7. DISCUSSION

Calcium plays a number of critically important roles in physiology and pathology. Circulating calcium levels directly promote vascular calcification.[211] Different type of calcium channel shows selective permeability to calcium ions. Out of this calcium channel, one non-voltage gated Store operated Calcium entry (SOCE) channel is activated through increased intracellular calcium concentration. Role of the SOCE in cardiovascular disorders are less clear-cut. Numerous calcium channel blocker are available for the treatment of cardiovascular disease with their side effect.

Herbs have been used as medical treatments since the beginning of civilization and some derivatives (eg, aspirin, reserpine, and digitalis) have become backbones of human pharmacotherapy.[212] A number of plant extract and phytochemicals have calcium channel blocking activity (e.g, Panax ginseng root extract, Ammi visnaga, Foeniculum vulgare., Apigenin, Dicumarol, Ostruthin) which have been beneficial in cardiovascular disorders through preventive action and as add on therapy. The multi-faceted role of the phytochemicals is mediated by its structure-function relationship and can be considered as leads for cardiovascular drug design in future.[213] *Piper betle Lin.* and *Rubia cordifolia Lin.* have calcium channel blocking action and used in ischemia induced free radical generation, coronary artery disease and congestive heart failure. Both plants have strong antioxidant activity. In line with above notion leaves of *Piper betle* and roots of *Rubia cordifolia* were selected for the studying their efficacy in cardiovascular disease. Moreover, Piper betle ethyl acetate extract and Rubia cordifolia hydro alcoholic extract have been reported by Gillani et al, 2000 and 1994 for the presence of calcium channel blocking action in tissue experiment using rat aortic strip.[214-217]

All the plant material is usually put in seclusion store and therefore it remains for a long time. As the time of storage of plant material or crude drug, it is necessary to maintain proper ventilation, suitable temperature, humidity control and light to sustain their pharmacological action. Any alter action in storage condition may change its original characteristics. To assess the stability of storage condition, the crude drugs should be tested for the various tests as per WHO guideline. Thus, a proximate analysis of the

obtained sample of plant material was done in the current study and it was found to match the available standards.

The total phenolics, total flavonoids and gas chromatography data, affirmed that the antioxidant and free radical scavenging activity of PBEA and RCHA might be attributed to its major phenolics compound and flavonoids compound. In-vitro assays manifested that PBEA have high impregnable reducing power than the RCHA. PBEA and RCHA has high level of inhibition of β-carotene oxidation, strong high superoxide anion radical scavenging activities and also possessed scavenging activity of hydrogen peroxide and hydroxyl radicals at very low concentration.

Eugenol was selected in the present study because of it has calcium channel blocking activity, antioxidant and antithrombotic activity. In addition, *Piper betle* ethyl acetate extract's calcium channel blocking action is might be due to the eugenol. 2-APB is specific SOCE blocker with antioxidant activity and it could protect liver, kidney and ovary ischemia-reperfusion injury by reducing cellular and mitochondrial calcium uptake.

Dose of eugenol was selected on the experimental evidence of oral pre-cotreatment with eugenol is endowed with antithrombotic, anti-inflammatory and preventive properties against cardiac remodeling. But it was necessary to select the dose and time of administration of 2-APB. Hence, it was necessarily to perform a pharmacokinetic study of 2-APB in animals. For the same an analytical method development was required for determines 2-APB in animal biological sample was required.

Thus, simple, specific, accurate and precise RP-HPLC method was developed for estimation of 2-APB. The developed method was validated according to ICH guidelines. The value of %RSD for intra-day and inter-day precision was found less than 2. This value confirms that method is precise. 93 % Recovery for this method shows that the method is accurate. Pharmaco-kinetic study was performed in rat and plasma $t_{1/2}$ was found to be 8.92 h. On the basis of the pharmacokinetic data the time interval of 2-APB administration in ISO induced myocardial infarction was fixed after every 12 hr interval.

Thus, PBEA, RCHA, Eugenol and 2-APB were selected for the studying the pharmacological modulation of calcium in selected cardiovascular disorders and also evaluating their role in inhibiting store operated calcium channel.

Circulating calcium is a risk factor for vascular disease. Polymorphisms of the calcium-sensing receptor associated with small elevations of serum calcium are also associated with cardiovascular disease, suggesting that calcium plays a causative role.[211] Calcium deposition in the vasculature is a consistent feature of vascular disease and is predictive of adverse cardiovascular events. SOCE plays a fundamental role in providing the complex spatiotemporal Ca^{2+} signals mandatory for the regulation of various cellular functions such as vascular reactivity, myotubes differentiation, skeletal muscle fatigue and endurance, cardiac homeostasis and pacemaking.[218] SOCE also seems relevant for processes involved in muscle diseases such as cardiovascular remodeling and different myopathies.

Secondly, a mutual interplay between calcium and ROS signaling systems seems to have important implications for fine tuning cellular signaling networks. Interactions among ROS and calcium signaling can be considered as bidirectional, wherein ROS can regulate cellular calcium signaling, while calcium signaling is essential for ROS production.[219] Thus, increased levels of Ca^{2+} activate ROS-generating enzymes and formation of free radicals.

Thirdly, different antioxidant components are present as antioxidant defense systems and their antioxidant capacities depend upon which free radicals or oxidizers are generated in the body. Defense mechanisms against oxidative injury plays pivotal role in the cardiac cell injury and it is protected by enzymes (SOD, CAT and GP_x) and non-enzymes defenses (GSH, vitamins C, E and β-carotene).[220] Many reports have demonstrated that transient vulnerability of H_2O_2 is an experimental source of oxygen-derived free radicals and produced significant oxidative damage in heart.[221] The concomitant H_2O_2 induced oxidative stress caused a variety of cellular derangements, lethal sarcolemmal disruption and cardiomyocyte integrity that could be conserved by antioxidants.

In view of the above role of Ca^{+2}, SOCE and their interaction with antioxidant defense led us to first evaluate the protective role of the drugs under investigation PBEA, RCHA, Eugenol and 2-APB against oxidative stress. Studies were hence designated to evaluate cell viability of cardiac H9c2 cell line under presence and absence of H_2O_2 induced oxidative stress, intracellular ROS level, in-vitro antioxidant assay and apoptosis.

Direct assessment of intracellular ROS is a good indicator of the oxidative damage in living cells. Cell permeable 2,7-Dichlorofluorescein diacetate (DCFH) gets directly oxidize to fluorescent DCF by pro-oxidant. In present study, treatment with 2-APB, Eugenol, PBEA and RCHA on H9c2 cells reduced ROS generation in the percentages of 98.9%, 91.05%, 99.9% and 98.9%, respectively. Dose of 2-APB (150 and 300 µM), Eugenol (50 mM), PBEA (10 and 25 µg/ml) and RCHA (100 ng/ml) treatment were adequate to provide a significantly protective effect against H_2O_2 induced cytotoxicity in H9c2 cell. This outcome proves that treatment group (2-APB, Eugenol, PBEA and RCHA) contain natural antioxidants preventing or delaying complimentary oxidative insults through decrease the steady-state generation of ROS in H9c2 cells culture. In addition, sequestering ROS and/or maintaining the cell and cellular machinery in their appropriate redox state by antioxidant enzymes and their changeable activity can be measured as biomarkers of antioxidant response. In cellular antioxidant assay it was observed that pre-incubation of cells with 2-APB, Eugenol, PBEA and RCHA before H_2O_2 exposure increased the cell viability and thus indicate that the integrity of treatment group (2-APB, Eugenol, PBEA and RCHA) treated cells against the oxidative affront.

Further, several researcher demonstrated that H_2O_2 is a suitable apoptosis inducer.[222] Moreover, Harsdorf [223] reported that H_2O_2 induced cardiomyocyte apoptosis is mediated by ROS. Transient exposure of H_2O_2 significantly manifested an elevation of apoptosis in H9c2 cell culture via double staining annexin-V and PI. In this study, the percentage decline in apoptosis of treatment group (2-APB, Eugenol, PBEA and RCHA) noted were 63.5%, 59.1%, 45.7% and 74.3% respectively. This effect might be through detoxification of free radical, through the ROS-dependent signaling pathway and by enhancing the intracellular antioxidant defense grid.

Taken together, these data strongly suggest that treatment with 2-APB, Eugenol, PBEA and RCHA protects H9c2 cells against H_2O_2 induced oxidative cardiac injury through decreasing ROS production, apoptosis and increasing antioxidant defense system.

Intracellular calcium plays a crucial role in the regulation of cardiovascular functions.[224] Many of these studies also prospectively assessed the association between serum calcium and global ischemia or cardiovascular disease. The regulation of blood pressure is complex with several organs being involved. An increased influx of calcium into the vascular smooth muscle cells leads to an augmental muscular tone and therefore to an increased vascular resistance and rise in blood pressure.[224]

Blood pressure is a mechanism that an individual has to sustain a constant blood flow, for nutrient supply and transport of respiratory gas. It is regulated essentially by two factors, cardiac output (CO) and total peripheral resistance (TPR). The CO is the total amount of blood pumped in to the aorta each minute by the heart and the vascular resistance is the obstacle to blood flow in a blood vessel.[225] The pharmacological model used in this study has an acute intravenous administration of Ag-II, thereby triggering vasoconstriction reaction through simulating an alteration of TPR and increasing blood pressure.

The vagotomy of the rats abolished the cardiovascular reflexes, without impeding the heart rate and normal blood pressure, allowing Ag-II induce acute rise in blood pressure without any involvement of the sympathetic and parasympathetic response. The ganglionic-blockade was used to block the transmission of impulses through the autonomic ganglia, impairing the vasculature to compensate blood pressure when administered Ag-II or the drug under investigation.[226] As per report of Chapman, Ag-II $t_{1/2}$ in conscious rat was 14.8 s and in anesthetic rat it was increased by 60%.[227] The percentage rise in SBP and DBP of Ag-II control group were noted 259.65% and 167.3% respectively as compare to control group. This can be excused through cellular events including endorsement of phospholipase C and phosphatidylinositol hydrolysis, enhancement of intracellular free calcium concentration, activation of protein kinase C and decreased release of NO. Treatment with 2-APB, Eugenol, PBEA and RCHA decreased SBP and DBP by 31.02%, 38.8%, 85.65%, 55.93%, 55.9%, 58.49%, 88.2%

and 26.81%, respectively between 0 min to until baseline pressure (~ 6 min) in Ag-II administrated rats.

The consequences obtained from this model allowed us not only to prove the antihypertensive effect of this drug but also to recognize the probable mechanism of action. This mechanism of action thus can be attributed to modulation of blood pressure through direct antagonism of the AT1 receptor or by the intrusion on calcium fluxes activated by Ag-II, impeding the immediate vasoconstriction response by the administration of AG II.[226]

Moreover, Ag-II has been reported to have a facilitating effect on Ca^{++} mobilization from the sarcoplasmic reticulum and endoplasmic reticulum by depletion of calcium store through IP3.[228] Ag-II initiated second messenger system, also is reported to upgrade the efflux of sarcoplasmic calcium that activate the store operated calcium channels thus allows more extracellular calcium to enter the cell and form a calcium-calmodulin complex that ultimately ends in vascular contraction.[229] This leads to activation of STIM1 and Orai1 protein expression which cause more extracellular calcium in to cytoplasm. Here, we thus depict that, inhibition of STIM1 and Orai1 bottles up Ag-II triggered acute hypertension, indicate a necessity for SOCE for this remodeling response.

In the current study, serum calcium level at different time point (0.5 and 1 min) was found to be significantly increased in Ag-II administration rats and treatment with 2-APB, Eugenol, PBEA and RCHA brought about a significant reduction (17.8%, 21.1%, 18.0% and 20.8%) in serum calcium level at 0.5 min respectively and it could be attributed to inhibition of calcium influx. Serum sodium level at 0.5 min was significantly increased in Ag-II administration rats which attributed to increase Na-H exchange by Ag-II, increasing sodium reabsorption. Increased levels of sodium in the body acts to increase the osmolarity of the blood, leading to a shift of fluid into the blood volume and extracellular space. The reduction of sodium was observed on treatment with 2-APB, Eugenol, PBEA and RCHA at 16.6%, 10.9%, 19.4% and 16.6%, respectively. In addition, Ag-II administrative rats serum magnesium level was decreased at 0.5 min, this magnesium deficiency may lead to an Ag-II induced rise in blood pressure, aldosterone concentration, and elaboration of vasoconstrictive prostaglandins. However, treatment

with 2-APB, Eugenol, PBEA and RCHA resulted in significantly increase in percentage of serum magnesium level 64.0%, 19.8%, 38.2% and 29.4%, respectively. Calcium channel blocking action of PBEA and RCHA in aorta rings against calcium concentration was observed at 1 mg/ml and 0.3 mg/ml, respectively.

In present study, Ag-II administration in rats showed marked elevation of heart and aorta STIM1 and Orai1 expression which may be reflected to calcium influx and oxidative stress in cellular organelles. Nonetheless, 2-APB and Eugenol administration in Ag-II administrated rats suppresses expression of aorta STIM1 in the percentage of 45.5% and 70.2%, respectively but only PBEA and RCHA treatment suppresses expression of aorta Orai1 in the percentage of 65.8% and 40.4%, respectively on account of potent SOCE blocking activity with antioxidant effect. In addition, the percentage of suppression of heart STIM1expression in treatment group (2-APB, Eugenol, PBEA and RCHA) were noted 40.3%, 78.8%, 18.8% and 72.1%, respectively and heart Orai1 expression were noted 61.1%, 64.5%, 55.3% and 63.1%, respectively. ***In this study, we provide evidence that deregulated SOCE through STIM1 and Orai1 is the main calcium entry mechanism during Ag-II induced acute hypertension.***

There is also increasing evidence that oxidative stress and consecutively the production of free radicals may play an important role in Ag-II mediated effects. MDA, SOD, Catalase and GSH level were measured in heart and kidney tissue. In this manner, result confirmed that increased ROS generation through the activation of NAD(P)H oxidase is an essential step in Ag- II administrated rats. However, heart MDA level percentage decrease in 2-APB, Eugenol, PBEA and RCHA treated animals were 1.68%, 34.74%, 45.15% and 32.41% respectively and kidney MDA level percentage decrease in 2-APB, Eugenol, PBEA and RCHA treated animals were 46.46.%, 41.64%, 34.62% and 32.34% respectively as compared to Ag-II control group. Moreover, The percentage rise in heart SOD level by 2-APB, Eugenol, PBEA and RCHA were 60.83%, 68.0%, 72.35% and 62.02% respectively and kidney SOD level by 2-APB, Eugenol, PBEA and RCHA were 86.70%, 129.81%, 114.58% and 97.55% respectively as compared to Ag-II control group. Treatment control group in heart and kidney significantly increased Catalase level as compared to Ag-II control group. The percentage of catalase rise noted was 39.68%,

45.78%, 47.36% and 35.06% in heart and 60.02%, 78.98%, 90.28% and 74.37% in kidney respectively. The percentage rise in heart GSH level with PBEA and RCHA were 74.46%, 69.72% as well as kidney GSH level with Eugenol and PBEA were 67.82%, 73.48% respectively as compared to model group. No significant changes were observed in 2-APB and Eugenol treated heart as well as 2-APB and RCHA treated kidney Catalase activity.

Hypertension is one of the major risk factors for global ischemia, but it is still unclear as to how high blood pressure and the pathologic changes that occur in hypertensive patients contribute to the development of coronary heart disease. The link between hypertension and myocardial ischemia should be considered from two points of view: (1) common risk factors like genetic risk, insulin resistance, sympathetic hyperactivity, and vasoactive substances such as angiotensin II are well reported for the two diseases and (2) linking factors that are induced by hypertension and contribute to the development of atherosclerosis and myocardial ischemia.[230] Physiological and pathophysiological role as well as prevalence of SOCE in myocardial cell was debated previously. Inhibition of STIM1 and Orai1 bottles up Isoproterenol (ISO) triggered ischemia; indicate a necessity for SOCE for this remodeling response.[231] Based on these results, we resolved that stress triggered STIM1 and Orai1 re-expression, and consequent SOCE activation, are decisive factors in the upstream, calcium dependent control of global ischemia and hence should be appropriately evaluated.

Increases in cytosolic free calcium concentration ([Ca2+]I) may play an important role in myocardial ischemic injury. An early effect of the rise in [Ca2+]I may be impaired postischemic contractile function if the ischemic myocardium is reperfused during the reversible phase of ischemic injury; furthermore, if the rise in [Ca2+]I is prolonged, a cascade of events may be initiated which ultimately results in lethal injury.[232]

Administration of ISO causes functional and morphological changes in the myocardium eventually contributing to cardiac ischemia, hypoxia and cell death coupled with decreased cardiac compliance, inhibitory effect on systolic and diastolic function and an exact similarity to pathological alterations examined in human myocardial infarction. Versatile mechanisms of ISO exhibited ischemia have been described by the researcher

till date.[233, 234] Now, numerous researchers have tried to linked SOCE pathway with those mechanisms. Elucidating the role of STIM1 and Orai1 in ISO induced myocardial ischemia is unknown. Typically, ISO acts through the GPCR by activating PLC (Phospholipase C) that breakdowns the PIP_2 (Phosphotidylinositol 4,5 bisphosphate) in to DAG (Diacylglycerol) and IP_3 (Inositol 1,4,5, triphosphate). This IP_3 is attached to IP_3R located on the ER membrane and by virtue of it, ER calcium store is depleted.[235] Depleted store can sensitize and activate the Ca^{+2} sensor protein STIM1 by its multimerization. Activated STIM1 is then translocated to puncta near the Plasma memebrane and it interacts with C & N–terminal of Orai1. Plasma membrane Orai1 channel is activated and extracellular calcium is entering via Orai1 channel in to the cytoplasm. Cytoplasmic intracellular concentration of calcium is increased.[236, 237] Moreover, upon store depletion STIM1 is also engaged with the other proteins like SERCA, Na^+/K^+ ATPase and PMCA.[238] Oxidative stress generated at interface of ER/SR-mitochondrial has entailed the myocardial ischemia through imbalance between ROS and calcium.[219]

In the present experiment, significant increase in heart weight to body weight ratio in ISO administrated rats was noted along with unchanged relative body weight of animals. Increased heart weight in ISO group can be caused by the overall protein synthesis accompanied by myocardial and mitochondrial oedematous or over expression of gene encoding contractile protein, invasion of inflammatory cell in necrotic myocardium and increased water content.[239] In addition, increased cytoplasmic calcium concentration has an essential role in aforementioned process during myocardial ischemia. Small (3%) increased myocardial water content has been colligated with (30%) ventricular systolic and diastolic dysfunction.[240] Treatment with 2-APB, Eugenol, PBEA and RCHA reduced heart weight to bodyweight ratio by 16.3%, 19.07%, 18.7% and 18.5%, respectively thus indicating myocardial protection against over expression, invasion and Ca^{+2} concentration as well as decrease in the myocardial water content.

The surface electrocardiography and its correct interpretation along with physical examination, biochemical estimation and clinical observation, is gold standard clinical test for earlier and subsequent diagnosis of patient with chest pain for distinguishing the

presence and spot of acute myocardial ischemia. Variations in the ionized serum calcium concentration produce the characteristic ECG changes that occur with hypercalcemia and hypocalcemia.[241] ISO administration in rats reported to cause ST-segment elevation pointing towards the membrane potential difference between non-ischemic and ischemic cells, induced by the ions accumulating vector towards the healthy cells and consequent deprivation of cell membrane of ions, contributes further to oxidative insult leading to myocardial necrosis.[242] This is affirmed with the comment of numerous reports that myocardial ischemia shows an ST-segment elevation in the injured myocardium region.[243] In the present study, minute pathological Q wave was seen in ISO groups which indicated the transmural myocardial ischemia in the LV wall. ECG findings on Q wave suggest that when infracted cardiac muscle lacks electrical activity.[244] Moreover, the experiment shows significant changes in ECG characteristic like decreased in P wave intensity, R-R intervals and QRS complex along with elevation of QT-intervals and heart rate. All of these alterations could be due to sequential plasma membrane loss in infracted muscle. Prolong QT interval reflects ventricular arrhythmia, on account of electrical heterogeneity of M cells located in ventricular mid myocardium, temperature reduction in the epicardium, acidosis and alteration in kinetics of Na^+ channel by ischemia generated lysophosphotidylecholine, resulting in the prolongation of repolarization.[245] Treatment with 2-APB, Eugenol, PBEA and RCHA in ISO injected rats terminate acute fatal complication and showed obvious improvement against diversified ECG pattern through the prevention of cell membrane loss.

Induction of myocardial ischemia by ISO administration in rats caused changes in hemodynamic parameter due to the structural injury by excessive content of MDA, which is similar as human acute myocardial infarction.[246] These changes represented by significantly decreased mean blood pressure, LV iontropic and LV lucitropic effect as well as significantly elevated Left ventricular end diastolic pressure (LVEDP). The $+dp/dt_{max}$ was extremely depressed (57.49%) indicating a more systolic left ventricular dysfunction as compare to diastolic function. In addition, elevated LVEDP usually indicate reduction of myocardial contractility, cardiac constriction or myocardial restriction, volume overload, increasing ventricular wall stiffness and ventricular failure.[247] Indeed, it is very crucial to improve the myocardial contractility along with

decreased LVEDP. Nevertheless, Treatment with 2-APB, Eugenol, PBEA and RCHA improved coronary flow by 123.8%, 94.7%, 116.3% and 104.1%, respectively as well as other hemodynamic parameter and contractility of left ventricular in course of the infraction caused by ISO.

Apart from hemodynamic function, subcutaneous ISO injection also elevated escape of cytosolic cardiac marker enzymes like CK-MB and LDH, as an index of myocardial injury. The ample concentrations of cardiac biomarkers are present in heart and when it metabolically damaged, its content exhausted in to extracellular fluid volume. The elevated calcium levels during ISO induced global ischemia leads to alteration of plasma membrane permeability through increased phospholipaseA2 activity in cell.[248] Primary biochemical estimation during the diagnosis of MI is CK-MB assay because of presence of high extent of this enzyme in cardiac tissue and its concomitant sensitivity. Cellular enzymes namely CK-MB and LDH activity in serum ruminates the alteration of cell membrane integrity.[249] In the present experiment, elevated activity of CK-MB and LDH in serum was observed due to myocardial necrosis by ISO intoxication, which is in consistence with previous reports.[244, 249] Moreover, Treatment with 2-APB, Eugenol, PBEA and RCHA in ISO injected rats seems to uphold the morphological and functional permeability of plasma membrane and thereby let down activities of these cardiac markers in serum. The percentage decline of CK-MB and LDH were noted 31.8%, 45.8%, 40.6%, 31.4%, 31.3%, 37.9%, 33.6% and 23.6%, respectively.

Oxidative stress causes Ca^{2+} influx into the cytoplasm from the extracellular environment and from the endoplasmic reticulum or sarcoplasmic reticulum (ER/SR) through the cell membrane and the ER/SR channels, respectively. Rising Ca^{2+} concentration in the cytoplasm causes Ca^{2+} influx into mitochondria and nuclei. In mitochondria Ca^{2+} accelerates and disrupts normal metabolism leading to cell death. In nuclei Ca^{2+} modulates gene transcription and nucleases that control cell apoptosis.[250]

The balance between free radical and antioxidant is essential for removal of oxidative stress from the cells.[251] However, pathophysiological condition like myocardial infarction, increasing ROS along with decreased level of antioxidant enzyme is observed.[252] Lipid peroxidation is an important pathological event for cell damage as well

as cell death and elevated MDA level in cell brings to increased ROS formation and /or decreased cellular defense activity.[253] In this study, ISO injected rats demonstrated significant elevation of MDA content, which consistent with similar findings in number of earlier research studies.[244, 251] The percentage decrease in MDA level with 2-APB, Eugenol, PBEA and RCHA were 48.68%, 26.20%, 51.84% and 43.41% respectively as compared to ISO control group which can be assigning to antioxidant activity through blockage of cytosolic calcium overload.

Antiperoxidative enzyme (SOD and Catalase) activities were decreased in heart tissue of ISO administered rats. SOD produced H_2O_2 and molecular oxygen through dismutation of two O_2^-. Hence, generated H_2O_2 is deactivated by the Catalase or by GSH redox system.[254] However, Treatment control group (2-APB, Eugenol, PBEA and RCHA) increased SOD and Catalase level by 93.13%, 101.46%, 155.87%, 118.94%, 67.17%, 113.94%, 98.77% and 80.21% respectively as compared to ISO control group which could be due to removal of ROS by blockage of intracellular calcium overload.

Body's master antioxidant GSH is present as non-enzymatic tripeptide antioxidant in our body. This decreased GSH level is responsible for high level of GSH used to protect –SH group containing proteins from free radical[255] and there by reduced availability of GSH affect the activity of GPx as well as GST like phase-II enzyme.[239] Nunes et al., mentioned that the ratio of oxidant to antioxidant and the level of oxidative stress may be crucial factors in fine-tuning beneath the SOCE core machinery.[256] In addition, Orai1 channel has a major role in the detrimental Ca^{++}influx caused by oxidative stress.[257] However, The percentage rise noted in GSH level with 2-APB, Eugenol, PBEA and RCHA was 62.98%, 67.31%, 86.48%, 99.25% and Gpx activity was 37.46%, 44.13%, 43.53% and 68.19% respectively as compared to ISO control group. In addition, Eugenol, PBEA and RCHA increased GST activity above 100% as compared to model group. These results may be attributed to cell survival promoting action of treatment through inhibitory effect on detrimental Ca^{++} influx during oxidative stress.

Calcium is an important ion for the normal function of heart, enzymes activity, cell membrane integrity and coagulation of blood.[258] ATPase are located in to the plasma membrane and enrolled in the energy requiring translocation of Ca^{++}, Na^+, K^+ and Mg^{++}.

ISO causes the opening of calcium channel through cAMP phosphorylation at several site on c-terminal chain of calcium channel, which might be the reason of increased Ca^{++}ATPase activity and increased cytosolic Ca^{++} concentration in infracted myocardium.[259] Intracellular calcium overload can trigger off a cascade of event that can contribute to ROS formation, which evoked that contractile dysfunction in myocardial ischemia might be due to involvement of calcium and ROS.[219] Soboloff et al. reported that both STIM1 and PMCA4 (Ca^{++}ATPase type 4) are up regulated during T cells activation and the subsequent interaction of PMCA with STIM1 reduces PMCA mediated [Ca2+]i clearance. Moreover, high cytosolic Ca^{2+} level in store-depleted Jurkat cells due to the STIM1-POST complex inhibited PMCA activity.[260] In addition, Na^{+}/K^{+} ATPase (Lipid dependent enzyme) contain -SH group and elevated level of free fatty acid during myocardial ischemia caused the oxidation of protein by attack on the –SH group. Inactivated Na^{+}/K^{+} ATPase increase intracellular sodium ion and subsequently, activates the Na^{+}- Ca^{++} exchanger (NCX) there by accumulation of calcium in to cell.[261] In the present study, ISO administered rats showed increased Ca^{++}ATPase activity and decreased Na^{+}/K^{+} ATPase and Mg^{++} ATPase activities, which is in consistent with the former report.[239, 248, 261] Nevertheless, Treatment control group (2-APB, Eugenol, PBEA and RCHA) administration decreased Ca^{++}ATPase activity and increased Na^{+}/K^{+} ATPase and Mg^{++} ATPase activities, which might be attribute to inhibition of STIM1 and direct antioxidant effect of treatment, thereby protects –SH group of oxidative insults.

Intracellular calcium signaling could contribute to the inflammatory response.[262] Pro-inflammatory cytokines like TNF-α involved in to reduced myocardial contractility, damage of cell membrane integrity, myocardial compliance, reduced antioxidant capacity and myocardial necrosis, thereby leads to myocardial ischemia- reperfusion, post myocardial infarction, myocardial hypertrophy and heart failure.[263] Jia et al., reported that increased TNF- α level constitutive upregulate the STIM1 aggregation that contributes SOCE in human air way smooth muscle and increased the basal [ca^{+2}]i as well as decreased SR Ca^{+2}.[264] Pleiotropic cytokine like IL-6 in myocytes also depressed basal contractility, turned on hypertrophic gene, LV enlargement and cardiac function.[265] Gao et al., demonstrated that SOCE can stimulated IL-6 and TNF-α production and Orai1 play a crucial role in cytokine production in spinal astrocytes.[266] Infracted myocardium by

ISO, raised level of TNF-α and IL-6 in serum, which might be due to possible mechanism of interplay between cytokine and SOCE machinery. However, The percentage decreased of TNF-α and IL-6 of Treatment control group (2-APB, Eugenol, PBEA and RCHA) were noted 50.8%, 46.1%, 44.7%, 55.8%, 24.7%, 37.9%, 29.9% and 41.62%, respectively through the possible suppression of STIM1expression.

Furthermore, heme containing enzyme like myeloperoxidase release in to extracellular space during inflammatory condition. Earlier reports have shown that higher concentration of MPO increased activity of platelet that contribute to formation of pathological thrombus and it has predominant role in the crucial events including myocardial infarction, arthrosclerosis and other cardiovascular complication.[267] Gorudko et.al reported that MPO bind to the plasma membrane of platelet, caused actin cytoskeleton reorganization and mechanical stiffness of platelet is affected. Thereby resulting in markedly activation of SOCE with increased $[Ca^{+2}]i$ leads to platelet aggregation.[268] In present experiment, increased activity of MPO in cardiac tissue was observed in ISO induced MI, which is in line with the former report. [244] Treatment control group (2-APB, Eugenol, PBEA and RCHA) blunted MPO activity in the percentage of 19.4%, 42.1%, 48.7% and 62.7%, respectivrely which may be speculate that treatment drugs suppressed the neutrophil infiltration in myocardium.

STIM1/Orai1- generated calcium signaling link to gene transcription and subsequent phenotypic modifications colligated with the processes of cardiac and vascular remodeling. Earlier epidemiological study revealed that disturbed SOCE signaling and its molecular machinery is connected to a multiple pathological condition including ischemia–reperfusion, hypoxia, intracellular calcium overload, oxidative stress, inflammation and hypertrophy.[269] These multiple mechanism are engaged with MI and hence the possible involvement of STIM1 and Orai1in ISO induced global ischemia was evaluated in current study. Mobile protein STIM1 along with SOCE activation play crucial role in the antioxidant response and demanded in regulating spatial and temporal panorama of Ca^{+2} signaling as well as cellular energy production. Over expression of STIM1 brings out the sub-cellular calcium microsphere near to STIM1 cluster thus resulting in myofibrillar protein (actin, myosin and troponin) degradation and

displacement of focal adhesion molecule, often resulting in cell detachment and death subsequently.[270] STIM1 as redox sensor, activate calcium- release activated calcium (CARC) channel under oxidative stress which facilitate an increased in calcium level and this raised sustained calcium leads to mitochondrial Ca^{+2} overload, subsequently changes in cellular bioenergetics which overtime can trigger cell death. Moreover, Orai1 channel has a prominent role in detrimental Ca+2 influx caused by oxidative stress.[271] Henke and their group reported that Orai1 might not only be activated by STIM1, but also via other possible factors like the Ca^{2+}-independent phospholipase A_2 and direct influence of the cellular redox state on Orai1 activity seems possible.[257] GSH is a decisive regulator of STIM1 signaling throughout oxidative stress.[272] Xin, Yuan and their group demonstrated that Ca^{+2} mobilizations along with SOCE (Orai1 mediated Ca^{+2} influx) play key role in inflammatory response through regulating neutrophil infiltration in vessels wall and endothelial activation.[273] In present study, ISO intoxicated rats showed markedly elevation of STIM1 and Orai1 expression which may be reflected to oxidative stress and calcium influx in cellular organelles during myocardial infarction. Nonetheless, Treatment control group (2-APB, Eugenol, PBEA and RCHA) administration in ISO injected group suppresses expression of Orai1 in the percentage of 35.2%, 32.5%, 32.7% and 63.0% and 64.3%, 49.5%, 67.2% and 28.6% suppresses expression of STIM1 on account of potent SOCE blocking activity with antioxidant effect. In this study, we thus provide evidence that ***dysregulated SOCE through STIM1 and Orai1 is the main calcium entry mechanism during ISO induced global ischemia, hinting that SOCE inhibition might be a precious tool in the treatment of oxidative stress associated disease.***

Areas of infarction indicate loss of membrane integrity which might be due to significant leakage of lactate dehydrogenase enzymes. Moreover, increase ROS and RNS enlarged infract size in ISO induced ischemia.[274] Present experiment showed significantly increased infracts size as compare to control group. That could be reviled by Treatment control group (2-APB, Eugenol, PBEA and RCHA) in the percentage of 64.7%, 29.8%, 50.7% and 24.2%, respectively. That might be attributed to their potent antioxidant activity which prevents leakage of lactate dehydrogenase enzymes.

During our histoarchitectural examination of heart, control rats displayed well defined, intact uniform architectural of myocardium with no evidence of inflammatory cell infiltration and focal necrosis; ISO injected rats call forth adverse histoarchitectural alterations in rats heart letting in coagulative necrosis, extensive infiltration of neutrophil, vacuolation of cardiac myocytes and separation of cardiac myofiber that are sign of myocardial ischemia. However, Treatment with 2-APB, Eugenol, PBEA and RCHA in ISO injected groups exhibited more and less similar architecture of heart as observed in control rats (without necrosis and inflammatory cell infiltration, but with intact myofibrils), which indicates prevention of necrosis by inhibitory action on calcium influx and antioxidant effect of treatment drugs.

In light of the above results, we evaluated the role of 2-APB, Eugenol, PBEA and RCHA on left anterior descending coronary artery ligation (LAD) induced myocardial reperfusion injury. Reperfusion of the ischemic heart has been shown to result in a marked increase in myocardial Ca^{2+} content. Overloading of mitochondria with Ca^{2+} under conditions of ischemia–reperfusion injury is considered to lower the energy status of cardiomyocytes.[275] Ligation of the LAD occludes oxygen supply and nutrients to the cardiac muscle, resembling the actions of a heart attack in human being. Such occlusion consequences in the muscle weakness, changes to the overall structure of the heart and cardiac dysfunction. As this model closely mimics heart failure disease progression in humans, it has proven to be a valuable model for the investigation of the mechanisms of heart failure and for the preclinical testing of pharmacological therapies.[276] LAD ligation leads to myocardial ischemia and necrosis. CK-MB is a cardiac-specific biomarker of acute myocardial infarction or myocardial tissue injury would leak into serum from cardiac myocytes. Treatment with 2-APB, Eugenol, PBEA and RCHA administration restrained the increase of CK-MB activity in the percentage of 39.2%, 40.8%, 48.7% and 38.3%, respectively which confirmed the protective effects of treatments against myocardial ischemic damage through protection of plasma membrane integrity. Recently there has been a rapid growth of ideas concerning the involvement of calcium in the series of events that are precipitated by an ischemic episode and become exacerbated upon reperfusion and reoxygenation. In the ischemic and reperfused myocardium, we postulate that the depletion of tissue stores of ATP is of sufficient magnitude to cause an

energy-depletion-induced failure of the mechanisms that are normally responsible for maintaining intracellular homeostasis with respect to Ca2+.[277] In that phenomenon, STIM1 and Orai1 protein expression which is significantly increased in LAD control as compare to control group. Nonetheless, Treatment with 2-APB, PBEA and RCHA suppresses the expression of STIM1 in the percentage of 53.8%, 13.7% and 50.8%, respectively and suppresses expression of Orai1 in the percentage of 41.1%, 65.9% and 74.5%, respectively on account of potent SOCE blocking activity coupled with antioxidant effect. There was no change of both protein expressions in Eugenol group.

Now on a safety concern, experiments have noticeably revealed a protective effect of treatment group (2-APB, Eugenol, PBEA and RCHA), which is recognized to have antioxidant effect, on H9c2 cells. In addition, 2-APB (25~300 μM), Eugenol (25~150 mM), PBEA (0.1~25 μg/ml) and RCHA (1~800 ng/ml) had no cytotoxic effect in H9c2 cells.

Doxorubicin is one such agent which backs the generation of free radical through metabolism of its quinone structure. This effect combined with induction of apoptotic and necrotic pathways leads to the development of irreversible cardiotoxicity. The variety of mechanisms include membrane alterations through lipid peroxidation, generation of free radicals, raising of myocardial sodium and calcium concentrations, induction of cardiomyocyte apoptosis and induction of ischemia-reperfusion injury.[277] Treatment with 2-APB, Eugenol, PBEA and RCHA restrained the increase of LDH activity, which confirmed the protective effects of treatment control group against cardiotoxicity through protection of plasma membrane integrity. Doxorubicin-mediated ROS generation and apoptosis can be inhibited by using a Ca^{2+} chelator. Nonetheless, Treatment with Eugenol suppressed 50.2% expression of STIM1 and PBEA treatment suppresses 60.4% expression of Orai1. Other treatment group did not change the elevated protein expression in this model. These results suggest cardio protection against doxorubicin by Eugenol and PBEA and not by RCHA and 2-APB.

Acute toxicity testing was carried out to determine the effect of a single dose on a particular animal species. The essence of toxicity testing is not just to check how safe a test substance is; but to characterize the possible toxic effects it can produce. The guiding

principles of toxicity testing is to check the effect of the test substances on laboratory animals and its direct toxic effect on human and secondly, the exposure of laboratory animals to high doses in order to evaluate its possible hazard on human that are exposed to much lower dose.[278] Acute toxicity test OECD 423 of PBEA and RCHA was performed, in which we found 2000 mg/kg dose of PBEA was not toxic and 2000 mg/kg dose of RCHA was toxic. Moreover, sub-acute toxicity study of PBEA in high dose (800 mg/kg) of female serum calcium level was significantly decreased as compare to control group but this value was within the range so there was no adverse effect. At last, histopathological finding suggest that sub-acute treatment of PBEA had no toxic effect.

In conclusion, Treatment with 2-APB, Eugenol, PBEA and RCHA has potent antioxidant effect and calcium channel blocking action depend upon the severity of cardiovascular disease. Ag-II induced acute hypertension regulated intracellular calcium level through SOCE and made up calcium calmodulin complex that can leads to vasoconstriction. Moreover, it cause increase production of free radical and oxidative stress. SBP and DBP rank order potency were PBEA > RCHA > Eugenol > 2-APB and PBEA > Eugenol > 2-APB > RCHA, respectively. Eugenol > RCHA > PBEA > 2-APB rank order potency was noted in the calcium level modulation and Eugenol >PBEA > RCHA >2-APB rank order potency was noted in the kidney elevated SOD antioxidant activity. Heart STIM1 and Orai1 expression rank order potency were Eugenol > RCHA > 2-APB > PBEA and Eugenol > RCHA > 2-APB > PBEA, respectively. ISO induced global ischemia leads to β-receptor modulation, oxygen deficiency and calcium influx there by SOCE activation. 2-APB > PBEA > RCHA > Eugenol rank order potency was noted in heart calcium reduction. Heart SOD and GST increased activity were noted PBEA > RCHA > Eugenol > 2-APB and RCHA > PBEA > Eugenol > 2-APB, respectively. Heart STIM1 and Orai1 expression rank order potency were PBEA > 2-APB > Eugenol > RCHA and RCHA > 2-APB > PBEA > Eugenol, respectively. LAD induced reperfusion injury leads to marked increase in myocardial calcium concentration that may modify cardiac gene expression like STIM1 and Orai1. 2-APB > RCHA > PBEA > Eugenol and RCHA > PBEA > 2-APB > Eugenol rank order potency were noted in heart STIM1 and Orai1, respectively. In addition, PBEA is safe in sub-acute

administration as compare to RCHA. From the above mentioned rank order, PBEA is more potent and safe drug in cardiovascular disorders.