2-treated group. Cells exposed to H_2O_2 (100µM) showed a significant reduction in viability compared to the untreated control. Treatment with PVA AgNPsPg of concentration ranging from 6.25–100 µg/mL significantly restored cell viability in a dose-dependent manner, with maximum protection observed at 25 µg/mL.

Figure 9. Effect of PVA-encapsulated silver nanoparticles on cell viability.

H_2O_2 alone treated cells effectively inhibited the SOD, CAT, GSH and GPX activities respectively. The obtained values exhibited a promising cardioprotective activity of PVA AgNPs Pg with 25ug/ml in relation to the cardioprotective effect in H_2O_2 induced human cardiomyocytes model by enhancing the enzyme activities.

Data are expressed as mean ± SD (n = 3). Statistical comparisons between groups were performed using student's *t*-test. A value of $p < 0.05$ was considered statistically significant. H_2O_2 exposure significantly decreased SOD activity compared to control ($p < 0.001$). Treatment with Digoxin (10µM) and PVA-AgNPs (25µg) significantly restored SOD levels compared to H_2O_2-treated cells ($p < 0.001$). PVA-AgNPs showed a modest but significant increase compared to Digoxin ($p < 0.05$). There is a significant decrease in CAT levels in the H_2O_2-treated group compared to the control (***p < 0.001). Treatment with Digoxin (10µM) and PVA-AgNPs (25µg) significantly restored CAT activity compared to H_2O_2 alone (**p < 0.01 and ***p < 0.001, respectively). Similarly, a significant reduction in GSH levels is observed in the H_2O_2-treated group compared to the control (***p < 0.001). Treatment with Digoxin (10µM) and PVA-AgNPs (25µg) significantly restored

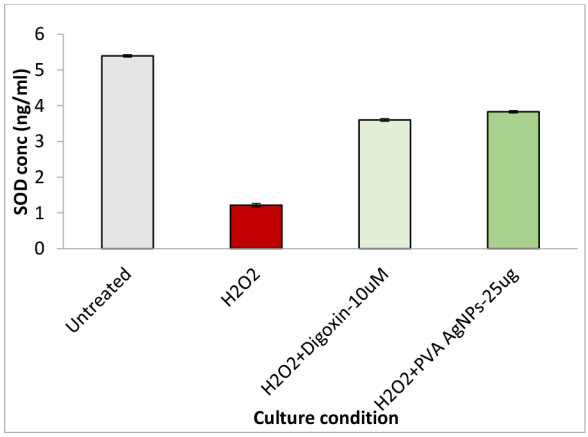


Figure 10. SOD levels observed in untreated, H_2O_2 alone, H_2O_2 +Digoxin with 10uM/ml and H_2O_2 +PVA AgNPs with 25ug/ml treated HCM cells after the incubation period of 24hrs.

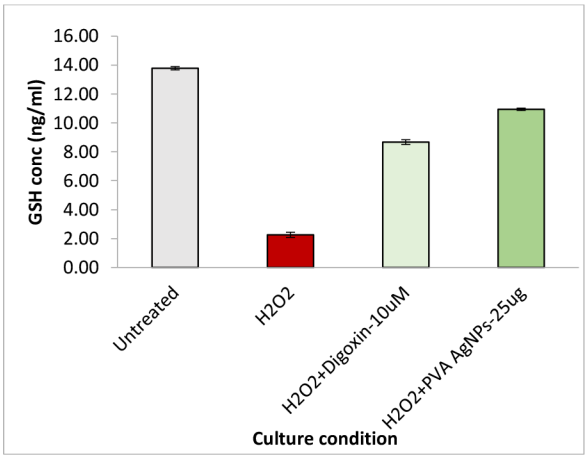


Figure 12. GSH levels observed in untreated, H_2O_2 alone, H_2O_2 +Digoxin with 10uM/ml and H_2O_2 +PVA AgNPs with 25ug/ml treated HCM cells after the incubation period of 24hrs.

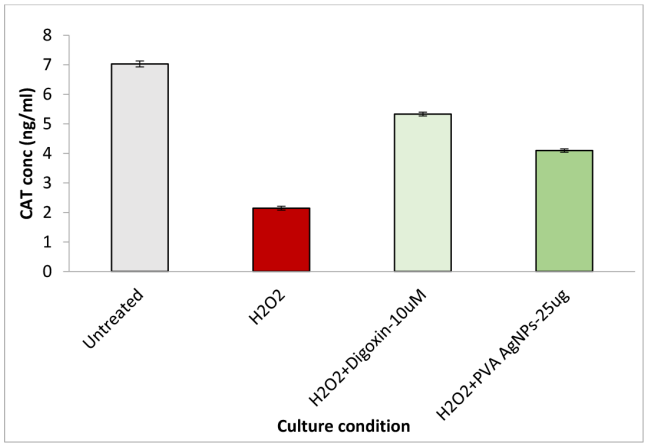


Figure 11. Catalase levels observed in untreated, H_2O_2 alone, H_2O_2 +Digoxin with 10uM/ml and H_2O_2 +PVA AgNPs with 25ug/ml treated HCM cells after the incubation period of 24hrs.

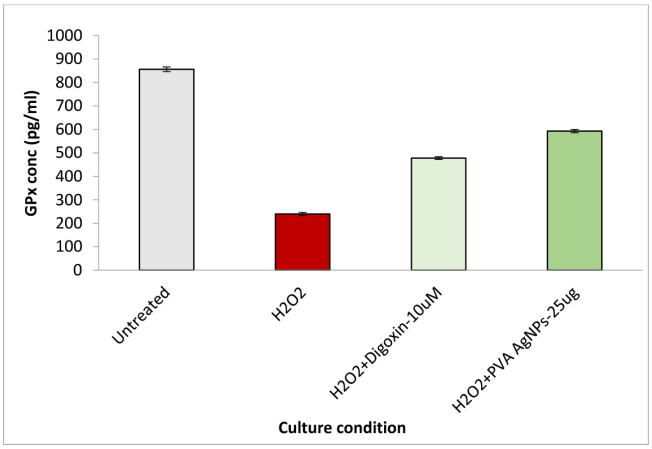


Figure 13. GPX levels observed in untreated, H_2O_2 alone, H_2O_2 +Digoxin with 10uM/ml and H_2O_2 +PVA AgNPs with 25ug/ml treated HCM cells after the incubation period of 24hrs.

GSH levels compared to H_2O_2 alone (**p < 0.01, ***p < 0.001, respectively). H_2O_2 exposure significantly decreased GPx activity compared to control (p < 0.001). Treatment with Digoxin (10μM) and PVA-AgNPs (25μg) significantly restored GPx levels compared to H_2O_2-treated cells (p < 0.001). PVA-AgNPs showed significantly higher GPx activity than Digoxin (p < 0.01).

3.4 Detection of Intracellular Reactive Oxygen Species (ROS) in *Pisonia grandis* Extract Treated Cells Using 2′, 7′-Dichloro Dihydro Fluorescein Diacetate (H2DCFDA)

The occurrence of ROS can be noticed and calculated by fluorescent stain 2′,7′-dichloro dihydro fluorescein diacetate (H2DCFDA), which is transformed into fluorescently energetic esters 2′,7′-dichlorofluorescein. As the concentration of 25μg of PVA Ag NPs showed active antioxidant defence against H_2O_2 like to control, according to the antioxidant assays, the same concentration was used to measure intracellular ROS levels. Oxidative stress elevates intracellular ROS, triggering cell death. Cells treated with 100μM H_2O_2 showed intense green DCF fluorescence that specifies reactive oxygen species formation. Green fluorescence is reduced in cells treated with extract (25 μg/ml). The results are shown in Figure 14. The DCF expression study of untreated, H_2O_2 with 100uM alone and combination of H_2O_2 followed by either digoxin with 10uM/ml or PVA AgNPsPg with 25ug/ml on HCM cells was carried out by means of BD FACS Calibur, Cell Quest Pro Software (Version: 6.0).

DCF histograms (Figure 15) of the gated HCM singlets differentiate cells at the M1 and M2 phases. Gating of M1 and M2 phases is relative and can be distinguished by software (Cell Quest Software, Version 6.0) examination. Cells expressed in the M2 region were measured for the current study. The FACS plots of different control groups are given in Figure 16. Under oxidative stress, mitochondria produce ROS that diffuse into the cytoplasm. Hydrogen peroxide, a type of ROS, is broken down by enzymatic antioxidants like catalase into water (H_2O)[8]. From the earlier studies, it was stated that the ethyl acetate fraction of *Dracocephalum moldavica* L. extracts significantly reduced the ROS production in H_2O_2 induced oxidative damage observed in rat H9c2 cells[22]. Rosamultin, a chief constituent identified from *Potentilla anserina* L, significantly reduced the ROS levels in pretreatment groups of H9c2 cardiomyocyte, signifying the plant's powerful cardioprotective ability in H_2O_2 generated toxicity[23]. Studies on *N. nucifera* indicate that pre-treatment with its methanolic leaf extract (NNLE) may offer cytoprotection by regulating H_2O_2-induced oxidative stress. Antioxidant assays, lipid peroxidation, and DNA fragmentation analyses demonstrated the notable defensive effect of *N. nucifera.* extract on cardiomyocytes, with optimal protection contrary to H_2O_2-induced oxidative stress observed at 50 μg/ml[9].

The results were expressed as mean ± SD. Statistical comparisons were performed using Student's t-test, with significance calculated relative to the H_2O_2-treated group. Intracellular ROS expression in HCM cells following H_2O_2 (100 μM) induction and treatment with digoxin or PVA-AgNPs. ROS levels were quantified using DCF fluorescence and expressed as mean ± SD. Statistical significance was determined using Student's t-test, where p < 0.05, p < 0.01, and p < 0.001 compared with H_2O_2 treated cells. Exposure of HCM cells to H_2O_2 (100 μM) resulted in a highly significant increase in intracellular ROS levels compared with untreated control cells, confirming oxidative stress induction. Treatment with digoxin significantly reduced ROS generation in H_2O_2-induced cells, indicating moderate antioxidant protection. Notably, treatment with PVA-AgNPsPg significantly attenuated ROS levels compared with the H_2O_2 treated group, demonstrating effective ROS-scavenging activity.

3.5 Mito Tracker Green FM Expression (Mitochondrial Damage) Study on H_2O_2 Induced HCM Cell Lines by Confocal Microscopy

The statistical data of the MTG expression study by confocal microscopy suggest that in HCM cells, the MTG expression is high in the untreated group and relatively low in the treated groups. Figure 17 showed the relative mean fluorescence intensity of MTG in untreated, H_2O_2 and a combination of H_2O_2 followed by either digoxin or PVA AgNPs on HCM cells. Figure 18 clearly explains that the given test compound, PVA AgNPs Pg, treated cells considerably enhanced the MTG expression in H_2O_2 induced HCM cells, similar

to the reference standard, digoxin. The control cells showed better expression of MTG dye as mitochondria were healthy and showed prominent expression,

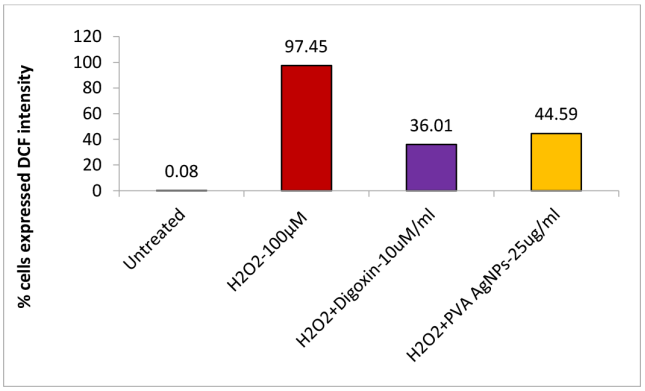


Figure 14. ROS expression-H_2O_2 induced HCM cells.

whereas in the H_2O_2 alone-treated group, the cells showed a very low rate of MTG intensity, confirming the mitochondrial damage. Studies reported the activity of astaxanthin, a carotenoid, on H_2O_2 induced HCM cell lines. It was found that Astaxanthin (AST) removed the cytotoxicity of H_2O_2 and Doxorubicin (DOX), while cell viability was amplified, and the mitochondrial volume had no difference from the control[24].

The results are expressed as mean ± SD. Statistical comparisons were performed using Student's *t*-test, with significance calculated relative to the H_2O_2-treated group. Fluorescence intensity is expressed as mean ± SD. Statistical significance was determined using Student's *t*-test, where $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared with H_2O_2-treated cells.

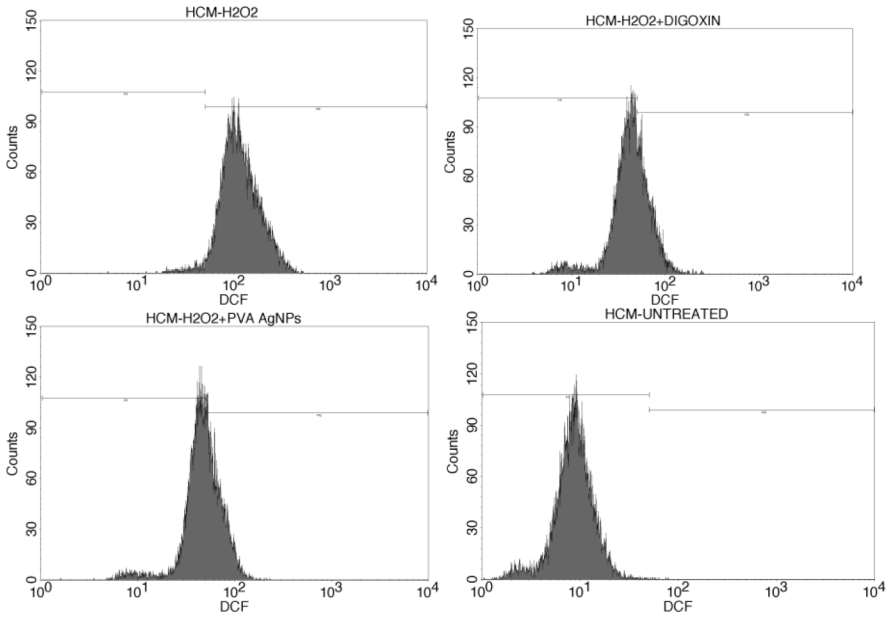


Figure 15. DCF histogram of different experimental groups.

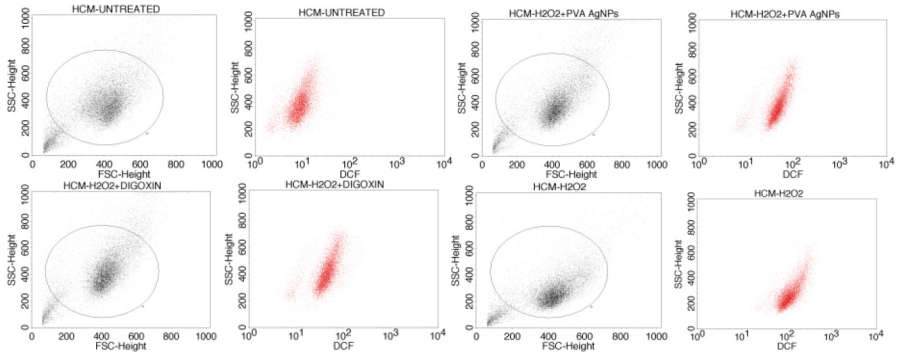


Figure 16. FACS plots of different experimental groups.

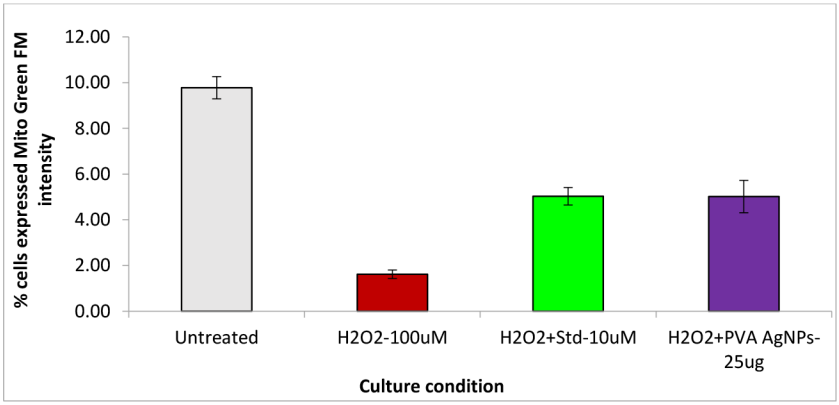


Figure 17. Mitotracker green FM expression in HCM cells in different experimental groups.

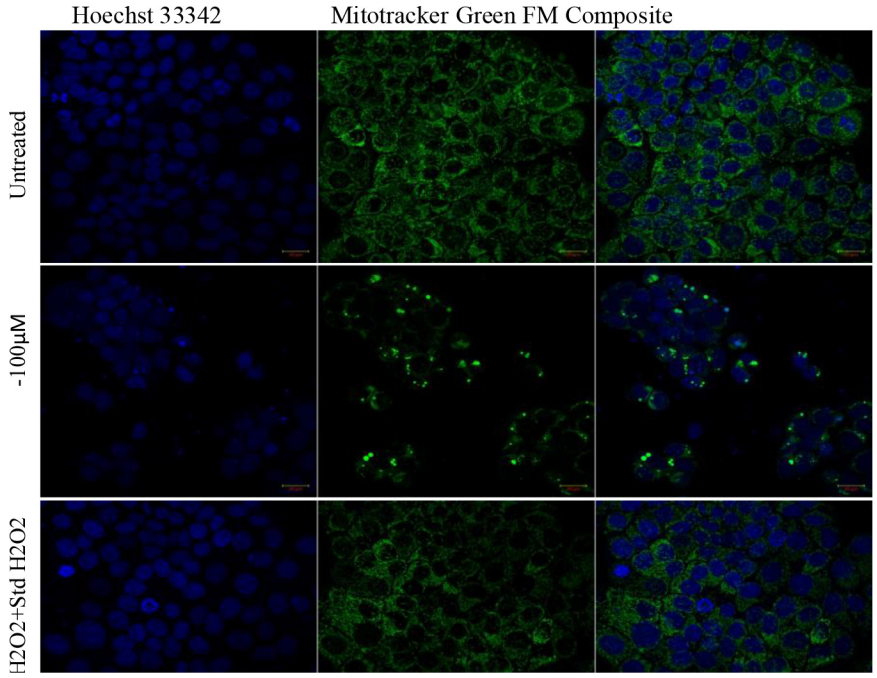


Figure18. MTG expression of H_2O_2 induced HCM cell lines by the confocal fluorescence microscope.

Exposure of HCM cells to H_2O_2 (100 µM) caused a highly significant reduction in Mitotracker Green FM fluorescence intensity compared with untreated control cells, indicating severe mitochondrial damage. Treatment with the standard drug (10µM) significantly increased Mitotracker Green FM expression relative to the H_2O_2-treated group, suggesting partial restoration of mitochondrial integrity. Notably, treatment with PVA-AgNPs (25µg) also resulted in a significant improvement in Mitotracker Green FM intensity compared with H_2O_2 alone, demonstrating a protective effect against oxidative stress–induced mitochondrial dysfunction.

4. Conclusion

Various diseases can impair the delicate balance between antioxidant defence mechanisms and the generation of ROS, resulting in oxidative stress and subsequent cellular dysfunction. Cell viability, as well as morphological and biochemical characteristics, may be impacted. Therefore, the discovery of an efficient chemical that may shield the heart against oxidative stress injury is of tremendous importance. The results of cytotoxicity studies suggested that the given test compound, PVA AgNPs with 25ug/ml concentration, does not show any sign of toxicity and, in combination with H_2O_2,

followed by PVA AgNPs treatment effectively inhibited the cytotoxic potency caused by H_2O_2, thus proving the cardioprotective ability of the PVA AgNPs compound. The cellular antioxidant results suggest that the given test compound, PVA AgNPs showed significant cardio-protective activity by enhancing the cellular oxidant enzymes.PVA AgNPs effectively inhibited the ROS expression and confirmed the protective effect in H_2O_2 induced HCM cells, similar to the reference std drug, digoxin, by analysing the DCF expression. The results on Mitotracker green FM expression studies clearly substantiated the cardioprotective ability of PVA AgNPs Pg by remarkably suggesting the MTG dye expression similar to the cardioprotective drug, digoxin and confirmed the cardioprotective effect by inhibiting the H_2O_2 caused Mitochondrial damage and recovering the mitochondrial health.

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Author Contribution

All authors contributed equally to the conception, design, experimentation, data analysis, and writing of this manuscript. All authors have read and agreed to the published version of the manuscript.