Chapter 3

ARCE mediated alleviation of hydrogen peroxide induced oxidative damage and apoptosis in H9c2 cells

3.1 INTRODUCTION

Oxidative stress is a well-known factor promoting apoptosis and has been implicated in the pathogenesis of various diseases including myocardial infarction, ischemia/reperfusion injury, and heart failure. Apoptosis in cardiomyocytes is due to oxidative stress, intracellular Ca^{2+} overload, and mitochondrial dysfunction. Oxidative stress is usually defined as the non-homeostatic states wherein production of reactive oxygen species (ROS) exceeds cellular detoxification or utilization (Dhalla et al., 2000; Imlay, 2003). Abnormal accumulation of intracellular ROS may cause detrimental modification of important cellular macromolecules.

Epidemiological studies had reported about correlation between certain dietary habits and cardiovascular health. Hence, functionality of dietary components in improving the pathologies of CVDs can support the development of functional foods and nutraceuticals. Anthocyanins (a member of flavonoid family) are polyhydroxyl and polymethyl derivatives of flavynium salts and have been extensively reported from various natural sources. Also, anthocyanin had been reported for their therapeutic potentials against alzheimer (shih et al., 2010), hyperlipidemia (Qin et al., 2009), hyperglycemia (Guo et al., 2012), cardiovascular diseases (Hidalgo et al., 2012) and diabetic retinopathy (Shim et al., 2012).

H9c2 is well studied for the presence of ion channels and cardiac like phenotype to be used as a substitute to primary culture of cardiomyocytes (Branco et al., 2015; Hescheler et al., 1991; Reeve et al., 2005; Wang et al., 1999; Watkins et al., 2011; Zordoky & El-Kadi, 2007). H9c2 is widely used as valuable *in vitro* model to study the key targets involved in progression of cardiac damage or cardioprotection (Chen

et al., 2011; Shan et al., 2008); role of individual drug or functional group or crude extract as cardioprotectant (Cao et al., 2006; Sheng et al., 2010; Wang et al., 2007).

Based on the findings of other research groups and our lab it has been evidenced that RC extract has anti inflammatory, anti-hyperlipidemic and anticancer potentials and can improve hyperglycemia and hyperthyroidism. In these studies anthocyanin and their role has not been discussed in detail. H_2O_2 induced cellular oxidative stress is a widely used model to assess cytoprotective/therapeutic potential of a test extract to gather prima facie evidence (Chen et al., 2002; Ryter et al., 2007; Xing & Jian, 2011). H_2O_2 induced oxidative stress in H9c2 cells is also an accepted and widely used model to asses cardioprotective potential of test extracts (Eguchi et al., 2008; Han et al., 2004; Ihara et al., 2006; Oyama et al., 2011). The same was used in our study and the data is envisaged in this chapter. The rationale was based on high content of anthocyanin (Chapter 2) observed in previous study and to investigate the role of anthocyanin in RC extract as cardioprotectants.

Aim: *To decipher the mechanism of ARCE mediated protection against oxidative stress and apoptosis in H9c2 cells.*

3.2 MATERIALS AND METHODS

Preparation of extract

Same as mentioned in Chapter 2

Procurement and maintenance of H9c2 cells

H9c2 cells were procured from National Centre for Cell Science (NCCS, Pune) and maintained in T25 flasks (TPP, Switzerland) at 37°C with 5% CO_2 in DMEM (Hi-media Laboratories, Mumbai, India) containing 10% serum (Gibco, Invitrogen, USA) and 1% antibiotic-antimycotic solution (Hi-media Laboratories, Mumbai, India). Cells were trypsinized using 1X trypsin phosphate versene glucose (TPVG; Hi-media Laboratories, Mumbai, India) at three day interval.

The experimental groups for this study were: Control (untreated cells), ARCE treated, H_2O_2 (100 µM/ml) and ARCE+ H_2O_2 (pre-treated with ARCE for 24 h and then with H_2O_2 for 12 h).

Cell Viability assay

H9c2 cells were seeded in 96 well plate (1×10^4 cells per well) and allowed to grow overnight. Cells were pre-treated with ARCE (10, 50, 100, 250, 500 and 750 µg/ml) for 12 h or 24 h and dosed with 100µM concentration of hydrogen peroxide (Merck, USA) for 12 h. MTT (SRL Pvt. Ltd., Mumbai, India) was added to each well at concentration of 0.5 mg/ml and incubated in dark for 4 h and resulted insoluble purple formazan crystals were dissolved by adding 150 µl DMSO (SRL Pvt. Ltd., Mumbai, India). The absorbance was measured at 540 nm by using ELX800 universal

microplate reader (Bio-Tek instruments, Inc., Winooski, VT) and % cell viability was calculated.

Analysis of intracellular ROS in H9c2 cells

Intracellular ROS status was determined by staining with 0.0075 mM of 2', 7'-dichlorfluorescein-diacetate (DCF-DA; Sigma Aldrich, USA) for 30 minutes. DCF-DA a non-fluorescent probe get converted into highly fluorescent 2′,7′-dichlorofluorescein (DCF) stain upon oxidation with peroxyl free radical. H9c2 cells were seeded in a 6 well plate for overnight. Cells were pre-treated with ARCE for 24 h and then with 100 uM H_2O_2 for 12 h. After incubation media was removed, washed with PBS and stained with DCF-DA. Nucleus was counterstained with 0.6 ug/ml of DAPI (Sigma Aldrich, USA). Excess stain was removed by 4-5 washes of PBS and observed in Floid cell imaging station (Invitrogen, USA).

Analysis of mitochondrial membrane potential in H9c2 cells

Intracellular mitochondrial membrane potential ($\Delta\psi_m$) was assessed by Rhodamine 123 (RHO-123; Sigma Aldrich, USA) dye. RHO-123 is lipophilic cationic dye that equilibrate across membranes in a nernstian fashion and accumulate into the negative (i.e., more polarized) mitochondrial membrane matrix space. A more negative $\Delta\psi_m$ will accumulate more dye in the case of healthy cells, while in apoptotic cells depolarization lead to loss of dye. H9c2 cells were seeded in 6 well plate for overnight. Cells were pre-treated with ARCE and then with 100 uM H_2O_2 for 12 h. Media was removed after incubation and washed with PBS. After washing RHO-123 stain (10 ug /ml) was added, incubated for 15 min. Nucleus was counterstained with 0.6 ug/ml of DAPI. Excess stain was removed by 4-5 washes of PBS and observed in floid cell imaging station (Invitrogen, USA).

Apoptosis assay using Annexin V-PI staining

Annexin-V specifically binds to phosphotidylserine which get flipped to outer leaflet from cell membrane during early apoptosis and propidium iodide is a DNA binding dye which can only enter in damaged cell and bind to DNA. Cells (1×10^4) of various groups were trypsinized, centrifuged and washed with PBS. Control and treated cells were stained with annexinV-alexa 488 and propidium iodide (Invitrogen, USA) for 15 min at 37 °C in dark and subjected to flow cytometric analysis. The data were acquired using BD FACSAria™ III (BD Biosciences, USA).

Isolation of total mRNA

RNA (Ribonucleic acid) of each groups were isolated using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol. TRIzol Reagent is a ready-to-use reagent for isolating RNA from cells and tissues. It involves four steps viz. homogenization, phase separation, RNA precipitation and RNA wash. TRIzol works by maintaining RNA integrity during tissue homogenization and cell lysis. Briefly, growth media was removed and 1ml of trizol reagent for 1×10^5 - 1×10^7 cells was added in culture dish (Homogenization). After 5 min, 0.2 ml Chloroform was added to separate the cell lysate into aqueous and organic phases. Lysate from each group was incubated for 2-3 min and centrifuged at 12000 xg for 15 min (Phase seperation). Aqueous phase was collected and precipitated with 1ml isopropyl alcohol (RNA precipitation). Precipitated RNA was pellet down at 12000 xg for 15 min and washed with 75 % ethanol. Ethanol was removed by centrifugation at 7500 xg for 10 min and resultant pellet was air dried at 37°C in closed chamber to remove excess ethanol. Later, pellet was dissolved in RNase-free water and purity was detected by analysing 260/280 ratio in UV-Vis spectrometer (Bio-Tek instruments, Inc.,

Winooski, VT). OD at 260 nm was further used to calculate concentration of RNA (µg/µl) in sample using formula given below.

$$\frac{\boldsymbol{OD\ at\ 260\ nm \times 40 \times 200}}{\boldsymbol{1000}}$$

Where, 40 = extinction coefficient, representing 1 = 40 µg/ml of RNA
200 = dilution factor for 5 µl of RNA in 995 µl of RNase free water
1000 = factor for conversion from µg/ml to µg/µl

Gene expression studies in H9c2 cells

mRNA expression levels of intracellular enzymatic antioxidants (superoxide dismutase; *sod* and *catalase*), apoptotic and anti-apoptotic marker (*bax* and *bcl-2* respectively) and cardiac marker (*caveolin-3*) were studied using reverse transcriptase PCR (RT-PCR), wherein cDNA was synthesized by reverse transcription of 1 µg of total RNA using iScript cDNA synthesis kit (BIORAD, USA). GAPDH was used as internal control. PCR was carried out using dream taq green PCR master mix (Thermo Scientific, USA) in T100 thermal cycler (BIORAD, USA). Total volume was 25 µl: 2 µl c-DNA, 12.5 µl Dream Taq™, 1 µl of each primer and 8.5 µl ultrapure water. Cycler conditions were as follows: Initial denaturation at 95°C for 3 min followed by PCR cycle (35 cycles) of Denaturation at 95 °C for 30 sec, annealing at 55.4°C (*gapdh*), 53.1°C (*bax*), 55°C(*bcl-2*), 55°C (*caveolin-3*), 55.2°C (*catalase*), 53.4°C (*sod-2*) for 30 s and strand extension at 72 °C for 30 s and ends with final extension at 72 °C for 8-10 min. The PCR products were separated on ethidium bromide stained 2% agarose gel, imaged in Gel Doc^{TM} EZ (BIORAD, USA) and quantified by measuring band intensity using ImageJ software. Statistical analysis was done by using ANOVA. Primers were designed using NCBI website and listed in Table 1.

Table 3.1: List of primer used for mRNA expression study.

Gene Name	Accession number	Forward Primer (5'→3')	Reverse Primer (5'→3')
GAPDH	NM_017008.4	actttggcatcgtggaaggg	acttggcaggtttctccagg
sod	NM_ 017051.2	gacattgtgcctctgggttt	gccctgcatactttgtccat
catalase	NM_012520.2	gaggaaacgcctgtgtgaga	ttggcagctatgtgagagcc
bax	NM_0170592	gctggacactggacttcctc	ctcagcccatcttcttccag
bcl-2	NM_016993.1	tctcatgccaaggggaaac	tatcccactcgtagcccctc
caveolin-3	NM_019155.2	ggcacggatcatcaaggaca	acacgccatcgaagctgtaa

Statistical analysis

The data were expressed as mean ± SEM 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.

3.3 RESULTS

Cell viability assay

ARCE pre-treatment for 24 h significantly prevented H_2O_2 induced cell death in H9c2 cells similarly as untreated group (Figure 3.1).

H_2O_2 induced 50% cell mortality was reduced to 47-35% in a dose dependent manner by 12 h pre-treatment of H9c2 cells with ARCE (Figure 3.2A) while, 24 h pretreatment with ARCE reduced H_2O_2 induced cell mortality to 35-5% in a dose dependent manner (Figure 3.2B). Henceforth, 250 µg/ml of ARCE pre-treatment for 24 h was used for this study.

ARCE mediated prevention of H_2O_2 induced apoptosis in H9c2 cells

ARCE treatment accounted for less number of apoptotic cells (12.3%) as compared to H_2O_2 treated cells (57.3%). However, ARCE+H_2O_2 group accounted for a decrement in apoptosis (19.1%) comparable to that of control or ARCE treated cells (Figure 3.3A).

Also, in H_2O_2 treated group mRNA expression level of anti-apoptotic marker (*bcl-2*) decreases with an increase in apoptotic marker (*bax*). However, in ARCE+ H_2O_2 group upregulated levels of *bcl-2* was observed with decreased levels of *bax* and is comparable to that of control or ARCE group (Figure 3.3B).

ARCE mediated reduction of H_2O_2 induced intracellular oxidative stress in H9c2 cells

H_2O_2 treated cells showed green fluorescence due to free radical species mediated break down of DCF-DA in fluorescent compound DCF. However, no green fluorescence was observed in cells of ARCE+H_2O_2 group and control group (Figure 3.4A).

Also, H_2O_2 treated group recorded decrease in mRNA expression level of intracellular enzymatic antioxidants (*sod* and *catalase*). While, in ARCE+ H_2O_2 group *sod* and *catalase* levels were upregulated and were comparable to that of control or ARCE group (Figure 3.4B).

ARCE mediated regulation of H_2O_2 induced imbalance in mitochondrial membrane potential and caveolin-3 in H9c2 cells

In ARCE+H_2O_2 group, presence of intracellular green fluorescence evident active mitochondria and intact mitochondrial membrane potential this is comparable to that of control. While in H_2O_2 group loss of intracellular green fluorescence evident the loss of mitochondrial membrane potential. Corrected cell fluorescence was measured and quantified using ImageJ software and further data was analysed using one way ANOVA (Figure 3.5A).

Also, mRNA expression level of cardiac specific caveolin (*caveolin-3*) decreases in H_2O_2 treated group. While, in ARCE+ H_2O_2 group *caveolin-3* levels were upregulated and were comparable to that of control or ARCE group (Figure 3.5B).

3.4 FIGURES

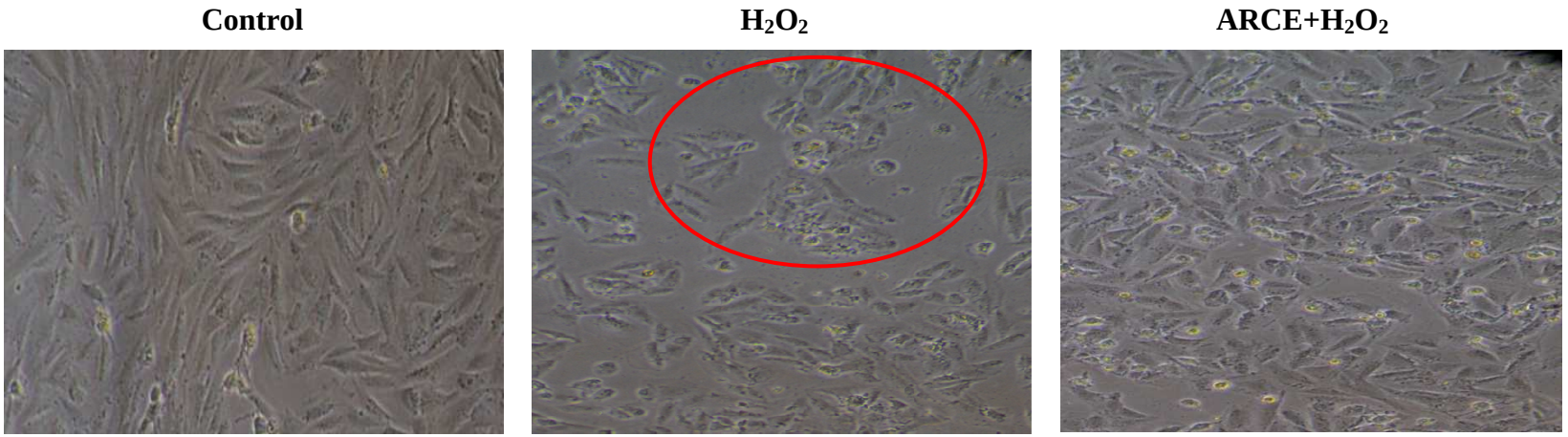

Figure 3.1: ARCE protects H9c2 cells from H_2O_2 induced cell death. Phase contrast imaging of H9c2 cells subjected to various treatment (magnification = 100X). Encircled area indicates the effect of H_2O_2 on H9c2 cells.

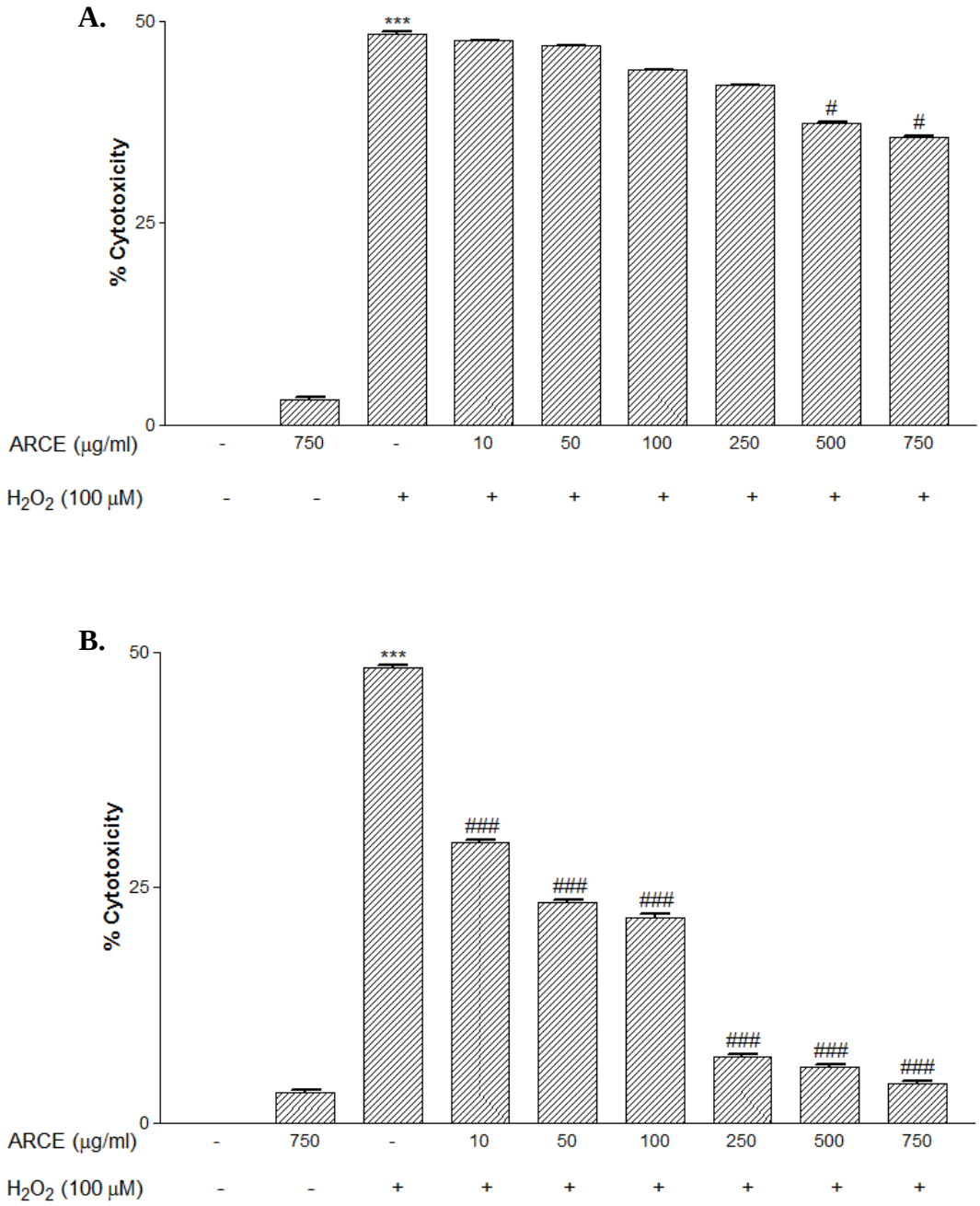

Figure 3.2: ARCE prevents H_2O_2 induced cytotoxicity in H9c2 cells. (A) H9c2 cells were treated with H_2O_2 for 12 h with or without pretreatment of ARCE for 12 h. **(B)** H9c2 cells were treated with H_2O_2 for 12 h with or without pretreatment of ARCE for 24 h. Untreated cells were used as control. % cytotoxicity was determined using MTT assay. ***P<0.001 vs. control group; ###P<0.001 vs. H_2O_2 group.

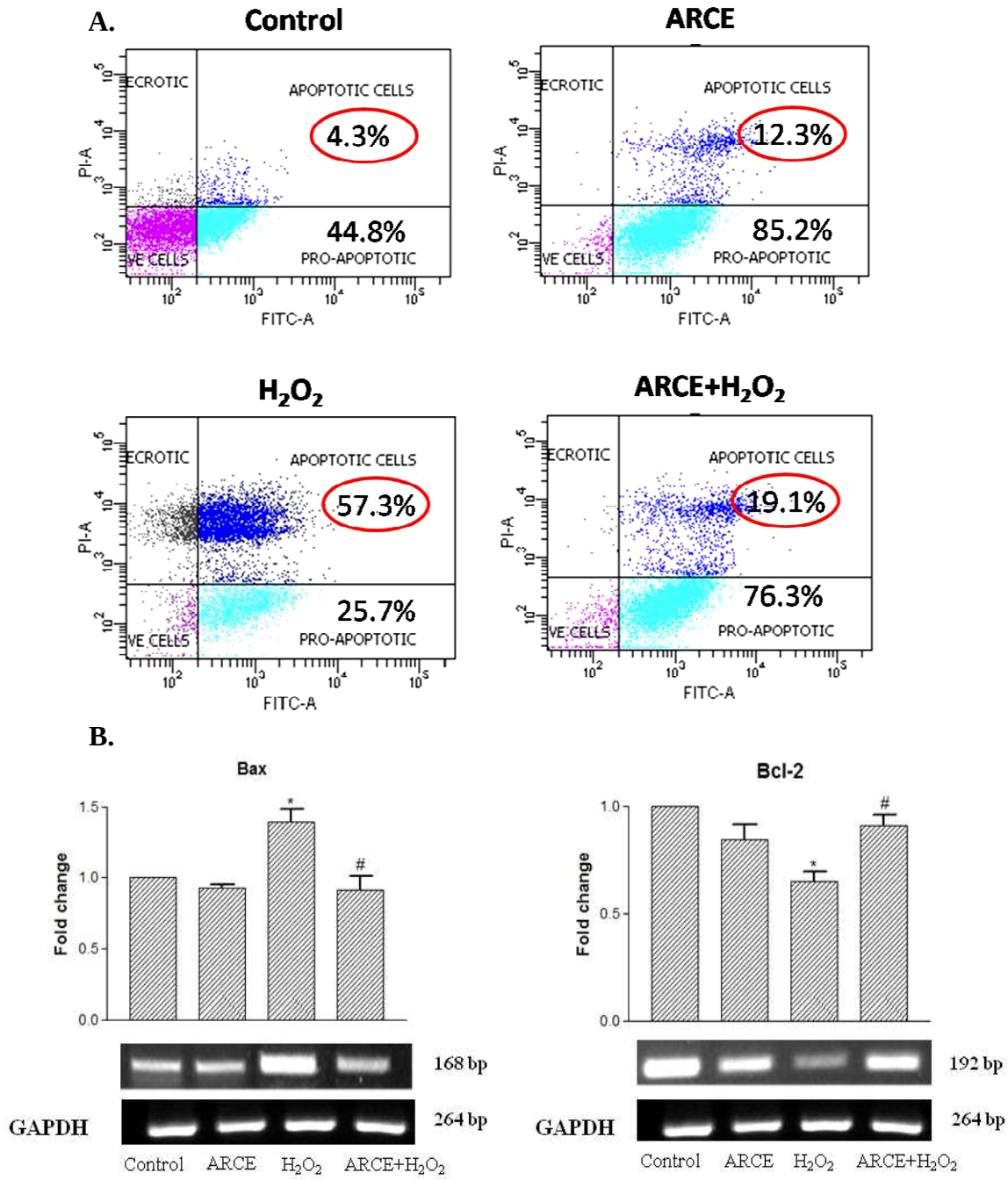

Figure 3.3: ARCE mediated prevention of H_2O_2 induced apoptosis in H9c2 cells. **(A)** H9c2 cells subjected to various treatment were stained with Annexin V-PI and analyzed by flow cytometry. Untreated cells were used as control. Double positive events indicate apoptotic cells encircled in red and double negative events indicate viable cells. **(B)** mRNA levels of pro-apoptotic (*bcl-2*) and anti-apoptotic (*bax*) genes in control and treated H9c2 cells. **P<0.01 and *P<0.05 vs. control group; #P<0.05 vs. H_2O_2 group.

A.

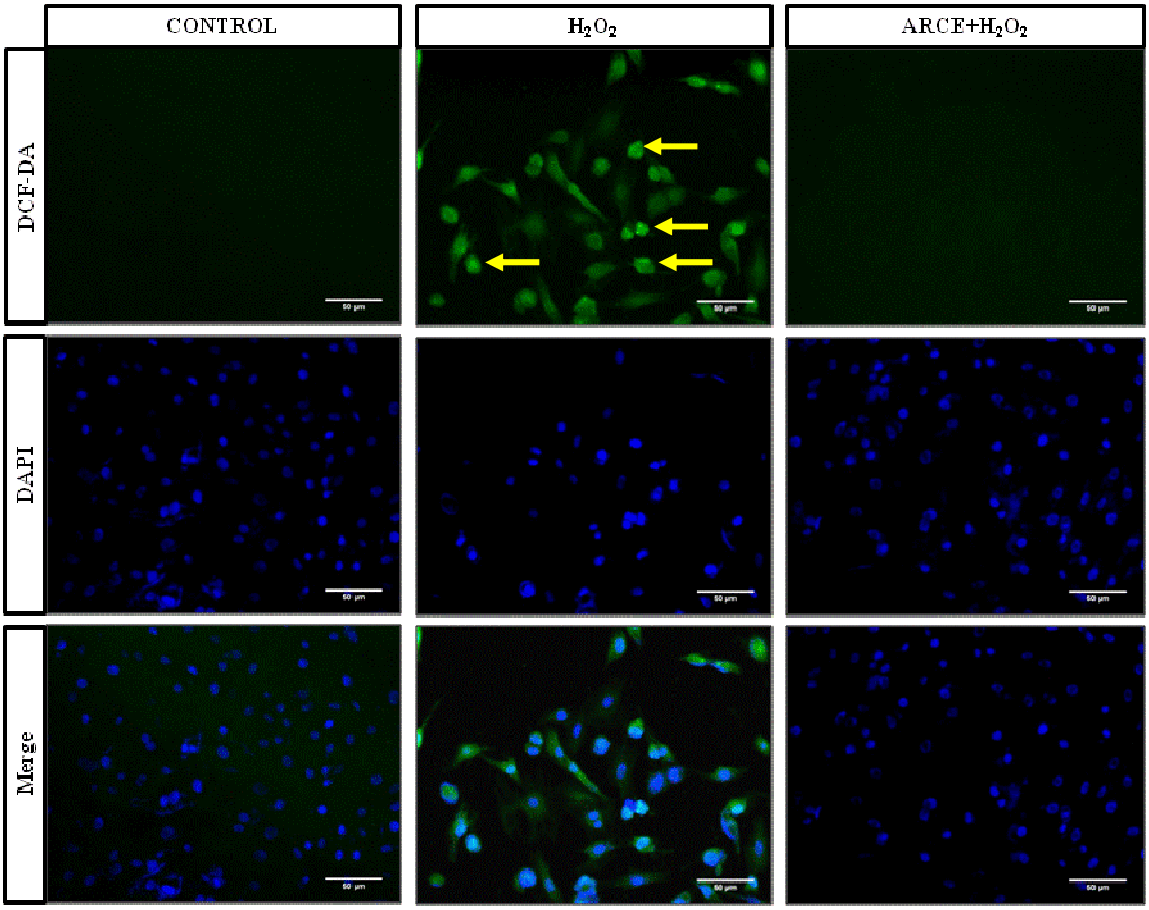

Figure 3.4: ARCE mediated reduction of H_2O_2 induced intracellular oxidative stress in H9c2 cells. (A) H9c2 cells subjected to various treatment were stained with DCF-DA and DAPI. Cells were imaged using Floid cell imaging station. Arrow represents the effect of H_2O_2 on H9c2 cells (DCF-DA positive cells). (Scale bar = 50µm)

B.

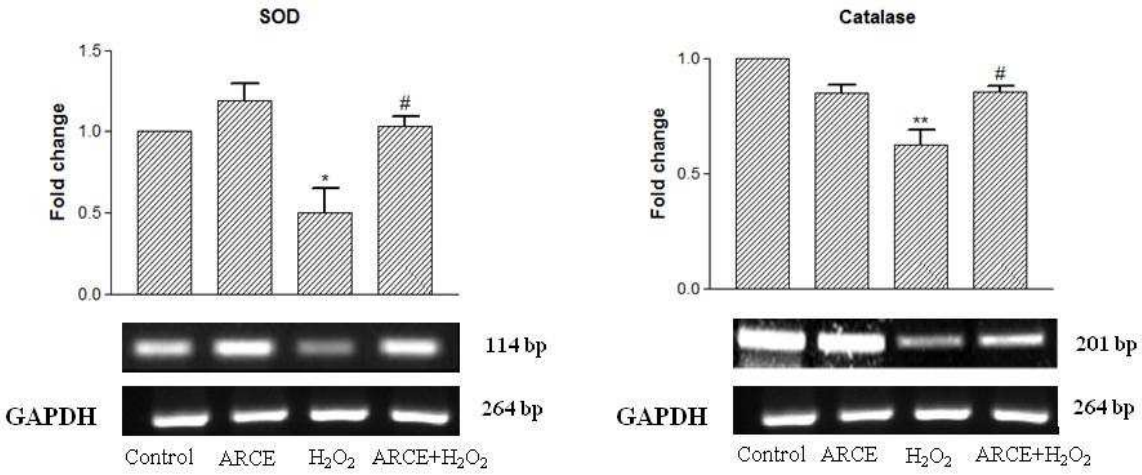

Figure 3.4: ARCE mediated reduction of H_2O_2 induced intracellular oxidative stress in H9c2 cells. (B) mRNA levels of antioxidant genes (*sod* and *catalase*) in control and treated H9c2 cells. **P<0.01 and *P<0.05 vs. control group; #P<0.05 vs. H_2O_2 group.

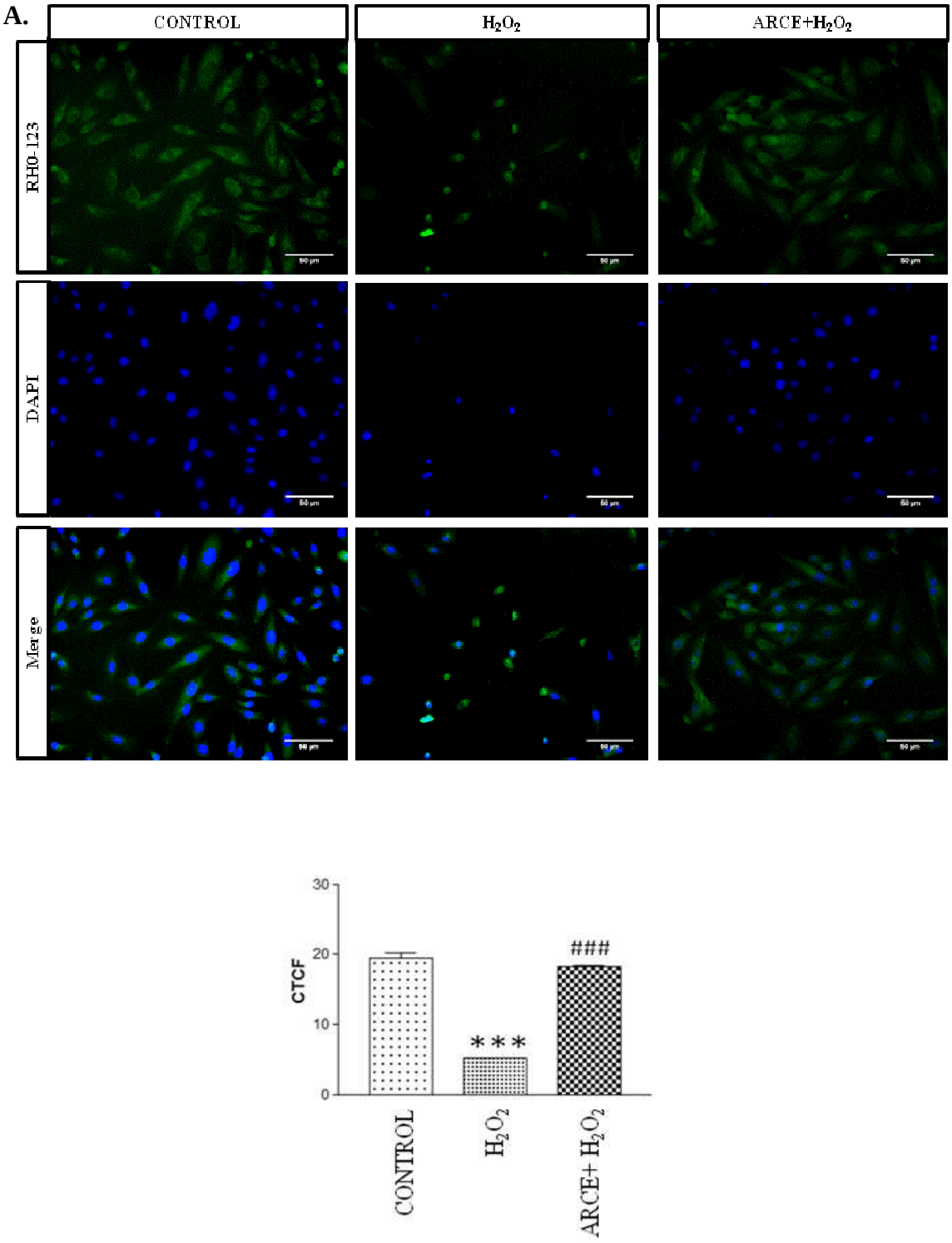

Figure 3.5: ARCE mediated regulation of H_2O_2 induced imbalance in mitochondrial membrane potential. (A) H9c2 cells subjected to various treatments were stained with RHO-123 and DAPI. Cells were imaged using floid cell imaging station. (Scale bar = 50µm). Corrected cell fluorescence (CTCF) was quantified using Image J software.

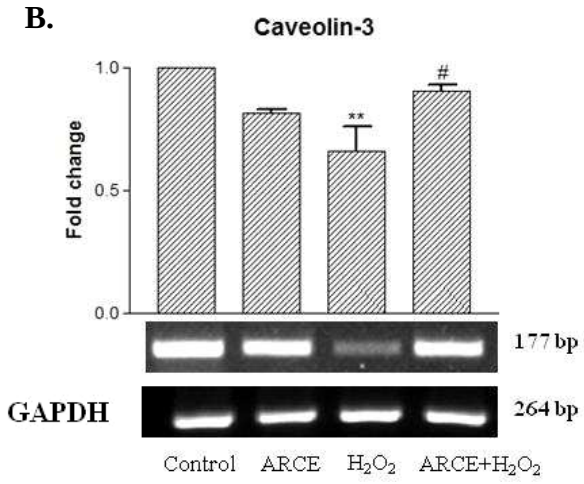

Figure 3.5: ARCE mediated regulation of H_2O_2 induced imbalance in *caveolin-3*. **(B)** mRNA levels of *caveolin-3* in control and treated H9c2 cells. ***P<0.001, **P<0.01 and *P<0.05 vs. control group; ###P<0.001 and #P<0.05 vs. H_2O_2 group.

3.5 DISCUSSION

Reactive oxygen species (ROS) are formed either enzymatically or non-enzymatically in mammalian cells and can cause cell damage by modulating cell signaling pathways (Dhalla et al., 2000). Endothelial cells, cardiomyocytes and neutrophils are the site for production of ROS in heart (Fabiani et al., 1993; Hess & Manson, 1984). Endogenous free radicals are by-products of the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase (XO), metabolism of the arachidonic acid and cytochrome P-450 (CYP) (Ambrosio et al., 1993; Griendling et al., 1994; Mohazzab-h et al., 1997; Thannickal & Fanburg, 1995). Stimulated production of ROS have substantial effect on predisposing conditions of cardaic heart failure (CHF) like ischemia/reperfusion injury, cardiac remodelling after myocardial infarction and left ventricular hypertrophy by causing cell damage and modulating intracellular signaling pathways (Finkel, 1999; Seddon, Looi, & Shah, 2007; Shah & Channon, 2004). Therefore, inhibition of oxidative stress mediated cardiomyocyte apoptosis is one of the key targets for devising therapeutic strategies.

ARCE was found to reduce ROS induced plasma protein carbonylation, oxidation and TBARS level of human plasma in dose dependent manner (5, 10 and 15 µM) (Kolodziejczyk et al., 2011). In our study, dose dependent decrease in H_2O_2 induced cytotoxicity was observed. Also, ARCE could prevent H_2O_2 induced intracellular oxidative stress and loss of mitochondrial membrane potential as evidenced by DCF-DA and RHO-123 staining respectively. These evidences explain the role of ARCE in preventing oxidative damage and cell death.

The Bcl-2 proteins are the major regulators of mitochondrial permeabilization, which includes pro-apoptotic (e.g., Bax and Bak) and anti-apoptotic (e.g., Bcl-2 and Bcl-xL) members. Bax undergoes oligomerization at the outer mitochondrial membrane and lead to the release of pro-apoptotic molecules in cytosol due to transmembrane pore formation, whereas bcl-2 forms heterodimers with a variety of pro-apoptotic proteins and prevent bax oligomerization (Kuwana & Newmeyer, 2003). In our study, H_2O_2 treatment recorded increase in *bax* expression levels with decrease in *bcl-2* expression level. However, ARCE pretreatment increased the expression of *bcl-2*, with the decrease in the expression of pro-apoptotic *bax* against H_2O_2 treatment. Hence, ARCE exerts protective effects by maintaining mitochondrial function and modulating the balance between anti-apoptotic *bcl-2* and pro-apoptotic *bax*.

The present study also showed that ARCE could prevent the decrease in intracellular enzymatic antioxidants (*sod* and *catalase*) of H9c2 cells against H_2O_2 treatment. While, alone ARCE treatment shows regulated levels of *sod* and *catalase* same as that of untreated group (Control). Similarly, Sankhari et al., (2012) and Majeed & Al-Azzawie 2012 had shown increase in plasma SOD and catalase levels after RC treatment in hyperthyroid and hyperlipidemic animal models. It seems that ARCE might play a role in modulating the expression of endogenous antioxidant enzyme (sod and catalase) or play a synergetic role with intracellular antioxidants against ROS and prevent the exhaustion of intracellular antioxidants due to ROS overload (Soengas et al., 2011).

Caveolae are being recognized as rather complex organelles with important role not only in endocytosis but also in lipid homeostasis, signal transduction, and tumorigenesis. In addition, they seem to play specific roles in distinct cell types,

making these structures one of the most interesting and multi-functional entities in cells. The caveolin protein family is composed of three distinct proteins, caveolin-1, -2 and -3. These proteins are expressed in tissues with a high abundance in caveolae; caveolin-1 and -2 are co-expressed in many cell types with especially high levels in endothelial cells, adipocytes, and type I pneumocytes; while caveolin-3 is exclusively expressed in skeletal and cardiac muscle cells. The main exception is smooth muscle cell, wherein intriguingly all three proteins are expressed (Rothberg et al., 1992; Scherer et al., 1996; Song et al., 1996; Tang et al., 1996). Previous scientific findings like: the inhibition of hypertrophy by adenovirus mediated overexpression of caveolin-3, association of caveolin-3 with the T-tubule biogenesis in sarcolemma and loss of caveolin-1 and caveolin-3 during aging and after myocardial infarction evidenced the role of caveolin-3 in cardioprotection (Koga et al., 2003; Parton et al., 1997; Patel & Insel, 2009; Ratajczak et al., 2003). In our study, H_2O_2 treatment altered the *caveolin-3* levels in H9c2 cells. Whereas, ARCE pretreatment improved the levels of *caveolin-3* in H9c2 cells as compared to the control group.

This study concludes that ARCE could modulate the expression of endogenous enzymatic antioxidants, minimize the deleterious effects on caveolin-3 and prevent oxidative stress induced apoptosis in H9c2 cells.

3.6 SUMMARY

Present study demonstrates that pre-treatment with ARCE protects H9c2 cells against H_2O_2 induced apoptosis by decreasing ROS generation, preventing the loss of mitochondrial membrane potential and inhibiting expression of *bax*. Furthermore, ARCE maintained mitochondrial function by regulating *bcl-2* levels and by improving the levels of *sod* and *catalase.* Also, ARCE was found to be potent in preventing H_2O_2 induced damage on *caveolin-3* which is cardiac specific caveolae protein and involved in signaling process.