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ORIGINAL ARTICLE

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Artemisia argyi mitigates doxorubicin-induced cardiotoxicity by inhibiting mitochondrial dysfunction through the IGF-IIR/Drp1/GATA4 signaling pathway

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Abstract

Doxorubicin (DOX) is mostly utilized as a wide range of antitumor anthracycline to treat different cancers. The severe antagonistic impacts of DOX on

Abbreviations: AA, *Artemisia argyi*; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; DOX, doxorubicin; ERK, extracellular signal-regulated kinase; IGF-IIR, insulin-like growth factor II receptor; MMPs, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling.

Chih-Yang Huang and Tsung-Jung Ho contributed equally to this work.

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cardiotoxicity constrain its clinical application. Many mechanisms are involved in cardiac toxicity induced by DOX in the human body. Mitochondria is a central part of fatty acid and glucose metabolism. Thus, impaired mitochondrial metabolism can increase heart failure risk, which can play a vital role in cardiomyocyte mitochondrial dysfunction. This study aimed to assess the possible cardioprotective effect of water-extracted *Artemisia argyi* (AA) against the side effect of DOX in H9c2 cells and whether these protective effects are mediated through IGF-IIR/Drp1/GATA4 signaling pathways. Although several studies proved that AA extract has benefits for various diseases, its cardiac effects have not yet been identified. The H9c2 cells were exposed to 1 μM to establish a model of cardiac toxicity. The results revealed that water-extracted AA could block the expression of IGF-IIR/calcineurin signaling pathways induced by DOX. Notably, our results also showed that AA treatment markedly attenuated Akt phosphorylation and cleaved caspase 3, and the nuclear translocation markers NFATC3 and p-GATA4. Using actin staining for hypertrophy, we determined that AA can reduce the effect of mitochondrial reactive oxygen species and cell size. These findings suggest that water-extracted AA could be a suitable candidate for preventing DOX-induced cardiac damage.

KEYWORDS
apoptosis, *Artemisia argyi*, cardiac hypertrophy, doxorubicin, IGF-IIR signaling pathways, mitochondrial biogenesis

1 | INTRODUCTION

Nowadays, with the increasing number of advanced techniques in cancer treatment, the survival rate has been very high since the 20th century.[1] However, a medical survey shows that nearly one-third of cancer survivor patients die due to chronic heart failure, because chemotherapy drugs can promote myocardial dysfunction years after chemotherapy completion.[2] The main complication with current chemotherapeutics is that they can cause nonspecific induction of apoptosis in non-cancer tissues, especially myocardium tissue. Most tissues in the human body are composed of replicating epithelial cells and can easily recover from side effects of chemotherapeutics, but cardiac tissues are terminally differentiated and have narrow ranges of replicative ability.[3,4] Doxorubicin (DOX) is a well-known chemotherapeutic agent that has been developed to combat several kinds of cancer diseases; however, the increasing number of harmful side effects, especially in the liver, brain, kidney, and heart,[5,6] has reduced its clinical usage. DOX is particularly known for its cardiac toxicity.[7] Mitochondrial abnormalities and DNA damages are major causes of DOX-induced cardiac toxicity.[8] However, the mechanism underlying the cardiac failure induced by DOX is still to be elucidated. Increasing evidence suggests that excess production of free radicals or reactive oxygen species (ROS) causing mitochondrial damage and impaired calcium homeostasis are involved in different processes of DOX metabolism. These processes accelerate myocardial injury development and progression.[9–11] Dexrazoxane is the first-line drug that is approved by European Medicines Agency and the US Food and Drug Administration (FDA) for alleviating the cardiac toxicity induced by DOX. However, it can hinder the anticancer activity of antibiotics such as anthracycline, and may also enhance the myelosuppressive effect of DOX.[12,13]

Cardioprotective agents with mitochondrial protective properties may be one of the best approaches to alleviate DOX-induced cardiac dysfunction. Nature produces a seemingly unlimited range of diverse metabolites that exhibit biological activities against various diseases. Many traditional Chinese medicines have a tremendous role in cardiac protection, and their phytochemical properties can be exploited to develop medicines worldwide.[14] The extract of *Artemisia argyi* (AA), a plant species belonging to the genus *Artemisia*, has flavonoids with biological activity and can be used in modern medicine. More than 260 species have been identified in *Artemisia*, and they contain an abundance of metabolites such as flavonoids and

sterols.[15] These phytochemicals play a vital role as antioxidant, cytotoxic, neuroprotective, anti-inflammatory, and antimicrobial agents.[16] Although *Artemisia* plants have been used for more than 200 years to treat various diseases, very little research has been done on the cardioprotective effects of this plant in the modern era, mainly in mainland China, Taiwan, Japan, and Hong Kong.

To date, no targeted strategy exists for treating DOX-induced cardiac toxicity. As very little research has been carried out regarding the impact of AA on cardiac diseases and DOX-induced cardiac toxicity, we aimed to examine the cardioprotective effect of AA extract against DOX in H9c2 cardiac cells and determine the underlying mechanisms involved.

2 | MATERIALS AND METHODS

2.1 | Reagents

For our experiment, doxorubicin hydrochloride (D1515) 98.0%–102.0% (HPLC) was purchased from Sigma Aldrich. A terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) staining kit was purchased from Roche and Western blot antibodies against atrial natriuretic peptide (ANP) (sc-515701), Akt 1(B-1, sc-5298), p-Akt (sc-514032), Bax (P-19, sc-526), Bcl-2 (#610539), Bcl-xL (H-5, sc-8392), calcineurin (#610259), caspase-3 (H-277, sc-7148), caspase-9-p35 (H-170, sc-8355), cleaved caspase-3 (Asp175) (5A1, #9664), cleaved caspase-9 (Asp353, #9509), COX IV (OxPhos complex IV, #11967), p-DRP1(ser 616, #3455), p-DRP1(ser 637, GTX50911), DRP1 (C5, SC-271583), p-ERK (E-4, sc-7383), p-Erk1(T202/Y204)/2(T185/Y187, AP0974), GAPDH (6C5, sc-32233), p-GATA-4 (H-4, sc-377543), GATA-4 (G-4, sc-25310), Insulin-like growth factor II receptor (IGF-IIR) (H-300, sc-25462), JNK (FL, sc-571), p-JNK (G-7, sc-6254), MFN1 (D-10, SC-166644), MFN2 (XX-1, SC-100560), MCU1 (HPA016480), NFATC3 (F-1, sc-8405), p53 (1C12, #2524), PARP (#9542), and p-p53 (Ser15, #9284) were purchased. For actin staining, rhodamine phalloidin (R415) was obtained from Invitrogen (Thermo Fisher Scientific). For all antibodies obtained from Santa Cruz Biotechnology, the dilution factor was 1:500, and for those from other sources, it was 1:1000. MitoSOX Red Mitochondrial Superoxide Indicator and MitoProbe JC-1 Assay Kit (Green-fluorescent JC-1) were purchased from Thermo Fisher Scientific.

2.2 | Cell culture and cell viability assay

The H9c2 cardiomyoblast cell line was obtained from the American Type Culture Center (ATCC). The standard protocol of the media data sheet was followed in terms of cell culture conditions and reagents used. The cells were cultured in low glucose Dulbecco's modified Eagle medium (DMEM; Gibco) low glucose with 3.5 g/L additional glucose, 10% fetal bovine serum (FBS), and penicillin and streptomycin (100 units/mL, Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. For experimental purposes, we also used serum-free media. Cell washing was performed using phosphate-buffered saline (PBS) and cell harvesting using trypsin buffer. Every 2–3 days, the cells were passaged according to the cells' health condition.

We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent for cell viability analysis according to the manufacturer's instructions.[17] Briefly, H9c2 cells were cultured in 24-well plates. When the cells became 70%–80% confluent, they were treated with the desired drug for 24 h, after which the drug-containing medium was discarded, and 0.5% MTT reagent in PBS was added to each well. The cells were incubated for 2 h, and then dimethyl sulfoxide (DMSO) was added. The color developed was analyzed using an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm. Relative cell viability was estimated as follows: Absorbance at 570 nm of treated cells/absorbance at 570 nm of untreated cells. Three individual experiments (three samples per experiment) were conducted for statistical analysis.

H9c2 cells were treated with DOX, and the effect of AA on DOX-induced cell death was analyzed by observing cell morphological alterations under a light microscope at different magnifications.

2.3 | Immunofluorescence staining

For immunofluorescence staining, we used a chamber slide in which the cells were grown at approximately 60% confluence. First, the cells were fixed with paraformaldehyde for 30 min at room temperature and were washed thrice with PBS. The slides were immersed in 0.1% Triton X in PBS for 2 min (cooled with an ice pack) for cell permeabilization, followed by washing thrice with PBS. Then, we blocked the cells with 2% bovine serum albumin (BSA) for 1 h at room temperature and washed them thrice with PBS. The cells were then incubated with the target antibody overnight at 4°C under constant shaking conditions. After incubation, the cells were washed thrice with PBS and incubated with the desired secondary antibodies, such as dye-conjugated Alexa Fluor 594- and Alexa Fluor 488-conjugated secondary antibodies (1:100 dilution; Thermo Fisher Scientific). Finally, after washing thrice with PBS, 4′,6-diamidino-2-phenylindole (DAPI) staining was performed for 10 min. The slides were mounted using

a mounting solution, and a coverslip was placed on the slide. Fluorescence images of the cells were obtained using the Axio Observer A1 digital fluorescence microscope (Olympus). Three independent experiments were conducted, with one result per experiment being used for analysis.

2.4 | ROS and actin staining

The steps in Section 2.3 were performed until the sample blocking step. After blocking, the samples were incubated with 5 μM Mitosox Red mitochondrial superoxide indicator and rhodamine phalloidin for 30 min at room temperature, followed by washing with PBS. The samples were incubated with DAPI for 10 min and washed with PBS. The slides were mounted using a mounting solution, and the coverslip was placed on the slide. Red staining indicates ROS and actin, while blue indicates the DAPI-stained nucleus. The JC-1 kit was used after the actin staining protocol.

For the deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, we followed the method in Section 2.3 until the fixation step and then added the TUNEL reagent. After that, we observed the samples under a fluorescence microscope; green indicates TUNEL, and blue indicates DAPI.

2.5 | Flow cytometry

Cells were cultured in a 10-cm petri dish plate. After they reached 60% confluence, they were treated with DOX. Then trypsin was used to collect the cells from the plates. The cells were suspended in the buffer, and Annexin V conjugated to FITC was added according to the manufacturer's instructions. We transferred the cells into a sterile strainer tube. Apoptotic cells were detected with a FACSCalibur or FACSVerse flow cytometer (BD Bioscience) using CellQuest or FACSuite software (Becton-Dickinson). Data were collected through different channels.

2.6 | Western blotting assay

The Western blotting experiment was performed following the method described by Cheng et al.[18–20] Cells were cultured in a 10-cm petri dish plate or six-well plates. After treatment with DOX, we washed the cells with PBS, followed by trypsin treatment to harvest the cells. The cells were resuspended in RIPA buffer containing sodium chloride (5 M), Tris-HCl (1 M, pH 8.0), N-P-40, sodium deoxycholate (10%), and sodium dodecyl sulfate (SDS, 10%). Then, 30 μg protein from the cell lysate was resolved via 10%–12% SDS-polyacrylamide gel electrophoresis. After that the gel containing protein was transferred to a polyvinylidene difluoride membrane, subjected to blocking, and incubated overnight at 4°C with primary antibodies under shaking conditions. After 16 h, we added the secondary antibody and incubated the blot under shaking conditions at room temperature. The blot was washed three times with a 10-min interval between the washing step. The protein was detected via chemiluminescence, and the band intensities were quantified using the ImageJ software.

2.7 | Statistical analysis

Statistically significant values were determined by the *t*-test using GraphPad Prism 8, and significance was set at $p < 0.05$. Each experiment was conducted three times. ImageJ software was used for Western blot analysis and immunofluorescence intensity measurement.

3 | RESULTS

3.1 | The protective effect of AA in DOX-treated H9c2 cells and improve cardiomyoblasts

The viability of DOX-treated H9c2 cardiomyoblasts was determined at different doses of DOX (0.5, 1.0, 1.5, and 2.0 μM) compared with that of untreated cells using the MTT assay (Figure 1A). DOX decreased the cell viability in a dose-dependent manner. Treatment with 1.0 μM DOX caused a significant reduction in viability by nearly 25% and almost 50% with 2.0 μM DOX. Western blotting analysis was performed to detect DOX-induced changes to the expression of multiple protein markers such as mitochondrial calcium uniporter (MCU), calcineurin, COX-IV, mitochondrial protein drp1, p-Drp1 ser 616, and p-Drp1 ser 637 (Figure 1B). The protein expression levels of MCU1 and calcineurin were increased with increasing DOX concentration; in contrast, COX-IV protein expression levels were decreased marginally. First, a dose–response curve was plotted to verify the toxicity level of water-extracted AA in H9c2 cells (Figure 1C). The cells were treated with different concentrations of AA (5, 10, 20, 40, 80, 100, and 120 μg) for 24 h, and AA produced no toxic side effects. The cells were then treated with AA at the same concentration range in the presence of 1.0 μM DOX, and a cell viability assay was performed (Figure 1D). AA at 20–80 μg induced a 20%–25% increase in cell viability after DOX damage. Interestingly, cell viability was improved significantly by subjecting the cells to different concentrations of AA post treatment with DOX. However, high concentrations of AA (100 and 120 μg)

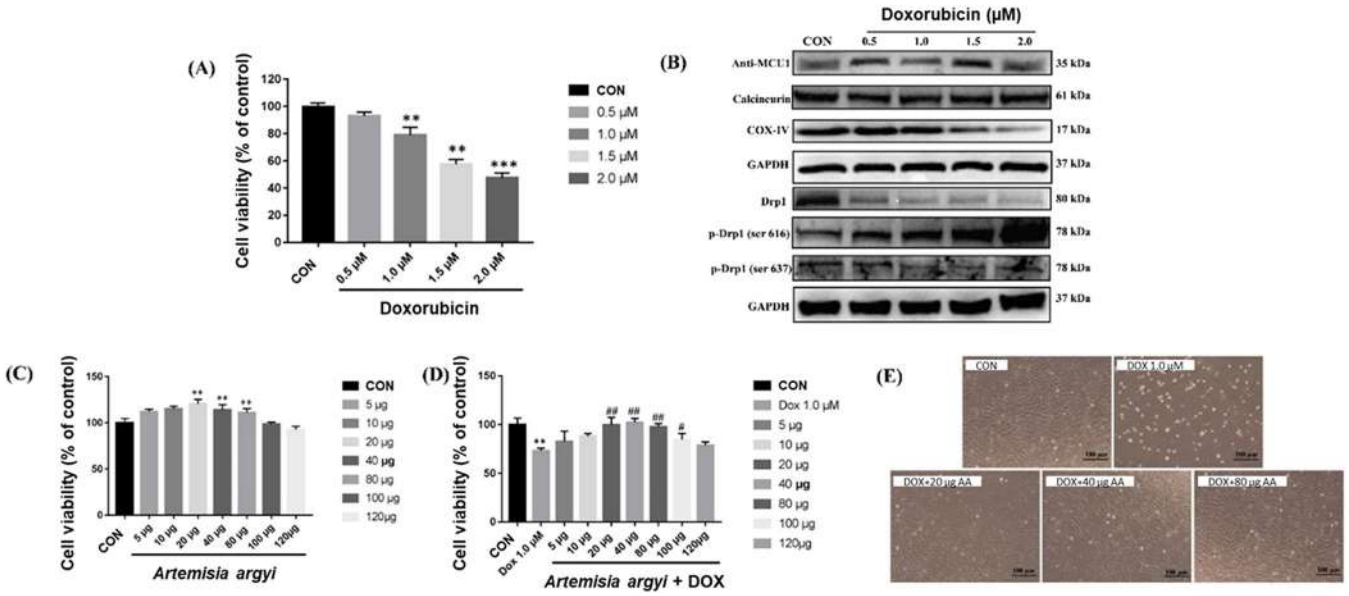

FIGURE 1 *Artemisia argyi* (AA) attenuated DOX-induced cytotoxicity in H9c2 cardiomyoblast cells as determined by MTT assay. (A) Effect of DOX on the viability of H9c2 cells observed by MTT assay. H9c2 cells were treated with dose-dependent increasing concentration of DOX (from 0.5 to 2.0 μM) for 6 h. Cell viability was determined as follows: A570 nm of treated cells/A570 nm of untreated cells ×100. (B) Western blot analysis of different markers with different concentrations of DOX treatment. (C) Effect of AA on the viability of H9c2 cells determined by MTT assay concentrations 5–120 μg. (D) DOX challenge with AA. H9c2 cells were cultured in serum-free Dulbecco's modified Eagle medium (DMEM) media and DOX treated 6 h and then AA treatment with 18 h, respectively. (E) Morphological analysis of H9c2 cells after treatment captured by light microscopy.

showed no significant effect on the viability of DOX-treated cells. The subsequent experiment revealed that a low concentration of the water extract of AA is more beneficial against DOX-induced toxicity than a high concentration of AA (100 and 120 μg). Phase-contrast microscopy was used to analyze the cell morphology (Figure 1E). DOX-treated cells were observed to shrink, whereas DOX-treated cells subjected to AA regained their original morphology in a dose-dependent manner.

3.2 | AA attenuates DOX-induced mitochondrial ROS overproduction and imbalance of mitochondrial fission and fusion

The intracellular ROS production was determined via an immunofluorescence assay using Mitosox red staining. The results of the assay indicated that DOX treatment markedly increased ROS production compared with untreated H9c2 cells (Figure 2A), as red cells around the nucleus were observed in the former. Treatment with the water extract of AA at 20, 40, and 80 μg for 18 h after treatment with DOX significantly decreased the production of ROS levels. ROS production increased twice with DOX treatment compared to control and was reduced with AA treatment (Figure 2B). Moreover, Drp1 staining data demonstrated that in DOX-treated H9c2 cells, Drp1 was localized in the cytoplasm, but AA treatment at different doses could recover Drp1 localization to that of the control cell (Figure 2C). According to Western blotting analysis, DOX-induced changes to MFN1 and MFN2 protein expression levels were irreversible. Furthermore, Ser 616 p-Drp1 expression was increased due to DOX, whereas ser 637 p-Drp1 expression decreased significantly. In both cases, AA treatment reversed the changes in a dose-dependent manner (Figure 2D). Moreover, statistical data analysis also supports these interpretations (Figure 2E). Matrix metalloproteinases (MMPs) play a role in cardiac repair and remodeling. As expected, DOX increased the expression of both MMP2 and MMP9, whereas the water extract of AA significantly decreased the MMP2 and MMP9 levels in a dose-dependent manner (Figure 2F). An approximately two-fold increase during the DOX treatment and a similar level of reduction with AA treatment were observed (Figure 2G).

3.3 | Effect of AA on mitochondrial integrity and membrane potential changes induced by DOX

Mitochondrial dysfunction is indicated by reduced mitochondrial membrane potential (MMP). Our study used a

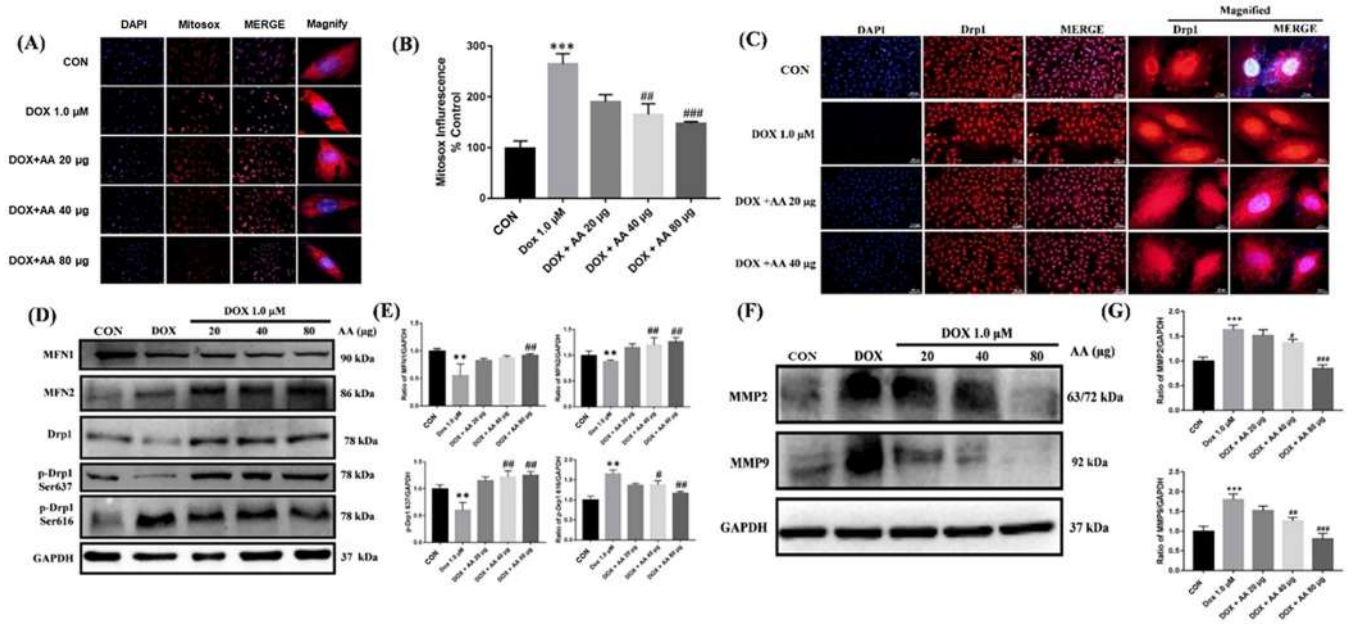

FIGURE 2 *Artemisia argyi* (AA) attenuated the imbalance of mitochondrial function in H9c2 cardiomyoblast cells induced by DOX. (A) Mitochondrial ROS determined by immunofluorescence assay using Mitosox. Mitochondrial ROS was induced in H9c2 cells after incubation with 1.0 μM DOX for 6 h, at which time mitochondrial ROS (red color) around the nucleus increased marginally were observed instead. Water extracted AA completely reduced or prevented the ROS at different concentrations of AA. (B) Statistical analysis showed the Mitosox intensity. DOX-induced mitochondrial florescence increased almost 280% significantly, where dose-dependent AA reduced marginally. (C) Drp1 mediated DOX-induced H9c2 cells staining. Mitochondrial localization of Drp1 was assessed under florescence microscopy. Nuclei were stained with 6-diamidino-2-phenylindole (DAPI) (blue). Differential interference contrast (DIC) images were observed such as 20× and 40× magnification. (D) Western blot analysis of mitochondrial-dependent pathways. Mitochondrial fission and fusion MFN1 and MFN2, mitochondrial dynamics-related protein Drp1 and phosphorylation group p-Drp1 ser 616 and ser 637 protein expression were determined by Western blot analysis. (E) Statistic analysis of Western blot mean value ± SD. (F) Western blot analysis of MMP-2 (matrix metalloproteinase-2), MMP-9 (matrix metalloproteinase-9) compared with GAPDH as control. (G) Statistical analysis of Western blot.

10 μM JC-1 staining solution to measure the MMP in H9c2 cells. We found that DOX induced a reduction in MMP (disappearance of red color), which was partially recovered after treatment with the water extract of AA, with significant inhibition of DOX action (Figure 3A). Analysis of MMP was performed using the ImageJ software; a minimum of 30 cells were analyzed for each group (Figure 3B). We also observed the structural alteration of mitochondria. In the untreated H9c2 cells, mitochondria exhibited a tubular structure, as evidenced by JC-1 staining. However, the DOX treatment group showed small and fragmented mitochondria, which were recovered to some extent with AA treatment (Figure 3B). According to Western blotting analysis, the DOX treatment group exhibited a high expression of calcineurin, MCU1, and COX-IV, which was significantly reduced with AA treatment (Figure 3C,D). The ratio of this three-protein expression level also quantifies and reveals the same significant value. Further, in DOX-treated H9c2 cells, ser 637 p-Drp1 was present in the cytoplasm and the nucleus, whereas ser 616 p-Drp1 was absent in the nucleus (Figure 3E,F). In contrast, the water extract of AA at different doses significantly recovered these proteins to that of the control cells.

3.4 | Effect of water extract of AA on DOX-induced reduction in cell survival

IGF-IIR is involved in regulating the survival, proliferation, differentiation, and metabolism of cardiac cells. Our previous study revealed that Akt/Cas-3/Bcl family is an essential pathway for the survival of cardiac cells.[21,22] Consequently, our results showed the role of the IGF-IIR-dependent Akt pathway in exhibiting the protective effect of AA water extract against the cardiac toxicity induced by DOX. The DOX treatment group exhibited a significant increase in the IGF-IIR and PARP levels, which was blocked by AA (Figure 4A). In contrast, p-Akt expression levels were decreased in the DOX treatment group. The p-Akt and Akt ratio decreased by approximately one-third (Figure 4B). These results indicate that the water extract of AA has an anti-apoptotic effect through the regulation

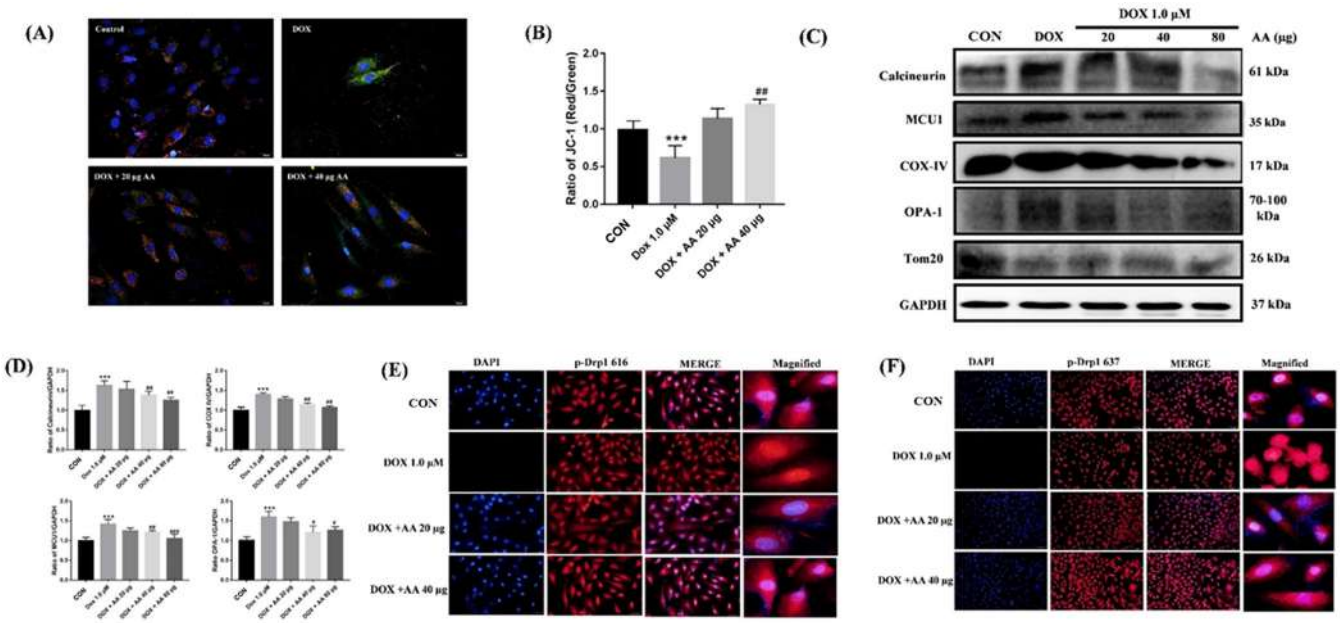

FIGURE 3 Effect of *Artemisia argyi* (AA) on DOX-induced mitochondrial membrane potential and mitochondrial fission and fusion in H9c2 cardiomyoblast cells. (A) JC-1 staining to check the mitochondrial membrane potential. Green florescence shows the JC-1 monomer, which normally moves into the cytosol following the mitochondrial membrane depolarization. Usually, red fluorescence indicates the JC-1 aggregation, which is accumulated in the inner membrane of mitochondria. (B) Quantitive analysis of JC-1 mitochondrial membrane potential. (C) Western blot analysis of calcineurin, MCU1, and COX-IV. (D) Western blot quantification done by ImageJ software. Every blot has three different experiments and quantifications done. (E and F) Immunofluorescence staining mitochondrial fission and fusion protein p-Drp1 ser 616 and p-Drp1 637, respectively.

of apoptosis-related protein. Several studies revealed that extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) are associated with promoting ROS-mediated cell death. Based on our data, DOX treatment induced irreversible effects on p-JNK and both ratios, and AA treatment significantly decreased these levels (Figure 4C). Statistical data analysis showed that both ratios were irreversibly affected by DOX and AA treatments (Figure 4D). Furthermore, the expression of apoptosis-related BCL-2 family and Cas-3 were downregulated due to DOX-induced apoptosis, whereas Bax and p53 expressions were increased (Figure 4E). In contrast, the water extract of AA prevented apoptosis and promoted cell survival (Figure 4E).

3.5 | AA protects H9c2 cells from apoptosis induced by DOX

DOX-induced apoptosis in cardiomyoblasts may be exacerbated by oxidative stress and uncontrolled inflammation. Double staining for FITC-annexin V and cellular DNA using propidium iodide (PI) was used for flow cytometry analysis. In our study, the water extract of AA reduced the apoptosis rate (early and late apoptosis) compared to the DOX-treated group at different doses (Figure 5A). Furthermore, the TUNEL assay determined that more TUNEL-positive cells were present in the DOX treatment group than in the untreated group (Figure 5B). However, after treatment with AA, the TUNEL-positive nuclei were significantly decreased by approximately 50% of that of the DOX treatment group.

3.6 | Effect of water extract of AA on GATA4-mediated pathways induced by DOX

Overexpression of ROS and increased oxidation stress can directly cause cardiac hypertrophy. GATA4 is a hypertrophy signaling factor overexpressed in the heart. NFATC3/GATA4 gene activation results in cardiac hypertrophy or heart failure. To verify our hypothesis, we used F-actin to analyze the actin filament and changes to the surface area of H9c2 cells, and we found that DOX-treated cardiac cells were enlarged compared to the control group (Figure 6A). In contrast, the water extract of AA reduced the cell size in a dose-dependent manner. Cell size quantification using the ImageJ software revealed that the cells in the DOX treatment group were larger by two-fold compared to the control group. Several studies have shown that the endocrine heart produces ANP/BNP (B-type

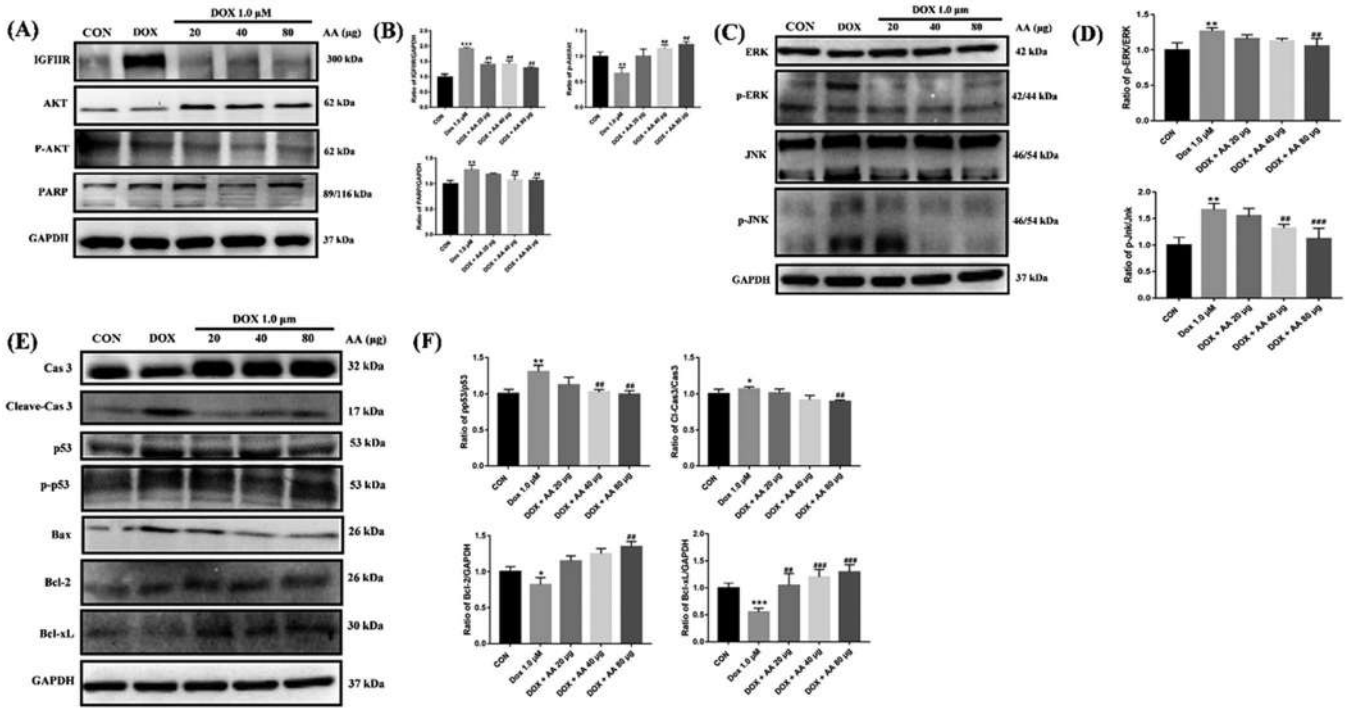

FIGURE 4 *Artemisia argyi* (AA) enhances the cell survival mechanism. AA protects cardiomyoblasts from DOX-induced cell death. H9c2 cells were grown in serum-free Dulbecco's modified Eagle medium (DMEM) media for 6 h, followed by DOX treatment with 6 h, and then 18 h AA different dose treatments, respectively. (A) AA decrease the expression of IGF-IIR and PARP survival pathways in H9c2 cells. The expression of upregulating gene AKT, p-Akt, IGF-IIR, PARP was analyzed by Western blotting. GAPDH indicated the internal control or loading control. (B) Quantitively analysis of Western blot represents three independent experiments and statistical significance. (C) Representative Western blot showing the MAPK signaling proteins (ERK and JNK) in H9c2 cells. The protein level of ERK, p-ERK, JNK, p-JNK was detected by Western blot analysis. The protein level was normalized by GAPDH as internal control. (D) Qualitative analysis of Western blot. *Significant difference compared to control ($p < 0.05$), #significance difference compared to the DOX ($p < 0.05$). (E) The protein expression level of Cas-3, Cleaved Cas-3, p53, pp53, Bax, Bcl-2 family were measured by Western blot experiment and normalized to GAPDH as internal control. The expression level is indicated to those of control. (F) Quantitive analysis of Western blot. *Significant difference compared to control ($p < 0.05$), #significance difference compared to the DOX ($p < 0.05$).

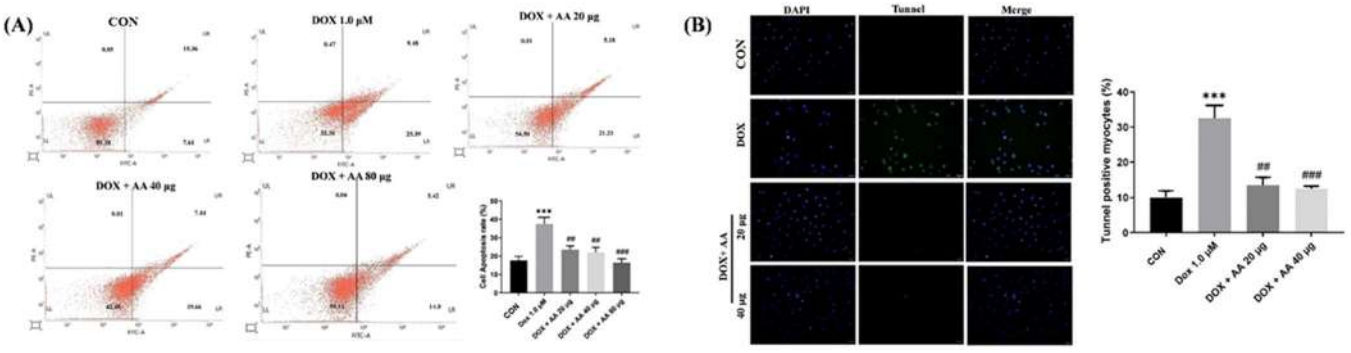

FIGURE 5 *Artemisia argyi* (AA) inhibits DOX-induced apoptosis in H9c2 cardiomyoblast cells using flow cytometry and TUNEL assay. (A) The apoptosis rate was detected by flow cytometry assay. Flow cytometric analysis of annexin V-FITC/PI-stained H9c2 cells. Viable cells are annexin V-FITC− and PI− early apoptotic cells are annexin V-FITC+ and PI−, and late apoptotic cells are annexin V-FITC+ and PI+. The quantitative results are represented as the percentage of annexin V-FITC+/PI+ cells among total cells. *Significant difference compared to control ($p < 0.05$), #significance difference compared to the DOX ($p < 0.05$). (B) TUNEL assay containing terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP for 1 h at room temperature. The apoptosis-positive cells were counted in six randomly selected fields from each slide. The results were recorded as the ratio of TUNEL-positive nuclei and analyzed via fluorescence microscopy. *Significant difference compared to control ($p < 0.05$), #significance difference compared to DOX ($p < 0.05$).

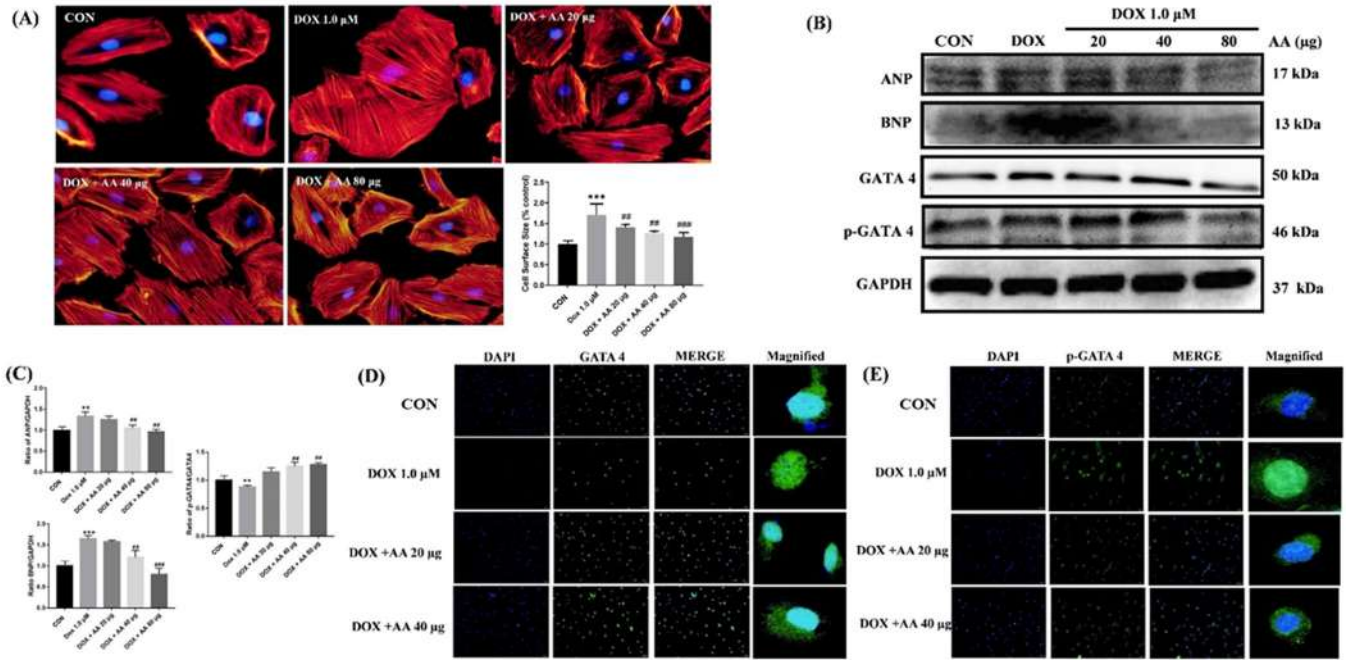

FIGURE 6 Effect of *Artemisia argyi* (AA) on DOX-induced cardiac hypertrophy in H9c2 cardiomyoblast cells. (A) Cells were stained with phalloidin rhodamine for 30 min to visualize F-actin staining and counterstained used DAPI. The cell surface size was determined by using ImageJ software. (B) Western blot analysis. The level of protein expression of ANP, BNP, GATA4, p-GATA4 determined by Western blot analysis. GAPDH was used as loading control. (C) Quantitive analysis of Western blot. *Significant difference compared to control ($p < 0.05$), #significance difference compared to DOX ($p < 0.05$). (D and E) Transcription factor GATA4 and p-GATA4, respectively, staining in H9c2 cells. The images are taken individually in three different fields of each slide or dose of treatment.

natriuretic peptide), cardiac hormones that play a vital role in cardiac diseases. GATA4 and ANP can increase heart failure because compensatory homeostatic response leads to myocardial overproduction. Consequently, we found that the water extract of AA could be inhibited by GATA4 and ANP/BNP in a dose-dependent manner (Figure 6B). The ratio of GATA4/p-GATA4 was also decreased in the DOX treatment group compared to the control group (Figure 6C). Furthermore, p-GATA4 staining was observed from the cytoplasm to the nucleus in the DOX treatment group compared to the control group; GATA4 also revealed the same result but was stronger (Figure 6D,E). However, the water extract of AA recovered GATA4 expression in a dose-dependent manner. Thus, the current study result showed that the water extract of AA has a protective effect against cardiac diseases, especially cardiac hypertrophy induced by DOX.

4 | DISCUSSION

DOX chemotherapy is a major reason behind the development of cardiac myopathy in the human body.[23,24] DOX is one of the major drugs used in a spectrum of cancers, including solid tumors and leukemia. Most importantly, among all the anthracycline classes, DOX usage is minimal because of cardiotoxicity, and if the dosage increases more than 300 mg/m^2, it can cause heart failure.[25] Many reports also revealed that even a lower dose of therapy had a risk of heart failure.[26] Therefore, the need of the hour is to search for suitable adjuvant therapy that protects the heart without interfering with tumor suppression. Cancer patients could be benefited from adjusting anthracycline dosage and preventing cardiomyopathy with the help of such drugs.[27,28] Researchers are yet to interpret the primary mechanism of the anthracycline drug DOX in inducing cardiac toxicity.

A wide range of apoptosis-causing effects like ATP consumption, damage to the DNA, excessive production of free oxygen species, contractile protein degradation, faulty regulation of transcription protein, calcium misutilization, and lipid peroxidation are directly related to anthracycline treatment.[7] Some of these processes have been targeted, but the effect is very low or nonexistent. For instance, DOX induces cardiac toxicity and overproduction of ROS; some clinical trials proved that common drug antioxidants such as *N*-acetylcysteine and α-tocopherol have no role in cardiac patients.[29,30] It is also proved that ROS may not be inciting factor of the chemotherapy drug DOX to cause cardiac toxicity.

Therefore, we need an urgent drug to reduce the side effect of DOX. AA is a famous plant belonging to the genus

Artemisia, and has lots of clinical benefits due to its anti-inflammatory, antioxidant, and anti-bacterial[31–33] effects. Previously, some studies showed that AA affects ulcer diseases, cancer diseases, and inflammatory diseases,[32,34,35] but there are no reports that tell us about the effect on cardiac diseases. To the best of our knowledge, we are the first research group to show that the water extract of AA has a vital role in alleviating DOX-induced cardiac failure. In the present study, we first checked the viability and morphology of DOX-treated H9c2 cells. Our data showed that AA at 20–80 μg acted against 1.0 μM DOX inducer, and, most importantly, the water extract could increase the viability of the damaged cells by 22%. Mitochondrial ROS overproduction is a major threat that leads to cardiac dysfunction or cardiomyopathy induced by DOX.[36–38] Growing evidence indicates that cardiac apoptosis happens through mitochondrial-dependent pathways,[39] and it leads to the high production of ROS. Furthermore, MFN1/MFN2 works as irreversible mitochondrial fission and fusion of DOX-treated cardiomyocytes.[40] Some groups showed that the absence of MNF2 results in mitochondrial fragmentation and damage to the mitochondria. It has been demonstrated that a wide range of post-translocation modifications, such as SUMOylation, phosphorylation, and ubiquitination, were regulated by mitochondrial protein Drp1.[41,42] Regarding the phosphorylation of drp1, some previous studies showed that the reduced phosphorylation of Drp1 637 and increased phosphorylation of Drp1 616 could be accelerated by the translocation of Drp1 from the cytosol to mitochondria and its interaction with mitochondria undergoing fission. Therefore, we focused on this issue and resolved the problem with the water extract of AA in DOX-treated cardiac cells. Our study revealed that the overproduction of ROS could be decreased with AA treatment, and Drp1 translocation occurring upon DOX treatment could be reversed with AA treatment. Our western blot data demonstrated that DOX induced apoptosis via mitochondria-dependent pathways, which was inhibited by the water extract of AA by alleviating mitochondrial dysfunction in terms of both fission and fusion proteins. It has been reported that overexpression of MMP-2 and MMP-9 can cause myocardial infarction.[43,44] They found a very small amount of collagen accumulation in infracted place in an animal model. The role of MMP2 in cardiac disease is still controversial because some groups found that MMP2 overexpression can lead to myofilament lysis through proteolyzing cardiac titin,[45] and others demonstrated that blocking MMP2 leads to cardiac fibrosis in diabetic cardiomyopathy.[46] The present study demonstrates that DOX-induced overexpression of MMP2 and MMP9 was reversed by the water extract of AA, thereby balancing cardiac function, and ameliorating myocardial remodeling.

It has been reported that calcineurin-mediated signaling pathways could play a vital role in the activity of the mtDNA mutation, reduction of the electron transport chain system, and most importantly, disturbance of MMP.[47–50] Notably, mitochondria play a crucial role in Ca^{2+} homeostasis by transiently removing the Ca^{2+} released from cell organelles and helping regulate Ca^{2+} levels in the cytosol.[51,52] There are two basic reasons for mitochondrial dysfunction: loss of membrane potential and the loss of Ca^{2+} uptake system.[53] From our study, we found that MMP was lost due to treatment with DOX, but AA at different doses could help recover MMP. Moreover, calcineurin and mitochondrial uniporter protein expression were increased significantly in the DOX treatment group. At the same time, water extract of AA could reduce the expression level.

IGF-IIR is translocated to the plasma membrane and contributes to cardiac hypertrophy and apoptosis.[54–56] Most importantly, it may play a role in fetal growth and heart development. A previous study proved that ANG-II might activate IGF-IIR and cardiomyocyte apoptosis.[57,58] In this study, we demonstrated that ERK/JNK possibly activates the IGF-IIR signaling pathway induced by DOX because of translocation into the plasma membrane, leading to apoptosis. Nevertheless, AA at different doses could inhibit this pathway, proving that this Chinese herbal medicine could affect apoptosis-related pathways. Moreover, it also blocks the expression of caspase-3 cleavage and PARP-mediated cardiac apoptosis. The Bcl-2 family normally works as an anti-apoptotic protein, where it can inhibit the release of cytochrome C from mitochondria and plays a major role in protecting heart cells from cell death and oxidative stress.[22,59] Our study showed that the down-regulation of cl-2 family and Bcl-xL induced by DOX could be suppressed by the water extract of AA in H9c2 cells. Taken together, our data revealed that the water extract of AA can play a major role in DOX-induced cardiac toxicity. Furthermore, our flow cytometry data revealed that late and early apoptosis also decreased marginally by using AA, and TUNEL-positive cell numbers were reduced upon treatment with AA.

In human heart function, there are two types of natriuretic peptides, ANP and BNP, which typically contribute to the endocrine regulation of blood pressure.[60] The common receptor, guanylyl cyclase A (GC-A), can be activated and work in kidneys and vasculature.[61] It is well known that different types of pathological issues, such as adrenergic overactivation, myocardial infarction, and hypertension, may result in a hypertrophic response in the human body. These symptoms involve cell-size increase and protein synthesis; two major proteins can be stimulated, BNP and ANP, by this cardiac gene.[62] In the human heart, NFACT3 is normally attached to

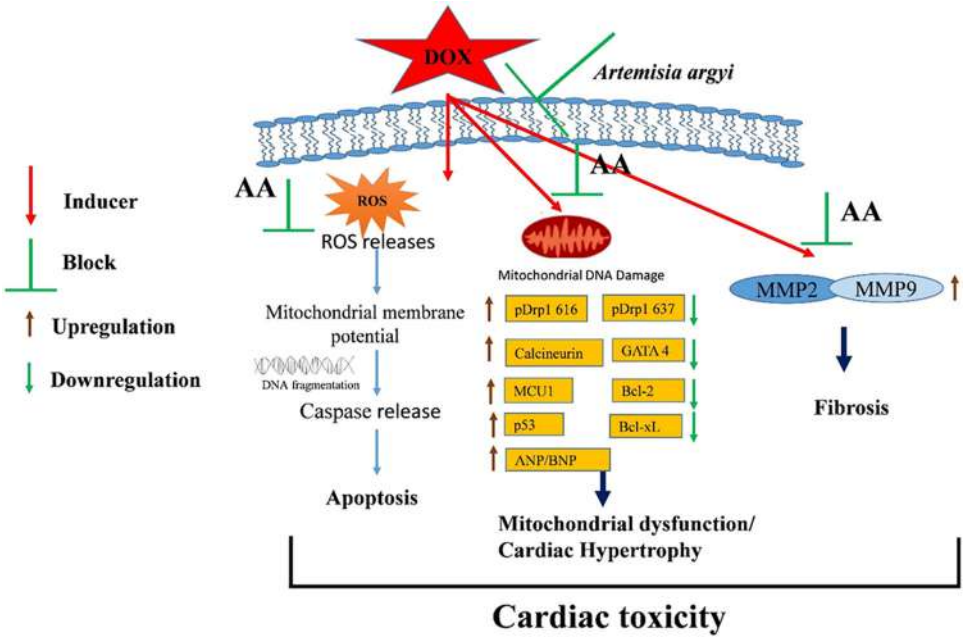

FIGURE 7 Schematic representation showing DOX-induced cardiac damage involving mitochondrial pathways.

transcription factor GATA4 and activates the cardiac hypertrophy gene.[63] Some research groups mentioned that GATA4 is required for controlling cardiomyocyte hypertrophy and its survival.[64] Our study revealed that DOX plays a vital role in inducing cardiac hypertrophy response, and increasing the expression of cardiac hypertrophy gene ANP and BNP. It is imperative to note that water extract AA promoted a protective effect against hypertrophy induced by DOX and a significant reduction in the level of a hypertrophic gene. In addition, our immune fluorescence staining data give us evidence of DOX-induced transcript cytosol of the nucleus where water extract AA is considerable to work as a control cell. Taken together, water extract AA exhibits important effects of cardiac hypertrophy.

In summary, our finding highlights the key role of water extract AA in terms of myocardial protection from DOX-induced acute cardiac toxicity by mitochondrial signaling pathways and consequent activation of some cell survival markers. Furthermore, our study gives evidence of the optimum modulation of apoptotic cell death pathways, which favors the salvage of cardiomyoblasts. This study reinforces the hypothesis that water-extracted AA might have an important role against side effect of DOX to assess prognosis and stratify patients by establishing a new target therapy and probably a new therapeutic window in clinical diseases (Figure 7).

5 | CONCLUSION

The intriguing possibility of our study is the involvement of water-extracted AA, which could be used as a novel target therapy against mitochondrial dysfunction induced by the chemotherapy drug DOX and DOX-induced cardiac injury. Our study will help to open a new entry point to investigate the fundamental differences between DOX-induced cardiac cells and their protective effect. Further, the animal experiment will focus to elucidate the role of AA against DOX chemotherapeutic and toxicity mechanism in more detail. In addition, in vivo experiment will give us very important information on whether our water-extract AA cardioprotective effect can extend to other models of cardiac injury, like other cardiac toxic chemotherapy and ischemia-reperfusion. Thus, it can be concluded that plant extract could have an important role to improve cardiac therapy.

AUTHOR CONTRIBUTIONS

Chien-Hao Wang, Chih-Yang Huang, and **Wei-Wen Kuo**: conceived and designed the study. **Md. Nazmul Islam** and **Marthandam Asokan Shibu**: performed experiments. **Chia-Hua Kuo** and **Dennis Jine-Yuan Hsieh**: collected samples. **Pi-Yu Lin** and **Shinn-Zong Lin**: performed the statistical data analysis. **Md. Nazmul Islam**: wrote the manuscript. **Samiraj Ramesh**: wrote the manuscript; professionally edited and thoroughly revised the manuscript. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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