2. 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 re-absorption, 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 signaling to the myofilaments occurs in global ischemia, atherosclerosis, 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 cardio inhibitory 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] Angiotensin-II (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 re-oxygenation.[17] Experimentally induced global ischemia has multi-factorial mechanism 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 tri-phosphate 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] Several mechanisms were suggested for DOX-induced cardio-toxicity, such as oxidative stress, produced by increased levels of ROS, and intracellular iron and decreased levels of antioxidants e.g. glutathione (GSH). Increased intracellular calcium by oxidative stress and acceleration of lipid peroxidation damages the cell membrane and other cellular components. The highly active metabolic process and the poorly developed antioxidant system in cardiac muscle cells makes it more vulnerable to such toxic mechanism[19]

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 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.[20] Dysfunctional calcium load and oxidative stress via mitochondria and endoplasmic reticulum in cardiac myocytes 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.[21]

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.[22] 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.[23] 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 over-expressed in conditions of ischemia/reperfusion and can protect from cardiac injury.[24] Moreover, overwhelming cardiomyocytes' enzymatic defenses, ROS can also alter gene expression through their interactions with regulatory proteins. ROS can affect the function of membrane-bound proteins such as the G-proteins, via lipid peroxidation. ROS can alter a protein's tertiary structure by oxidizing S-S bonds.[25] Perhaps, most critically for the myocardium, ROS can induce the release of Ca2+ ions.

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.[26, 27] 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.[28] 2-APB and thapsigargin both are act as SOCE modulator depending upon its concentration.[29] Another SOCE inhibitors like Synta 66, ML-9, Diethylstilbestrol, Carboxyamidotriazole, RO2959, Linoleic acid, 1-Phenyl-3-(1-phenylethyl)urea derivatives.[30] 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.[31]

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 ontogenetic 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 deregulated Ca^{2+} influx. [31]

It is therefore, the need of the hour to scientifically generated data to project herbal medication heaving 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, global ischemia and cardio toxicity 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.[32] Capsaicin has been demonstrated to inhibit high-voltage-activated calcium channel (HVACC) currents.[33] 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 has been proved. 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^{++}.[34] Eugenol exerted a strong inhibition of ACE activity in vitro and in diabetic rats. Eugenol inhibits neuronal excitotoxicity or oxidative injury and has protective effects against N-methyl-D-aspartate–induced neurotoxicity.[35] 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.[36]

Piper betle which has antioxidant, anti-platelet, anti-inflammatory activity and calcium channel blocker.[37] Furthermore, Piper betle is also anti-infective, analgesic, anticancer, anti-diabetic, hepatoprotective, immuno-modulatory. *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 anti-platelet effects, without effects on haemostatic functions.[38] The plant has been known to contain several chemicals including eugenol. Interestingly, eugenol has been reported to exhibit a smooth muscle relaxing action possibly through an inhibitory action on the intracellular release and entry of extracellular calcium. It is likely, therefore, that the calcium channel antagonist effect of the plant extract or its ethyl acetate fraction may be partly due to the presence of eugenol. There has been some indication for the presence of calcium channel antagonist(s) in the betel quid.[39]

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.[40] *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 decrease lactate dehydrogenase enzyme which is highly secreted during cardiovascular disorders. These extract may exhibit spasmolytic activity similar to that of varapamil suggestive of possible presence of calcium channel blocker(s) in rubia cordifolia.[41]

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 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.[42] The calcium pump of the endoplasmic reticulum (SERCA) is also blocked by 2-APB at very high concentrations.[43] 2-APB protects against liver, kidney and ovary ischemia-reperfusion injury by reducing cellular and mitochondrial calcium uptake.[44-46]

With this background, the present study hence was undertaken to carry out evaluating the role of SOCE and its inhibitors with special reference to hypertension, myocardial ischemia reperfusion injury and cardio toxicity using different animal models for better health prospects in the individuals suffering from CVD with the following objectives:

1. Preparation, phytochemicals screening and standardization of ethyl acetate extracts of *Piper betle* and hydro alcoholic extract of *Rubia cordifolia.*
2. Evaluating the cell viability, antioxidant status and anti-apoptosis effect of treatment drug on H9c2 cell line.
3. Development of RP-HPLC method and to find out time as well as efficacy study of 2-APB.

4. Effect of selected drug on Ag-II induced acute hypertension in vagotomized rat and evaluating molecular aspect of SOCE.
5. Effect of selected drug on isoproterenol induced global ischemia as well as coronary artery ligation induced reperfusion injury in rat and evaluating molecular aspect of SOCE.
6. Safety profile of selected drug against cardio toxicity as well as acute and sub-acute toxicity study through OECD guideline.