CHAPTER 3

Protective Effects of *Anethum graveolens* L. seeds extract against H_2O_2 induced Oxidative Stress in Rat Cardiac H9C2 Cells

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INTRODUCTION

An imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage, is termed as 'oxidative stress'. Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions (Sies, 1997 and Rahal *et al*., 2014). Antioxidant defense involves several strategies, both enzymatic and non-enzymatic (Nimse and Dilipkuma, 2015). Oxidative stress has been proven in the pathogenesis of many human diseases such as acute myocardial infarction, atherosclerosis, cancer, diabetes, liver damage, rheumatoid arthritis, cataract, Parkinson's disease, motor neuron disease and conditions associated with premature birth (Sádabaa *et al*., 2016 and Hosseinia and Rajabianba, 2016). Oxidative stress can activate apoptotic pathways via various mechanisms such as lipid peroxidation, oxidation of DNA and proteins (Ghorbani *et al*., 2015; Asadpour *et al*., 2014 and Hosseinia and Rajabianba, 2016).

H9C2 cardiomyoblasts are clonal cell line derived from embryonic BD1X rat heart tissue (Kimes and Brandt, 1976 and Witek *et al*., 2016) which exhibits many of the properties of skeletal muscles. H9C2 also has several characteristics of cardiac muscles such as electrophysiology, ion channels, biochemical properties of both skeletal and cardiac muscles (Branco *et al*., 2012 and, Hosseinia and Rajabianba, 2016) except beating property. Hence, H9C2 cells have been used as model in several *in vitro* cardiac studies and cardiac tissues developmental studies (Zykova *et al*., 2010).

H_2O_2 is known to produce reactive oxygen species (ROS) and is used as a toxicant to mimic oxidative stress-induced injury in *in vitro* studies. H_2O_2 can trigger mitochondrial dysfunction via oxidative stress that in turn decreases Bcl-2 with the release of cytochrome C and activation of caspases (Yang *et al*., 2008) leading to apoptosis. Antioxidants might be effective to prevent or may delay ROS-induced apoptosis in these cells.

In our previous study, we had reported cardioprotective property of *Anethum graveolens* L. seed aqueous extract (AG) due to lipid lowering and free radical scavenging activity in isoproterenol induced myocardial infarction in rat. The aim of the present work was to study the protective effects of the *Anethum graveolens* L. seed aqueous extract against H_2O_2 induced oxidative damage in H9C2 cells.

MATERIALS AND METHODS

Chemicals

Propidium iodide (PI), Rhodamine 123, 4',6-diamidino-2-phenylindole (DAPI) and 2',7' –dichlorofluoresceindiacetate (DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) was purchased from Merck (Darmstadt, Germany). Fetal bovine serum (FBS) was purchased from Biosera (Ringmer, East Sussex UK). Dulbeco's Modified Eagle Medium (DMEM), Trypsin Phosphate Verses Glucose (TPVG) solution, antimycotic-antibiotic solution and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Dimethyl sulfoxide (DMSO) was purchased from the Sisco Research Laboratories Pvt. Ltd. (Mumbai, India).

Preparation of Extract

As shown in chapter 1.

Cell Culture and Treatment Protocol

H9C2 cell line was obtained from National Centre for Cell Sciences, Pune, India. Cells were seeded (1.0 $x10^5$ cells/ml in T-25 Flask) and cultured in DMEM containing 10% fetal bovine serum and 1% antimycotic-antibiotic solution at 37°C. Sub-culturing of cardiomyoblasts was performed at every third day by trypsinization using 0.25% TPVG solution. Media and all the required reagents used for experiment and sub culturing were filtered through 0.22 μm filter (Laxbro Bio-Medical Aids Pvt. Ltd.) prior to use.

Confluent H9C2 cells were treated with 100 mM H_2O_2 in presence or absence of AG extract (10–150 µg/ml) for 24 h and used for further analysis.

Cytotoxicity Assay by MTT

H9C2 cells (7.0 x 10^3cells/well) were plated in 96 well cell culture plates (Tarson India Pvt. Ltd) for a period of 24 h as mentioned earlier. Cells were treated with various concentrations of AG extract for 24 h. At the end of incubation period, 10 µl of MTT (5 mg/ml) was added to each well. Cells were incubated for 4 h at 37°C. After 4 h incubation period, the culture media was removed and all the wells were washed with phosphate buffer saline. After washing, 100 µl of DMSO was added to each well and incubated for further 30 min. Absorbance was read at 540 nm in ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT) and cell viability was calculated (Devkar *et al*., 2012).

Lactate Dehydrogenase (LDH) Release Assay

H9C2 cells (7.0 x10^3 cells per well) were plated in 96 well cell culture plates. Cells were treated with H_2O_2 in presence and absence of AG extract for 24 h as mentioned earlier. After incubation, supernatant from each well was collected in 1 mL centrifuge tubes and activity levels of LDH were assayed using commercially available kit (Reckon Diagnostics Pvt. Ltd., Baroda, India). Readings were recorded on Merck micro lab L300 semi autoanalyzer.

Lipid Peroxidation (LPO) Assay

H9C2 cells (1.0 x 10^5 cells per well) were seeded in 6 well cell culture plates (Tarson India Pvt. Ltd.) and treated with H_2O_2 in presence and absence of AG extract for 24 h as mentioned earlier. Subsequently, cells were harvested using TPVG solution and collected in 2 ml centrifuge tubes. Levels of malondialdehyde (MDA) accumulated were assayed using thiobarbituric acid–trichloro acetic acid–hydrochloric acid reagent (Buege and Aust, 1978) in cardiomyoblasts cell suspension.

Intracellular Reactive Oxygen Species (ROS) Generation

H9C2 cells (1.0 x 10^5 cells per well) were grown on cover slips in 12 well cell culture plates. Cells were treated with H_2O_2 in presence and absence of AG extract for 24 h as mentioned earlier. After 24 h, adhering cells were washed with DPBS and incubated with 7.5 mM CM-H_2DCFDA (5-(and-6)-chloromethyl-20, 70-dichlorodihydrofluoresceindiacetate, acetyl ester) at 37°C for 30 min in dark. After incubation period, cardiomyoblasts were observed under fluorescent microscope (Leica DMRB florescence microscope) (Jadeja *et al*., 2011).

Mitochondrial Membrane Potential (MMP)

Mitochondrial membrane potential of cardiomyoblasts was measured according to Patel *et al*., 2012 using the fluorescent cationic dye Rhodamine 123 (rho123). H9C2 cardiomyoblasts (1.0 x 10^5 cells/well) were plated in 6 well culture plates. Cells were treated with H_2O_2 in presence and absence of AG extract for 24 h as mentioned earlier. After treatment period, cells were incubated with 1 mM rho123 for 10 min at 37°C. The

fluorescence intensity was determined using spectrofluorometer (Jasco FP-6350) at excitation and emission wavelengths of 485 and 530 nm, respectively.

Acridine Orange/Ethidium Bromide Staining

H9C2 cells (1.0 x 10^5 cells per well) were seeded in 6 well cell culture plates (Tarson India Pvt. Ltd.) and treated with H_2O_2 in presence and absence of AG extract for 24 h as mentioned earlier. After treatment, cardiomyoblasts were collected using TPVG solution in 15 mL centrifuge tubes and mixed with one µl of acridine orange (AO) and ethidium bromide (EB) dye mixture (1 mg/ml AO and 1 mg/ml EB in phosphate buffer saline). Cell suspension of different treatment groups was immediately examined under Leica DMRB florescence microscope and photographed (Devkar *et al*., 2012).

Propidium Iodide (PI) Staining

H9C2 cells (1.0 x10^5 cells per well) were grown on glass cover slips in 12 well cell culture plate and treated with H_2O_2 in presence and absence of AG extract for 24 h as mentioned earlier. After treatment period, cells were washed with ice cold DPBS twice and incubated with PI staining solution (5 µg/ml in 10 mmol/L PBS) for 3-5 minutes at room temperature in dark. Cells were observed under Leica DMRB florescence microscope and photographed (Canon cybershot 72 digital camera) (Patel *et al*., 2012).

Statistical Analysis

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni's multiple comparison tests. The results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, SanDiego California USA.

RESULTS

Cytotoxicity Assay

Cytotoxicity assay of H9C2 cells with AG extract (10, 20, 50, 100, and 150 μg/mL) revealed a non-significant alteration in cell viability as compared to the control group. However, H_2O_2 treated cell showed significant reduction in the cell viability upto 17.60% as compared to the control group (untreated cells). However, AG extract significantly reduced the effect of H_2O_2 treatment in form of dose dependent improvement in cell viability (Table 3.1a and 3.1b; Figure 3.1a and 3.1b).

LDH Release Assay

LDH level was significantly increased in the supernatant of H9C2 cells treated with H_2O_2 as compared to untreated cells. However, it was significantly reduced in presence of AG in dose dependent manner. The highest AG treatment (150 μg/ml) showed the most significant decrement ($p<0.001$) in LDH release as compared to cells treated with H_2O_2 alone (Table 3.2; Figure 3.2).

Lipid Peroxidation Assay

H9C2 cells treated with H_2O_2 showed significant increment ($p<0.001$) in LPO levels as compared to untreated cells. However, H_2O_2+AG treated cells showed dose dependent decrement in LPO levels and it was highest in cells treated with 150 μg/ml of AG as compared to H_2O_2 alone treated cells ($p<0.001$) (Table 3.3; Figure 3.3).

Mitochondrial Membrane Potential

Mitochondrial membrane potential ($p<0.001$) was significantly reduced with H_2O_2 treated H9C2 cells as compared to untreated cells (control group). However, this decrement was significantly reduced with AG treatment. The highest dose (150 μg/ml) accounted for significant increment ($p<0.001$) compared to H_2O_2 treated cells (Table 3.4; Figure 3.4).

Intracellular Reactive Oxygen Species (ROS) Generation

The fluorescence microscopy data showed that the fluorescence intensity of DCF-DA was significantly increased in cells treated with H_2O_2. Increased fluorescence is an indicator of increased O_2^{-} and NO radicals, compared to the control. The enhanced fluorescence intensity of DCF-DA was significantly reduced by AG treatment as compared to H_2O_2 treated cells (Figure 3.5).

Acridine Orange/Ethidium Bromide Staining

Acridine orange/ethidium bromide (AO/EB) staining of H_2O_2 treated H9C2 cells showed higher number of EB positive (red) and lesser number of AO positive (green) cells as compared to the control wherein, the highest number of AO positive (green) cells were observed in control group. AG treatment to the cells showed lesser number of EB positive and more AO positive cells which is an indicator of its protective effects (Figure 3.6).

Propidium iodide Staining for Nuclear Morphology

H_2O_2 treated H9C2 cells showed higher amount of nuclear condensation and fragmented nuclei, whereas H_2O_2+AG treatment resulted in less number of cells with condensed or fragmented nuclei as compared to H_2O_2 treated cells. The highest concentration of AG extract seemed to be very effective in H_2O_2 mediated cellular damage (Figure 3.7).

TABLES AND FIGURES

Table 3.1: Effects on Cell Viability of H9C2

3.1 a: The Cell Viability of H9C2 Cells Treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 24 h

Concentrations of AG Extract (µg/ml)	% Cell Viability
0	100.00 ± 0.10
10	99.60 ± 0.76^{ns}
20	99.27 ± 0.67^{ns}
50	99.61 ± 0.64^{ns}
100	100.00 ± 0.60^{ns}
150	99.35 ± 0.86^{ns}

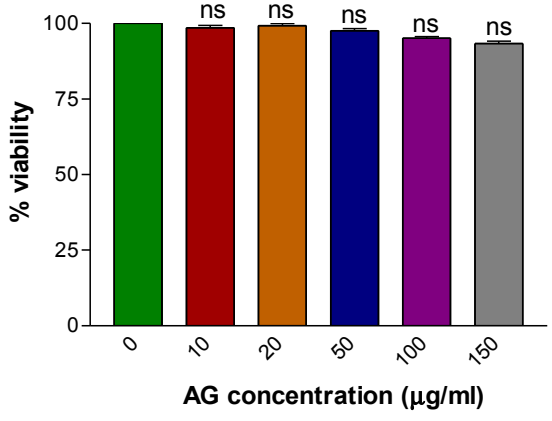
3.1 b: The Cell Viability of H9C2 Cells Pre-treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 3 h and then treated with 100 µM of H_2O_2 for 24 h

Concentrations of AG Extract + H_2O_2	% Cell Viability
0 µg/ml + 0 µM	100.00 ± 0.10
0 µg/ml + 100 µM	17.60 ± 0.62$^{###}$
10 µg/ml + 100 µM	26.27 ± 0.47***
20 µg/ml + 100 µM	28.62 ± 0.47***
50 µg/ml + 100 µM	41.09 ± 0.52***
100 µg/ml + 100 µM	60.35 ± 0.76***
150 µg/ml + 100 µM	87.43 ± 1.07***

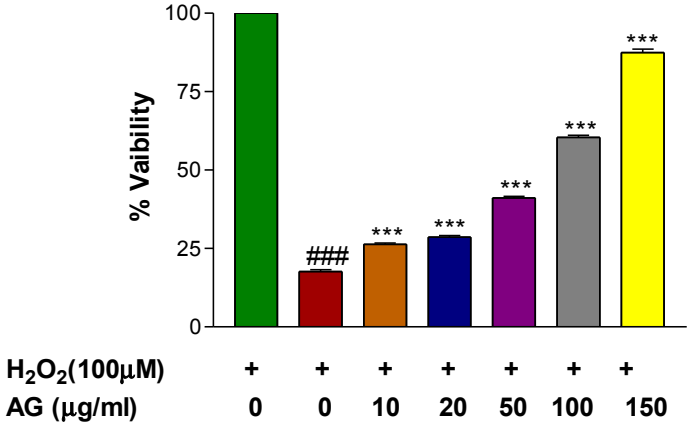
Results are expressed as mean ± S.E.M for n=3. Where, ns = non-significant, ### p< 0.001 compared to cells deprived of H_2O_2 and AG. **p < 0.01 and ***p < 0.001 compared to cells treated with H_2O_2.

Figure 3.1: Effects on Cell Viability of H9C2

3.1 a: The Cell Viability of H9C2 Cells Treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 24 h



3.1 b: The Cell Viability of H9C2 Cells Pre-treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 3 h and then Treated with 100 µM of H_2O_2 for 24 h



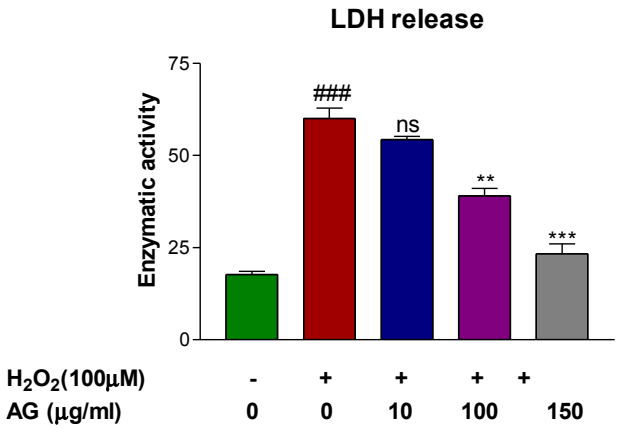
Results are expressed as mean ± S.E.M for n=3. Where, ns = non-significant, ### p< 0.001 compared to cells deprived of H_2O_2 and AG. ***p < 0.001 compared to cells treated with H_2O_2.

Table 3.2: LDH Release from H9C2 Cells Pre-treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 3 h and then treated with 100 µM of H_2O_2 for 24 h

Concentrations of AG Extract + H_2O_2	Enzyme Activity
0 µg/ml + 0 µM	17.67 ± 0.88
0 µg/ml + 100 µM	$60.00 \pm 2.88^{###}$
10 µg/ml + 100 µM	54.33 ± 0.88^{ns}
100 µg/ml + 100 µM	$39.00 \pm 2.08^{**}$
150 µg/ml + 100 µM	$23.3 \pm 2.72^{***}$

Results are expressed as mean ± S.E.M for n=3. Where, ns = non-significant, ### $p < 0.001$ compared to cells deprived of H_2O_2 and AG. **$p < 0.01$ and ***$p < 0.001$ compared to cells treated with H_2O_2.

Figure 3.2: LDH Release from H9C2 Cells Pre-treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 3 h and then treated with 100 µM of H_2O_2 for 24 h



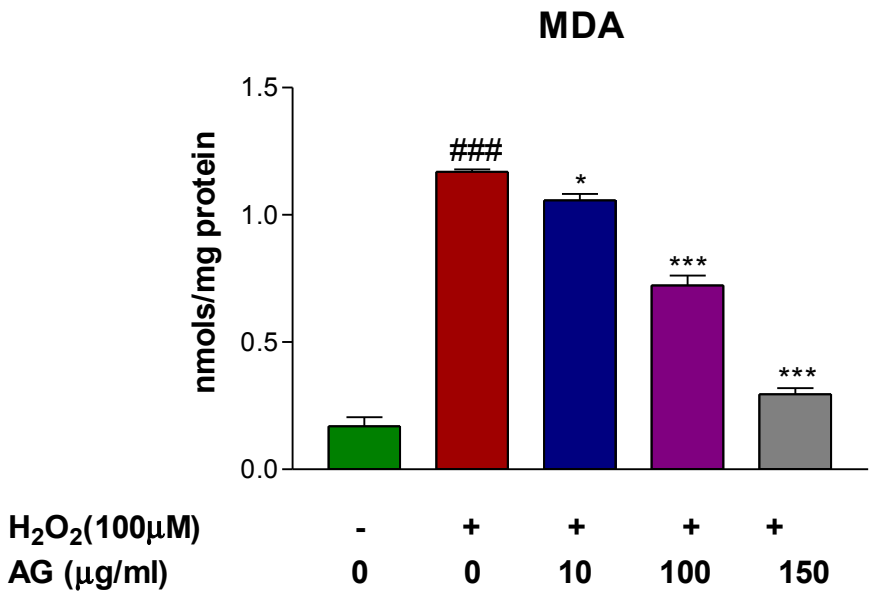
Results are expressed as mean ± S.E.M for n=3. Where, ns = non-significant, ### p< 0.001 compared to cells deprived of H_2O_2 and AG. **p < 0.01 and ***p < 0.001 compared to cells treated with H_2O_2.

Table 3.3: Lipid Peroxidation levels in H9C2 Cells Pre-treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 3 h and then treated with 100 µM of H_2O_2 for 24 h

Concentrations of AG Extract + H_2O_2	nmols/mg Protein
0 µg/ml + 0 µM	0.16 ± 0.03
0 µg/ml + 100 µM	1.16 ± 0.01$^{###}$
10 µg/ml + 100 µM	1.05 ± 0.02*
100 µg/ml + 100 µM	0.72 ± 0.03***
150 µg/ml + 100 µM	0.29 ± 0.02***

Results are expressed as mean ± S.E.M for n=3. Where, ns = non-significant, ### p< 0.001 compared to cells deprived of H_2O_2 and AG. **p < 0.01 and ***p < 0.001 compared to cells treated with H_2O_2.

Figure 3.3: Lipid Peroxidation levels in H9C2 Cells Pre-treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 3 h and then treated with 100 µM of H_2O_2 for 24 h



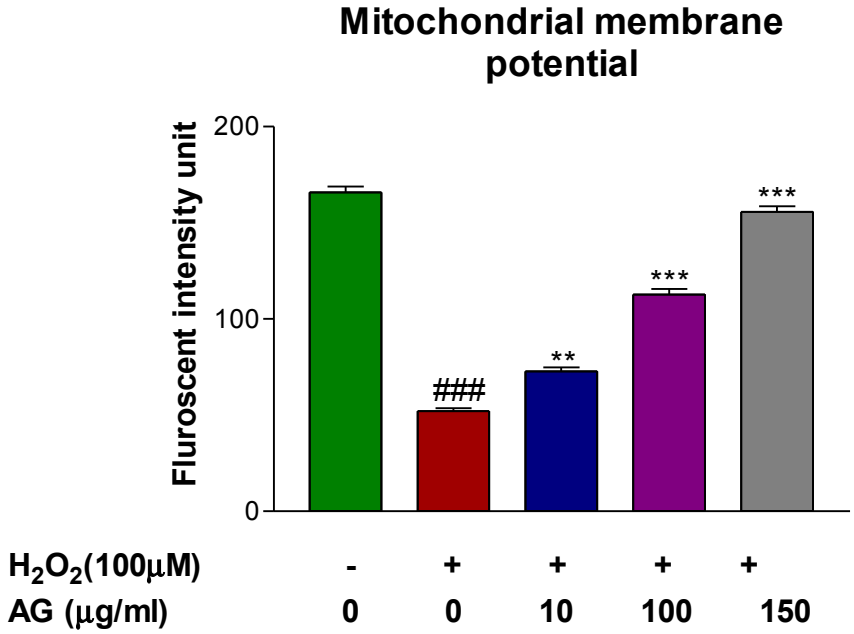
Results are expressed as mean ± S.E.M for n=3. Where, ns = non-significant, ### p< 0.001 compared to cells deprived of H_2O_2 and AG. * <0.05, **p < 0.01 and ***p < 0.001 compared to cells treated with H_2O_2.

Table 3.4: Mitochondrial Membane Potential of H9C2 Cells Pre-treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 3 h and then treated with 100 µM of H_2O_2 for 24 h

Concentrations of AG Extract + H_2O_2	Fluroscence Intensity
0 µg/ml + 0 µM	165.70 ± 3.18
0 µg/ml + 100 µM	52.00 ± 1. 52$^{###}$
10 µg/ml + 100 µM	72.67 ± 2.18**
100 µg/ml + 100 µM	112.70 ± 2.90***
150 µg/ml + 100 µM	155.70 ± 2.96***

Results are expressed as mean ± S.E.M for n=3. Where, ns = non-significant, ### p< 0.001 compared to cells deprived of H_2O_2 and AG. **p < 0.01 and ***p < 0.001 compared to cells treated with H_2O_2.

Table 3.4: Mitochondrial Membane Potential of H9C2 Cells Pre-treated with 10, 20, 50, 100 and 150 µg/mL of AG Extract for 3 h and then treated with 100 µM of H_2O_2 for 24 h



Results are expressed as mean ± S.E.M for n=3. Where, ns = non-significant, ### p< 0.001 compared to cells deprived of H_2O_2 and AG. **p < 0.01 and ***p < 0.001 compared to cells treated with H_2O_2.

Figure 3.5: Photomicrographs of DCF-DA stained H9C2 cells; untreated (CN), treated with 100 µM H_2O_2 (H_2O_2), treated with 100 µM H_2O_2 in presence of 150 µg/ml of AG (H2O2+AG)

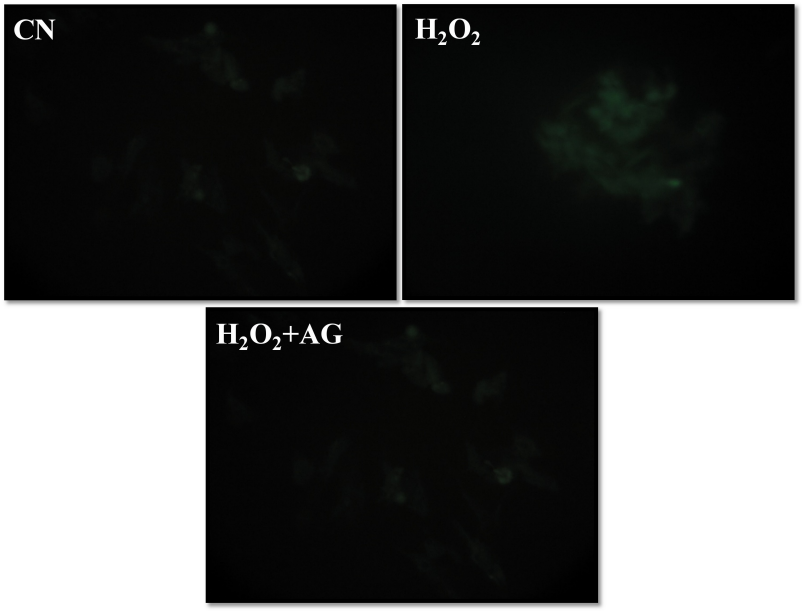


Figure 3.6: Photomicrographs of AO-EB stained H9C2 cells; untreated (CN), treated with 100 µM H_2O_2 (H_2O_2), treated with 100 µM H_2O_2 in presence of 150 µg/ml of AG (H_2O_2+AG)

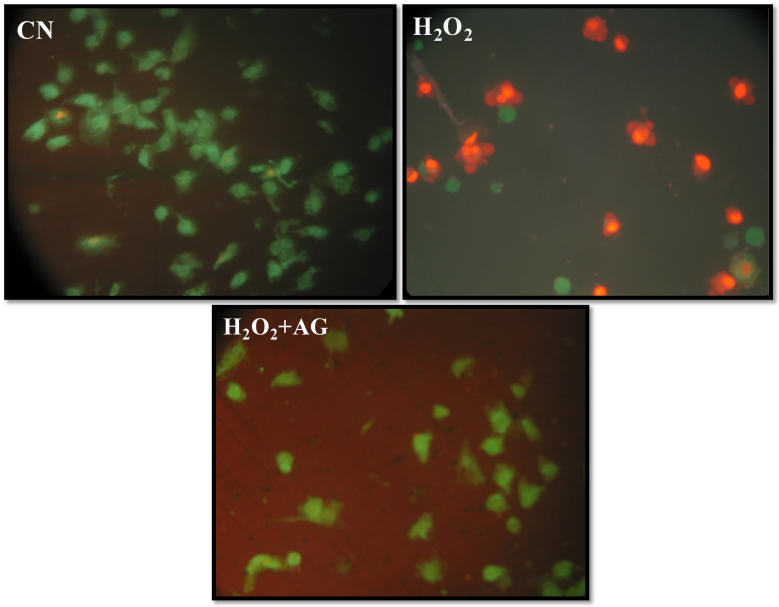
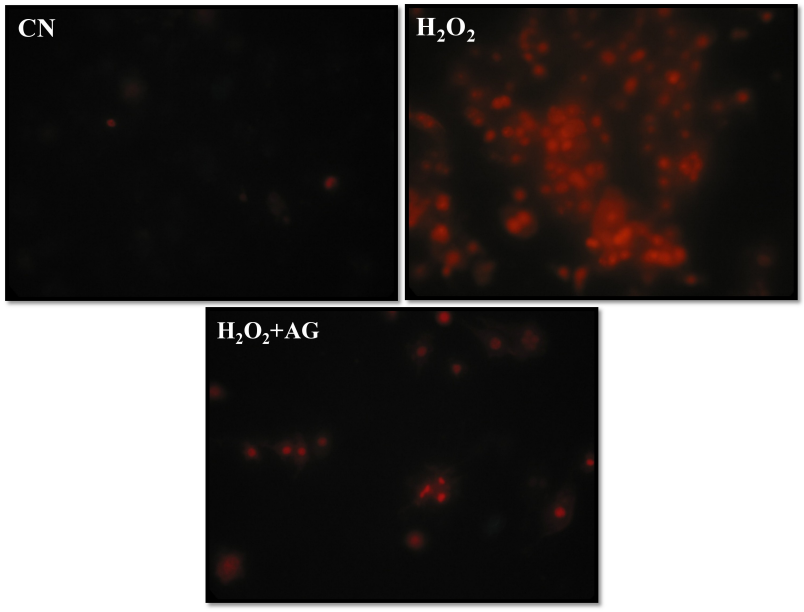


Figure 3.7: Photomicrographs of PI stained H9C2 cells; untreated (CN), treated with 100 µM H_2O_2 (H_2O_2), treated with 100 µM H_2O_2 in presence of 150 µg/ml of AG (H_2O_2+AG)



DISCUSSION

Oxidative stress is a condition that is generated by imbalance between reactive oxygen species (ROS) or free radicals and lower cellular antioxidant levels. During myocardial infarction, the ROS level increases and compromises the antioxidant defence system of cardiomyocytes. ROS in cardiomyocytes can be generated from mitochondrial electron transport chain, nitric oxide synthase (NOS), NADPH oxidase, xanthine oxidase, and lipoxygenase/cyclooxygenase during myocardial infarction (Change *et al*., 2013). In acute myocardial infarction ischemic or reperfusion injury may cause mitochondrial dysfunction in heart cells (Misra *et al*., 2009).

Hydrogen peroxide (H_2O_2) induced oxidative stress is well known model to study ROS mediated cellular damage during ischemic condition. Exposure of H9C2 cardiomyoblasts to H_2O_2 causes significant activation of oxidative stress in the cells, which can be characterized by reduced cell viability, increased intracellular ROS and lipid peroxidation, and reduced intracellular antioxidant milieu (Devkar *et al*., 2012). In the present study, we observed that H_2O_2 treatment to H9C2 cells showed the significant decrement in cell viability with marked increased LDH release as compared to the control group. The concurrent treatment of AG extract with H_2O_2 to H9C2 cardiomyoblasts showed significant increment in cell viability and decrement in LDH release in the test medium. Lipid peroxidation level was significantly increased with H_2O_2 treatment to H9C2 cells while AG extract treated cells showed dose dependent decrement in LPO levels. These results imply towards a protective effect of AG extract against H_2O_2 mediated cell damage to H9C2 cells.

Mitochondrial dysfunction was also observed following H_2O_2 exposure to H9C2 cells and is reflected by the loss of mitochondrial membrane potential and

intracellular ATP levels. These pathophysiological processes can lead to apoptosis by activation of the intrinsic apoptotic pathway in cardiomyocytes (Liu *et al*., 2009). H_2O_2 treatment to H9C2 cells showed significant decrement in mitochondrial membrane potential as compared to the control group. AG extract treated group showed dose dependent increment in mitochondrial membrane potential which indicated the protective effect of AG extract on the mitochondrial dysfunction.

Fluorescent dye CM-H_2DCFDA is a chloromethyl derivative of H_2DCFDA and its intensity is an indirect reflection of intracellular reactive oxygen species (ROS). CM-H_2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases. Thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols and undergoes oxidation to produce a fluorescent adduct (Vyas *et al*., 2014). AG treatment showed a dose dependent decrement in H_2O_2 mediated increment in fluorescent intensity. This result is attributable to the antioxidant property of AG extract that provides protection against ROS mediated damage to the cardiomyoblasts.

These results are also in accordance with the Acridine Orange (AO) and Ethidium Bromide (EtBr) and propidium iodide (PI) staining. AG treatment accounted for a higher population of live cells and lesser number of cells with condensed and fragmented nuclei as compared to H_2O_2 treated H9C2 cells.

These results are in accordance with previous reports from our lab (Devkar *et al*., 2012; Patel *et al*., 2012) and other research groups (Madamwar *et al*., 2015; Madamwar *et al*., 2015) wherein extract or pure compounds of herbal or algal origin respectively have been shown to manifest therapeutic potential due to their strong antioxidant properties. The relevance of AG as a functional food is highlighted

through this study because of the observed cardioprotective potential and adds value to its well reported medicinal properties.

SUMMARY

Reactive oxygen species (ROS) plays pivotal role during myocardial infarction. The aim of the present study was to evaluate the protective effect of *Anethum graveolens* L. (AG) against H_2O_2 induced oxidative stress in H9C2 cells. The H9C2 cells were incubated with 10 mM H_2O_2 and different concentrations of AG extract for 24 h. Cell viability and LDH release assays showed AG had successfully prevented H_2O_2 induced cardiomyoblasts death and prevention on LDH enzyme leakage from damage cells. Reduced levels of lipid peroxidation and intracellular ROS production indicated that the presence of antioxidants in AG were capable to prevent H_2O_2 mediated ROS production and lipid peroxidation. AG extract also prevented H_2O_2 mediated reduction in mitochondrial membrane potential. Acridine Orange/Ethidium Bromide and Propidium iodide staining of cardiomyoblasts showed that AG prevented H_2O_2 induced cardiomyoblasts cell death. Based on the results of the present study, it can be concluded that AG extract has strong antioxidant property and it successfully prevents H_2O_2-induced cytotoxicity.