Annexure – IX

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project- Cellular and molecular investigation on cardioprotective potential of anthocyanin rich red cabbage using *in vivo* and *in vitro* experimental models

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR
Dr. Ranjitsinh V. Devkar
Division of Phytotherapeutics and Metabolic Endocrinology,
DST-FIST Department of Zoology, Faculty of Science,
The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat.

3. NAME AND ADDRESS OF THE INSTITUTION
Department of Zoology, Faculty of Science, The M. S. University of Baroda, Vadodara.

4. UGC APPROVAL LETTER NO. AND DATE: **F.No. 41-89/2012(SR) 12th July 2012**

5. DATE OF IMPLEMENTATION: **1st August 2012**

6. TENURE OF THE PROJECT: **36 months**

7. TOTAL GRANT ALLOCATED: **10,70,800/-**

8. TOTAL GRANT RECEIVED: **9,27,300/-**

9. FINAL EXPENDITURE: **9,37,044/-**

10. TITLE OF THE PROJECT - **Cellular and molecular investigation on cardioprotective potential of anthocyanin rich red cabbage using *in vivo* and *in vitro* experimental models**

11. OBJECTIVES OF THE PROJECT
- *In vitro* assessment cardioprotective potential of ARCE using cultured H9C2 cells (rat cardiomyocytes).
- *In vivo* potential of ARCE in mitigating isoproterenol induced myocardial infarction in rats.

12. WHETHER OBJECTIVES WERE ACHIEVED
(GIVE DETAILS)
The ones planned till date has been achieved and further work are in progress.

RESULTS:

Anthocyanin content in red cabbage extract:

Total anthocyanin concentration in ARCE was 86.004 ± 3.1038 mg/100gm measured using pH differential method. Presence of anthocyanin was confirmed by method given by Wagner. Also during HPTLC prominent peak was observed at 530 nm.

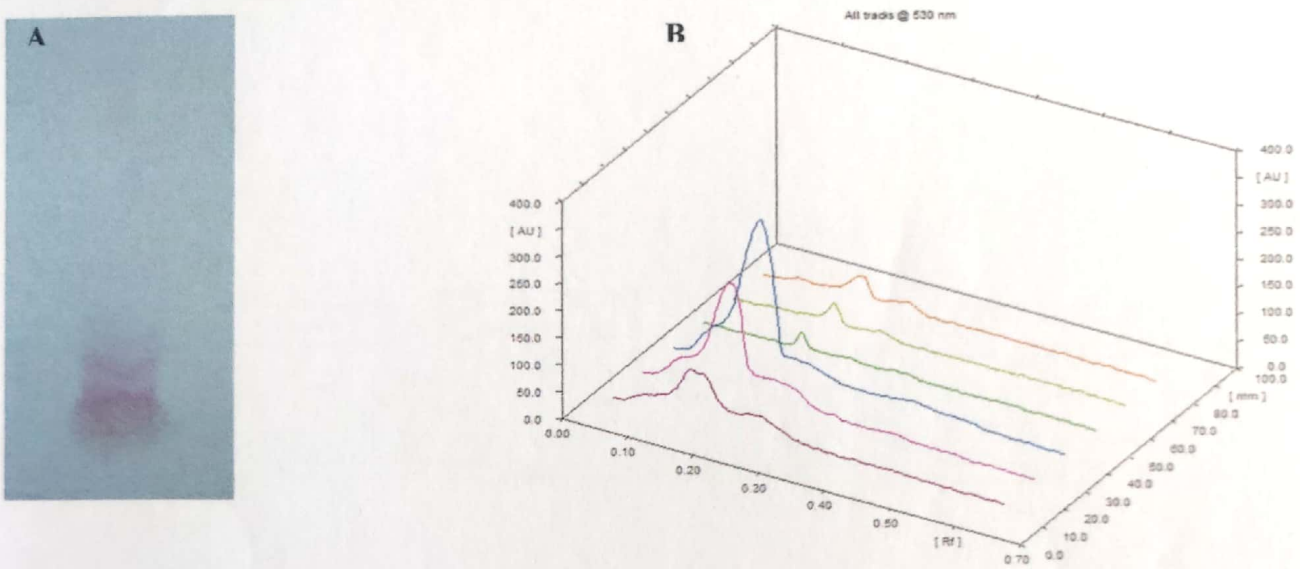
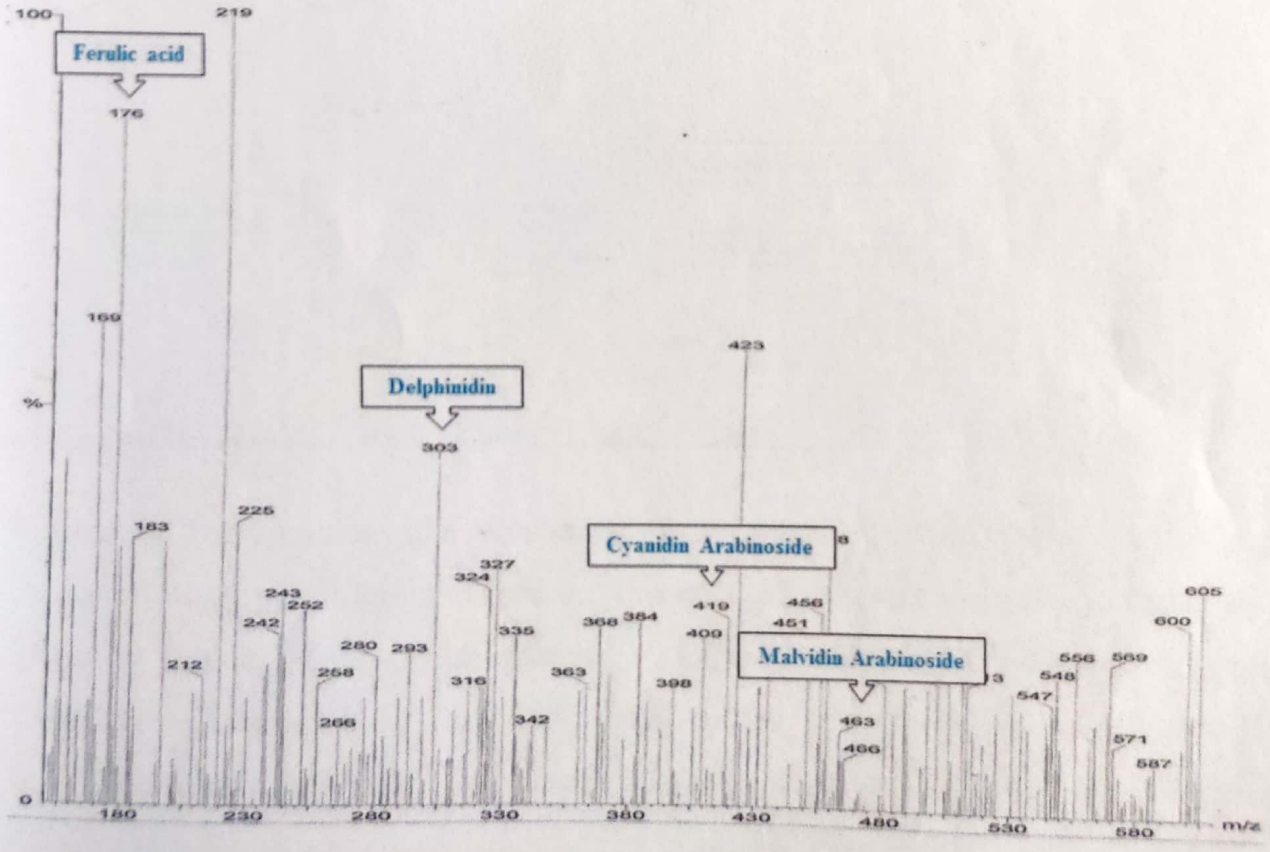
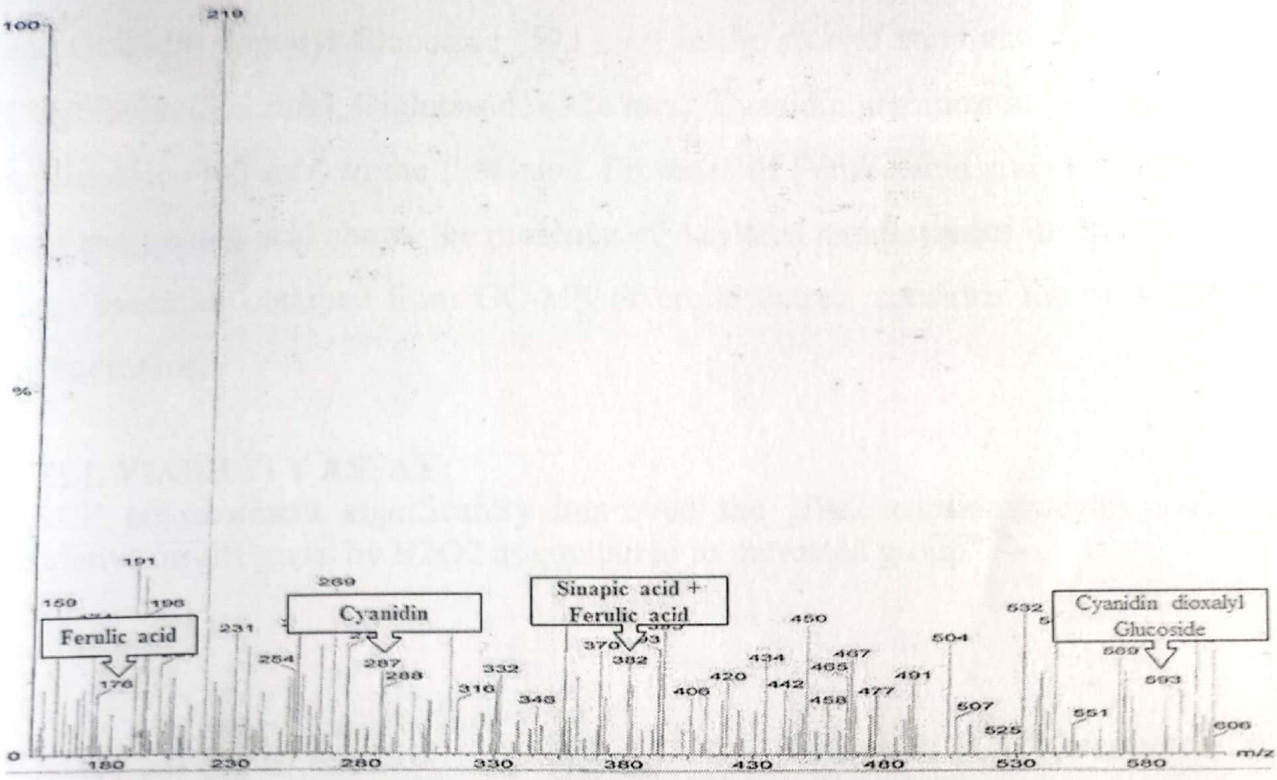


Figure 1: A. TLC chromatogram of Anthocyanin rich extract of Red Cabbage.
B. 3D densitogram of HPTLC measured at 530 nm in CAMAG Linomat5. First threee lanes are of extract dissolved in methanol and remaining three lanes are of extract dissolved in acidified methanol.





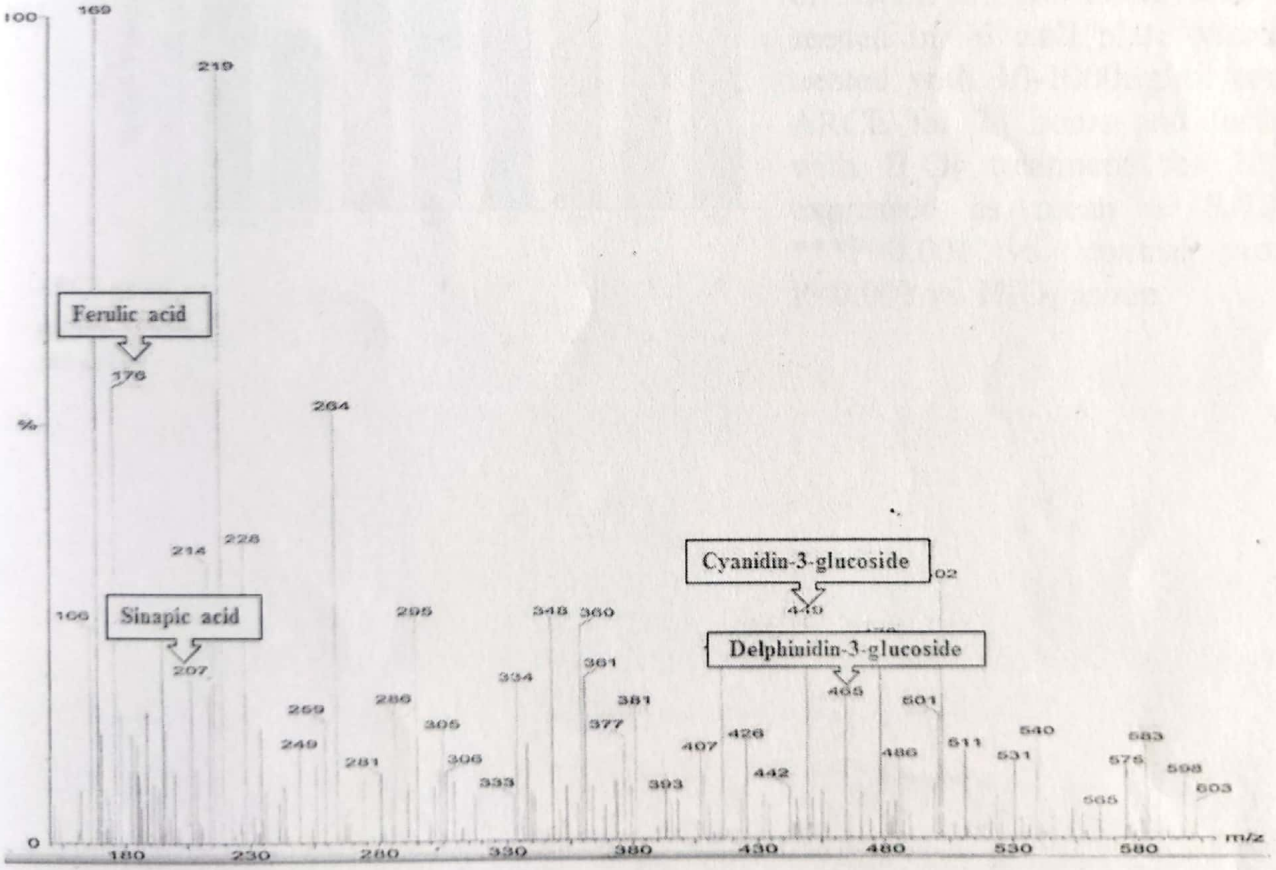


Figure 2: Total anthocyanin concentration in ARCE was 86.004 ± 3.1038 mg/100gm measured using pH differential method. Presence of anthocyanin was confirmed by method given by Wagner. Mass spectra obtained by GC-MS of crude extract shows the presence of Cyanidin-3-glucoside (449 m/z), Delphinidin-3-glucoside (465 m/z), Ferulic acid (176 m/z) and Sinapic acid (207 m/z). While mass spectra of bands separated on TLC showed the

presence of Cyanidin (287 m/z), Ferulic acid (176 m/z), Sinapicacid+Ferulic acid (382 m/z) and Cyanidin dioxalyl Glucoside (593 m/z) in the second band and Ferulic acid (176 m/z), Delphinidin (303 m/z), Diglucoside (324 m/z), Cyanidin arabinoside (419 m/z) and Malvidin arabinoside (463 m/z) in the first band. Presence of Ferulic acid and combination of Sinapic acid and Ferulic acid shows the presence of Acylated anthocyanins in *Brassica oleraecea*. A mass spectrum obtained from GC-MS of crude extract confirms the presence of acylated anthocyanins.

CELL VIABILITY ASSAY:

ARCE pre-treatment significantly improved the H9c2 cardiomyocytes viability against oxidative insults given by H2O2 as compared to untreated group.

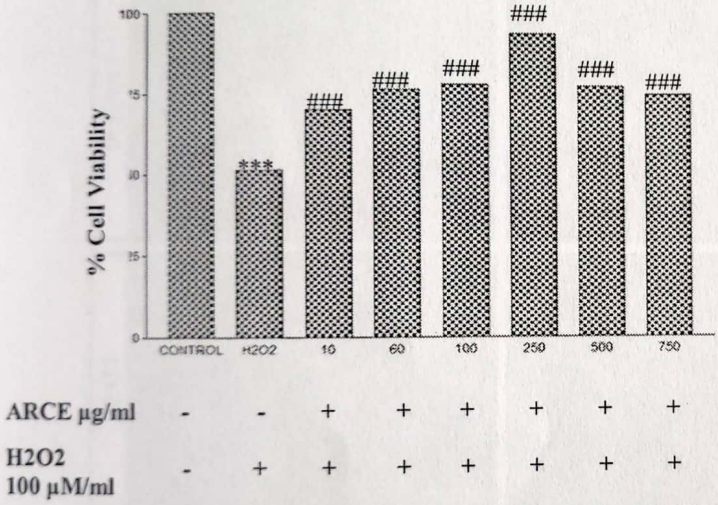
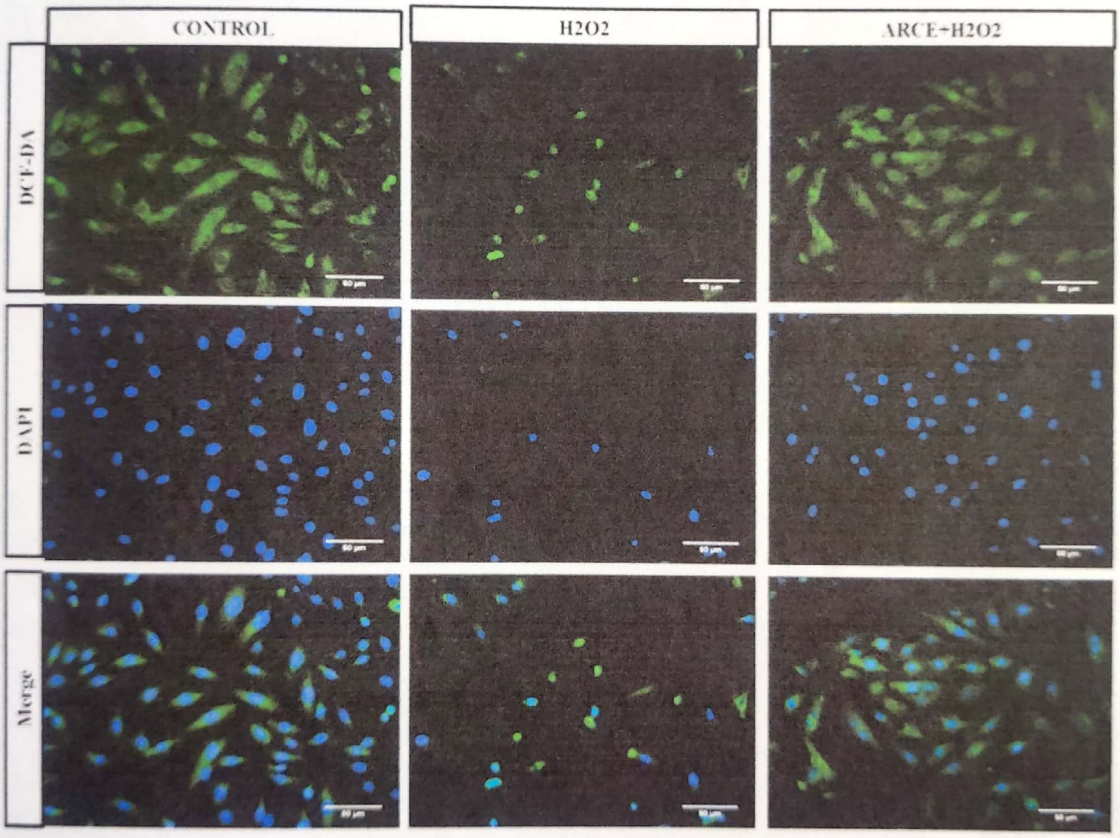


Figure 3: H2O2 accounted for decrement in cell viability that was alleviated in presence of ARCE extract. H9c2 cells ($1X10^4$) were seeded in 96 well plate overnight and pre-treated with 10-1000µg/ml concentration of ARCE for 24 hours and further proceeded with H_2O_2 treatment for 12 hours. Data expressed as mean ± S.E.M. for n=3. ***P<0.001 vs. control group and ### P<0.001 vs. H_2O_2 group

AMELIORATION OF LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL DUE TO OXIDATIVE STRESS:

Generation of reactive oxygen species in cell leads damage to several other organelles among them damage to mitochondrial membrane which further leads to uncontrolled damage to bioenergetics potential in mitochondria can be detected using Rhodamine 123. Rhodamine 123 is lipophilic stain which stains healthy cells. Pre-treatment with ARCE protected from loss in mitochondrial membrane potential due oxidative stress as observed in group C compared to group B which is not pre-treated with ARCE and given H2O2 to induce oxidative stress. Cells were counterstained with DAPI to avoid false interpretation. Corrected cell fluorescence was measured and quantified using Image J software and further data was analysed using one way ANOVA.

1.



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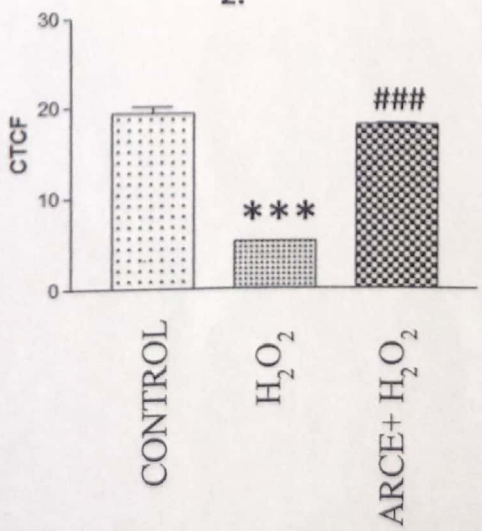

DETECTION OF PROTECTIVE EFFECTS AGAINST CYTOPLASMIC REACTIVE OXYGEN SPECIES:

Generation of cytoplasmic reactive oxygen species and amelioration of it by pre-treatment of ARCE has been detected by DCF-DA. As DCFDA comes in contact with free radical it undergoes oxidation and converted into highly fluorescent compound DCF.

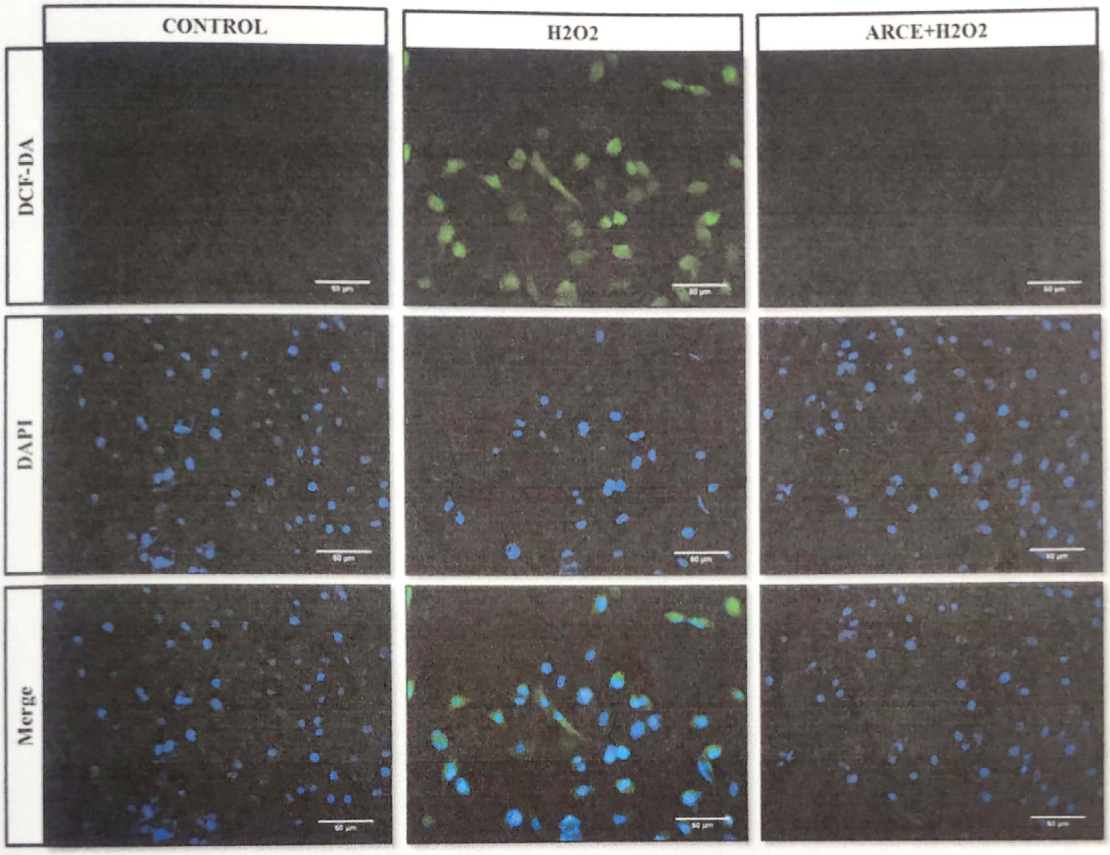

Figure 4: Intracellular detection of ROS using DCF-DA staining

Control H9c2 cells (untreated), H_2O_2 (100µM) induced stressed cardiomyocytes for 12 hours, ARCE+ H_2O_2 group pre-treated with ARCE (250µg/ml) for 24 hours and later stressed with H_2O_2 (100µM) for 12 hours.

Figure 5: Assessment of Mitochondrial membrane potential using Rhodamine 123 and nuclear characteristics using DAPI stains.

1) Control (untreated), H_2O_2 (100µM) induced stressed cardiomyocytes for 12 hours, ARCE+ H_2O_2 group pre-treated with ARCE (250µg/ml) for 24 hours and later stressed with H_2O_2 (100µM) for 12 hours.
2) Mean fluorescence Intensity measured using ImageJ software and statistical analysis was performed using ANOVA. ***P<0.001 vs. control group and ### P<0.001 vs. H_2O_2 group.

INCREMENT OF ANTI-APOPTOTIC AND ANTI OXIDANT ACTIVITY OF OXIDATIVELY STRESSED CARDIOMYOCYTES:

Oxidative damage caused due H2O2 results into decrease in antioxidant milieu (sod and catalase) which can be upregulated by pre-treatment with ARCE. Decrease in antioxidant capacity of cells further leads to apoptosis and increases in bax mRNA expression and decrease in antiapoptotic bcl-2 mRNA expression. These changes are further reversed by pretreatment with ARCE.

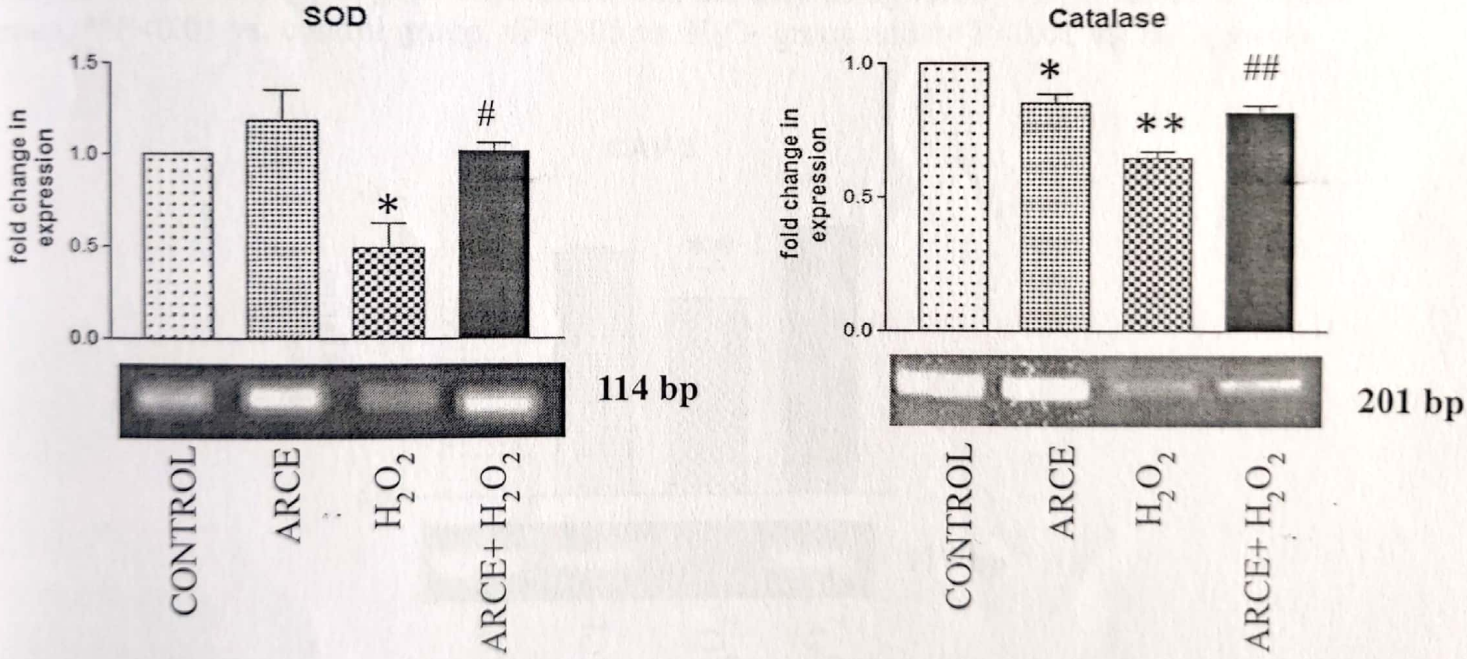

Figure 6: ARCE prevents depletion of intracellular antioxidants
ARCE mediated upregulation of (Catalase, SOD) enzymatic antioxidants. Statistical ananlysis of fold change in gene expression was done by using ANOVA. *P<0.05 vs. control group, **P<0.01 vs. control group, #P<0.05 vs. H_2O_2 group and ## P<0.01 vs. H_2O_2 group.

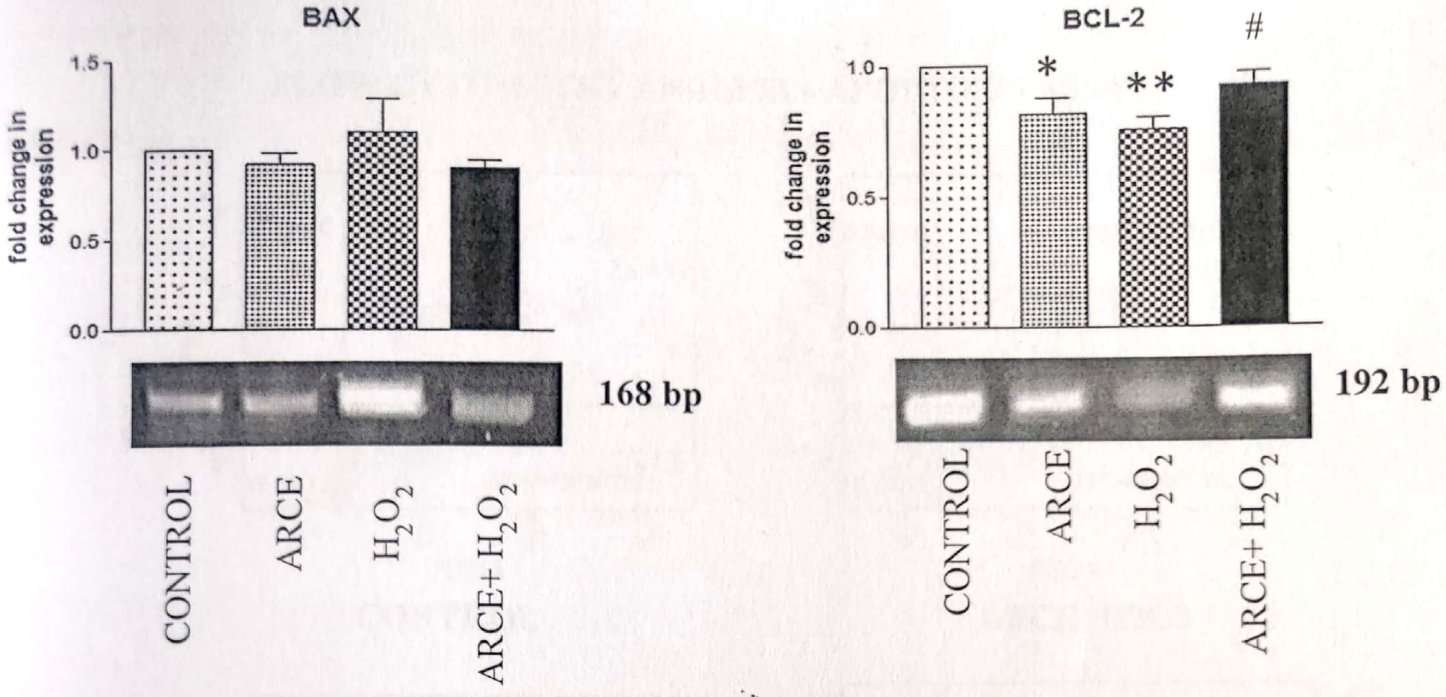

Figure 7: ARCE down-regulates apoptosis

ARCE mediated upregulation and downregulation of BCL2 and BAX respectively. Statistical analysis of fold change in gene expression was done by using ANOVA. *P<0.05 vs. control group, **P<0.01 vs. control group, #P<0.05 vs. H_2O_2 group and ## P<0.01 vs. H_2O_2 group.

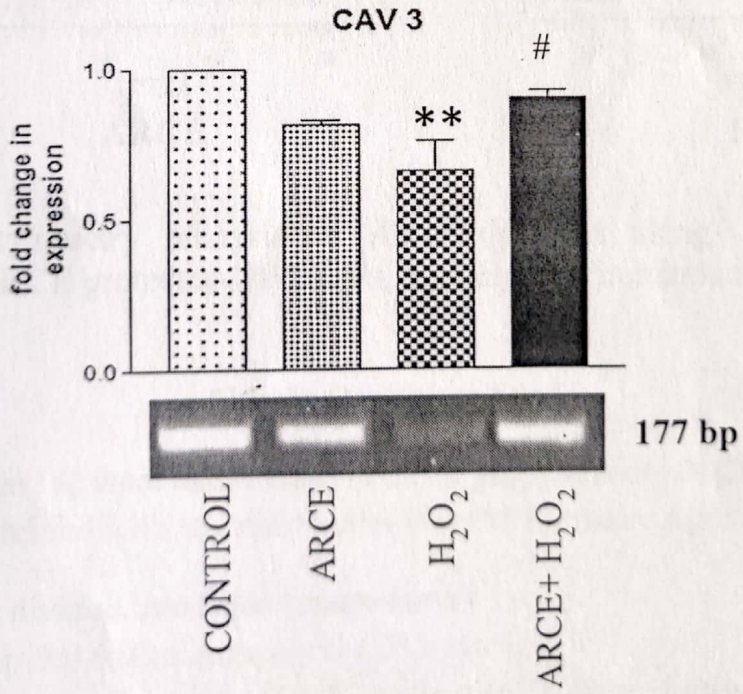

Figure 8: ARCE prevents down-regulation of Caveolin-3

ARCE mediated upregulation of Cav-3 cardiac marker genes. Statistical ananlysis of fold change in gene expression was done by using ANOVA. *P<0.05 vs. control group, **P<0.01 vs. control group, #P<0.05 vs. H_2O_2 group and ## P<0.01 vs. H_2O_2 group.

FLOW CYTOMETRY ANALYSIS-APOPTOSIS ASSAY

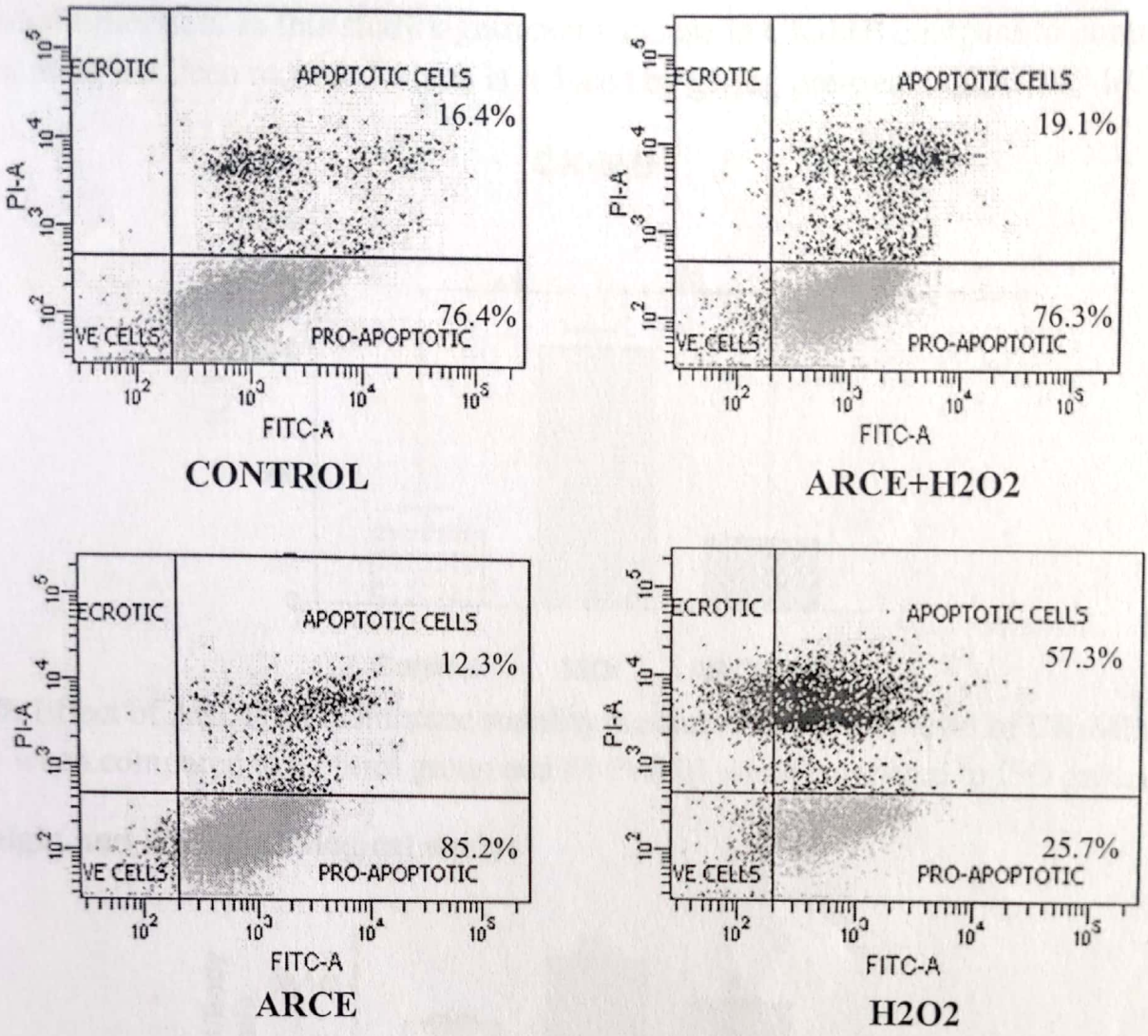

Figure 9: Flow cytometry analysis of Apoptotic cells using AnnexinV-PI staining. Pretreatment with ARCE protected H9c2 cells from H_2O_2 which induced apoptosis by 38%.

Whole Organism Study

Charles foster rats (n=18) were maintained in clean propylene cages (23±2°C, LD 12:12 and 45-50% humidity) and fed with standard pellet diet (M/S Pranav Agro Ltd. Baroda).

Rats were randomly divided into three groups (n=6)

1. Control group-fed with n.s. orally for 28 days
2. ARCE+ISO group-fed with ARCE orally for 28 days, further on 29th and 30th day dosed with isoproterenol 80mg/kg body weight subcutaneously)
3. ISO group-fed with n.s. orally for 28 days, further on 29th and 30th day dosed with isoproterenol 80mg/kg body weight subcutaneously)

On last day rats were starved for overnight. Blood was collected from retro-orbital sinus under mild ether anaesthesia. Heart was excised after sacrificing rats by cervical dislocation under mild ether anaesthesia and stored in RNA later solution at -20°C for the study of expression level of mRNA and in 10% paraformaldehyde for histopathological study.

Plasma marker of Cardiac injury: Isoproterenol (ISO) induced lipid peroxidation, membrane damage and leakage of CK-MB in plasma are prominent markers for identification of myocardial infarction. In this study significant increase in CK-MB compare to control due to ISO treatment has been recorded which is reduced by giving pre-treatment with ARCE.

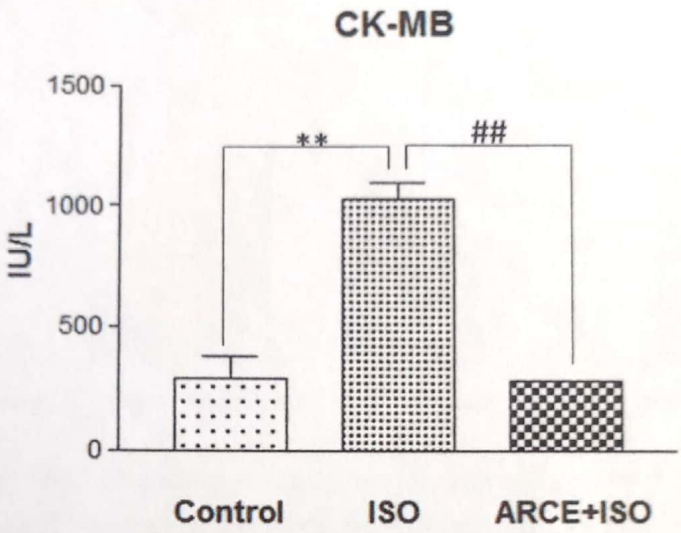

Figure 10: Effect of ARCE on membrane stability measured in form of level of CK-MB. **P<0.01 when compared to Control group and ## P<0.01 when compared to ISO group.

Heart weight and Histopathological study:

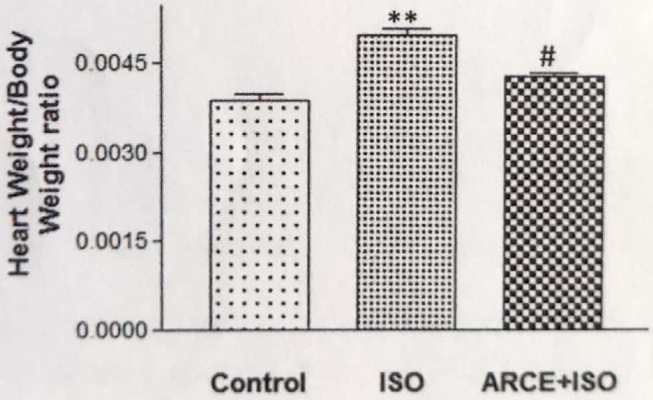

Figure : Effect of ARCE pre-treatment on ratio of heart weight to body weight. **p<0.01 compared to control and #p<0.05 compared to ISO group.

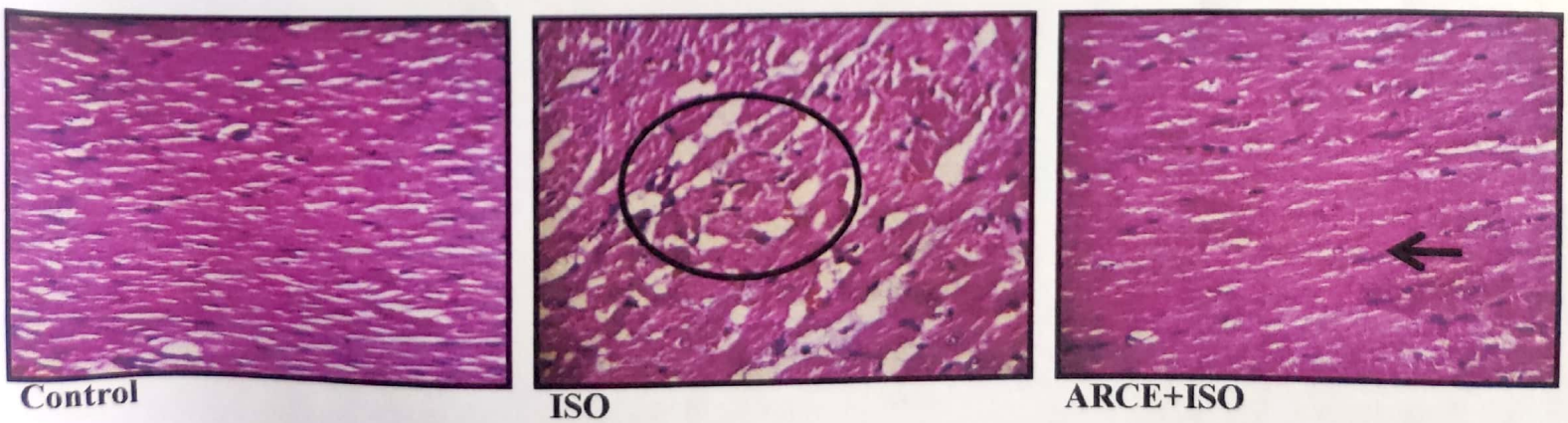

Figure 11: Effect of ARCE on cardiac histopathology of cardiac tissue. Tissue sections were stained with hematoxylin-eosin (400x). Encircled area indicates focal myocardial necrosis whereas, arrows indicate healthy myofibers.

Study of cardiac Antioxidants and Apoptotic cascade:

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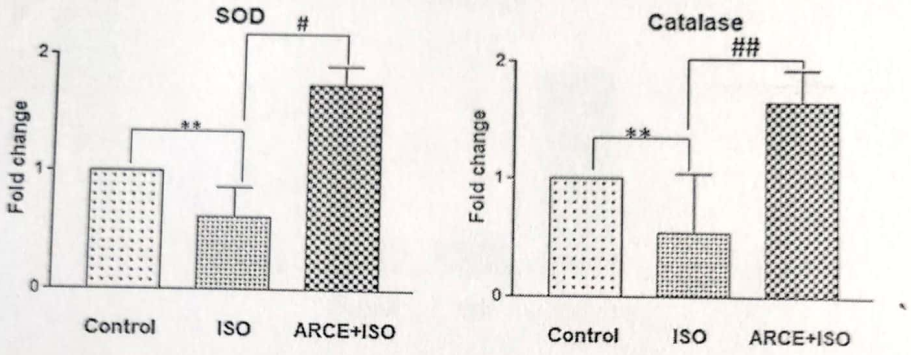

Figure 11: ARCE mediated changes in enzymatic antioxidants- Pre-treatment with ARCE for 28 days maintained and increased the antioxidant milleu of cells to protect against damage caused due to ISO. CT values were normalized with GAPDH and further with Control. **P<0.01, *p<0.05 compared to control, ## P<0.01 compared to ISO.

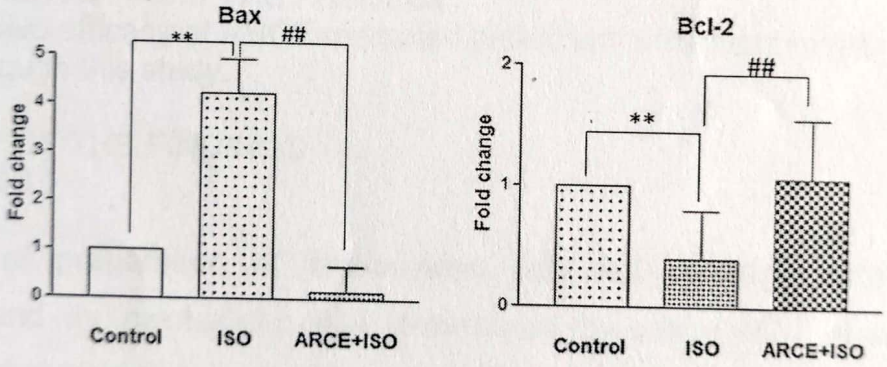

Figure 12: ARCE mediated changes in Proapoptotic and antiapoptotic genes- Pre-treatment with ARCE prevented apoptosis due to ISO by inhibiting the expression of Pro-apoptotic gene (Bax) and maintained the expression level of Anti-apoptotic gene (Bcl-2). CT values were normalized with GAPDH and further with Control. **P<0.01, *p<0.05 compared to control, ## P<0.01 compared to ISO.

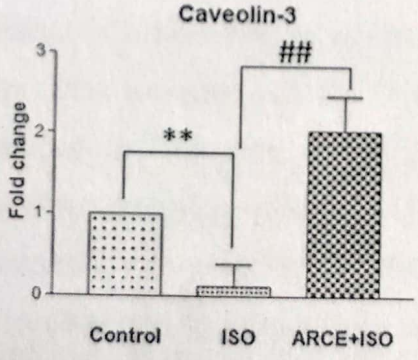

Figure 13: ARCE mediated changes in cardiomyocyte membrane integrity: Pre-treatment with ARCE protected membrane integrity by increasing two fold gene expression of caveolin-3. Caveolin-3 is a protein which plays important role as chaperone, in caveolae formation and signal transduction. CT values were normalized with GAPDH and further with Control. **P<0.01, compared to control, ## P<0.01 compared to ISO.

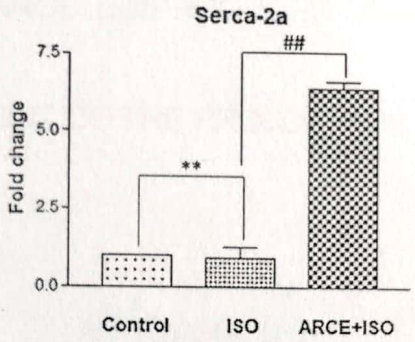

Figure 13: ARCE mediated changes in cardiomyocyte membrane integrity- Pre-treatment with ARCE restored the expression level of Serca-2a which is involved in maintaining calcium cycle in cardiomyocytes. CT values were normalized with GAPDH and further with Control. **P<0.01 compared to control, ## P<0.01 compared to ISO.

13. ACHIEVEMENTS FROM THE PROJECT

In vitro and In vivo efficacy of ARCE mediated protection to cardiomyocytes has been established through this study.

14. SUMMARY OF THE FINDINGS
(IN 500 WORDS)

The process of preparation of anthocyanin rich red cabbage extract (ARCE) was standardized and its cytotoxicity was determined by using MTT assay. Also specific concentration of extract to be dosed against H2O2 induced oxidatively stressed H9C2 cells was standardized and further MTT assay was performed. As a part of standardization it was observed that pre-treatment for 2 hours did not account for protection to the cells than and hence, 6 hour and 24 hour treatment schedules were tried out. It was found that 24 hour pretreatment was effective in imparting required protection against 100uM H2O2 stress for 12 hours. The same was further confirmed via expression based studies ofapoptotic genes (Bax, Bcl-2), caveolin 3, caveolin1α, SOD and catalase. It was observed that 24 hours pre-treatment with ARCE was effective in preventing apoptosis and oxidative stress. The same was further confirmed using cell cycle analysis and DCFHDA staining of cells. Staining with Rhodamine123 provided evidences on the role of ARCE in providing protection to the mitochondria against H2O2 induced oxidative stress. Further efficacy of ARCE to protect myocardium *In-vivo* was assessed by measuring CK-MB Level, histopathological studies and real time PCR to detect the changes in intracellular antioxidants (SOD and Catalase), membrane integrity (caveolin-3 and carp) and antiapoptotic and apoptotic genes (bcl-2 and bax). It was observed that pretreatment with anthocyanin prevented the cardiac necrosis

caused due Isoproterenol which causes oxidative stress by auto-oxidation, membrane damage, calcium overloading, increase in ionotropic and chronotropic responses and .

15. CONTRIBUTION TO THE SOCIETY: Basic research that will establish the mechanism of action of ARCE mediated cardioprotection.

16. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT: Yes
Name: Sarmita Jana

17. NO. OF PUBLICATIONS OUT OF THE PROJECT -NIL

(PRINCIPAL INVESTIGATOR) HEAD DEAN REGISTRAR

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Department of Zoology
The Maharaja Sayajirao University of Baroda, Vadodara - 390 002

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