Synopsis of the thesis entitled

Anthocyanins in *Brassica olereacea* L. and their cardioprotective potential

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Introduction:

Cardiovascular diseases (CVDs) are the largest cause of morbidity and mortality among other non-communicable diseases (NCDs). CVDs are expected to be the fastest growing chronic illnesses at the rate of 9.2% annually. A more worrying fact is that the incidences of CVDs have gone up significantly for people between the age of 25 and 69, which means we are losing more productive people to these diseases. India is not just the diabetes capital but also has the highest prevalence of metabolic syndrome, obesity and hypertension (Shokeen & Aeri, 2015). These factors together have accelerated the growth of CVDs and eventually mortality in India. Formation and abnormal accumulation of reactive free radical species, causes significant alterations in activity levels of key enzymes; structural modification of proteins; DNA damage; lipid peroxidation and thus play central role in development of several disease. Lipid peroxidation leads to structural and functional changes in cellular membrane; sulfhydryl and amino oxidation of proteins results to the loss of enzymatic activity and DNA damage triggers mutation (Santos, Anilkumar, Zhang, Brewer, & Shah, 2011). Thus, these events results into onset and progression of cardiovascular diseases such as myocardial infarction, ischemia/reperfusion injury and heart failure (Lee & Gustafsson, 2009). Therefore, regulation of the apoptotic cascade by reducing intracellular ROS is one of the key targets of research in preventing cardiovascular diseases.

Indigenous therapeutic herbs (Patel, Desai, Gajaria, Devkar, & Ramachandran, 2013; Thounaojam et al., 2011), spices (Gajaria, Patel, Devkar, & Ramachandran, 2015), functional foods (Guo, Guo, Jiang, Li, & Ling, 2012) and fruits (Jadeja, Thounaojam, Patel, Devkar, & Ramachandran, 2010) are the main sources of antioxidants, dietary fibres and trace elements vital to our body systems to ease metabolic stress. Reports on changes in lifestyle and exercise coupled with consumption of diets rich in antioxidants have been reported to lower the risk of heart and brain stroke (Enkhmaa, Surampudi, Anuurad, & Berglund, 2015; Willett et al., 2006). Anthocyanins (a member of flavonoid family) are polyhydroxyl and polymethyl derivatives of flavynium salts that have been extensively reported from various natural sources with reported therapeutic potentials against Alzheimer (Shih, Chan, Liao, Wang, & Yen, 2010), Hyperlipidaemia (Qin et al., 2009), Hyperglycaemia (Guo et al., 2012), cardiovascular diseases (Hidalgo et al., 2012),

diabetic retinopathy (Shim, Kim, Choi, Kim, & Park, 2012) and lowering blood pressure (Jennings et al., 2012). Anthocyanins gained importance among food and breverages due to its colour imparting property. Red cabbage anthocyanins are found to be thermostable at < 80ºC; showed less photodegradation quantum at pH-7 and are coloured at wide range of pH (pH-3 Pink; pH-5 Violet; pH-7 Blue) than anthocyanins from grape skin, black current and elder berry (Dyrby, Westergaard, & Stapelfeldt, 2001).

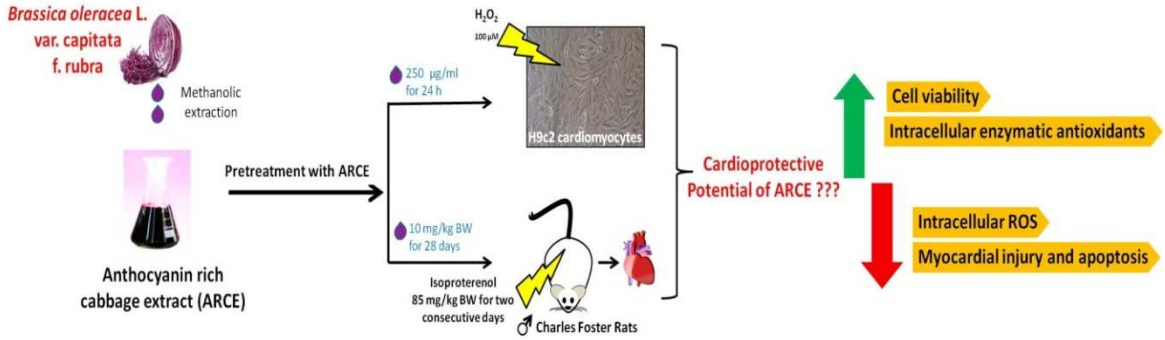
Red cabbage (*Brassica oleracea L.*), a rich source of cyanidin derivatives of anthocyanin is commonly consumed in Europe and Asia due to its low calorie-high fibre composition (Draghici et al., 2013; Wiczkowski, Szawara-Nowak, & Topolska, 2013). Red cabbage was found to be potent in improving diabetic neuropathy (Kataya & Hamza, 2008), preventing lipid peroxidation (Igarashi, Kimura, & Takenaka, 2000) and stabilizing erythrocyte membrane in hypercholestremic patients (Duchnowicz, Bors, Podsędek, Koter-Michalak, & Broncel, 2012). Previous studies conducted in our lab have shown that Anthocyanin rich Red Cabbage extract (ARCE) co-supplementation prevented cardiac and hepatic oxidative stress in rats fed with atherogenic diet (Sankhari, Thounaojam, Jadeja, Devkar, & Ramachandran, 2012). Also, ARCE was efficient in lowering intracellular oxidative stress and improving mitochondrial membrane potential in oxidatively stressed rat cardiomyoblasts (Devkar, Pandya, & Shah, 2012). These results formed the basis of our present study wherein, mechanism of ARCE mediated prevention of myocardial infarction were investigated through a series of carefully scripted *in-vivo*, *in-vitro* and *in silico* models.

Rationale

Anthocyanins have been extensively reported for their therapeutic potential by several research groups. In this regard anthocyanin rich fruits are the most preferred topics of research for treating life style disorder including cardiovascular disease. Rationale behind this study is to scrutinize red cabbage anthocyanins that qualify as a functional food in imparting cardioprotection.

Hypothesis

Death of cardiomyocytes and subsequent myocardial infarction forms the basis of cardiovascular diseases with mitochondrial function occupying the epicentre of the sequence of the events taking place herein. It is hypothesized in this study that the anthocyanins in ARCE provides cardioprotection via multiple mechanisms such as preventing depletion of intracellular antioxidants, scavenging free radicals and preventing subsequent membrane damage, safe guarding mitochondrial function and preventing apoptosis the same will be accessed via a series of protocols.



Objective

1. Profiling of anthocyanins present in Red cabbage extract.
2. Deciphering mechanism of Anthocyanin rich red cabbage mediated decrement in risk of myocardial damage
3. In-Silico study with using molecular docking of chosen anthocyanins.

Objective 1: Profiling of anthocyanins present in Red cabbage extract.

Result: Red cabbage(*Brassica oleracea* L. var. Capitata f. rubra DC.) was procured from Spencer's mall, Vadodara, Gujarat, India (22º 19' 21" N, 73º 10' 32" E), identified and authenticated by Dr. Vinay Raole, Department of Botany and voucher specimen (accession no. 213) was submitted to departmental herbarium (BARO), The M.S. University of Baroda, Vadodara, Gujarat. The resultant yield (7.1% w/w) was diluted with distilled water and the total Anthocyanin content was measured spectrophotometrically using molar extinction coefficient of Cyanidin-3,5-diglucoside (26,300 M^{-1} cm^{-1}).

Total anthocyanin content in ARCE was found to be 86.004 ± 3.103 mg/100gm. TLC of ARCE revealed two bands with R_f value 0.26 and 0.31 respectively. These values were in the R_f value range of 0.2-0.35 and which indicate the presence of monoglucosides of delphinidin and cyanidin (Delphinidin-3-glucoside and Cyanidin-3-glucoside (Figure 1A). The GC-MS spectra provided information regarding the structural identification of anthocyanin pigments. The m/z ratio of the daughter and parents ions, confirmed the presence of anthocyanins. Analysis of crude extract (Figure 1B) showed presence of cyanidin-3-glucoside (449 m/z) and Delphinidin-3-glucoside (465 m/z). Whereas, analysis of bands obtained from TLC showed presence of daughter ions of (epi) gallocatechin delphinidin (303 and 481 m/z), (epi) gallocatechin peonidin glucoside (605 m/z), peonidin glucoside (463 m/z) in the first band (Figure 1C) and Cyanidin (287 m/z), Cyanidin-3(6“-acetyl glucoside) (491 m/z), Cyanidindioxalyl Glucoside (593 m/z), Delphinidin-3(6“-acetyl glucoside)(507 m/z) and delphinidin-3-glucoside (465 m/z) in second band (Figure 1D). Overall, presence of cyanidin and delphinidin monoglucosides were recorded in ARCE.

Objective 2: Deciphering mechanism of Anthocyanin rich red cabbage mediated decrement in risk of myocardial damage

Result: Cell culture

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 (10% serum and 1% antibiotic, antimycotic solution 100X). Cells were trypsinized using 1X trypsin phosphate versene glucose (TPVG) at three day interval [24]. The experimental groups for this study were: Control (untreated cells), ARCE treated, H_2O_2 (100µM/ml) treated and ARCE+ H_2O_2 (pre-treated with ARCE for 24 h followed by H_2O_2 treatment for 12 h) treated.

H_2O_2 induced cytotoxicity of H9c2 cells

H_2O_2 (100 µm) induced toxicity in H9c2 cells was reduced by ARCE pretreatment in dose dependent manner. Also, ARCE alone at highest dose was nontoxic to H9c2 cells (Figure 2).

H_2O_2 induced peroxyl radical formation, loss of mitochondrial membrane potential and apoptosis in H9c2 cells

DCFDA (2’,7’ –dichlorofluorescin diacetate) a non-fluoroscent probe get converted into highly fluorescent 2′,7′-dichlorofluorescein (DCF) stain upon oxidation with

peroxyl free radical. 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 and formation of peroxyl radicals in ARCE+H_2O_2 group compare to H_2O_2 group. In DCFDA staining, green colour in cells confirms the H_2O_2 induced formation and presence of intracellular reactive oxygen species. While, presence of more number of green colour cells in ARCE+H_2O_2 group compare to H_2O_2 group stained with RHO123 represents the active mitochondrial membrane potential due to pretreatment of ARCE. Cells were counterstained with DAPI (nuclear stain) to avoid false interpretation. Corrected cell fluorescence was measured and quantified using ImageJ software. AnnexinV-PI stain was used for apoptosis study. 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 enters in damage cell and bind to DNA. Pretreatment with ARCE protected cardiomyocytes from apoptosis (38.2%) compare to H_2O_2treatedgroup. However ARCE treatment recorded 12.3% apoptosis, similar to control (Figure 3).

Gene expression studies in H9c2 cells and rat cardiac tissue

In the present study H_2O_2treated H9c2 cells were used as a disease control model to study the potential of ARCE to prevent onset and progression of reactive free radical species induced damage in H9c2 cardiomyoblast. In H9c2 cells, apoptotic (*bax*) and anti-apoptotic (*bcl-2*) mRNA levels in H_2O_2 group were found to be reversed by pre-treatment with ARCE. RNA levels of intracellular antioxidants (*sod and catalase*) were found to be significantly low in the above mentioned disease control group. But the same were significantly upregulated in ARCE+H_2O_2 treated group. Further, RNA levels of *caveolin-3* were reduced in H_2O_2 treated group but were comparable to that of control in ARCE+ H_2O_2 treated group.

ISO treated Charles foster male rats were used as *in-vivo* disease control model to study the myocardial infarction ameliorating potential of ARCE. Isoproterenol induces myocardial infarction by inducing the overstimulation of beta-1 adrenergic receptor, formation of its auto-oxidation by-products (ROS). Thus, cardiac membrane caveolae specific marker (*caveolin-3*); intracellular antioxidative enzymes (*sod* and *catalase);* apoptotic marker (*bax* and *bcl-2)*; sarcoplasmic reticulum calcium transporter (*SERCA2a*); cardiac ankryin repeat protein (*CARP*) were studied to

accesss the ARCE mediated prevention of myocardial infarction. RNA levels of intracellular antioxidants (*sod* and *catalase*) were found to be significantly low in the above mentioned disease control group. But the same were significantly upregulated in ARCE+ISO treated group. Further, RNA levels of *caveolin-3, SERCA2a* were reduced in ISO treated group but were comparable to that of control in ARCE+ISO treated group. Also in ISO treated rats the levels of *bax* and *ANKRD1* was upregulated wherein, *bcl-2* was down regulated as compared to the control. However, ARCE pretreatment accounted for the five fold and three fold decrease in expression level of *bax* and *ANKRD1* respectively with increase in *bcl-2* expression level.

Gross evaluation, histology of cardiac tissue and plasma CK-MB

ISO treatment accounted for significant increment in Heart weight : Body weight ratio (HW:BW) and plasma CK-MB levels ($P<0.01$). However, these parameters in ARCE+ISO treated group were comparable to control. The TTC stained sections of ventricle of control rats showed brick red color indicating healthy tissue whereas, that of ISO treated rats was pale yellow to white in color suggestive of necrotic patches. However, necrotic tissue was minimal in ARCE+ISO group and was comparable to that of control. Haematoxylin-Eosin stained sections of ventricular tissue of ISO treated rats showed gross derangement of myocardial fibres Whereas, ARCE+ISO treated group showed intact multinucleated fibres as observed in control.

Objective 3: In-Silico study with using molecular docking of chosen anthocyanins.

Result: The sequence of Rat ß1 adrenergic receptor (β_1AR) was retrieved from NCBI sequence database (accession number NP_036833 XP_001063787) and the 3D model was generated using CPHmodels-3.2 Server. Further the stereochemical quality of the modelled structure was evaluated through Ramachandran plot. Molecular docking of delphinidin-3-glucoside and cyanidin-3-glucoside with Rat β_1AR model was performed using Glide program in Schrodinger and calculations were done using Extra Precision (XP) method. The protein and the ligand molecules were prepared for docking using Protein Preparation Wizard and LigPrep respectively, available in Schrodinger suite (Christopher et al., 2013; Laskowski & Swindells, 2011). A 20 Å grid box was generated at the active site of the β_1AR using three active site residues N352, S228 and D138. Information of these three residues was retrieved from the co-

crystal structure of quinoline with Turkey β_1AR (PDB ID: 3ZPR). PyMol was used for visualization of molecular interactions.

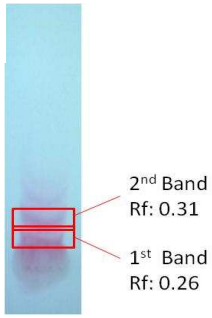
Thermo-stabilised Turkey β_1 Adrenergic Receptor bound to quinoline served as a template showing 69.9% alignment with query Rat β_1AR sequence. Further, D138, S228 and N352 residues in turkey $\beta_1$were found to be conserved in Rat β_1AR as per the query template alignment. The 3D structure of modelled protein consisted of seven helical structures in bundled formation with flexible loops between Helix-5 (H5) and Helix-6 (H6), residue numbers viz. 258 to 298. Ramachandran plot analysis of the model showed that 91.5% residues were in the most favored regions (Figure 4). Molecular docking of Cyanidin-3-glucoside and Delphinidin-3-glucoside with Rat β_1AR showed that they were well accommodated within the active site and interacted through the hydrophobic and electrostatic bonds at a distance of 2.5 to 3.2 Å. These two anthocyanins accounted for Glide XPG (docking) scores of -8.7 kcal/mol and -8.5 kcal/mol respectively (Figure 5).

Conclusion

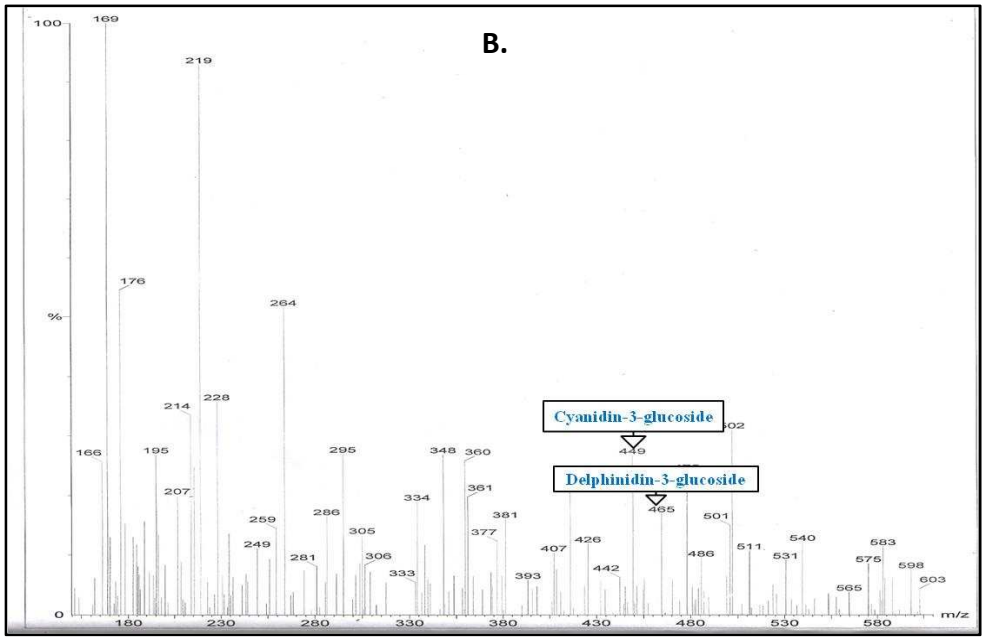
From this study it can be concluded that ARCE manifests therapeutic effects not only by improving the intracellular antioxidant status but also can alleviate the ISO induced changes in *caveolin-3, SERCA2a* and *ANKRD1*which plays an important role in regulating membrane bound signaling channels; intracellular calcium cycling; transcription of cardiac specific genes and cardiac muscle integrity. Molecular docking scores of cyanidin-3-glucoside and delphinidin-3-glucoside provide insights on their stable interaction with β_1 adrenergic receptor and ARCE mediated prevention of myocardial damage.

Figures:

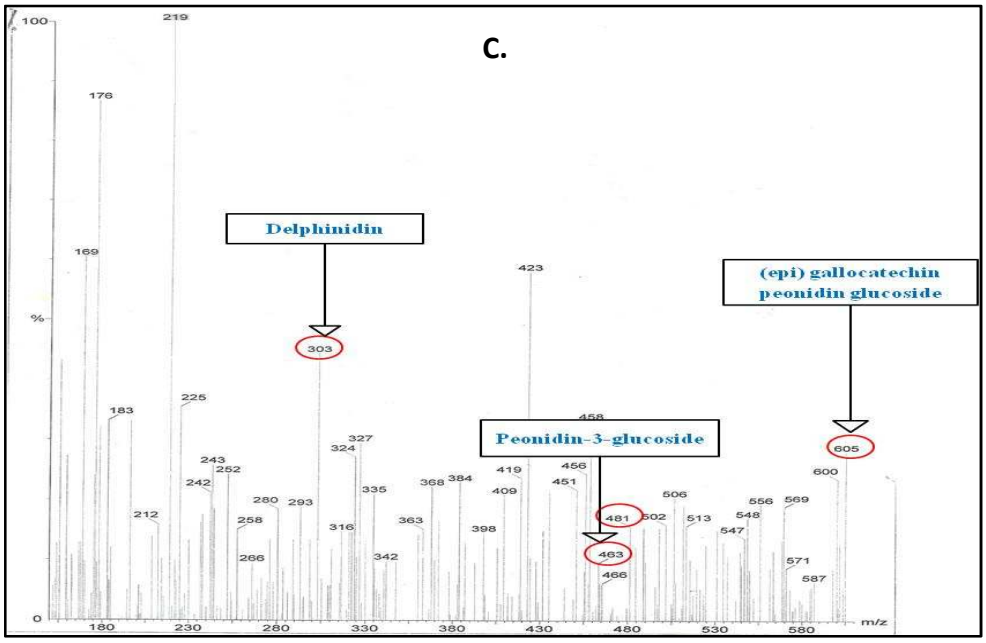
A.


2nd Band
Rf: 0.31
1st Band
Rf: 0.26

B.


Cyanidin-3-glucoside
Delphinidin-3-glucoside
m/z

C.


Delphinidin
(epi) gallocatechin
peonidin glucoside
Peonidin-3-glucoside
m/z

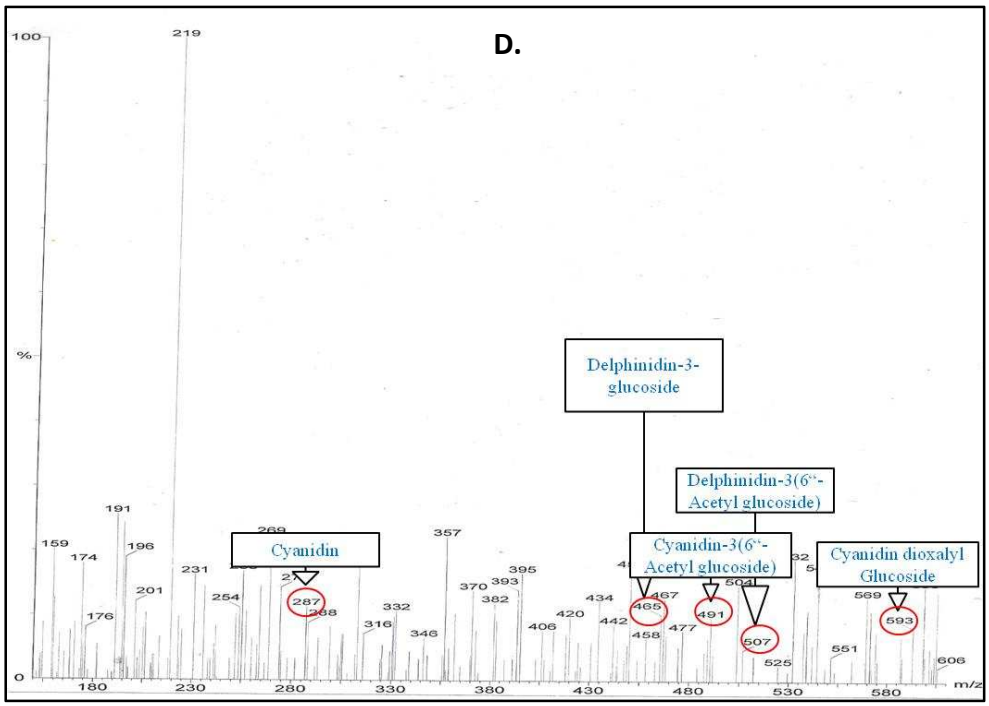


Figure 1: A. TLC chromatogram of ARCE. B. GC-MS analysis of crude extract. C. GC-MS analysis of TLC 1st band. D. GC-MS analysis of 2nd band.

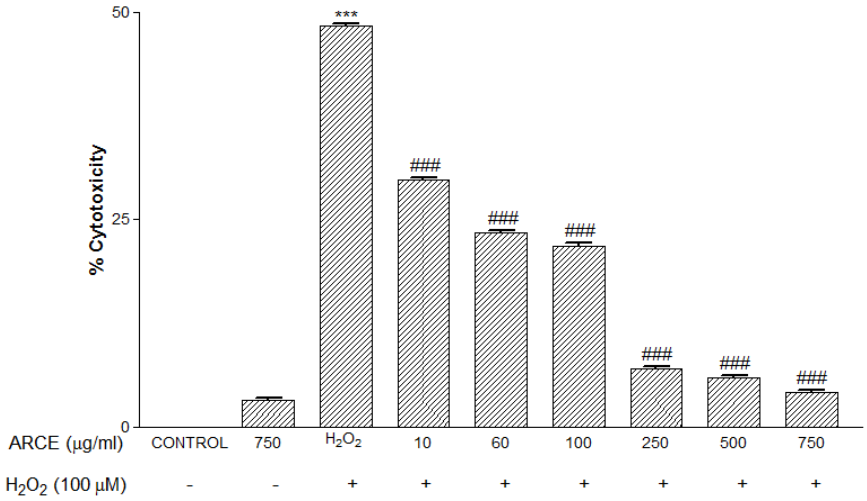


Figure 2: ARCE mediated prevention of H_2O_2 induced cytotoxicity. % Cytotoxicity was determined by MTT Assay.Thedata were represented asmean ± SEM,for three independent experiments. ***P<0.001 vs. control group and ### P<0.001 vs. H_2O_2 group.

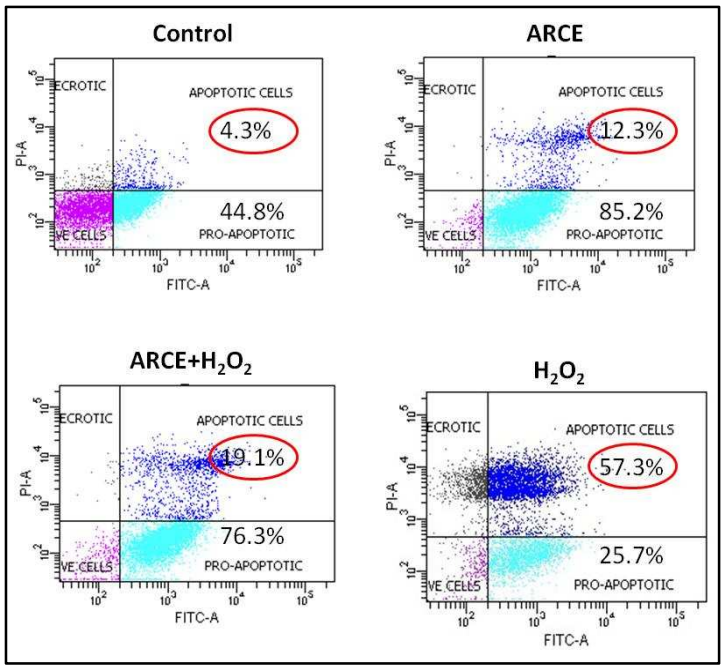


Figure 3: ARCE mediated prevention of H_2O_2 induced Apoptosis in H9c2 cells. Double positive events indicate apoptotic cells (valuesencircled in red) and double negative events indicate viable cell population.

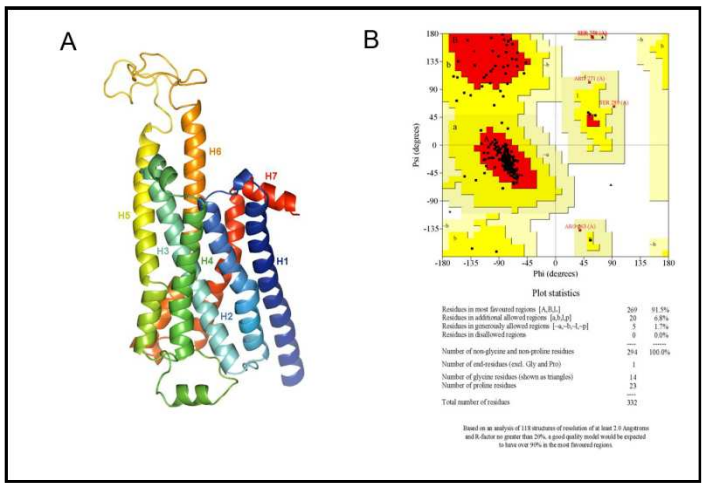


Figure 4: Homology model of β_1AR. (A) 3D model of Rat β_1AR showing a bundle structure constitutes of seven helices, the order of helices are marked from N-C terminal as per their respective colours. (B) Ramachandran plot of modelled Rat β_1 AR showing stereochemical parameters of each residues present in the structure.

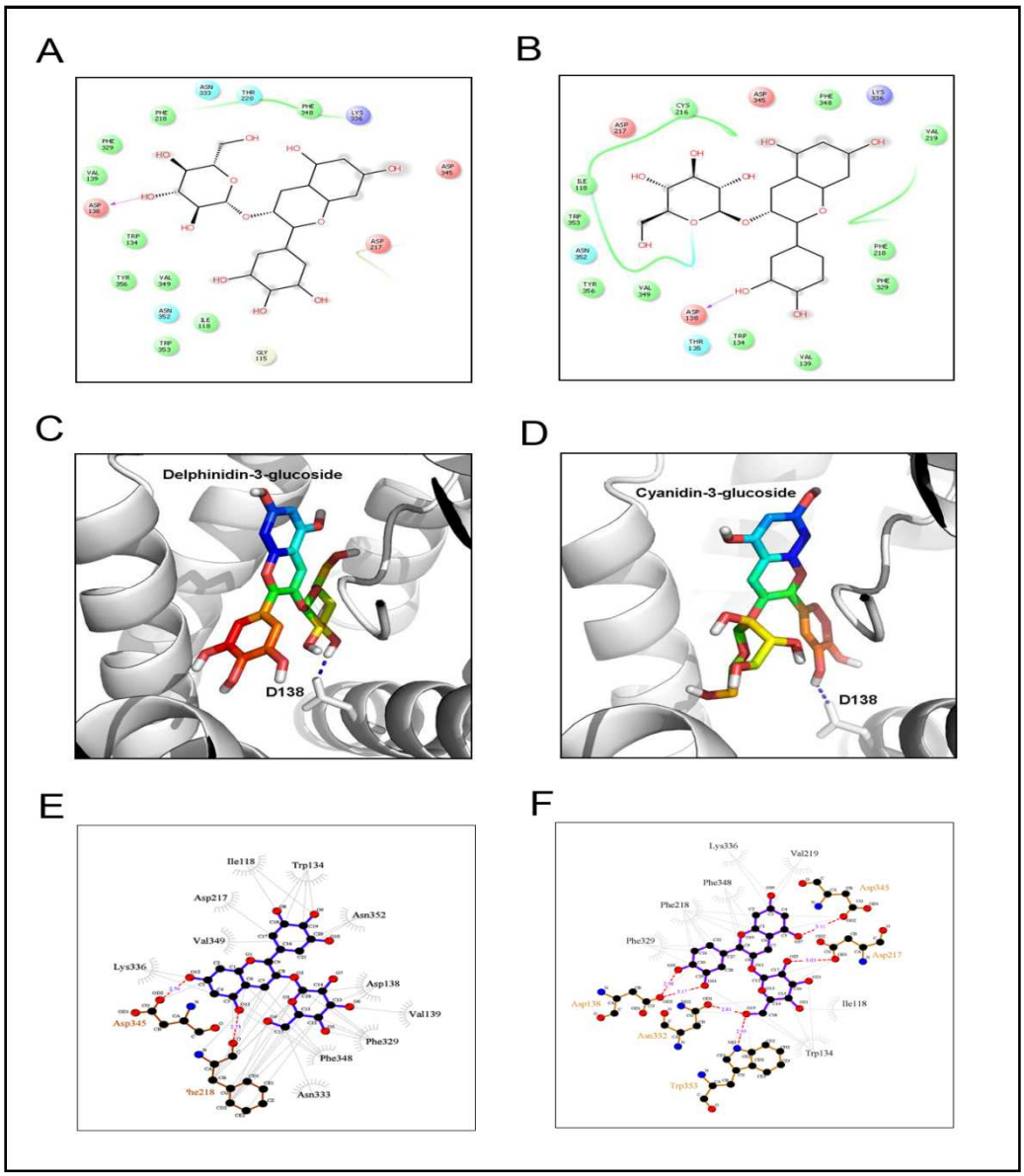


Figure 5: Molecular interaction of Delphinidin-3-glucoside and Cyanidin-3-glucoside with Rat $\beta_1$adrenergic receptor.2D representation of Rat β_1AR with (A) Delphinidin-3-glucoside and (B) Cyanidin-3-glucoside. Residues in green spheres are hydrophobic, blue spheres are positively charged, cyan spheres are polar, and red spheres are negatively charged. The ligand atoms involved in hydrophobic interactions are marked in gray. The purple arrows and their directions represent hydrogen bonds between the ligand and the protein. 3D representations of the interactions are shown for (C) Delphinidin-3-glucoside and (D) Cyanidin-3-glucoside,

the Hydrogen boding with D138 is showing through blue dotted line. (E) LigPlot diagram of Delphinidin-3-glucoside and (F) Cyanidin-3-glucoside.

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Candidate **Research Guide**

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