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RESEARCH ARTICLE

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A combination of isoliquiritigenin with *Artemisia argyi* and *Ohwia caudata* water extracts attenuates oxidative stress, inflammation, and apoptosis by modulating Nrf2/Ho-1 signaling pathways in SD rats with doxorubicin-induced acute cardiotoxicity

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Abstract

Ohwia caudata (Thunb.) H. Ohashi (Leguminosae) also called as "Evergreen shrub" and *Artemisia argyi* H.Lév. and Vaniot (Compositae) also named as "Chinese mugwort" those two-leaf extracts frequently used as herbal medicine, especially in south east Asia and eastern Asia. Anthracyclines such as doxorubicin (DOX) are commonly used as effective chemotherapeutic drugs in anticancer therapy around the world. However, chemotherapy-induced cardiotoxicity, dilated cardiomyopathy, and congestive heart failure are seen in patients who receive DOX therapy, with the mechanisms underlying DOX-induced cardiac toxicity remaining unclear. Mitochondrial

Abbreviations: AA, Artemisia argyi; OC, *Ohwia caudata*; DOX, doxorubicin; ISL, isoliquiritigenin; CVD, cardiovascular diseases; FDA, Food and Drug Administration; IL, interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; DRP1, dynamin-related protein 1; PI3K, phosphoinositide 3-kinases; ROS, reactive oxygen species.

Michael Yu-Chih Chen and Chih-Yang Huang contributed equally to this study.

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dysfunction, oxidative stress, inflammatory response, and cardiomyocytes have been shown to play crucial roles in DOX-induced cardiotoxicity. Isoliquiritigenin (ISL, 10 mg/kg) is a bioactive flavonoid compound with protective effects against inflammation, neurodegeneration, cancer, and diabetes. Here, in this study, our aim is to find out the *Artemisia argyi* (AA) and *Ohwia caudata* (OC) leaf extract combination with Isoliquiritigenin in potentiating and complementing effect against chemo drug side effect to ameliorate cardiac damage and improve the cardiac function. In this study, we showed that a combination of low (AA 300 mg/kg; OC 100 mg/kg) and high-dose(AA 600 mg/kg; OC 300 mg/kg) AA and OC water extract with ISL activated the cell survival-related AKT/PI3K signaling pathway in DOX-treated cardiac tissue leading to the upregulation of the antioxidant markers SOD, HO-1, and Keap-1 and regulated mitochondrial dysfunction through the Nrf2 signaling pathway. Moreover, the water extract of AA and OC with ISL inhibited the inflammatory response genes *IL-6* and *IL-1β*, possibly through the NFκB/AKT/PI3K/p38α/NRLP3 signaling pathways. The water extract of AA and OC with ISL could be a potential herbal drug treatment for cardiac hypertrophy, inflammatory disease, and apoptosis, which can lead to sudden heart failure.

KEYWORDS
antioxidant, apoptosis, *Artemisia argyi*, doxorubicin, ISL, mitochondrial dysfunction, *Ohwia caudata*

1 | INTRODUCTION

Traditional Chinese drugs have become increasingly popular worldwide due to their efficacy in treating several different diseases has been demonstrated, Chinese traditional drug and herbal therapies have become increasingly popular in the western countries among the world recently.[1] Isoliquiritigenin (ISL; 2′,4,4′-trihydroxy chalcone)[2] is a most common herbal medicine that has been widely used in clinical applications.[3] Actually, ISL is a major component of *Glycyrrhiza uralensis* (*G. uralensis*), which is a flavonoid with a chalcone structure (2′,4,4′-trihydroxy chalcone) and plays a vital role in multiple chronic diseases[4,5] including inhibitory effect of cancer cell,[6] anti-angiogenic effect,[7] anti-oxidant effect,[8] and anti-inflammatory effect.[9]

Ohwia caudata (OC) and *Artemisia argyi* (AA) are shrubs endogenous to Asian subcontinental countries, including China, Japan, Taiwan, Hong Kong, Korea, Russia, India, and some African countries.[10–14] According to previous literature, *Ohwia caudata* (OC) and *Artemisia argyi* (AA) locally called chinse herbal medicines derived from these plants are used to treat several common human diseases, including bacillary dysentery and rheumatic arthritis which is known as folk medicine. *Ohwia caudata* (OC) belongs to the Fabaceae family under the genus *Ohwia* formally *Desmodium* (*Desmodium caudatum*).[10,11,15] *Artemisia argyi* (AA) leaf extract is commonly used as traditional Chinese herbal medicine for several diseases, such as eczema. Moreover, it contains various components, including polysaccharides.[10,16] To the best of our knowledge, there are no reports investigating the effects of combination of OC and AA on inflammation, oxidative stress, mitochondrial dysfunction, and cardiac hypertrophy.

Doxorubicin (DOX) is one of the anticancer drugs in the anthracycline group and is widely used clinically.[17,18] However, patients treated with DOX are frequently reported to have dilated cardiomyopathy and congestive heart failure[19] with the rate of morbidity and mortality increasing over time.[20] According to the FDA, dexrazoxane is the only drug approved for DOX-induced cardiomyopathy; however, it increases secondary malignant neoplasms.[21] A few reports have described the use of angiotensin II receptor blockers, beta-adrenoreceptor blockers, and angiotensin-converting enzyme inhibitors for DOX-induced cardiomyopathy. According to previous study, it has been proved that numerous mechanisms are involved in doxorubicin-induced cardiac toxicity. One of the most common mechanisms is over expression of free radical oxygen species which normally leads to endogenous expression of lipid peroxidation, DNA damage, regulatory cell death, and inflammatory signaling.[22] The side effect of Dox shown a significant alteration of inflammatory mediator cytokine and pro-inflammatory cytokine in myocardium cells.[23] It is well-known mechanism activating pro-apoptotic signaling pathway by activating mitogen-activating protein (MAPK) and nuclear factor kappa-B (NF-κB). The apoptosis showed by doxorubicin via c-Jun N-terminal kinases (JNKs) and MAP kinase signaling pathways also reported previously.[24] Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor protein which can be regulated by Nuclear factor kappa B (NF-κB), and most importantly it is a master

regulator of oxidative stress because it can be augmentation of antioxidant defense mechanism and activation of hemeoxygenase-1 (HO-1).[25] Due to multifactorial nature of DOX-induced cardiotoxicity, naturally several kinds of agents can mitigate inflammation, oxidative stress, and apoptosis which shown to improve endogenous antioxidant as well as inflammatory response by promoting cell signaling pathways and cell survival.[26] Therefore, a longer time window is needed for better performance of pharmacotherapies and to prevent cardiac toxicity.

The present study determined whether an ISL formulation with AA and OC could protect against DOX-induced cardiomyopathy in SD rats in vivo and cells in vitro using multiple experimental approaches.

2 | MATERIALS AND METHODS

2.1 | Preparation of aqueous extract from AA and OC

AA and OC were identified in the eastern Taiwan island, verified by special experts (Lévl et Vant was identified and verified by Dr. Tamilselvi Shanmugam), and deposited to the Department of Chinese Medicine, Tzu Chi Hospital Research Institution, Aerial plant parts (leaves from both plants) were collected and washed rigorously with distilled water to remove outer surface dirt. AA and OC extracts were prepared by grinding the dried leaves to a coarse powder using a blender, then boiling 100 g of leaf powder in 1 L distilled water at 100°C for 30 min. The solution was cooled at room temperature and centrifuged at 2500 rpm at 4°C for 30 min. The supernatant was collected and centrifuged again at 1245×*g* at for 10 min. The clear supernatant was collected and passed through a 0.2-μm Millipore syringe filter and stored at 4°C for prolonged use. Final concentration was obtained using the dry Petri plate method and it was 50 and 65 mg/mL for AA and OC, respectively.

2.2 | Antibodies and reagents

DOX (Catalog number: 44583) was purchased from Sigma Aldrich, St. Louis, MO. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (purchased from Sigma-Aldrich (St. Louis, MO), a probe for echocardiography. Primary antibodies against the following were used: Nrf2, phospho-Nrf2 (Abcam, Cambridge, UK), caspase-3, Cleaved caspase-3, Bak, Bcl-2, β-actin, AKT, phospho-AKT, phospho-ERK1/2, SOD-1, SOD-2 (Santra Cruz, Biotechnology, USA), Keap-1, Ho-1, NLRP-3, IL-6, IL-1β, Drp1, phospho-Drp1 637, GATA-4, phospho-GATA-4, iNOS, Cox-2, T-NFκB 65, phospho-NFκB 65, IκBa, phospho-IκBa, phospho-JNK, JNK, p38, phospho-p38, PI3K, p53, phospho-p53, PARP, caspase-9, Cleaved caspase-9, and cytochrome c (Cyt-c; Cell Signaling Technology (Danvers, MA). Secondary antibodies, including anti-rabbit immunoglobulin G (IgG), anti-goat immunoglobulin G, and anti-mouse immunoglobulin G HRP-conjugated antibodies, were purchased from Santa Cruz Biotechnology, Dallas, TX.

2.3 | Cell culture

H9c2 cardio myoblast cells, derived from rat heart tissue, were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, MA) with low glucose supplemented with 10% fetal bovine serum (FBS, Cosmo Bio, Japan), 1 U/mL penicillin/streptomycin (Thermo Fisher Scientific), 2 mM glutamine (Sigma-Aldrich), 1 mM pyruvate (Sigma-Aldrich), and 100 mg/mL streptomycin (Thermo Fisher Scientific) in a humidified chamber (5% CO_2) at 37°C. The cells were grown to reach 70% confluence before using them for drug treatment or passaging.

2.4 | MTT assay

As previously described,[27,28] briefly the cytotoxic effects of AA and OC on H9c2 cells were tested using the MTT assay (Catalog number: M6494, Thermo Fisher Scientific), and cell viability was assessed after a DOX challenge. The cells (1×10^4 cells/well) were seeded in 96-well plates and treated with increasing concentrations of AA (5–120 μg) and OC (20–600 μg), DOX challenge, and combined AA and OC and incubated for 24 h. The media was removed and replaced with the MTT solution (0.5 mg/mL) and incubated for 2 h at 37°C. The solution was replaced with DMSO, and the cells were shaken for 10 min in the dark. Absorbance was measured at 570 nm using an automated microplate reader (Thermo fisher scientific).

2.5 | Animal experiment design and administration of DOX and AA and OC extracts

All experiments involving animals were performed according to the NIH guide for the use of laboratory animals and care, with approved protocols from the Institutional animal care and the committee of Hualien Tzu Chi Hospital, Taiwan (IACUC approval no: 109-02). Sprague Dawley (SD) male rats were used for animal experiments. Ten-week-old SD rats (weighing 250–300 g) were acclimatized for 2 weeks at the Tzu Chi University, Taiwan core facility before experimental use. The animals were provided by the National Animal Breeding and Research Center (Taipei, Taiwan) for experimental use only. Rats were housed in individual cages under constant temperature (22°C) and a 24 h light–dark cycle with sufficient diet and tap water (Lab diet 5001; PMI Nutrition International, Inc). The animals were divided into four groups ($n = 4$): control group (without any drug treatment), DOX group (30 mg/kg/4 weeks), DOX challenge with a low dose of AA and OC (AA 300 mg/kg; OC 100 mg/kg) along with 10 mg/kg ISL, and DOX challenge with a high dose of AA and OC (AA 600 mg/kg; OC 300 mg/kg) along with 10 mg/kg ISL. DOX was

injected peritoneally, and combined AA and OC with ISL was administered orally.

2.6 | Echocardiography

Echocardiography was used to analyze cardiac function. All images and calculations were evaluated according to the instructions of the American Society of Echocardiography using a 12 MHz linear transducer and 5–8 MHz sector (Vivid 3, General Electric Medical Systems Ultrasound). Isoflurane inhalation was used for anesthesia, measurements were made using M-mode, and two-dimensional images were obtained from six independent different cardiac cycles parasternal long and short axes. The basic cardiac parameters, including left ventricular posterior, interventricular septal thickness, and LVD, were estimated during diastole and systole. Furthermore, ejection fraction (EF) and fractional shortening (FS) were obtained from echocardiography and calculated using the following formula: (% $EF = (LVDd)^3 - (LVDs)^3/(LVDd)^3 \times 100$) for the EF, (%$FS = LVDd - LVDs/LVDd \times 100$) for the FS.

2.7 | Tissue extraction

The left ventricle tissue was extracted and isolated. Tissue samples (0.5 mg) were measured and dissolved in tissue lysis buffer (50 mM Tris–HCL, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) up to 1 mL. Tissues were homogenized for 5 min using sonication and incubated at −20°C overnight, followed by temperature normalization at 4°C and centrifugation at 10 000 rpm for 30 min at 4°C. The supernatant was collected and stored at −80°C for prolonged use.

2.8 | Immunohistochemical staining

Cardiac tissue sections were rehydrated in a graded alcohol series and deparaffinized with xylene as previously described.[29] For intact heart tissue sections use hematoxylin and eosin (H&E). The tissues were embedded in formalin and paraffin. The section was xylene overnight and subsequently dyed with hematoxylin for 5 min. The section is usually washed with distilled water (Millipore) and soaked with 85% EtOH and in the end-graded series of alcohol (100%, 95%, and 75%). For Masson's trichrome stain (ScyTek Laboratories, Inc., Logan, UT) for detection of fibrotic changes and collagen deposition. Tissues were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS), blocked with horse serum for 1 h at room temperature, and washed with PBS. Tissues were incubated with primary antibodies overnight, followed by washing with PBS and incubating with secondary antibodies. Signal was developed using horseradish peroxidase (HRP)-conjugated avidin-biotin complex using the Vectastain Elite ABC Kit and Nova RED chromogen (Vector Laboratories), followed by hematoxylin and eosin staining. Images were acquired using an Olympus BX53 microscope (OLYMPUS Microscope, Tokyo, Japan).

2.9 | Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Sigma-Aldrich, USA) was performed on 3-μm thick cardiac tissue paraffin sections. Sections were deparaffinized, followed by the staining protocol. Samples were incubated with Proteinase K (20 μg/mL) and washed with PBS, followed by incubation with TUNEL reagent (90 min) and DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride, Cat D1306, Invitrogen Life Technologies) for 15 min. TUNEL-positive cardiomyocytes formed a green color. All experiments were performed using individual areas of cardiac tissue slides.

2.10 | Western blot assay

Tissues were extracted using the method described above, and the Lowry method was used for protein quantification. Protein samples were separated using 10%–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to polyvinylidene fluoride (PVDF; Millipore, Bedford, MD) or nitrocellulose membranes. Membranes were blocked for 1 h in skim milk (5–8%) and washed with TBST, followed by incubation with the respective primary antibodies at 4°C overnight with shaking. Membranes were washed three times with TBST (Tris Buffered Saline, with Tween 20) and incubated with secondary antibodies for 1 h, followed by washing with TBST. The signal was visualized using a chemiluminescence (ECL) kit (Millipore) and an imaging system (Fujifilm). The relative density band was measured by Image J software (NIH, Bethesda, MD).

2.11 | Statistical analysis

Data are presented as mean ± standard deviation (SD), and all experiments were performed in triplicate. GraphPad Prism 7 software was used for statistical analysis, and two-way analysis (ANOVA) was performed. Statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Effects on the viability of cardiac H9c2 cells treated with ISL and AA and OC water extract

ISL is an established natural antioxidant that could play a vital role against cardiac injury.[30] Figure 1A shows the chemical structure of ISL. To determine whether ISL can protect cardiomyocytes, we first analyzed the viability of cardiac H9c2 cells at different doses of ISL.

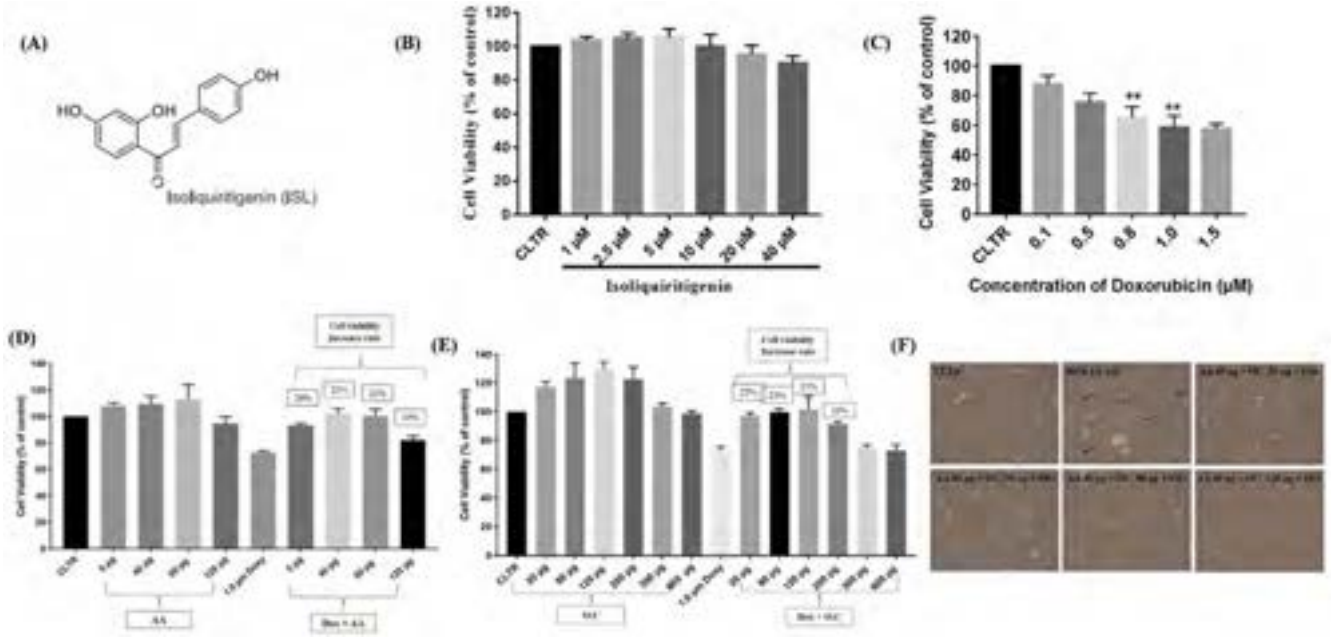

FIGURE 1 Isoliquiritigenin (ISL) with combination of water extract AA and OC attenuated DOX-induced cardiac toxicity in Cardio myoblast cell H9c2. (A) Chemical composition of ISL. (B) Effect of ISL on dose depended manner on the cell viability of H9c2 cardiomyocytes cells using MTT assay. ISL treated concentration (1–40 μM) in H9c2 cells for 24 h. Cell viability was quantified using as follows: (A570 nm of treated sample/A570 of control sample) × 100. (C) DOX treatment in H9c2 cells different doses from 0.1 to 1.5 μM). We also observed the significant value by statistically. All the results are representative of three independent experiments. (D, E) The effect of ISL combination with AA and OC along with DOX on the cell viability of H9c2 cells were examined using MTT assay. The cardiomyocytes cells were culture into serum-free media using 6 h DOX treatment and followed by the 18 h treatment with ISL combination with AA and OC. Different concentrations were used from 5 to 120 μg/mL for AA and from 20 to 600 μg/mL for OC. (F) H9c2 cells morphological analysis after the DOX and ISL combination of AA and OC treatment using Light microscopy. Error bar indicates the damaged cell using chemo drug DOX. CLTR, control; DOX, doxorubicin; ISL, isoliquiritigenin.

No cardiac toxicity was observed in response to up to 20 μM ISL, indicating that ISL can help to protect cardiac cells (Figure 1B). Several different doses of DOX were tested, and we found that 1.0 μM DOX can reduce around 40% of cardiac cells (Figure 1C). However, AA and OC water extract protected against DOX-induced damage in a dose-dependent manner (Figure 1D, E). Moreover, light microscopy analysis also revealed that low- and high-dose AA and OC combination significantly recovered the DOX-damaged H9c2 cells (Figure 1F). Our results give evidence that DOX-induced cardiac toxicity may be recovered by AA and OC in combination with ISL.

3.2 | Synergistic effect of ISL combined with AA and OC water extract against DOX challenge

We assessed whether a combination of ISL and AA and OC water extract rescued DOX-induced cardiac injury in an in vivo model. Before drug treatment, all rats exhibited normal body weight and blood glucose levels. Chorionic cardiac injury was induced in the DOX treatment group subjected to peritoneal DOX injection. After several weeks of DOX treatment, combined ISL with low and high doses of AA and OC were orally administered to SD rats. Echocardiography was performed to evaluate the cardiac function of experimental animals, and we observed that cardiac parameters related to cardiac function were increased compared to the untreated group, while low and high doses of combined ISL and AA and OC water extract had significantly decreased these parameters compared to the DOX group (Figure 2A). Figure 2B shows enlarged cardiac tissue due to hypertrophy in the DOX group compared with the control group, while it was marginally decreased in a dose-dependent manner in the group subjected to the combination of ISL with AA and OC water extract. Moreover, EF and FS were also increased in the DOX group compared to the control group (Figure 2C,D). The body weight was slightly increased in the low- and high-dose combination groups compared to the DOX group (Figure 3A). In addition, heart weight was marginally decreased compared to the DOX group (Figure 3B), and the ratio between heart weight and body weight was also slightly decreased in the low- and high-dose groups compared to the DOX group (Figure 3C). Besides, tibia length was decreased in the DOX group compared to the untreated group, while it was significantly increased in the ISL combination group (Figure 3D). Interestingly, left ventricular heart weight and the ratio of left ventricular weight and tibia length were decreased compared to the control group (Figure 3E,F).

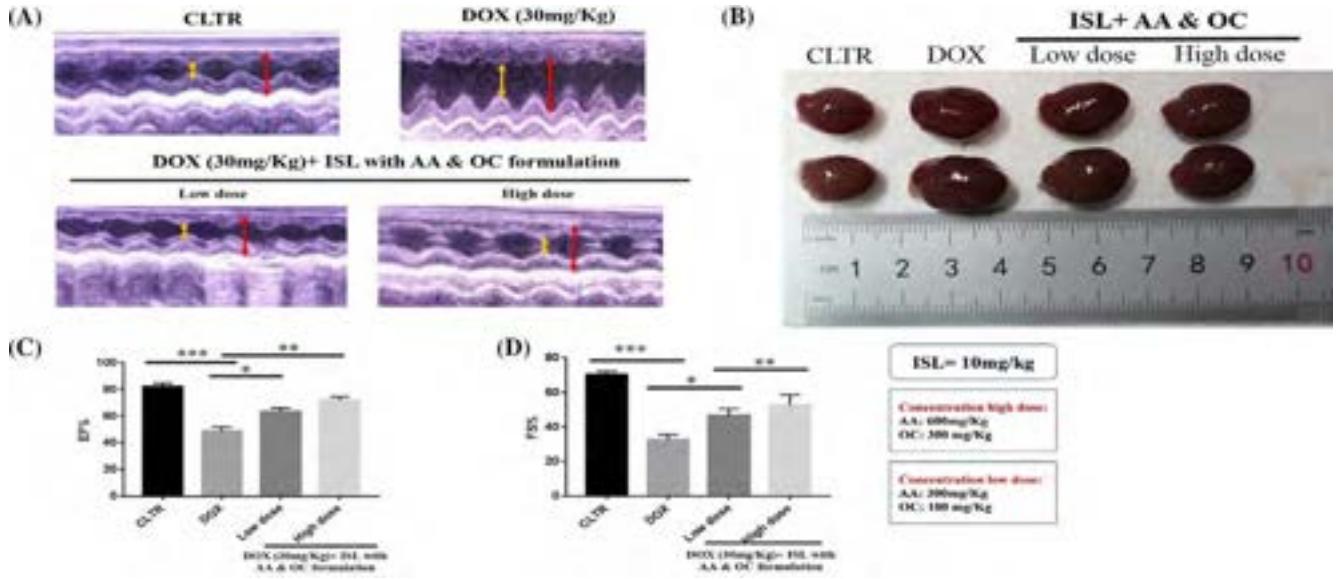

FIGURE 2 ISL with combination of AA and OC attenuated DOX challenge systolic dysfunction in SD rats' model. (A) Representative M-mode echocardiographs from Control, DOX, ISL with high dose, low dose. (B) Morphological assessment of heart tissue from different experimental group. (C, D) Ejection fraction (EF) and Fractional shortening (FS) were measured using echocardiograph machine. *, **, and *** denotes statistically significant ($p < 0.05$; $p < 0.01$; $p < 0.001$). Low dose (AA 300 mg/kg; OC 100 mg/kg; ISL 10 mg/kg) and high-dose(AA 600 mg/kg; OC 300 mg/kg; ISL 10 mg/kg). CLTR, control; DOX, doxorubicin; ISL, isoliquiritigenin.

3.3 | AA and OC water extract with ISL reverses DOX-induced cardiac morphology alterations in SD rats

We confirmed cumulative cardiac toxicity in the heart tissue of animals using histology. Hematoxylin and eosin staining revealed that there were no intercellular spaces and the cardiac tissues were compact with no damaged fibers in both the control as well as the low- and high-dose groups using bright field microscopy, while histopathological changes were observed in the DOX group (Figure 4). DOX-treated animals had ruptured cardiac muscle fibers, which led to cardiac cell apoptosis. Interstitial cardiac fibrosis was also exacerbated in the DOX group. Collagen detection using Masson's Trichrome staining also revealed an increase in the heart of DOX-treated rats compared to control rats. However, the low- and high-dose AA and OC water extract with ISL groups had significantly decreased collagen detection. These results indicate that DOX increased the apoptosis of cardiac myocytes.

3.4 | ISL combination inhibits cardiac apoptosis, oxidative stress, and mitochondrial dysfunction in rats

TUNEL staining was used to confirm apoptosis in cardiac tissue. As demonstrated in Figure 5, several green spots were detected in the DOX-treated group compared to the control group. Cell death was reduced in the low- and high-dose AA and OC water extract with ISL groups under light field electron microscopy (Figure 5). TUNEL-positive cells are shown in green color (thick white arrows). Immunofluorescence staining to detect nuclear translocation of Nrf2 revealed slightly decreased intensity and rupture sites in the DOX group, while the control and ISL combination groups showed the same. DOX-treated rats had a marked increase in nuclear Nrf2 staining, suggesting its activation upon DOX treatment. As we know that mitochondria are highly dynamic and play vital roles in fusion, fission, and migration between other organs to facilitate communication and interaction. Thus, cell mitochondrial morphology, function, and intracellular distribution can provide insight into the cell–cell energetic demands. Dynamin-related protein (Drp1) plays a crucial role in mitochondrial morphology and function. However, our study revealed that DOX-treated cardiac tissue had cytoskeleton muscle displacement, which was probably affected by Drp1 overexpression, while the control group showed no such rupture or damage under bright field microscopy. The group subjected to a low dose of AA and OC water extract with ISL had slightly improved mitochondrial function, and the group subjected to a high dose of AA and OC water extract with ISL significantly alleviated the DOX-induced cardiac damage in SD rats (Figure 5). In agreement with this evidence, we observed that low or high doses of AA and OC water extract with ISL combination synergistically alleviated DOX-induced mitochondrial dysfunction.

3.5 | ISL combination suppresses oxidative stress in DOX-treated SD rats by activating the Nrf2 signaling pathway

Oxidative stress induction in DOX-induced cardiac failure could be a major mechanism in its cardiac toxicity. We first analyzed the

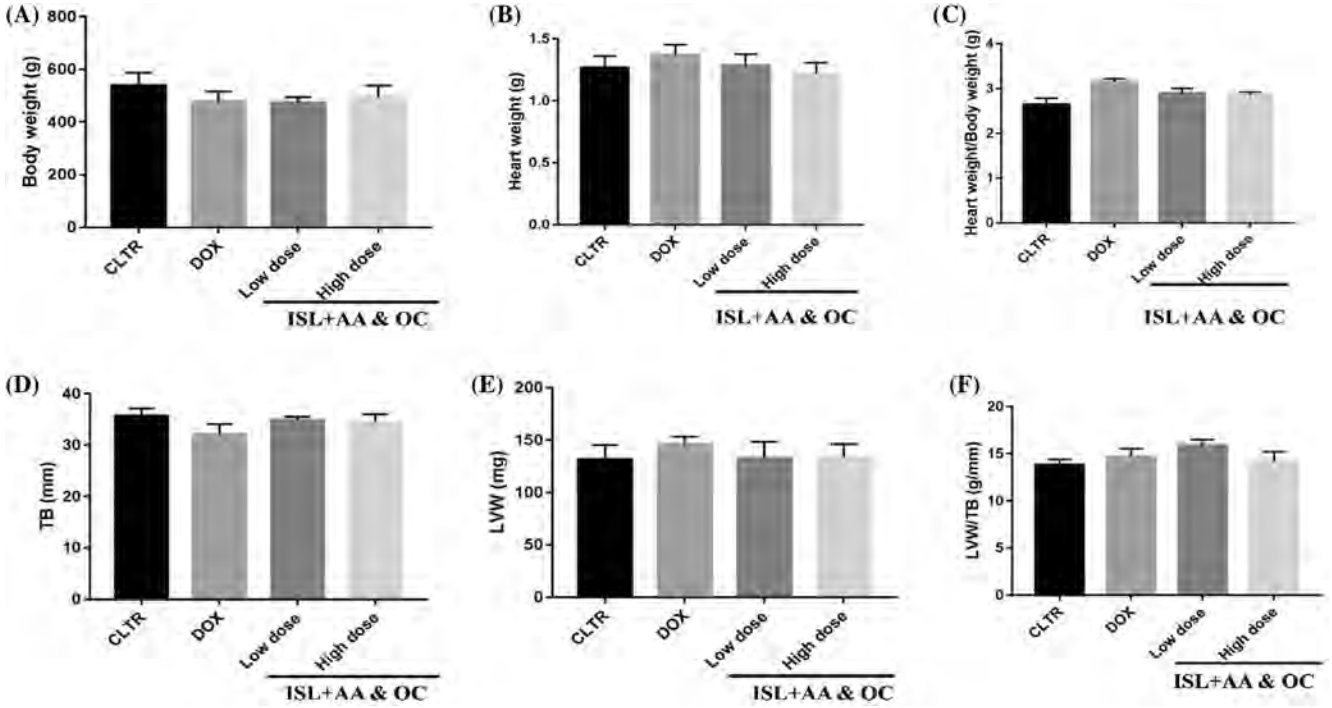

FIGURE 3 Morphological assessment analysis effect of ISL combination AA and OC on DOX-induced cardiac toxicity SD rats model. (A) Total body weight measure (B) Total heart weight measure. (C) fraction of heart weight versus body weight. (D) Measure the tibia length using scale. (E) Left ventricular heart weight measure. (F) Ratio of Left ventricular heart weight vs tibia length. DOX, doxorubicin; ISL, isoliquiritigenin.

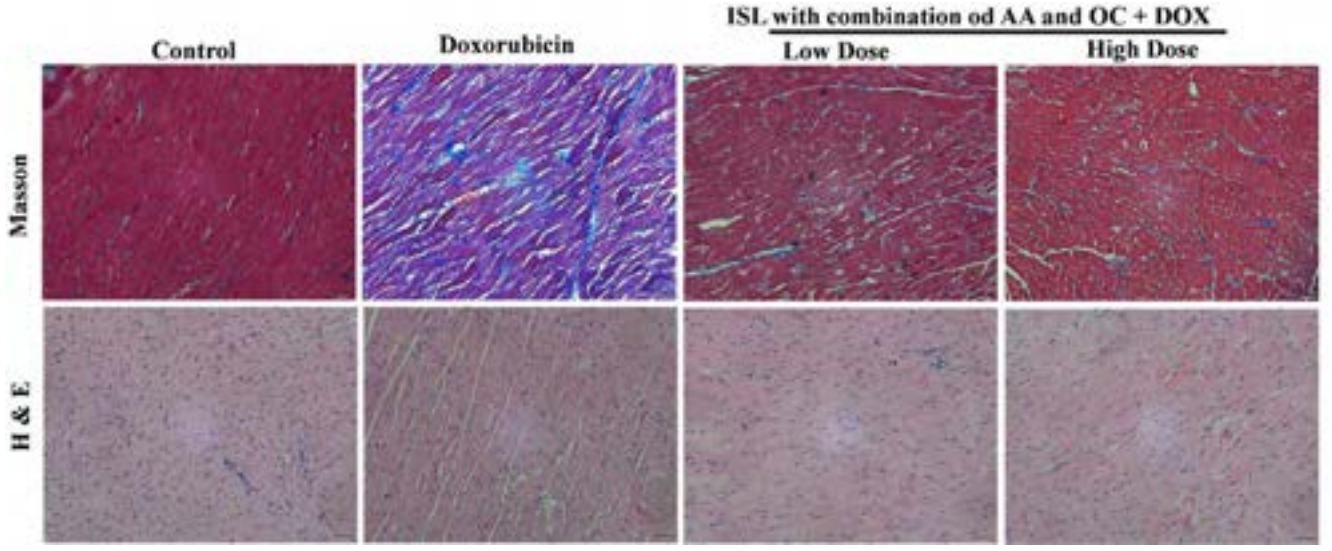

FIGURE 4 Histopathology of myocardium effect ISL combination of AA and OC ameliorated DOX-induced cardiac damage in SD rats morphological of cardiac tissue staining. Hematoxylin–eosin (HE) and Masson's trichrome (MT) were determined to evaluate cardiac morphology, fibrosis, collagen detection, and glycogen accumulation in different experimental groups. DOX, doxorubicin; ISL, isoliquiritigenin.

effects of DOX on oxidative stress induction in cardiac tissue by analyzing the levels of the myocardial antioxidant markers superoxide dismutase (SOD 1 and SOD 2), Nrf2, heme oxygenase-1 (HO-1), and kelch-like ECH associated protein (Keap-1). DOX-injected rats had significantly decreased levels of SOD-1 and SOD-2 compared with those in the untreated rat group, while their levels were significantly increased in the groups subjected to low or high doses of AA and OC water extract with ISL compared with those in the DOX

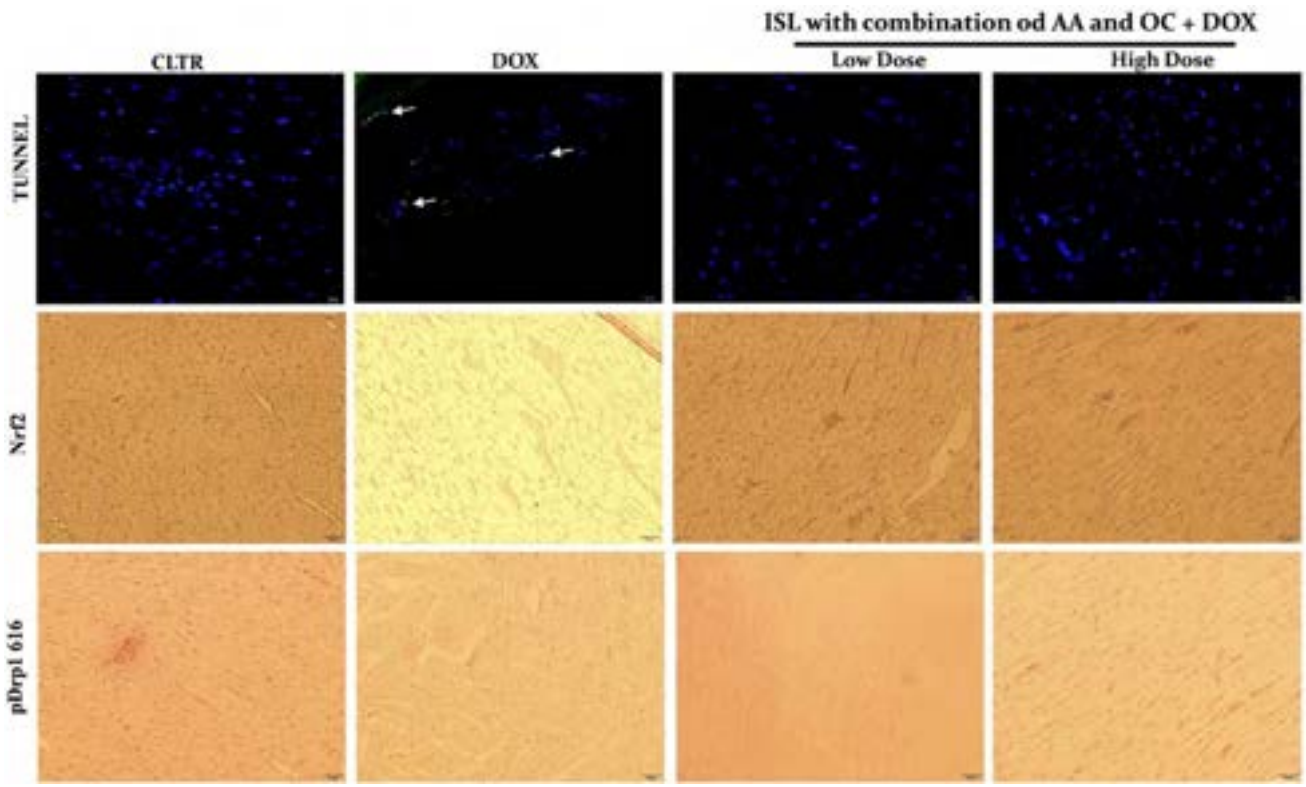

FIGURE 5 Effect ISL combination of AA and OC ameliorated DOX-induced cardiac damage in SD rats morphological of cardiac tissue staining. Tunnel assay staining, hematoxylin–eosin (HE), and Masson's trichrome (MT) were determined to be evaluated cardiac morphology, fibrosis, collagen detection, and glycogen accumulation in different experimental groups. The arrow is indicated that damaged cell in tunnel assay. DOX, doxorubicin; ISL, isoliquiritigenin.

group (Figure 6). Moreover, DOX-treated rats had marginally decreased expression of HO-1 and the nuclear factor Nrf2 compared with the control group, while their expression was significantly increased in the groups subjected to low or high doses of AA and OC water extract with ISL compared with that in the DOX-treated group. The expression of Keap-1 was significantly increased in the DOX-treated group, while the control group and the ISL combination groups had similar Keap-1 levels.

3.6 | ISL combination treatment attenuates the induction and release of pro-inflammatory cytokines

We used western blot analysis to determine the roles of the inflammasome and pro-inflammatory cytokine markers NLR family pyrin domain containing 3 (NLRP3), interleukin (IL)-6, and IL-1β in DOX-induced cardiac toxicity. DOX treatment increased NLRP3 expression levels, while the untreated group had no significant increase, and the groups subjected to low or high doses of AA and OC water extract with ISL had decreased NLRP3 expression levels (Figure 7). Moreover, DOX-treated SD rats showed an upward trend of both IL-6 and IL-1β protein levels, while the control and the ISL combination groups exhibited a downward trend as well as low and high dose of AA and OC water extract with ISL combination followed the same trends.

3.7 | Effect of ISL combined with water extract of AA and OC on DOX-induced mitochondrial dysfunction

Mitochondria play a vital role in cardiomyocyte cell functioning; thus, we evaluated the effects of DOX on mitochondria[31,32] by analyzing Drp1 expression. Excessive generation of reactive oxygen species (ROS) leads to the activation of this pathway.[33] Total Drp1 levels were decreased in the heart tissue of the DOX treatment group, while its levels were increased in the groups subjected to low or high doses of AA and OC water extract with ISL (Figure 8). Immunoblot of cardiac tissue also revealed that phosphorylated Drp1 on Ser 616 and 637 showed the opposite effects, as p-Drp1 Ser 637 inhibits mitochondrial fission. The DOX-treated group had decreased p-Drp1 Ser 637 levels, and the ISL combination and untreated rats groups also showed a significant decrease. In contrast, p-Drp1 Ser 616 levels slightly increased in the DOX-treated group compared with those in the water extract ISL combination group but marginally decreased in the control group. Thus, low or high doses of AA and OC water extract combined with ISL can inhibit

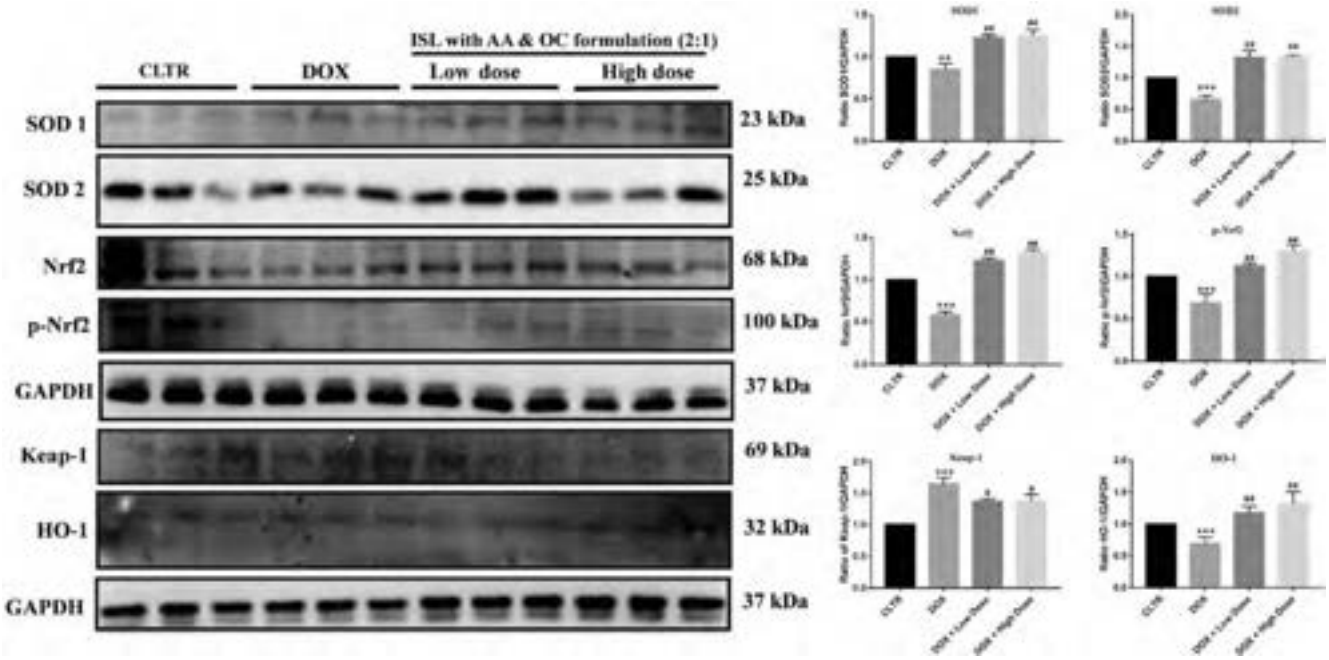

FIGURE 6 Effect of ISL formulation with AA and OC with low and high dose on the oxidative stress in the heart tissue induced by DOX. Western immune blot analysis and densitometry analysis of SOD1, SOD2, Nrf2, p-Nrf2, Keap-1, and HO-1. Values are presented as mean ± SEM. $^{*}p < 0.05$ versus normal control, $\#p < 0.05$ versus DOX control. Dox-induced cell death highly statistically significant by quantify western blot data and recover by low and high dox proved statistically significant. DOX, doxorubicin; ISL, isoliquiritigenin.

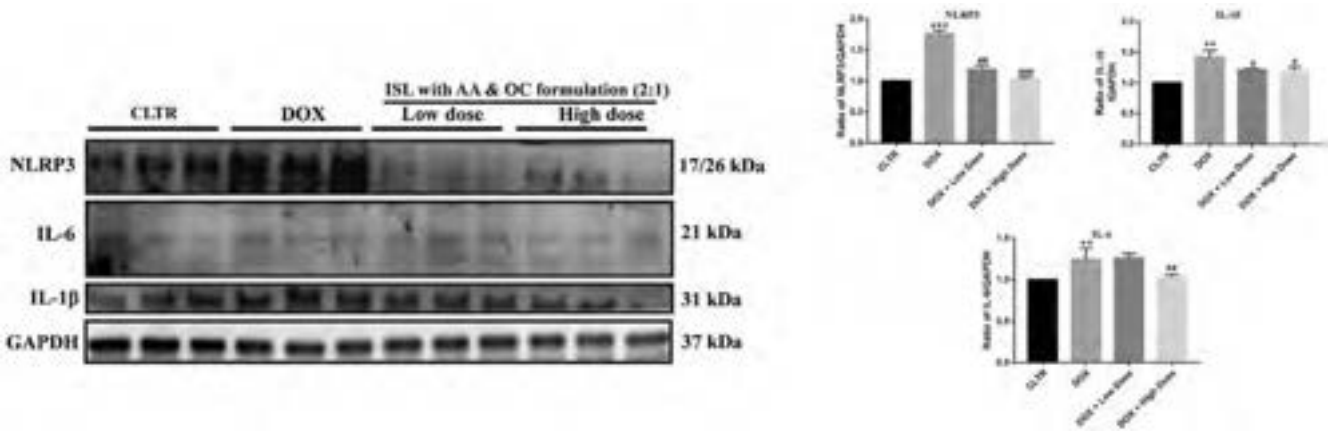

FIGURE 7 ISL formulation with AA and OC with low and high dose suppress the activation of NLRP3 and IL-1β inflammasome in the heart tissue induced by DOX. Western immune blot analysis and densitometry analysis of NLRP3, IL-6, and IL-1β. Values are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA coupled with a post hoc test. Significant differences were indicated as $^{*}p < 0.05$ versus normal control, $\#p < 0.05$ versus DOX control. Dox-induced cell death highly statistically significant by quantify western blot data and recovery by low and high dox proved statistically significant. DOX, doxorubicin; ISL, isoliquiritigenin.

mitochondrial damage can inhibit low and high dose of AA and OC water extract with ISL combination.

3.8 | Water extract of AA and OC combined with ISL inhibits cardiac hypertrophy in SD rats

The transcription factor GATA-4 is activated by DOX administration and is involved in cardiac hypertrophy. Western blot data revealed that p-GATA-4 expression was highly induced by DOX treatment, while water extracts ISL combination groups significantly inhibited the phosphorylation of GATA-4 (Figure 9). Interestingly, GATA-4 expression exhibited significant changes in both the untreated and treated groups. Therefore, our data showed that low or high doses of AA and OC water extract combined with ISL can prevent cardiac hypertrophy through the phosphorylation of GATA-4, the transcription regulatory gene in cardiac tissue.

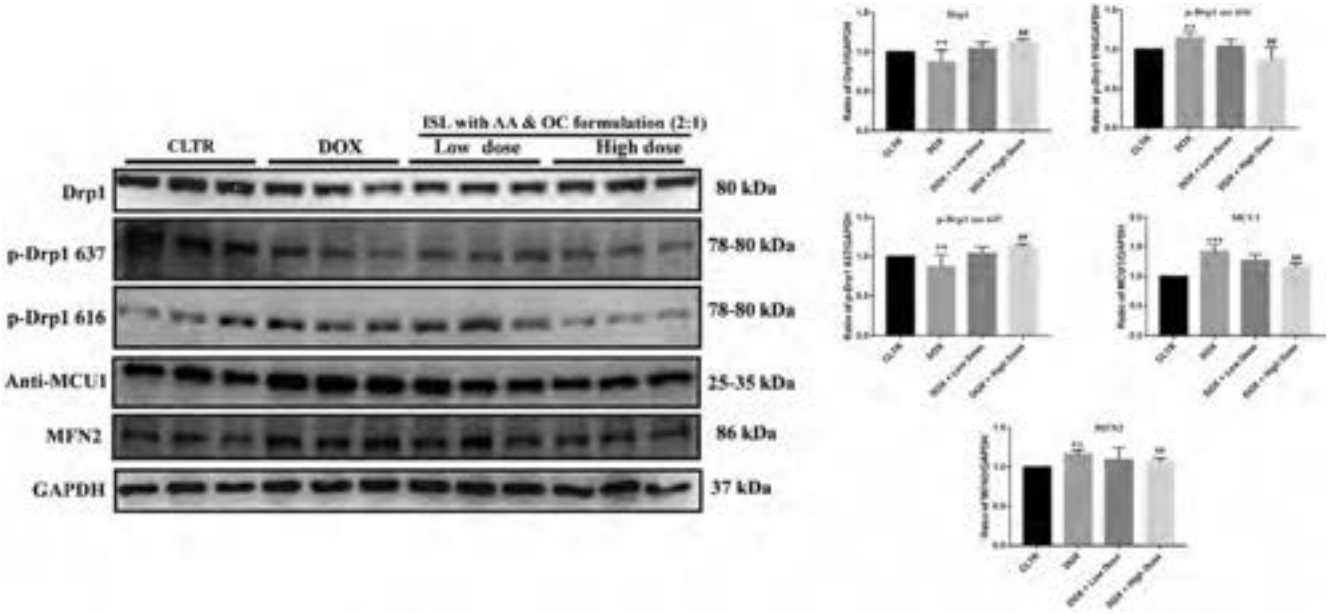

FIGURE 8 DOX induces mitochondrial fragmentation in a Drp1-dependent manner and ISL with formulation with AA and OC have effect against DOX. Mitochondrial dynamics machinery proteins, including Drp1, p-Drp1 ser 637, p-Drp1 ser 616, mitochondrial calcium uniport anti-MCU1, MFN2, and GAPDH were used as a loading control. Protein expression levels are shown as unit 1 is 100%. Data are presented as means ± SEM from three independent experiments. Significant differences were indicated as $^{*}p < 0.05$ versus normal control, $\#p < 0.05$ versus DOX control. Dox-induced cell death was highly statistically significant by quantify western blot data and recover by low and high dox proved statistically significant. DOX, doxorubicin; ISL, isoliquiritigenin.

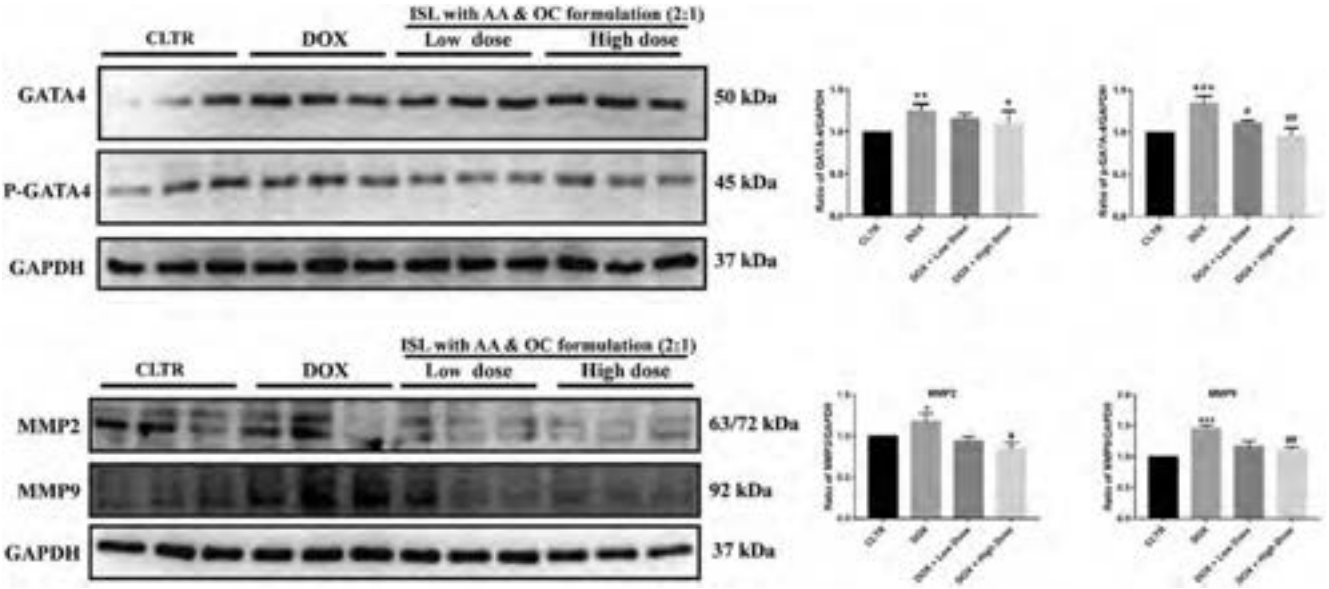

FIGURE 9 DOX induces cardiomyocytes death and cardiac hypertrophy through GATA4-dependent manner and ISL formulation with AA and OC have effect against DOX. Cardiac myocytes machinery proteins, including GATA4, p-GATA4, and GAPDH was used as a loading control. The protein expression level was detected by western blot assay. Protein expression levels are shown as unit 1 is 100%. Data are presented as means ± SEM from three independent experiments. Significant differences were indicated as $^{*}p < 0.05$ versus normal control, $\#p < 0.05$ versus DOX control. Dox-induced cell death highly statistically significant by quantify western blot data and recover by low and high dox proved statistically significant. Matrix metalloproteinases machinery proteins, including MMP2, MMP9, and GAPDH was used as a loading control. Protein expression levels are shown as unit 1 is 100%. Data are presented as means ± SEM from three independent experiments. Significant differences were indicated as $^{*}p < 0.05$ versus normal control, $\#p < 0.05$ versus DOX control. Dox-induced cell death highly statistically significant by quantify western blot data and recovery by low and high dox proved statistically significant. DOX, doxorubicin; ISL, isoliquiritigenin.

3.9 | Effect of ISL combined with water extract of AA and OC attenuates the activation of matrix metalloproteinase-2 and MMP-9

Increasing evidence suggests that the overproduction of mitochondrial ROS plays a vital role in matrix metalloproteinase (MMP) activation, activating NADPH (Nicotinamide adenine dinucleotide phosphate), which is essential for ROS generation. Several clinical studies showed that cardiac failure is associated with increased MMP-2 and MMP-9 levels.[34,35] We analyzed the levels of MMP-2 and MMP-9 using western blot assay was performed to check the effectivity against DOX-induced rats in the matters of ISL combination can block the expression level. As predictive and found that the DOX group had significantly upregulated MMP-2 and MMP-9 levels, while the water extract and control groups showed significant inhibition of this upregulation (Figure 10).

3.10 | Water extract of AA and OC combined with ISL attenuates inflammatory mediators and downregulates NF-$_K$B signaling pathways

Meanwhile, it is also found that DOX treatment of SD rats upregulated the protein levels of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), NF-κB65, p-NF-κB65, IκB kinase-α (IκB α), and p-IκBα compared to control rats, while the low or high doses of AA and OC water extract with significantly reduced the levels of these renal and inflammatory protein markers compared to the DOX-treated group (Figure 11).

3.11 | Effect of water extract of AA and OC combined with ISL on DOX-induced cell survival signal and MAPK signaling protein

The phosphoinositide-3-kinase PI3K/Akt cell survival signaling pathway plays a vital role in cardiac tissue the Phosphoinositide 3-kinase PI3K/Akt signaling pathway plays a vital role.[36] Consequently, our present study investigated the role of this signaling pathway in exhibiting the protective effects of AA and OC water extract combined with ISL against DOX-induced cardiac tissue damage. To further investigate this interesting phenomenon, we have performed an immunoblot assay and observed that DOX-treated rats had significantly increased levels of PI3K compared with those in controls and marginally decreased expression level of p-AKT compared to the untreated group (Figure 12). Remarkably, water extract of AA and OC combined with ISL showed the opposite effects.

Based on the previous study, p38 mitogen-activated protein kinase (MAPK) plays a vital role in DOX-induced cardiac tissue damage, and the MAPK family promotes ROS-induced cell death by extracellular signal-regulated protein kinase p38 and c-Jun N terminal kinase (JNK) signaling.[37] Based on western blot data revealed DOX-

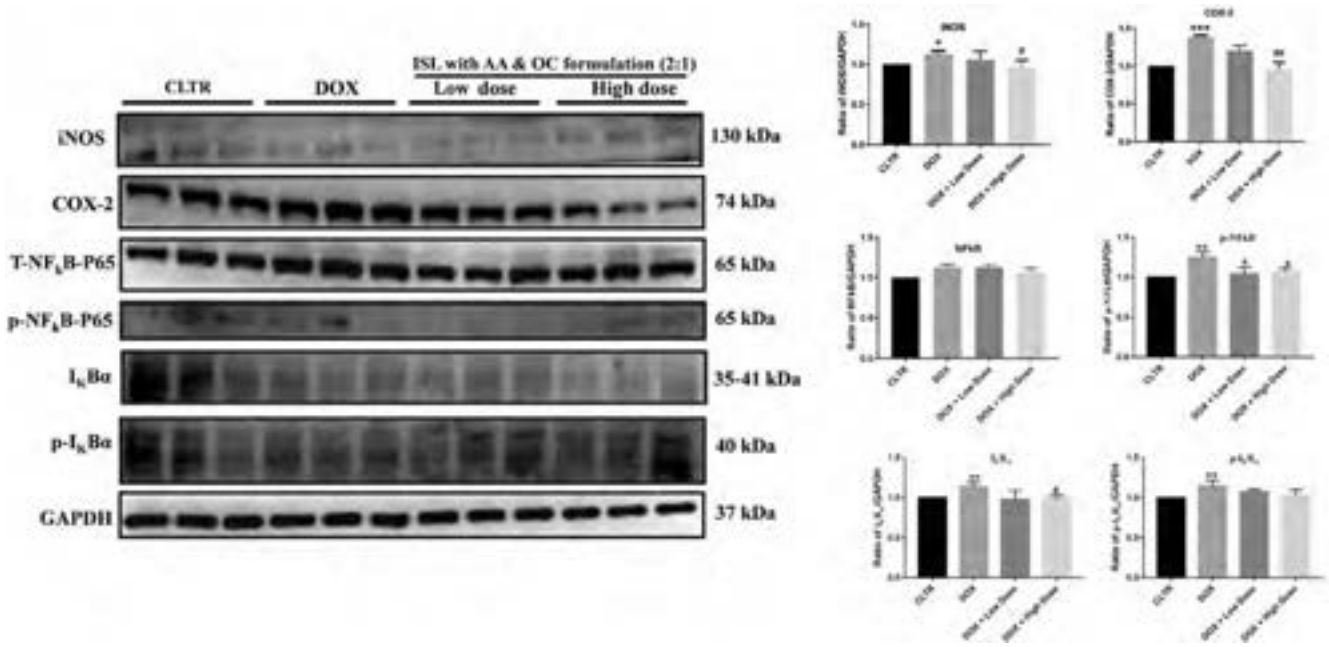

FIGURE 10 ISL formulation with AA and OC attenuates the inflammatory mediators and modulates altered nuclear factor Kappa B signaling pathways on DOX induce cardiac heart tissue. Western blot assay was performed on different protein markers, including iNOS, COX-2, NF$_K$B65, p-NF$_K$B65, I$_K$Bα, p-I$_K$Bα, and GAPDH was used as a loading control. Protein expression levels are shown as unit 1 is 100%. Data are presented as means ± SEM from three independent experiments. Significant differences were indicated as $^*p < 0.05$ versus normal control, $\#p < 0.05$ versus DOX control. Rats were administered with a 4 weeks intraperitoneal dose of DOX and orally treated with ISL formulation with AA and OC high and low dose. Dox-induced cell death highly statistically significant by quantify western blot data and recover by low and high dox proved statistically significant. DOX, doxorubicin; ISL, isoliquiritigenin.

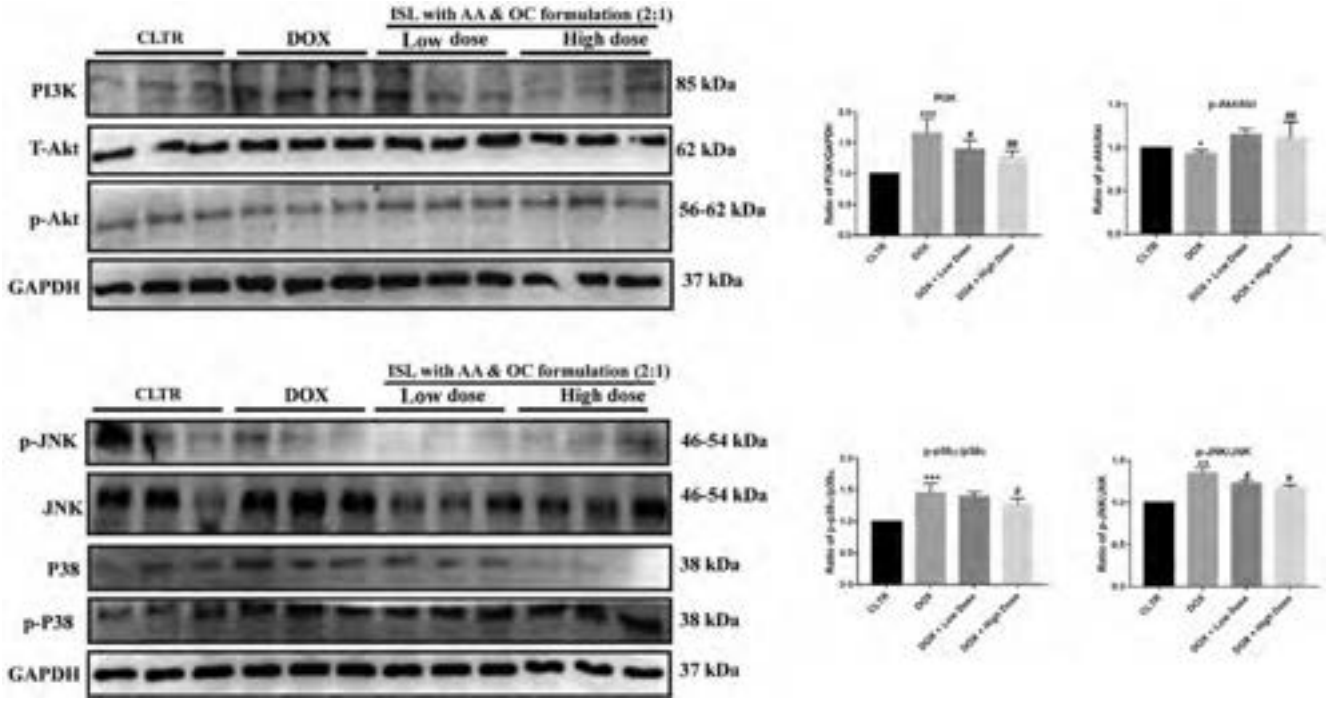

FIGURE 11 Effect of ISL formulation with AA and OC on cell survival mechanism and mitogen-activated protein kinase (MAPK) signaling pathways on DOX induce cardiac heart tissue. Western blot assay was performed on different protein marker, including PI3K, Akt, p-Akt, p-JNK, JNK, p38α, p-p38α, and GAPDH was used as a loading control. Represented Protein expression levels are shown as unit 1 is 100%. Data are presented as means ± SEM from three independent experiments. Significant differences were indicated as $*p < 0.05$ versus Normal Control, $\#p < 0.05$ versus DOX control. Rats were administered with a 4 weeks intraperitoneal dose of DOX and orally treated with ISL formulation with AA and OC high and low dose. Dox-induced cell death highly statistically significant by quantify western blot data and recovery by low and high dox proved statistically significant. DOX, doxorubicin; ISL, isoliquiritigenin.

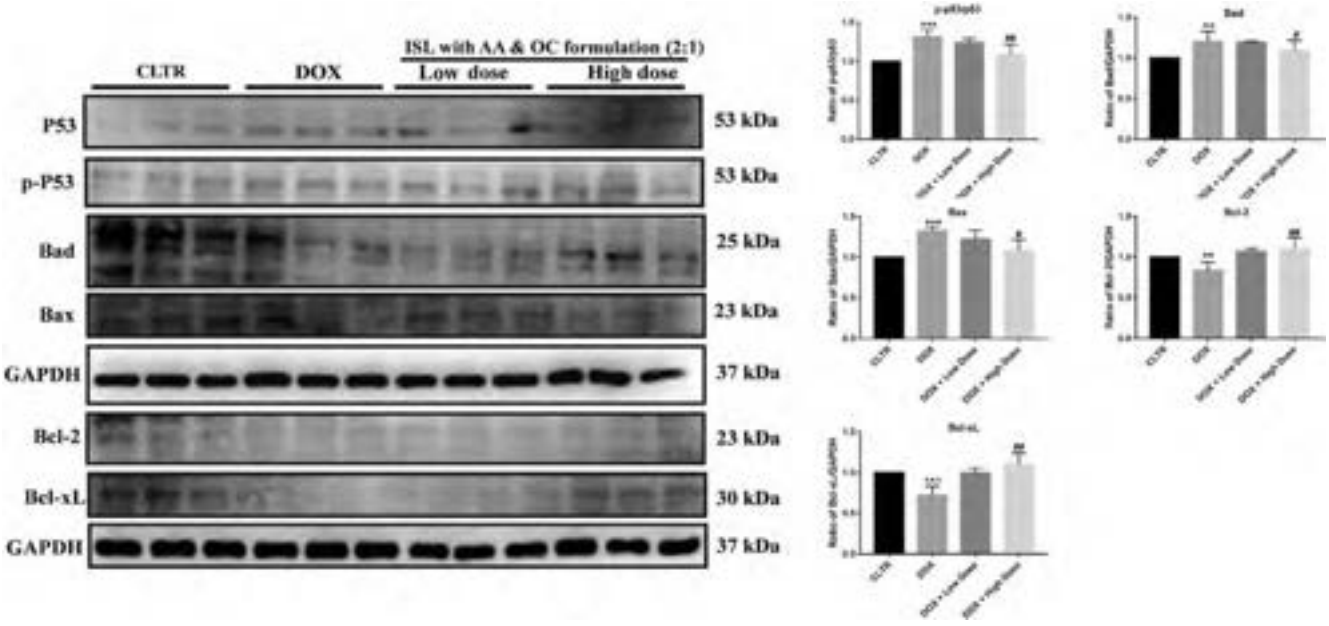

FIGURE 12 ISL formulation with AA and OC prevent DNA damages on DOX induce cardiac heart tissue. Western blot assay performed different protein markers, including P53, p-P53, Bad, Bax, Bcl-2, Bcl-xL, and GAPDH was used as a loading control. Represented Protein expression levels are shown as unit 1 is 100%. Data are presented as means ± SEM from three independent experiments. Significant differences were indicated as $*p < 0.05$ versus normal control, $\#p < 0.05$ versus DOX control. Rats were administered with a 4 weeks intraperitoneal dose of DOX and orally treated with ISL formulation with AA and OC high and low dose. Dox-induced cell death was highly statistically significant by quantify western blot data and recover by low and high dox proved statistically significant. DOX, doxorubicin; ISL, isoliquiritigenin.

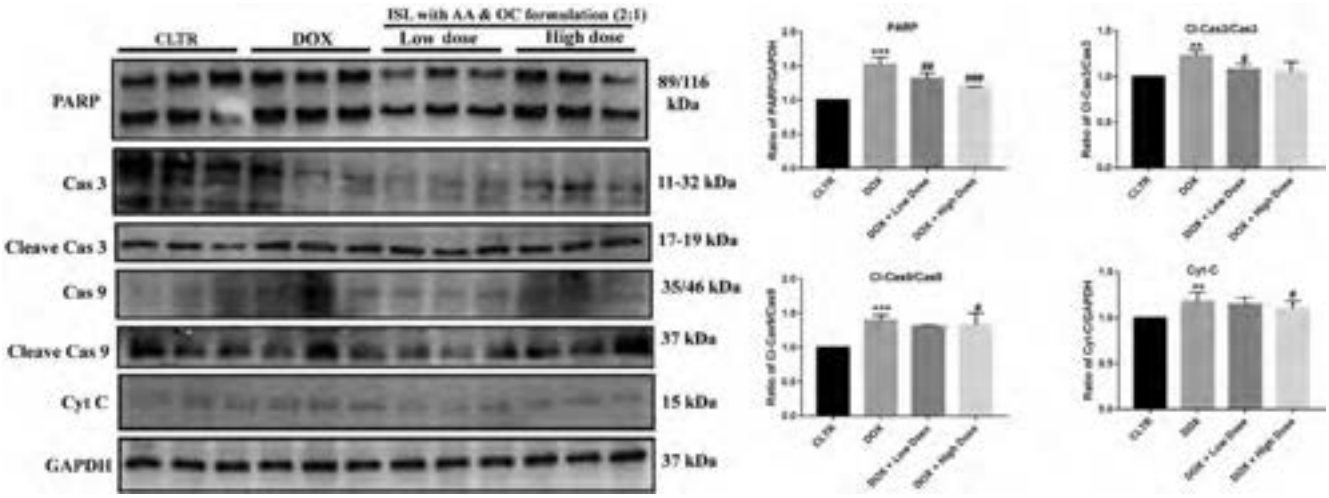

FIGURE 13 ISL formulation with AA and OC inhibit DOX induce apoptosis in cardiac heart tissue. Western blot assay was performed with different protein marker, including PARP. Cas3, Cleave Cas3, Cas 9, Cleave Cas 9, and GAPDH was used as a loading control. Represented Protein expression levels are shown as unit 1 is 100%. Data are presented as means ± SEM from three independent experiments. Significant differences were indicated as $^{*}p < 0.05$ versus normal control, $\#p < 0.05$ versus DOX control. Rats were administered with a 4 weeks intraperitoneal dose of DOX and orally treated with ISL formulation with AA and OC high and low dose. DOX, doxorubicin; ISL, isoliquiritigenin.

induced JNK pathway activation, indicating that cardiac hypertrophy was caused by hypertrophy while the water extract of AA and OC combined with ISL effectively blocked the hypertrophy-mediated pathways (Figure 12). Therefore, our data showed that low or high doses of AA and OC water extract combined with ISL inhibited the activation of p38 and JNK in hypertrophy pathways.

3.12 | ISL combined with water extract of AA and OC protects from DOX-induced apoptosis by modulating the Bcl-2 family

DOX-induced cardiac toxicity leads to cell death, which is regulated by the B-cell lymphoma-2 (Bcl-2) family that includes both the pro-apoptotic Bcl-2-like protein Bax and the anti-apoptotic protein Bcl-2.[38] Western blot analysis revealed that DOX treatment decreased the expression of anti-apoptotic Bcl-2 and increased the pro-apoptotic protein Bax/Bad. As expected, the levels of Bcl-2 and Bax in the cardiac tissue of rats subjected to low or high doses of AA and OC water extract with ISL did not differ significantly from those in normal cardiac tissue (Figure 13). Moreover, DOX-treated cardiac tissue had drastically increased p-p53 (ser 15) protein expression, whereas water extract with ISL combination inhibited these changes.

3.13 | ISL combined with water extract of AA and OC protects against DNA damage that leads to apoptosis

The release of Cyt-c from the mitochondria into the cytosol could trigger the caspase cascade leading to the activation of the apoptosis pathway, resulting in cardiac apoptosis and impairment of the contractile function of the heart.[39] Our study revealed that the expression of the proteolytic protein PARP, which is activated by both cytochrome-c and caspase-3, was upregulated in the DOX-treated group, while its expression was significantly blocked in the groups subjected to a combination of ISL with water extract of AA and OC. Moreover, we also evaluated caspase-9 activation and observed that low or high doses of AA and OC water extract combined with ISL enhanced caspase-9 activation compared to DOX-treated rats (Figure 14).

4 | DISCUSSION

Cardiovascular disease is a leading cause of mortality and morbidity worldwide and has become a major research focus among all generations. Regarding cardiac disease, currently, a new formulation strategy badly newer and better therapies against cardiac disease are needed to protect and prolong the human life span. Herbal medicine has long been used to treat hypertension, arthritis, inflammation, aging, and cardiac disease. AA and *OC* are popular herbal medicines in Southeast Asia. This plant extract has been used as an anti-cancer, anti-apoptotic, and anti-aging agent and for Parkinson's disease treatments.[12,14,40–42] ISL drug has also been used against multiple diseases such as cardiomyopathy and intracerebral hemorrhage.[43,44] To the best of our knowledge, this is the first study providing evidence about the combination of AA and OC with ISL against cardiac damage induced by DOX. Cardiac hypertrophy is a systemic chronic inflammation. Due to Dox chemotherapy, the major side effect is cardiac failure and lots of news in the last 50 years found that Dox-induced cardiac failure is chronic. ISL formulation is a new idea against such chemotherapy-reducing drug. It is also mentioned that Inflammation can be induced by several reprogramming of cellular energy

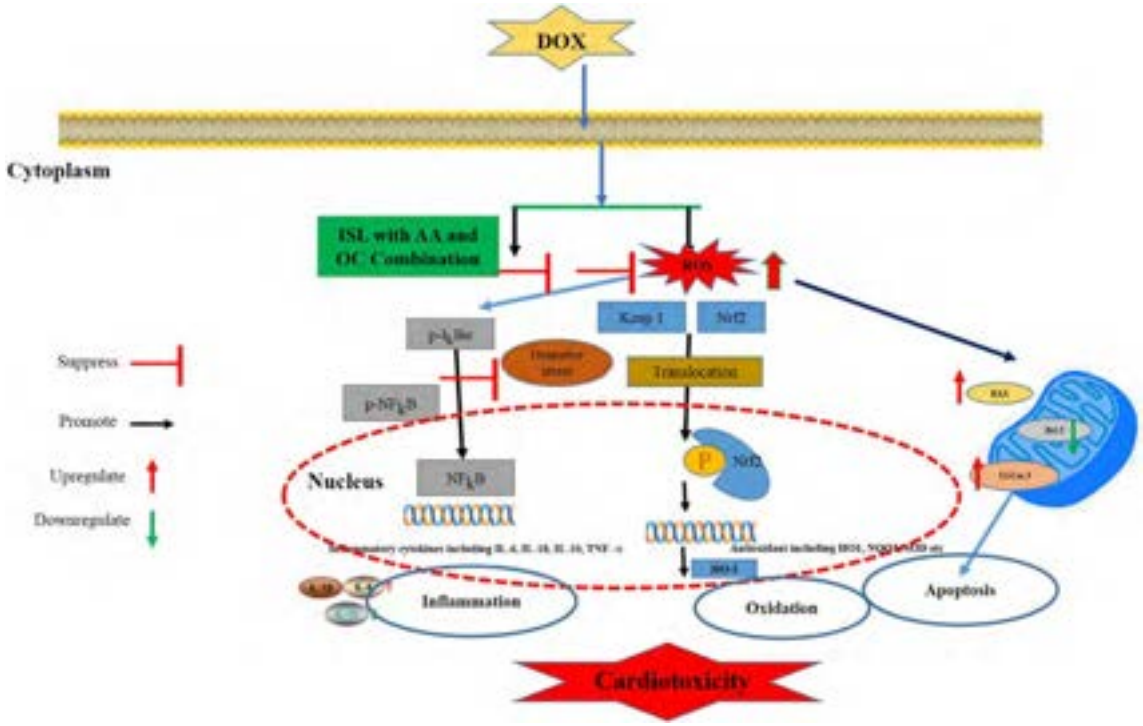

FIGURE 14 Graphical representative on the role of ISL formulation with AA and OC in DOX-induced cardiac failure. DOX, doxorubicin; ISL, isoliquiritigenin.

production which may play a vital role in biosynthesis to ensure faster ATP production, and this may damage repair by increasing glycolysis and promoting mitochondrial dysfunction and cause cell damage in cardiac tissue. However, in chinse leaf, it has been proved that there are lots of verity of chemical composition present which can block the target substance.[45]

Side effects of DOX may cause serious damage in the left and right ventricles, as confirmed by the significant reduction in NO content, which plays a vital role in vascular function. Extracellular matrix deposition impairs the oxidant and antioxidant homeostasis and leads to the development of atherosclerosis, which causes cardiovascular disease.[46] Cardiac fibrosis is a pathological marker of exacerbation of different underlying chronic heart diseases, which may cause prolonged heart tissue damage with high mortality and morbidity. In terms of heart tissue, excessive proliferation and abnormal deposition in heart tissue may lead to changes in abnormal heart structure and functional damages.[47] ISL formulation can help to reduce fibrosis effect in heart tissue. Similar changes happen in the oxidative load sensor Nrf2, its downregulated target protein HO-1, and its receptor Keap-1, confirming the presence of oxidative injury. Another study reported that the antioxidant SOD, catalase, or lipid peroxidation after DOX injection in rats could cause oxidative damage.[48] DOX-treated rats are susceptible due to the excess production of free radicals because of reduced antioxidant defenses and increased oxidative metabolism leading to myocardial injury. Our study also revealed that DOX-treated rats subjected to low- or high-dose AA and OC combined with ISL exhibit a normalization in the SOD levels and antioxidant mechanisms. Here, we demonstrated the antioxidant property of ISL combined with water extract of AA and OC, and our findings also support previous studies wherein ISL helped to prevent heart, lung, and brain damage.

The chemotherapeutic drug DOX causes myocardial inflammation, activating NFκB signaling pathways in the myocardial inflammatory response and contributing to inflammatory cytokine (IL-6, IL-1β, and NLRP3) production. A recent study reported the interesting role of NLRP3 inflammasome in DOX-induced myocardial dysfunction.[49,50] NLRP3 plays multiple roles in various diseases, including pathogen-associated molecular patterns.[51] This study strongly supported that DOX-induced cardiac dysfunction involved ROS/NLRP3 inflammasome and that DOX enhanced the activity of NLRP3, leading to subsequent hypersecretion of IL-1β. Consistent with this report, we showed that NLRP3 levels were significantly increased in DOX-treated rats compared to controls with significant elevations of IL-6 and IL-1β protein levels that enhanced apoptosis in cardiac tissue, while low or high doses of AA and OC water extract combined with ISL significantly reduced their levels.

Another recent study reinforced the idea that ROS generation in mitochondria during mitochondrial dysfunction is the main reason behind the activation of NLRP3 inflammasome In our study, we also observed phosphorylated Drp1 Ser 616 protein and ruptured tissue in DOX-treated rats compared to the ISL combination treatment group. Furthermore, western blot data supported that mitochondrial uniporter and fission and fusion mitochondrial proteins play a vital role in

DOX-induced cardiac damage, and most importantly, natural herbal medicine with ISL successfully alleviated the cardiac damage.

A previous study demonstrated that DOX-induced cardiac failure is important in hypertrophy-related signaling pathways, including GATA-4, ANP, and BNP.[52] In the nucleus, the transcript protein NFATC3 activates GATA-4, which plays a myocardial role during hypertrophy, and the downstream GATA-4 may activate other signaling pathways, such as calcineurin, which plays a vital role in the development of cardiac hypertrophy and survival.[52] Our study revealed that DOX treatment increased GATA-4 expression, while the ISL combination with AA and OC water extract inhibited its expression.

The caspase-mediated signaling pathway is important in cardiac apoptosis.[53,54] During apoptosis, activated caspase 3 modulates the mitochondrial transmembrane potential, Bcl-2 plays a vital role in mitochondrial function and structure, and downregulated Bcl-2 is associated with heart failure.[55] Western blot of apoptosis markers revealed that DOX increased the expression of the pro-apoptotic proteins Bax, Bcl-2, and Bad, the tumor suppressor p53, and apoptosis-related pathway AKT, and caspase 3/9, whereas anti-apoptotic protein levels were decreased after DOX treatment compared to the untreated group and ISL combination groups. Therefore, our results indicate that the inhibitory effect of low or high doses of AA and OC with ISL combination on DOX-induced apoptotic cell death happens by regulating several major signaling pathways, such as those involving the Bcl-2/Bax family and caspase protein activity (Figure 14). It also revealed that MMP-2/9 also shown the downregulated as previously mentioned.[56] However, our study has a few limitations, that is, we did not use a specific hypertrophy marker like ANP or BNP and other mitochondrial-related genes responsible for cardiac apoptosis and hypertrophy. Moreover, additional research is needed to clarify the molecular and cellular mechanisms involved in the effects of the combination of ISL with OC and AA water extract.

5 | CONCLUSION

Our result provides the primary evidence of the combination treatment of low and high doses of AA and OC with ISL in protecting against cardiac tissue damage and myocardial injury caused by DOX by modulating the PI3K/AKT/Nrf2/Drp1/GATA-4 signaling pathway, thereby enhancing antioxidant capacity and inhibiting mitochondrial imbalance. These results provide new strategies for formulating new drugs for the clinical management of patients with cardiac dysfunction using the chemotherapeutic drug DOX.

AUTHOR CONTRIBUTIONS

Dennis Jine Yuan Hsieh, Md. Nazmul Islam, Marthandam Asokan Shibu: conceptualization; **Wei-Wen Kuo, Marthandam Asokan Shibu:** data curation; **Dennis Jine-Yuan Hsieh, Md. Nazmul Islam:** formal **analysis; Pi-Yu Lin, Md. Nazmul Islam, Shinn-Zong Lin:** methodology; **Michael Yu-Chih Chen, Md. Nazmul Islam:** supervision; **Md. Nazmul Islam:** writing – original draft; **Md. Nazmul Islam, Chih-Yang Huang:** writing – review and editing. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The author has been declared there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The correspondence author is mainly responsible for availability of data on request.

CONSENT STATEMENT

Not applicable.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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