

8. SUMMARY AND CONCLUSION

8.1. Aim of the study

Calcium plays a number of critically essential roles in cardiovascular physiology and pathology. Calcium is a key messenger in the contraction of muscle, including the myocardium. Globally, cardiovascular diseases (CVD) are the number one cause of death and they are projected to remain so. The major causes of cardiovascular disease are tobacco use, physical inactivity, and an unhealthy diet. Despite the advancements in diagnosis and treatment modalities, the mortality and morbidity associated with CVD is massive. Changes in calcium homeostasis during CVD is to be attributed mainly to the energy 'crisis' and reactive oxygen species. However, the molecular mechanisms of store operated calcium entry (SOCE) in CVD are remaining unknown. This demands serious attention to unravel the molecular mechanisms to identify the therapeutic strategies either to prevent or to control CVD. With increasing enhancement of people's awareness of self-care and concerning on the inevitable adverse effects of conventional medicine, herbal medicines are favored by people with CVD all over the world for their unique advantages in preventing and curing diseases, rehabilitation, and health care. Consequently, an effort was made in the present assessment to recognize an effectively easy to get a regular herb and assess its potential in the treatment or aversion of CVD. The medicinal plants selected for the study were *Piper betle* and *Rubia cordifolia*. *Piper betle* have been reported to be associated with various properties such as antioxidant, anti platelet, anti-inflammatory activity and calcium channel blocker activity. This calcium channel blocking activity might be due to presence of Eugenol. It has been reported to exhibit a smooth muscle relaxing action possibly through an inhibitory action on the intracellular release and entry of extracellular calcium. *Rubia cordifolia* have been reported to be associated with various properties such as antioxidant, anti platelet, calcium channel blocker, anti diabetic and anti-inflammatory activity.

With this background, the present study was aimed to evaluate *Piper betle* and *Rubia cordifolia* in various CVD like Hypertension, global ischemia and reperfusion injury.

The objectives of the study were:

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 cardiotoxicity as well as acute and sub-acute toxicity study through OECD guideline.

8.2. Work Plan

- To investigate the photochemical screening of *Piper betle* ethyl acetate extract (PBEA) and *Rubia cordifolia* hydro alcoholic extract (RCHA) by different free radical scavenging and antioxidant in-vitro assay and standardize both extract with Eugenol.
- Development of RP-HPLC method of 2-APB and to find out half life of 2-APB by pharmacokinetic study.
- In present study, treatment group (2-APB, Eugenol, PBEA and RCHA) were screened for cytoprotective in H9c2cell line by MTT assay. These treatments were also evaluated for Intracellular ROS level and apoptosis assay. For calcium channel blocking action of PBEA and RCHA were performed in ring aorta.
- Treatment group (2-APB, Eugenol, PBEA and RCHA) were estimated in Ag-II induced acute hypertension in vagotomised rats, ISO induced global ischemia and LAD induced myocardial reperfusion injury along with mechanistic SOCE approach through the estimation of STIM1 and Orai1 protein expression by western blot analysis.
- Further, Safety concern of selected drug through cytotoxicity as well as cardiotoxicity and acute as well as sub acute toxicity study through OECD guideline.

8.3. Phytochemical screening and Standardization of PBEA and RCHA

- High amount of Flavonoids, Steroids, Alkaloids, Glycosides, Tannin and Phenolics compound were present in PBEA and RCHA. Total Phenolic content present in PBEA and RCHA were 76.75 ± 1.41 and 61.61 ± 0.68 mg of gallic acid/ g of dried extract. Moreover, Total Flavonoid content present in PBEA and RCHA were 45.22 ± 1.65 and 75.00 ± 2.11 mg of Quercetin/ g of dried extract.
- Quantity of Eugenol in PBEA and RCHA were 43.43 ± 1.46 and 0.414 ± 0.027 mg/gram of extract through standardization of PBEA and RCHA using GC-FID.
- In-vitro assay of PBEA and RCHA for DPPH Scavenging activity (IC_{50}) were found to be 100.1 and 32193 μ g/ml, respectively and % inhibition of β -carotene bleaching was 73.64 ± 0.84 and 69.11 ± 0.33 , respectively.
- Moreover, Superoxide anion radicals scavenging activity of PBEA and RCHA were found to be 98% and 99% and Hydrogen peroxide scavenging activity (IC_{50}) of PBEA and RCHA were found to be 46.17 and 73.10 μ g/ml, respectively and hydroxyl radical scavenging activity (IC_{50}) of PBEA and RCHA were found to be 0.6 and 0.42 μ g/ml.
- Reducing power of PBEA and RCHA were found to be 73.3 and 503 μ g/ml, respectively.

8.4. Development of RP-HPLC method and Pharmacokinetic study.

- Methanol: K_2HPO_4 (0.01% TEA) pH 8.2 Solvent system was used for estimation of 2-APB and symmetric well resolved peak was found at 7.3 RT. 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. Pharmacokinetic study was performed in rat at a dose of 4 mg/kg, I.V and got 8.92 h plasma $t_{1/2}$ which was utilized for the time interval of 2-APB administration in ISO induced myocardial infarction.

8.5. In-Vitro cell line study of selected drugs

- MTT assay of 2-APB, Eugenol, PBEA and RCHA drug were performed on different dose level against 100 μ M H_2O_2 on H9c2 cell and cytoprotectivity were

found to be at a dose of 150 μ M, 50 mM, 10 μ g/ml and 100 ng/ml. which indicated that drug has protective effect against oxidative cardiac injury.

- After that selected dose from the MTT assay, Intracellular ROS assay performed by DCFHDA assay. 2-APB, Eugenol, PBEA and RCHA all drugs significantly ($p < 0.001$) decreased ROS generation against H_2O_2 exposure on H9c2 cell. For targeting various antioxidant enzymes MDA, SOD, CAT and GSH were measured in H9c2 cell. PBEA were more significantly reduced MDA level and increased SOD level. Eugenol were more significantly increased CAT level and RCHA were more significantly increased GSH level. This indicated that all drug has high oxidant defense activity and gives the protection against cardiac injury.
- For anti apoptotic activity, Annexin –PI was performed in which RCHA found to be 74.3 % anti-apoptotic activity.
- Calcium channel blocking activity of PBEA and RCHA was performed by vascular reactivity assay in ring aorta. EC50 of PBEA and RCHA were found to be 0.07 ± 0.02 and 0.1 ± 0.02 , respectively.

8.6. In-vivo activity of Selected drugs

- In Ag-II induced acute hypertension, it was deduced that PBEA is superior to other drugs in averting hypertension. Blood pressure (SBP and DBP) was noted which was significantly reduced in the order proficiency of $PBEA > RCHA > Eugenol > 2-APB$ and $PBEA > Eugenol > 2-APB > RCHA$, respectively. This indicated calcium channel blocking activity.
- While addressing electrolyte concentration in serum, estimated calcium level at 0.5 and 1 min were significantly ($P < 0.05$) reduced in all drugs. Moreover, PBEA decreased 19.4% sodium level and 2-APB rise 64.0% magnesium level as compare to Ag-II control group.
- While focusing on antioxidant activity, PBEA and 2-APB reduced 45.1% and 46.4% heart and kidney MDA level, respectively. In addition, PBEA and Eugenol elevated 72.3% and 129.8% heart and kidney SOD level and PBEA significantly ($P < 0.01$) elevated heart and kidney CAT level and significantly ($P < 0.05$) elevated heart and kidney GSH level.

- While targeting to SOCE activity, Aorta STIM1 and Orai1 expression were inhibited by 2-APB (45.5%), Eugenol (70.2%) and PBEA (65.8%), RCHA (40.4%), respectively. Heart STIM1 expression were significantly ($P<0.01$) suppressed by Eugenol and RCHA but also significantly ($P<0.05$) reduced heart Orai1. There were no changes in Kidney STIM1 and Orai1 expression.
- ISO induce global ischemia, Cardiac markers CK-MB were 45.8% and 40.6% decline in Eugenol and PBEA, also LDH were 37.9% and 33.6% decline in Eugenol and PBEA. This gives the conformation of plasma membrane integrity.
- Hemodynamic and electrocardiography are much of importance in global ischemia. ST-segment elevation and heart rate were significantly ($P<0.001$) reduced in PBEA and 2-APB but not as compare to Eugenol and RCHA. Coronary flow was increased 123.8% and 116.3% in 2-APB and PBEA, respectively as well as LVEDP was decline 34.8% and 28.1% in PBEA and 2-APB. $+dp/dt$ max was significantly (59.8%) higher in 2-APB and $-dp/dt$ max was significantly (78.9%) higher in PBEA as compare to ISO control group.
- While focusing on electrolyte and membrane bounded ATPase enzymes, Serum calcium was significantly ($P<0.001$) reduced in all drugs. Na^+/K^+ ATPase activity and Mg^{++} ATPase activity were 113.2 % and 78.6% rised in PBEA. Ca^{++} ATPase activity was decreased in order of the proficiency 2-APB > RCHA > PBEA > Eugenol. This outcome can be attributed to calcium channel blocking action of plant extract for progression of myocardial injury.
- While Addressing the antioxidant status, PBEA was decline 51.8% MDA level and raise 155.8% SOD level which indicated high antioxidant activity that could protect against ischemia. Moreover, Eugenol elevated 113.9 % CAT level and RCHA elevated 99.2 % and 68.1% GSH and Gpx activity. All drugs raised GST activity above 100% which is effective against oxidative stress generated in ISO induced ischemia.
- For anti-inflammatory activity, RCHA was significantly ($P<0.001$) reduced $TNF-\alpha$, IL-6 cytokine and MPO activity. These outcomes can be attributed to anti- anti-inflammatory potential of extract which halt the progression of ischemia. Area of

infarction was significantly reduced in the order of proficiency 2-APB > PBEA > Eugenol > RCHA.

- While targeting to SOCE activity, PBEA was suppressed 67.2% STIM1 expression and RCHA was suppressed 63.0% Orai1 expression. This was indicated that both drug has good calcium channel blocking activity and reduced intra-cellular calcium level. The PBEA wins the race in handling global ischemia more efficiently.
- Now LAD induced myocardial injury, RCHA significantly ($P<0.01$ and $P<0.001$) reduced expression of both STIM1 and Orai1. This outcome indicated that late phase of myocardial injury can be prevented by RCHA.
- Thus, using three pharmacological models it can be concluded that PBEA and RCHA might be used in CVD.

8.7. Safety of Selected drugs

- In safety point of view, cytotoxicity of all drugs were found to be toxic beyond the dose of 2-APB (300 μ M), Eugenol (150 mM), PBEA (25 μ g/ml) and RCHA (800 ng/ml). In cardiotoxicity study, Eugenol and PBEA could handle Doxorubicin induced cardiotoxicity. Moreover, One time administration of PBEA and RCHA were safe at dose 2000 mg/kg and 300 mg/kg, respectively. So, there was a need to long term administration of PBEA and it was found to be safe at high dose level (800 mg/kg).
- Thus, it can be concluded that PBEA might be safest SOCE blocker used in CVD.