IAEC Approval of Animal Studies

Animal husbandry, handling and treatments were performed as per the CPCSEA guidelines. They were housed in polypropylene cages (47x34x20cm) with corn cob, renewed every 3 days under well-controlled conditions of temperature (22 ± 2°C), relative humidity (55 ± 10%) and 12h/12h light-dark cycle. Animals were provided pelleted diet (Pranav Agro Industries Ltd., Maharashtra, India) and purified R.O drinking water *ad libitium*. The animals were monitored daily for any sign of distress and mortality. All the mentioned studies were approved by the Institutional Animal Ethics Committee (IAEC), Faculty of Pharmacy, The M. S. University of Baroda *vide* the protocol number mentioned below:

MSU/IAEC/2015-16/1507 dated 24/08/2015

MSU/IAEC/2018-19/1801 dated 06/08/2018

1. Introduction

Calcium is the 5th of the most abundant elements in the earth crust and is also the most abundant mineral in human body. The human body contains approximately 1 kg of calcium with more than 99% deposit in the bone in the form of calcium phosphate. Through interaction with numerous proteins distributed in different cellular compartments, calcium is involved in varied aspects of life, such as muscle contraction, enzyme activation, cell differentiation, immune response, programmed cell death and neuronal activity.[1] Such broad functions are maintained by tightly controlled calcium concentration in extracellular fluid and cellular compartments. The concentrations of calcium in blood and extracellular fluid are usually maintained at 1 - 2 mM, while the concentration of intracellular calcium at resting state is maintained at 100 nM or less by calcium ATPase, channels, and exchangers located in plasma membrane and endoplasmic reticulum (ER) membrane.[2] During the signaling process of calcium, the concentration of intracellular calcium is increased to approximately 100µM which triggers calcium signaling through the activation or deactivation of an array of calcium-binding proteins. Extracellular calcium homeostasis is mainly controlled by three physiological modes, including intestinal calcium absorption, renal calcium reabsorption, and bone formation/resorption.[1]

Cellular movement of calcium is associated with an elaborate system including the glutamate receptor channels (divided into two subtypes: the ionotropic receptors NMDA, AMPA, and kainic acid receptor activation (KA) and the metabotropic receptors mGluR), voltage-dependent calcium channels (VDCCs) L, N, P/Q, T type VDCCs and the sodium calcium exchanger (NCX). More recently, transient receptor ion channels (TRPM and specifically TRPM7), acid-sensing ion channels (ASIC), and inward excitotoxic injury current (I_{EIC})–calcium-permeable channels have also been implicated in calcium influx.[3] In addition, the release and sequestering of calcium from organelles, namely the mitochondria and endoplasmic reticulum (ER), can also contribute to intracellular calcium overload following disease.[4] Store-operated calcium entry (also called capacitative calcium entry) refers to an influx of extracellular calcium across the plasma membrane via store-operated calcium channels (e.g., ORAI, TRP channels) in response to ER intracellular calcium release and store depletion.[5]

Disordered calcium signalling to the myofilaments occurs in myocardial infarction, athrosclerosis, arrhythmia, heart failure (HF) and cardiomyopathy. The role of calcium in vascular health is less clear-cut. There are calcium-sensing receptors on vascular smooth muscle cells and on platelets, calcium plays a role in smooth muscle contraction and its role in the electrophysiology of the heart and myocardial function have already been alluded to.[6] Calcium deposition in the vasculature is a consistent feature of vascular disease and is predictive of adverse cardiovascular events. Ca^{+2} homeostasis is of pivotal interest for the cell, reflecting the central importance of Ca^{+2} as a second messenger, regulating a variety of cellular processes such as metabolism, protein phosphorylation and dephosphorylation, cell proliferation, division and

differentiation, gene transcription, cell motility, muscle excitation-contraction and stimulus-secretion coupling, programmed cell death and neurotransmission. [7]

Calcium channel blockers (CCBs) reduces contraction of arteries by inhibiting calcium entry and by interacting with binding sites identified on voltage-dependent calcium channels.[8] This led to the denomination of calcium channel blockers. In short-term studies, by decreasing total peripheral resistance, CCBs lower arterial pressure. By unloading the heart and increasing coronary blood flow, CCBs improve myocardial oxygenation. In long-term treatment, the decrease in blood pressure is more pronounced in hypertensive than in normotensive patients.[9] There are two main types of CCBs: dihydopyridine and non-dihydropyridine; the first type is vascular selective. Dihydropyrines are indicated for hypertension, chronic, stable and vasospastic angina. Non-dihydropyridines (Non-DHP) have the same indications plus antiarrythmic effects in atrial fibrillation or flutter and paroxysmal supraventricular tachycardia. In addition, CCBs reduced newly formed coronary lesions in atherosclerosis. In order to reach recommended blood pressure goals, there is a recent therapeutic move by combination of CCBs with other antihypertensive agents particularly with inhibitors acting at the level of the Renin-angiotensin system. They are also combined with statins. CCBs are used as an additional treatment in patients with severe CAD or in patients who do not tolerate other drugs. The most often used third and fourth generation drugs are amlodipine, clinidipine, diltiazem and verapamil. The adverse effects of CCBs include palpitations, headache, hot flashes, edema, gingival growth and constipation. Non-DHP CCBs must not be used in patients with heart failure or marked bradycardia because of their cardioinhibitory actions, and careful consideration is necessary regarding their use in elderly patients with latent cardiac disorders or their concomitant use with digitalis or a β-blocker.[10]

The idea of store-operated calcium entry (SOCE) developed from studies of calcium signaling in the 1970s and 1980s. The field built on a considerable body of unsung work. Several conclusions had been firmly established by the early 1980s— that how calcium release, mobilize, refill in cells.[11] Revisiting the earlier CVD models with this SOCE pathway established that enhanced calcium uptake is independent not only of receptor occupancy but also of residual IP3, until internal calcium stores are refilled. The accumulated evidence set the stage for wide acceptance of a model that sensing of store content controls a plasma membrane calcium influx mechanism.[12] Now, understanding of the store-operated Ca^{2+} entry mechanism and its emerging roles in another CVD, areas of uncertainty in which further progress is needed, and recent findings that are opening new directions for research in this rapidly growing field.

As per Xiang Luo et al; STIM1-dependent store-operated Ca2+ entry is required for pathological cardiac hypertrophy. STIM1 expression is re-activated by pathological stress to trigger significant SOCE-dependent Ca2+ influx. STIM1 amplifies agonist-induced hypertrophy via activation of the calcineurin–NFAT pathway. Importantly, inhibition of STIM1 suppresses

agonist-triggered hypertrophy, pointing to a requirement for SOCE in this remodeling response. Stress-triggered STIM1 re-expression, and consequent SOCE activation, is critical elements in the upstream, Ca2+-dependent control of pathological cardiac hypertrophy.[13]

Endothelial dysfunctions are the central focus of most theories of hypertension pathophysiology. Early theories suggested the role of renal sodium retention, expanded vascular volume and increasing cardiac output in the pathophysiology of hypertension. The increased cardiac output was believed to lead to increased vascular resistance.[14] Another theory suggests that inherited cellular defects cause increased intracellular sodium, leading to increases in ionic calcium along with increased vascular tone and reactivity which leads to Hypertension.[15] Ag-II has been associated with pathological consequences such as hypertension and pressure overload. The multiple cardiac actions of Ag-II are mediated by changes in $[Ca^{2+}]_i$. Ag- II activates inwardly directed Ca^{2+} currents, giving rise to increased Ca^{2+}-induced Ca^{2+} release from cardiac sarcoplasmic reticular stores. This peptide also stimulates phospholipase C, which results in activation of protein kinase C and mobilization of intracellular Ca^{2+}. Increased $[Ca^{2+}]_i$ may contribute to the positive inotropic effect of Ag-II.[16] Recently there has been a rapid growth of ideas concerning the involvement of Ca^{2+} in the progression of events that are precipitated by an ischemic episode and become exacerbated upon reperfusion and reoxygenation.[17] Myocardial infarction is experimentally induced by isoproterenol which mechanism is multifactorial, but ischemia and intracellular calcium overload plays a central role. The rise in intracellular and mitochondrial Ca^{+2} concentrations in association with a decrease in adenosine triphosphate during ischemia is also known to play an important role in cell damage, causing phospholipase, nuclease, and protease activation, and thus an increase in reactive oxygen species (ROS).[18]

Calcium signaling regulation and ROS production can be deliberated as bidirectional. Acceleration of oxidative stress by the disparity between inadequate antioxidant defenses system and systemic manifestation of reactive oxygen species (ROS) that are continuously generated, transformed and consumed in all living organisms as an upshot of aerobic life. Calcium is converse with versatile system and pathway, among them also with ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) which can damage cellular proteins, RNA, DNA and lipids.[19] Dysfunctional calcium load and oxidative stress via mitochondria and endoplasmic reticulum in cardiacmyocytes develop fibrosis, apoptosis, inflammation, ischemia/reperfusion damage, hypertrophy and structural cardiac remodeling eventually leading to atherosclerosis, myocardial infarction, arrhythmias, hypertension, cardiomyopathy, heart failure and sudden death in the ageing population.[20]

Oxidative stress is a common denominator in many aspects of cardiovascular diseases. Despite the fact that mechanisms underlying hypertension are not yet fully elucidated, a large amount of evidence shows that oxidative stress plays a central role in its pathophysiology.[21] Oxidative stress leads to a decrease in nitric oxide bioavailability, which is the main factor responsible for

maintaining the vascular tone. Several vasoconstrictor peptides, such as angiotensin II, endothelin-1 and urotensin II, act through their receptors to stimulate the production of reactive oxygen species, by activating enzymes like NADPH oxidase and xanthine oxidase. ROS-induced vasoconstriction results from increased intracellular calcium concentration, thereby contributing to the pathogenesis of hypertension.[22] Vasomotor tone is dependent upon a delicate balance between vasoconstrictor and vasodilator forces resulting from the interaction of the components of the vascular wall and the blood, and both of them can be altered by oxidative stress.

During myocardial oxidative stress, the generation of ROS is enhanced and the defense mechanisms of myocytes are altered. The sources of ROS in cardiac myocytes could be mitochondrial electron transport chain, nitric oxide syntheses (NOS), NADPH oxidase, xanthine oxidase, and lipoxygenase/cyclooxygenase and the auto-oxidation of various substances, particularly catecholamine. In acute myocardial infarction (AMI), two distinct types of damage occur to the heart: ischemic injury and reperfusion injury, which lead to mitochondrial dysfunction in heart cells. During ischemia and reperfusion, ROS can be produced by both endothelial cells and circulating phagocytes. Ischemia also causes alterations in the defense mechanisms against ROS. Some proteins, including heat-shock proteins, are overexpressed in conditions of ischemia/reperfusion and can protect from cardiac injury.[23]

Lanthanides, Imidazole compounds like SKF-96365, econazole and miconazole, Diphenylboronate compounds like 2-Aminoethyldiphenyl borate (2-APB) and their derivatives DPB162-AE and DPB163-AE.[24, 25] The Pyrs including Pyr3, pyr6 and pyr10 and recently, several novel pyrazole compounds like the GSKs including GSK5498A, GSK-5503A and GSK-7975A are SOCE inhibitors.[26] 2-APB and thapsigargin both are act as SOCE modulator depending upon its concentration.[27] Another SOCE inhibitors like Synta 66, ML-9, Diethylstilbestrol, Carboxyamidotriazole, RO2959, Linoleic acid, 1-Phenyl-3-(1-phenylethyl)urea derivatives.[28] The CalciMedica series compound like CM2489 is the only SOCE channel inhibitor tested in human and has completed Phase I clinical trials for treating moderate-to-severe plaque psoriasis.[29]

Currently available most SOCE inhibitors by far have not reached clinical trials, primarily owing to their poor selectivity and high toxicity. The lanthanide salts of other multivalent anions and proteins are insoluble. Econazole and miconazole also exhibit a lack of specificity to CRAC channel. Synta 66 exerts no significant effect on a series of receptors, enzymes and ion channel targets. Diethylstilbestrol could not be used in clinical setting due to its activation on estrogen receptors. Although no SOCE channel inhibitors have reached the milestone of FDA approval and clinical use, the increasing attention paid by pharmaceutical companies, together with our deeper understanding of the activation and regulatory mechanisms of SOCE channel and the advent of novel optogenetic tools to manipulate SOCE channel activity, would certainly expedite the quest for new drugs that specifically target SOCE channels to treat human disorders associated with dysregulated Ca2+ influx. [29]

It is therefore, the need of the hour to scientifically generated data to project herbal medication heavily the therapeutic SOCE activity in a proper perspective and help them sustain in the global market. Given that SOCE channels have emerged as an attractive target for developing new therapies for cardiovascular disease. It was decided to evaluate the role of SOCE in modulation of calcium in hypertension and myocardial infarction and its inhibition by SOCE inhibitors.

Eugenol, a major phenolic component from clove oil has demonstrated several biological activities, such as anti-inflammatory activity by inhibiting the enzyme cyclooxygenase-II, analgesic activity due to selective binding at the capsaicin receptor, anti-oxidation activity, and antibacterial activity against both gram-positive and gram-negative microorganisms.[30] Capsaicin has been demonstrated to inhibit high-voltage-activated calcium channel (HVACC) currents.[31] Since the chemical structure of eugenol is similar to that of capsaicin, there is a possibility that eugenol modulates HVACC as capsaicin does. Calcium channel blocker (CCB) activity of Eugenol by inhibiting L and T type of Calcium channel. Eugenol has been reported to exhibit a smooth muscle relaxing action possibly through an inhibitory action on the intracellular release and entry of extracellular Ca^{++}.[32] Eugenol exerted a strong inhibition of ACE activity in vitro and in diabetic rats. Eugenol inhibits neuronal excitotoxic or oxidative injury and has protective effects against N-methyl-D-aspartate–induced neurotoxicity.[33] The effect of eugenol on lipid peroxidation and oxidation of low-density lipoprotein was studied. In view of its nonmutagenic and noncarcinogenic properties, eugenol is generally regarded as safe by the Food and Agricultural Organization of the United Nations, with an acceptable daily intake of up to 2.5 mg/kg body weight in humans.[34]

Piper betle which has antioxidant, antiplatelet, anti-inflammatory activity and calcium channel blocker.[35] Furthermore, Piper betle is also anti-infective, analgesic, anticancer, antidiabetic, hepatoprotective, immunomodulatory. *Piper betle* contain piperbetol, methylpiperbetol, piperol-A, piperol-B, hydroxychavicol and allylpyrocatechol like phytocostituent. Piperbetol, methylpiperbetol, piperol-A, piperol-B, is capable of protecting the myocardium against IR injury, partly mediated through inhibited platelet aggregation induced by platelet activating factor (PAF) in a concentration-dependent manner. Hydroxychavicol and allylpyrocatechol are capable of protecting the myocardium against IR injury, partly mediated through ROS scavenger. Hydroxychavicol could be a potential therapeutic agent for prevention and treatment of atherosclerosis and other cardiovascular diseases through its anti-inflammatory and antiplatelet effects, without effects on haemostatic functions.[36] The calcium channel antagonist effect of PBEA may be partly due to the presence of eugenol.[37] There has been some indication for the presence of calcium channel antagonist(s) in the betel quid.[38]

Rubia cordifolia which has antioxidant, anti-platelet, calcium channel blocker and anti-inflammatory activity. Furthermore, *Rubia cordifolia* is also known for diuretic, blood purifier, remove toxins, haemostatic, analgesic and anti-pyretic. The polyherbal prepration is also

prescribed by many ayurvedic practitioners in the treatment of myocardial infarction. Herbal marketed formulation (Body Revival) is prescribed for myocardial infarction and also contains rubia cordifolia.[39] *Rubia cordifolia* contain anthraquinone and naphthoquinone like rubiadin, mollugin and 1-hydroxytectoquinone. Rubiadin is capable of protecting the myocardium against IR injury, partly mediated through its own antioxidant properties. Mollugin is capable of protecting the myocardium against IR injury, partly mediated through inhibit Platelet activating factor. Hydroxytectoquinone is capable of protecting the myocardium against IR injury, partly mediated through inhibit inflammatory action. Hydro alcoholic extract of rubia cordifolia may exhibit the CCB activity due to possible presence of calcium channel blocker(s) in rubia cordifolia.[40]

2-APB was initially characterized as a membrane-permeable modulator of IP_3 receptors, which are localized at intracellular calcium stores, but later it was found to directly influence store-operated calcium entry (SOCE) mechanisms. The effect of 2-APB on SOCE activity is nevertheless quite complex. In general, 2-APB potentiates I_{CRAC} currents at low concentrations and inhibition is observed at higher concentrations. 2-APB was found to block cellular distribution of Orai1 and STIM1.[41] The calcium pump of the endoplasmic reticulum (SERCA) is also blocked by 2-APB at very high concentrations.[42] 2-APB protects against liver, kidney and ovary ischemia-reperfusion injury by reducing cellular and mitochondrial calcium uptake.[43-45]

With this background, the present study hence was undertaken to carry out evaluating the role of SOCE and its inhibitors with special reference to myocardial infarction and hypertension using different animal models for better health prospects in the individuals suffering from CVD.

2. Objective

a] Evaluate the role of SOCE in hypertension.

b] Evaluate the role of SOCE in myocardial infarction.

c] Preparation, phytochemicals screening, in-vitro antioxidant assay and standardization of ethyl acetate extracts of *Piper betle* and hydro alcoholic extract of *Rubia cordifolia.*

d] Evaluating the cell viability, antioxidant status and anti-apoptosis effect of treatment drug on H9c2 cell line.

e] Effect of selected drug on Ag-II induced acute hypertension in vagotomized rat and evaluating molecular aspect of SOCE.

f] Effect of selected drug on isoproterenol induced myocardial infarction in rat and evaluating molecular aspect of SOCE.

g] Development of RP-HPLC method and to find out time as well as efficacy study of 2-APB.

h] Safety study of ethyl acetate extracts of *Piper betle* and hydro alcoholic extract of *Rubia cordifolia.*

3. Experimental work

3.1. Materials

Piper betle leaves and *Rubia cordifolia* root part were procured from star coin sales, Ahmedabad. H9c2 cell line was procured from NCCS, Pune. Isoproterenol, Angiotensin-II, 2-APB, Propidium iodide and 2′,7′-Dichlorofluorescin diacetate (DCFHDA) were procured from Sigma Aldrich. Eugenol, MTT, DMSO, Culture media, fetal bovine serum, penicillin G streptomycin solution were procured from Himedia. Annexin V-FITC assay kit was procured from BD sciences. Creatine Kinase-MB (CK-MB), Lactate Dehydrogenase (LDH), Na^{+}, K^{+} estimation kits were purchased from Coral clinical system. Ca^{++} estimation kit was purchased from Siemens Ltd. Rabbit polyclonal STIM1 and Rabbit polyclonal Oria1 primary antibody were purchased from Santa Cruz Biotechnology. Human Plasma was obtained as gift samples from Suraktam blood bank, Vadodara.

Animals

Acute Toxic Class Method (OECD 423), healthy wistar albino female rats (nulliparous and non pregnant) weighing 160±25gm with 8-12 weeks age were obtained from Zydus Research Centre, Ahmedabad. Repeated Dose 28-Day Oral Toxicity Study (OECD 407), healthy wistar albino male and female rats (nulliparous and non pregnant) weighing 230+25gm and 170±25gm respectively, with 8-12 weeks age were obtained from Zydus Research Centre, Ahmedabad. The animals were housed in a group of 3 rats per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 10%) and 12hrs/12hrs light-dark cycle. The animals had free access to conventional laboratory diet and purified R.O drinking water *ad libitum*.

The experiment was 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. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Department, The Maharaja Sayajirao University of Baroda (MSU/IAEC/2018-19/1801).

Healthy wistar albino male rats weighing 300±15gm with 12-16 weeks and 150±10 gm with 6-10 weeks age were obtained from Zydus Research Centre, Ahmedabad for SOCE evaluation in hypertension and myocardial infarction respectively.

Healthy wistar albino male rats weighing 300±15gm with 12-16 weeks and 250±25gm with 8-12 weeks age were obtained from Zydus Research Centre, Ahmedabad for main study of hypertension and myocardial infarction.

3.2. Methods

3.2.1. Evaluating the role of SOCE in Hypertension and Myocardial infarction

3.2.1.1. Hypertension[46]

All the animals were randomized and control and Ag-II groups were prepared. 3 animals were used in each group. Control and Ag-II group animals received saline and Ag-II (2µg/kg, i.v.), respectively. Animals were euthanized humanely and kidney, aorta and heart were isolated and used for western blot analysis.

3.2.1.2. Myocardial infarction[47]

All the animals were randomized and control and ISO groups were prepared. 3 animals were used in each group. Control and ISO group animals received saline and ISO (85mg/kg, s.c.), respectively. Animals were euthanized humanely and heart was isolated and used for western blot analysis.

3.2.1.3. Western blot analysis[13]

All tissue were homogenized in RIPA buffer and centrifuged at 10000 rpm and supernant was collected. After that protein estimation was done by Bardford method and protein sample was denatured by adding Lamelli's buffer and heated at 69°c for 15 min. For western blotting, Protein samples containing an equal amount of protein (20 µg) were separated by 10% SDS–polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After being blocked with 5% non-fat dry milk in PBS containing 0.1% Tween 20 (PBST) for 1 h at room temperature, transferred membranes were incubated overnight at 4°C with respective primary antibodies (in PBST with 5% non-fat milk) (STIM1 1:500 dilution; Orai1 1:500 dilution). Four washes with PBST 15 min each were given on a rocker. The membranes were incubated with the appropriate HRP-conjugated secondary antibodies (Goat Anti rabbit/mouse 1:5000 dilution) in PBST with 5% non-fat milk for 1 h at room temperature. After four washes with PBST and two washes with PBS, target proteins were detected with enhanced chemiluminescence reagents for 2 min and visualized using chemidocumentation system. The immunoreactive protein bands were scanned and normalized with ponceau S bands.

3.2.2. Plant authentication

Piper betle leaves and *Rubia cordifolia* root were identified 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.

3.2.3. Preparation of extracts[37, 48]

Leaves of *Piper betle* and root of *Rubia cordifolia* powder (30g) were packed into Soxhlet column and extracted with 250 ml of ethyl acetate and 80 % methanol, respectively at 70°C for 12 h. The extract was concentrated under reduced pressure on a rotary evaporator. The extracts

were stored at 4°C in airtight container till further use and labeled as follows: Piper betle ethyl acetate extract (PBEA) and Rubia cordifolia hydro alcoholic extract (RCHA).

3.2.4. Phytochemical screening of extract[49, 50]

0.5% of PBEA and RCHA solutions were prepared in ethyl acetate and methanol respectively and filtered through WHATMAN No. 1 filter paper. These solutions were used for determination of alkaloids, flavonoids, glycosides, tannins, phenolics, steroids, quinines, carbohydrate, protein and amino acid test.

3.2.5. Standardization of extract

3.2.5.1. GC-FID[51]

A gas chromatography with flame ionization detector was used for the determination of eugenol in PBEA and RCHA. TotalChrom Navigator clarus 500 software was used to analyze the sample. Instrument is coupled with a split/splitless injector, operated in a split-mode and FID. The computer with TotalChrom Navigator clarus500 software has been used to control the gas chromatograph and ZB-5 capillary column (cross bond 5% diphenyl/95% dimethyl polysiloxane) with a length of 30 meters and an internal diameter of 250 µm was used throughout the study. The GC-FID parameters used in the analysis were based on the boiling point and affinity towards the stationary phase of the drug. PBEA, RCHA and Eugenol 10% solutions were prepared. Eugenol has a boiling point of about 254˚C. Manual split injection of 1 µL sample were performed at an inlet temperature and detector temperature of 240˚C and 300˚C, respectively. After injection, the oven temperatures was increased quickly from 60˚C and hold for 2 min. then programmed within 25min 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), hydrogen (45 mL/min) were fed to the FID. All the gases used in these studies were of pharmacopoeial purity.

3.2.5.2. HPTLC[52]

PBEA, RCHA (conc.100mg/ml) and Eugenol (conc.10mg/ml) solutions were prepared and applied using auto sampler on Pre-coated silica gel 60 F254 HPTLC aluminum plates. This plate was developed in the mobile phase Toluene: Ethyl acetate (9.3: 0.7), dried in air and scanned at 282 nm for Eugenol. The peak areas were recorded for all concentrations. Calibration curve of Eugenol was plotted as peak area versus concentration of Eugenol in µg/band applied in triplicate. The plate was derivatized by spraying with anisaldehyde reagent followed by photographing in visible mode. The amount of eugenol in PBEA and RCHA were quantified using calibration curve plotted with Eugenol standard.

3.2.6. Authentification of 2-APB

Authentification of 2-APB was done by IR spectra.

3.2.7. Vascular reactivity assay[46]

Healthy wistar albino male rats weighing 300±15gm with 12-16 weeks age were obtained from Zydus Research Centre, Ahmedabad. Descending thoracic aorta was dissected from euthanized animlas and surrounding connective tissue and fat were removed. The aorta was then cut into 2.5-mm rings, and these rings were suspended on systemic force transducers at 2.0 g resting tension in 30 mL temperature-controlled baths (37°C) containing Krebs solution that was continuously gassed with 95% O_2 and 5% CO_2. The rings were equilibrated at 2.0 g resting tension for 90 min, during which time the bathing solution was changed every 15 min. The denuded aortic rings were exposed to a calcium-free Kreb solution containing a high concentration of K+ (50 mM KCl). The calcium was added cumulatively to achieve a final bath concentration (from 0.1 to 100 mM) and response was recorded. After that The Different conc. of treatment drug was added directly to the calcium-free isotonic depolarizing solution containing a high concentration of KCl (50 mM) 30 min before the calcium (Ca2+)-induced contraction. The effect of treatment drug 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 treatment drug.

3.2.8. In- vitro antioxidant assay[53]

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 redical activity by Fenton reaction, Fe3+ reducing power method, Total phenolics content by Folin & Ciocalteau method and Total flavonoid content by AlCl3 method.

3.2.9. Effect of drug treatment on cell viability in H9c2 cell line.

3.2.9.1. Cell line and cell culture[54]

H9c2 cell line were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) -high glucose supplemented with 10% fetal bovine serum (FBS), 1% penicillin-G streptomycin solution. Cells were grown in 37° C in humidified incubator with 5% CO2.

3.2.9.2. MTT Assay[55]

H9c2 cells (10000/well) were seeded into each well of 96-well plates for overnight before being treated or untrated with different concentrations of drug treatment (2-APB, Eugenol, PBEA and RCHA) for 24 h before addition of 100 μM H_2O_2 for 1 h. The media containing extracts was discarded after specified time intervals and 5 mg/ml MTT solution (20 µl/well) was added to each well. The cells were incubated for 4 h at 37˚C. The supernatant was aspirated and DMSO (100 µl) was added to the wells. The absorbance was then measured at 570 nm by micro plate reader. The percentage of cell viability was calculated against control cell.

3.2.9.3. Intracellular ROS measurement[56]

To detect H_2O_2 induced intracellular ROS accumulation, the cells were plated in 12-wellplates and effective concentration (cytoprotective) of drug treatment were exposed for 24 h and 100μM H_2O_2 was added to the plate and incubated at 37°C for another 1 h. After staining for 30 min in the dark with 10µg/ml DCFH-DA and 1µg/ml DAPI, the cells were washed twice with PBS to remove the extracellular compounds. DCFH-DA green fluorescence was observed using florescent microscope. The relative cell fluorescence intensity was measured by Image J software.

3.2.9.4. Cellular antioxidant enzyme profile[53]

Cells were seeded at 1 x 10^6 cells/well and cultured overnight. Cells were cultured with or without selective dose of drug treatment in 24 well culture plates for 24 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 homogenized in cold 0.1 % Triton X-100 lysis buffer. Cells were again centrifuged at 10000g for 15 min. at 4°C and supernatant were used for assay of Lipid peroxidation, endogenous antiperoxidative enzymes like Superoxide dismutase (SOD) and Catalase, Reduced glutathione (GSH) were determined.

3.2.9.5. Detection of Apoptosis with Annexin V-PI[57]

1×10^5 cells were plated in 12-wellplates and exposed to selective dose of drug treatment for 24 h and 100 μM H_2O_2 was added to the plate and incubated at 37°C for another 1 h. 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 (RT) followed with addition of PI in the same tubes to a final concentration of 2 µg/ml for 15 min at RT as per manufacturers protocol. These, cells were observed using florescent microscope. The relative cell fluorescence intensity was measured by Image J software and then plotted.

3.2.10. Ag-II induced acute hypertension in vagotomized rats[46]

All the animals were randomized and 6 groups were prepared. 6 animals were used in each group. Acute hypertension in rats was induced by slow intravenous injection of 2 µg/kg body weight of Ag-II which was dissolved in normal saline.

The control (group 1) animals received saline. All groups except group 1 received Ag-II. Group 2 served as model control. Group 3 to 6 were test groups which received 2-APB (4 mg/kg, i.v. at just before Ag-II injection) as well as Eugenol (50 mg/kg, p.o), PBEA (200 mg/kg, p.o) and RCHA (200 mg/kg, p.o) 1 h before Ag-II injection. During experimental period, Blood pressure was measured at specific time. Blood was withdrawn from retro-orbital plexus under mild ether anesthesia without anticoagulant from each animal before drug administration and also from jugular vein at specific time. Animals were euthanized humanely for assessing different parameters.

3.2.10.1. Blood pressure[46]

Systolic BP and Diastolic BP difference (ΔP) of all animals were measured using pressure transducer attached with Power lab data acquisition system.

3.2.10.2. Electrolyte[58]

3.2.10.2.1. Calcium

Calcium was estimated by OCPC method as per kit manufacture instruction at different time point.

3.2.10.2.2. Sodium

Sodium was estimated by colorimetric method as per kit manufacture instruction.

3.2.10.2.3. Magnesium

Magnesium was estimated by calmagite method as per kit manufacture instruction.

3.2.10.3. Antioxidant parameter

10% homogenate of Heart and Kidney were prepared in Tris- HCL pH 7.2. Samples were centrifuged and supernant were collected and used for enzyme estimation. Protein concentration was measured by Lowry method.

3.2.10.3.1. Melondialdehyde[59]

0.75 ml of the tissue homogenate (supernatant) was added to 0.75 ml of freshly prepared TCA and the 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 and 0.75 ml of clear supernatant solution was mixed with 0.75 ml of freshly prepared TBA. The resulting solution was heated in a boiling water bath for 30 minutes. It was then immediately cooled in an ice bath for 5 minutes. The colour developed was measured at 532 nm against reagent blank. Different concentrations (0-512 ng/ml) of standard MDA were taken and processed as above for standard graph. The values were expressed as nmoles of MDA/mg protein.

3.2.10.3.2. Glutathione[60]

Equal volumes of tissue homogenate (supernatant) and 20% TCA were mixed. The precipitated fraction was centrifuged at 4000 rpm for 10 min at 4°C and to 0.25ml of supernatant, 2ml of DTNB reagent was added. The final volume was made up to 3ml with phosphate buffer. The colour developed was read at 412 nm against reagent blank. Different concentrations (1-128µg/ml) of standard glutathione were taken and processed as above for standard graph. The amount of reduced glutathione was expressed as nmoles of GSH/mg protein.

3.2.10.3.3. Superoxide dismutase[61]

0.25 ml of ice-cold ethanol and 0.15 ml of ice-cold chloroform was added to 0.5 ml of tissue homogenate. The 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 optical density/minute was measured at 480 nm against reagent blank. SOD activity was expressed as units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. Calibration curve was prepared by using 1-320 units of SOD.

3.2.10.3.4. Catalase[62]

Dilute homogenate 10 or 20 times with phosphate buffer (50 mmol/l; pH 7.0). To 1 ml of diluted sample, 0.5 ml of hydrogen peroxide (30 mmol/l) was added to initiate the reaction. The blank was prepared by mixing 1 ml of diluted sample (similar dilution) with 0.5 ml of phosphate buffer (50 mmol/l; pH 7.0). The decrease in absorbance was measured at 240 nm. Catalase activity was expressed as units/mg protein. Calibration curve was prepared by using 0.2-16 units of Catalase.

3.2.10.4. Western blot analysis[13]

All groups of animal samples were analyzed for SOCE by western blots. Procedure was same as described above in **Section 3.2.1.3.**

3.2.11. Isoproterenol induced myocardial infarction[47]

Myocardial infarction in rats was induced by subcutaneous injection of 85 mg/kg body weight of ISO at an interval of 24 hours for 2 days. ISO was dissolved in normal saline.

Pilot study of PBEA and RCHA, three graded (50, 100, 200 mg/kg) dose was selected and best dose of treatment was selected for main study. All the animals were randomized and six groups were prepared. The control (group 1) animals received saline. All groups except group 1 received ISO. Group 2 served as model control. Group 3 to 6 were test groups which received 2-APB (4 mg/kg, i.v. at 12 h intervals for 2 days during before ISO injection), Eugenol (50 mg/kg, p.o), PBEA (200 mg/kg, p.o) and RCHA (200 mg/kg, p.o). At the end of experimental period, Blood pressure and electrocardiography was measured. Blood was withdrawn from retro-orbital plexus under mild ether anesthesia with or without anticoagulant from each animal. Animals were euthanized humanely for assessing different parameters.

3.2.11.1. Drug efficacy study[63]

Based on the pharmaco-kinetic study data, we administered 2-APB at the dose of 4 mg/kg i.v. of 12 h interval in Isoproterenol (85 mg/kg) administered rats. Blood was withdrawn from retro orbital plexus at different time point (0 to 48 h) and serum was separated. Calcium level was estimated at different end point.

3.2.11.2. Blood pressure[64]

Systolic BP, Diastolic BP and Mean BP were measured at the end of treatment period by Tail cuff method using LE 5002 storage pressure transducer attached with Biopac MP36 data acquisition system.

3.2.11.3. Electrocardiogram[65]

After 48 hours of the first injection of isoproterenol, ECG was recorded under light ether anesthesia through needle electrodes (Lead II) using Biopac MP36 data acquisition system. The changes in ST interval, QT interval, RR interval, QRS complex, P wave amplitude and Heart rate were determined from ECG.

3.2.11.4. Hemodynamic parameter[66]

Rats were intraperitoneal (i.p) injected with 500 IU heparin and anesthetized by i.p. injection of 50-80 mg/kg pentobarbital. After the rat became unconscious and lost pedal reflex activity, the 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, the 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 flow rate of the modified Krebs-Henseleit buffer saturated with carbogen (95% O2 and 5% CO2), and its temperature were controlled at 37°C. In order to measure pharmacodynamic response, a latex balloon tied to the end of a polyethylene tube, which was connected with pressure transducer attached with Biopac MP36 data acquisition system, was carefully inserted into a left ventricle of the isolated heart.

3.2.11.5. Biochemical parameter[65]

3.2.11.5.1. Creatinine kinase muscle brain (Ck-MB)

Ck-MB was estimated by IFCC method as per kit manufacture instruction.

3.2.11.5.2. Lactate dehydrogenase (LDH)

LDH was estimated by IFCC method as per kit manufacture instruction.

3.2.11.5.3. Calcium

Calcium was estimated by OCPC method as per kit manufacture instruction.

3.2.11.5.4. Potassium

Potassium was estimated by colorimetric method as per kit manufacture instruction.

3.2.11.5.5. Sodium

Sodium was estimated by colorimetric method as per kit manufacture instruction.

3.2.11.5.6. Tumor necrosis factor –alpha (TNF-α)

TNF- α was estimated by ELISA method as per kit manufacture instruction.

3.2.11.5.7. Interlukin-6 (IL-6)

IL-6 was estimated by ELISA method as per kit manufacture instruction.

3.2.11.6. Gravimetric analysis[65]

Gravimetric analysis was measured using heart weight divided by body weight.

3.2.11.7. Antioxidant parameter[65]

10% homogenate of heart were prepared in Tris- HCL pH 7.2. Samples were centrifuged and supernant were collected and used for enzyme estimation. Protein concentration was measured by Lowry method.

3.2.11.7.1. Melondialdehyde (MDA)

Melondialdehyde was measured as same procedure described in **Section 3.2.10.3.1.**

3.2.11.7.2. Glutathione (GSH)

Glutathione was measured as same procedure described in **Section 3.2.10.3.2.**

3.2.11.7.3. Glutathione peroxidase (Gpx)

0.2 ml each of EDTA, sodium azide, reduced glutathione, hydrogen peroxide, 0.4ml of phosphate buffer and 0.1ml of homogenate were 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 of supernatant, 4ml of disodium hydrogen phosphate and 1 ml of DTNB were added and the color developed was read at 420 nm immediately using Shimadzu UV spectrophotometer. Graded concentration of the standards (1-64 U/ml) was also treated similarly. GPx is expressed as U/mg protein.

3.2.11.7.4. Glutathione –S-Transfarse (GST)

To 1 ml of phosphate buffer, 0.1 ml of homogenate, 1.7 ml of water and 0.1 ml of CDNB were added and incubated at 37ºC for 15min. After incubation, 0.1 ml of reduced glutathione was added. The increase in optical density was measured against that of the blank at 340 nm. All reagents except homogenate served as blank. GST activity was expressed as nmoles of CDNB conjugated/min/mg protein.

3.2.11.7.5. Superoxide dismutase (SOD)

SOD was measured as same procedure described in **Section 3.2.10.3.3.**

3.2.11.7.6. Catalase

Catalase was measured as same procedure described in **Section 3.2.10.3.4.**

3.2.11.8. Membrane bounded enzymes

3.2.11.8.1. $Na^{+/}k^{+}$ ATPase[67]

1.0ml of tris-hydrochloride buffer and 0.2 ml each of magnesium sulphate, sodium chloride, potassium chloride, EDTA, ATP were added to test tube containing 0.2ml of homogenate. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0 ml of 10% TCA, mixed well and centrifuged at 4000 rpm for 10 min at 4°C. The phosphorus

content of the supernatant was estimated by Fiske and Subbarow method. The enzyme activity was expressed as μM of inorganic phosphorus liberated/h/mg protein.

3.2.11.8.2. Ca^{++} ATPase[68]

The incubation mixture contained 0.1 ml each of tris-hydrochloride buffer, calcium chloride, ATP and homogenate 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 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated by Fiske and Subbarow method. The enzyme activity was expressed as μM of inorganic phosphorus liberated/h/mg protein

3.2.11.8.3. Mg^{++} ATPase[69]

The incubation mixture contained 0.1 ml each of tris-hydrochloride buffer, magnesium chloride, ATP and homogenate 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 10% TCA, mixed well and centrifuged at 4000 rpm for 10 min at 4°C. The phosphorus content of the supernatant was estimated by Fiske and Subbarow method. The enzyme activity was expressed as μM of inorganic phosphorus liberated/h/mg protein.

3.2.11.9. Myeloperoxidase enzyme[65]

The myocardial tissue was homogenized in 50 mM K2HPO4 buffer (pH 6) containing 0.5% hexadecyl-trimethylammonium bromide using a Polytron tissue homogenizer. After freeze-thawing three times, the samples were centrifuged at 15,000 rpm for 30 min at 4°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% H2O2.The change in absorbance at 460 nm was measured for 3 min with the spectrophotometer (Shimadzu 1800 UV-Vis Spectrophotometer). Myeloperoxidase activity was expressed as U/gm tissue.

3.2.11.10. Western blot analysis[13]

All groups of animal samples were analyzed for SOCE by western blots. Procedure was same as described above in **Section 3.2.1.3.**

3.2.11.11. Infract size[70]

Infract size was measured by TTC staining method described by Warltier et al. and % infraction was calculated by image J.

3.2.11.12. Histopathology[65]

Histopathology was processing by Sakshi histopathology Laboratory, Vadodara.

3.2.12. Development of RP-HPLC method and Pharmaco-kinetic study of 2-APB

3.2.12.1. Selection and Optimization of Chromatographic conditions[71]

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 could be 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.

3.2.12.2. Preparation of 2-APB solution

100 ppm standard solution of 2-APB was prepared in methanol and 0.05, 0.1, 0.5, 1, 2, 4, 6, 8 and 10 ppm working standard solution were made from standard solution by serial dilution with double distilled water. All solutions were prepared in ice cold condition.

3.2.12.3. Selection of Detection Wavelength

2-APB was scanned between 200-400nm in uv-visible spectrophotometer and 2-APB showed good absorbance at 230 nm.

3.2.12.4. Bioanalytical method of 2-APB in Human plasma

3.2.12.4.1. Preparation of Spiking Solutions of 2-APB

Spiking Solutions of 2-APB were prepared using different volume of standard solutions was madeup to 2 ml with plasma. Calibration curve of plasma was prepared by protein precipitation method.

3.2.12.4.2. Pharmaco-kinetic study[72]

2-APB was administrated at dose of 4 mg/kg i.v. in rats (n=6). Blood was withdrawn at different time point of 0 to 24 h. and plasma was separated by centrifuge. 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.

3.2.13. Toxicity study of plant extracts[73]

All the animals were randomized and four groups were prepared for OECD 423. 3 animals of 2 sets were used for each group. 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.

All the animals were randomized and six groups were prepared for OECD 407. 10 animals (five female and five male) were used in each group. The control and s-control (group 1 and 5) animals received olive oils. Group 2 to 4 were test groups which received PBEA- 50, 100, 200 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.

4. Results and Discussion

4.1. Evaluating expression of STIM1 and Orai1 in Hypertension and Myocardial infarction

4.1.1. Evaluating expression of STIM1 and Orai1 in Ag-II induced acute hypertension in vagotomized rat

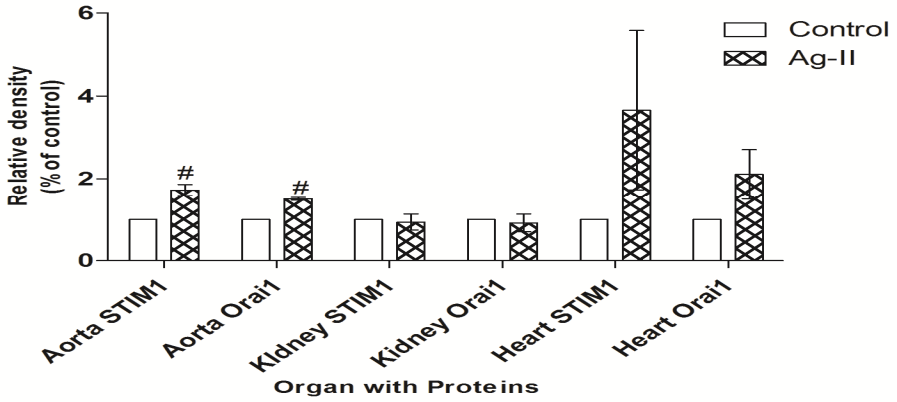

Figure 1: Effect of Ag-II on STIM1 and Orai1 expression of Aorta, Kidney and Heart.

Values are expressed as Mean ± SD of 3 animals. Values are statistically evaluated using one tailed paired t- test. #P<0.05 Control vs Ag-II.

4.1.2. Evaluating expression of STIM1 and Orai1 in Isoproterenol induced myocardial infarction

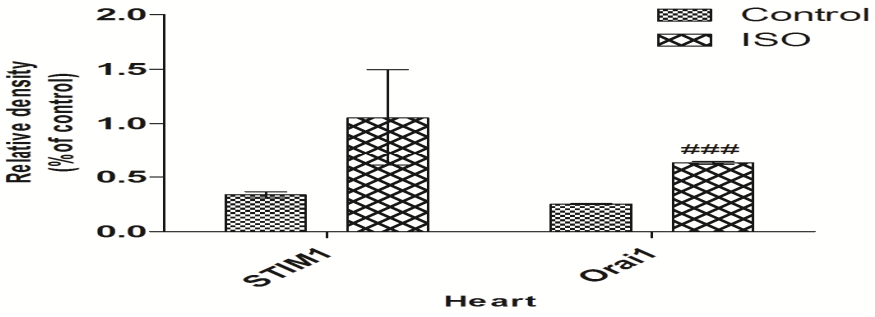

Figure 2: Effect of ISO on STIM1 and Orai1 expression of heart.

Values are expressed as Mean ± SD of 3 animals. Values are statistically evaluated using one tailed unpaired t- test. ###P<0.001 Control vs ISO.

4.2. %Yield and Phytochemical screening of PBEA and RCHA

As per previously mentioned process parameter of extraction, extractive yield of PBEA and RCHA was found to be 6.8% and 8.0% respectively. PBEA contain steroids, tannins, phenolics, carbohydrate, fat and oils. RCHA contain steroids, cardiac glycosides, anthraquinone glycoside, saponin glycoside, Tannin, phenolics, carbohydrate, proteins, fat and oils.

4.3. Standardization of extracts

Quantification of Eugenol by GC-FID method we got 43.43±1.46 mg Eugenol /g of PBEA was found in PBEA and 0.414±0.027 mg Eugenol / g of RCHA was found in RCHA. Quantification of Eugenol by HPTLC method we got 1.11±0.24 mg Eugenol /g of PBEA was found in PBEA and Eugenol didn't detect in RCHA. On the basis of these results we can say that GC-FID is good method for qualitative and quantitative determination of Eugenol.

4.4. IR spectrum of 2-APB

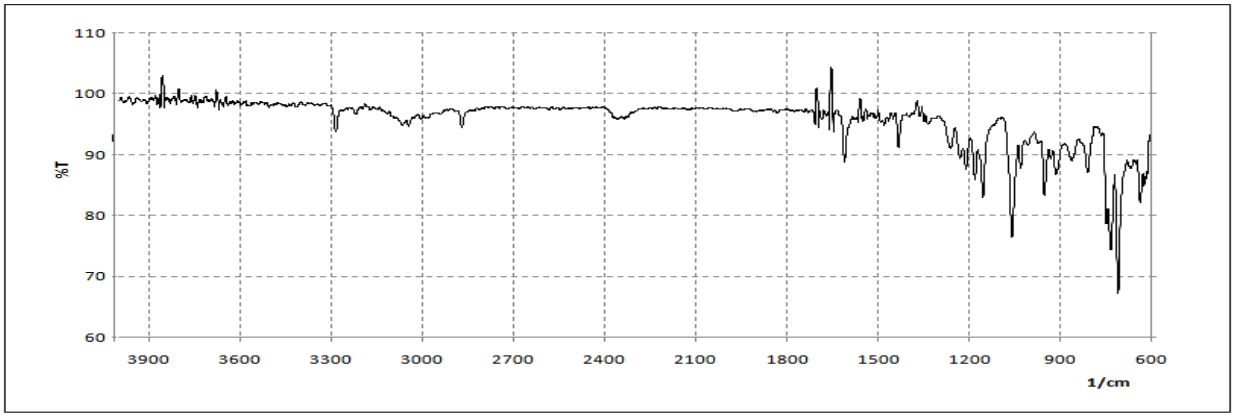

Figure 3: Infrared spectrum of 2-APB.

4.5. Effect of PBEA and RCHA on calcium induced contraction in isolated ring aorta

In accordance with previous reports, PBEA (1000µg/ml) significantly attenuated ($P<0.05$) the contraction induced by the cumulative addition of calcium (0.1 to 100 mM) compared with the rings untreated with PEBA in the calcium-free isotonic depolarizing solution containing 50 mM KCl. In addition, RCHA (300 µg/ml) significantly attenuated ($P<0.01$) the contraction induced by the cumulative addition of calcium (0.1 to 100 mM) compared with the rings untreated with RCHA.

Calcium	EC_{50} value	Extracts	EC_{50} value
Control-1	0.057 ± 0.004	PBEA	0.039 ± 0.006*
Control-2	0.006 ± 0.001	RCHA	0.109 ± 0.020**

Table 1: Effect of extracts on calcium induced contraction in isolated ring aorta

Values are expressed as Mean ± SEM of 5-6 animals. Values are statistically evaluated using unpaired t- test. *P<0.05 Control vs drug; **P<0.01 Control vs drug.

4.6. Effect of PBEA and RCHA on antioxidant activity

Both of this extract has strong antioxidant activity and free radical scavenging activity depends upon its concentration as well as it has high flavonoids and phenolics content.

Assay	PBEA	RCHA
DPPH assay (IC50 µg/ml)	100.1 ± 0.32	32193±2.28
Hydrogen peroxide scavenging activity (IC50 µg/ml)	46.17±0.28	73.10±0.07
Superoxide anion scavenging activity (IC50 µg/ml)	132.2±0.25	46.1±0.38
Hydroxyl radical scavenging activity (IC50 µg/ml)	0.61±0.08	0.42±0.12
Reducing power assay ($RP_{0.5}$AU µg/ml)	73.33	503.00
β-carotene bleaching assay (% inhibition)	73.64±0.84	69.11±0.33
Total phenolics (mg of gallic acid/ g of dried extract)	76.75 ± 1.41	61.61 ± 0.68
Total flavonoids (mg of Quarcetin/ g of dried extract)	45.22 ± 1.65	75.00 ± 2.11

Table 2: Effect of extracts on In-vitro antioxidant activity

4.7. Effect of drug on cell viability in H9c2 cell

In present study, cytotoxicity was observed in H9c2 cell line which was found to be dose dependent. IC_{50} value of 2-APB, Eugenol, PEBA and RCHA were found to be 2.6 M, 8533 M, 48.56 µg/ml and 5.86 µg/ml, respectively. Cytoprotectivity was observed in H9c2 cell line against 100µM H_2O_2 was also dose dependent. Best cytoprotective dose of 2-APB, Eugenol, PEBA and RCHA were found to be 150µM, 50mM, 10µg/ml and 100ng/ml, respectively.

4.8. Effect on intracellular ROS measurement in H9c2 cell line

Excessive production of ROS may induce cell damage via apoptosis in any cell type, and such effects can be blocked or delayed by a wide variety of antioxidants. Control group cells showed no fluorescence which indicates no intracellular ROS accumulation in cell. When cell is treated with 100µM H_2O_2, cells accumulate ROS which was significantly higher as compare to control cells. After pretreatment of 2-APB, EU, PBEA and RCHA intracellular ROS was significantly decreased as compare to H_2O_2 group cells.

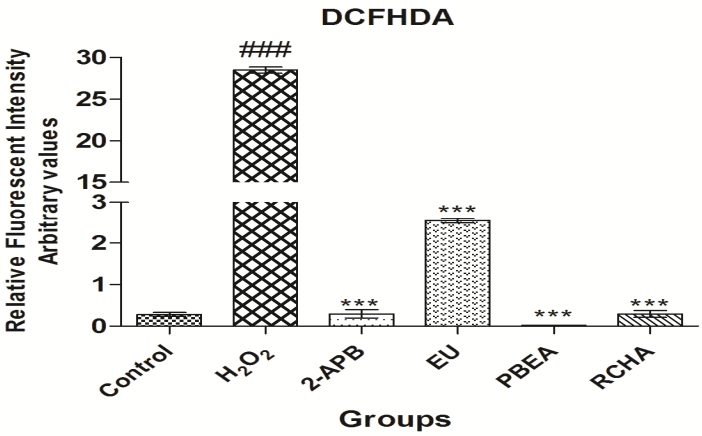

Figure 4:Effect of drug on intracellular ROS measurement in H9c2 cell line

Values are expressed as Mean ± SEM of 3 data. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ###P<0.001 control vs H_2O_2; ***P<0.001 treatments vs H_2O_2.

4.9. Effect on cellular antioxidant status in H9c2 cell line

H_2O_2 treated cells, lipid peroxidation was significantly (P<0.001) increased and endogenous antioxidants were significantly (P<0.001) decreased as compare to control groups cells. Pretreated cells with treatment showed significant effect on oxidative insults against H_2O_2 induced oxidative insults. This finding can be correlated with free radical scavenging activity and intra cellular ROS inhibition.

Groups	MDA (nmoles/mg proteins)	GSH (nmoles/mg proteins)	SOD (U/mg proteins)	CAT (U/mg proteins)
Control	1.18±0.09	461.9±16.38	22.38±1.48	6.31±0.22
H_2O_2	$4.10\pm0.30^{\#\#\#}$	$184.3\pm7.782^{\#\#\#}$	$6.377\pm1.27^{\#\#\#}$	$2.54\pm0.12^{\#\#\#}$
2-APB	$1.84\pm0.09^{***}$	$264.1\pm19.27^{**}$	9.634±0.70	$4.60\pm0.62^{*}$
EU	$2.84\pm0.18^{***}$	$274.5\pm10.45^{**}$	$12.12\pm0.67^{**}$	$5.80\pm0.64^{***}$
PBEA	$1.59\pm0.06^{***}$	$298.0\pm15.85^{***}$	$13.91\pm0.68^{***}$	$4.33\pm0.40^{*}$
RCHA	$2.15\pm0.10^{***}$	$305.7\pm19.47^{***}$	$10.90\pm0.70^{*}$	$4.31\pm0.10^{*}$

Table 3:Effect of drug on cellular antioxidant status in H9c2 cell line

Values are expressed as Mean ± SEM of 3 data. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ###P<0.001 control vs H_2O_2; *P<0.05 treatments vs H_2O_2; **P<0.01 treatments vs H_2O_2; ***P<0.001 treatments vs H_2O_2.

4.10. Effect on cellular apoptosis in H9c2 cell line

Transient exposure of H_2O_2 significantly manifested an elevation in the extant of apoptosis in H9c2 cell culture via double staining with annexin-V and PI. Treatment control all groups protects H9c2 cells against apoptosis induced by H_2O_2 through detoxification of free radical, through the ROS-dependent signaling pathway and by enhancing the intracellular antioxidant defense grid.

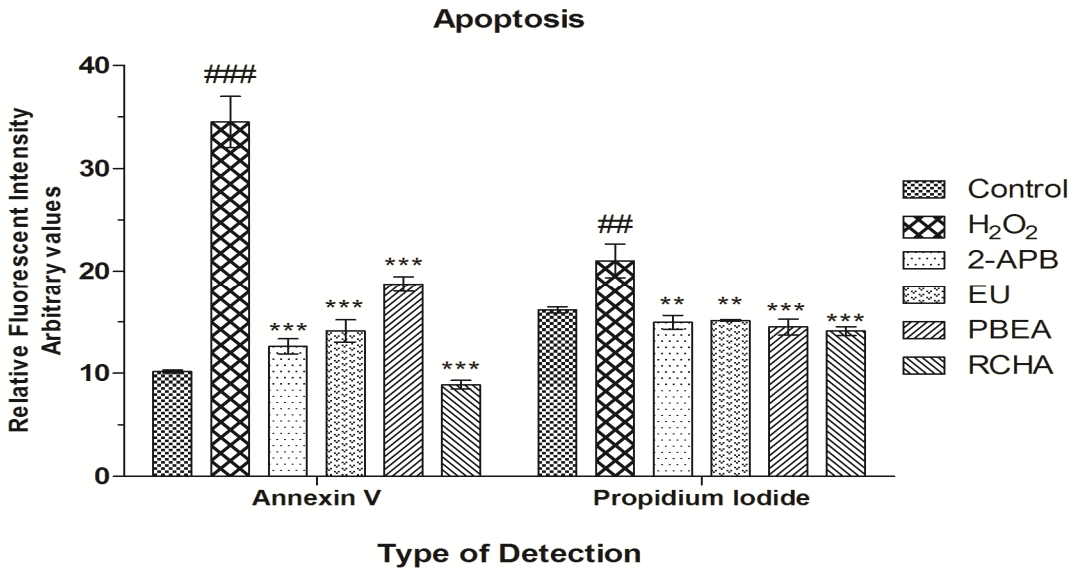

Figure 5: Effectof drug on cellular apoptosis in H9c2 cell line

Values are expressed as Mean ± SEM of 3 data. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ##P<0.01 control vs H_2O_2; ###P<0.001 control vs H_2O_2; **P<0.01 treatments vs H_2O_2; ***P<0.001 treatments vs H_2O_2.

4.11. Effect of drug in Ag-II induced acute hypertension in vagotomized rat

4.11.1. Effect on blood pressure in Ag-II induced acute hypertension in vagotomized rat

Angiotensin-II increase VSMC intracellular Ca2+ concentration [Ca2+] i and thereby activate the multifunctional Ca2+/calmodulin-dependent kinase II (CaMKII). CaMKII has been shown to activate L-type Ca2+ channel (LTCC) current (ICa) in excitable tissues. In present study, Ag-II group systolic blood pressure (SBP) and diastolic blood pressure (DBP) difference was significantly (P<0.001) elevated than control groups. In treatment control 2-APB, Eugenol, PBEA and RCHA groups SBP and DBP difference was significantly (P<0.001) lower than Ag-II group. This finding can be correlated with the calcium blocker activity in vascular reactivity assay.

Groups	SBP (ΔP)	DBP (ΔP)
Control	5.53 ± 0.13	6.60 ± 0.65
Ag-II	19.88 ± 0.98$^{###}$	17.64 ± 0.78$^{###}$
2-APB	13.71 ± 0.84***	7.76 ± 0.55***
EU	12.16 ± 0.86***	7.32 ± 0.65***
PBEA	2.85 ± 0.55***	2.08 ± 0.62***
RCHA	8.76 ± 1.32***	12.91 ± 0.96***

Table 4: Effect of drug on blood pressure difference in Ag-II induced acute hypertension in vagotomized rat

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ###P<0.001 control vs. Ag-II, ***P<0.001 treatments vs Ag-II.

4.11.2. Effect on electrolyte level of blood in Ag-II induced acute hypertension in vagotomized rat

4.11.2.1. Effect on calcium level at different time point in Ag-II induced acute hypertension in vagotomized rat

Ang II-stimulated Ca^{+2} signaling is complex and occurs via multiple pathways to elicit an integrated Ca^{+2} signal. Ang II typically mediates a biphasic $[Ca^{+2}]i$ response comprising a rapid initial transient phase and a sustained plateau phase.[74] Exact mechanisms whereby Ang II stimulates Ca^{+2} influx are unclear but may involve voltage-dependent calcium channels, which are directly or indirectly activated by Ang II, Ca^{+2} -permeable, nonspecific dihydropyridine-insensitive cation channels, receptor-gated Ca^{+2} channels, Ca^{+2}-activated Ca^{+2} release channels, and activation of the Na1/ Ca^{+2}exchanger. Ag-II group, calcium level at 0.5 and 1 min was significantly ($P< 0.01$ and $P< 0.05$) increased as compare to control group. In treatment control 2-APB, Eugenol, PBEA and RCHA groups calcium level at 0.5 and 1 min was significantly ($P< 0.05$) decreased as compare to Ag-II group. This result can be correlated with blood pressure.

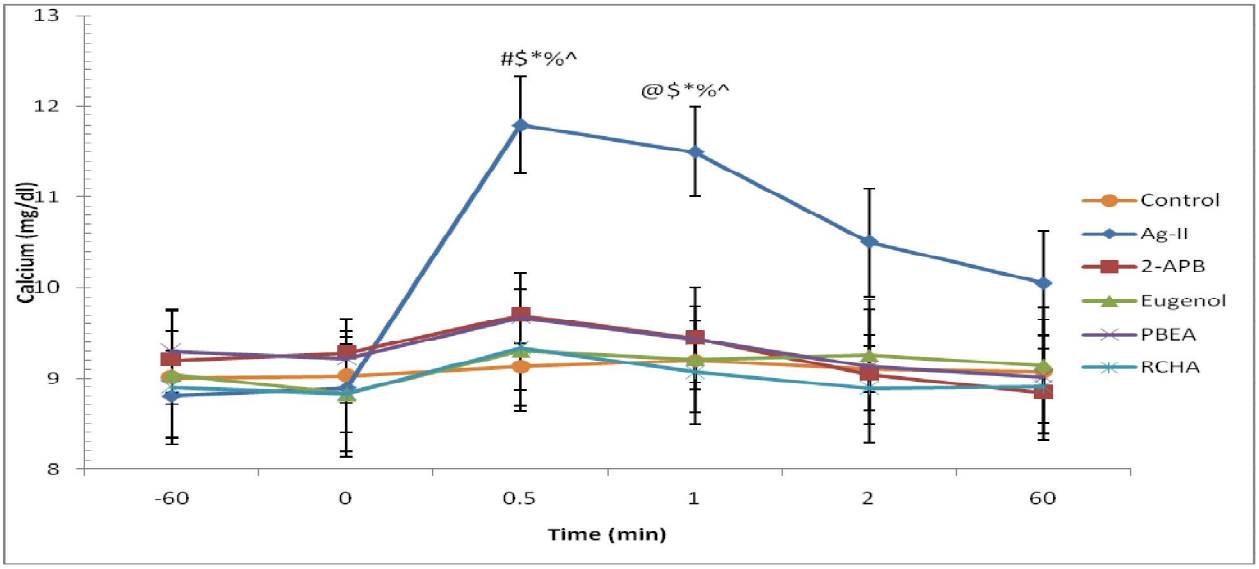

Figure 6: Effect of drug on calcium level at different time point in Ag-II induced acute hypertension in vagotomized rat

Each bar represents Mean ± SEM of 6 animals. Values are statistically evaluated using One Way ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with #P<0.01 vs. control; @P<0.05 vs. control; $P<0.05 vs.2-APB;*P<0.05 vs.Eugenol; %P<0.05 vs.PBEA; ^ P<0.05 vs RCHA.

4.11.2.2. Effect on sodium and magnesium level at 0.5 min in Ag-II induced acute hypertension in vagotomized rat

In addition to increasing $[Ca^{+2}]i$ and pHi, Ang II raises $[Na^{+}]i$ and reduces $[Mg^{+2}]i$ in a concentration-dependent fashion in vascular smooth muscle cells. Ang II-stimulated increase in $[Na^{+}]i$ and $[Mg^{+2}]I$ reduction in influence vascular smooth muscle contraction directly or indirectly by modulating $[Ca^{+2}]i$. Ag-II group, sodium level was significantly (P< 0.01) increased as well as magnesium level was significantly (P< 0.001) decreased as compare to control group. In treatment control, 2-APB, PBEA and RCHA groups, sodium level was significantly (P< 0.05) decreased as well as 2-APB and PBEA groups, magnesium level was significantly (P< 0.001 and P< 0.05) increased as compare to Ag-II groups.

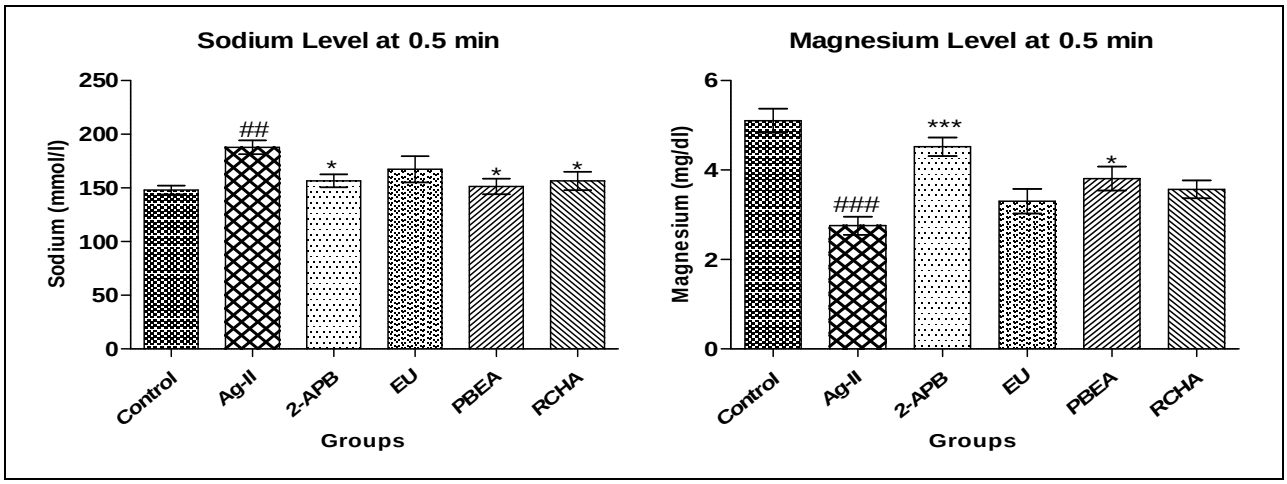

Figure 7: Effect of drug on sodium and magnesium level at 0.5 min in Ag-II induced acute hypertension in vagotomized rat

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ##P<0.01 control vs. Ag-II; ###P<0.001 control vs. Ag-II; *P<0.05 treatments vs Ag-II; ***P<0.001 treatments vs Ag-II.

4.11.3. Effect on antioxidant defense system in Ag-II induced acute hypertension in vagotomized rat

Reactive oxygen species such as superoxide anions and hydrogen peroxide act as intercellular and intracellular second messengers that may play a physiological role in vascular tone and cell growth, and a pathophysiological role in inflammation, ischemia-reperfusion, hypertension, and atherosclerosis. [19]Ang II, which increases $O_2^{\cdot}$ and H_2O_2 production in cardiac, vascular smooth muscle, endothelial, adventitial, and mesangial cells and generation of reactive oxygen species has been implicated in the pathogenesis of Ang II-induced but not catecholamine-induced hypertension

4.11.3.1. Effect on heart antioxidant enzymes in Ag-II induced acute hypertension in vagotomized rat

Ag-II group, heart MDA level was significantly (P<0.01) increased as well as GSH level, SOD and CAT activity was significantly (P<0.01) decreased as compare to control group. In treatment control, EU and RCHA groups, heart MDA level was significantly (P<0.05) decreased as well as PBEA group, heart MDA level was significantly (P< 0.001) decreased as compare to Ag-II group. Further, PBEA and RCHA groups, heart GSH level was significantly (P< 0.05) increased as compare to Ag-II group. Moreover, all treatment groups were significantly restores heart SOD and CAT activity as compare to Ag-II groups.

Groups	MDA (nmoles/mg proteins)	GSH (nmoles/mg proteins)	SOD (U/mg proteins)	CAT (U/mg proteins)
Control	1.12 ± 0.20	20.35 ± 2.26	12.02 ± 1.24	21.85 ± 1.15
Ag-II	1.79 ± 0.03$^{\#\#}$	11.20 ± 1.54$^{\#\#}$	7.13 ± 0.68$^{\#\#}$	14.83 ± 1.46$^{\#\#}$
2-APB	1.76 ± 0.12	15.93 ± 0.94	11.47 ± 0.72*	20.71 ± 1.16**
EU	1.16 ± 0.10*	16.58 ± 1.96	11.98 ± 1.37**	21.62 ± 0.48**
PBEA	0.98 ± 0.16***	19.54 ± 2.90*	12.29 ± 0.91**	21.85 ± 1.38**
RCHA	1.21 ± 0.12*	19.01 ± 1.22*	11.55 ± 0.45*	20.03 ± 1.50*

Table 5: Effect of drug on heart antioxidant enzymes in Ag-II induced acute hypertension in vagotomized rat

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ##P<0.01 control vs. Ag-II; *P<0.05 treatments vs Ag-II; **P<0.01 treatments vs Ag-II; ***P<0.001 treatments vs Ag-II.

4.11.3.2. Effect on kidney antioxidant enzymes in Ag-II induced acute hypertension in vagotomized rat

Ag-II group, kidney MDA level was significantly (P<0.05) increased as well as GSH level, SOD and CAT activity was significantly (P<0.01 and P<0.001) decreased as compare to control group. In treatment control, 2-APB and EU groups, kidney MDA level was significantly (P<0.05) decreased as compare to Ag-II group. Further, EU and PBEA groups, kidney GSH level was significantly (P< 0.05) increased as compare to Ag-II group. Moreover, all treatment groups were significantly restores kidney SOD and CAT activity as compare to Ag-II groups.

Groups	MDA (nmoles/mg proteins)	GSH (nmoles/mg proteins)	SOD (U/mg proteins)	CAT (U/mg proteins)
Control	1.47 ± 0.26	84.71 ± 5.44	73.35 ± 6.55	334.62 ± 15.63
Ag-II	2.54 ± 0.25$^{\#}$	46.17 ± 4.02$^{\#\#}$	32.62 ± 4.81$^{\#\#\#}$	157.68 ± 12.95$^{\#\#\#}$
2-APB	1.36 ± 0.36*	68.54 ± 5.38	60.90 ± 6.25**	252.34 ± 27.90*
EU	1.48 ± 0.15*	77.48 ± 10.27*	74.97 ± 4.70***	282.23 ± 19.94**
PBEA	1.66 ± 0.21	80.10 ± 5.77*	70.00 ± 6.45***	300.05 ± 31.39 ***
RCHA	1.71 ± 0.22	71.51 ± 9.99	64.45 ± 6.60**	274.96 ± 28.74**

Table 6: Effect of drug on heart antioxidant enzymes in Ag-II induced acute hypertension in vagotomized rat

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with

#P<0.05 control vs. Ag-II; ##P<0.01 control vs. Ag-II; ###P<0.001 control vs. Ag-II; *P<0.05 treatments vs Ag-II; **P<0.01 treatments vs Ag-II; ***P<0.001 treatments vs Ag-II.

4.11.4. Effect on expression of STIM1 and Orai1 in Ag-II induced acute hypertension in vagotomized rat

4.11.4.1. Effect on STIM1 and Orai1 expression of aorta in Ag-II induced acute hypertension in vagotomized rat

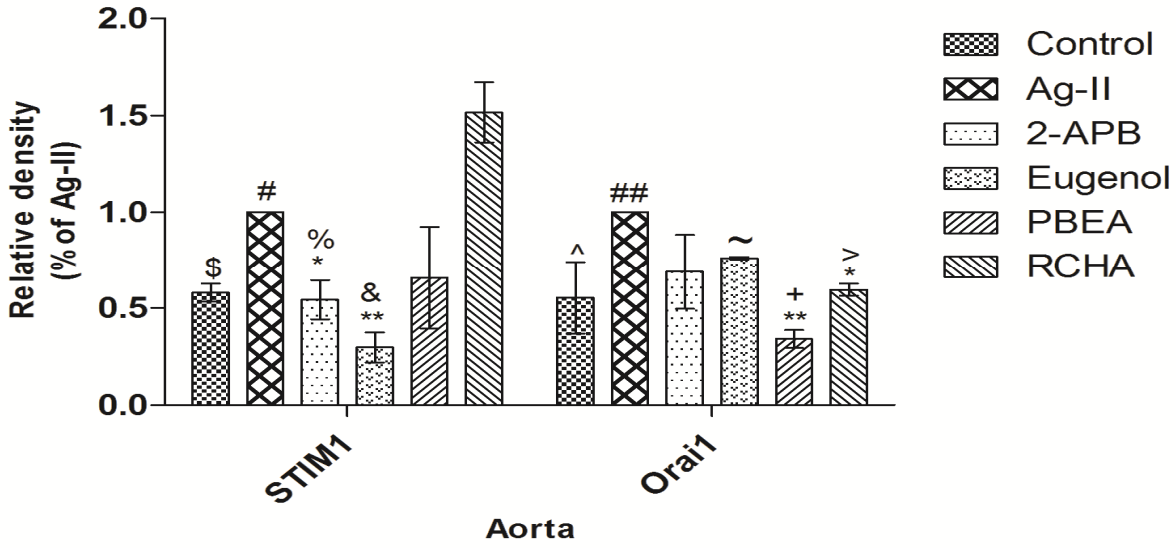

Figure 8: Effect of drug on STIM1 and Orai1expression of aorta in Ag-II induced acute hypertension in vagotomized rat

Values are expressed as Mean ± SEM of 3 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test and one tailed paired t test. Significant values were compared with #P<0.05 control vs. Ag-II; ##P<0.01 control vs. Ag-II; *P<0.05 treatments vs Ag-II; **P<0.01 treatments vs Ag-II; $P<0.05 control vs Ag-II; %P<0.05 Ag-II vs 2-APB; &P<0.05 Ag-II vs Eugenol; ^P<0.05 control vs Ag-II; ~P<0.01 Ag-II vs Eugenol; +P<0.05 Ag-II vs PBEA; > P<0.05 Ag-II vs RCHA.

4.11.4.2. Effect on STIM1 and Orai1 expression of kidney in Ag-II induced acute hypertension in vagotomized rat

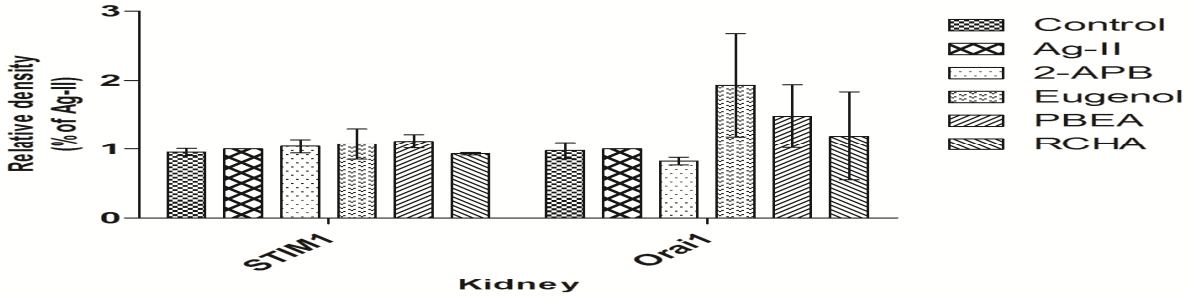

Figure 9: Effect of drug on STIM1 and Orai1 expression of kidney in Ag-II induced acute hypertension in vagotomized rat

Values are expressed as Mean ± SEM of 3 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test and one tailed paired t test.

4.11.4.3. Effect on STIM1 and Orai1 expression of heart in Ag-II induced acute hypertension in vagotomized rat

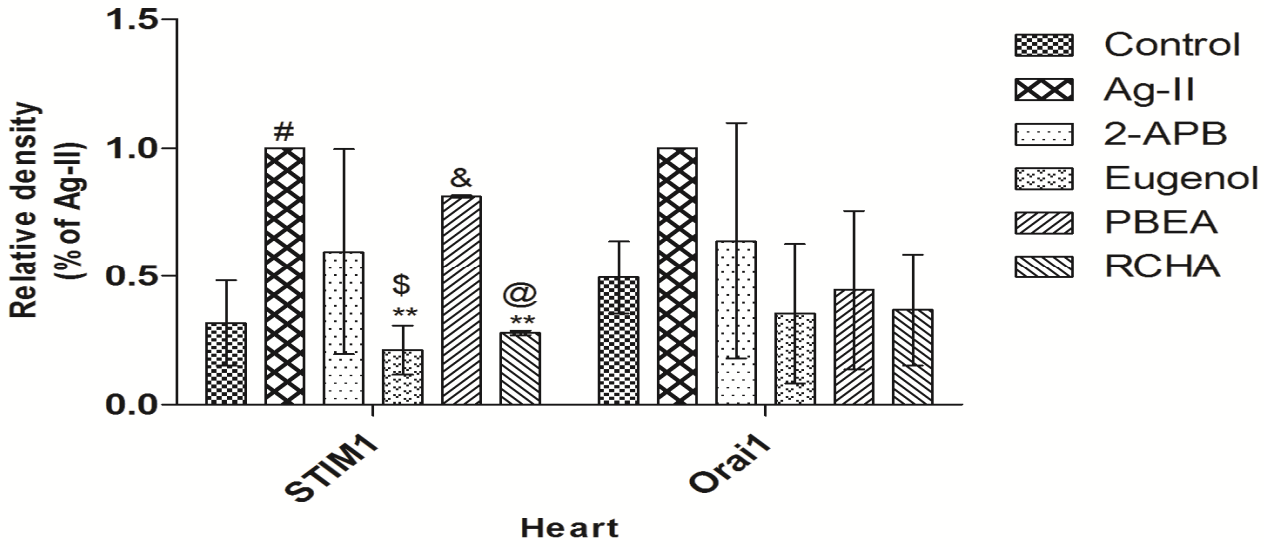

Figure 10: Effect of drug on STIM1 and Orai1 expression of heart in Ag-II induced acute hypertension in vagotomized rat

Values are expressed as Mean ± SEM of 3 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test and one tailed paired t test. Significant values were compared with #P<0.05 control vs. Ag-II; **P<0.01 treatments vs Ag-II; $P<0.05 Ag-II vs Eugenol; &P<0.05 Ag-II vs PBEA; @P<0.05 Ag-II vs RCHA.

4.12. Effect of drug in isoproterenol induced myocardial infarction

4.12.1. Drug efficacy study of 2-APB in isoproterenol induced myocardial infarction

ISO Group, calcium level was significantly elevated after 2 h of administration of ISO till 48 h continuously as compare to control group. In 2-APB group, calcium level was significantly reduced after 2 h of administration of ISO till 48 h continuously as compare to ISO group. This result shows that, 2-APB has static calcium channel blocking effect until 48 hr which is required in Myocardial infarction model.

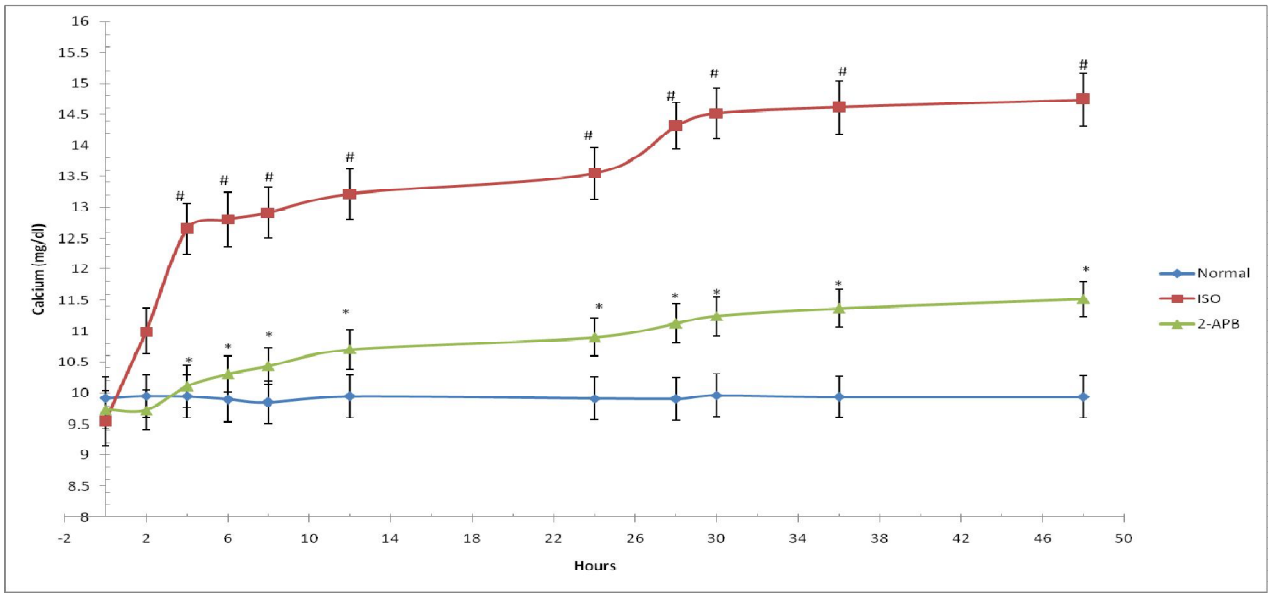

Figure 11: Effect of 2-APB on calcium level at different time point in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Multiple Comparison post hoc test. Significant values were compared with #P<0.001 control vs. ISO; *P<0.001 2-APB vs.ISO.

4.12.2. Effect on blood pressure in isoproterenol induced myocardial infarction

ISO group, blood pressure (SBP, DBP and MBP) was significantly (P<0.01 and P<0.001) decreased as compare to control group. In treatment control, EU and PBEA groups were significantly increased SBP and DBP, respectively as compare to ISO group. Moreover, 2-APB, EU and PBEA groups were significantly increased MBP as compare to ISO group.

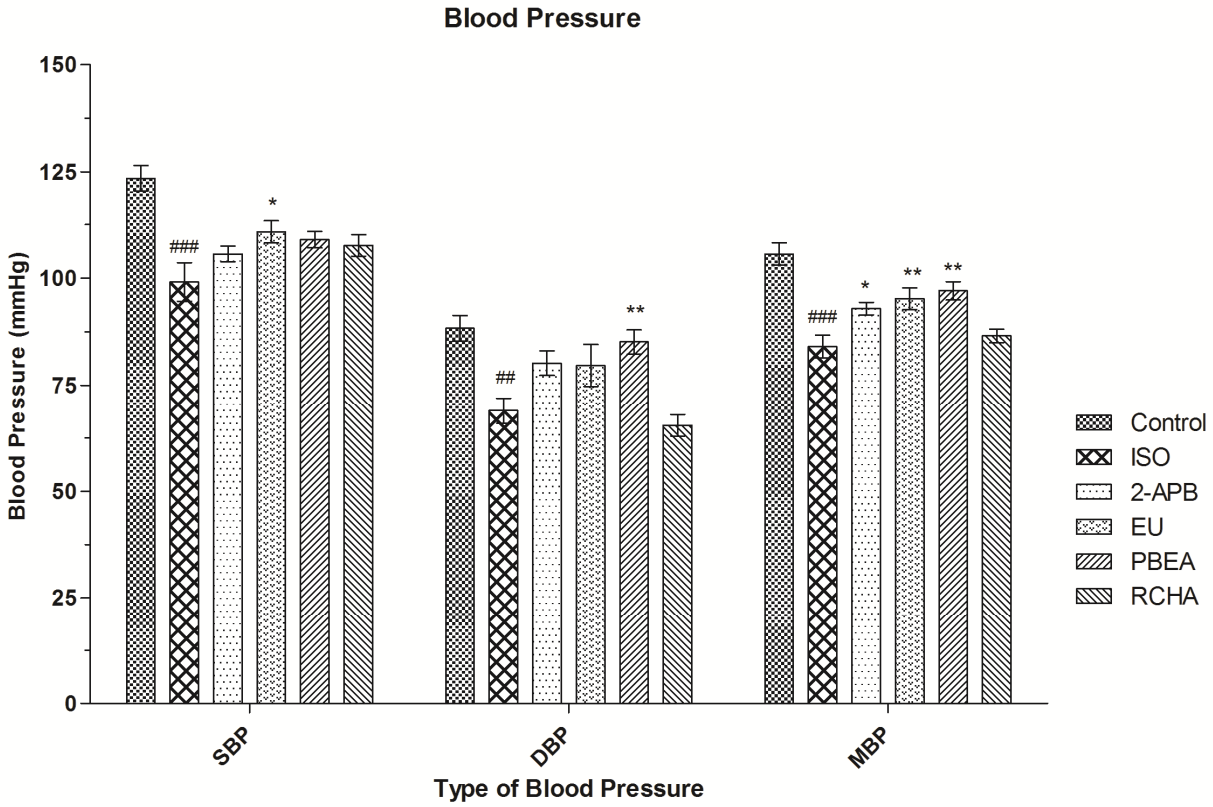

Figure 12: Effect of drug on SBP, DBP and MBP in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ##P<0.01 control vs. ISO; ###P<0.001 control vs.ISO; *P<0.05 treatments vs ISO; **P<0.01 treatments vs ISO.

4.12.3. Effect on electrocardiographic parameter in isoproterenol induced myocardial infarction

The ECG is considered the single most important initial clinical test for diagnosis of myocardial ischemia and infarction. Its correct interpretation is usually the basis for immediate therapeutic interventions and/or subsequent diagnosis tests.These changes could be due to the consecutive loss of cell membrane in injured myocardium.[65] It has been demonstrated that an increase in heart rate is responsible for increased oxygen consumption leading to accelerated myocardial necrosis. ST segment elevation reflects the potential difference in the boundary between ischemic and non-ischemic zones and consequent loss of cell membrane function, whereas decreased R-amplitude might be due to the onset of myocardial edema.ISO groups, electrocardiographic parameter (Heart rate, ST segment and QT intervals) were significantly (P<0.001) increased as well as other electrocardiographic parameter (P wave, QRS complex and R-R intervals) were significantly (P<0.05 and P<0.001) decreased as compare to control group.

In treatment control, all group restores electrocardiographic parameters except P wave were significantly changes as compare to ISO group.

Groups	Heart rate (BPM)	ST segment elevation (mv)	P wave (msec)	QRS complex (msec)	QT intervals (msec)	R-R intervals (msec)
Control	304 ± 14.86	0.18 ± 0.02	37.73 ± 2.81	41.88 ± 1.62	81.45 ± 1.48	177.25 ± 2.08
ISO	494.16 ± 12.57$^{###}$	0.39 ± 0.02$^{###}$	30.15 ± 1.28$^{#}$	29.90 ± 2.19$^{###}$	95.15 ± 1.36$^{###}$	132.95 ± 3.50$^{###}$
2-APB	364.16 ± 22.86***	0.24 ± 0.01***	37.26 ± 0.98*	38.88 ± 0.66***	85.14 ± 1.24***	159.25 ± 3.31***
EU	403.83 ± 20.12**	0.26 ± 0.01***	32.1 ± 1.59	35.63 ± 0.99*	88.47 ± 1.15**	150.58 ± 3.36**
PBEA	365.33 ± 20.06***	0.24 ± 0.00***	37.3 ± 1.13*	37.81 ± 1.50**	86.38 ± 1.95***	158.9 ± 3.82***
RCHA	399.83 ± 16.11**	0.27 ± 0.00***	33.1 ± 2.37	35.66 ± 1.23*	89.19 ± 1.07*	149.21 ± 3.85**

Table 7: Effect of drug on electrocardiographic parameter in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with #P<0.05 control vs. ISO; ###P<0.001 control vs.ISO; *P<0.05 treatments vs ISO; **P<0.01 treatments vs ISO; ***P<0.001 treatments vs ISO.

4.12.4. Effect on hemodynamic parameters in isoproterenol induced myocardial infarction

Increase in (+)LVdP/dt and (-)LVdP/dt reflects an overall enhancement of myocardial contractility and relaxation respectively. Another consequence of the reduction in LVEDP is to increase blood flow through the sub-endocardial region of the ventricular muscle that bears the maximum brunt of the ischaemic insult.[75] Under ischaemic conditions, there is a disproportionate reduction in blood flow to the subendocardial regions of the heart, which are subjected to the greatest extra-vascular compression during systole ISO group, Hemodynamic parameters like coronary flow, +dp/dt max and -dp/dt min were significantly (P<0.001) decreased as well as LVEDP was significantly elevated as compare to control group. In treatment control, all group was significantly increased coronary flow (P<0.001) as well as +dp/dt max and -dp/dt min (P<0.05, P<0.01 and P<0.001) as compare to ISO group. Moreover, treatments control all group restored LVEDP was significantly changes as compare to ISO group.

Groups	Coronary flow (ml)	LVEDP (mmHg)	+dp/dt max (mmHg/s)	-dp/dt min (mmHg/s)
Control	19.15 ± 0.10	6.26 ± 0.27	3048.57 ± 196.46	2273.5 ± 178.66

ISO	7.33 ± 0.84$^{###}$	15.14 ± 0.77$^{###}$	1752.67 ± 157.28$^{###}$	1032 ± 130.43$^{###}$
2-APB	16.41 ± 0.49***	10.88 ± 0.51***	2801.83 ± 134.43***	1745.17 ± 142.70*
EU	14.28 ± 0.64***	12.03 ± 0.83**	2595.83 ± 235.35**	1642.67 ± 150.85*
PBEA	15.86 ± 0.44***	9.86 ± 0.63***	2746 ± 140.74**	1847 ± 169.14**
RCHA	14.96 ± 0.54***	12.6 ± 0.31*	2484.5 ± 154.27*	1683.33 ± 133.40*

Table 8: Effect of drug on hemodynamic parameters in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ###P<0.001 control vs.ISO; *P<0.05 treatments vs ISO; **P<0.01 treatments vs ISO; ***P<0.001 treatments vs ISO.

4.12.5. Effect on Biochemical parameter in isoproterenol induced myocardial infarction

Myocardium contains plentiful concentrations of diagnostic markers of myocardial infarction and once metabolically damaged, it releases its contents into the extracellular fluid. Of all the macromolecules that leak from the damaged tissue, enzymes because of their tissue specificity and catalytic activity are the best markers of tissue damage. ISO group, biochemical parameters (Ck-MB, LDH, sodium, calcium, TNF-α and IL-6) significantly (P<0.001) increased as well as potassium level was significantly (P<0.001) decreased as compare to control group. The beneficial effect on cardiac marker enzymes, Electrolytes and cytokine levels were observed in treatment control's all groups.

Parameters	Control	ISO	2-APB	EU	PBEA	RCHA
Ck-MB	103.0 ± 8.32	246.3± 14.31$^{###}$	167.8 ±25.10**	133.3±7.524***	146.3±9.241***	168.9±19.67**
LDH	490.7±36.13	1260±82.15$^{##}$$^{#}$	864.4±36.17***	781.0±68.39***	835.8±62.04***	961.0±45.05**
Calcium	9.76±0.38	13.49±0.54$^{###}$	10.46±0.24***	10.20±0.22***	10.29±0.34***	11.02±0.46***
Potassium	9.54±0.75	3.73±0.66$^{###}$	9.79±0.60***	9.05±0.77***	9.16±0.63***	7.86±1.23**
Sodium	148.4±4.46$^{##}$$^{#}$	196.9±5.65***	148.4±8.38***	132.1±7.90***	137.3±8.61***	141.0±9.72***
TNF-α	424.2±38.05	1291±48.13$^{##}$$^{#}$	635.0±47.92***	695.2±21.71***	713.7±18.28***	569.7±37.87***
IL-6	91.50±11.43	272.7±19.23$^{##}$$^{#}$	205.2±24.34*	169.2±17.64***	191.0±9.74**	159.2±8.69***

Table 9: Effect of drug on Biochemical parameter in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with

###P<0.001 control vs.ISO; *P<0.05 treatments vs ISO; **P<0.01 treatments vs ISO; ***P<0.001 treatments vs ISO.

4.12.6. Effect on gravimetric analysis in isoproterenol induced myocardial infarction

ISO treated animal's heart weight was significantly increased and body weight significantly decreased as compare to control group animals. So, the ratio of heart weight by body weight was significantly increased in ISO groups. Increase in heart weight might be attributed to increased water content, edematous intramuscular space and increased protein content. In treated animals the ratio of heart weight by body weight was significantly decreased as compare to ISO groups. Increase in heart weight might be attributed to increased water content, edematous intramuscular space and increased protein content.

Groups	Heart weight	Body weight	Heart weight/ Body weight
Control	0.89 ± 0.00	345.17 ± 18.21	0.0026 ± 0.00
ISO	1.09 ± 0.03$^{\#\#}$	266.17 ± 3.05$^{\#\#\#}$	0.0041 ± 0.00$^{\#\#\#}$
2-APB	0.95 ± 0.02*	328.33 ± 17.35**	0.0030 ± 0.00***
EU	0.95 ± 0.03*	319.67 ± 5.73*	0.0030 ± 0.00***
PBEA	0.94 ± 0.03*	313.67 ± 8.23*	0.0030 ± 0.00***
RCHA	0.95 ± 0.04*	323.33 ± 13.22*	0.0030 ± 0.00***

Table 10: Effect of drug on gravimetric analysis in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ##P<0.01 control vs.ISO; ###P<0.001 control vs.ISO; *P<0.05 treatments vs ISO; **P<0.01 treatments vs ISO; ***P<0.001 treatments vs ISO.

4.12.7. Effect on antioxidant parameters in isoproterenol induced myocardial infarction

Free radical scavenging enzymes such as SOD, catalase and GPx are the first line of cellular defense system against oxidative stress, eliminating reactive oxygen radicals such as superoxide and hydrogen peroxide and preventing the formation of more deteriorating hydroxyl radicals. The equilibrium between antioxidants and free radicals is an important process for the effective removal of oxidative stress in intracellular organelles. Isoproterenol auto-oxidation leads to generation of enormous amounts of reactive oxygen species. These reactive oxygen species may attack any type of molecules, but their main target appears to be polyunsaturated fatty acids (PUFAs) within membranes forming peroxyl radicals. These radicals then attack adjacent fatty acids within membranes causing a chain reaction of lipid peroxidation. ISO group, lipid peroxidation was significantly (P<0.001) increased and endogenous antioxidants were significantly (P<0.001) decreased as compare to control groups. In treatment control, all groups showed significant effect on oxidative insults against ISO induced myocardial infarction. This finding can be correlated with free radical scavenging activity and intra cellular ROS inhibition.

Groups	MDA (nmoles/mg proteins)	GSH (nmoles/mg proteins)	GPx (U/mg proteins)	GST (nmoles of CDNB consumed/ min/mg proteins	SOD (U/mg proteins)	CAT (U/mg proteins)
Control	0.82 ± 0.11	26.15 ± 1.34	7.95 ± 0.41	378.67 ± 52.04	9.71 ± 1.23	24.75 ± 3.04
ISO	5.17 ± 0.25$^{###}$	12.60 ± 1.64$^{###}$	4.29 ± 0.50$^{###}$	112.96 ± 24.75$^{###}$	3.77 ± 0.48$^{###}$	10.80 ± 0.55$^{###}$
2-APB	2.65 ± 0.27***	20.54 ± 2.58*	5.91 ± 0.26*	219.08 ± 36.11	7.29 ± 0.58*	18.06 ± 1.00*
EU	3.81 ± 0.22**	21.09 ± 2.02*	6.19 ± 0.33*	249.19 ± 32.78*	7.61 ± 0.49**	23.11 ± 2.22***
PBEA	2.49 ± 0.30***	23.50 ± 2.17**	6.17 ± 0.19**	308.77 ± 23.46**	9.67 ± 0.98***	21.47 ± 1.79**
RCHA	2.92 ± 0.28***	25.11 ± 1.65***	7.23 ± 0.36***	339.19 ± 40.28***	8.27 ± 0.76**	19.47 ± 0.88**

Table 11: Effect of drug on antioxidant parameters in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ###P<0.001 control vs.ISO; *P<0.05 treatments vs ISO; **P<0.01 treatments vs ISO; ***P<0.001 treatments vs ISO.

4.12.8. Effect on membrane bounded enzymes in isoproterenol induced myocardial infarction

ATPases are intimately associated with the plasma membrane and participates in the energy requiring translocation of sodium, potassium, calcium and magnesium. ISO treated animals, Na+/K+ ATPase and Mg++ ATPase activity were significantly decreased as well as Ca++ ATPase activity was significantly increased as compare to control group. In treatment control, all groups showed significant changes in Na+/K+ ATPase and Ca++ ATPase but Mg++ ATPase activity was significantly (P<0.05) increased in only PBEA group, which may be attributed to the direct antioxidant activity and antioxidant enzyme stimulatory effect of drug which thereby protects the –SH group from oxidative damage.

Groups	Na^{+}/K^{+} ATPase	Ca^{++} ATPase	Mg^{++} ATPase
Control	5.16 ± 0.88	2.64 ± 0.30	2.91 ± 0.30
ISO	2.33 ± 0.25$^{##}$	4.64 ± 0.58$^{###}$	1.36 ± 0.17$^{##}$
2-APB	4.49 ± 0.51*	2.62 ± 0.08***	1.69 ± 0.28
EU	4.31 ± 0.37*	3.04 ± 0.30**	1.93 ± 0.23
PBEA	4.98 ± 0.22**	2.92 ± 0.11**	2.43 ± 0.30*
RCHA	4.67 ± 0.55*	2.74 ± 0.35**	1.70 ± 0.30

Table 12: Effect of drug on membrane bounded enzymes in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ##P<0.01 control vs.ISO; ###P<0.001 control vs.ISO; *P<0.05 treatments vs ISO; **P<0.01 treatments vs ISO; ***P<0.001 treatments vs ISO.

4.12.9. Effect on myeloperoxidase enzyme in isoproterenol induced myocardial infarction

Myeloperoxidase, an enzyme that is associated with the azurophilic granules of neutrophils, has been measured to form an index of the infiltration of neutrophils into inflamed tissue. Animals treated with isoproterenol significantly (P<0.001) increased myeloperoxidase enzyme because of inflammatory markers are increased in myocardial infarction. This enzyme was significantly (P<0.05 and P<0.001) decreased in treatment control groups.

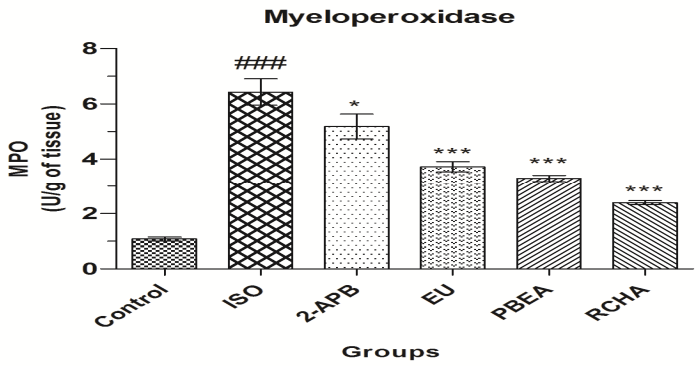

Figure 13: Effect of drug on myeloperoxidase enzyme in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ###P<0.001 control vs.ISO; *P<0.05 treatments vs ISO; ***P<0.001 treatments vs ISO.

4.12.10. Effect on expression of STIM1 and Orai1 in isoproterenol induced myocardial infarction

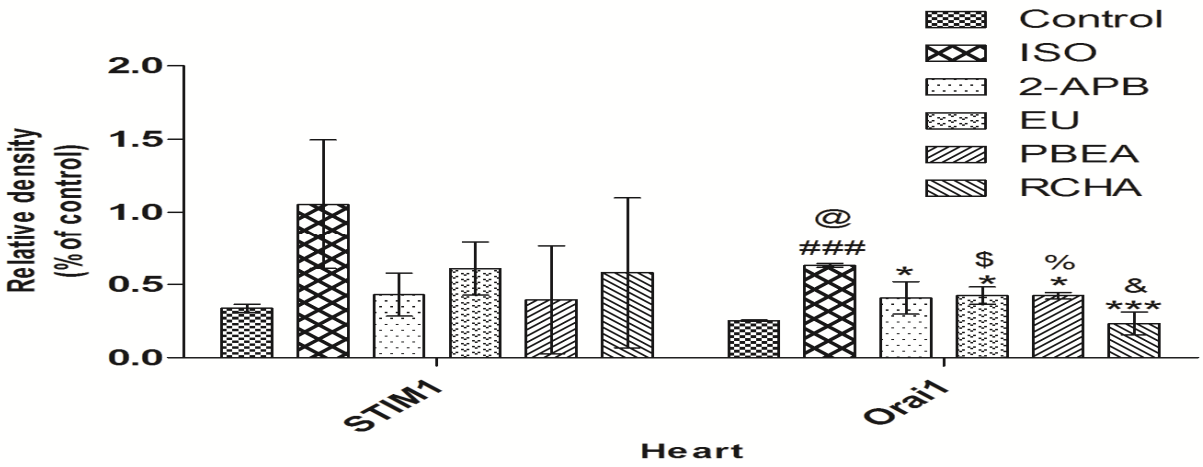

Figure 14: Effect on expression of STIM1 and Orai1 in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 3 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test and one tailed unpaired t test. Significant values were compared with ###P<0.001 control vs. ISO; *P<0.05 treatment vs ISO; ***P<0.001 treatment vs ISO; @P<0.001 Control vs ISO; $P<0.05 ISO vs Eugenol; %P<0.05 ISO vs PBEA; &P<0.05 ISO vs RCHA.

4.12.11. Effect on infract size in isoproterenol induced myocardial infarction

Area of infarction indicates loss of membrane integrity which might be due to leakage of lactate dehydrogenase enzymes and increase in ROS production lead to an enlarged infarct size. Isoproterenol treated rats showed 43.35% infract size. In treatment control, 2-APB and PBEA groups showed 15.28% and 21.36% infract size as well as EU and RCHA groups showed 30.40% and 32.85% infract size as compare to ISO injected rats.

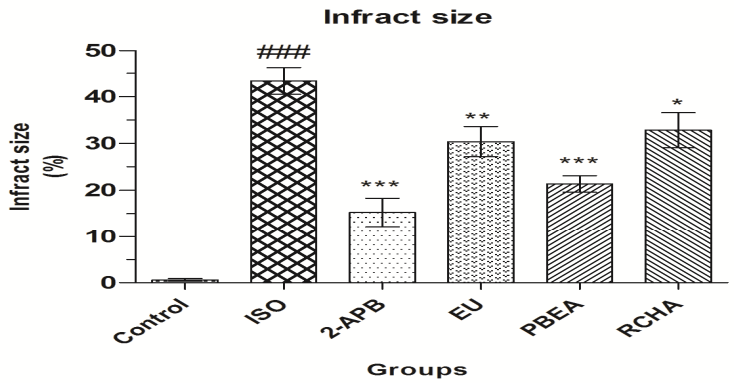

Figure 15: Effect of drug on infract size in isoproterenol induced myocardial infarction

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's post hoc test. Significant values were compared with ###P<0.001 control vs.ISO; *P<0.05 treatments vs ISO; **P<0.01 treatments vs ISO ***P<0.001 treatments vs ISO.

4.12.12. Effect on Histopathology in isoproterenol induced myocardial infarction

The control heart exhibited clear integrity of myocardial membrane and showed normal cardiac fibers without any infarction and infiltration of inflammatory cells. Enormous changes are seen in heart of model control rats. Isoproterenol injected rats showed widespread myocardial structure disorder and subendocardial necrosis with capillary dilatation and leukocyte infiltration. After treatment with 2-APB, PBEA and RCHA, normal cardiac fibers with no signs of infarction can be seen and the neutrophil infiltration was not observed. Treatment with EU showed less infracted cardiac fiber with capillary dilation but the neutrophil infiltration was not observed.

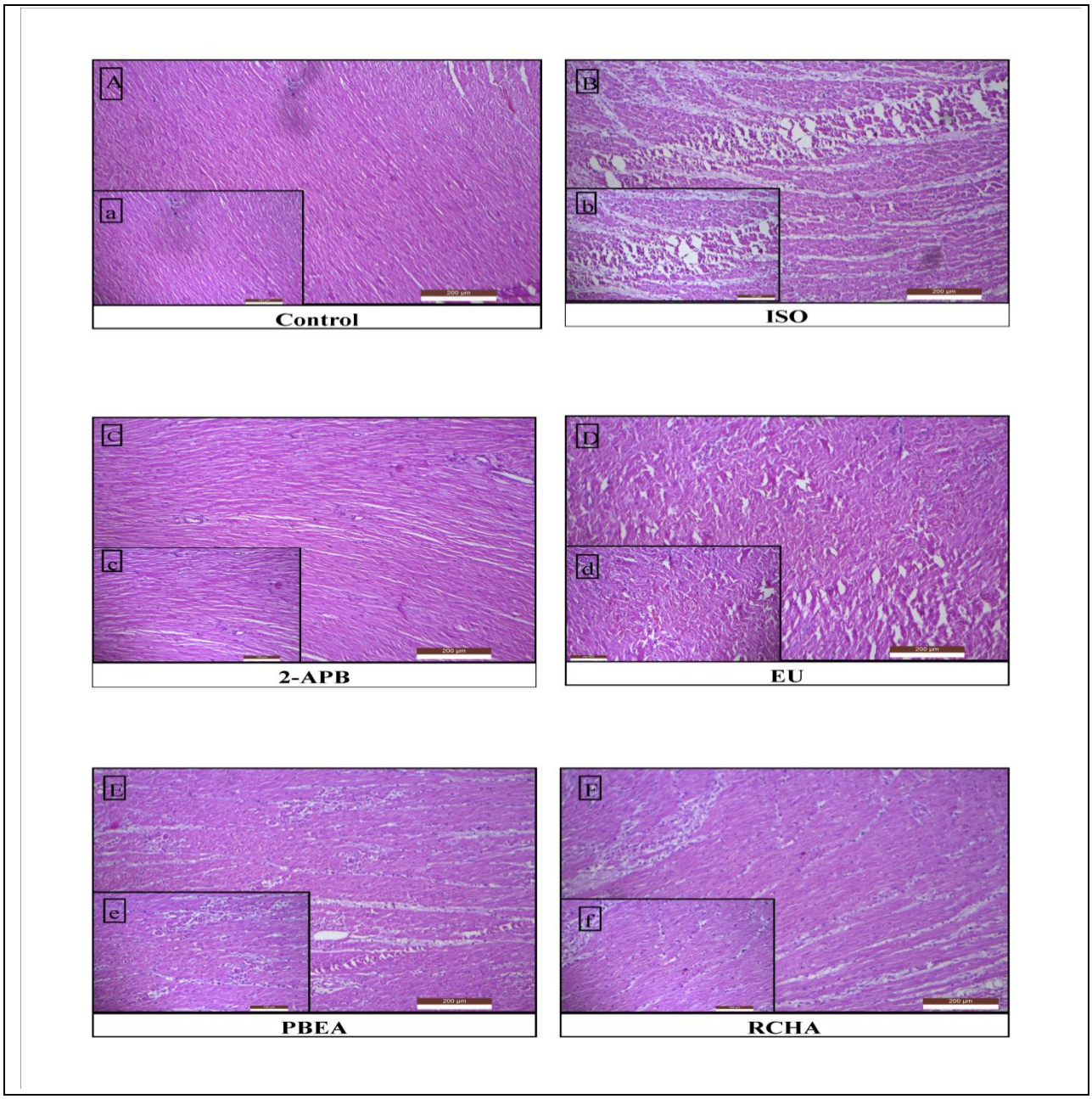

Figure 16: Effect of drug on histopathology of heart in ISO induced myocardial infarction

a) Normal heart: normal structure of cardiac fiber and clear integrity of myocardial membrane; b) model control heart: necrosis with capillary dilatation and leukocyte infiltration; c) 2-APB treated heart: no signs of infarction and no neutrophil infiltration; d) EU treated heart: less infracted cardiac fiber with capillary dilation. e) PBEA treated heart: normal cardiac fibers f) RCHA treated heart: clear integrity of myocardial membrane. Magnification: 10 and 20x

4.13. Development of RP-HPLC method for 2-APB

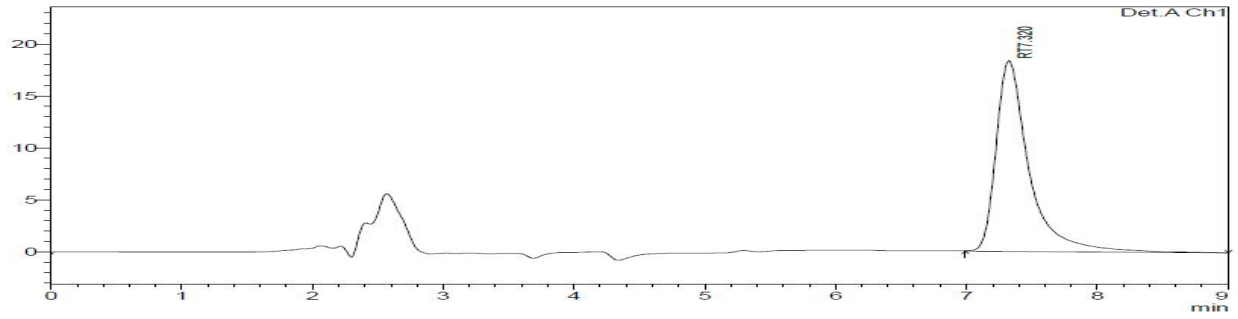

Figure 17: RP-HPLC Chromatogram of 2-APB

The calibration curve was found linear in the concentration range of 0.05 to 10µg/ml. The linearity regression equation was Y= 32528x+9.417 with correlation coefficient (R^2) = 1 that indicate linearity of the plot.

Drug concentration (PPM)	Mean of Area	RSD	%RSD
0.05	1636.49	27.298108	1.6680889
0.1	3249.6467	63.567917	1.9561486
0.5	16261.567	303.46417	1.8661435
1	32563.133	578.58275	1.7768031
2	65092.933	1179.1404	1.8114722
4	130085.87	2433.008	1.8703093
6	195198.8	3595.302	1.8418668
8	260205.07	4839.7058	1.8599583
10	325314.67	6004.744	1.8458264

Table 13: Data of calibration curve of 2-APB at 230 nm λ_{max}

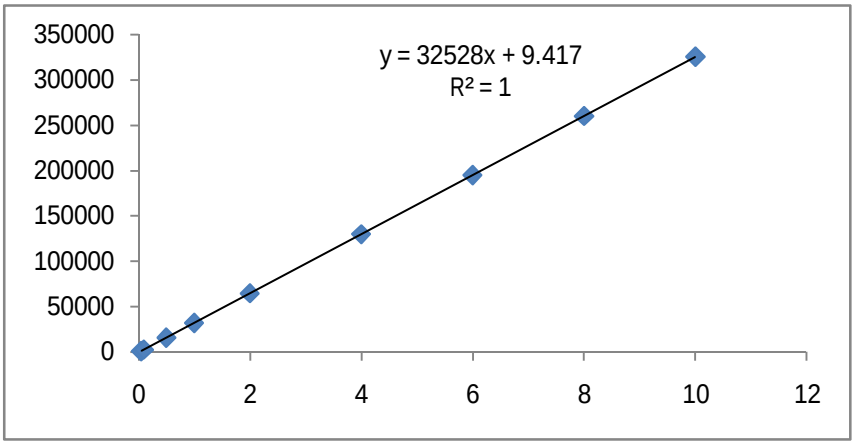

Figure 18: Calibration plot of 2-APB

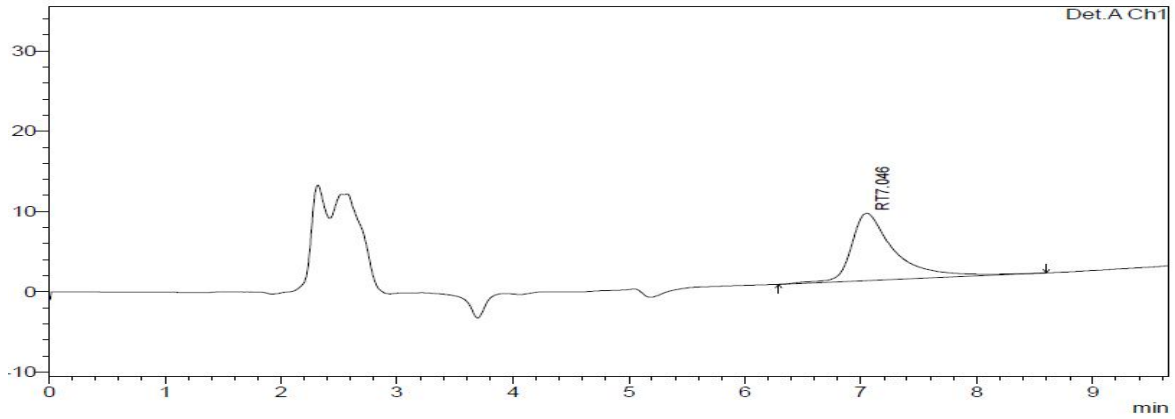

Figure 19: RP-HPLC chromatogram of bioanalytical Method of 2-APB

The calibration curve of bioanalytical method was found linear in the concentration range of 0.05 to 10µg/ml. The linearity regression equation was Y= 30012x-9.55 with correlation coefficient (R^2) = 1 that indicate linearity of the plot.

Drug concentration (PPM) in Plasma	Mean of Area	RSD	%RSD
0.05	1424.851	17.63119	1.237407
0.1	2926.169	64.68038	2.210412
0.5	14957.16	328.7839	2.19817
1	29950.76	603.1653	2.013857
2	60042.94	1006.776	1.676759
4	120738.5	2024.494	1.676759
6	179470.5	3866.501	2.154393
8	240124.5	1643.333	0.684367
10	300155.6	2054.167	0.684367

Table 14: Data of bioanalytical method calibration curve of 2-APB at 230 nm λ_{max}

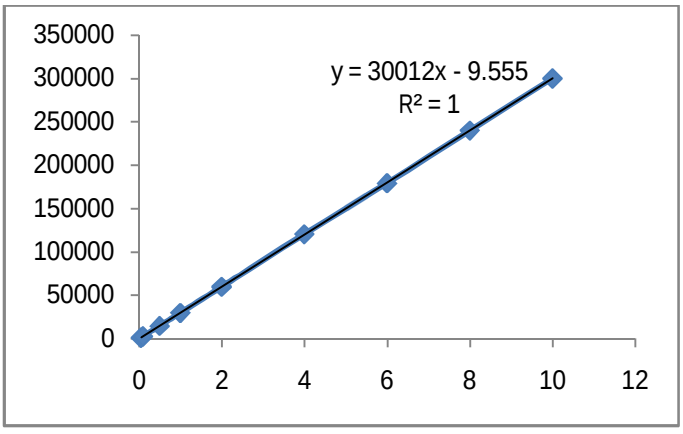

Figure 20: Calibration plot of bioanalytical method of 2-APB

4.14. Pharmacokinetic study of 2-APB

Pharmacokinetic study of 2-APB was performed using previous mentioned bioanalytical method and different time point containing drug concentration was found. Pharmacokinetic parameter was evaluated using kinetica software.

Parameters	Value
C_{max}	0.5266 µg/ml
T_{max}	0.08 h
AUC_{Total}	2323.51 µg/l*h
$t_{1/2}$	8.92 h
MRT	10.45 h

Table 15:Pharmacokinetic parameters of 2-APB

4.15. Toxicity Study of PBEA and RCHA by OECD 423

Changes in body weights are a clear indicative of damage caused by the substance test while the hippocratic screening provides a general estimate of pharmacological and toxicological nature. After the acute toxicity test, the dose of 300 and 2000 mg/kg of PEBA did not cause the death of any animal. The dose of 300 mg/kg of RCHA did not cause the death of any animal but 2000 mg/kg of RCHA did cause the death of 2 animals. The female rats exposed both dose of PEBA presented no behavioral changes during the treatment period, as well as no changes in water and food consumption and ponderal evolution, in relation to the control group. 300 mg/kg of RCHA results was same as PEBA but 2000mg/kg of RCHA animal presented lethargy and bradycardia. No abnormality was found in the organs at necropsy in PEBA both groups and RCHA 300mg/kg group but abnormality was found in heart and kidney of RCHA 2000mg/kg group (Table 16). In this study, it was observed that the lethal oral toxicity of PEBA and RCHA was estimated to be higher than 5000 mg/kg and 2000mg/kg, classified as category 5 or unclassified and category 5 respectively, according to OECD Guide 423, indicating a certain safety margin associated with the use of PBEA and RCHA as therapeutic agent.

Parameter (Female)	PBEA		RCHA	
	300 mg/kg	2000 mg/kg	300 mg/kg	2000 mg/kg
Initial Body weight (g)	178.66 ± 1.45	181.16 ± 2.66	174.66 ± 4.55	163.5 ± 2.49
Final Body weight (g)	204.33 ± 2.65	207.16 ± 4.69	200.5 ± 3.78	189 ± 3.33
Body weight gain (%)	14.37 ± 1.19	14.29 ± 0.93	14.90 ± 0.86	10.89 ± 3.72
Food intake (g)	102.28 ± 7.83	99.35 ± 8.26	107.92 ± 4.36	93 ± 10.86
Water intake (ml)	174.57 ± 12.61	186.42 ± 13.04	172.85 ± 13.82	151.92 ± 12.79
Necropsy	No abnormality	No abnormality	No abnormality	Abnormality was found in heart

				and kidney.

Table 16: Body weight gain and food and water consumption of rats treated orally with PBEA and RCHA

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using unpaired t- test.

4.16. Toxicity study of PEBA by OECD 407

Toxicological evaluations after repeated exposures are required by regulatory agencies to characterize the toxicological profile of any substance. In the present study, after subacute exposure, the animals were active and responsive to stimuli, with no clinical signs that could be associated with local or systemic toxic effects observed. There were no deaths and the behavior of animals remained normal for the species. However, the consumption of water and food for the both geneder treated with PBEA, at all doses, not significantly differ to the control group. Similarly, the % body weight gain and relative weights of all organs examined did not vary significantly among groups (Table 17), corroborating the hypothesis of low toxicity of the oil after sub-acute exposure.

Parameter (Female)	**Control**	**50 mg/kg**	**200 mg/kg**	**800 mg/kg**	**S-control**	**Satellite**
Initial Body weight (g)	191.6 ± 3.85	175.2 ± 2.27***	176.2 ± 1.85**	177.6 ± 2.54**	1.74 ± 3.31	176.4 ± 1.60
Final Body weight (g)	244.4 ± 6.28	228 ± 4.30	227.4 ± 5.28	224.4 ± 8.02*	228.6 ± 2.46	226.4 ± 2.34
Body weight gain (%)	27.76 ± 4.28	30.22 ± 2.97	29.15 ± 3.60	26.33 ± 3.93	31.17 ± 1.36	28.34 ± 0.64
Food intake (g)	100.10 ± 5.11	90.85 ± 9.44	90.17 ± 8.52	87.71 ± 7.96	104.83 ± 5.00	94.28 ± 6.15
Water intake (ml)	164.71 ± 8.20	151.92 ± 9.48	147.64 ± 9.54	139.57 ± 10.45	154.71 ± 7.68	144.21 ± 8.13
Parameter (Male)	**Control**	**50 mg/kg**	**200 mg/kg**	**800 mg/kg**	**S-control**	**Satellite**
Initial Body weight (g)	245 ± 6.10	250.6 ± 3.80	247.4 ± 2.79	247.2 ± 2.01	247.6 ± 4.45	249.6 ± 4.38
Final Body weight (g)	322.2 ± 14.47	321.0 ± 12.78	318.8 ± 8.14	312.0 ± 8.57	330.4 ± 2.73	328.4 ± 3.85
Body weight gain (%)	31.42 ± 4.29	28.28 ± 5.86	29.07 ± 4.69	26.31 ± 4.11	33.67 ± 3.23	31.81 ± 3.61
Food intake (g)	129.21 ± 8.94	125.64 ± 8.25	127.35 ± 8.13	118.75 ± 7.29	128.33 ± 7.14	120.71 ± 6.39

Water intake (ml)	210.67 ± 5.85	198.35 ± 11.37	201.71 ± 7.66	186.28 ± 7.68	208.76 ± 5.12	191.21 ± 9.11

Table 17:Body weight gain and food and water consumption of rats treated orally with PBEA.

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using Bonferroni's Multiple Comparison Post hoc test. Significant values were compared with *P<0.05 PBEA vs control; **P<0.01 PBEA vs control; ***P<0.001 PBEA vs control.

Organ weight (g)/100 g of body weight(Female)	**Control**	**50 mg/kg**	**200 mg/kg**	**800 mg/kg**	**S-control**	**Satellite**
Liver	3.86 ± 0.11	3.95 ± 0.04	3.90 ± 0.05	3.84 ± 0.04	4.00 ± 0.04	3.79 ± 0.08
Kidney	0.37 ± 0.01	0.38 ± 0.02	0.39 ± 0.01	0.38 ± 0.00	0.38 ± 0.00	0.38 ± 0.01
Heart	0.36 ± 0.01	0.38 ± 0.01	0.37 ± 0.01	0.35 ± 0.01	0.37 ± 0.00	0.34 ± 0.02
Brain	0.61 ± 0.03	0.64 ± 0.02	0.60 ± 0.02	0.60 ± 0.03	0.65 ± 0.03	0.62 ± 0.04
Spleen	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.00	0.18 ± 0.00
Lung	0.52 ± 0.03	0.55 ± 0.04	0.53 ± 0.02	0.56 ± 0.01	0.54 ± 0.02	0.55 ± 0.02
Uterus	0.17 ± 0.00	0.16 ± 0.01	0.17 ± 0.00	0.17 ± 0.01	0.18 ± 0.00	0.17 ± 0.01
Organ weight (g) /100 g of body weight(Male)	**Control**	**50 mg/kg**	**200 mg/kg**	**800 mg/kg**	**S-control**	**Satellite**
Liver	3.92 ± 0.16	4.09 ± 0.07	3.82 ± 0.17	4.14 ± 0.25	3.98 ± 0.10	3.91 ± 0.18
Kidney	0.39 ± 0.02	0.39 ± 0.03	0.38 ± 0.01	0.40 ± 0.02	0.38 ± 0.01	0.38 ± 0.01
Heart	0.40 ± 0.02	0.41± 0.02	0.42 ± 0.01	0.41 ± 0.02	0.40 ± 0.01	0.41 ± 0.01
Brain	0.62 ± 0.02	0.64 ± 0.03	0.61 ± 0.01	0.63 ± 0.02	0.60 ± 0.00	0.60 ± 0.01
Spleen	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.02	0.15 ± 0.01	0.15 ± 0.00	0.14 ± 0.01
Lung	0.46 ± 0.01	0.45 ± 0.02	0.47 ± 0.02	0.48 ± 0.03	0.45 ± 0.01	0.45 ± 0.02
Testis	0.45 ± 0.02	0.46 ± 0.02	0.45 ± 0.02	0.45 ± 0.02	0.45 ± 0.01	0.42 ± 0.01

Table 18: Relative organ weight (g/100 g of body weight) of rats treated orally with PBEA

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using Bonferroni's Multiple Comparison Post hoc test.

The hematopoietic system is one of the most susceptible targets to toxic substances and is an important parameter for assessing the physiological and pathological status in humans and animals. Although, WBC showed a statistical difference in the groups treated with 800 mg/kg PBEA, the other hematological parameters were similar among groups (Table 19). The observed variations are not biologically meaningful, since the values are within the normal range for the species, indicating that the PBEA provided no adverse effects on circulating blood cells or on their production.

Hematological Parameter (Female)	Control	50 mg/kg	200 mg/kg	800 mg/kg	S-control	Satellite
Hemoglobin (g/dl)	12.92 ± 0.43	14.02 ± 0.49	12.74 ± 0.53	13.06 ± 0.82	13.00 ± 0.54	13.46 ± 0.72
Hematocrit(%)	38.52 ± 1.20	41.58 ± 1.52	37.58 ± 1.59	38.86 ± 2.36	39.32 ± 1.12	39.86 ± 2.29
RBC(10^6 /µL)	6.73 ± 0.06	7.15 ± 0.14	6.76 ± 0.20	7.08 ± 0.30	6.55 ± 0.18	6. 46 ± 0.38
WBC (103 /µL)	4.53 ± 0.43	5.91 ± 0.32	6.09 ± 0.55	6.72 ± 0.42**	4.54 ± 0.29	5.33 ± 0.55
Neutrophils (%)	23.98 ± 2.57	25.64 ± 2.78	22.52 ± 1.12	25.12 ± 2.67	23.00 ± 0.71	22.58 ± 0.53
Lymphocytes (%)	71.74 ± 2.40	70.57 ± 2.70	73.14 ± 1.17	71.25 ± 2.67	73.06 ± 0.89	73.67 ± 0.61
Eosinophils (%)	1.15 ± 0.29	1.04 ± 0.25	1.18 ± 0.13	0.99 ± 0.20	1.09 ± 0.11	1.03 ± 0.02
Monocytes (%)	3.08 ± 0.38	2.66 ± 0.40	3.08 ± 0.37	2.60 ± 0.29	2.78 ± 0.32	2.68 ± 0.14
Basophils (%)	0.04 ± 0.02	0.08 ± 0.03	0.08 ± 0.03	0.04 ± 0.02	0.06 ± 0.01	0.061 ± 0.01
Plateletes (10^3 /µL)	745.40 ± 41.34	768.20 ± 46.11	789.40 ± 50.79	787.60 ± 59.16	875.00 ± 33.22	854.2 ± 57.22
Hematological Parameter (Male)	**Control**	**50 mg/kg**	**200 mg/kg**	**800 mg/kg**	**S-control**	**Satellite**
Hemoglobin (g/dl)	15.20 ± 0.50	15.08 ± 0.41	14.56 ± 0.91	14.12 ± 0.43	14.5 ± 0.51	14.8 ± 0.5
Hematocrit(%)	45.66 ± 1.18	44.00 ± 1.24	43.08 ± 2.57	42.18 ± 1.12	42.26 ± 1.34	43.92 ± 1.67
RBC(10^6 /µL)	8.15 ± 0.16	7.81 ± 0.17	7.54 ± 0.44	7.35 ± 0.17	7.32 ± 0.14	7.28 ± 0.23
WBC (103 /µL)	5.20 ± 0.50	5.18 ± 0.51	5.21 ± 0.73	6.23 ± 0.44	6.51 ± 0.67	6.35 ± 0.31
Neutrophils (%)	20.99 ± 1.07	18.96 ± 2.11	19.46 ± 1.38	19.74 ± 2.37	19.32 ± 2.18	17.85 ± 1.25
Lymphocytes (%)	75.94 ± 1.03	78.30 ± 2.16	76.88 ± 1.84	77.82 ± 2.08	77.85 ± 1.62	78.72 ± 2.19
Eosinophils (%)	1.23 ± 0.40	1.07 ± 0.22	1.61 ± 0.43	0.91 ± 0.27	1.15 ± 0.24	1.63 ± 0.24
Monocytes (%)	1.78 ± 0.25	1.63 ± 0.25	2.03 ± 0.24	1.50 ± 0.32	1.60 ± 0.24	1.73 ± 0.20
Basophils (%)	0.06 ± 0.02	0.05 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.06 ± 0.018	0.06 ± 0.02
Plateletes (10^3	809.60 ±	865.40 ±	910.60 ±	861.80 ±	860.4 ±	903.4 ±

/µL)	60.04	66.49	20.49	38.30	34.00	23.24

Table 19: Hematological parameters of rats treated orally with PBEA

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using Bonferroni's Multiple Comparison Post hoc test. Significant values were compared with **P<0.01 PBEA vs control.

In biochemical study, there was no statistical difference in liver or renal parameters between the treated and control groups (Table 20).

Biochemical Parameter (Female)	Control	50 mg/kg	200 mg/kg	800 mg/kg	S-control	Satellite
AST (U/L)	97.24 ± 7.48	85.57 ± 12.95	107.49 ± 19.94	105.02 ± 18.50	93.70 ± 7.05	96.18 ± 12.31
ALT(U/L)	28.29 ± 3.83	22.63 ± 4.45	20.51 ± 2.28	37.13 ± 2.31	31.82 ± 4.58	29.70 ± 11.0
Total Protein (g/dl)	6.42 ± 0.31	6.38 ± 0.23	6.00 ± 0.11	6.25 ± 0.30	6.64 ± 0.15	6.53 ± 0.10
Albumin (g/dl)	4.37 ± 0.17	4.35 ± 0.18	4.04 ± 0.08	4.32 ± 0.19	4.49 ± 0.12	4.56 ± 0.21
Globulin (g/dl)	2.06 ± 0.21	2.03 ± 0.12	1.96 ± 0.12	1.93 ± 0.14	2.16 ± 0.04	1.97 ± 0.15
Albumin/globulin Ratio	2.21 ± 0.21	2.17 ± 0.17	2.09 ± 0.15	2.27 ± 0.13	2.08 ± 0.05	2.41 ± 0.29
Total Bilirubin (mg/dl)	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.12 ± 0.02	0.11 ± 0.00	0.10 ± 0.00
Direct Billirubin (mg/dl)	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
Indirect Billirubin (mg/dl)	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.06 ± 0.00	0.05 ± 0.01
Blood Urea Nitrogen (mg/dl)	19.89 ± 0.88	20.29 ± 2.51	22.30 ± 1.44	20.29 ± 1.90	17.22 ± 1.39	20.03 ± 2.82
Total Cholesterol (mg/dl)	80.41 ± 4.90	83.12 ± 4.67	76.75 ± 2.43	81.63 ± 2.59	64.27 ± 2.53	66.31 ± 3.09
Creatinine (mg/dl)	0.37 ± 0.09	0.42 ± 0.09	0.34 ± 0.07	0.36 ± 0.07	0.35 ± 0.02	0.36 ± 0.05
Sodium (mmol/l)	147.23 ± 1.91	145.06 ± 3.45	145.14 ± 3.76	147.92 ± 6.03	149.83 ± 1.02	144.19 ± 3.07
Potassium (mmol/l)	5.88 ± 0.73	5.62 ± 0.61	5.85 ± 0.99	5.85 ± 0.53	5.81 ± 0.46	5.09 ± 0.36
Calcium (mg/dl)	11.29 ± 0.87	9.59 ± 0.48	9.02 ± 0.46	8.80 ± 0.30*	11.76 ± 0.65	9.75 ± 0.68

Biochemical Parameter	Control	50 mg/kg	200 mg/kg	800 mg/kg	S-control	Satellite

(Male)						
AST (U/L)	90.17 ± 13.10	97.59 ± 9.61	102.90 ± 8.24	102.19 ± 11.16	100.78 ± 8.27	110.32 ± 14.67
ALT(U/L)	30.76 ± 4.05	29.70 ± 3.42	27.58 ± 4.13	30.06 ± 2.31	35.36 ± 7.23	33.24 ± 10.60
Total Protein (g/dl)	6.25 ± 0.15	6.32 ± 0.11	5.94 ± 0.09	6.39 ± 0.13	6.52 ± 0.18	6.46 ± 0.13
Albumin (g/dl)	4.21 ± 0.06	4.14 ± 0.06	3.91 ± 0.03	4.20 ± 0.10	4.30 ± 0.18	4.40 ± 0.11
Globulin (g/dl)	2.05 ± 0.20	2.18 ± 0.09	2.03 ± 0.12	2.19 ± 0.19	2.23 ± 0.07	2.06 ± 0.13
Albumin/globulin Ratio	2.15 ± 0.23	1.91 ± 0.09	1.95 ± 0.11	1.99 ± 0.21	1.94 ± 0.12	2.18 ± 0.18
Total Bilirubin (mg/dl)	0.10 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	0.12 ± 0.02	0.10 ± 0.01	0.09 ± 0.01
Direct Billirubin (mg/dl)	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
Indirect Billirubin (mg/dl)	0.06 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	0.04 ± 0.01
Blood Urea Nitrogen (mg/dl)	17.10 ± 0.68	17.84 ± 0.78	19.33 ± 0.55	18.27 ± 0.27	18.56 ± 1.71	21.36 ± 2.74
Total Cholesterol (mg/dl)	77.30 ± 4.41	79.86 ± 3.28	80.28 ± 2.54	85.39 ± 2.71	72.41 ± 3.78	76.34 ± 2.36
Creatinine (mg/dl)	0.29 ± 0.08	0.39 ± 0.06	0.41 ± 0.09	0.43 ± 0.07	0.35 ± 0.06	0.43 ± 0.09
Sodium (mmol/l)	148.27 ± 2.91	143.32 ± 3.18	146.88 ± 4.06	143.58 ± 5.81	142.89 ± 3.84	147.66 ± 4.66
Potassium (mmol/l)	4.65 ± 0.38	4.45 ± 0.23	4.75 ± 0.38	4.54 ± 0.27	4.98 ± 0.50	4.71 ± 0.36
Calcium (mg/dl)	11.01 ± 0.61	9.87 ± 0.79	10.60 ± 0.28	10.17 ± 0.71	10.80 ± 0.77	9.08 ± 0.79

Table 20: Biochemical parameters of rats treated orally with PBEA

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using Bonferroni's Multiple Comparison Post hoc test.

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