5. MATERIAL AND METHODS

5.1. Material

5.1.1. Drugs:

Isoproterenol hydrochloride (ISO), Angiotensin-II (Ag-II) and 2-Aminoethyldiphenyl borinate (2-APB) was purchased from Sigma Aldrich, St. Louis, MO, USA. Doxorubicin hydrochloride (DOXO) was obtained as gift samples from Sun Pharmaceutical Industry Ltd., Vadodara, India. Eugenol was purchased from Hi-Media Company, Vadodara, India. *Piper betle* Lin. leaf and *Rubia cordifolia* Lin. root powder were purchased from a commercial supplier. *Piper betle* leaves and *Rubia cordifolia* roots were authenticated by Dr. Padmanabhi S. Nagar (Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Gujarat, India). A voucher specimen (BARO 20610 and 20611) was deposited in herbarium of Botany Department, The Maharaja Sayajirao University of Baroda, Gujarat, India. (Annexure-I)

5.1.2. Chemicals:

- Superoxide Dismutase, Catalase, 2,3,5-Triphenyl tetrazolium chloride (TTC), 1, 1', 3,3'-Tetraethoxypropane, Hexamethonium chloride Phenazine Methosulphate, 2,2-diphenyl-1-picrylhydrazyl, 2′,7′-Dichlorofluorescin diacetate, Quercetin, Sulfanilamide, Naphthylenediamine dihydrochloride, Igepal (NP-40), Linoleic acid, and Propidium iodide were purchased from Sigma Aldrich, St. Louis, M.O., U.S.A.
- Annexin –FITC was purchased from BD Bioscience, Mumbai, India.
- Epinephrine bitartrate, Thiobarbituric acid, Sucrose, Adenosine triphosphate (ATP), Reduced glutathione (GSH), 5, 5'-dithiobis (2-nitro benzoic acid) (DTNB), Sodium azide, Bovine serum albumin (BSA), Trichloro acetic acid (TCA), Nitro blue tetrazolium (NBT), 1-chloro, 2, 4-dinitrobenzene (CDNB), 1-Amino 2-naphthol 4-sulphonic acid (ANSA), Hexadecyltrimethylammonium Bromide (HTAB), O-Dianisidine hydrochloride, MTT (3-4,5- dimethylthiazol-2,5 biphenyl tetrazolium bromide), Trypsin- EDTA, Sodium dodecyl sulphate (SDS), β-carotene, Ascorbic acid, Butyrtaed hydroxy anisole (BHA), Gallic acid, Olive oil were purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

- Barford reagent, Acrylamide/bis, Tetramethylethylenediamine (TEMED), Ultrasensitive enhanced chemiluminescence reagent, Ammonium persulfate and Ponceau S were purchased from Bio-Rad, CA, USA.
- Pen strep was purchased from Thermo Pierce, Waltham, Massachusetts, U.S.
- HPLC grade Methanol, Acetonitrile, Acetone, Dimethyl sulphoxide, Tert-butyl MethylEther, Chloroform, Ethyl acetate, Dichloromethane and Triethylamine (TEA) were purchased from Spectrochem Laboratories Pvt. Ltd., Mumbai, India.
- Hydrogen peroxide, Disodium hydrogen orthophosphate (Na_2HPO_4), Potassium dihydrogen orthophosphate (KH_2PO4), Ethylene diamine tetra acetic acid (EDTA-K^+), Magnesium sulphate, Potassium chloride, Sodium chloride, Calcium chloride, Magnesium chloride, Ammonium molybdate reagent, Sodium metabisulphite, Sodium sulphite, Sodium hydroxide, Potassium hydroxide, Copper sulphate, Lead acetate, Zinc dust, Sodium potassium tartarate, Sodium carbonate, Sulphosalicyclic acid, Lithium lactate, Sodium nitroprusside, Ferric chloride, Tween 80, Potassium ferricyanide (III) [$K_3Fe(CN)_6$], Aluminum chloride hexahydrate ($AlCl_3.6H_2O$), Potassium acetate (CH_3COOK), Potassium permanganate, 2-deoxy-Dribose, Chloroform, Methanol, Ethyl acetate, Sulphuric acid, Glacial acetic acid, Hydrochloric acid, Orthophosphoric acid (OPA), Nitric acid, Perchloric acid, Isopropanol, Acetone, Diethyl ether, Sodium deoxy cholate and Folin's phenol reagent were purchased from S.D. Fine Chemicals, Mumbai, India.
- Gelatin and Potassium dichromate were purchased from Sulab, Vadodara, India.

5.1.3. Plasma:

Human Plasma was obtained as gift samples from Suraktam Blood Bank, Vadodara.

5.1.4. Cell line:

For in-vitro assays, embryonic cardiomyocyte H9c2 cell line was procured from National center for cell science (NCCS) cell repository. (Annexure-II)

5.1.5. Antibodies:

- Rabbit polyclonal STIM1 and Rabbit polyclonal Oria1 primary antibody were purchased from Santa Cruz Biotechnology, USA.

- Goat anti-rabbit IgG secondary antibody was obtained as gift samples from Biochemistry Department, The Maharaja Sayajirao University of Baroda.

5.1.6. Kits:

- Creatine Kinase-MB (CK-MB), Lactate Dehydrogenase (LDH), Na^{+}, K^{+} and Mg^{++} estimation kits were purchased from Coral clinical system, Goa, India.
- The rat IL-6 and TNF-α ELISA kit were purchased from Krishgen biosystems, Mumbai, India.
- Ca^{++} estimation kit was purchased from Siemens Ltd, Vadodara, India.
- Cholesterol, Bilirubin, Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Blood urea nitrogen, Creatinine, Total protein, Albumin were purchased from Span Diagnostic ltd. Surat, India.

5.1.7. Animals:

All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Department, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda. (MSU/IAEC/2015-16/1507, MSU/IAEC/2018-19/1801). (Annexure-III) All experiments described in present study were carried out as per guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and The Prevention of Cruelty to Animals act (PCA), 1960. The group of 3 rats were housed in polypropylene cages (47x34x20cm) lined with corn cob, renewed every 7 days under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 10%) and 12h/12h light-dark cycle. The animals were fed on a conventional laboratory diet (Pranav Agro Industries Ltd., Maharashtra, India) and purified R.O drinking water *ad libitum*. The pellet diet consisted of crude protein (20.30%), crude oil (4.20%), crude fibre (4.54%) and ash (6.10%) which provides the energy of 3630 Kcl/Kg.

5.2. Methods

5.2.1. Prepration and Evaluation of extracts of Piperbetle and Rubia cordifolia.

5.2.1.1. Preparation of Plant Extract [147, 148]

- **Piper betle Lin.**

Powder leaves of *Piper betle Lin.* (30g) was packed into soxhlet column and extracted with 250 ml of ethyl acetate at 60°C till colorless solvent was seen in siphon tube. The extract was concentrated to dark greenish black sticky residue under reduced pressure on a rotary evaporator (Büchi® Rotavapor R-100), with an approximate 3.6 % w/w yield. The extract was labeled with Piper betle ethyl acetate extract (PBEA) and stored at 4°C in airtight container till further use.

- **Rubia Cordifolia Lin.**

Powder roots of *Rubia cordifolia Lin.* (30g) was packed into soxhlet column and extracted with 250 ml of 80% v/v methanol at 60°C till colorless solvent was seen in siphon tube. The extract was concentrated to dark reddish brown sticky residue under reduced pressure on a rotary evaporator (Büchi® Rotavapor R-100), with an approximate 7.6 % w/w yield. The extract was labeled with Rubia cordifolia hydroalcoholic extract (RCHA) and stored at 4°C in airtight container till further use.

5.2.1.2. Proximate analysis[149]

The crude drugs should be tested for the following tests as per WHO guideline for the effect of storage condition on original characteristic.

- **Loss on drying**

About 5 g of powdered drug was accurately weighed, placed in petridish and dried in hot-air oven at 110°C for four hours. After cooling, it was placed in a desiccator, later the loss in weight was recorded and the procedure was repeated till constant weight was obtained.

- **Ash value**

About 2g of crude drug powder was accurately weighed in a tarred and previously ignited silica crucible. It was incinerated gradually by increasing the heat, not exceeding dull red heat, until free from carbon, cooled and weighed. The percentage of ash was calculated.

a) Acid insoluble ash: The ash from the above experiment was boiled for 10 min with 25 ml of diluted hydrochloric acid, and the insoluble matter was collected in a silica crucible (previously ignited and weighed). The percentage of acid-insoluble ash was calculated.
b) Water soluble ash: The total ash was boiled for 5 min with 25 ml of water. The insoluble matter was collected in a crucible, washed with hot water, ignited and weighed. The percentage of water soluble ash was calculated.

5.2.1.3. Analysis of Phytoconstituents

A. Identification of Phytoconstituents by chemical test

- **Preparation of Extract sample:**

An accurately weighed 100 mg of PBEA and RCHA were mixed with 20 ml ethyl acetate and 80% methanol respectively, and 0.5% of these solutions were filtered through WHATMAN No. 1 filter paper. Following tests were performed for Phytochemical screening in which 0.5 ml of 0.5% solution was used for all tests.

Qualitative Phytochemical screening tests for Alkaloids, Flavonoids, Glycosides, Cardiac glycosides, Cytogenetic glycosides, Anthraquinone glycosides, Saponins glycosides, Tannins and Phenolics, Steroids, Quinones, Fat and oils, Carbohydrates and Proteins with amino acids were carried out according to the method of Harborne and Kokate [148, 150].

B. Determination of Total Phenolic Content[151]

Reagents:

1. Folin-Ciocalteu reagent (FCR) (1:10 v/v): 1ml of FCR was diluted in 9 ml of distilled water.
2. Carbonate buffer (7.5% w/v): 7.5 g of Na_2CO_3 was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water.

3. Gallic acid (GA) (1000 µg/ml): 10 mg GA was dissolved in 10 ml of methanol solution.
4. Sample preparation: An accurately weighed 10 mg PBEA and RCHA were dissolved in 10 ml of ethyl acetate and 80% methanol solution, respectively to obtain 1000 µg/ml stock solution. Serial dilutions of stock solution (1000 µg/ml) were prepared into different concentration with the same solvent.

0.5 ml of sample stock solution was mixed with 2.5 ml FCR. 10 min later, 2 ml of Na_2CO_3 solution was added. The mixture was allowed to stand for 90 min at room temperature and the changes of dark blue color due to phenol oxidation were determined by spectrophotometer (1800, Shimadzu, Japan, 2008-09) at 765 nm. GA was chosen as a standard. Using a six point standard curve (8-80µg/ml), the levels of total phenolic contents in sample was determined in triplicate and the results were expressed as gallic acid equivalents (mg gallic acid/g dried extract).

C. **Determination of Total Flavonoids content**[152]

Reagents:

1. Ethanol (95%): 95 ml of ethanol was diluted in 5 ml of distilled water.
2. Aluminum chloride hexahydrate ($AlCl_3.6H_2O$) (10%): 10 gm of $AlCl_3$ was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water.
3. Potassium acetate (CH_3COOK) (1M): 98.15 g of Potassium acetate was dissolved in 800 ml of distilled water. The volume was then made up to 1000 ml with distilled water.
4. Quercetin (1000 µg/ml): 10 mg Quercetin was dissolved in 10 ml of methanol solution.
5. Sample preparation: An accurately weighed 10 mg PBEA and RCHA were dissolved in 10 ml of ethyl acetate and 80% methanol solution, respectively to obtain 1000 µg/ml stock solution. Serial dilutions of stock solution (1000 µg/ml) were prepared into different concentration with the same solvent.

0.5 ml of sample stock solution was mixed with 1.5 ml ethanol, 0.1 ml of aluminum chloride hexahydrate ($AlCl_3.6H_2O$) (10%), 0.1 ml of CH_3COOK, and 2.8 ml of deionized

water. After incubation at room temperature for 40 min, absorbance of the mixture was then determined by spectrophotometer (1800, Shimadzu, Japan, 2008-09) at 415 nm against deionized water as blank. Quercetin was chosen as a standard. Using an eight point standard curve (10–80 µg/ml), the levels of total flavonoid contents in sample were determined in triplicate and the results were expressed as Quercetin equivalents (mg Quercetin/g dried extract).

D. Quantitative determination of Eugenol by GC-FID[153]

- **Instrument**

Instrument Name: Perkin Elmer GC clarus 500

Detector: FID (Flame ionization detector)

Software: TotalChrom Navigator clarus500

- **Preparation of Standard and Sample Solutions**

Standard solution of Eugenol: Standard stock solution (100 mg/ml) of eugenol was prepared by dissolving 1000 mg of accurately weighed eugenol in methanol and volume was made up to 10 ml. From the standard stock solution, working standards (1-10000 µg/ml) were prepared by appropriate dilutions of the stock solution with methanol.

Sample solution of PBEA: 1000 mg of PBEA was weighed accurately in 10 ml volumetric flasks and it was dissolved in ethyl acetate followed by sonication for 5 to 10 minutes and the volume was made up to 10 ml using the same solvent. Solution was filtered using 2 µm syringe filter.

Sample solution of RCHA: 1000 mg of RCHA was weighed accurately in 10 ml volumetric flasks and it was dissolved in 80% methanol followed by sonication for 5 to 10 minutes and the volume was made up to 10 ml using the same solvent. Solution was filtered using 2 µm syringe filter.

- **Experimental and Analytical Conditions**

Oven Program:

Initial Temp:	60°C
Initial Hold:	2.00 min

Total Run Time:	35 min
Maximum Temperature:	350°C
Equilibration Time:	0.3 min
Ramp 1:	10°C/min to 310°C hold for 8.00 min
Injector Temperature:	340°C
Carrier Gas Velocity:	32 cm/sec
Detector:	300°C

Gas Flow		**Column**	
Air:	450.0 mL/min	Column A length:	30.00 m
H_2:	45.0 mL/min	Diameter:	250 µm
Carrier gas:	N_2	Split Ratio:	80.0: 1

- **Chromatographic procedure**

The GC-FID parameters used in the analysis were based on the boiling point and affinity towards the stationary phase of the drug. Eugenol's boiling point is about 254˚C. Manual split injection of 1 µL sample and the standard was performed at an inlet temperature and detector temperature of 240˚C and 300˚C, respectively. After injection, the oven temperature were increased quickly from 60˚C at hold for 2 min then programmed within 25 min to 310˚C at a rate of 10˚C per min and hold for 8 min. Nitrogen at a flow rate of 0.942 ml/min was used as a carrier gas. Synthetic air (flow rate of 450 ml/min) and hydrogen (45 ml/min) were fed to the FID. All the gases used in these studies were of pharmacopoeial purity. The peak areas were recorded for all concentrations. Five point calibration curve of eugenol (1-10000 µg/ml) was plotted as peak area versus concentration of eugenol in µg/ml injected in triplicate. The amount of eugenol in PBEA and RCHA were quantified using a calibration curve plotted with eugenol standard.

E. Quantitative determination of Eugenol by HPTLC[154]

- **Instrument**

Sample applicator **:** CAMAG Linomat 5, automatic sample applicator

Development**:**	CAMAG Twin trough chamber 10×10
Detection **:**	CAMAG TLC Scanner 3 with D2 and Hg lamp
Software **:**	WinCATS Planar Chromatography Manager and CAMAG software, Reprostar 3 with G5 digital camera and WinCATS integration software.

- **Preparation of Standard and Sample Solutions**

Standard solution of Eugenol: Standard stock solution (10 mg/10 ml) of eugenol was prepared in methanol. Working solution of eugenol (1000µg/ml) was prepared by appropriate dilutions of the stock solution with methanol.

Sample solution of PBEA: An accurately weighed 500 mg of PBEA was mixed with 5 ml ethyl acetate. The solution was filtered using 2 µm syringe filter.

Sample solution of RCHA: An accurately weighed 500 mg of RCHA was mixed with 5 ml methanol. The solution was filtered using 2 µm syringe filter.

- **Experimental Conditions**

Stationary phase :	Pre-coated silica gel 60 F254 HPTLC aluminum plates (10×10 cm, 0.2 mm thick), Merck
Mobile Phase :	Toluene : Ethyl acetate (9.3 : 0.7)
Application position :	20.0 mm
Band length :	8.0 mm
Preconditioning :	Saturation with mobile phase for 20 min
Solvent front position :	70.0 mm
Measurement mode :	Absorption/ Fluorescence

- **Chromatographic procedure**

Priority was given to presence of eugenol in PBEA and RCHA against eugenol R_f value and after conformation, main plates were prepared. These plates were developed in the mobile phase, dried in air and scanned at 282 nm for eugenol as per the chromatographic conditions mentioned above. The peak areas were recorded for all concentrations. Ten

point calibration curve of Eugenol was plotted as peak area versus concentration of eugenol. The plate was derivatized by spraying with Anisaldehyde Sulphuric acid reagent (ASR) followed by photographing in UV visible mode. The amount of Eugenol in PBEA and RCHA were quantified using calibration curve plotted with Eugenol standard.

5.2.1.4. Evaluating antioxidant activity of PBEA and RCHA: In-vitro

To ascertain free radical scavenging property of PBEA and RCHA, *in vitro* methods were performed, namely radical scavenging activity by DPPH reduction (DPPH assay), β-carotene bleaching method, Hydrogen peroxide scavenging activity, Superoxide radical scavenging activity in PMS-NBT system, Nitric oxide scavenging activity, Hydroxyl radical activity by Fenton reaction and Fe3+ reducing power method.

Reagents:

1. **Standard preparation:** An accurately weighed 10 mg (α-tochopherol, Ascorbic acid, Butyrated hydroxyl anisol (BHA) and Quercetin were dissolved in 10 ml of methanol solution to obtain 1000 µg/ml stock solution. Serial dilutions of stock solution (1000 µg/ml) were prepared into different concentration with the same solvent.
2. **Sample preparation:** An accurately weighed 10 mg PBEA and RCHA were dissolved in 10 ml of ethyl acetate and 80% methanol solution, respectively to obtain 1000 µg/ml stock solution. Serial dilutions of stock solution (1000 µg/ml) were prepared into different concentration with the same solvent.

5.2.1.4.1. DPPH radical scavenging assay[155]

Reagent:

1. DPPH solution: 2.5 mg of 2, 2-diphenyl- 1-picrylhydrazyl radical (DPPH) was dissolved in 25 ml of methanol.

In each reaction, one ml of solutions (Sample & Standard) and methanol (Blank) were mixed with 1.5 ml of 0.25 mM DPPH. The mixture was shaken vigorously for 2 minutes and allowed to reach a steady state at room temperature for 30 min in dark place. Ascorbic acid and α-tochopherol were used as standard and for the blank, sample was

substituted by methanol. Decolorization of DPPH was determined by measuring the absorbance at 517 nm against methanol with a spectrophotometer (1800, Shimadzu, Japan, 2008-09). The DPPH radicals scavenging activity was calculated according to the following equation: Scavenging activity = $[A_0\text{-}A_1/A_0] \times 100$ where A_0 is the absorbance of the control (blank, without extract) and A_1 is the absorbance of the extract or standard sample.

5.2.1.4.2. β-carotene bleaching assay[156]

Reagent:

1. β-carotene-linoleic acid mixture: 0.5 mg of β-carotene in 1 ml of chloroform was added to 25 µL of linoleic acid and 200 mg of Tween 80 emulsifier mixture. After that chloroform was evaporated under vacuum and 100 ml of distilled water saturated with oxygen was added slowly by vigorous shaking.

4 ml of this mixture was added into different test tubes containing 0.2 ml fixed concentration (500 µg/ml) of the sample and standard. As soon as the zero time absorbance was measured at 470 nm using a spectrophotometer (Shimadzu-1800). The emulsion integral was incubated for 2 h at 50°C. After incubation 120 min absorbance was measured. A blank, devoid of b-carotene, was prepared for background subtraction. Ascorbic acid, Quercetin, Curcumin and α-tochopherol were used as standards. The bleaching rate (R) of b-carotene was calculated according to the following equation: R= ln(a/b)/t where ln is the natural log, a is the absorbance at time 0, b is the absorbance at time t (120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using following equation: AA $= [Rcontrol - Rsample/Rcontrol] \times 100$

5.2.1.4.3. Superoxide anion radical scavenging activity[157]

Reagents:

1. Tris-HCl buffers (16mM, pH-8.0): 1.93 g Tris base was dissolved in 800 ml of distilled water. The pH of the solution was adjusted to 8.0 with 1N hydrochloric acid (HCl). The volume was then made up to 1000 ml with distilled water.

2. Nitro blue tetrazolium (NBT) (50µM): 40.85mg of NBT was dissolved in 800 ml of Tris-HCl buffer. Then volume was then made up to 1000ml with Tris-HCl buffer.
3. Phenazine methosulphate (PMS) (10µM): 3.06mg of PMS was dissolved in 800 ml of distilled water. Then volume was made up to 1000ml with distilled water.
4. NADH (78 µM): 55.33 mg of NADH was dissolved in 800 ml of Tris-HCl buffer. The volume was then made up to 1000ml with Tris-HCl buffer.

In the experiment, O_2^- anion were generated in 3 ml of Tris–HCl buffer containing 1 ml of NBT, 1 ml of NADH and 1 ml of different concentrations (10, 25, 50, 100, 200 and 500 µg/ml) of sample and standard. The reaction was initiated by adding 1 ml of PMS solution in the reaction mixture and after 5 min incubation at 25°C; absorbance was measured at 560 nm against the corresponding blank. The capacity of scavenging anion radical was calculated using the following equation:

$$\% O_2^- \text{ anions scavenging activity} = [A_{control}-A_{sample}/A_{control}] \times 100$$

Where $A_{control}$ was the absorbance without extract and A_{sample} was the absorbance with extract.

5.2.1.4.4. Hydroxyl redical scavenging activity[158, 159]

Reagents:

1. KH_2PO_4-KOH buffer (20 mM) (pH-7.4): (a) 1.55g of KOH was dissolved in 800 ml of distilled water. The volume was then made up to 1000ml with distilled water. (b) 1.16ml of H_3PO_4 was dissolved in 800 ml of distilled water. The volume was then made up to 1000ml with distilled water. (a) was treated with (b) until pH was adjusted 7.4
2. 2-deoxy-Dribose (28 mM): 3.75g of 2-deoxy-Dribose was dissolved in 800 ml of KH_2PO_4-KOH buffer. The volume was then made up to 1000 ml with KH2PO4-KOH buffer.

3. Ethylenediaminetetraacetic acid (EDTA) (1.04mM): 303.88mg of EDTA was dissolved in 800 ml of distilled water. The volume was then made up to 1000ml with distilled water.
4. Ferric chloride ($Fecl_3$) (200µM): 32.44mg of $Fecl_3$ was dissolved in 800 ml of distilled water. The volume was then made up to 1000ml with distilled water.
5. Hydrogen peroxide (1.0mM): 0.113 ml of H_2O_2 was dissolved in 800 ml of distilled water. The volume was then made up to 1000ml with distilled water.
6. Ascorbic acid (1mM): 0.176g of ascorbic acid was dissolved in 800 ml of distilled water. The volume was then made up to 1000ml with distilled water.
7. Thiobarbituric acid (TBA) (1%): 1.0 g of TBA was dissolved in 80 ml of hot & slight acidic (adjusted by drop of acetic acid) distilled water. The volume was then made up to 100ml with hot distilled water.
8. Trichloro acetic acid (TCA) (2.8%): 2.8 g of TCA was dissolved in 80 ml of distilled water. The volume was then made up to 100ml with distilled water.

The Fenton reaction mixture (1.0 ml) consist of 100 µL of 2-Deoxy-Dribose, 500 µL of samples and standards (0.05,0.1, 0.2,0.3,0.4,0.5,0.6,0.7,0.8,0.9,1.0 µg/ml), 200 µL of $FeCl_3$: EDTA (1:1 v/v), 100 µL of H_2O_2 and 100 µL of ascorbic acid was incubated at 37 °C for 1 h. Following incubation, 1.0 mL of TCA and 1 ml of TBA were added to the reaction mixture and placed in a boiling water bath for 20 min for color development. For, control sample deionized water was used instead of methanol because methanol itself as Hydroxyl radical scavenger. After cooling, absorbance was measured by spectrophotometer (1800, Shimadzu, Japan, 2008-09) at 532 nm, against a blank sample containing devoid of deoxyribose. Ascorbic acid and α-tochopherol were used as standard and % inhibition was calculated by comparing test and blank sample.

5.2.1.4.5. Hydrogen peroxide scavenging activity[160]

Reagent:

1. Phosphate buffer saline (PBS) (pH-7.0): 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 were dissolved in 800 ml of distilled water. The pH of the

solution was adjusted to 7.4 with 1N hydrochloric acid (HC1). The volume was then made up to 1000 ml with distilled water.

2. A solution of hydrogen peroxide (40 mM) was prepared by adding 410µL H_2O_2 in phosphate buffer saline (PBS) (pH 7.4) at 20°C and concentration was determined spectrophotometrically at 230 nm.

Appropriate stock solution of (Samples and Standards) were taken, and then added to the H_2O_2 solution at a final concentration of 10, 25, 50, 100, 200 and 500 µg/ml at 20°C. After 10 min absorbance of H_2O_2 was determined in a Shimadzu spectrophotometer with 230nm against blank solutions containing stock solution of (sample-1 and standard-1) (10, 25, 50, 100, 200 and 500 µg/ml) and (sample-2 and standard-2) (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µg/ml) in PBS without H_2O_2. Ascorbic acid and BHA were used as standards. The percentage of hydrogen peroxide scavenging is calculated as follows: % scavenged = $[A_0\text{-}A_1/A_0] \times 100$ where A_0 is the absorbance of the control and A_1 is the absorbance of the extract or standard sample. Here, Sample-1 is PBEA, Sample-2 is RCHA, Standard-1 is Ascorbic acid and Standard-2 is BHA.

5.2.1.4.6. Reducing power method[161]

Reagents:

1. Phosphate buffer (0.2M, pH 6.6): (a) 2.76 gm of sodium dihydrogen orthophosphate (NaH_2PO_4. H_2O) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water, (b) 5.36 gm of disodium hydrogen orthophosphate (Na_2HPO_4. $7H_2O$) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water. 62.5 ml of (a) was mixed with 37.5 ml of (b). The pH of the mixture was adjusted to 6.6 with NaOH or H_3PO_4.
2. Potassium ferricynide [$K_3Fe(CN)_6$] (1% w/v): 1.0 g of Potassium ferricynide was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water.
3. Trichloroaceticacid (TCA) (10.0%): 10.0 g of TCA was dissolved in 80 ml of distilled water. The volume was then made up to 100 ml with distilled water.

4. Ferric chloride ($FeCl_3$) (0.1%): 0.1 g of $FeCl_3$ was dissolved in 80 ml of distilled water. The volume was then made up to 100 ml with distilled water.

750µL of sample and standard (10, 25, 50, 100, 200 and 500 µg/ml) were mixed with 750µL of phosphate buffer and 750µL of [$K_3Fe(CN)6$] (III), followed by incubating at 50 °C in a water bath for 20 min. The reaction was stopped by adding 750µL of TCA solution and then centrifuged at 4000 rpm for 10 min. 1.5 milliliter of the supernatant was mixed with 1.5 ml of distilled water and add 100µL of $FeCl_3$ solution. After 10 min. absorbance at 700 nm was determined with spectrophotometer (1800, Shimadzu, Japan, 2008-09).

5.2.2. Pharmacokinetic Study of 2-APB

5.2.2.1. Development of RP-HPLC method of 2-APB[162]

- **Preparation of 2-APB solution**
  - **(a)** 2-APB 1st stock solution (1000 ppm): Accurately weighed 10 mg 2-APB was taken in 10 ml volumetric flask and then diluted with methanol up to the mark.
  - **(b)** 2-APB working solution (100 ppm): Prepared by transferring 1 ml from 2-APB stock solution to 10 ml volumetric flask and then diluted with methanol up to the mark.
  - **(c)** 2-APB detection solution (10 ppm): Prepared by transferring 1 ml from 2-APB working solution to 10 ml volumetric flask and then diluted with methanol up to the mark. All solutions were prepared in cold condition.
- **Selection of detection wavelength by UV**

10 ppm 2-APB detection solution was scanned between 200-400 nm in uv-visible spectrophotometer (Shimadzu-1800).

- **Selection and optimization of chromatographic conditions**

To optimize the chromatographic conditions, the effect of chromatographic variables such as composition of mobile phase, ratio of mobile phase and flow rate were studied. The resulting chromatograms were recorded and the chromatographic parameters such as

capacity factor, asymmetric factor, resolution and theoretical plates were calculated. The conditions that gave the best resolution, symmetry and theoretical plate were selected for estimation. Different mobile phase and pH were trialed. Finally, a simple and inexpensive method was developed by using a simple combination of 10mM K_2HPO_4 buffer and methanol (52.5: 47.5) with 0.01% Triethyl amine (TEA) and pH was adjusted to 8.2 with O-Phosphoric acid which was finally filtered with 0.2 µm Nylon membrane filter. Resulting solution was degassed by ultra sonication for 5 minutes.

5.2.2.1.1. Method validation

- **Linearity and Range**

The linearity study was carried out for 2-APB at six different concentration levels from the 2-APB working solution. The calibration curve constructed was linear over the concentration range of 10-100 µg/ml.

- **Precision**

Solutions containing different concentrations of 2-APB were prepared with mobile phase. 20 µL solutions were injected and chromatograms were recorded. The peak area of 2-APB was calculated for each trial. The experiment was repeated 3 times in a day for Intra-day precision and on 3 different days for inter-day precision.

- **Sensitivity**

To study the sensitivity of optimized method LOD and LOQ was determined using blank replicates injection and by equation 1 and 2.

LOD =3.3 σ/S …….. (1)

LOQ =10 σ/S ……..... (2)

- **Accuracy**

Accuracy of the method was confirmed by recovery study from prepared Laboratory sample at 3 level of standard addition (80%, 100%, and 120%) of label claim. Recovery greater than 98 % with low SD justified the accuracy of the method.

- **System suitability**

Retention time, Theoretical plate per meter and Tailing factor were calculated.

5.2.2.1.2. Bioanalytical method of 2-APB in Human plasma

- **Preparation of Spiking Solutions of 2-APB**

10 ppm spiking solutions of 2-APB was prepared using 0.2 ml of standard solutions made up to 2 ml with plasma.

- **Method development by Protein Precipitation Extraction**

Prepared sample was precipitated by the 3 times addition of Methanol, Acetonitrile, Acetone along with vortex for 1min and centrifuged at 5000 rpm for 5 min at 4°C.

5.2.2.1.3. Method validation

- **Linearity and Range**

The linearity of the method was determined over calibration range of 0.05 µg/ml to10 µg/ml. The calibration standards were prepared by spiking known concentration of 2-APB working standard solution. A linearity curve containing six non-zero concentrations was analyzed. Back-calculated the concentrations of each level and plot the graph of back-calculated concentration against drug area ratio. Calculate the slope, y-intercept and correlation coefficient curve by suitable linear regression analysis. Calibration curve of plasma was prepared by protein precipitation method.

- **System Suitability**

System suitability was performed before start of every new batch. It was performed by injecting six replicates of MQC of 2-APB. Retention time, Tailing Factor and Theoretical Plate of 2-APB were calculated.

- **Precision and Accuracy**

Precision and Accuracy was measured on the samples spiked with known amounts of the analyte. Accuracy and precision were determined by replicate analysis of six

determinations of four concentration levels which covers the calibration range: LLOQ, LQC, MQC, and HQC. These QC samples were analyzed against the calibration curve and obtained concentrations compared with the nominal value. Precision is expressed as the % coefficient of variation (% CV).

- **Selectivity**

Specificity and selectivity was carried out using six plasma samples.

- **Recovery**

Recovery of 2-APB in plasma was evaluated by comparing the mean peak responses of at least six injection of each low, medium and high quality control sample, prepared in plasma, to mean peak responses of non-spiked samples prepared in elution solvent and external spiked matrix extracted sample.

5.2.2.1.4. Pharmaco-kinetic study of 2-APB[163, 164]

Healthy male wistar albino rats (8-12 weeks) weighing 250 ± 25 g were obtained from Zydus Research Centre, Ahmedabad for pharmaco-kinetic study. 2-APB was administrated at dose of 4 mg/kg i.v. in rats (n=6). Blood was withdrawn from retro orbital plexus under light anesthesia and collected with anticoagulant at different time point of 0.08, 0.16, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 8, 12, 18 h. Plasma was separated by centrifuge at 4000 rpm for 10 min at 4°C (CPR 30, REMI Centrifuge, India, 2011-12) and stored at -80°C (EIE 414, Deep freezer, CryoScientific, India, 2008-09). Sample was analyzed by developed precipitation of RP-HPLC method. Concentration of 2- APB was find out with time profile, this data was interpreted by Pharmacokinetic software and to elucidate half life of 2-APB.

5.2.3. Preliminary in-vitro Safety and efficacy studies of 2-APB, eugenol, PBEA and RCHA.

5.2.3.1. Cell-viability assay[165]

Reagents:

1. Dulbecco's Modified Eagles' Medium (DMEM): Total 100 ml of DMEM contain 10 ml of FBS, 1 ml of 100 IU/mL penicillin and 100 µg/mL streptomycin. Stored at 2-8°C.
2. Phosphate buffer saline (PBS) (pH-7.4): 8.0g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g of KH_2PO_4 were dissolved in 800 ml of distilled water. The pH of the solution was adjusted to 7.4 with 1N hydrochloric acid (HCl). The volume was then made up to 1000 ml with distilled water and autoclaved.
3. Trypsin-EDTA solution (0.25%):1 ml aliquots were prepared and stored at -20°C.
4. Trypan Blue (0.4% w/v): 0.4g of trypan blue dye was dissolved in PBS. Constant stirring was required for complete dissolution of powder. The solutions were sterilized by filtering through a sterile membrane filter with porosity of 0.22 microns or less.
5. 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) solutions: 5.0 mg of MTT was dissolved in 1 ml of PBS. The solutions were sterilized by filtering through sterile membrane filter with porosity of 0.22 microns or less.
6. H_2O_2 (100µM):1.02µL of H_2O_2 was added immediately to 10 ml media (100 mM) and made to 100 µM with dilution of stock solution.
7. Dimethylsulphoxide (DMSO): Sterile DMSO was used for Drug and formazan crystal dissolution.
8. Drug Dilution:

   Eugenol (mM) and 2-APB (µM): Different concentrations (0, 25, 50, 75, 100, 150, 300 and 500) were prepared in media.

   PBEA: 0.1, 1, 10, 25, 50, 100 and 500µg/ml were prepared in media.

   RCHA: 0.001, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 6.0, 12.0 and 24.0µg/ml were prepared in media.

- **Cell culture maintenance**

Cardiomyoblasts (H9c2) is a sub-clone of original clonal cell line derived from embryonic heart tissue (BD1X) of rat. Fusion occur faster if the serum concentration in the medium reduced to one percent. Cells were maintained in DMEM (Dulbecco's Modified Eagle's medium-high glucose with 2mM glutamine) with 10% heat in-activated

FBS (Fetal Bovine Serum), 100 U/ml Penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2.

- **Sub-culture procedure**

Laminar cabinet was disinfected and all the components were allowed to dry for 30 minutes and media was removed from the flask in sodium hypochlorite. 1 ml of warm (37°C) trypsin-EDTA was added to the culture flask containing H9c2 cells at a sub confluent stage in monolayer and washed; further 1ml warm trypsin-EDTA was added and incubated for 1 min at 37°C so as to complete detachment of cells from the surface of the flask. To confirm the complete detachment, trypsin treated cells were observed under microscope (cell change to spherical from spindle shape). After complete detachment of cells from the surface, trypsin-EDTA containing cells was transferred into 2 ml centrifuged tubes, spun at 1000 rpm for 3-5 min, the supernatant was discarded and the pellet was resuspended in 500 µl to 1 ml DMEM depending on the cell density and subculture requirements. 500 µl volumes of the cells were added to a culture flask containing DMEM. Atmospheric conditions of 37°C temperature, 95% relative humidity, and 5% Carbon dioxide concentration were maintained in CO_2 incubator.

- **Cell counting**

50 µl of cell suspension was added to 50 µl PBS in a micro-centrifuge tube; to this 100 µl 0.4% of trypan blue was added and incubated for 2-3 minutes. Cells were counted on improving Neubauer chamber of Haemocytometer.

Calculation of number of cells and cell viability was done as below:

Total no of cells (L+D) in 4 $1mm^2$ (A,B,C,D)	Cells per mm^2 (0.1 µl or 10^{-4} ml)	Dilution factor (D)	Cells per ml	% viability = no. of live cells *100
X	X/4	X/4*D=Y	$Y*10^4$	L/X*100

The improved Neubauer haemocytometer consists of a glass that fits onto the adjustable stage of a microscope. A grooved gird is observed through the microscope on the haemocytometer surface. A cell suspension was put onto the gird by touching the end of capillary tube (can be pipette tip or pasteur pipette) containing the cell suspension at the edge of the cover-slip placed on the upper surface of the haemocytometer. Then cells were counted in a standard volume (usually 5 × 0.1 µl) as defined by the area of the gird. Trypan blue was added to the cell suspension before counting. The dye penetrates the membrane of nonviable cells, which were stained blue and were therefore distinguished from viable cells.

- **Treatment protocol:**

H9c2 cells were seeded to 60-80% confluency in 96 well plates within a growth period of 24 hours. Upon reaching 80% confluency, H9c2 cells were treated with 100 µL of various concentration of sample or without sample for another 24 h, followed by incubation with or without H_2O_2 (100μM) for 1 h. Different concentrations of sample were used which was mentioned above and untreated cells were considered as control. All the experiments were prepared with triplicates for statistical analysis. After 1 hours of H_2O_2 exposure, cells were washed with PBS and again 200 µl of media was added in well plate.

5.2.3.1.1. MTT assay for cell viability[166]

After 1 h of H_2O_2 exposure, 20µl of MTT was added in 96 well plates and to made final well concentration 500µg/ml. Plate was wrapped with aluminum foil and incubated 4 hours in a humidified atmosphere at 37°C. Media was removed from the well leaving formazan crystals at the bottom of the well, crystals were dissolved in 100µl of DMSO in each well. Viability was then evaluated by measuring an absorbance at wavelength of 570 nm using Microplate reader (680XR, Bio-Rad, USA, 2009-10). OD values of each well were normalized against the control well which contain maximum viability as no treatment is given to them. Viability of cells treated with sample was calculated as mean of OD at 570 nm and plotted against sample concentration.

5.2.3.2. Intracellular reactive oxygen species (ROS) levels assay[167]

Reagents:

1. 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA)(1mg/ml): 1mg of DCFHDA was dissolved in 1 ml DMSO.
2. 4′,6-diamidino-2-phenylindole (DAPI) (0.1 mg/ml): 1mg of DAPI was dissolved in 10 ml distilled water.
3. Phosphate buffer saline (PBS) (pH-7.4): 8.0g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24g of KH_2PO_4 were dissolved in 800 ml of distilled water. The pH of the solution was adjusted to 7.4 with 1N hydrochloric acid (HCl). The volume was then made up to 1000 ml with distilled water and autoclaved.
4. Drug Concentration: Based on the results of cell viability assay.
5. H_2O_2 (100µM):1.02 µL of H_2O_2 was added immediately to 10 ml media (100mM) and made to 100 µM with dilution of stock solution.

H9c2 cells were seeded and maintained in 24-well plates for 24 h and exposed to best concentration of sample (2-APB-150µM, Eugenol-50mM, PBEA-10µg/ml, RCHA-100 ng/ml) or without sample for 24 h followed by incubation with 100 µM H_2O_2 for 1 h. Before H_2O_2 exposure medium was aspirated. After H_2O_2 treatment cells were washed with PBS and incubated with (10 µg/ml) of DCFHDA and (1 µg/ml) of DAPI for 30 min at 37°C in the dark. Cells were washed immediately with 3×PBS to remove the extracellular compound. DCFH-DA green fluorescence was observed using florescent microscope (CLSM 510, Zeiss, Germany, 2006-07). The relative cell fluorescence intensity was measured by Image J software (NIH, Bethesda, MD).

5.2.3.3. Cellular antioxidant enzyme profile

Reagent:

1. 0.1% Triton X-100: 10 µl of Triton X-100 was dissolved in 10 ml of distilled water.

H9c2 cells were seeded at 2.5 x 10^6 cells/well and cultured overnight. Cells were exposed to best concentration of sample (2-APB-150µM, Eugenol-50mM, PBEA-10µg/ml, RCHA-100 ng/ml) or without sample for 24 h followed by incubation with 100 µM H_2O_2

for 1 h. Adherent cells were harvested in 1.5 ml centrifuge tubes after 1 h H_2O_2 exposure and then subjected to centrifugation at 1000 rpm for 10 min at 4°C. Cell pellets were sonicated in 2 ml of cold 0.1 % Triton X-100 lysis buffer. Cells were again centrifuged at 10000rpm for 15 min at 4°C and supernatant was used for assay of Lipid peroxidation[168], endogenous antiperoxidative enzymes like Superoxide dismutase (SOD)[169] and Catalase[170], Reduced glutathione (GSH)[171] were determined and detailed procedure are discussed in section **5.2.4.1.5, 5.2.4.1.6, 5.2.4.1.7** and **5.2.4.1.8.**

Note: Sample size is to be reduced 10 times.

5.2.3.4. Annexin-FITC apoptosis assay[172]

5.2.3.5. Reagents:

1. Phosphate buffer saline (PBS) (pH-7.4): 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 were dissolved in 800 ml of distilled water. The pH of the solution was adjusted to 7.4 with 1N hydrochloric acid (HC1). The volume was then made up to 1000 ml with distilled water and autoclaved.
2. Binding Buffer (1X): 10 mm HEPES (pH 7.4), 150 mm NaCl, 2.5 mm $CaCl_2$ dissolved in PBS (pH 7.4).
3. AnnexinV-FITC: Per sample 5µL solution was used.
4. Propidium Iodide (PI): 2 mg of PI dissolved in 1 ml distilled water.
5. Drug Concentration: Based on the results of cell viability assay.

H9c2 cells were seeded and maintained in 24-well plates for 24 h and exposed to best concentration of sample (2-APB-150µM, Eugenol-50mM, PBEA-10µg/ml, RCHA-100 ng/ml) or without sample for 24 h followed by incubation with 100 µM H_2O_2 for 1 h. Before H_2O_2 exposure medium was aspirated. After H_2O_2 treatment cells were washed with cold PBS and resuspended in 1X binding buffer, after which they were incubated with Annexin-V for 15 min in dark at room temperature followed with addition of PI in the same wells to a final concentration of 10µg/ml for 15 min at room temperature as per manufacturer's protocol. These, cells were observed using florescent microscope (Zeiss CLSM 510). The relative cell fluorescence intensity was measured by Image J software (NIH, Bethesda, MD) and then plotted.

5.2.3.6. Vascular reactivity assay (Calcium channel blocking action on aorta)[173, 174]

2-APB and Eugenol have been reported in literature for their calcium channel blocking action and hence only PBEA and RCHA were evaluated here.

Reagent:

1. Krebs solution: 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.4 mM CaCl2, 25 mM NaHCO3, 11 mM glucose and 0.03 mM EDTA were dissolved in 1000 ml of distilled water.

Healthy male wistar albino rats (8-12 weeks) weighing 250 ± 25 gm were obtained from Zydus Research Centre, Ahmedabad for vascular reactivity assay. Animals were sacrificed by an overdose of sodium pentobarbital (50 mg/kg). The descending thoracic aorta was isolated, and the adjacent connective tissue and fat were removed. All aortas were denuded of endothelium film by gentle mechanical procedure and, finally, cut into rings of about 4-5mm of width. The rings were tied to stainless steel hooks with silk thread and these rings were immersed on systemic force transducers (PL3504, AD Instruments, Australia, 2012-13) at 2.0 g resting tension in 30 ml reservoir of temperature-controlled baths (VJ Instruments, India, 2008-09) (37°C) containing Krebs solution that was continuously oxygenated (O_2/CO_2, 95: 5%). The rings were stabilized at 2.0 g resting tension for 120 min, during which the bathing solution was changed every 15 min. This solution was then replaced with a calcium-free isotonic depolarizing solution containing a high concentration of K^+ (100 mM KCl). The sample (PBEA/RCHA) and standard (Verapamil) was added directly to the calcium-free isotonic depolarizing solution containing a high concentration of KCl (100 mM) 30 min before the Ca^{2+}-induced contraction. Finally, the calcium was added cumulatively to achieve a final bath concentration (from 0.1 to 100 mM). The effect of sample and standard on the concentration-response curve for calcium was assessed by comparing the contractile response induced by the addition of calcium in the presence and absence of sample (PBEA-1.0 mg/ml and RCHA-0.3 mg/ml) and standard (5×10^{-6}M) or (2.2 mg/ml).

5.2.4. Pharmacological evaluation of 2-APB, eugenol, PBEA and RCHA in selective cardiovascular disorders.

Note: For dose fixation of test compound (PBEA and RCHA) a pilot study comprising of three dose level (50, 100 and 200 mg/kg) was performed on ISO induced global ischemia in rats. Based on the findings 200 mg/kg dose for the test compound was selected for all experimental models involved in main study.

5.2.4.1. Angiotension-II induced Acute hypertension in vagotomized rats.[173, 175]

- Drug Preparation

PBEA: PBEA was dissolved in olive oil.

RCHA: RCHA was dissolved in water.

Eugenol (EU): Eugenol was dissolved in olive oil.

2-APB: 2-APB was initially dissolved in pure DMSO and further diluted with normal saline so as to make the final concentration of DMSO not exceeding 1%. 2-APB were kept in aluminum foil wrapped micro centrifuge tubes, as they are light sensitive drugs and stored in ice during dosing.

Angiotensin (Ag-II): Ag-II was dissolved in normal saline at the time of injection. Ag-II was kept in aluminum foil wrapped micro centrifuge tubes, as it is light sensitive drug. All the drug solutions were prepared freshly.

Healthy male wistar albino male rats (12-16 weeks) weighing 300 ± 15g were obtained from Zydus Research Centre, Ahmedabad for hypertensive study. All the animals were randomized according to weight in to six groups and each group contain 6 animals. Groups were labeled as control and negative control (Ag-II control) and positive control (2-APB, Eugenol, PBEA and RCHA control). The control (group I) animals received saline. All groups except group I received Ag-II (2µg/kg, i.v.). Group II served as Ag-II control. Group III was test group which received 2-APB (4 mg/kg, i.v.) immediately before Ag-II injection. Group IV to VI were test groups which received eugenol (50 mg/kg, p.o), PBEA (200 mg/kg, p.o) and RCHA (200 mg/kg, p.o), respectively 1 h before Ag-II injection. Animals were put under surgical anesthesia with urethane (1.5 g/kg, *i.p.*)

and tracheotomy was performed followed by severing cervical vagus nerve. Femoral vein and jugular vein were cannulated in order to allow drug administration and blood collection, respectively. The left common carotid artery was cannulated using a PE50 cannula, for the measurement of blood pressure using indirect blood pressure transducer attached with Power lab (PL3504, AD Instruments, Australia, 2012-13). In all animals, hexamethonium chloride (ganglion-blocking agent) was administered at 0.1 g/kg, i.v. dose. 60 min before Ag-II administration blood was withdrawn from retro-orbital plexus under mild anesthesia without anticoagulant from each animal. After BP stabilization (95 ± 4.2 mmHg), blood was collected (labeled as 0 min) just before the administration of Ag-II (2µg/kg, i.v) in negative control, positive control. During experimental period, systolic and diastolic blood pressure difference (ΔP) was measured at 20sec time interval between specific times (0 min to 6 min). Blood was also withdrawn from jugular vein at specific time (0, 0.5, 1, 2, 60 min) after Ag-II administration. Animals were euthanized humanely after 1 h of Ag-II administration for assessing different parameters. 0.1 ml of blood was collected at each point and serum was separated by centrifugation at 4000 rpm for 15 min at 4^{o}C (CPR 30, REMI Centrifuge, India, 2011-12) for estimation of serum electrolytes. Aorta, heart and both kidneys were excised immediately in ice-chilled normal saline and washed with ice-chilled phosphate buffered saline. They were blotted free of blood and tissue fluids, and were weighed on balance (ATX 224, Shimadzu, India, 2013-14) and stored at - 80^{0}C (EIE 414, Deep freezer, CryoScientific, India, 2008-09) for estimation of STIM1 and Orai1 by western blot analysis and antioxidant enzymes.

5.2.4.1.1. Measurement of Blood pressure[173]

Systolic and diastolic blood pressure differences (ΔP) were measured using pressure transducer attached with Power lab data acquisition system (PL3504, AD Instruments, Australia, 2012-13).

- **Biochemical parameters in serum**

The collected serum from control and experimental animals were used as serum electrolytes for assessment of biochemical parameters like Calcium, Sodium and Magnesium.

5.2.4.1.2. Assay of Calcium[176]

Wavelength / Filter: 570nm

Procedure:

Addition Sequence	B (ml)	S (ml)	T (ml)
Buffer Reagent	0.5	0.5	0.5
Color reagent	0.5	0.5	0.5
Distilled water	0.02	-	-
Calcium Standard	-	0.02	-
Sample	-	-	0.02

Prepared samples were mixed well and incubated at room temperature (25^{o}C) for 5 minutes. Absorbances of standard and test samples were measured against the blank, within 60 min.

Calculation: Calcium in mg/dl = $\left(\frac{AbsT}{AbsS}\right) * 10$

5.2.4.1.3. Assay of Sodium[177]

Wavelength / Filter: 530nm

Procedure:

1. Precipitation

Test tubes were labeled as Standard (S) and Test (T) and serum samples and reagents were added as follows:

Addition Sequence	S (ml)	T (ml)
Precipitating Reagent	1.0	1.0
Na^{+}/ K^{+} Standard (S)	0.02	-
Sample	-	0.02

2. Color Development:

Test tubes were labeled as Blank (B), Standard (S) and Test (T) and serum samples and reagents were added as follows:

Addition Sequence	B (ml)	S (ml)	T (ml)

Acid Reagent	1	1	1
Supernant from step 1.		0.02	0.02
Precipitating Reagent	0.02		
Color Reagent	0.1	0.1	0.1

Prepared samples were mixed well and incubated at room temperature (25ºC) for 5 minutes. Absorbances of blank, standard and test samples were measured against distilled water within 15 min.

Calculation: Sodium in mmol/L = $\frac{\text{Abs B} - \text{Abs T}}{\text{Abs B} - \text{Abs S}} * 150$

5.2.4.1.4. Assay of Magnesium[178]

Wavelength / Filter: 510 nm

Temperature: RT

Light Path: 1 cm

Pipette into clean micro centrifuged tubes labeled as Blank (B), Standard (S) and Test (T) and serum samples and reagents were added as follows:

Addition Sequence	B (ml)	S (ml)	T (ml)
Buffer Reagent (L1)	0.5	0.5	0.5
Colour Reagent (L 2)	0.5	0.5	0.5
Distilled water	0.01	-	-
Magnesium Standard (S)	-	0.01	-
Sample			0.01

Prepared samples were mixed well and incubated at room temperature (25ºC) for 5 minutes. Absorbance of standard and test samples was measured against blank within 30 min.

Calculation: Magnesium in mEq/L = $\frac{\text{Abs T}}{\text{Abs S}} * 2$

- **Biochemical parameters in Tissues**

✓ **Homoginization of tissue**

Heart and kidney tissue of control and experimental animals were cross chopped with surgical scalpel into fine slices and were chilled in cold 0.25 M sucrose, quickly

blotted on a filter paper. Tissues were minced and 10% w/v homogenate was prepared separately in 10 mM tris-HCl buffer, pH 7.4 with 25 strokes of tight teflon pestle of glass homogenizer (RQ 127 A, Remi Motors, India, 2005-06) at a speed of 2500 rpm. Tissue homogenate was centrifuged (CPR 30, REMI Centrifuge, India, 2011-12) at 10000rpm for 15 min at 4°C. The clear supernatant was used for antioxidant enzyme estimation.

- **Assessment of Lipid Peroxidation and Endogenous Antioxidants**

5.2.4.1.5. Assay of Lipid Peroxidation (Malonedialdehyde [MDA] content)[168]

Reagents:

1. Thiobarbituric acid (0.67% w/v): 0.67 g of thiobarbituric acid was dissolved in 50 ml of hot distilled water and the final volume was made up to 100 ml with hot distilled water.

2. Trichloroacetic acid (10% w/v): 10 g of TCA was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

3. Standard Malonedialdehyde stock solution: A standard MDA stock solution was prepared by mixing 56 µl of 1, 1′, 3, 3′-tetraethoxypropane with 10 ml with distilled water. 1.0 ml of this stock solution was diluted up to 100 ml to get solution containing 51.20 µg of malondialdehyde/ml. One ml of this stock solution was diluted up to 100 ml to get a working stand]ard solution containing 512 ng of malondialdehyde/ml.

Procedure:

Equal volume of supernatant and ice-cold 10% w/v TCA (0.75 ml: 0.75 ml) were mixed and mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation at 3000 rpm for 15 min at 4°C. Equal volume of clear supernatant solution and freshly prepared TBA was mixed. The resulting solution was heated at 95°C in a boiling water bath for 20 minutes. It was then

immediately cooled in an ice bath for 5 minutes. The color developed was measured at 532 nm against reagent blank. Different concentrations (2-512 ng/ml) of standard MDA were taken and processed as above for standard graph. The values were expressed as nmoles of MDA/mg protein.

5.2.4.1.6. Assay of Superoxide Dismutase (SOD)[169]

Reagents:

1. Carbonate Buffer (0.05 M, pH 10.2): 16.8 g of sodium bicarbonate and 22 g of sodium carbonate was dissolved in 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.

2. Ethylenediaminetetra acetic acid (EDTA) solution (0.49M):1.82 gm of EDTA was dissolved in 200 ml of distilled water and the volume was made up to 1000 ml with distilled water.

3. Hydrochloric acid (0.1 N): 8.5 ml of conc. hydrochloric acid was mixed with 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.

4. Epinephrine solution (3 mM): 0.99 gm epinephrine bitartarate was dissolved in 100 ml of 0.1 N hydrochloric acid and the volume was adjusted to 1000 ml with 0.1 N hydrochloric acid.

5. Superoxide Dismutase (SOD) standard (320 U/ml):

Bottle contain 2050 Units/mg of solid. 2554 Units/mg of protein.

7.3 mg of SOD from bovine liver was dissolved in 1.46 ml of carbonate buffer to prepared 5mg/ml stock solution-A which was same as 14965 Units/1.46 ml. Stock solution-B (1000 U) was prepared from 98 µl of Stock solution-A was dissolved in 902 µl of carbonate buffer. Working solution (320 U/ml) was prepared from the stock solution-B.

Procedure:

0.25 ml of ice-cold ethanol and 0.15 ml of ice-cold chloroform were added in 0.5 ml of supernatant. The reaction mixture was mixed well using cyclomixer for 5 minutes and centrifuged at 2500 rpm for 10 min at 4°C. To 0.5 ml of supernatant, 1.5 ml of carbonate buffer and 0.5 ml of EDTA solution were added. The reaction was initiated by the addition of 0.4 ml of epinephrine and the change in absorbance/minute was measured at 480 nm against reagent blank. SOD activity was expressed as units/mg protein. Calibration curve was prepared by using 1-320 units of SOD.

5.2.4.1.7. Assay of Catalase (CAT)[170]

Reagents:

1. Phosphate Buffer (50 mmol/l, pH 7.0): (a) 6.81g of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in distilled water and made up to 1000 ml. (b) 8.90g of disodium hydrogen orthophosphate (Na_2HPO_4) was dissolved in distilled water and made up to 1000 ml. The solutions (a) and (b) were mixed in the proportion of 1: 1.5 (v/v).

2. Hydrogen Peroxide (30 mmol/l): 0.34ml of 30% hydrogen peroxide was diluted with phosphate buffer to 100 ml. Freshly prepared solution was used every time.

3. Catalase standard (2500 U/mg protein; 1 mg protein/ml): Crystalline beef-liver catalase suspension was centrifuged to isolate the crystals of the enzyme that was dissolved in 0.01 M phosphate buffer (pH 7.0) to give a final concentration of 2.0 mg protein/ml. Before assay, it was diluted with distilled water to obtain 100 U/ml.

Procedure:

To 1 ml of diluted supernatant 0.5 ml of H_2O_2 was added to initiate the reaction. Blank was prepared by mixing 1 ml of diluted supernatant (similar dilution) with 1 ml of phosphate buffer. Dilution should be such that initial absorbance should be approximately 0.500. Decrease in absorbance was measured at 240 nm. Catalase activity was expressed as U/mg proteins. Calibration curve was prepared by using 0.2-16 units of CAT.

5.2.4.1.8. Assay of Reduced Glutathione (GSH)[171]

Reagents:

1. Trichloroacetic acid (20% w/v): 20 g of TCA was dissolved in 60 ml distilled water and the final volume was made up to 100 ml with distilled water.

2. Phosphate Buffer (0.2 M, pH 8.0): 0.2 M sodium phosphate was prepared by dissolving 30.2 g sodium phosphate in 600 ml of distilled water and pH was adjusted to 8.0 with 0.2 M sodium hydroxide solution and final volume was adjusted up to 1000 ml with distilled water.

3. DTNB reagent: 23.76 mg of 5, 5'-dithiobis (2-nitro benzoic acid) was dissolved in 50 ml of buffer and the final volume was adjusted to 100 ml with buffer.

4. Standard Glutathione: 10mg of reduced glutathione was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

Procedure:

Equal volumes of supernatant and 20% TCA (0.5 ml: 0.5 ml) were mixed. The precipitation was separated by centrifugation at 4000 rpm for 10 min at 4°C. 0.25ml of clear supernatant was added in to 2 ml of DTNB reagent. The final volume was made up to 3ml with phosphate buffer. Color developed was read at 412 nm against reagent blank. Different concentrations (1-128 µg/ml) of standard glutathione were taken and processed as above for standard graph. The amount of reduced glutathione was expressed as µg of GSH/mg protein.

5.2.4.1.9. Measurement of STIM1 and Orai1 protein expression in heart, kidney and aorta by Western blot analysis

- **Preparation of Tissue lysates[179]**

Reagent:

1. RIPA buffer (Semi denaturing buffer): Tris –HCL (pH-7.4) (50mM), NaCl (150mM), Sodium deoxycholate (0.5%), Igepal (NP-40) (1%), EDTA (2 mM),

Na-Floride (50mM), SDS (0.1%) was added in 100 ml of distilled water. Storage at 4°C for 6 months.

Aorta, kidney and heart issues were cross-chopped with surgical scalpel in to slices, then minced and homogenized (RQ 127 A, Remi Motors, India, 2005-06) in chilled RIPA lysis buffer. Lysates were sonicated 3 cycles at 45% amplitude with total time 20sec containing 2 sec on and 0.2 sec off interval on ice in sonicator (3000 Ultra sonic homogenizer, Biologics, USA, 2012-13). The supernatant obtained by centrifugation (CPR 30, REMI Centrifuge, India, 2011-12) at 10,000 rpm for 10 min at 4°C was aliquoted and stored at -80°C (EIE 414, Deep freezer, CryoScientific, India, 2008-09).

- **Estimation of Total Protein[180]**

Total protein was estimated by the Bradford Method. 10µL Sample was mixed with 190 µL of Bradford reagent in well plate and absorbance was recorded at 595nm using Microplate reader (680 XR, Biorad, USA, 2008-09). 0-600µg/ml concentration of Bovine serum albumin (BSA) was used as standard.

- **Estimation of STIM1 and Orai1 by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) [181, 182]**

Reagents:

1. Laemmli Buffer: To 10 ml Tris (1.0M, pH 6.8), 4.0 g SDS, 20 ml Glycerol, 10 ml β-mercaptoethanol and 0.1 g Bromophenol blue were added. The total volume was made up to 50 ml with distilled water.
2. Resolving gel:
    - 10% gel: 3.8 ml H_2O, 2.6 ml Acrylamide/bis (30% 37.5:1; Bio-Rad), 2.6 ml Tris–HCl (1.5M, pH 8.8), 100 µL SDS (10%), 10 µL *N*,*N*,*N*′,*N*′-tetramethylethylene-diamine (TEMED) (Bio-Rad), 100 µL Ammonium persulfate (APS) (10%).
    - 8 % gel : 4.6 ml H_2O, 2.6 ml Acrylamide/bis (30% 37.5:1; Bio-Rad), 2.6 ml Tris–HCl (1.5 M, pH 8.8), 100 µL SDS (10%), 10 µL *N*,*N*,*N*′,*N*′-

tetramethylethylene-diamine (TEMED) (Bio-Rad), 10 µL Ammonium persulfate (APS) (10%).

After adding TEMED and APS to the SDS-PAGE separation gel solution, the gel polymerize quickly, so these two reagents was added to SDS page only when the entire system was ready to pour. Gel was poured, leaving ~2 cm below the bottom of the comb for the stacking gel, making sure to remove bubbles. The top of the gel was layered with water. This helped to remove bubbles at the top of the gel and also presented the polymerized gel from drying out. In ~30 min, the gel was completely polymerized and the water layer was removed.

3. Stacking gel:

3. 4% gel: 2.97 ml H_2O, 0.67 ml Acrylamide/bis (30% 37.5:1; Bio-Rad), 1.25 ml Tris–HCl (0.5M, pH 6.8), 0.05 ml SDS (10%), 0.005 ml *N*,*N*,*N*′,*N*′-tetramethylethylene-diamine (TEMED) (Bio-Rad), 0.05 ml Ammonium persulfate (APS) (10%).
Stacking gel was poured on top of the separation gel. Wells were made with comb. In ~30 min, the stacking gel was completely polymerized.

4. Tris-glycin buffer (Running buffer): Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H_2O. Store the running buffer at room temperature and dilute to 1X before use.

Different concentration (for Heart and Kidney- 8% and for Aorta- 10%) of polyacrylamide Resolving gel was poured between sealed glass plates with spacers. A stacking gel was overlaid on the polymerized resolving gel. Sample was mixed with 4x Laemmli buffer and denatured by heating on controlled water bath at 67ºC for 15 min before loading on to the gel. 20µg of total protein was loaded into wells along with dual color molecular weight marker (Hi-media, India) was run in one lane as a reference. The proteins were separated using Tris-glycine buffer under electrophoresis at constant 100 V for 90 min.

- **Estimation of STIM1 and Orai1 expression by Western blotting[183, 184]**

Reagents:

1. Transfer buffer: 25 mM Tris-HCl (pH 7.6), 192 mM glycine, 20% methanol and 0.03% sodium dodecyl sulfate (SDS)
2. Phosphate buffer saline (PBS) (pH-7.4): 8.0g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g of KH_2PO_4 was dissolved in 800 ml of distilled water. The pH of the solution was adjusted to 7.4 with 1N hydrochloric acid (HC1). The volume was then made up to 1000 ml with distilled water and autoclaved.
3. Phosphate buffer saline with tween-20 (PBS-T) (pH-7.4): 1 ml of tween-20 was added in to 1000 ml of PBS.

Following SDS-PAGE, protein bands were transferred from gel to a nitrocellulose membrane using Bio-Rad transfer system at 100 V for 90 min in 1X Transfer buffer supplemented with 20% v/v methanol. The nitrocellulose membrane was then stained for 5 min with non-fixative Ponceau S stain solution to confirm complete protein transfer. Then the membrane was rinsed with PBS to remove Ponceau S. Non-specific binding was blocked by incubating the membranes in 5% fat free skimmed milk with 0.1% Tween-20 in PBS/TBS at room temperature for 1 h. Blots were subsequently incubated with STIM1and Orai1 protein (1:500 dilutions in 5% fat free skimmed milk) primary antibodies overnight at 4ºC on gentle agitation. Blots were washed 4 times at 15 min intervals with TBS/PBS containing 0.1% Tween (TBS/PBS-T) and incubated in appropriate secondary antibody (Got anti-rabbit-HRP conjugated, 1:10000 dilution) at room temprature for 1 hour with gentle agitation and again washed 4 times with TBS/PBS containing 0.1% Tween (TBS/PBS-T) (15 min each). After four washes, two washes with PBS/TBS; specific bands of immune-reactive proteins were visualized using ultrasensitive enhanced chemiluminescence reagent and images were captured on chemigenious gel documentation system (Q9, Uvitech, UK, 2015-16). The immunoreactive protein bands were scanned and normalized with ponceau S bands.

5.2.4.2. Isoproterenol induced global ischemia[185, 186]

- **Drug Preparation:**

PBEA: PBEA was dissolved in olive oil.

RCHA: RCHA was dissolved in water.

Eugenol (EU): Eugenol was dissolved in olive oil.

2-APB: 2-APB was initially dissolved in pure DMSO and further diluted with normal saline so as to make the final concentration of DMSO not exceeding 1%. 2-APB were kept in aluminum foil wrapped micro centrifuge tubes, as they are light sensitive drugs and stored in ice during dosing.

Isoproterenol (ISO): Isoproterenol was dissolved in normal saline at the time of injection. (Because after 45 min drug is oxidized and pink colour is appeared) ISO was kept in aluminum foil wrapped micro centrifuge tubes, as it is light sensitive drug. All the drug solutions were prepared freshly.

- **Fixation of optimum dosage of drug:**

Dose of ISO (85mg/kg), 2-APB (4 mg/kg), and EU (50 mg/kg) were selected on the basis of reported dose. Dose of PBEA and RCHA were selected based on after performing pilot study.

- **Pilot study**

Pilot study of PBEA and RCHA, Three increasing graded dose (50, 100, 200 mg/kg) were selected and best treatment dose was selected for main study on the basis of Ck-MB and LDH level as well as MDA level and GSH activity.

- **Drug efficacy study of 2-APB[175]**

Healthy male wistar albino rats (8-12 weeks) weighing 250 ± 25g were obtained from Zydus Research Centre, Ahmedabad. All the animals were randomized according to weight with a given mean of (243.34 ± 48.2g) in to three groups and each group contain 6 animals. Control (group I) animals received saline. All groups except group I received ISO (85 mg/kg, s.c.) at an interval of 24 h for two constitutive days. Group II served as ISO control. Based on pharmaco-kinetic study data, Group-III received 2-APB at the dose of 4 mg/kg i.v. of 12 h interval along with ISO (85 mg/kg, s.c.). 0.1 ml blood was withdrawn from retro orbital plexus at different time point (0, 2, 4, 6, 8, 12, 24, 28, 30, 36

and 48 h) and serum was separated. Calcium level was estimated at different end point by the method described in the section **5.2.4.1.2**

- **Experimental design**

Healthy male wistar albino rats (8-12 weeks) weighing 250 ± 25g were obtained from Zydus Research Centre, Ahmedabad for global ischemia study. All the animals were randomized according to weight in to six groups and each group contain 6 animals. Groups were labeled as control and negative control (ISO control) and positive control (2-APB, Eugenol, PBEA and RCHA). Control (group I) animals received saline. All groups except group I received ISO (85mg/kg, s.c.) on two constitutive days at 24 h interval, respectively. Group II served as ISO control. Group III was test group which received 2-APB (4 mg/kg, i.v.) at 12 h intervals for 2 days just before ISO injection. Group IV to VI were test groups which received eugenol (50 mg/kg, p.o), PBEA (200 mg/kg, p.o) and RCHA (200 mg/kg, p.o), respectively for 15 days. At the end of experimental period, blood pressure and electrocardiogram were measured. After 48 h of 1st injection of ISO, blood was withdrawn from retro-orbital plexus. Serum and plasma were separated by centrifugation at 4000 rpm for 15 min at 4^{o}C (CPR 30, REMI Centrifuge, India, 2011-12) for estimation of serum cardiac markers, electrolytes and plasma interleukins. Rats were euthanized humanly. Heart of three animals was subjected to Langendorff assembly for hemodynamic parameter measurements. Heart tissue were excised immediately in ice-chilled normal saline and washed with ice-chilled phosphate buffered saline. They were blotted free of blood and tissue fluids, and were weighed on balance (ATX 224, Shimadzu, India, 2013-14) and stored at - 80^{0}C (EIE 414, Deep freezer, CryoScientific, India, 2008-09) for estimation of STIM1 and Orai1 by western blot analysis, antioxidant enzymes, membrane bounded enzymes, infract size and Histopathology.

5.2.4.2.1. Blood pressure measurement by non-invasive method (Tail cuff method)[187]

For arterial blood pressure measurements tail cuff method was used which needs elevated ambient temperature. Rat regulates their body temperature by controlling the amount of

blood that flows through their tail. When body temperature is elevated above normal; either by elevated ambient temperature or exercise; the flow of blood in the tail increases dissipating the extra heat. When measuring blood pressure in rats, the temperature was significantly increased (36 to 40 ºC) by lamp than the thermo stress level was also increased in order to detect pulses. When blood flows increased throughout the tail, the diameter of the tail changes. The form of pulse detection is critical and sensor selection determines this method. When an external physical force is applied to sensor it generates electrical signals. Rats were trained for at least one week until the blood pressure was recorded with minimal stress and restraint. Systolic BP, Diastolic BP and Mean BP were measured at the end of treatment period (Tail cuff) using LE 5002 storage pressure meter (MP 36, Biopac Systems, USA, 2012-13).

5.2.4.2.2. Electrocardiography[188]

After 48 h of the first injection of isoproterenol, needle electrodes (SS2L) were inserted under the skin of animals under light ether anesthesia in lead II position [Right arm (-ve)][Left arm (+ve)][Right leg (neutral)]. ECG recordings were made using computerized Biopac data acquisition system (MP 36, Biopac Systems, USA, 2012-13) and changes in ECG pattern were considered. The changes in ST interval, QT interval, RR interval, QRS complex, P wave amplitude and heart rate were determined from ECG.

5.2.4.2.3. Hemodynamic parameter[189]

Reagent:

1. Modified Krebs–Henseleit buffer: NaCl (118mM), KCl (4.7 mM), $CaCl_2$ (1.5 mM), $MgSO_4$ (1.66 mM), $NaHCO_3$(24.88 mM), KH_2PO_4 (1.18 mM), Glucose (5.55mM), Na Pyruvate (2mM) and Bovine Albumin (0.1%) were dissolved in 1000 ml of Distilled water.

Control and experimental animals were injected 500 IU heparin by intraperitoneal (i.p) and anesthetized by 80 mg/kg ketamine and 20 mg/kg xylazine (i.p). After rat became unconscious and lost pedal reflex activity, heart surgery was started. A midsternal thoracotomy was performed to open its chest. Second, the heart was hastened to excise

and to transfer into oxygenated ice-cold modified Krebs-Henseleit buffer. Then, cannula filled with oxygenated modified Krebs-Henseleit buffer was tied to its aorta. Finally, the heart attached cannula will be rapidly switched to connect with the Langendorff perfusion apparatus. The apparatus had been previously set for constant 20 ml/min flow rate of the modified Krebs-Henseleit buffer saturated with carbogen (95% O2 and 5% CO2), and its temperature was controlled at 37°C. In order to measure Pharmacodynamics response, a latex balloon tied to the end of a polyethylene tube, which was connected with pressure transducer attached with Biopac data acquisition system (MP 36, Biopac Systems, USA, 2012-13), was carefully inserted into a left ventricle of the isolated heart. Coronary flow, LVEDP, +dp/dt max and -dp/dt min were measured and calculated.

5.2.4.2.4. Gravimetric Analysis[190]

Heart weight to body weight ratio was calculated by final body weight along with heart weight was recorded at last day of experiment. From this values heart weight to body weight ratio was calculated by dividing heart weight (g) by body weight (g) and multiplying it with 100.

- **Biochemical analysis in serum and plasma**

The collected serum from control and experimental animals were used for biochemical parameter assessment of cardiac tissue injury marker enzymes and cytokine profile.

- **Assessment of serum cardiac tissue injury marker enzymes**

5.2.4.2.5. Assay of Creatine Kinase-MB (CK-MB)[191]

Procedure:

Wavelength/filter: 340 nm

Temperature: 30 °C

Light path: 1 cm

Pipette into a clean micro centrifuged tube labeled as Test (T):

Addition Sequence	(T) 30 °C

Enzyme Reagent (L1) Sample	0.8 ml 0.05 ml
Incubate at the assay temperature for 1 minute and add	
Starter Reagent (L2)	0.2 ml

Mix well and read the initial absorbance A and repeat the absorbance reading after every 1, 2, & 3 minutes. Calculate the mean absorbance change per minute (ΔA/ min).

Calculation: CK - MB activity in U/L (30 °C) = ΔA/ min x 6666

5.2.4.2.6. Assay of Lactate Dehydrogenase (LDH)[192]

Procedure:

Wavelength/filter: 340 nm

Temperature: 30 °C

Light path: 1 cm

Pipette into a clean micro centrifuged tubes labeled as Test (T)

Addition Sequence	(T) 37 °C
Buffer Reagent (L1) Sample	0.8 ml 0.05 ml
Incubate at the assay temperature for 1 minute and add	
Starter Reagent (L2)	0.2 ml

Mix well and read the initial absorbance A and repeat the absorbance reading after every 1, 2, & 3 minutes. Calculate the mean absorbance change per minute (ΔA/ min).

Calculation: LDH activity in U/L (37 °C) = ΔA/ min x 8095

- **Assessment of serum electrolytes**

5.2.4.2.7. Assay of Calcium[176]

The detailed procedure is described in section **5.2.4.1.2**

5.2.4.2.8. Assay of Potassium[177]

Wavelength / Filter: 630nm

Test tubes were labeled as Blank (B), Standard (S) and Test (T) and serum samples and reagents were added as follows:

Addition Sequence	B (ml)	S (ml)	T (ml)
Potassium Reagent	1	1	1
Deionised water	0.02		
Na^+/ K^+ Standard (S)		0.02	
Sample			0.02

Prepared samples were mixed well and incubated at room temperature (25ºC) for 5 minutes. Absorbance of the standard and test samples was measured against the blank within 15 min.

Calculation: Potassium in mmol/L = $\frac{\text{Abs T}}{\text{Abs S}} * 100$

5.2.4.2.9. Assay of sodium[177]

The detailed procedure is described in section **5.2.4.1.3**

- **Assessment of plasma Inflammation Marker**

5.2.4.2.10. Assay of Tumor Necrosis Factor – Alpha (TNF-α)[193]

Reagents:

1. Wash Buffer (1X): To make wash Buffer (1X), add 5ml of wash Buffer (20X) to 95ml of deionized (DI) water. This is working solution.
2. Assay diluents (1X): To make assay diluents (1X), add 1ml of assay diluents (5X) to 4ml of DI water. This is working solution.
3. Detection Antibody (50 µl): Add 24µl of detection antibody solution to 5796 µl of assay diluents (1X) to make final volume to 6 ml.
4. Concentrated Streptavidin-HRP 50µl: Add 6µl of Streptavidin-HRP solution to 5994µl of assay diluents (1X) to make final volume to 6 ml.
5. Standard (recombinant Rat TNF-α ,1 µl/ml) (20 µl): Upon first use, thaw 1µg tube of recombinant standard and quick-spin, aliquot into polypropylene vials, and store at -20°C.

Top standard solution (3000pg/ml) was prepared by adding 6 µL of the recombinant protein in to 1994 µL of assay diluents (1X).

Procedure:

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
2. Add 100µl/well of Standards and plasma to the plate. Perform two-fold serial dilutions of the 3000pg/ml top standard, either within the plate or in separate tubes. Thus, the Rat TNF-alpha standard concentrations are 3000pg/ml, 1500pg/ml, 750pg/ml, 375pg/ml, 187.5pg/ml, 93.75pg/ml and 46.87pg/ml. Assay diluents (1X) serves as the zero standard (0 pg/ml). Seal plate and incubate for 2 hours at Room temperature (18-25 °C).
3. Aspirate and wash plate 4 times with Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.
4. Add 100µl of diluted detection antibody solution to each well, seal plate and incubate for 1 hour at Room temperature (18-25 °C).
5. Wash plate 4 times with Wash Buffer (1X) as in step 3.
6. Add 100µl of diluted Streptavidin-HRP solution to each well, seal plate and incubate for 30 minutes at Room Temperature (18-25 °C).
7. Wash plate 4 times with Wash Buffer (1X) as in step 3. For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
8. Add 100µl of TMB Substrate solution and incubate in the dark for 15 minutes. Positive wells should turn bluish in color. It is not necessary to seal the plate during this step.
9. Stop reaction by adding 100µl of Stop Solution to each well. Positive wells should turn from blue to yellow.
10. Read absorbance at 450 nm within 30 minutes of stopping reaction.

11. Calculate concentration of the plasma from regression by plotting standard curve.

5.2.4.2.11. Assay of Interlukin-6 (IL-6)[194]

Reagents:

1. Wash Buffer (1X): To make wash Buffer (1X), add 5ml of wash Buffer (20X) to 95ml of DI water. This is the working solution.
2. Assay Diluent (1X): To make assay diluents (1X), add 1ml of assay diluents (5X) to 4ml of DI water. This is the working solution.
3. Detection Antibody (13 µL/vial): Add 12.5 µL of detection antibody solution to 4987.5 µL of assay diluents (1X) to make final volume to 5 ml.
4. Concentrated Streptavidin-HRP 50µl: Add 50µl of Streptavidin-HRP solution to 9950µl of assay diluents (1X) to make final volume to 10 ml.
5. Standard (recombinant Rat IL-6, 1 µL/ml) (25 µL): Upon first use, thaw 1µg tube of recombinant standard and quick-spin, aliquot into polypropylene vials, and store at -20°C.

Top standard solution (5000pg/ml) was prepared by adding 10 µL of the recombinant protein in to 1990 µL of assay diluents (1X).

Procedure:

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
2. Add 100µl/well of standards and plasma to the plate. Perform two-fold serial dilutions of the 5000pg/ml top standard, either within the plate or in separate tubes. Thus, the rat IL-6 standard concentrations are 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 312.5pg/ml, 156.25pg/ml and 78.13pg/ml. Assay diluents (1X) serves as the zero standard (0pg/ml). Seal plate and incubate at Room temperature for 2 hours.
3. Wash plate 4 times with wash buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the

bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.

4. Add 100µl of diluted biotin conjugated detection antibody solution to each well, seal plate and incubate at Room temperature for 2 hour.
5. Wash plate 4 times with wash Buffer (1X) as in step 3.
6. Add 100µl of diluted Streptavidin-HRP solution to each well, seal plate and incubate at Room temperature for 30 minutes.
7. Wash plate 4 times with wash buffer (1X) as in step 3. For this final wash, soak wells in wash buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
8. Add 100µl of TMB substrate solution and incubate in the dark for 15 minutes.
9. Positive wells should turn bluish in color. It is not necessary to seal the plate during this step.
10. Stop reaction by adding 100µl of stop solution to each well. Positive wells should turn from blue to yellow.
11. Read absorbance at 450nm within 15 minutes of stopping reaction.
12. Calculate concentration of the plasma from regression by plotting standard curve.

- **Biochemical Parameters in Heart Tissue**

➢ **Assessment of Lipid Peroxidation and Endogenous Antioxidants**

5.2.4.2.12. Assay of Lipid Peroxidation (Malonedialdehyde [MDA])[168]

The detailed procedure is described in section **5.2.4.1.5**

5.2.4.2.13. Assay of Superoxide Dismutase (SOD)[169]

The detailed procedure is described in section **5.2.4.1.6**

5.2.4.2.14. Assay of Catalase (CAT)[170]

The detailed procedure is described in section **5.2.4.1.7**

5.2.4.2.15. Assay of Reduced Glutathione (GSH)[171]

The detailed procedure is described in section **5.2.4.1.8**

5.2.4.2.16. Assay of Glutathione Peroxidase (GPx)[195]

Reagents:

1. Phosphate buffer (0.32 M, pH 7.0): 0.2 M sodium phosphate was prepared by dissolving 27.59 g sodium phosphate in 600 ml of distilled water, pH was adjusted to 7.0 with 0.2 M sodium hydroxide solution and the final volume was adjusted up to 1000 ml with distilled water.

2. EDTA (0.8 mM): 2.9 mg of EDTA was dissolved in 10 ml of distilled water.

3. Sodium azide (10 mM): 65 mg of sodium azaide was dissolved in 100 ml of distilled water.

4. Standard Glutathione (3 mM): 9.21 mg was dissolved in 10 ml of distilled water.

5. Hydrogen peroxide (2.5 mM): 28 µl of 30% H2O2 was added in 100 ml of distilled water.

6. Disodium hydrogen phosphate (0.3 M): 42.6 g of Na2HPO4 was dissolved in 1000 ml of Distilled water.

7. DTNB (1mM): 39.6 mg DTNB in 100ml of phosphate buffer.

8. Trichloroacetic acid (10%): 10 gm of TCA was dissolved in 100 ml of distilled water.

Procedure:

Reaction mixture containing 0.2 ml each of EDTA, sodium azide, reduced glutathione, hydrogen peroxide, 0.4ml of phosphate buffer and 0.1ml of homogenate was mixed and incubated at 37ºC for 10 min. The reaction was arrested by the addition of 0.5ml of 10% TCA and the tubes were centrifuged at 10,000 rpm for 10 min at 4°C. To 0.5 ml of clear supernatant, 4ml of disodium hydrogen phosphate and 1 ml of DTNB were added and the color developed was read at 420 nm immediately using

spectrophotometer (Shimadzu-1800). Graded concentrations of the standards (1-64 U/ml) were also treated similarly. GPx is expressed as μmoles of glutathione oxidised/min/mg protein. Where, 1 U/ml is equal to 1 μmoles of glutathione oxidised/min/mg protein.

5.2.4.2.17. Assay of Glutathione S-Transferase (GST)[196]

Reagents:

1. Phosphate buffer (0.1 M, pH 6.5): (a) 0.1 M sodium phosphate was prepared by dissolving 14.2 g Na_2HPO4 in 1000 ml of distilled water. (b) 0.2 M Potassium phosphate was prepared by dissolving 27.2 g KH_2PO4 in 1000 ml of distilled water. 8 ml of (a) was mixed in to 28 ml of (b), the pH was adjusted to 6.5 with sodium hydroxide solution and the final volume was adjusted up to 1000 ml with distilled water.

2. 1-chloro, 2,4-dinitrobenzene (CDNB) (30 mM): 0.6 g of CDNB was dissolved in 4 ml of hot 95% ethanol and made up to 100 ml of phosphate buffer.

3. Standard Glutathione (3mM): 9.21 mg glutathione was dissolved in 10 ml of distilled water.

Procedure:

To 1 ml of phosphate buffer, 0.1 ml of homogenate, 1.7 ml of water and 0.1 ml of 1-chloro, 2, 4-dinitrobenzene in 95% ethanol were added and incubated at 37ºC for 15min. After incubation, 0.1 ml of reduced glutathione was added. Increase in optical density was measured against the blank at 340 nm. GST activity was expressed as nmoles of CDNB conjugated/min/mg protein. 1U = 1 micromoles of CDNB conjugate with reduced glutathione per min at pH 6.5 at 25 °C.

- **Assessment of Membrane Bound Phosphatases (ATPases)**

5.2.4.2.18. Sodium-Potassium Dependent Adenosin Triphosphatase (Na^+K^+ATPase)[197]

Reagents:

1. Tris hydrochloride buffer (92 mM, pH 7.5): 11.4 g of tris buffer was dissolved in 900 ml of distilled water and pH was adjusted to 7.5 with 1 M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.
2. Magnesium sulphate solution (5 mM): 1.232 g of magnesium sulphate dissolved and made up to 1000 ml with distilled water.

3. Potassium chloride solution (5 mM): 0.372 g of potassium chloride was dissolved in 400 ml of distilled water and the final volume was made up to 1000ml with distilled water.

4. Sodium chloride solution (60 mM): 3.506 g of sodium chloride was dissolved in 400 ml of distilled water and the final volume was made up to 1000ml with distilled water.

5. EDTA solution (0.1 mM): 36.25 mg of EDTA (Na salt) was dissolved in 400 ml of distilled water and the final volume was made upto 1000 ml with distilled water.

6. Adenosine triphosphate (ATP) solution (40 mM): 202.87 mg of ATP was dissolved in 4 ml of distilled water and the final volume was made up to 10 ml with distilled water.

7. Trichloroacetic acid (10% w/v): 10 g of TCA was dissolved in sufficient quantity of distilled water and the final volume was made up to 100 ml with distilled water.

Procedure:

1.0 ml of tris-hydrochloride buffer and 0.2 ml each of magnesium sulphate, sodium chloride, potassium chloride, EDTA and ATP was added to test tube containing 0.2 ml of homogenate. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0 ml of TCA, mixed well and centrifuged at 4000 rpm for 10 min at 4°C. The phosphorus content of the supernatant was estimated as described in section **5.2.4.2.21.** The enzyme activity was expressed as µM of inorganic phosphorus liberated/mg protein/min.

5.2.4.2.19. Calcium Dependent Adenosine Triphosphatase (Ca^{2+}ATPase)[198]

Reagents:

1. Tris hydrochloride buffer (125 mM, pH 7.5): 15.6 g of tris base was dissolved in 900 ml of distilled water and pH was adjusted to 7.5 with 1 M hydrochloric acid. The resulting solution was diluted to 1000 ml with distilled water.

2. Calcium chloride solution (50 mM): 5.55 g of calcium chloride was dissolved in 400 ml of distilled water and the final volume was made upto 1000 ml with distilled water.

3. Adenosine triphosphate (ATP) solution (10 mM): 50.71 mg of ATP was dissolved in 4 ml of distilled water and the final volume was made up to 10 ml with distilled water.

4. Trichloroacetic acid (10% w/v): 10 g of TCA was dissolved in sufficient quantity of distilled water and the final volume was made up to 100 ml with distilled water.

Procedure:

The incubation mixture contained 0.1 ml each of tris-hydrochloride buffer, calcium chloride, ATP and supernatant in a test tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0 ml of TCA, mixed well and centrifuged at 4000 rpm for 10 min at 4°C. The phosphorus content of the supernatant was estimated as described in section **5.2.4.2.21.** The enzyme activity was expressed as µM of inorganic phosphorus liberated/mg protein/min.

5.2.4.2.20. Magnesium Dependent Adenosine Triphosphatase (Mg^{2+}ATPase)[199]

Reagents:

1. Tris hydrochloride buffer (374 mM, pH 7.6): 46.72 g of tris was dissolved in 900 ml of distilled water and pH was adjusted to 7.6 with 1 M hydrochloric acid. The resulting solution was diluted to 1000 ml with distilled water.

2. Magnesium chloride solution (25 mM): 2.38 g of magnesium chloride was dissolved in 400 ml of distilled water and the final volume was made up to 1000 ml with distilled water.

3. Adenosine triphosphate (ATP) solution (10 mM): 50.71g of ATP was dissolved in 4 ml of distilled water and the final volume was made up to 10 ml with distilled water.

4. Trichloroacetic acid (10% w/v): 10 g of TCA was dissolved in sufficient quantity of distilled water and the final volume was made up to 100 ml with distilled water.

Procedure:

The incubation mixture contained 0.1 ml each of tris-hydrochloride buffer, magnesium chloride, ATP and supernatant in a test tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0 ml of TCA, mixed well and centrifuged at 4000 rpm for 10 min at 4°C. The phosphorus content of the supernatant was estimated as described in section **5.2.4.2.21**. The enzyme activity was expressed as μM of inorganic phosphorus liberated/mg protein/min.

5.2.4.2.21. Determination of Inorganic Phosphorus (P_i):

Reagents:

1. Ammonium molybdate reagent (2.5% w/v): 2.5 g of ammonium molybdate was dissolved in 100 ml with 3 M sulphuric acid.

2. 1-Amino 2-naphthol 4-sulphonic acid (ANSA) reagent (0.25%): (a) 0.25% w/v of ANSA reagent in 15% w/v of sodium metabisulphite and 20% w/v of sodium sulphite. (b) Accurately weighed 15 g of sodium metabisulphite and 20 g of solium sulphite were dissolved in 50 ml of distilled water separately. 250mg of ANSA was dissolved in 50 ml of sodium metabisulphite and 50 ml of 20% w/v of sodium sulphite, mixed well and stored at room temperature.

3. Standard Phosphorus: 35.1 mg of potassium dihydrogen orthophosphate was dissolved in 100ml of distilled water. This contained 80μg of phosphorus/ml.

Procedure:

1ml of the supernatant was taken and the volume was made up to 5.0 ml with distilled water. To this, 1ml of ammonium molybdate reagent and 0.5 ml of ANSA reagent were added. The color developed was read for 8 h at interval of 2 h using blank containing water instead of sample at 620 nm. A standard graph was prepared taking different concentrations of standard phosphorus (2-32 µg/ml). The values were expressed as µM of inorganic phosphorus liberated/mg protein/min.

5.2.4.2.22. Estimation of Tissue Total Proteins[200]

Reagents:

1. Sodium hydroxide (0.1M): 4 g of sodium hydroxide was dissolved in 400 ml of distilled water and the final volume was made up to 1000 ml with distilled water.

2. Lowry C reagent: (a) Copper sulphate in 1% sodium potassium tartarate (1% w/v). 0.5 g of copper sulphate was dissolved in 1% sodium potassium tartarate (Prepared by dissolving 1g of sodium potassium tartarate in 100 ml of distilled water). (b) Sodium carbonate in 0.1 M sodium hydroxide (2% w/v) 2 g of sodium carbonate was dissolved in 100 ml of 0.1 M sodium hydroxide. 2 ml of solution (a) was mixed with 100 ml of solution (b) just before use.

3. Standard Protein (Bovine serum albumin): 20 mg of bovine serum albumin was dissolved in 80 ml of distilled water and few drops of sodium hydroxide were added to aid complete dissolution of bovine serum albumin and to avoid frothing. Final volume was made up to 100 ml with distilled water and stored overnight in a refrigerator.

4. Folin's phenol reagent: Folin's phenol reagent was diluted with distilled water in the ratio of 1:2. (i.e. 1ml of Folin's phenol reagent was mixed with 2ml of distilled water).

Procedure:

0.1 ml supernatant was taken in test tubes. To this, 0.8 ml of 0.1 M sodium hydroxide and 5 ml of Lowry C reagent was added and the solution was allowed to stand for 15 minutes. Then 0.5 ml of Folin's phenol reagent was added and the contents were mixed

well on a vortex mixer. Color developed was measured at 640 nm against reagent blank containing distilled water instead of sample. Different concentrations (20-640 µg) of Bovine serum albumin was taken and processed as above for standard graph. The values were expressed as mg of protein/ ml.

5.2.4.2.23. Measurement of Heart Tissue Nitrite Level[201]

Reagents:

1. Griess reagent: (a) Ortho- phosphoric acid (2.5% v/v) prepared by 2.84 ml of OPA (minimum assay 88%) was added in 97.15 ml of distilled water. (b) Sulphanilamide (1% w/v) prepared by 1 g of salphanilamide was dissolved in 50 ml of prepared 2.5% OPA. (c) NEDA (0.1% w/v) prepared by 0.1 g of NEDA in 50 ml of prepared 2.5% OPA. To prepare 100 ml of griess reagent by 50 ml of (b) was mixed with 50 ml of (C) when used.
2. Sodium Nitrate: 10 mg of sodium nitrate was dissolved in 100 ml of water.

Procedure:

Briefly equal volumes of supernatant and Griess reagent (1:1 ml) were mixed and incubated at room temperature for 10 min and the absorbance was determined at 540 nm wavelength. Different concentrations (10-320 ng/ml) of standard were taken and processed as above for standard graph. The levels of heart tissue nitrite were expressed as nmoles/mg of protein.

5.2.4.2.24. Measurement of Myeloperoxidase (MPO) activity[202]

Reagents:

1. Potassium phosphate buffer (50 mM pH 6.0): 3.402 g KH_2PO_4 was dissolved in 450 ml of distilled water, pH was adjusted to 6.0 and final volume was made up to 500 ml.

2. Extraction Buffer: 0.5 g HTAB (hexadecyl trimethyl ammonium bromide) was dissolved in 100 ml phosphate buffer.

3. Substrate solution (0.167 mg/ml dianisidine): Dissolve 16.7 mg o-dianisidine di-HCl in 100 ml phosphate buffer. If there is a precipitate in solution filter using 0.2µm filter. Cover with aluminum foil as this substrate is light sensitive and store at 4°C. Approximately 1 week solution was stored. Discard if solution becomes slightly brownish. On the day of assay, warm solution up to room temperature and add hydrogen peroxide (100 µL of 0.5% per 100 ml substrate solution) approx 30 mins before use.

Procedure:

The myocardial tissue was homogenized in 50 mM K_2HPO_4 buffer (pH 6) containing 0.5% hexadecyl-trimethylammonium bromide using a Polytron tissue homogenizer. Every three freeze-thawing cycles, samples were sonicated in ice bath. After sonication, samples were centrifuged at 15,000 rpm for 30 min at 40°C, and the resulting supernatant was assayed spectrophotometrically for myeloperoxidase determination. 40 µL of sample was mixed with 960 µL of 50 mM phosphate buffer (pH 6), containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% H_2O_2. The change in absorbance for 3 min at 460 nm was measured with the spectrophotometer (1800, Shimadzu, Japan, 2008-09). One unit of enzyme activity was defined as the amount of myeloperoxidase present that caused a change in absorbance measured at 460 nm for 3 min. Myeloperoxidase activity were expressed as U/gm tissue.

5.2.4.2.25. Determination of infarct Size (Macroscopic enzyme mapping)[203]

The heart was washed rapidly in ice- cold PBS to remove excess blood, taking care not to macerate the tissue. The excess epicardial fat was lightly trimmed off. Heart was allowed to get frozen at -80°C for 2 hours. The frozen heart was transversely cut across the left ventricle to obtain slices no more that 0.2cm in thickness. Heart slices were placed in covered, darkened glass dish containing pre warmed (1%) TTC solution in phosphate buffer (pH-7.4) and the dish was incubated at 39ºC for 40 min. The heart slices were turned over once after 20 min to make certain that it remains immersed and covered by 1cm of the TTC solution. At the end of incubation period, the heart slices was placed in 10% formalin solution which enhances the color contrast

developed. The % infarction was measured using Image J Software system (NIH, Bethesda, MD).

5.2.4.2.26. Histopathology study[190]

After the treatment period, animals were sacrificed and organs were excised and blotted free of blood and tissue fluids and preserved in 10% v/v formalin in Phosphate buffered saline solution (pH-7.0). The specimens were given for further processing to Sakshi Histopathological Laboratory, Vadodara where routine procedure for sectioning, staining and mounting was observed by the laboratory personnel. Briefly, after a week tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, tissues were cleaned in xylene and embedded in paraffin wax. Sections of 5µm thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinised in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The haematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada balsam. The stained sections were examined and photograph under optical microscope (BX10, Olympus, Japan, 2010-11) attached with camera (DP12, Olympus, Japan, 2010-11). The pathologist, blinded to the treatment protocols, analyzed the heart sections and interpreted.

5.2.4.2.27. Measurement of STIM1 and Orai1 protein expression in heart by Western blotting [179-184]

The detailed procedure is described in section **5.2.4.1.9**

5.2.4.3. Coronary artery ligation induced Reperfusion injury[204, 205]

- **Drug Preparation:**

PBEA: PBEA was dissolved in olive oil.

RCHA: RCHA was dissolved in water.

Eugenol (EU): Eugenol was dissolved in olive oil.

2-APB: 2-APB was initially dissolved in pure DMSO and further diluted with normal saline so as to make the final concentration of DMSO not exceeding 1%. 2-APB were kept in aluminum foil wrapped micro centrifuge tubes, as they are light sensitive drugs and stored in ice during dosing.

- **Experimental design**

Healthy male wistar albino rats (8-12 weeks) weighing 250 ± 25g were obtained from Zydus Research Centre, Ahmedabad for coronary artery ligation study. All the animals were randomized according to weight in to two groups and each group contain 5 animals. Groups were labeled as control and negative control (LAD control) and positive control (2-APB, Eugenol, PBEA and RCHA). Control (group I) animals received saline. All groups except group I performed LAD coronary artery ligation. Group II served as LAD control. Group III was test group which received 2-APB (4 mg/kg, i.v.) just before ischemia generation. Group IV to VI were test groups which received eugenol (50 mg/kg, p.o), PBEA (200 mg/kg, p.o) and RCHA (200 mg/kg, p.o), respectively for 15 days.

The Reperfusion injury was produced by LAD coronary artery ligation. Rats were anesthetized with ketamine HCl (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.). The trachea was then cannulated and connected to a rodent ventilator (7025, UGO BASILE Rodent Ventilator, Italy, 2005-06) for artificial respiration. Rats were ventilated at 67 breaths/min and 12 ml/kg tidal volume with room air. The body temperature was maintained at 37±1°C during the experiment. Left thoracotomy was performed at the fifth intercostal space. A pericardiotomy was then performed and a 6-0 silk suture, attached to a 10-mm micropoint reverse-cutting needle, was passed below the LAD coronary artery close to the origin immediately below the left atrial appendage to the right portion of the left ventricle. The ends of the suture were threaded through a polyethylene tube to form a snare to allow occlusion and re-opening of the vessel to induce ischemia and reperfusion respectively. Occlusion was elicited for a period of 30 min by pulling on the snare and clamping the snare onto the epicardial surface using a hemostat. This resulted in left ventricular ischemia. Reperfusion was achieved by unclamping the hemostat and loosening the snare and was elicited for 2 hours. After reperfusion, blood was withdrawn from retro-orbital plexus. Serum was separated by centrifugation at 4000 rpm for 15 min

at 4^{o}C (CPR 30, REMI Centrifuge, India, 2011-12) for estimation of serum cardiac markers. Rats were euthanized humanly. Heart tissue were excised immediately in ice-chilled normal saline and washed with ice-chilled phosphate buffered saline. They were blotted free of blood and tissue fluids, and were weighed on balance (ATX 224, Shimadzu, India, 2013-14) and stored at - 80^{0}C (EIE 414, Deep freezer, CryoScientific, India, 2008-09) for estimation of STIM1 and Orai1 by western blot analysis.

- **Biochemical analysis in serum**

The collected serum from control and experimental animals were used for biochemical parameter assessment of cardiac tissue injury marker enzymes.

➢ **Assessment of serum cardiac tissue injury marker enzymes**

5.2.4.3.1. Assay of CK-MB[191]

The detailed procedure is described in section **5.2.4.2.5**

- **Biochemical analysis in tissue**

5.2.4.3.2. Measurement of STIM1 and Orai1 protein expression in heart by western blot analysis[179-184]

The detailed procedure is described in section **5.2.4.1.9**

5.2.5. Safety study of drug

5.2.5.1. Cellular Toxicity

H9c2 cell were treated with only treatment drug without H_2O_2. The detailed procedure is described in section **5.2.3.1.1**

5.2.5.2. Doxorubicin induced Cardiotoxicity[206, 207]

- **Drug Preparation**

PBEA: PBEA was dissolved in olive oil.

RCHA: RCHA was dissolved in water.

Eugenol (EU): Eugenol was dissolved in olive oil.

2-APB: 2-APB was initially dissolved in pure DMSO and further diluted with normal saline so as to make the final concentration of DMSO not exceeding 1%. 2-APB were kept in aluminum foil wrapped micro centrifuge tubes, as they are light sensitive drugs and stored in ice during dosing.

Doxorubicin (DOX): DOX was dissolved in normal saline at the time of injection. DOX was kept in aluminum foil wrapped micro centrifuge tubes, as it is light sensitive drug. All the drug solutions were prepared freshly.

- **Experimental design**

Healthy male wistar albino rats (8-12 weeks) weighing 250 ± 25g were obtained from Zydus Research Centre, Ahmedabad for cardiotoxicity study. All the animals were randomized according to weight in to six groups and each group contain 6 animals. Groups were labeled as control and negative control (DOXO control) and positive control (2-APB, Eugenol, PBEA and RCHA).

The control (group I) animals received saline. All groups except group I received DOXO (10 mg/kg, i.p.). Group II served as DOXO control. Group III was test groups which received 2-APB (4 mg/kg, i.v.) at 12 h intervals for 4 days during before DOXO injection. Group IV to VI were test groups which received Eugenol (50 mg/kg, p.o), PBEA (200 mg/kg, p.o) and RCHA (200 mg/kg, p.o), respectively for 15 days. After 96 h of DOXO injection, blood was withdrawn from retro-orbital plexus under mild ether anesthesia without anticoagulant and serum was separated by centrifugation at 4000 rpm for 15 min at 4^{o}C (CPR 30, REMI Centrifuge, India, 2011-12) for serum cardiac biomarker. Heart tissue were excised immediately in ice-chilled normal saline and washed with ice-chilled phosphate buffered saline. They were blotted free of blood and tissue fluids, and were weighed on balance (ATX 224, Shimadzu, India, 2013-14) and stored at - 80^{0}C (EIE 414, Deep freezer, CryoScientific, India, 2008-09) for western blot analysis.

- **Biochemical analysis in serum**

The collected serum from control and experimental animals were used for biochemical parameter assessment of cardiac tissue injury marker enzymes.

- **Assessment of serum cardiac tissue injury marker enzymes**

5.2.5.2.1. Assay of Lactate Dehydrogenase (LDH)[192]

The detailed procedure is described in section **5.2.4.2.6**

- **Biochemical analysis in tissue**

5.2.5.2.2. Measurement of STIM1 and Orai1 protein expression in heart by Western blotting[179-184]

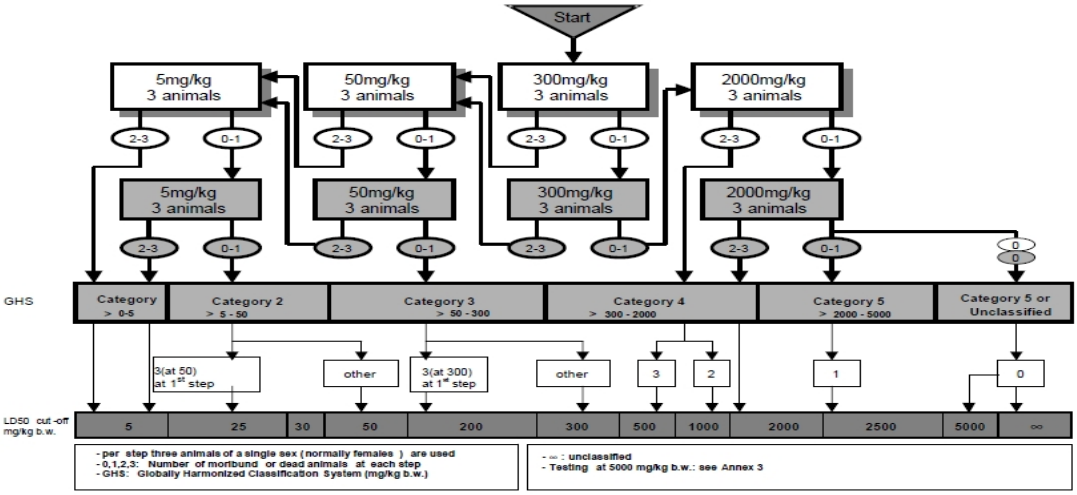
The detailed procedure is described in section **5.2.4.1.9**

5.2.5.3. Acute Toxicity Study OECD 423[208]

Acute Toxic Class Method (OECD 423), healthy female wistar albino rats (8-12 weeks) (nulliparous and non pregnant) weighing 160 ± 25 g were obtained from Zydus Research Centre, Ahmedabad. (MSU/IAEC/2018-19/1801).

All the animals were randomized according to weight in to two set and each set contain 3 animals. When there is no information on a substance to be tested, for animal welfare reasons it is recommended to use the starting dose of 300 mg/kg body weight. Rats were fasted prior to dosing. Following the period of fasting, rats were weighed and PBEA and RCHA were administered. After PBEA and RCHA administration, food was withheld for a further 4 hours in rats. The time interval between treatment groups was determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose was delayed until one is confident of survival of the previously dosed animals.

On day 0, Group 1 and 2 were PBEA groups which received PBEA 300 and 2000 mg/kg (p.o) respectively. Group 3 and 4 were RCHA groups which received RCHA 300 and 2000 mg/kg (p.o) respectively. Animals were observed individually after dosing with special attention given at 0.5,1, 2, 4 h and daily thereafter, for a total of 14 days and



clinical sign were recorded. Body weight (weekly) and food and water intake (daily) were measured. At the end of experimental period, animals were euthanized humanely for assessing gross necropsy.

5.2.5.4. Acute Toxicity Study OECD 407[209]

Repeated Dose 28-Day Oral Toxicity Study (OECD 407), healthy male and female wistar albino rats (8-12 weeks) (nulliparous and non pregnant) weighing 230 ± 25 g and 170 ± 25 g respectively, were obtained from Zydus Research Centre, Ahmedabad.

All the animals were randomized according to weight in to six groups and each group contains 10 animals (five female and five male). The control and satellite control (s-control) (group I and V) animals received olive oils. Group II to IV were test groups which received PBEA- 50, 200, 800 mg/kg (p.o) respectively. Group 6 were satellite group which received PBEA-800 mg/kg (p.o). The treatment was given for 28 days. General clinical observations were made at 11 a.m. after dosing. Body weight (weekly) and food and water intake (daily) were measured. At the end of experimental period, with or without anticoagulant blood was withdrawn from retro-orbital plexus under mild ether anesthesia from each animal. Hematological and biochemical parameters were

performed. Animals were euthanized humanely for assessing gross necropsy, relative organ body weight and histopathology examination.

- **Hematological Parameters**

At the end of the experiment, blood was withdrawn from retro-orbital plexus. Hemoglobin (g/dl), Hematocrit%, RBC (10^6/µL), WBC (10^3/µL), Neutrophils%, Lymphocytes%, Eosinophils %, Monocytes %, Basophils% and Plateletes (10^3/µL) were analyzed by Vet Auto Hematology Analyzer (BC-2800, Mindray, China, 2014-15).

- **Biochemical Parameters**

5.2.5.4.1. Assay of Total Cholesterol[210]

Procedure:

Working wavelength: 505 nm

Pipette into tubes marked	Blank	Standard	Test
Serum/plasma	-	-	10 µL
Reagent 2 (cholesterol standard)	-	10 µL	-
Reagent 1(cholesterol mono-reagent)	1000 µL	1000 µL	1000 µL

All the above solutions were mixed well and incubated at 37°C for10min or at room temperature (15-30˚C) for 30 minute and read absorbance of test and standard against reagent blank at 505nm. It is documented as fold change.

Calculation: Total Cholesterol (mg/dL) = $\frac{\text{Abs Test}}{\text{Abs Stanadrd}} \times 200$

5.2.5.4.2. Assay of Total Billirubin[210]

Procedure:

Pipette into tube marked	Sample Blank(AB)	Test(AT)
Sodium nitrite reagent	-	50µl
Sulphanilic acid reagent	100µl	50µl

Mix properly		
Working reagent	1000 µl	1000 µl
Sample	50µl	50µl

Working reagent: prepare working reagent by diluting equal volume of caffeine reagent with purified water. Mixed well and incubate at room temperature (+15 to +30°C) for 5 minutes. Measure the absorbance of the sample blank and test at 546 nm. Blank the analyzer with purified water.

Calculation: Total bilirubin (mg/dL) = $\frac{\text{Abs Test}}{\text{Abs Stanadrd}} \times 5$

5.2.5.4.3. Assay of Alanine Aminotransferase (ALT)[210]

Procedure:

Working wavelength= 340nm

Reagents were reconstituted and allowed to attain room temperature.

Pipette into tube marked	Test
Sample	100µL
Working ALT reagent	1000µL

Working reagent preparation: Add substrate to buffer in 1:4 ratios, i.e. 1mL of substrate + 4ml of buffer. Sample and reagent mixed well and aspirate immediately for measurement. Read the absorbance after 60 seconds. Repeated reading after every 30 seconds i.e. up to 120 sec. at 340 nm wavelength.

Calculation: ALT activity (IU/L) =ΔA/minute × Kinetic factor

Where, ΔA/minute = Change in absorbance per minute and Kinetic factor (K) =1768

5.2.5.4.4. Assay of Aspartate aminotransferase (AST)[210]

Procedure:

Working wavelength: 340nm

Reagents were reconstituted and allowed to attain room temperature

Pipette into tube marked	Test
Sample	100µL
Working AST reagent	1000µL

Working reagent preparation: Add substrate to buffer in 1:4 ratios, i.e. 1mL of substrate + 4ml of buffer. Sample and reagent mixed well and aspirate immediately for measurement. Read the absorbance after 60 seconds. Repeated reading after every 30 seconds i.e. up to 120 sec. at 340 nm wavelength.

Calculation: AST activity (IU/L) =ΔA/minute * Kinetic factor

Where, ΔA/minute = Change in absorbance per minute and Kinetic factor (K) =1768

5.2.5.4.5. Assay of Creatinine[210]

Procedure:

Working wavelength: 505 nm

Pipette into tubes marked	Standard	Test
Serum	-	100 µl
Reagent-3 (creatinine standard)	100 µl	-
Working reagent	1000 µl	1000 µl

Working reagent prepared by mixing equal volume of reagent-1 (picrate reagent) with reagent-2 (sodium hydroxide) to make up the desired volume and mix gently for 2 minutes. Blank the analyzer with purified water. Measure initial absorbance of the standard i.e. AS1 after 30 sec and final absorbance AS2 after interval of another 120 sec. Same way measure the absorbance of test and calculate concentration according to following equation.

Serum Creatinine concentration (mg/dl) = $\frac{AT2 - AT1}{AS2 - AS1} \times 2$

5.2.5.4.6. Assay of Total Protein[210]

Procedure:

Working wavelength: 578 nm

Pipette into marked tubes	Blank	Standard	Test
Serum	-	-	10 µl
Reagent -2 (total protein standard)	-	10 µl	-
Reagent-1 biuret reagent	1000 µl	1000 µl	1000 µl

Mix well and incubate at 37˚C for five minute. Programme the analyzer with reagent blank. Measure absorbance of the standard followed by the test.

Calculation: Total protein concentration (g/dl) = $\frac{\text{Abs Test}}{\text{Abs Stanadrd}} \times 6.5$

5.2.5.4.7. Assay of Albumin[210]

Procedure:

Working wavelength: 578 nm

Pipette into tube marked	Blank	Standard	Test
Serum	-	-	10µl
Albumin standard	-	10µl	-
Albumin reagent	1000µl	1000µl	1000µl

Sample, reagent mixed well and incubated at room temperature (+15 to +30°C) for 1 minute. Read absorbance at 630 nm wavelength.

Calculation: Albumin (g/dL) = $(\frac{\text{Abs T}}{\text{Abs S}}) * 4$

5.2.5.4.8. Assay of Globulin

Globulin was calculated from total protein and albumin

Globulin = total protein – albumin

5.2.5.4.9. Assay of Blood Urea Nitrogen[210]

Procedure:

Working wavelength: 505 nm

Pipette into marked tubes	Blank	Standard	Test
Purified water	500 µl	-	-
Reagent -1 (O- Phthaldehyde)	-	500 µl	500 µl
Standard	-	50 µl	-
Serum	-	-	50 µl
Mix properly			
Reagent-2 (NED reagent)	-	500 µl	500 µl

Mix well, program the analyzer with purified water. Measure initial absorbance of standard i.e. AS1 after 60 sec and final absorbance AS2 after an interval of 60 sec. Same way take reading of test.

Calculation: Serum urea concentration (mg/dl) = $\frac{AT2-AT1}{AS2-AS1} \times 50$

5.2.5.4.10. Assay of Sodium[177]

The detailed procedure is described in section **5.2.4.1.3**

5.2.5.4.11. Assay of Potassium[177]

The detailed procedure is described in section **5.2.4.2.8**

5.2.5.4.12. Assay of Calcium[176]

The detailed procedure is described in section **5.2.4.1.2**

5.2.5.4.13. Necropsy and Organ Body weight

All living male and female animals of all treated groups were euthanized on day 29 using diethyl ether. Necropsy and related findings were carried out for all animals including the dead and moribund animals. Post mortem necropsy findings were made by systemic approach (i.e. gross changes in organ size, shape and any visible lesions). Detailed post

mortem lesions from all the animals were recorded. For gross (macroscopic) lesions brain (cerebellum, cerebrum, mid brain), eyes, spleen, thymus, adrenal gland, lung, heart, aorta, oesophagus, stomach, duodenum, jejunum, ileum, colon, rectum, liver, kidney, urinary bladder, testes, ovary, uterus, skin and mesenteric lymph node were collected and examined after opening the body of sacrificed experimental animals.

Weight of the Liver Kidney, Heart, Brain, Spleen, Lung and Testis organs were recorded for each animal. Organs were collected, cleaned using filter paper and then weighed on balance (ATX 224, Shimadzu, India, 2013-14).

5.2.5.4.14. Histopathology[190]

Full histopathology was carried out on the preserved organs of all animals in the control and high dose groups. The detailed procedure is described in section **5.2.4.2.26**

5.3. Statistical analysis

The results are expressed as mean ± SEM. Statistical difference between the means of the various groups were analyzed by using one way Analysis of Variance (ANOVA) followed by dunnett's and bonferroni's post hoc test and two tailed unpaired t-test with P value < 0.05.