Chapter 4

Investigation of cellular and molecular mechanism of ARCE in preventing experimentally induced myocardial infarction

4.1 INTRODUCTION

Hypertension, arrhythmia, coronary artery disease, myocardial infarction and cardiac failure are leading cause of death worldwide (Hennekens, 1998). Myocardial infarction (MI) is marked by rapid development of myocardial necrosis, due to imbalance between the oxygen demand versus supply in myocardium. Increased production of free radicals with decrease in intracellular antioxidant levels of myocardium plays the major role in pathologies of heart diseases such as ischemic heart disease, atherosclerosis, congestive heart failure, cardiomyopathy and arrhythmias (Das and Maulik, 1995). Free radical production is an enzyme catalyzed as well as electron transfer process, which is an important phenomenon in the cell metabolism. Although, free radicals are important for the maintenance of normal physiological function, over abundance or uncontrolled chain reactions of the same can be potentially lethal to a cell (Kaul et al., 1993).

Zbinden & Bagdon (1963) was the first to induce cardiac damage using isoproterenol (ISO). Thereafter, ISO induced myocardial infarction model is widely used to study the protective effects of compounds. Myocardial infarction is the rapid development of myocardial necrosis caused by critical imbalance between the oxygen supply and the demand of the myocardium. Several events are responsible for ISO induced myocardial damage: First, an imbalance between demand versus supply of oxygen to cardiomyocytes, resulting into myocardial hyperfunction. This occurs due to an increase in both chronotropism and inotropism as well as decrease in coronary vascular resistance (John C Yeager & Marvin E Whitehurst, 1982). Secondly, an elevation in intracellular Ca^{2+} with an imbalance in calcium transport protein (Sherman Bloom & Delbert L Davis, 1972; Shibata et al., 2011). In addition, ISO is

also related to the activation of the adenylate cyclase and depletion of ATP levels (Singal et al., 1982).

Dietary factors play a key role in the development and improvement of various human diseases, including cardiovascular disease. It is well documented that certain natural substances have the potential to reduce the detrimental effect of cardiovascular risk factors. Epidemiological studies have shown that fruits (Jadeja et al., 2010; Lopera et al., 2013), herbs (Hsieh et al., 2014; Patel et al., 2012; Menaka C Thounaojam et al., 2011), spices (M. Padmanabhan & P. S. M. Prince, 2006) or pure compound from functional food (Rajadurai & Prince, 2007) are associated with reducing the risk of experimentally induced myocardial infarction. Wherein, improvement in plasma enzymatic and non-enzymatic antioxidants, TBARS and histopathological findings were observed with regulated levels of HDL and LDL. Also, combinatorial use of traditional Chinese herbs (Cortex Moutan and Radix Salviae) enhanced antioxidant defence mechanism by activating Nrf2 signaling in ISO induced myocardial infarction rat model and prevented apoptosis by regulating bax, bcl-2 and caspase-3 expression levels (Li et al., 2012).

Reports on hepatoprotective (Igarashi et al., 2000), membrane stabilizing (Duchnowicz et al., 2012) and neuroprotective (Kataya & Hamza, 2008) potentials of red cabbage are attributed to the high content of anthocyanins. Previous studies in our lab had reported that co-supplementation of anthocyanin rich red cabbage extract (ARCE) prevents cardiac and hepatic oxidative stress in atherogenic diet fed rats (Sankhari et al., 2012) and improved mitochondrial membrane potential in oxidatively stressed cardiomyoblasts (Devkar et al., 2012). Though, consumption of anthocyanin in reducing cardiovascular risks and myocardial infarction (Cassidy et al., 2013) has

been reported, ARCE has not been investigated in detail for its cardioprotection.

Also, none of the previous studies were focused on benefits of functional foods on β-adrenergic (AR) stimulated modifications like Ca^{2+} handling protein, cardiac specific membrane marker (caveolin-3) and ANKRD1. Hence the present study was designed to decipher the potential of ARCE in improving the intracellular homeostasis during myocardial infarction.

Aim: *To evaluate the protective role of ARCE against experimentally induced myocardial infarction in rat model.*

4.2 MATERIALS AND METHODS

Preparation of extract

Same as mentioned in Chapter 2.

Experimental animals

Adult male Charles foster rats (n=36, 160-180 g) were obtained from Department of Biochemistry, The M.S. University of Baroda, Vadodara, India. Throughout the study, rats were maintained in clean polypropylene cages and controlled conditions (23±2ºC, LD 12:12 and 45-50% humidity with food and water *ad libitum*) as per standard guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experimental protocol (P.N.3approval no. 827/ac/04/CPCSEA) was approved by the Institutional Animal Ethics Committee (IAEC) and the committee for the purpose of control and supervision of experiments on animals (reg. no. 827/ac/04/CPCSEA) of the Department of Zoology, The M.S. University of Baroda, Vadodara, Gujarat, India.

Experimental design

Rats were randomly divided into three groups of six animals each. Group 1 (Control): Rats were fed with normal saline (*orally*) daily for 30 days. Group 2 (Disease control): Rats were fed with normal saline (*orally*) daily for 28 days followed by ISO (ISO: 85 mg/kg body weight *s.c.*) on 29th and 30th day. Group 3 (ARCE+ISO): ARCE (250 mg/kg body weight *orally*) daily for 28 days and ISO (ISO: 85 mg/kg body weight *s.c.*) on 29th and 30th day. At the end of the experimental period (31st day) rats

were fasted overnight (12 h). The next day, blood samples were collected from retro-orbital sinus puncture under mild ether anesthesia. Blood samples were centrifuged at 3000 rpm for 10 min at 4°C and plasma was stored at -20°C till further analysis. Animals were sacrificed and whole hearts (6 per group) were excised and weighed. Ratio of heart: body weight (cardiosomatic index) was calculated (Joseph, 1908). Some part of the ventricular tissue (~50 mg) was cut from each heart and stored in RNAlater (Invitrogen, USA) stabilization solution at -20°C.The remaining ventricular tissue was used for histopathology and study of infracted area respectively.

Plasma CK-MB

Plasma samples were thawed and activity levels of CK-MB enzyme were estimated in control and treated samples as per the instruction manual of ENZOPAK CK-MB kit (Reckon Diagnostics Pvt. Ltd., Gujarat).

Microscopic and Macroscopic evaluation of cardiac tissue

Fresh transverse ventricular slices (1-2 mm) were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Aldrich, USA) at 37°C for 20 min and photographed using Canon power S70 shot digital camera. The % infarct area of the ventricles was measured using Image J software (NIH, USA).Whereas, the ventricular thickness was measured using an occulometer.

Tissue samples of ventricle (control and treated) were fixed in 10% buffered paraformaldehyde after autopsy. Later, tissue samples were dehydrated with series of graded alcohol and embedded in paraffin wax. Tissue sections (5 µm) were cut, mounted onto slides and stained with picrosirius red (0.1% Sirus red in saturated aqueous picric acid; Sigma Aldrich, USA) as described by Junqueira et al. (1979). Separate set of slides of the same experimental groups were stained with

haematoxylin and eosin (HXE; Sigma Aldrich, USA) and photographed (Leica DM 2000) at 100X and 400X.

Isolation of total mRNA

Cardiac tissue samples collected in RNA later stabilization solution were washed with DEPC water and total RNA was isolated using TRIzol reagent, similarly as mentioned in Chapter 2.

Gene expression studies of rat ventricular tissue

cDNA was synthesized by reverse transcription of total RNA (1 µg) using iScript cDNA Synthesis kit (BIORAD, USA). Further, mRNA levels of enzymatic antioxidants (superoxide dismutase; *sod* and *catalase*), apoptotic genes (*bax* and *bcl-2*), myocardium specific caveolae protein (*caveolin-3*), sarco/endopalsmic reticulum calcium ATPases (*SERCA2a*) and cardiac ankyrin repeat protein (*ANKRD1*) with *GAPDH* as an internal control were evaluated by quantitative PCR as elucidated herein.

Quantitative PCR analysis (QuantStudio 12K Flex, Life Technologies, CA, USA) was performed using SYBR Select Master Mix. The reaction mixture consisted of cDNA (0.8 µl), forward and reverse primers (0.4 µl each), SYBR green master mix (5 µl) and ultrapure water (3.4 µl). Melting curve of each sample was measured to ensure the specificity of the products. The data were normalized to the internal control *GAPDH* and analysed using $2^{-\Delta\Delta CT}$method. Primers were obtained from NCBI website. Primers used in this study are listed in Table-3.1 of Chapter 3 and primer other than that are listed below (Table-4.1).

Table 4.1: List of primer for mRNA expression study.

Gene Name	**Accession number**	**Forward Primer (5'→3')**	**Reverse Primer (5'→3')**
carp	NM_013220.1	cagccacaagagggaaaaacat	actgttggctggaagtgtctt
serca2a	NM_001110139.2	caacacatcttccagccctct	acttggctgatggcttctgtt

4.3 RESULTS

ISO induced changes in cardiosomatic index and plasma CK-MB levels

ISO treatment accounted for significant increment in heart: body weight ratio (HW:BW) and plasma CK-MB levels. However, these parameters were comparable to control and ARCE+ISO treated group (Figure 4.1A and B; Table 4.2).

ARCE mediated prevention of ISO mediated histopathological changes and apoptosis

TTC stained sections of ventricular tissue of control rats showed brick red color indicating healthy tissue whereas, that of ISO treated rats was pale in color with white (necrotic) patches. However, necrotic tissue was minimal in ARCE+ISO group (Figure 4.2A). Image analysis of TTC stained ventricular sections in ISO treated rats showed higher percentage of infarct areas compared to ARCE+ISO treated rats. Control group did not show any infarct tissue (Figure 4.2B). Ventricles of ISO treated rats showed hypertrophy and accounted for more thickness than the control. However, in ARCE+ISO treated rats, it was comparable to that of control (Figure 4.2C). Picrosirus red stained sections of ventricular tissue of ISO treated rats showed distorted arrangement of collagen fiber. While ARCE+ISO treated group showed intact collagen arrangement in ventricular tissue identical to that of control (Figure 4 .3A). HXE stained sections of ventricular tissue of ISO treated rats showed gross derangement of myocardial fibers. Whereas, ARCE+ISO treated group showed intact multinucleated fibers identical to that of control (Figure 4.3B). Pro-apoptotic gene (*bax*) showed upregulation following ISO treatment with decrement of anti-apoptotic gene (*bcl-2*). ARCE+ISO treatment could negate the increment of *bax* and decrement of *bcl-2* with levels comparable to control group (Figure 4.4A).

ARCE prevented ISO induced alterations in intracellular enzymatic antioxidant, calcium signaling, *caveolin-3* and *ANKRD1*.

mRNA levels of intracellular antioxidants (*sod* and *catalase*) were found to be significantly low in ISO treated rats but the same were significantly upregulated in ARCE+ISO group (Figure 4.4B). mRNA levels of *caveolin-3* and *SERCA2a* show a significant decrement in cardiac tissue following ISO treatment whereas, supplementation of ARCE prevented the said decrement as seen in Figure 4.5. Also, ISO treatment showed upregulation of cardiac ankyrin repeat protein 1 (*CARP/ANKRD1*) whereas, ARCE+ISO treatment could negate the increment of *ANKRD1* (Figure 4.5).

Statistical analysis

The data were expressed as mean ± SEM from two independent experiments, n=6 in each experiments and analyzed by one way analysis of variance (ANOVA) using Graph Pad Prism 3.0 (CA, USA). $P<0.05$ were considered to be significant. ***$P<0.001$ and **$P<0.01$ vs. control group; ###$P<0.001$, ##$P<0.01$ and #$P<0.05$ vs. ISO group.

4.4 FIGURES

Table 4.2: Values represent level of plasma CK-MB and cardiosomatic index of control and treated groups.

Groups	CK-MB (IU/L)	Cardiosomatic index (HW/BW)
Control	291.46	0.003872
ISO	1023.77	0.004975
ARCE+ISO	279.42	0.004292

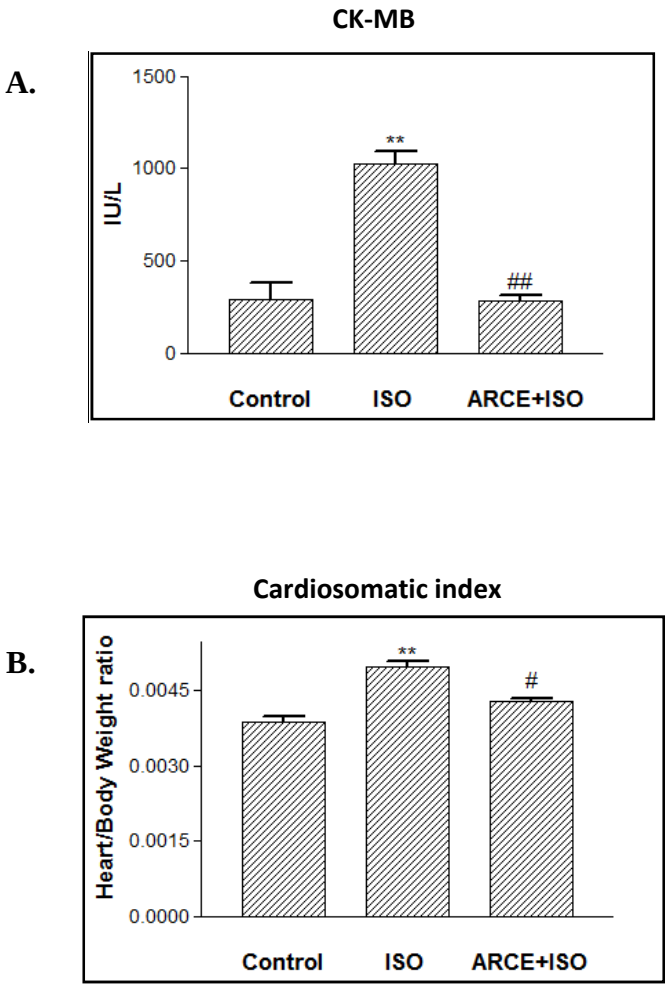

Figure 4.1: ARCE mediated regulation of ISO induced imbalance in (A) plasma CK-MB - Activity levels of plasma CK-MB of control and treated rats **(B) Cardiosomatic index -** Heart weight : body weight ratio of control and treated rats. Untreated rats were used as control. **P<0.01 vs. control group; ##P<0.01 and #P<0.05 vs. ISO group.

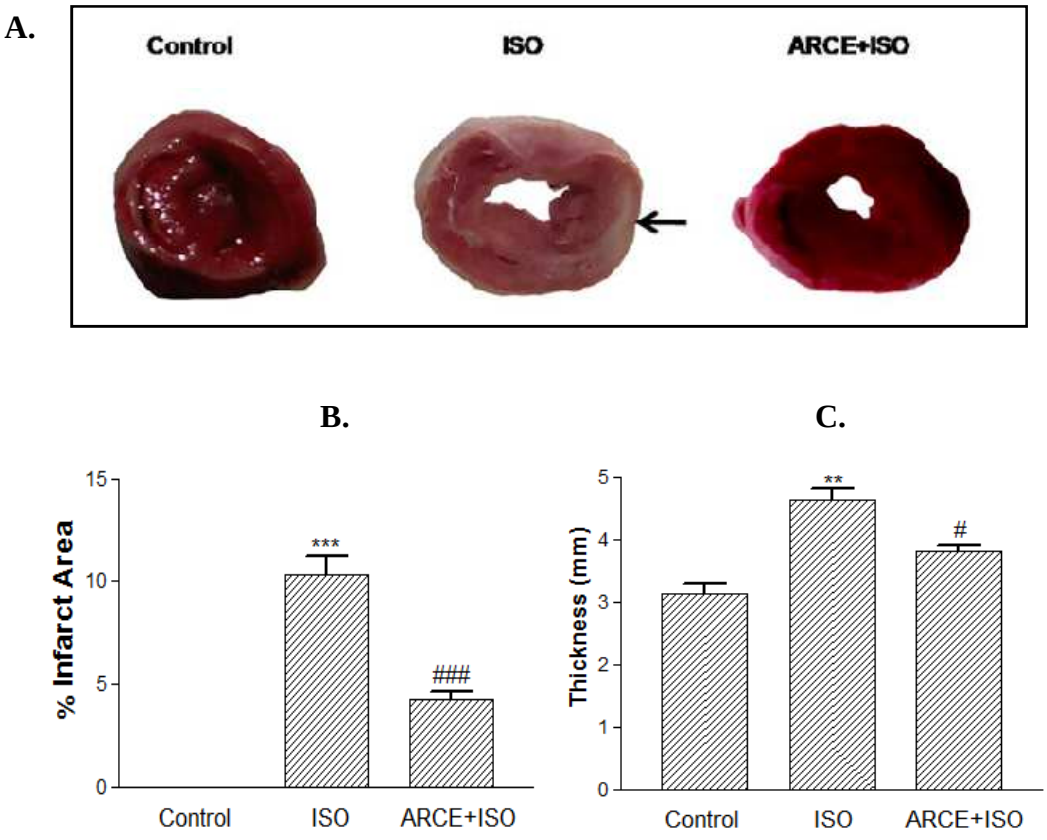

Figure 4.2: ARCE prevented ISO induced cardiac damage-Macroscopic observations. (A) Representative images of the ventricular tissue sections (control and treated) stained with TTC. Arrows indicate infarcted regions. **(B)** The plot represents % infarct area as measured from the TTC stained sections using Image J software. **(C)** Ventricular wall thickness (mm) of control and treated group. ***P<0.001 and **P<0.01 vs. control group; ###P<0.001, ##P<0.01 and #P<0.05 vs. ISO group.

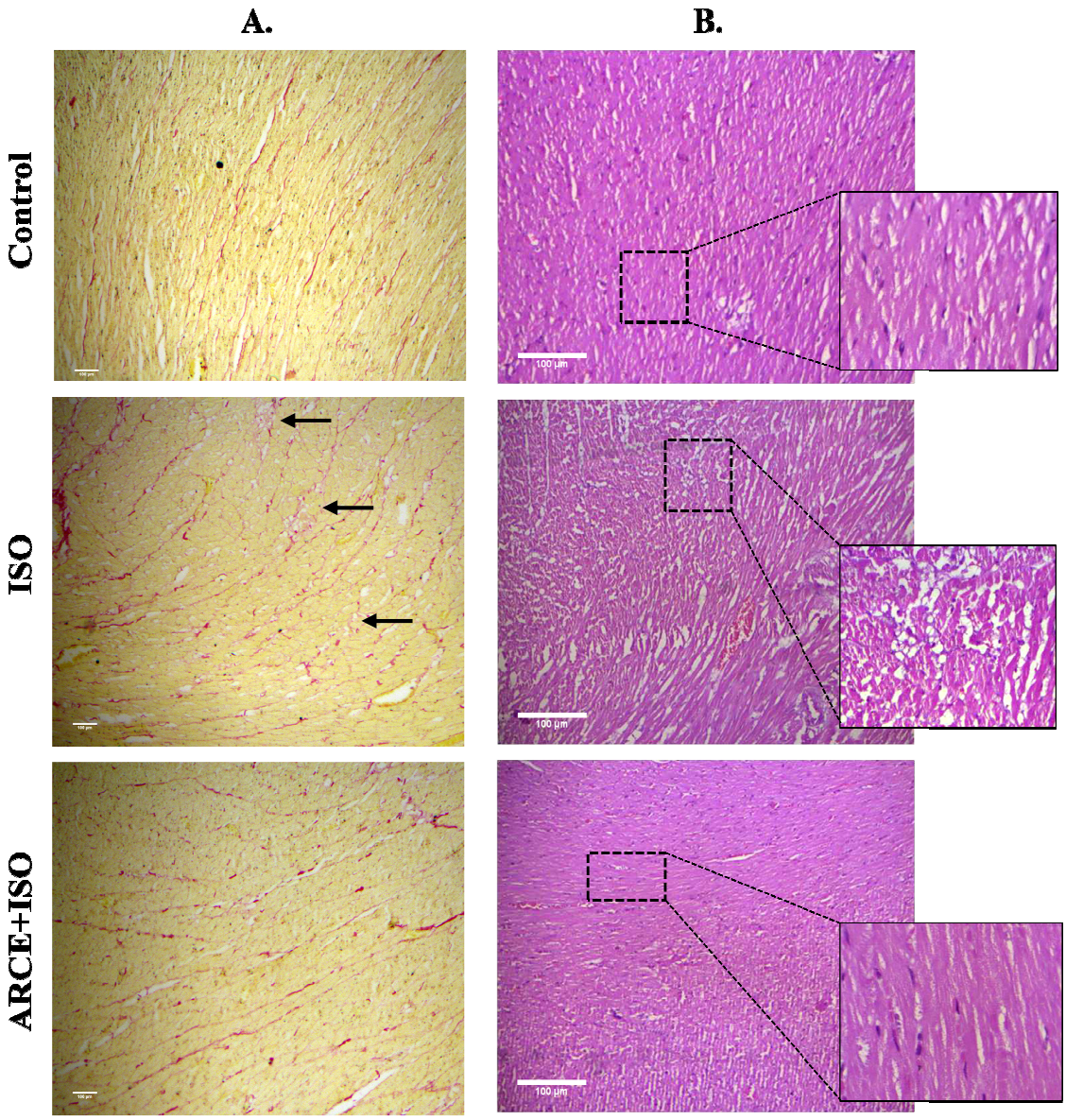

Figure 4.3: ARCE prevented ISO induced cardiac damage-Microscopic observations. (A) Photomicrograph of ventricular tissue sections stained with Picrosirus red. Collagen is stained red with pale yellow background and nuclei in brown to black. Arrow represents distorted arrangements of collagen **(B)** Representative images of the ventricular tissue sections stained with HXE. Magnification= 100X (400X for inset).

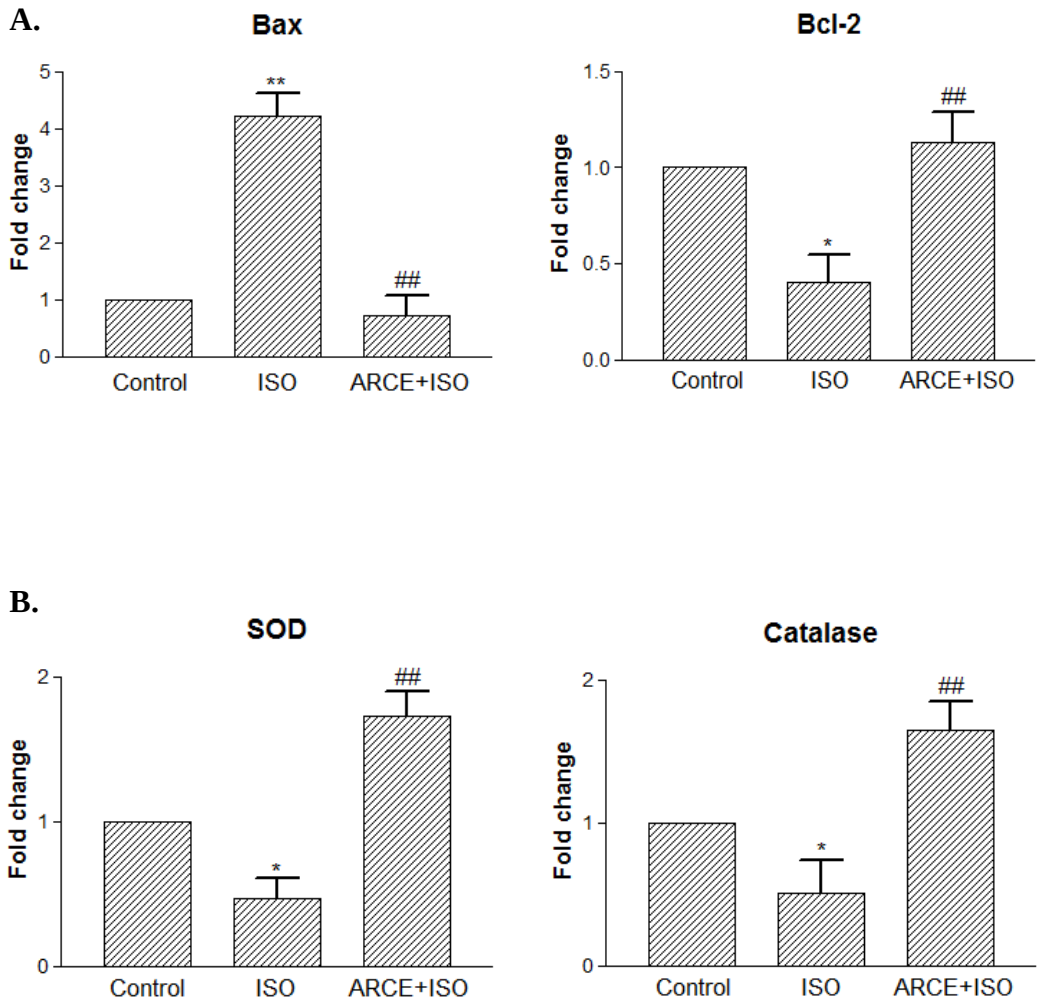

Figure 4.4: ARCE prevented ISO induced oxidative stress and apoptosis. mRNA levels of **(A)** *bax* and *bcl-2* **(B)** *sod* and *catalase* in cardiac ventricular tissue of control and treated rats, were analyzed by quantitative PCR. *P<0.05 vs. control group; ##P<0.01 vs. ISO group.

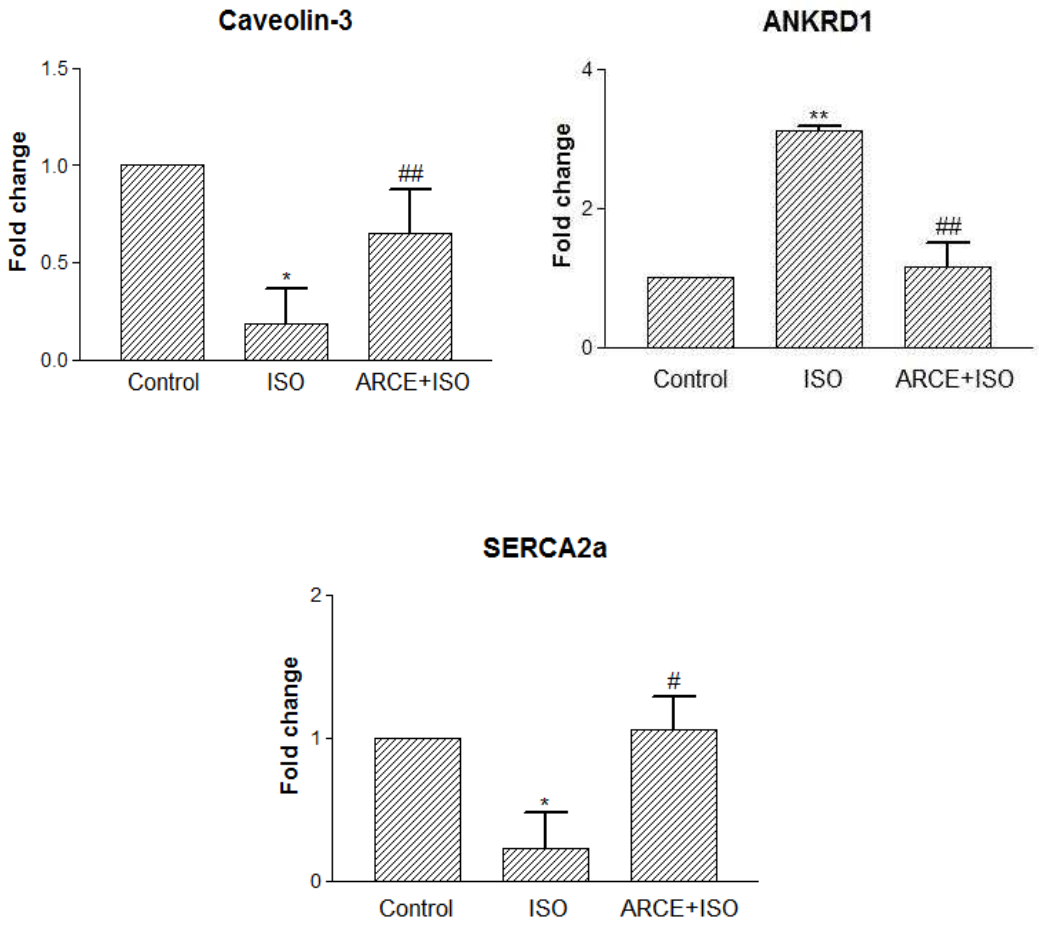

Figure 4.5: ARCE prevented ISO induced modulations in gene expressions in rat heart tissues. mRNA levels of *caveolin-3*, *ANKRD1* and *SERCA2a* in cardiac ventricular tissue of control and treated rats were analyzed by quantitative PCR. **P<0.01 and *P<0.05 vs. control group; ##P<0.01 and #P<0.05 vs. ISO group.

4.5 DISCUSSION

Plant anthocyanin have been extensively studied and reported for their therapeutic properties in human diseases but *in vivo* stability of anthocyanin is always a concern. However, anthocyanins from red cabbage have been reported to have *in vivo* stability as evidenced by the presence of its metabolic byproducts in human urine samples (Charron et al., 2007). Red cabbage is rich in acylated anthocyanins with strong antioxidant activity, stability and therapeutic properties (Wiczkowski et al., 2013)

Previous studies in our lab with safety evaluations of ARCE had revealed it to be non-toxic to H9c2 cells (10-100 µg/ml) and Swiss albino mice (1000-3000 mg/kg) (Devkar et al., 2012; Thounaojam et al., 2011). Keeping these findings as a background, the present study was initiated to decipher the underlying mechanism of ARCE mediated cardioprotection.

In rats, ISO had been reported to increase cellular oxygen demand, deplete ATP levels, cause calcium overload and undergo auto-oxidation leading to formation of free radicals (S. Bloom & D. L. Davis, 1972; Singal et al., 1982; J. C. Yeager & M. E. Whitehurst, 1982). Eventually, several metabolic products like quinones originated from ISO generates aminochrome such as adrenochrome from cathecholamine which further undergoes oxidation and leads to the formation of free radicals (Singal et al., 1982). These oxidized products are highly reactive and interact with sufhydryl group of proteins, DNA and lipids; with sequential changes in microsomal permeability, Ca^{2+} handling protein (SERCA2a), ATP levels, mitochondrial dysfunction and apoptosis.

In such a scenario, enzymatic antioxidants (*sod* and *catalase*) have been known to undergo degradation and subsequent exhaustion that furthers the magnimity of cellular oxidative damage (Nimse & Pal, 2015). SOD plays an important role in protecting the cells from oxidative damage by converting superoxide radicals into hydrogen peroxide, which is further metabolized by catalase to molecular oxygen and water. The decrease in the activity of these antioxidant enzymes might be due to myocardial cell damage. Superoxide radical formed at the site of damage, modulates SOD and catalase enzyme activity, which further results in accumulation of superoxide anion in myocardium.

According to the Loper et al. (1961) in both MI and unstable angina, there is increased production of lipid peroxidation and a transient inhibition of protective enzymes such as superoxide dismutase (SOD). Also, rats treated with ISO have been reported to undergo increase in heart weight due to increased water retention and oedema in intramuscular spaces culminating in extensive necrotic changes. Therefore, in cardiac tissue ISO induced decrease in activities of SOD and catalase, lipid peroxidation, membrane damage and leakage of CK-MB in plasma are markers of experimentally induced myocardial infarction (Padmanabhan & Prince, 2006). In our study, ARCE pretreatment was instrumental in providing cardioprotection as evidenced by heart: body weight ratio, decreased circulating levels of CK-MB, improved levels of enzymatic antioxidants (*sod* and *catalase*) and apoptotic markers (*bax* and *bcl*-2).

TTC is the redox indicator which gets enzymatically reduced to brick red precipitates of formazan dye or TPF (1,3,5-triphenylformazan) in metabolically active cells and tissues (Altman, 1976). Reduced coenzymes in active mitochondria is responsible for the reduction of TTC to TFP in all tissues including the cardiac tissue

(Ramkissoon, 1966). Collagen plays major role in structural organization of heart. Distorted arrangement of cardiac collagen fibers with loss of nuclei represents damaged cardiac tissue (Lattouf et al., 2014). In our study ISO treated rats recorded distorted collagen fibers whereas, in ARCE+ISO treated rats less distortion with intact nuclei was observed which is comparable to that of control rats. Cardiac tissue sample of ISO treated rats showed extensive derangement of cardiac syncitium (HXE staining) and prominent infarcted area (TTC staining). These changes were less pronounced in ARCE+ISO group that also corroborates with the higher levels of mRNA of *sod, catalase* and *bcl-2* in this group. Hence, ARCE contributes towards imparting overall cytoprotection to the myocardial tissue.

Signaling molecules involved in cardiac protection are known to exist within caveolae or interact directly with caveolins. Caveolin is a protein that functions as chaperones and forms little caveolae. Since, last 10 years it has been reported that (1) caveolae and the caveolin isoforms 1 and 3 are essential for cardiac protection from myocardial ischemia/ reperfusion injury, (2) stimuli for cardiac myocytes preconditioning including brief periods of ischemia/ reperfusion and exposure to volatile anesthetics, alter the number of membrane caveolae, and (3) cardiac myocyte specific overexpression of caveolin-3 can produce innate cardiac protection from myocardial ischemia/reperfusion injury (Li et al., 2006; Roth & Patel, 2011). In our study, ISO treatment lead to the deterioration of caveolin-3 expression level, while ARCE+ISO treatment prevented the down regulation of caveolin-3 expression. This result is attributable to the membrane stabilizing property of ARCE against damaging oxidative reactions (Duchnowicz et al., 2012).

Sarcoplasmic Reticulum Calcium ATPase cardiac isoform 2a (*SERCA2a*) play a critical role in the control of spatio-temporal patterns of intracellular calcium

signaling, that controls contraction, proliferation/ hyperthrophic growth and apoptosis. ISO mediated stimulation of β-AR receptor activates protein kinase A (PKA) that phosphorylates calcium channels and increases calcium overload within cytoplasm. Ryanodine receptor (RyR) increases net calcium load in cytoplasm due to its efflux from sarcoplasmic reticulum resulting in muscle contraction. SERCA2a is instrumental in restoring calcium in sarcoplasmic reticulum therefore, decrement in expression of SERCA2a increases cytoplasmic calcium load causing arrhythmia and myocardial damage (Bers, 2008; Lipskaia et al., 2009). This includes altered contraction, hypertrophic growth and apoptosis of cardiomyocytes that have been known to be reverted by upregulation of *SERCA2a*. Hence, merits of *SERCA2a* are also debated as a pharmacotherapeutic target in preventing myocardial infarction (Lipskaia et al., 2010). In the present study, ISO treated rat recorded decrement in *SERCA2a* expression whereas; the ones pre-treated with ARCE showed restored *SERCA2a* levels.

Muscle ankyrin repeat protein have dual nuclear-cytoplasmic localization in cell but differ in tissue origin. It consist of three members, cardiac ankyrin repeat protein 1 (CARP/ANKRD1) specific to cardiac tissue; Ankyrin repeat domain 2 (ANKRD2) specific to skeletal muscles and Diabetes-related ankyrin repeat proteins (DARP) equally present in both. Dual location of ANKRD1 was confirmed by its role in regulating mechanosensing machinery in cardiac myocytes by binding to the two myofibrillar proteins (Titin and myopalladin) within I-band of sarcomere, wherein after sensing stretch ANKRD1 translocates to nucleus and regulate gene transcription. Activation of sympathetic nervous system and chronic β1AR stimulation is involved in ANKRD1 mediated negative regulation of several cardiac gene expressions like myosin light chain, β-myosin heavy chain and cardiac troponin C. Thus, β1AR

stimulated overexpression of *ANKRD1* deteriorates contractile function. Similarly, in our study β1AR stimulated over expression of *ANKRD1* was evidenced in ISO treated rats and the ones pre-treated with ARCE showed restored *ANKRD1* levels.

Overall, this study concludes that ARCE is potent to regulate the ISO induced changes by improving the histopathological changes, intracellular enzymatic antioxidants, intracellular calcium signaling and regulating mechano-sensing machinery.

4.6 SUMMARY

Present study provides an insight into the molecular and cellular mechanism of ARCE mediated cardioprotection against ISO induced myocardial damage. ISO treatment resulted in decrement of intracellular enzymatic antioxidants (*sod and catalase*), scar tissue formation and apoptosis, wherein ARCE pretreatment prevented scar tissue formation and apoptosis with improved levels of the intracellular enzymatic antioxidants. Experimental evidences on alleviation of ISO induced modulations of *caveolin-3* and *SERCA2a* in cardiac tissue by ARCE explains its role in regulating membrane integrity and intracellular calcium signaling. Also, ARCE was found instrumental in preventing the increase in levels of *ANKRD1* and *bax*, thus explaining its role in regulating mechanosensing machinery and preventing apoptosis in cardiomyocytes.

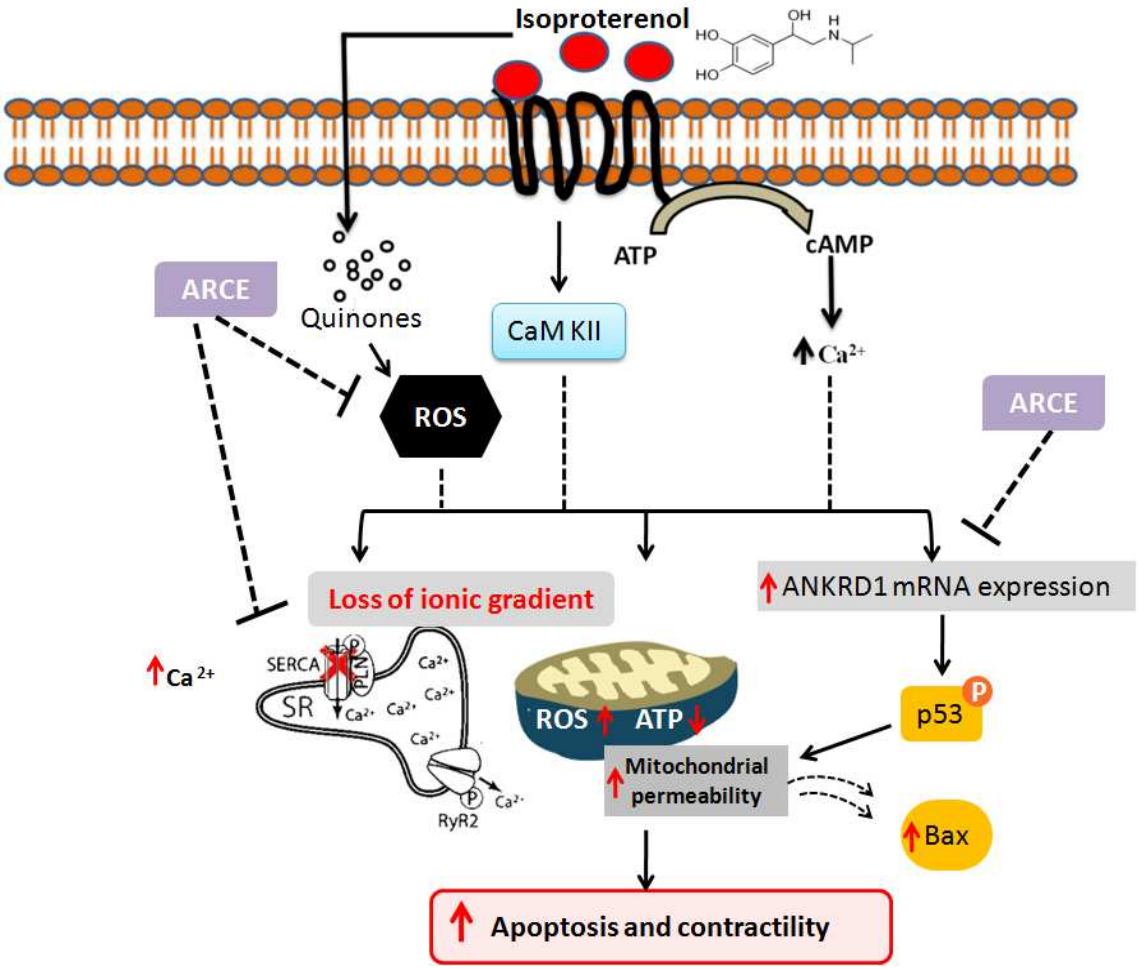

Figure 4.6: Graphical abstract depicting the mode of action of ARCE mediated prevention of experimental induced myocardial infarction.