6. RESULTS

6.1 Authentification of *Piper betle* and *Rubia cordifolia* powder

- **Piper betle Lin.**

  Microscopic study of *piper betle* powder reveals the presence of epidermal cell, trichomes, stomata, calcium oxalate crystal, xylem vessels and mucilage canal. (Figure 6.1)

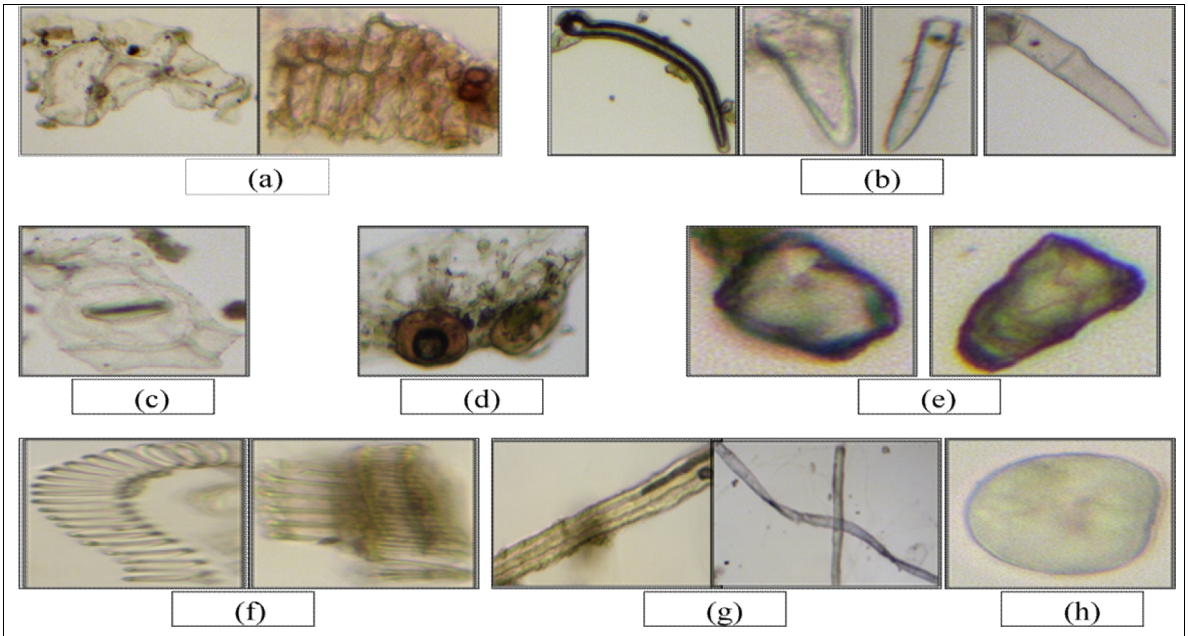


Figure 6.1 Powder microscopy of *Piper betle* Lin.

(a) Fragments of epidermal cell (b) Unicellular and multicellular trichomes (c) Anisocytic stomata (d) Epidermal cell with oil cell (e) Prismatic crystals of calcium oxalate (f) Xylem vessels with annular and spiral thickening (g) Long fibers (h) Presence of mucilage canal.

- **Rubia cordifolia Lin.**

  Microscopic study of *rubia cordifolia* powder reveals the presence of starch grains, calcium oxalate crystal, lignified vessels and cork cells. (Figure 6.2)

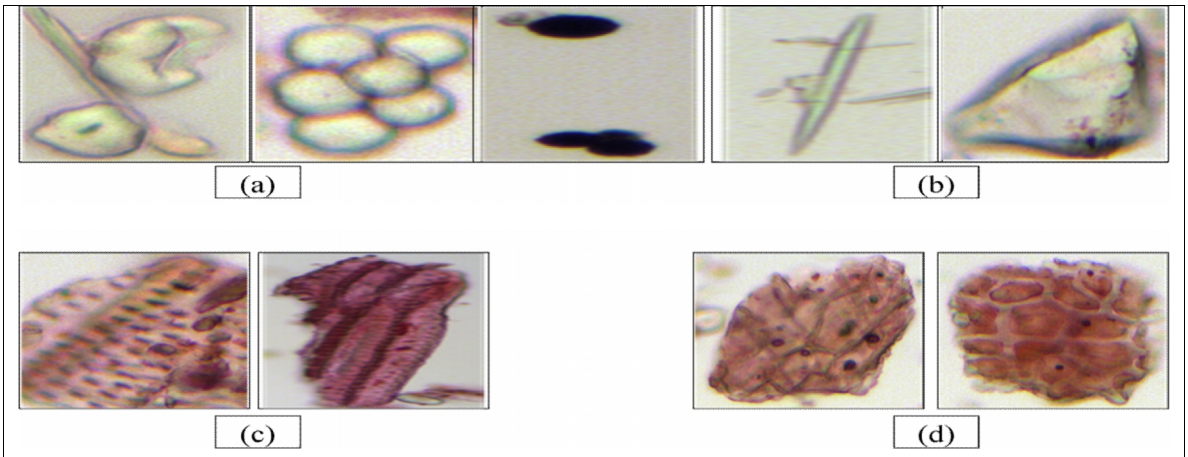


Figure 6.2 Powder microscopy of *Rubia Cordifolia* Lin.

(a) Starch grins (b) Acicular crystals and Crystals of calcium oxalate (c) Lignified vessels and trachids (d) Cork cells

6.2 Prepration and evaluation of extract of *Piper betle* and *Rubia cordifolia.*

6.2.1 Extarction yield

As per section **5.2.1.1**, process parameter of extraction, we observed 6.8% w/w PBEA and 7.6% w/w RCHA extraction yield.

6.2.2 Proximate analysis

The results of the proximate analysis parameters are given values expressed as percentage of air-dried material and are presented in Table 6.1

Table 6.1 Piper betle Lin. and Rubia cordifolia Lin. proximate analysis.

Sr. No	Parameters	Piper betle Lin.	Rubia Cordifolia Lin.
1	Moisture content (%)	9.52	7.75
2	Total Solids (%)	90.47	92.24

3	Total Ash (%)	6.2 ± 0.09	8.15
4	Acid insoluble ash (%)	2.35	3.1
5	Water soluble ash (%)	1.04	1.1

6.2.3 Phytochemical screening test

PBEA contain flavonoids, steroids, alkaloids, glycosides and phenolics and RCHA contain same phytochemicals class along with carbohydrate, protein and amino acid which is present in Table 6.2

Table 6.2 PBEA and RCHA phytochemical screening test.

Sr. No	Chemical Test	Results of PBEA	Results of RCHA
Flavonoids			
1	Alkaline reagent test:	P (+)	P (+)
2	Lead acetate test:	P (++)	P (++)
3	Zinc Hydrochloride reduction test:	P (++)	P (+++)
4	Shinoda test (Magnesium hydrochloride reduction test):	P (+)	P (+)
Steroids			
1	Salkowski test:	P (++)	P (+)
2	Libermann-Buchard test:	P (+)	P (++)
3	Libermann's reaction:	P (++)	N
Alkaloids			
1	Dragendorff's test:	P (+)	P (+)
2	Meyer's test:	P (+)	N
3	Wagner's test:	N	N
4	Hager's test:	P (+)	P (+)
5	Tannic acid test:	P (+)	P (+)
6	Murexide test for purine alkaloids:	N	N
Glycosides			
1	Raymond's test:	P (+)	P (+)

2	Legal's test: (test for Cardenoloids)	P (++)	P (++)
3	Bromine water test:	N	P (+)
Cardiac Glycoside			
1	Kellar Kiliani test: (Test for deoxy sugars)	P (+)	P (+)
2	Concentrated Sulphuric acid test:	N	P (++)
3	Xanthoprotein test: (For tyrosin and tryptophan)	P (+)	P (++)
4	Kedde test:	P (+)	P (+)
Anthraquinones glucosides			
1	Borntrager's test:	N	P (+)
2	Modified Borntrager's test for C-glycoside:	N	P (++)
Saponin glycoside			
1	Foam test:	N	P (+)
Tannins and Phenolic compounds			
1	Gelatin test:	N	N
2	5% Fecl3 solution Test:	P (++)	P (+++)
3	Lead acetate solution Test:	N	N
4	Bromine water Test:	N	N
5	Acetic acid solution Test:	P (+)	N
6	Potassium Dicromate solution Test:	P (+)	N
7	Dilute iodine solution Test:	P (++)	P (+)
8	Dilute HNo3 Solution Test:	P (+)	N
9	Dilute NH_4OH and Potassium Ferricyanide solution Test:	P (+)	N
10	Diulte Potassium permegenate solution:	P (++)	N
Carbohydrate			
1	Molisch test:	P (+)	P (+)
2	Benedict's test:	N	P (+)
3	Camnelisation:	N	P (+)
4	Selwinoff's test:	N	N

5	Fehling's test:	N	P (+)
Proteins and Amino acid			
1	Mellons test:	N	P (+)
2	Cysteine test (Sulphur containing amino acid):	N	N
3	Protein contining sulphur:	N	P (+)

Where, P- Positive and N- Negative; (+) Low, (++) Moderate and (+++) High

6.2.4 Total Phenolics content

Being plant secondary metabolites, the phenolics or polyphenols reveal significant free radical scavenging and antioxidant activities by chelating redox-active metal ions, terminating lipid free radical chains, and inhibiting the transfer of hydroperoxide into reactive oxyradicals. Total phenolics contents of extracts were estimated by the method of Folin–Ciocalteau's assay.

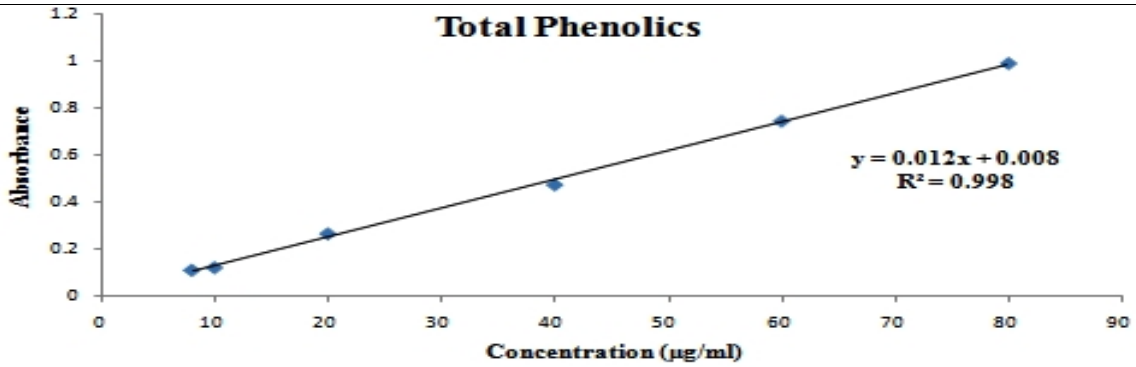


Figure 6.3 Calibration curve of Gallic acid.

Total phenolics contents of extracts was determined through a linear Gallic acid standard curve ($y = 0.012x + 0.008$; $r^2 = 0.998$) and articulated in mg of Gallic acid equivalents. As shown in Table 6.3, PBEA and RCHA have 76.75 mg and 61.61mg Gallic acid equivalent.

Table 6.3 Total phenolics content in extracts.

Sr. No	Extracts	mg of gallic acid/ g of dried extract
1	PBEA	76.75 ± 1.41

2	RCHA	61.61 ± 0.68

6.2.5 Total Flavonoids content

Flavonoids and its subclass have powerful antioxidant activity which depends on the existence of free OH groups, especially both 3-OH and 5-OH. Total flavonoids contents of extracts were estimated by the method of aluminum chloride assay, respectively.

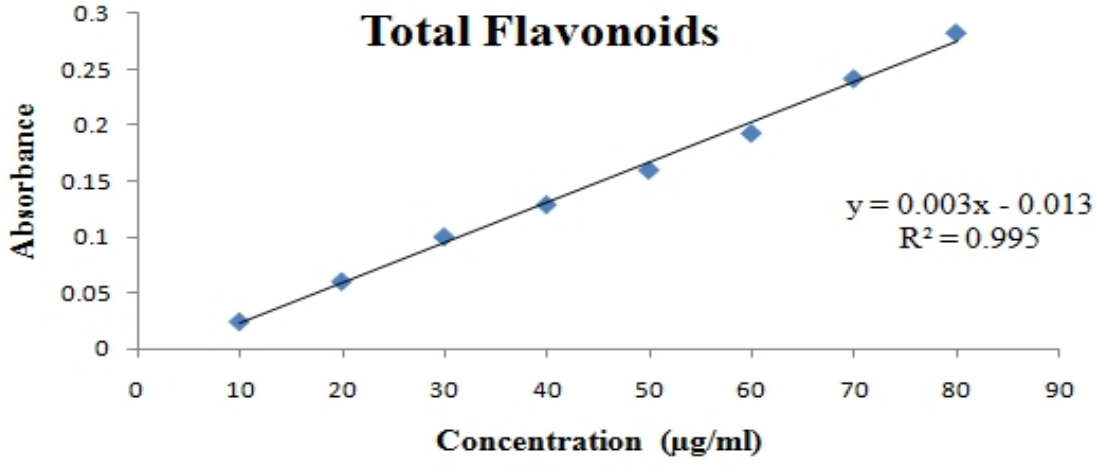


Figure 6.4 Calibration curve of Quarcetin.

Total flavonoids contents of extracts was determined through a linear Quercetin standard curve (y = 0.003x - 0.013; r^2= 0.995) and articulated in mg of Quercetin equivalents. As shown in Table 6.4, PBEA and RCHA have 45.22 mg and 75.00 mg Quercetin equivalent.

Table 6.4 Total Flavonoids content of extracts.

Sr. No	Extracts	mg of Quercetin/ g of dried extract
1	PBEA	45.22 ± 1.65
2	RCHA	75.00 ± 2.11

6.2.6 Quanitative determination of Eugenol by GC-FID

The Eugenol in plants was identified by spiking their retention time with authentic Eugenol standard. Here, Eugenol was found as the more (43.43±1.46) in PBEA. The area from the chromatogram was noted. The identified Eugenol with area are given in Table

6.5 and the figure representing the Eugenol profiling with retention time are given in Figure 6.5, Figure 6.7 and Figure 6.8.

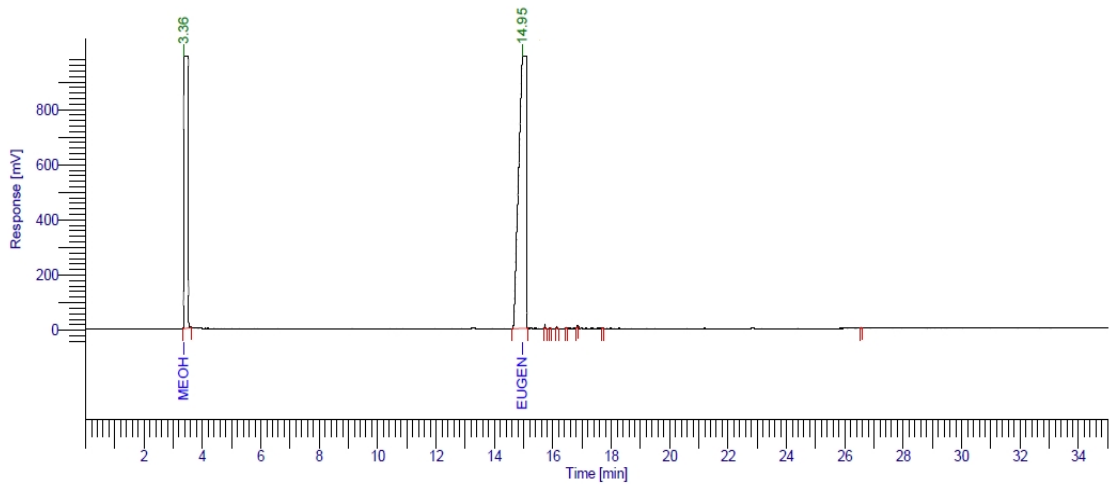


Figure 6.5 Chromatogram of Eugenol.

Table 6.5 Calibration data of Eugenol

Sr. No	Concentration (PPM)	Mean Area
1	10	4931.33
2	100	46073
3	1000	358055.3
4	10000	1838443
5	100000	17734136

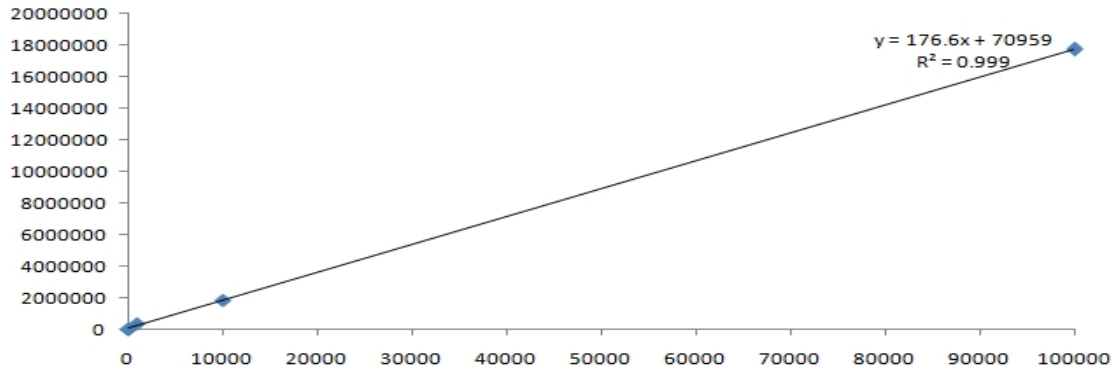


Figure 6.6 Calibration curve of Eugenol by GC-FID.

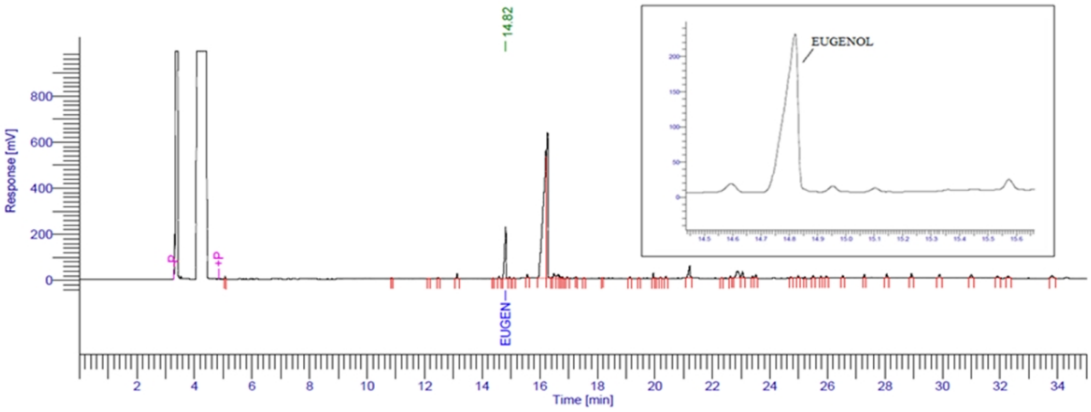


Figure 6.7 Chromatogram of PBEA.

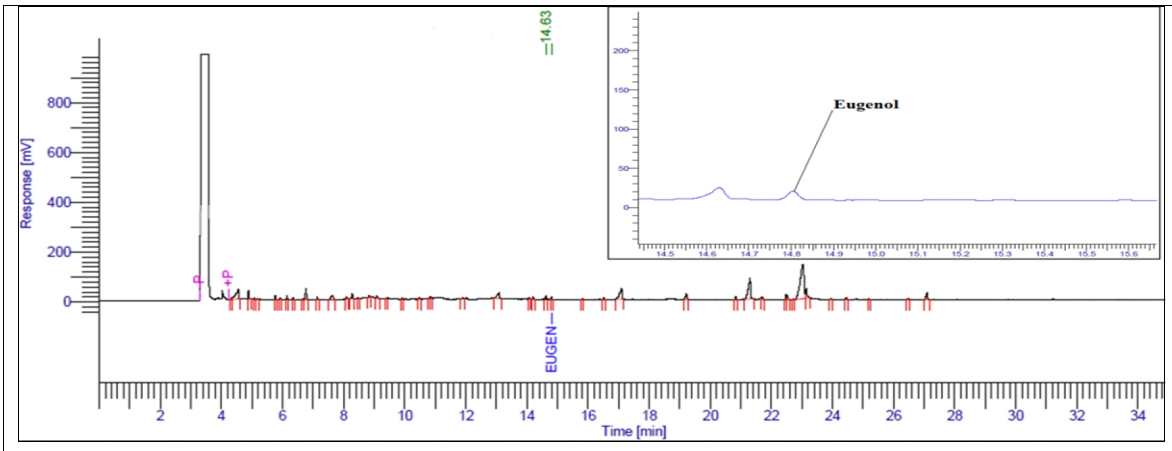


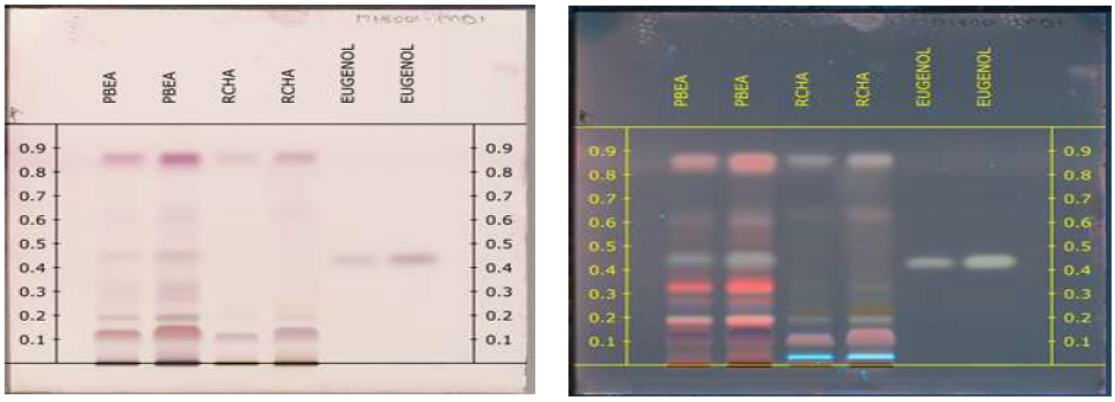
Figure 6.8 Chromatogram of RCHA.

Table 6.6 Quantification of eugenol in plant extracts.

Sr. No	Extarcts	Mean area	mg/g of extarct
1	PBEA	765079.88	43.43±1.46
2	RCHA	22139.06	0.414±0.027

6.2.7 Quanitative determination of Eugenol by HPTLC

The amounts of eugenol in plant extarcts were quantified using a developed HPTLC method. The experiment was carried out by the method as given in section **5.2.1.3.E** of material and methods. Ascending development with toluene: ethyl acetate (9.3:0.7 v/v) mobile phase gave a sharp, symmetrical well and resolved band at the R_f value of 0.45±0.001 in only PBEA that didn't found in RCHA during primary screening.



(b)

Figure 6.9 Primary screening of Eugenol and its identification in PBEA and RCHA.

(a) Image under white light after derivitizing (b) Image under 366 nm after derivitizing.

On the basis of primary screening, HPTLC chromatograms of Eugenol and PBEA were shown in Figure 6.11 and Figure 6.13.

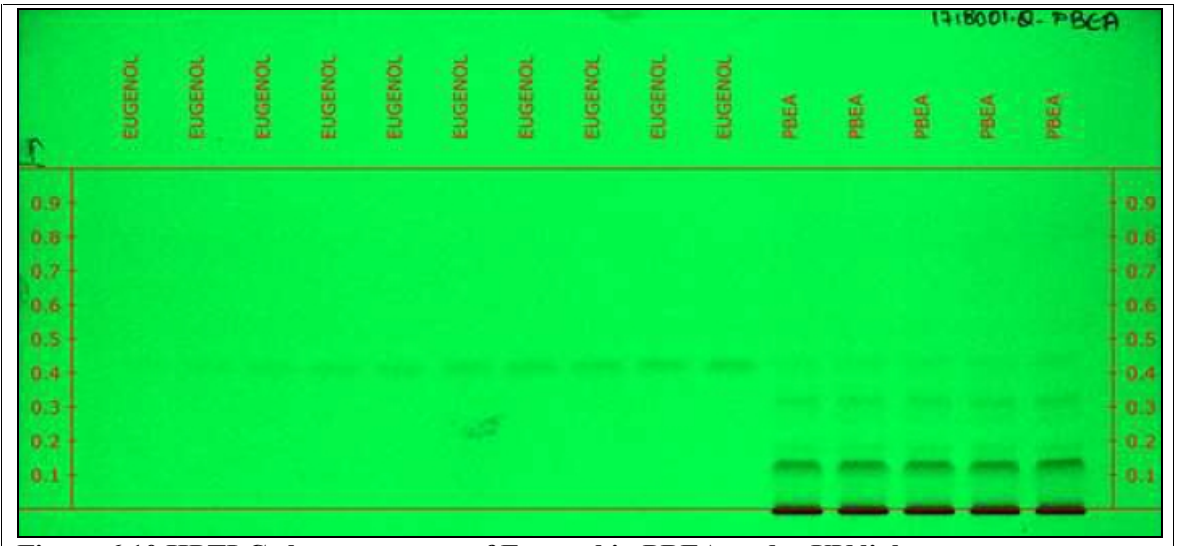


Figure 6.10 HPTLC chromatogram of Eugenol in PBEA under UV light.

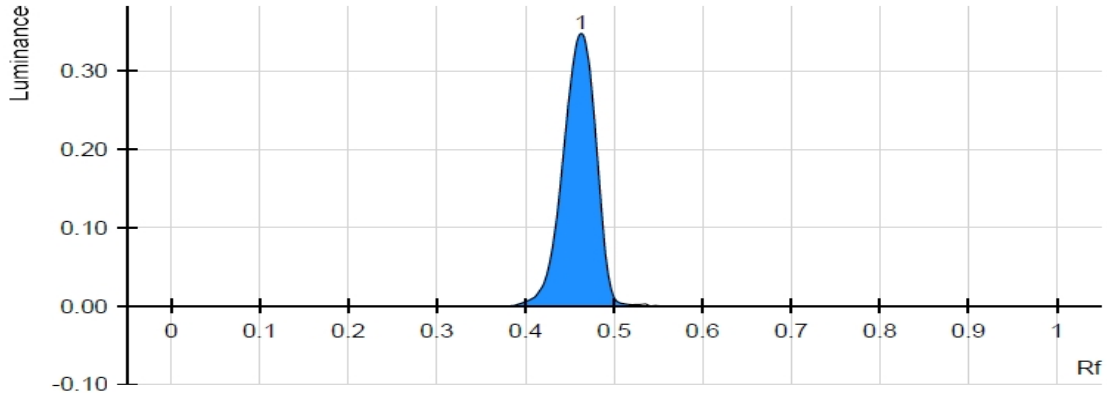


Figure 6.11 HPTLC Chromatogram of Eugenol.

The calibration curve area versus concentration (µg/spot) was found to be linear in the range of 0.2-1.8 µg/spot. The linear regression data for the calibration curve (Figure 6.12) showed a good linear relationship over the concentration ranges of 0.2-1.8 µg/spot with respect to peak area, as shown in Table 6.7. The R_f was found to be 0.45±0.001.

Table 6.7 Calibration data of Eugenol.

Sr. No	Concentration (PPM)	Mean Area
1	0.2	0.0028
2	0.4	0.0050
3	0.6	0.0070
4	0.8	0.0087
5	1.0	0.0103
6	1.2	0.0114
7	1.4	0.0126
8	1.6	0.0139
9	1.8	0.0151

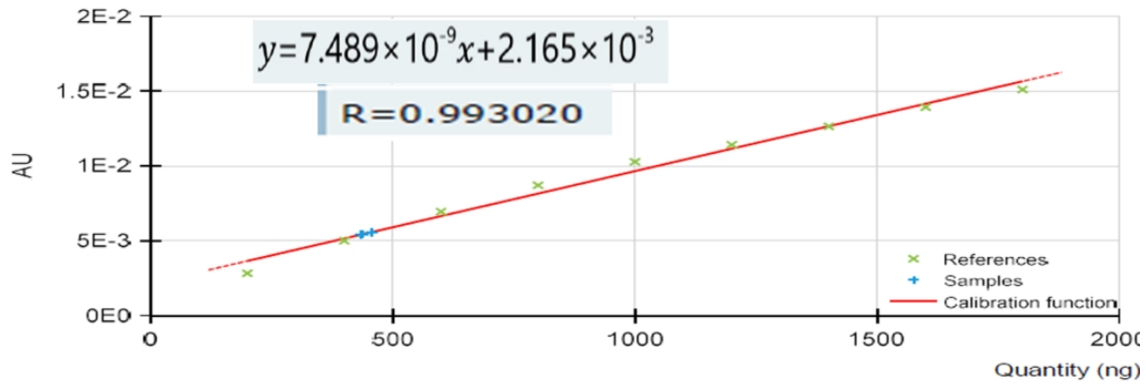


Figure 6.12 Calibration curve of Eugenol by HPTLC.

The linearity range of Eugenol was obtained as 0.2-1.8 µg/spot, as shown in Table 6.7. The regression equation was $y = 7.489\times10^{-9}x+2.165\times10^{-3}$, with a correlation coefficient (R^2) of 0.993020.

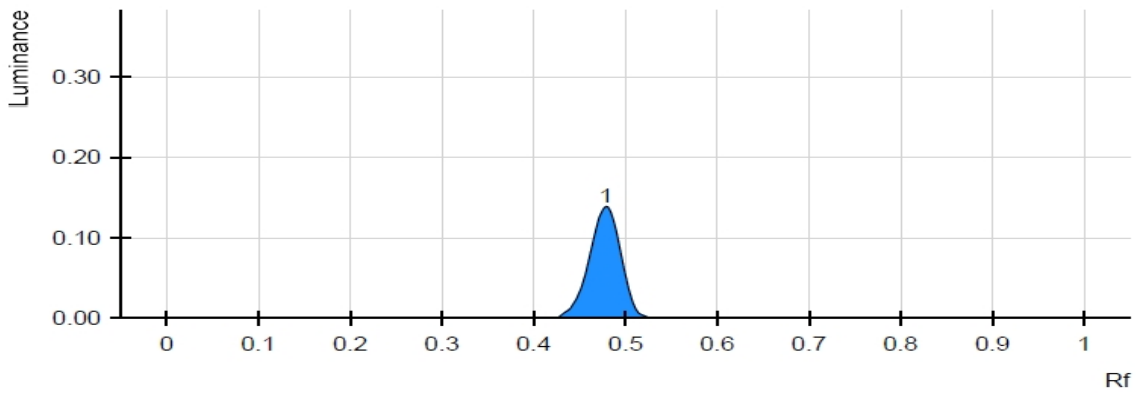


Figure 6.13 HPTLC Chromatogram of PBEA.

6.2.8 Antioxidant activity of PBEA and RCHA: In-vitro

In order to implement more than one antioxidant methods, is required to understand the various oxidation aspects during the evaluation of antioxidant activity. In this circumstance, the antioxidant activity of plant extracts and well known antioxidant were compared. Furthermore, different antioxidant assays vary in terms of assay principle and experimental conditions. For instant, some methods use organic radical producers e.g. DPPH and some use metal ions for oxidation e.g. FRAC assay technique. The time factor associated with their chemical reactions to produce free radicals by oxidation reaction also different from each other. Since the procedure and experimental conditions are

different for different techniques, the various antioxidants are considered as a standard for different assay techniques according to their rate and time of scavenging. In addition, antioxidants could be polar e.g. phenolics, flavanoids etc. or non-polar e.g. vitamin E in nature and they can act as radical scavenger by electron donating mechanism or by hydrogen donating mechanism. Therefore, different standard antioxidants were used for different antioxidant assays.

6.2.8.1 DPPH Assay

Free radical scavenging (hydrogen donors), antioxidant activity of foods and complex biological system was evaluated by the simple and rapid 1,1-diphenyl2-picrylhydrazyl (DPPH) assay. The IC_{50} value (the concentration with scavenging activity of 50%) of PBEA, Ascorbic acid, Quercetin, α-tocopherol, Curcumin and RCHA was found to be 100.1, 53.66, 11.41, 19.96, 22.83 and 32193 µg/ml; respectively for DPPH radical scavenging activity (Figure 6.14). Electron transfer/hydrogen donating ability of extracts contributing radical scavenging activity due to presence of phenolics compounds.

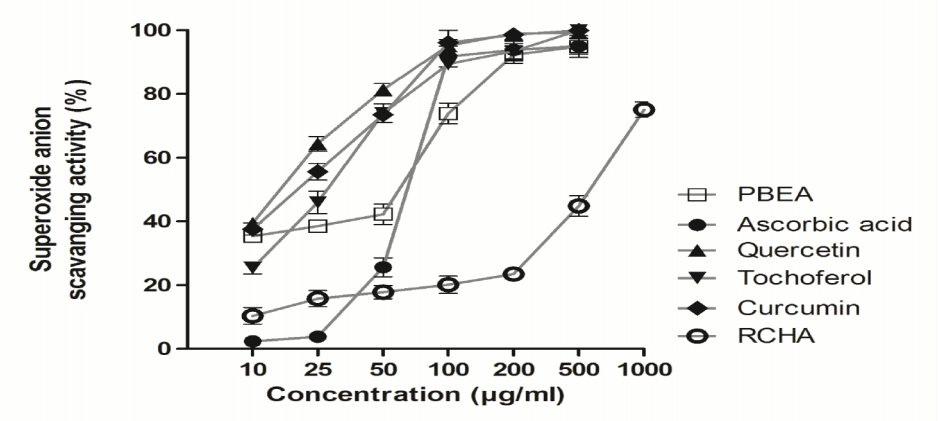


Figure 6.14 DPPH assay of Plant extracts and standards.

6.2.8.2 β-carotenebleaching assay

In another perspective, β-carotene bleaching assay, Potency of extracts for inhibiting the formation of dienehydroperoxides from linoleic acid oxidation was estimated based on the discoloration of yellowish color of a β-carotene solution. In this experiment, the effect of extracts on oxidation of β-carotene/linoleic acid is shown (Table 6.8). Result indicated

that the oxidation of β- carotene by hydroperoxides reduced through the presence of antioxidants in the extracts. Thus, antioxidant activity of the extracts might be attributed to decrease degradation rate of β-carotene.

Table 6.8 β-carotene bleaching assay of Plant extracts and standards

Sample	β-carotene bleaching (% inhibition)
Ascorbic acid	89.31 ± 1.19
Quarcetin	80.6 ± 2.17
α-Tochoferol	83.21 ± 0.93
Curcumin	91.58 ± 1.21
PBEA	73.64 ± 0.84
RCHA	69.11±0.33

6.2.8.3 Superoxide anion radicals scavenging assay

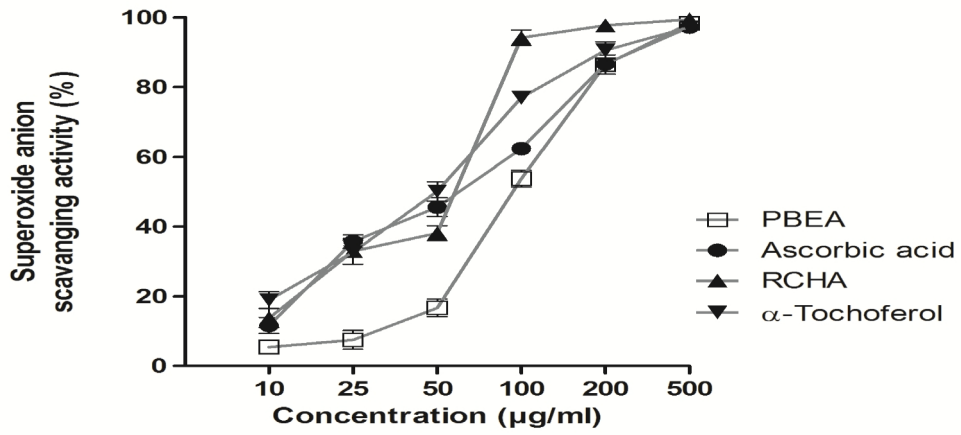


Figure 6.15 Superoxide anion radical scavenging assay of plant extracts.

Superoxide anion radicals engage in the production of powerful and dangerous other reactive oxygen species such as hydroxyl radical, hydrogen peroxide, and singlet oxygen in living system. The results depicted in Figure 6.15, the scavenging activity increased with increasing concentration up to a certain limit (between 100–500µg/ml for PBEA). The highest scavenging activity was found to be 98%, 97%, 99% and 97% at 500µg/ml concentration for PBEA, Ascorbic acid, RCHA and α-Tochoferol; respectively. Those results recommend that the extracts could prevent or ameliorate oxidative stress via superoxide anion radical scavenging activity.

6.2.8.4 Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of extracts was performed via deoxy-ribose degradation. Hydroxyl radical is generating carcinogenesis, mutagenesis, and cytotoxicity through damaging all bio-molecule found in the living cells and leads to DNA strand breakage. The results illustrated in Figure 6.16, the scavenging activity increased with increasing concentration up to a certain limit (between 0.6 – 1 µg/ml for PBEA and RCHA), and over the limit reach a plateau state (data not shown). The IC_{50} value of PBEA, RCHA, and α-tochoferol as a positive control was found to be 0.6, 0.42 and 0.4µg/ml, respectively was extremely efficient on hydrogen radical scavenging.

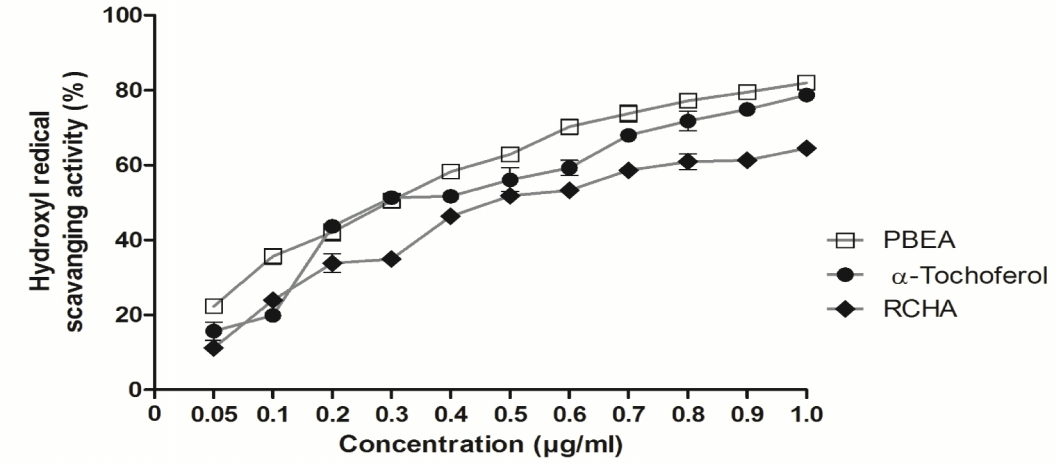


Figure 6.16 Hydroxyl radical scavenging activity of plant extracts.

6.2.8.5 Hydrogen peroxide scavenging activity

H_2O_2 is very toxic if it goes through cellular membranes, which can generate hydroxyl radicals through combine with reactive iron in the cells. The scavenging of hydrogen peroxide by extracts was augmented in a dose-dependent manner as illuminated in Figure 6.17. The IC_{50} value of PBEA, Quercetin, RCHA and BHA was found to be 46.17, 84.57, 73.10 and 52.58µg/ml; respectively. These results imply that PBEA having abundant antioxidative phytochemicals (electron donating/radical quenching) could also shield against cell lysis.

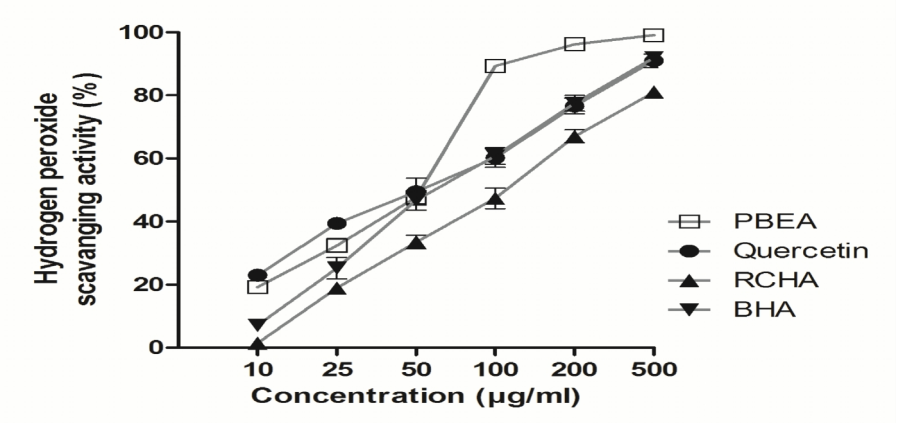


Figure 6.17 Hydrogen peroxide scavenging activity of plant extracts.

6.2.8.6 Reducing Power assay

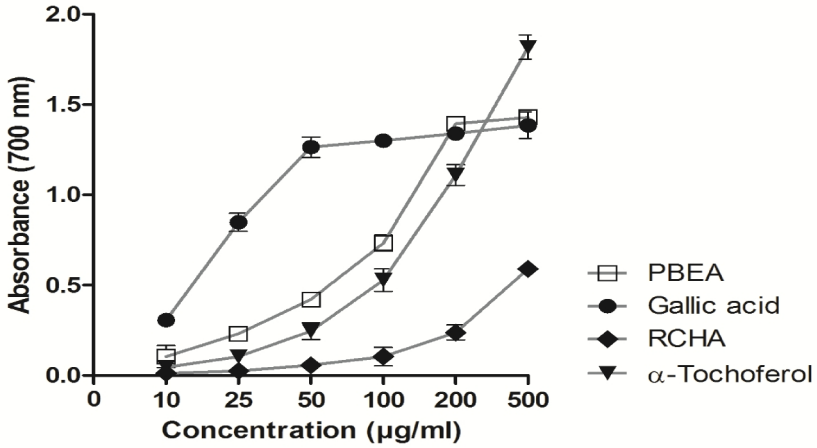


Figure 6.18 Reducing power assay of plant extracts.

For the measurement of the reducing power, antioxidant potency of extracts was carried out via reducing capacity of the Fe^{3+}/ferricyanide complex in the presence of extracts. As mentioned in Figure 6.18, showed the reductive capacity (as indicated by absorbance at 700 nm) of PBEA proportional to Gallic acid as standard and RCHA proportional to α-tochoferol as standard. Reducing activity of PBEA increased in dose-dependent manner to certain level and subsequently leveled off with more augment in concentrations with high correlation index (r^2= 0.998) which is similar to the antioxidant activity. Moreover, Reducing activity of RCHA increased in dose-dependent manner with high correlation index (r^2= 0.999) which is similar to the antioxidant activity. The reducing power, $RP_{0.5}$

$_{AU}$ (defined as the effective concentration (µg per ml) that produces 0.5 absorbance unit at 700 nm for reducing power) of PBEA and RCHA were 73.3 and 503 µg/ml; respectively. The outcome elucidate that polyphenolic content of the extracts which may act as good electron and hydrogen donors, thereby producing more stable products and terminate radical chain reaction.

6.3 Evaluation of 2-APB and Eugenol

6.3.1 IR spectra of 2-aminoethyl diphenyl borinate (2-APB)

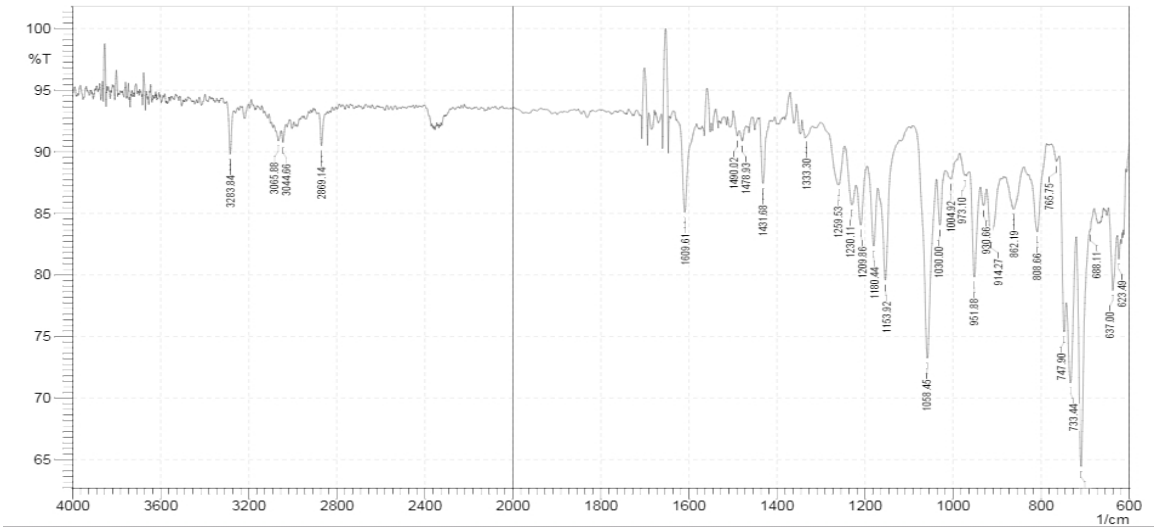


Figure 6.19 IR spectra of 2-APB.

2-APB IR spectrum exhibited N-H stretching vibration at 3283 cm^{-1} and very sharp C-O bond stretching at 1058 cm^{-1}**.**

6.3.2 Selection of Detection Wavelength by UV

The detection wavelength should be the one where drug shows considerable absorbance for the purpose of obtaining good sensitivity. UV spectra of 2-APB showed in Figure 6.20. As can be seen, at 232 nm, drug is having appreciable absorbance and hence 232 nm is selected for detection.

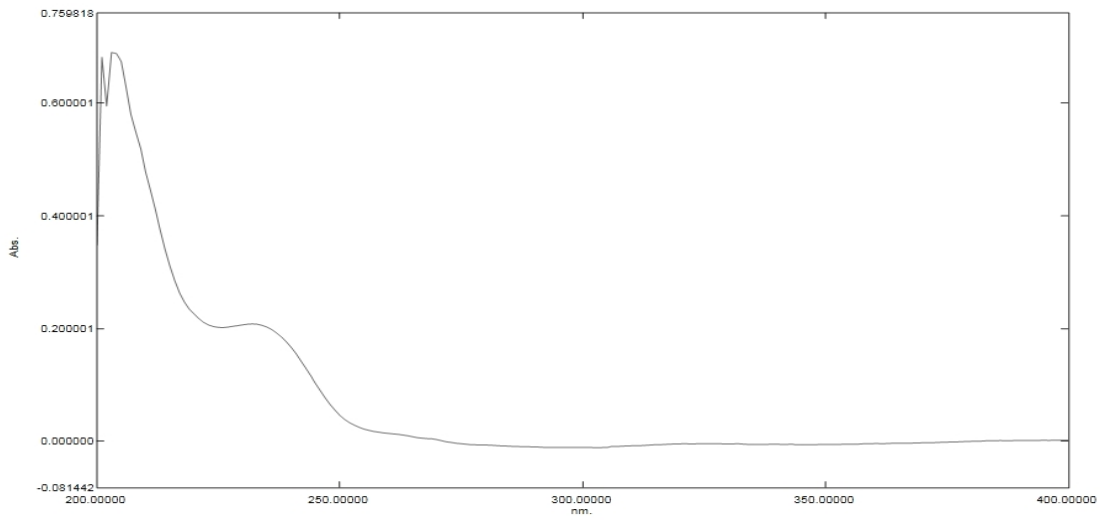


Figure 6.20 Wavelength detection of 2-APB by UV

6.3.3 Optimization of mobile phase

Table 6.9 Different trials of mobile Phase

Sr. No	Mobile Phase	Ratio	Flow rate	RT (min)	Peak shape
1	Methanol: K_2HPO_4(0.01% TFA) pH 3.2	70:30	1	6.3	Splited peak
2	ACN:NH_3PO_4 (pH- 4.0)	80:20	1	8.9	Asymmetric peak
3	ACN:NH_3PO_4 (pH- 8.0)	90:10	1	7.4	Asymmetric peak
4	Methanol: ACN: NH_3PO_4 (pH- 7.0)	40:40: 20	1	8.4	Asymmetric peak
5	ACN: K_2HPO_4(0.01% TFA) pH 3.2	60:40	1	10.3	Bifurcated peak
6	ACN:NH_3PO_4 (pH- 6.0)	90:10	1	7.0	Asymmetric peak
7	Methanol: K_2HPO_4(0.01% TFA) pH 5.0	30:70	1	9.3	Asymmetric peak
8	Methanol:$C_2H_7NO_2$	65:35	1	5.14	Asymmetric peak
9	Methanol: K_2HPO_4 (0.01% TFA) pH 6.2	85:15	1	3.8	Tailing
10	Methanol: K_2HPO_4 (0.01% TEA) pH 8.2	52.5: 47.5	1	7.3	Symmetric well resolved peak

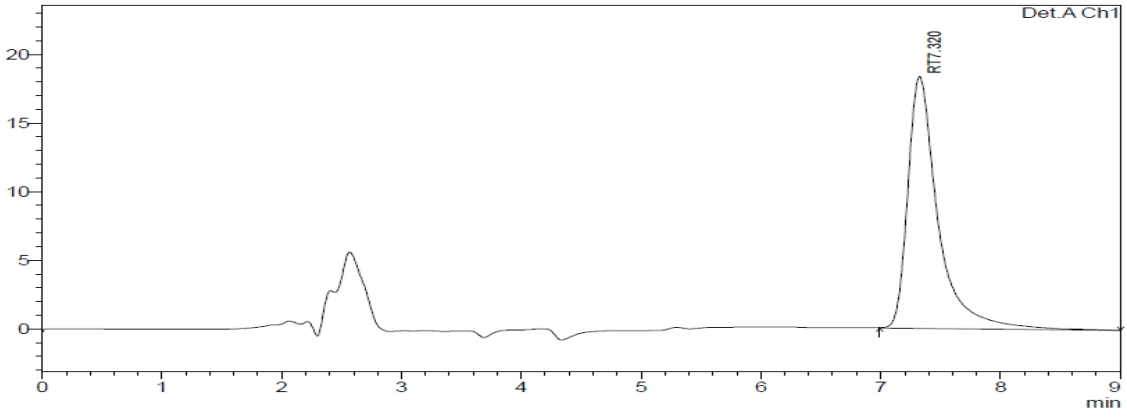


Figure 6.21 Chromatogram of 2-APB by HPLC.

6.3.4 Method validation of 2-APB

6.3.4.1 Linearity and Range

The linearity study was carried out for 2-APB at six different concentration levels. The calibration curve constructed was linear over the concentration range of 10-100 µg/mL 2-APB with correlation co-efficient, slope and intercept values are mentioned in the Figure 6.22.

Table 6.10 Calibration data of 2-APB

Sr. No	Concentration (µg/ml)	Mean area	RSD	%RSD
1	10	325298	6017.43	1.849821
2	20	681565.7	1234.877	0.181182
3	40	1369541	2150.721	0.15704
4	60	2083990	2112.276	0.101357
5	80	2805338	2910.943	0.103764
6	100	3520007	10265.02	0.291619

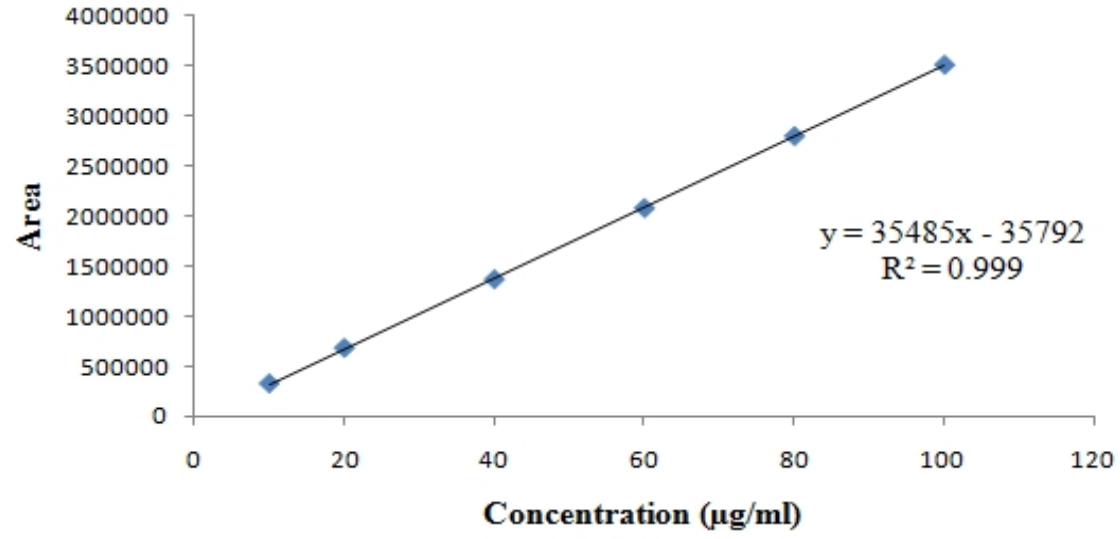


Figure 6.22 Calibration curve of 2-APB.

6.3.4.2 Precision

The average % RSD of intra-day and inter-day was found to be 0.656 and 0.83 respectively. The values confirm the precision of the method.

Table 6.11 Intraday precision for estimation of 2-APB

Sr. No	Concentration(PPM)	Mean	SD	%RSD
1	15	484400.2	3073.553	0.634507
2	50	1628053.07	15985.991	0.98190847
3	90	2932606.708	10650.10287	0.363161649

Table 6.12 Intraday precision for estimation of 2-APB

Sr. No	Concentration (PPM)	Mean	SD	%RSD
1	15	486373.7	3590.407	0.738199
2	50	1636386	22608.69	1.381623
3	90	2932607	14604.57	0.498006

6.3.4.3 Sensitivity

LOD and LOQ for2-APB were found to be 0.53µg/mL and 1.61µg/mL, respectively. These results show that method was enough sensitive for the analysis of formulation.

Hence the method was applicable to formulation. Results of LOD and LOQ are also incorporated in Table 6.13.

Table 6.13 LOD and LOQ of 2-APB

Parameter	Concentration (PPM)
LOD	0.531974
LOQ	1.612043

6.3.4.4 Accuracy

Recovery greater than 98 % with low SD justified the accuracy of the method.

Table 6.14 Recovery from Laboratory sample.

Sr. No	% Spiking	C_{Actual} (PPM)	C_{Found} (PPM)	% Recovery
1	80	36	35.3412074	98.1700206
2	100	40	39.6239736	99.0599339
3	120	44	44.3633258	100.82574

6.3.4.5 System suitability

Following parameters were calculated for system suitability of HPLC method.

Table 6.15 System Suitability Parameters

Sr. No	Parameter	Data Obtained
1	Retention time (min) ± SEM	7.3±0.04
2	Tailing factor ± SEM	1.2±0.03
3	Theoretical plate per meter ± SEM	2400±183.00

6.3.5 Bioanalytical method of 2-APB

Table 6.16 Protein Precipitation Extraction Method Development Trials

Extraction method	Sr. No	Precipitating agent	Results
Protein Precipitation	1	Methanol	Good recovery (about 93%)
	2	Acetonitrile	Low Recovery (Around 40-50%)

	3	Acetone	Low Recovery (Around 35-50%)

6.3.5.1 Method validation of bioanalytical method

6.3.5.1.1 Linearity and Range

Calibration curves were found to be consistently accurate and precise for 2-APB over 0.05-10 µg/ml. The correlation coefficient was 1. 2-APB concentration at each calibration level was back calculated from the calibration curves. The results obtained are shown in Table 6.17 and were meeting the acceptance criteria of $r^2 \geq 0.98$.

Table 6.17 Calibration data of 2-APB bioanalytical method

Sr. No	Concentration (PPM)	Mean	RSD	%RSD
1	0.05	1424.851	17.63119	1.237407
2	0.1	2926.169	64.68038	2.210412
3	0.5	14957.16	328.7839	2.19817
4	1	29950.76	603.1653	2.013857
5	2	60042.94	1006.776	1.676759
6	4	120738.5	2024.494	1.676759
7	6	179470.5	3866.501	2.154393
8	8	240124.5	1643.333	0.684367
9	10	300155.6	2054.167	0.684367

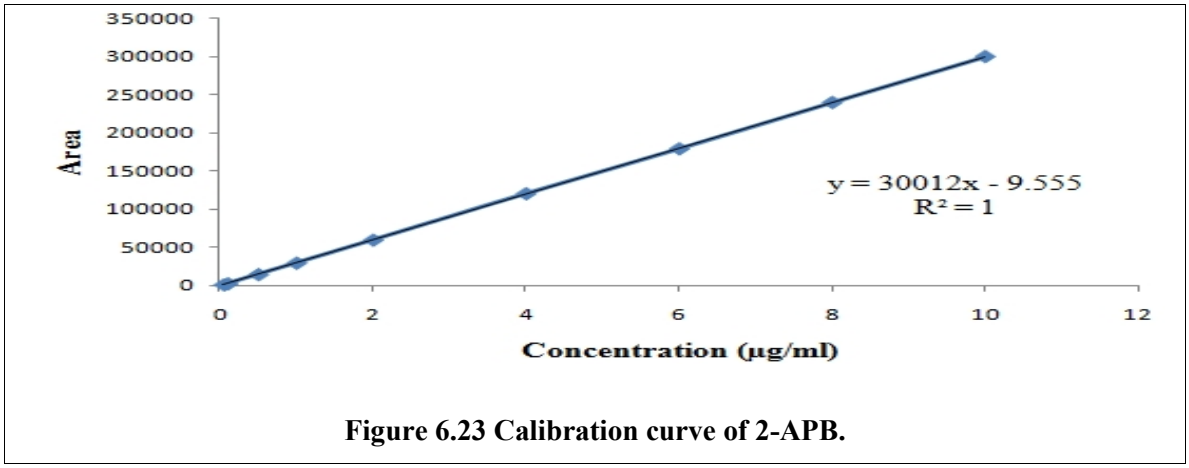


Figure 6.23 Calibration curve of 2-APB.

6.3.5.1.2 System Suitability

Table 6.18 System Suitability Parameters

Sr. No	Parameters	Data obtained
1	Retention time (min) ± SEM	7.31±0.04
2	Tailing factor ± SEM	1.17±0.02
3	Theoretical plate per meter ± SEM	2245±244.00

6.3.5.1.3 Precision

Table 6.19 Intraday precision of bioanalytical method of 2-APB.

Sr. No	Concentration(PPM)	Mean	SD	%RSD
1	0.05	1438.543	30.21059	2.100082
2	0.3	8970.215	115.4115	1.286608
3	3	89993.74	666.5043	0.740612
4	7	211947.5	4417.49	2.084238

Table 6.20 Intrerday precision of bioanalytical method of 2-APB.

Sr. No	Concentration(PPM)	Mean	SD	%RSD
1	0.05	1421.8	65.51911	4.608181
2	0.3	9205.253	225.3447	2.448001
3	3	90330.27	825.6273	0.91401
4	7	212345.3	7735.859	3.643056

6.3.5.1.4 Selectivity

Table 6.21 Selectivity of bioanalytical method of 2-APB

Blank Plasma	Area at LLOQ level	Area of blank plasma at RT min	% Interference
BP-1	1450.2	22.538	1.554130465
BP-2	1398.22	34.32	2.454549356
BP-3	1420.23	38.4	2.703787415
BP-4	1489.18	26.36	1.770101667

BP-5	1430.17	27.33	1.910961634
BP-6	1390.21	30.34	2.182404097

6.3.5.1.5 Recovery

Recovery of analyte were evaluated by injecting three replicates of the aqueous QC samples at each LQC, MQC and HQC concentrations and three replicates of the extracted QC samples at each LQC, MQC and HQC concentrations. Results are presented in Table 6.22.

Table 6.22 Recovery of bioanalytical method of 2-APB.

Replicate No	**HQC**		**MQC**		**LQC**	
	Pure drug	**Extracted drug**	**Pure drug**	**Extracted drug**	**Pure drug**	**Extracted drug**
1	9970.03	9045.23	98670.23	91598.34	232632.5	216289.45
2	9847.21	9123.45	98744.54	92895.12	231728.2	217674.45
3	9956.32	9157.87	98959.34	92985.23	230618.1	214765.45
Mean	9924.52	9108.85	98791.37	92492.89	231659.6	216243.11
SD	67.302	57.721	150.136	776.017	1008.917	0.263
RSD	0.678	0.633	0.151	0.839	0.435	0.000
%Recovery	91.78		93.62		93.34	

6.3.6 Pharmaco-kinetic study

According to pharmacology and toxicology review by CDER, based on plasma profiles, overall pattern of metabolism in humans most closely approximated the metabolite pattern seen in rats. Therefore the method can be easily extended in estimation of 2-APB in human plasma and pharmacokinetic studies.

Table 6.23 Peak area and corresponding concentrations of 2-APB at depicted time points obtained from calibration graph

Sr. No	Time (hr)	Area	Concentration (PPM)
1	0.08	15815.67	0.52666
2	0.16	15681.67	0.522195
3	0.25	13746.17	0.457704
4	0.5	13137.33	0.437418
5	0.75	10395.67	0.346065
6	1	9370.667	0.311912
7	1.5	7951.333	0.26462
8	2	5942.667	0.197691
9	4	2754.167	0.091451
10	8	2368.833	0.078611
11	12	1715.833	0.056853
12	18	1092.833	0.036095

The pharmacokinetic parameters were calculated with a Non-Compartmental model using **Thermo Kinetica PK/PD analysis software** (version 5.0 Thermo Fisher Scientific). The pharmacokinetic data is represented in Table 6.24.

Table 6.24Pharmacokinetic parameters

Sr. No	Parameter	Observed Value
1	Cmax	0.52 µg/ml
2	Tmax	0.08 h
3	AUC Total	2323.51 µg/L*h
4	AUMCTotal	24281 µg/L*(h^2)
5	$t_{1/2}$	8.92 h
6	MRT	10.450 h

6.3.7 IR spectra of Eugenol

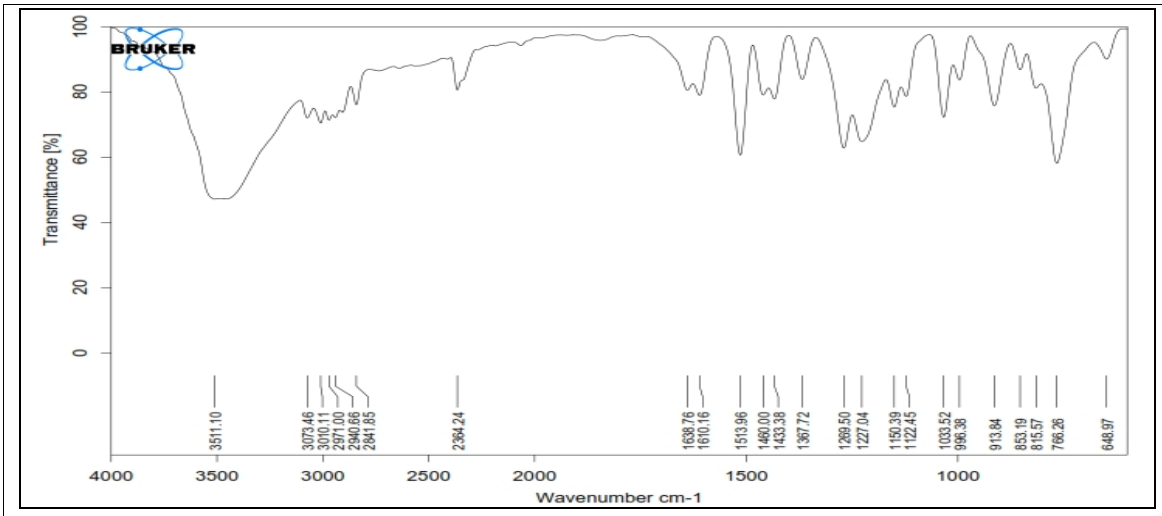


Figure 6.24 IR spectrum of eugenol.

6.4 In-vitro safety and efficacy study of 2-APB, Eugenol, PBEA and RCHA through cellular model.

6.4.1 Cellular studies

6.4.1.1 MTT assay

Cardio protective effect of drugs against H_2O_2 induced cell death of H9c2 cells was determined by MTT assays. The results demonstrated that the viability of cells exposed to 100 μM H_2O_2 for 1 h was 34.84 ± 0.67 % of the control group (Figure 6.24), while pretreatment with PBEA (0.1, 1.0, 10 or 25 μg/ml) protected cells from H_2O_2 induced damage, restoring cell survival to 53.48 ± 1.88%, 67.45 ± 2.09%, 101.81 ± 3.86%, and 110.19 ± 3.10%, respectively. However, the higher concentrations of PBEA were found ineffective because it contains second highest component Eugenol. It has been previously reported that higher concentration of Eugenol was cytotoxic in nature for fibroblasts and H9c2 cells are fibroblastic in nature. Cytotoxic effects of eugenol are due to the formation of a reactive intermediate, possibly a quinone methide.

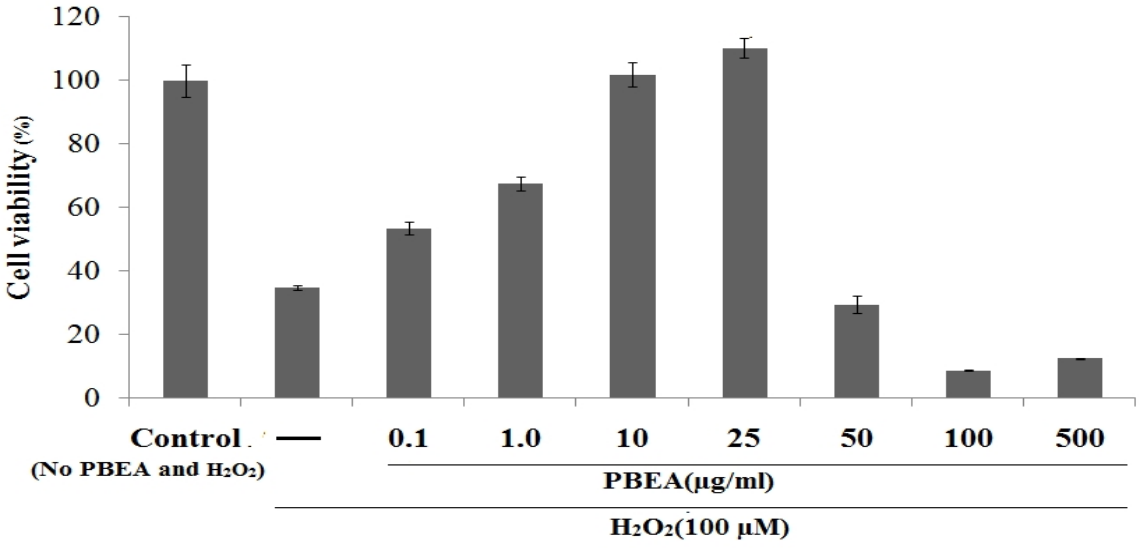


Figure 6.24 Cell cytoprotectivity assay of PBEA.

Moreover, the viability of cells exposed to 100 µM H_2O_2 for 1 h was 16.68 ± 2.68 % of the control group (Figure 6.25), while pretreatment with RCHA (1.0, 10, 50 and 100 ng/ml) protected cells from H_2O_2 induced damage, restoring cell survival to 49.13 ± 2.33%, 52.19 ± 2.53%, 53.67 ± 0.89%, and 62.71 ± 6.22 %, respectively. However, the higher concentrations of RCHA were found ineffective because it has been previously reported that higher concentration of anthraquinones glycoside was cytotoxic in nature.

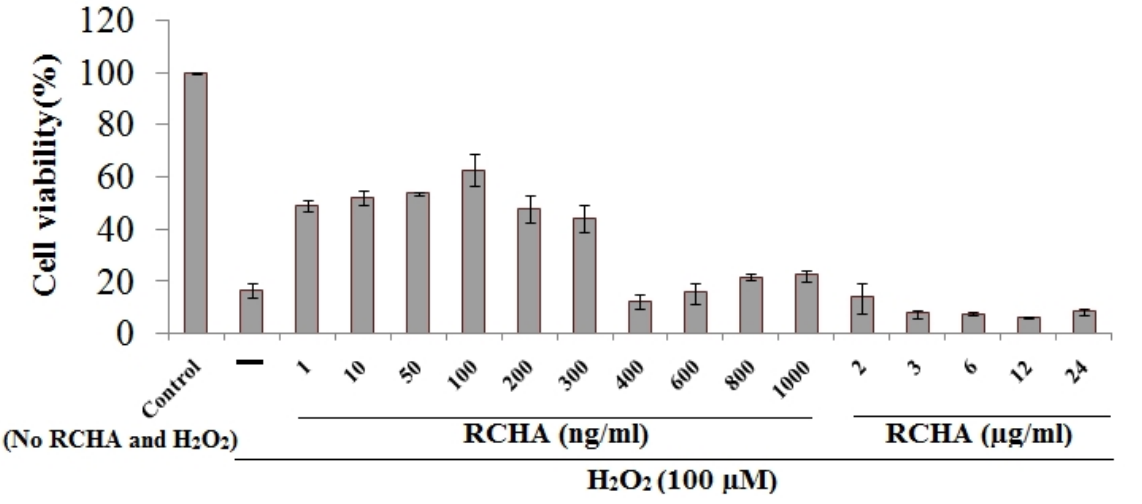


Figure 6.25 Cell cytoprotectivity assay of RCHA.

Moreover, the viability of cells exposed to 100 µM H_2O_2 for 1 h was 49.64 ± 1.51 % of the control group (Figure 6.26), while pretreatment with 2-APB (50, 75, 100, 150 and 300 µM) protected cells from H_2O_2 induced damage, restoring cell survival to 51.72 ± 1.00%, 58.00 ± 4.01%, 59.52 ± 5.59%, 81.06 ± 1.14% and 82.06 ± 3.87; respectively. However, the higher concentrations of 2-APB were found ineffective.

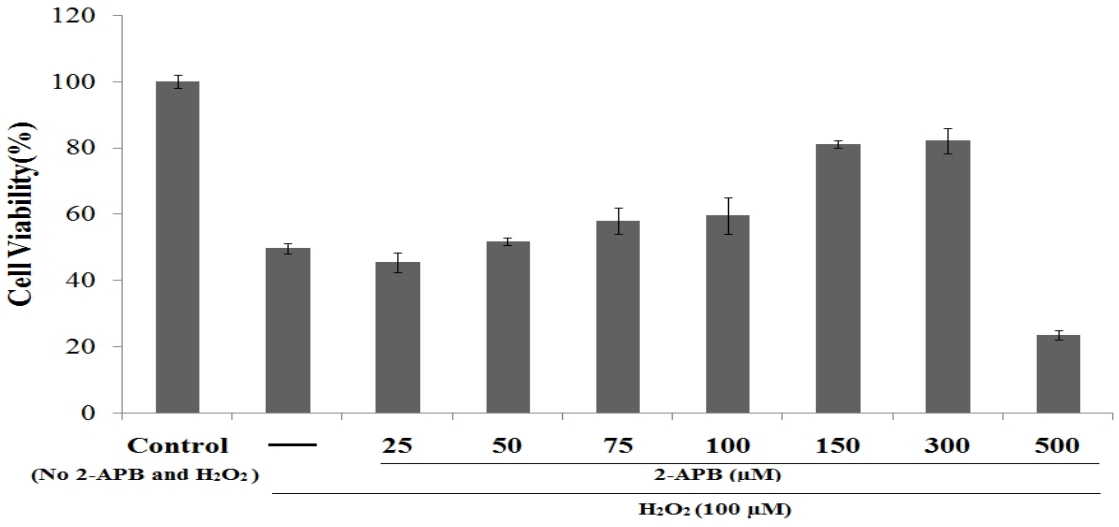


Figure 6.26 Cell cytoprotectivity assay of 2-APB.

Moreover, the viability of cells exposed to 100 µM H_2O_2 for 1 h was 52.63 ± 1.85 % of the control group (Figure 6.27), while pretreatment with Eugenol (25 and 50 mM) protected cells from H_2O_2 induced damage, restoring cell survival to 85.02 ± 1.88% and 98.19 ± 1.68%; respectively. However, the higher concentrations of Eugenol were found ineffective. It has been previously reported that higher concentration of Eugenol was cytotoxic in nature for fibroblasts and H9c2 cells are fibroblastic in nature. Cytotoxic effects of eugenol are due to the formation of a reactive intermediate, possibly a quinone methide.

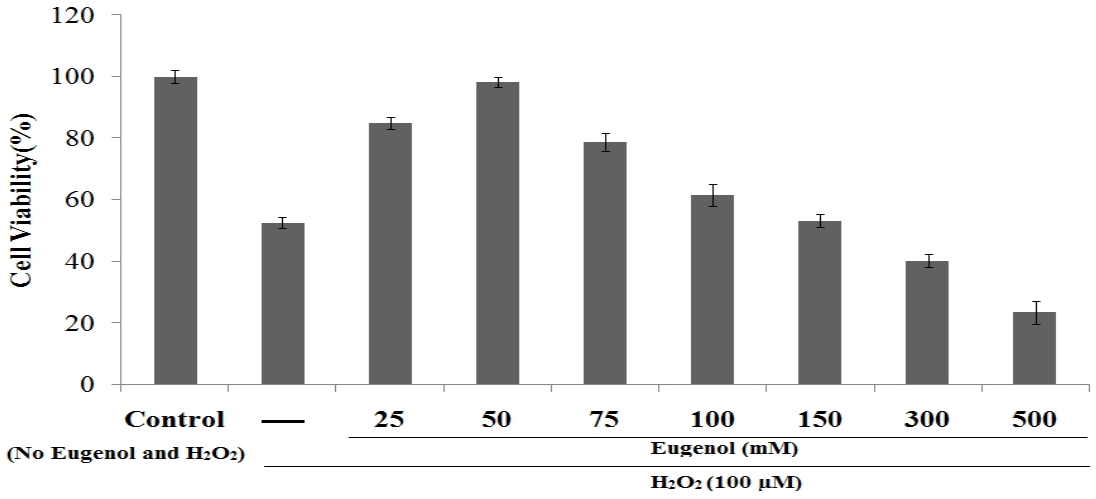


Figure 6.27 Cell cytoprotectivity assay of Eugenol.

6.4.1.2 Intra cellular ROS

H_2O_2 as the main resource of ROS is drawn in multiple factors induced oxidative stress in cardiovascular diseases. The intracellular ROS estimation explores the degree of cellular oxidative stress. In the present experiment, the intracellular ROS generation was significantly ($p < 0.001$) increased in H_2O_2 treated H9c2 cells compared to the untreated control. (Figure 6.28(a)) However, pretreatment of H9c2 cells cultured with 2-APB (150µM), Eugenol (50mM), PBEA (10µg/ml) and RCHA (100 ng/ml) for 24 h and followed by H_2O_2 exposure significantly ($p < 0.001$) decreased ROS generation as evidenced by decreased DCF fluorescence intensity. (Figure 6.28 (b))

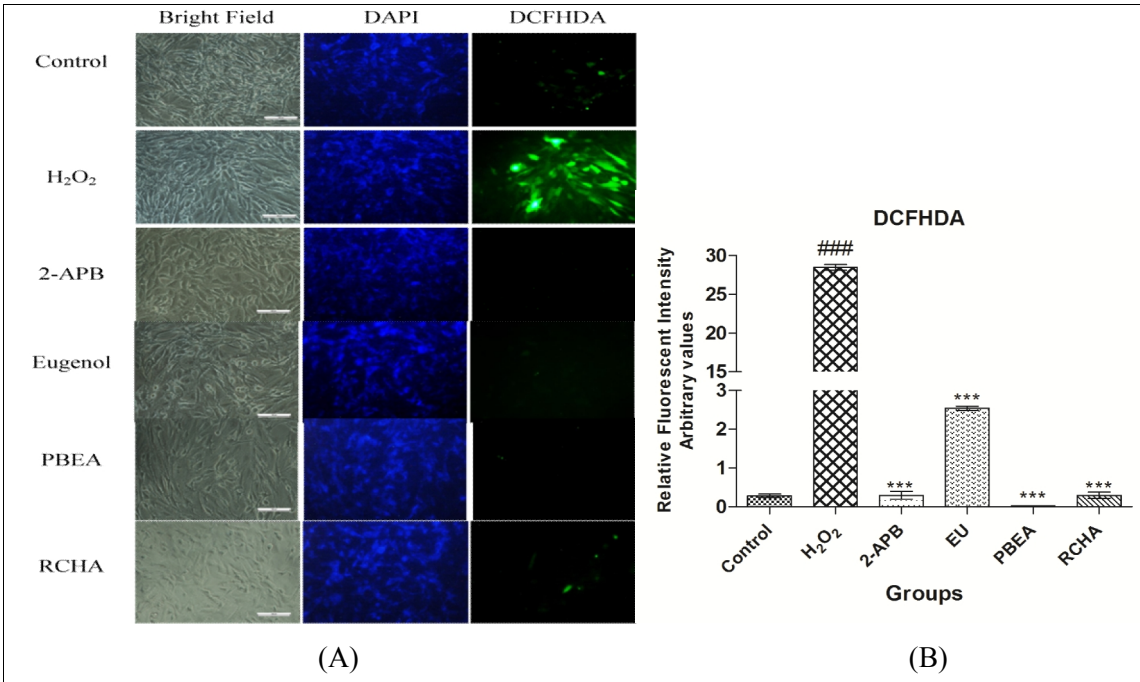


Figure 6.28 DCFH-DA assay of drugs.

(a) Fluorescence images of DCFH-DA (b) Relative fluorescence intensity

6.4.1.3 Cellular antioxidants

SOD, Catalase and GSH are critically involved in protection against various forms of oxidative injuries through the detoxification of ROS. As shown in Table 6.25, H_2O_2 exposed H9c2 cell showed significantly increased lipid peroxidation levels (246.36%) but decreased GSH (60.09%), SOD (71.50%) and Catalase (59.78%) activities. However, pre-incubation with 2-APB (150µM), Eugenol (50mM), PBEA (10µg/ml) and RCHA (100 ng/ml) at the indicated concentration for 24 hours showed decreased lipid peroxidation (55.12%), (30.78%), (61.05%) and (47.51%) whereas increased GSH content (43.27%), (48.89%), (61.65%) and (65.84%), SOD (51.08%), (90.05%), (118.21%) and (70.92%) and Catalase (81.32%), (128.48%), (70.69%) and (69.68%) activities were observed; respectively.

Table 6.25 Effect of drugs in cellular antioxidant enzymes.

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±0.30$^{###}$	184.3±7.782$^{###}$	6.377±1.27$^{###}$	2.54±0.12$^{###}$
2-APB	1.84±0.09***	264.1±19.27**	9.634±0.70	4.60±0.62*
EU	2.84±0.18***	274.5±10.45**	12.12±0.67**	5.80±0.64***
PBEA	1.59±0.06***	298.0±15.85***	13.91±0.68***	4.33±0.40*
RCHA	2.15±0.10***	305.7±19.47***	10.90±0.70*	4.31±0.10*

6.4.1.4 Apoptosis assay

Both apoptotic and necrotic cells were examined qualitatively and quantitatively by Annexin-V/PI dual staining because of corresponding previous observations regarding cell death by H_2O_2 among different cell line. H9c2 Control cell did not show Annexin-V staining with negligible apoptotic nuclei. On the other hand, cell when exposed to H_2O_2 for 1 hr, demonstrated significant increase in the apoptotic nuclei (red) with Annexin-V binding to phosphotidyl serine (green). Nevertheless, pre-incubating of H9c2 cells cultured with 2-APB (150µM), Eugenol (50mM), PBEA (10µg/ml) and RCHA (100ng/ml) for 24 h and later exposed to H_2O_2 significantly inhibited apoptosis (Figure 6.29 (a)). It was observed that both Annexin-V and PI fluorescent intensities when quantified manifested significant reduction after drug pretreatment (Figure 6.29 (b)).

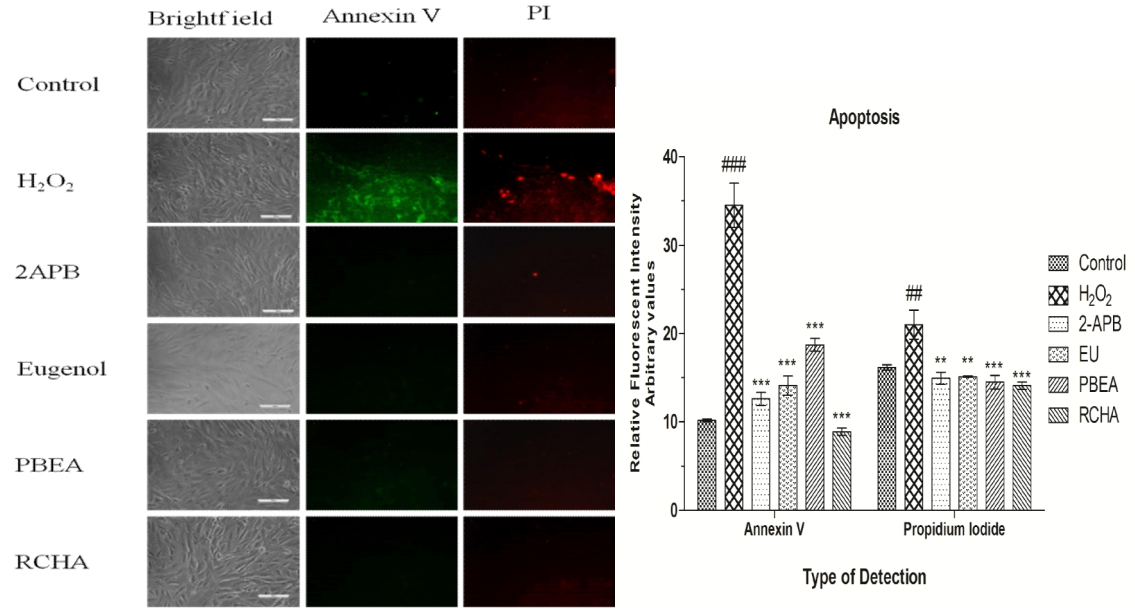


Figure 6.29 Apoptosis assay of drugs.

(a) Fluorescence images of Annexin- PI (B) Relative Fluorescence intensity.

6.4.1.5 Vascular reactivity assay

In the calcium-free isotonic depolarizing solution containing 100 mM KCl, high dose PBEA, RCHA and Verapamil (1 mg/ml, 0.3 mg/ml and 5 µM) significantly attenuated ($P < 0.05$, $P < 0.01$ and $P < 0.001$) the contraction induced by the cumulative addition of calcium (0.1 to 100 mM) compared with the rings untreated with extracts and standards. Concentrations dependent actions are present in Figure 6.30. EC_{50} values are presented in Table 6.26.

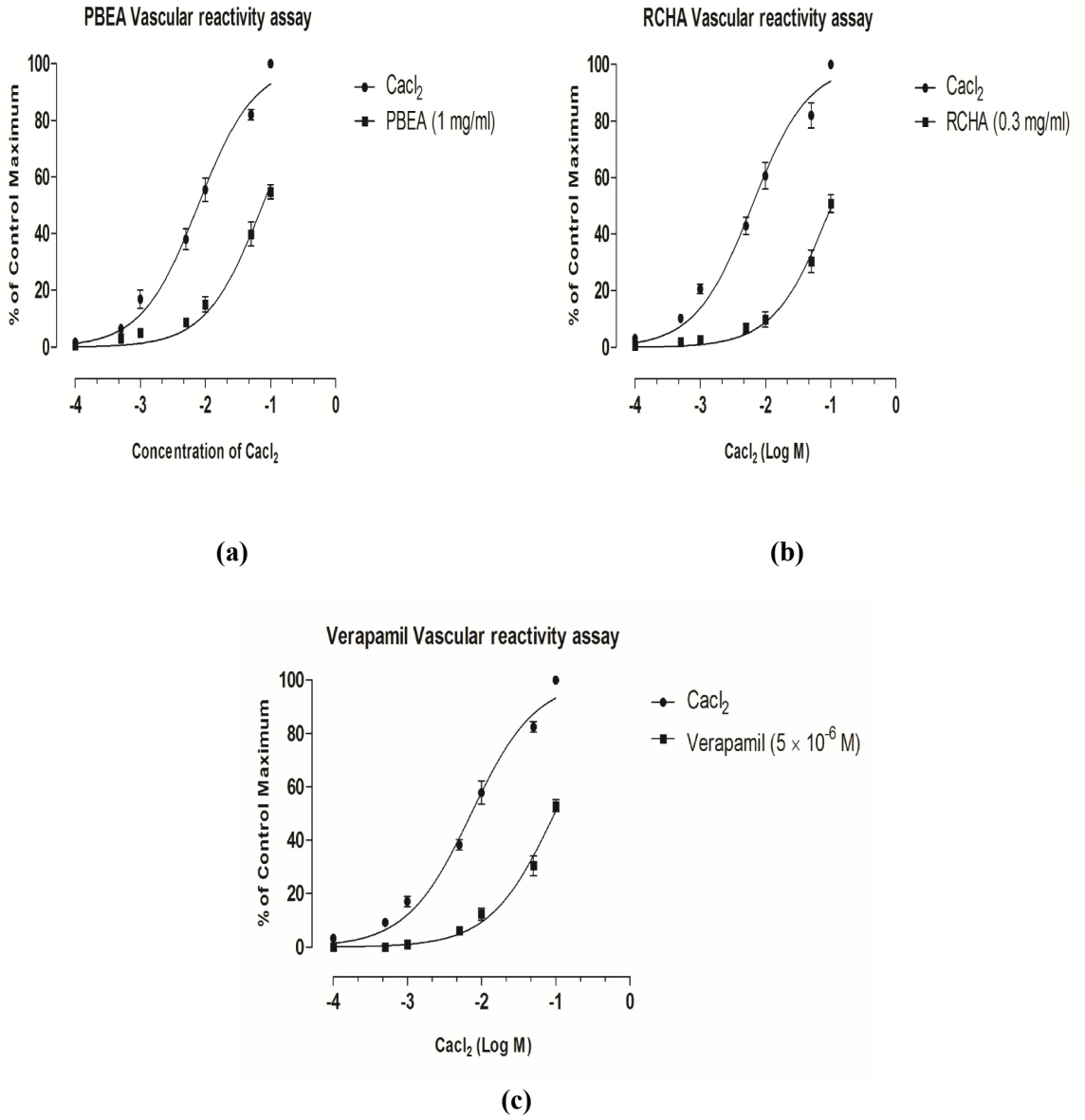


Figure 6.30 Vascular reactivity assay of extracts and standard.

Table 6.26 EC_{50} value of plant extracts

Sr. No	Extracts	EC_{50}
1	PBEA	0.07±0.02
2	RCHA	0.1±0.02
3	Verapamil	0.09±0.02

6.5 Pharmacological evaluation of 2-APB, Eugenol, PBEA and RCHA in selected cardiovascular disorders through in-vivo study.

6.5.1 Angiotensin-II induced acute hypertension in rats.

6.5.1.1 Effect of drugs on systolic blood pressure in Ag-II induced acute hypertension in rats.

Ag-II control group significantly (P<0.001) increased in SBP (19.89±0.98) as compared to control group (5.53 ±0.13). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.001) decreased blood pressure (13.72 ± 0.84, 12.17 ± 0.86, 2.85 ± 0.55 and 8.76 ± 1.33) as compared to Ag-II control groups. (Figure 6.31)

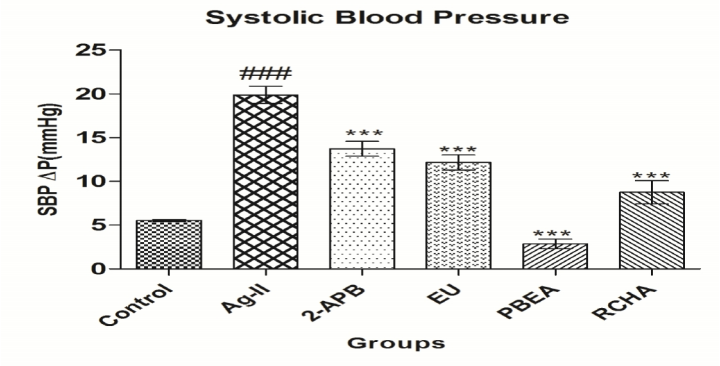


Figure 6.31 Effect of drugs on systolic blood pressure in Ag-II induced acute hypertension.

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 control vs. Ag-II control (###P<0.001); Ag-II control vs. Treatment groups (***P<0.001).

6.5.1.2 Effect of drugs on diastolic blood pressure in Ag-II induced acute hypertension in rats.

Ag-II control group significantly (P<0.001) increased in DBP (17.64 ±0.78) as compared to control group (6.60 ± 0.64). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.001) decreased blood pressure (7.76 ± 0.54, 7.32 ± 0.64, 2.08 ± 0.62 and 12.91 ± 0.96) as compared to Ag-II control groups. (Figure 6.32)

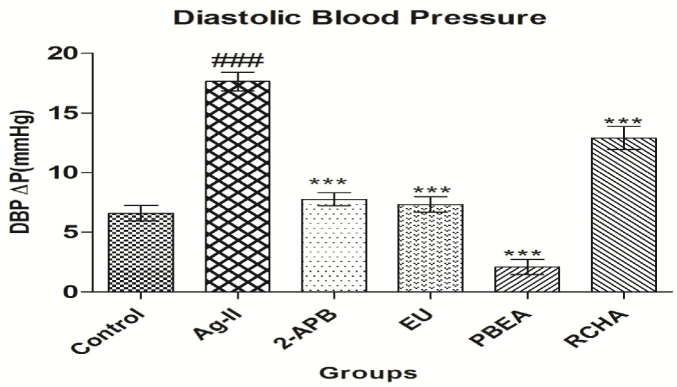


Figure 6.32 Effect of drugs on diastolic blood pressure in Ag-II induced acute hypertension.

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 control vs. Ag-II control (###P<0.001); Ag-II control vs. Treatment groups (***P<0.001).

6.5.1.3 Effect of drugs on calcium level at different time point in Ag-II induced acute hypertension in rats.

Calcium level in Ag-II control group at (-60, 0, 2 and 60 min) insignificantly increased (8.8±0.5, 8.89±0.53, 10.50±0.65 and 10.05±0.63) as compared to control group (9.0±0.29, 9.0± 0.29, 9.10±0.25 and 9.07±0.25). Moreover, Calcium level in Ag-II control group at (0.5 and 1 min) significantly increased in calcium level (11.80±0.58 and 11.50±0.54) as compared to control group (9.12±0.26 and 9.19±0.23). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05) decreased calcium level at 0.5 min (9.70± 0.48, 9.30±0.67, 9.67±0.48 and 9.33±0.64) and 1 min (9.45±0.51, 9.21±0.58, 9.44±0.56 and 9.07±0.57) as compared to Ag-II control groups. (Figure 6.33) In addition, Treatment control group (2-APB, Eugenol, PBEA and RCHA) insignificantly decreased in calcium level at -60 min (9.20±0.40, 9.04±0.70, 9.30±0.46 and 8.90±0.62), 0 min (9.28±0.40, 8.82±0.69, 9.21±0.44 and 8.83±0.63), 2 min (9.05±0.54, 9.26±0.61, 9.13±0.63 and 8.89±0.59) and 60 min (8.84±0.54, 9.14±0.63, 9.02±0.62 and 8.90±0.58); respectively as compared to Ag-II control group.

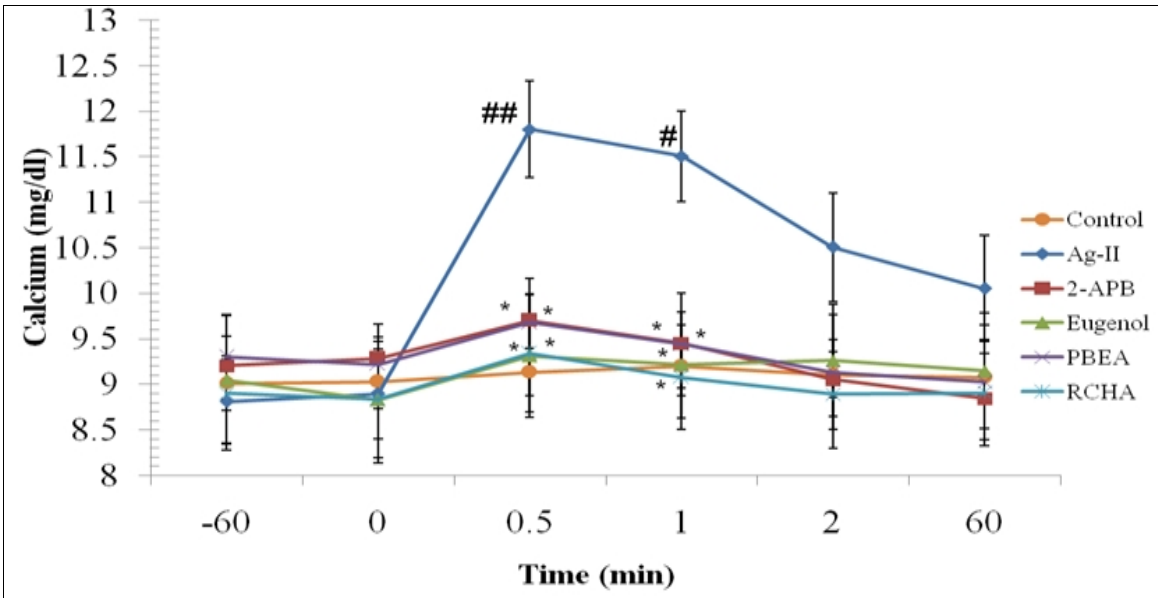


Figure 6.33 Effect of drugs on calcium level at different time point in Ag-II induced acute hypertension.

Each time point 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 control vs. Ag-II control (##P<0.01, ###P<0.001); Ag-II control vs. Treatment groups (*P<0.05).

6.5.1.4 Effect of drugs on sodium level in Ag-II induced acute hypertension in rats.

Ag-II control group significantly (P<0.01) increased in sodium level (187.8±6.40) as compared to control group (148.1±4.12). Treatment control group (2-APB, PBEA and RCHA) significantly (P<0.05) decreased sodium level (156.6±5.98, 151.4±7.21 and 156.5±8.65) as compared to Ag-II control groups. Moreover, Eugenol insignificantly decreased sodium level (167.3±12.13) as compare to Ag-II control group. (Figure 6.34)

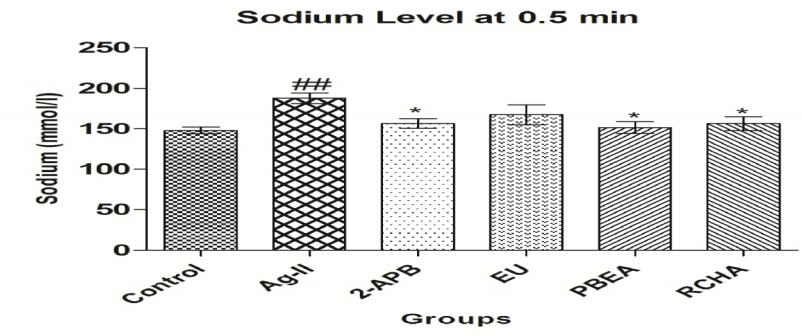


Figure 6.34 Effect of drugs on sodium level at 0.5 min in Ag-II induced acute hypertension.

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 control vs. Ag-II control (##P<0.01); Ag-II control vs. Treatment groups (*P<0.05).

6.5.1.5 Effect of drugs on magnesium level in Ag-II induced acute hypertension in rats.

Ag-II control group significantly (P<0.001) decreased in magnesium level (2.75±0.20) as compared to control group (5.10±0.26). Treatment control group (2-APB and PBEA) significantly (P<0.001 and P<0.05) increased magnesium level (4.52±0.20 and 3.80±0.26) as compared to Ag-II control groups. Moreover, Eugenol and RCHA showed insignificantly increased magnesium level (3.30±0.27 and 3.56±0.19) as compare to Ag-II control group.(Figure 6.35)

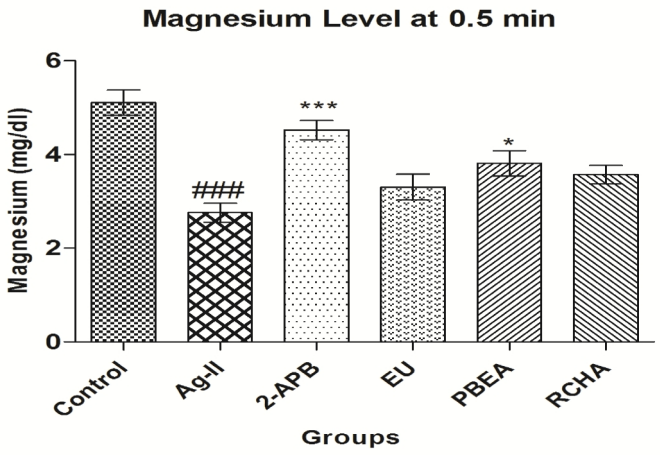


Figure 6.35 Effect of drugs on magnesium level at 0.5 min in Ag-II induced acute hypertension.

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 control vs. Ag-II control (###P<0.001); Ag-II control vs. Treatment groups (*P<0.05 and ***P<0.001).

6.5.1.6 Effect of drugs on heart and kidney malondialdehyde level in Ag-II induced acute hypertension in rats.

Ag-II control group significantly (P<0.01 and P<0.05) increased in heart and kidney MDA level (1.79±0.03 and 2.54±0.25) as compared to control group (1.12±0.20 and 1.47±0.26). Treatment control group (Eugenol, PBEA and RCHA) significantly (P<0.05, P<0.001 and P<0.05) decreased heart MDA level (1.17±0.10, 0.98±0.16 and 1.21±0.12) as compared to Ag-II control groups. In addition, Treatment control group (2-APB and Eugenol) significantly (P<0.05) decreased kidney MDA level (1.36±0.36 and 1.48±0.15) as compared to Ag-II control groups. Moreover, 2-APB didn't decreased heart MDA level (1.76±0.12) but PBEA and RCHA insignificantly decreased kidney MDA level (1.66±0.21 and 1.72±0.22) as compare to Ag-II control group.(Figure 6.36)

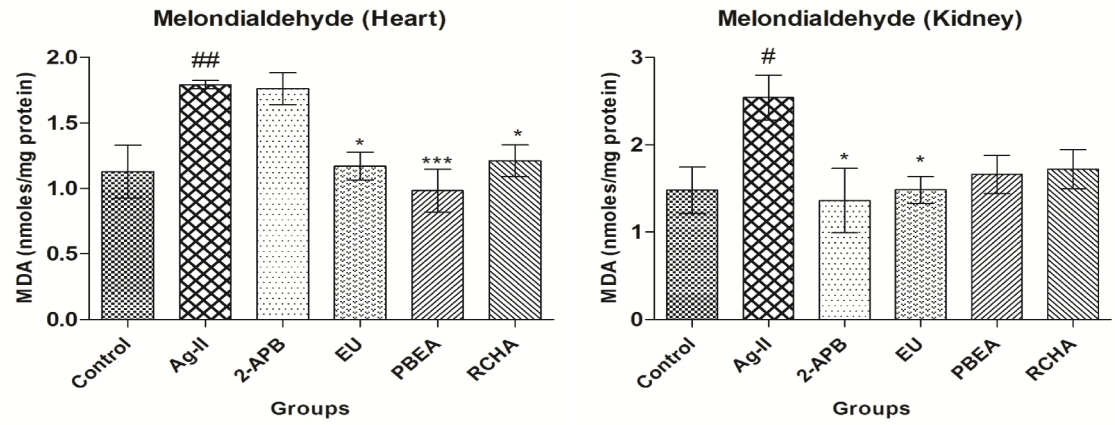


Figure 6.36 Effect of drugs on heart and kidney malondialdehyde level in Ag-II induced acute hypertension.

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 control vs. Ag-II control (##P<0.01 and ###P<0.001); Ag-II control vs. Treatment groups (*P<0.05, **P<0.01 and ***P<0.001).

6.5.1.7 Effect of drugs on heart and kidney superoxide dismutase level in Ag-II induced acute hypertension in rats.

Ag-II control group significantly (P<0.01 and P<0.001) decreased in heart and kidney SOD level (7.13±0.68 and 32.62±4.81) as compared to control group (12.21±1.24 and 73.36±6.55). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05 and P<0.01) increased heart SOD level (11.47±0.72, 11.98±1.37, 12.29±0.91 and 11.56±0.45) as compared to Ag-II control groups. In addition, Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.01 and P<0.001) increased kidney SOD level (60.91±6.25, 74.97±4.70, 70.0±6.45 and 64.45±6.60) as compared to Ag-II control groups. (Figure 6.37)

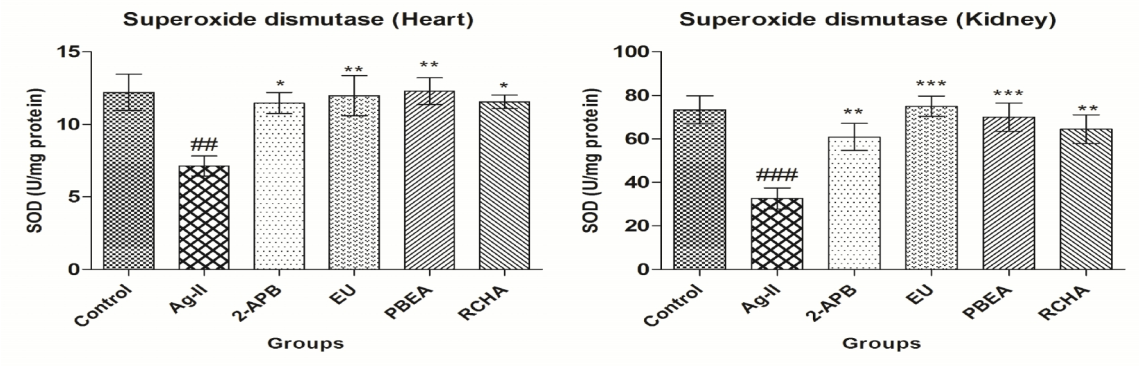


Figure 6.37 Effect of drugs on heart and kidney SOD level in Ag-II induced acute hypertension.

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 control vs. Ag-II control (##P<0.01 and ###P<0.001); Ag-II control vs. Treatment groups (*P<0.05, **P<0.01 and ***P<0.001).

6.5.1.8 Effect of drugs on heart and kidney catalase level in Ag-II induced acute hypertension in rats.

Ag-II control group significantly (P<0.01 and P<0.001) decreased in heart and kidney catalase level (14.83±1.46 and 157.7±12.95) as compared to control group (21.86±1.15 and 334.6±15.64). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05 and P<0.01) increased heart catalase level (20.72±1.16, 21.62±0.48, 21.86±1.38 and 20.03±1.50) as compared to Ag-II control groups. In addition, Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05 and P<0.01) increased kidney catalase level (252.3±27.90, 282.2±19.95, 300.1±31.40 and 275.0±28.74) as compared to Ag-II control groups. (Figure 6.38)

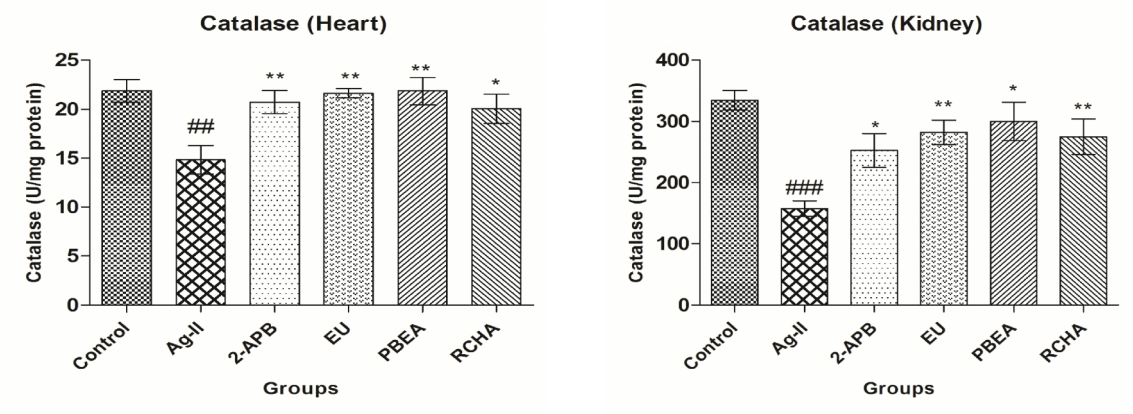


Figure 6.38 Effect of drugs on heart and kidney Catalase level in Ag-II induced acute hypertension.

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 control vs. Ag-II control (##P<0.01 and ###P<0.001); Ag-II control vs. Treatment groups (*P<0.05 and **P<0.01).

6.5.1.9 Effect of drugs on heart and kidney glutathione level in Ag-II induced acute hypertension in rats.

Ag-II control group significantly (P<0.01) decreased in heart and kidney GSH level (11.20±1.54 and 46.17±4.02) as compared to control group (20.35±2.26 and 84.72±5.44). Treatment control group (PBEA and RCHA) significantly (P<0.05) increased heart GSH level (19.54±2.90 and 19.01±1.22) as compared to Ag-II control groups. In addition, Treatment control group (Eugenol and PBEA) significantly (P<0.05) increased kidney GSH level (77.49±10.27 and 80.10±5.77) as compared to Ag-II control groups. Moreover, Treatment control group (2-APB and Eugenol) insignificantly increased heart GSH level (15.94±0.94 and 16.59±1.96) and another treatment control group (2-APB and RCHA) insignificantly increased kidney GSH level (68.54±5.38 and 71.51±9.99). (Figure 6.39)

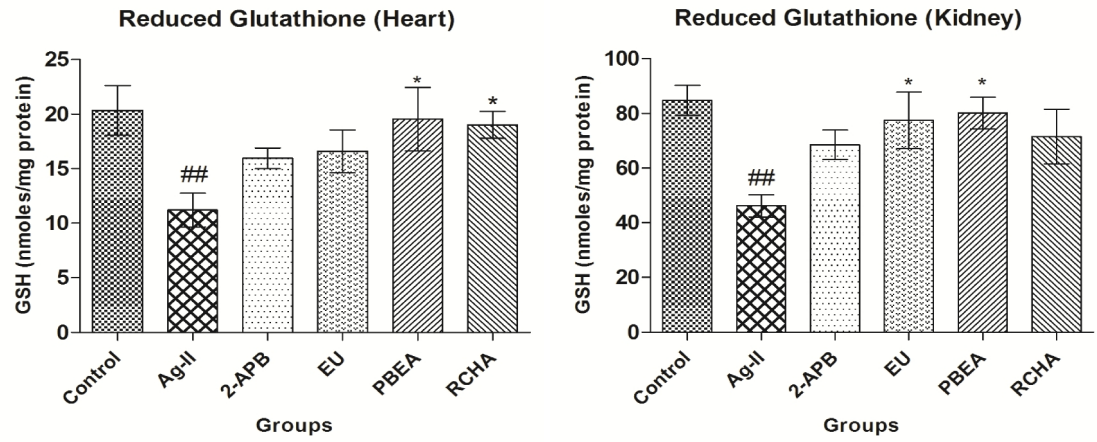
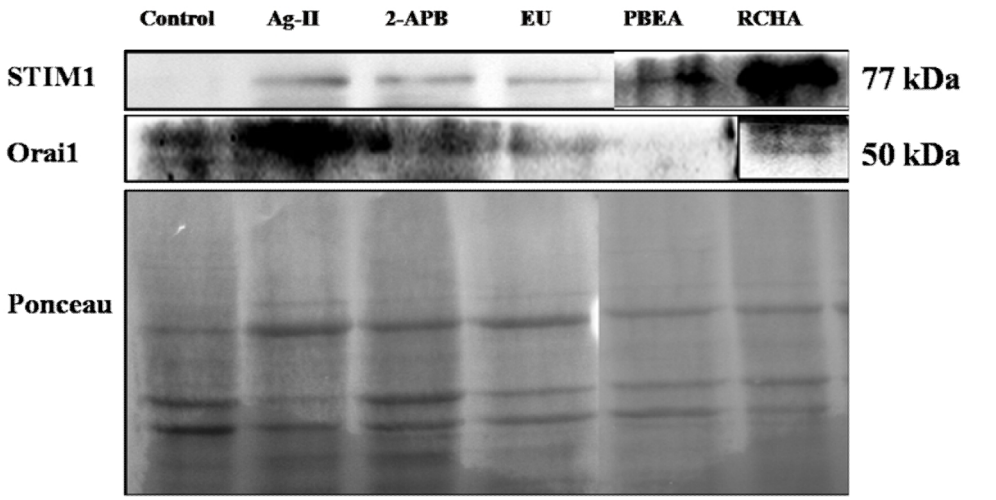


Figure 6.39 Effect of drugs on heart and kidney GSH level in Ag-II induced acute hypertension.

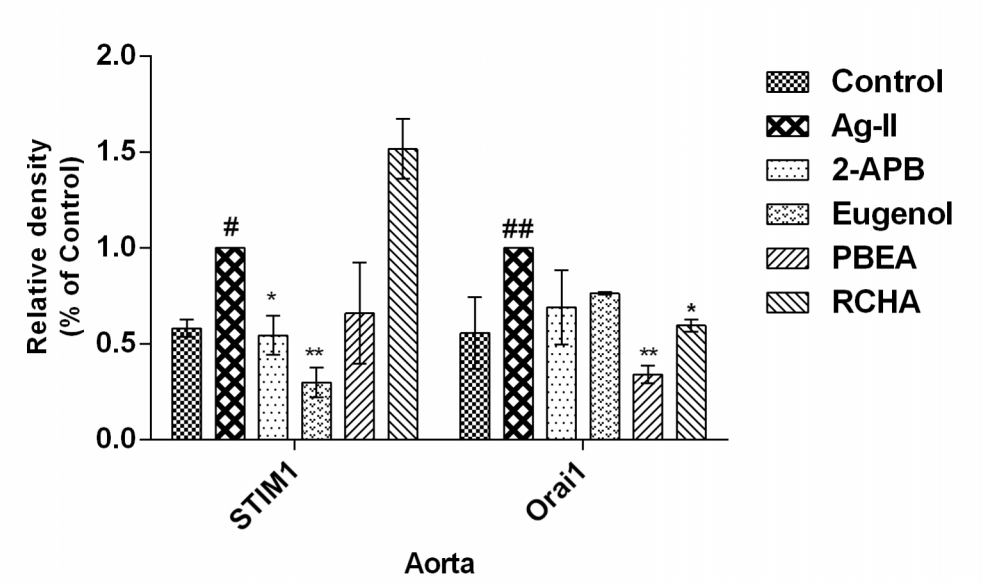
Each bar represents Mean ± SEM of 6 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were compared with control vs. Ag-II control (##P<0.01); Ag-II control vs. Treatment groups (*P<0.05).

6.5.1.10 Effect of drugs on STIM1 and Orai1 in Ag-II induced acute hypertension in rats.

Ag-II administration in rats significantly (P<0.05 and P<0.01) elevated STIM1 and Orai1expression in aorta as compare to control group which is an indication of SOCE activation. However, pretreatment group (2-APB and Eugenol) was significantly (P<0.05 and P<0.01) decreased STIM1 expression in aorta and another pretreatment group (PBEA and RCHA) didn't reverse the STIM1 expression in aorta. Moreover, pretreatment group (PBEA and RCHA) was significantly decreased Orai1 expression in aorta and another pretreatment group (2-APB and Eugenol) didn't reverse the Orai1 expression in aorta. (Figure 6.40)



(a)



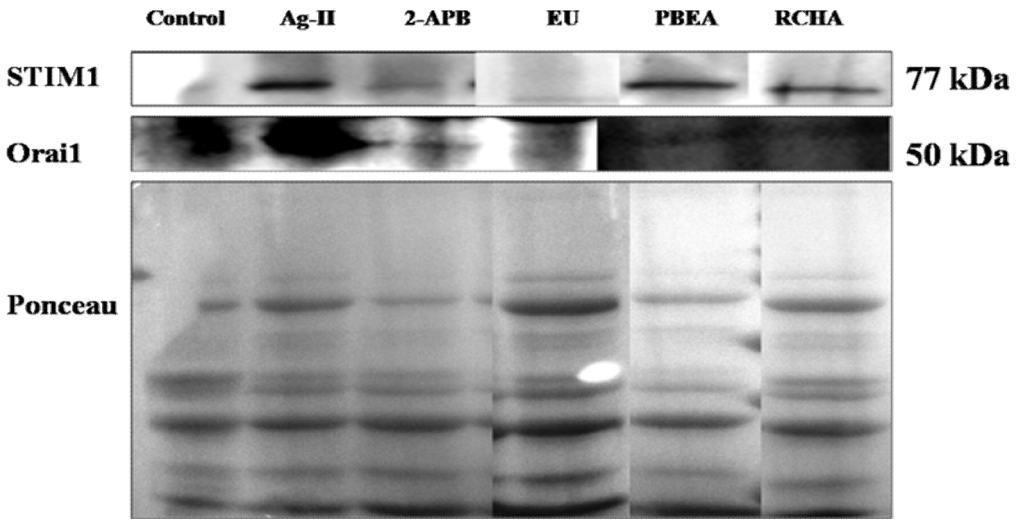
(b)

Figure 6.40 Effect of drugs on aorta STIM1 and Orai1 in Ag-II induced acute hypertension. Immunoblotting assay: (a) Western blot analysis for STIM1 and Orai1 proteins expression of Aorta in Ag-II induced acute hypertension. (b) Densitometry plot for STIM1 and Orai1 proteins.

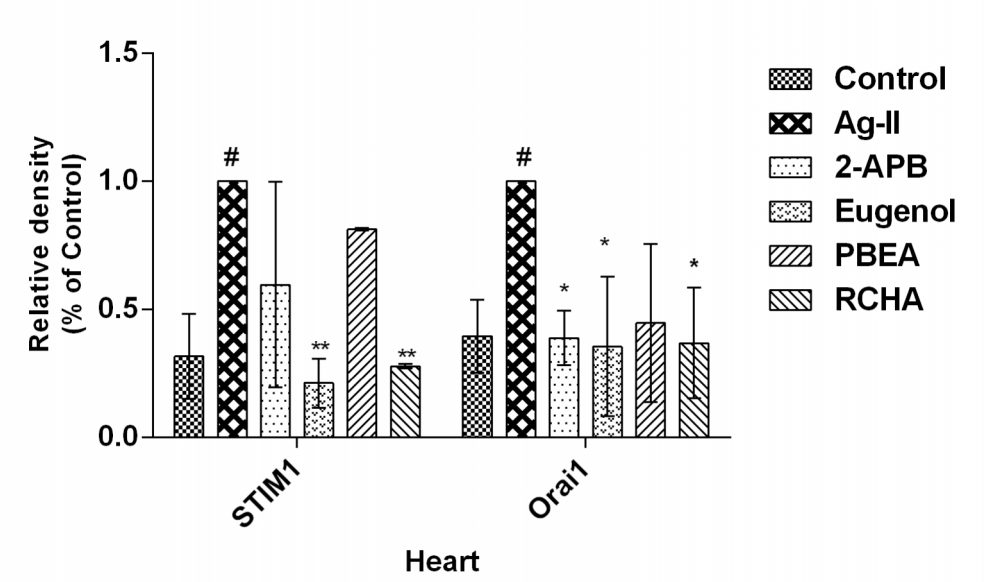
Each bar represents Mean ± SD of 3 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were

compared with control vs. Ag-II control (#P<0.05 and ##P<0.01); Ag-II control vs. Treatment groups (*P<0.05 and **P<0.01).

Ag-II administration in rats significantly (P<0.05) elevated STIM1 and Orai1 expression in heart as compare to control group which is an indication of SOCE activation. However, pretreatment group (Eugenol and RCHA) significantly (P<0.01) decreased STIM1 expression in heart and another pretreatment group (2-APB and PBEA) didn't reverse the STIM1 expression in heart. Moreover, pretreatment group (2-APB, Eugenol and RCHA) significantly (P<0.05) decreased Orai1 expression in heart and another pretreatment group (PBEA) didn't reverse the Orai1 expression in heart.(Figure 6.41)



(a)

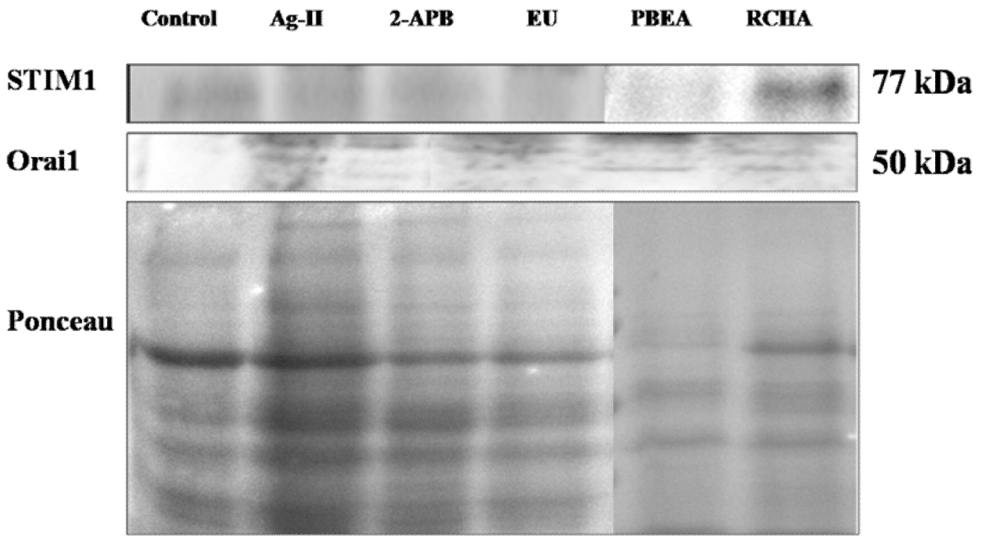


(b)

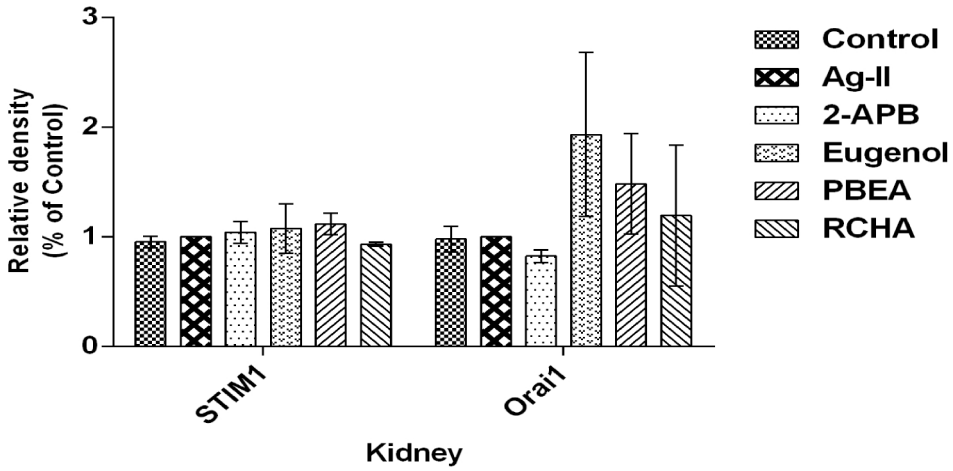
Figure 6.41 Effect of drugs on heart STIM1 and Orai1 in Ag-II induced acute hypertension. Immunoblotting assay: (a) Western blot analysis for STIM1 and Orai1 proteins expression of heart in Ag-II induced acute hypertension. (b) Densitometry plot for STIM1 and Orai1 proteins.

Each bar represents Mean ± SEM of 3 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were compared with control vs. Ag-II control (#P<0.05); Ag-II control vs. Treatment groups (* P<0.05 and **P<0.01).

Ag-II administration in rats didn't elevated STIM1 and Orai1 expression in kidney as compare to control group. (Figure 6.42)



(a)



(b)

Figure 6.42 Effect of drugs on kidney STIM1 and Orai1 in Ag-II induced acute hypertension.

Immunoblotting assay: (a) Western blot analysis for STIM1 and Orai1 proteins expression of Kidney in Ag-II induced acute hypertension. (b) Densitometry plot for STIM1 and Orai1 proteins.

Each bar represents Mean ± SEM of 3 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test.

6.5.2 Isoproterenol induced global ischemia.

6.5.2.1 Pilot studies

6.5.2.1.1 Effect of PBEA and RCHA on CK-MB in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in CK-MB activity (254.0±22.96) as compared to control group (65.92±10.37). PBEA control group (PBEA100 and PBEA200) significantly (P<0.01 and P<0.001) decreased CK-MB activity (165.9±9.62 and 111.1±3.84) and RCHA control group (RCHA 200) significantly (P<0.001) decreased CK-MB activity (142.2±5.87) as compared to ISO control groups. Moreover, another treatment group (PBEA50, RCHA50 and RCHA100) insignificantly decreased CK-MB activity as compare to ISO control groups.(Figure 6.43)

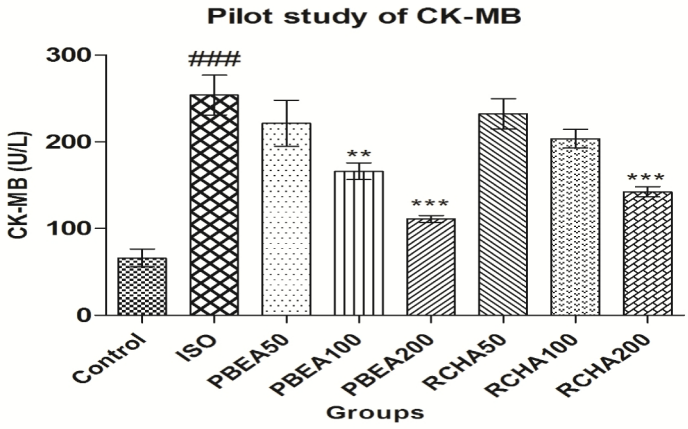


Figure 6.43 Effect of PBEA and RCHA on CK-MB in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (**P<0.01 and ***P<0.001).

6.5.2.1.2 Effect of PBEA and RCHA on LDH in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in LDH activity (842.9±22.60) as compared to control group (317.0±25.83). PBEA control group (PBEA200) significantly (P<0.001) decreased LDH activity (460.7±29.65) and RCHA control group (RCHA100

and RCHA200) significantly (P<0.05 and P<0.001) decreased LDH activity (668.1±32.56 and 542.2±68.86) as compared to ISO control groups. Moreover, another treatment group (PBEA50, PBEA100 and RCHA50) insignificantly decreased LDH activity as compare to ISO control groups. (Figure 6.44)

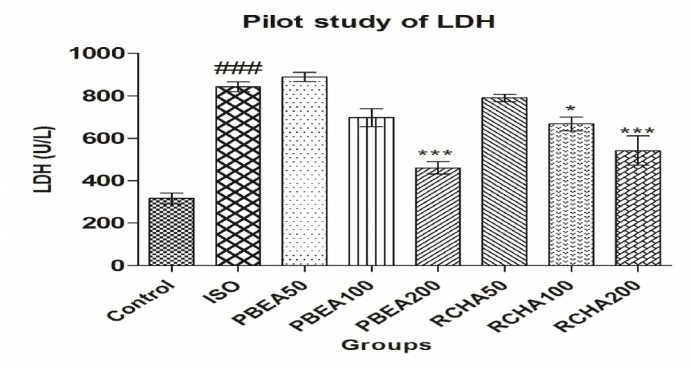


Figure 6.44 Effect of PBEA and RCHA on LDH in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (*P<0.05 and ***P<0.001).

6.5.2.1.3 Effect of PBEA and RCHA on malondialdehyde in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in MDA level (4.92±0.39) as compared to control group (0.71±0.21). PBEA control group (PBEA 100 and PBEA200) significantly (P<0.01 and P<0.001) decreased MDA level (2.99±0.45 and 1.78±0.16) and RCHA control group (RCHA100 and RCHA200) significantly (P<0.01 and P<0.001) decreased MDA level (2.90±0.12 and 2.43±0.42) as ISO control groups. Moreover, another treatment group (PBEA50 and RCHA50) insignificantly decreased MDA level as compare to ISO control groups. (Figure 6.45)

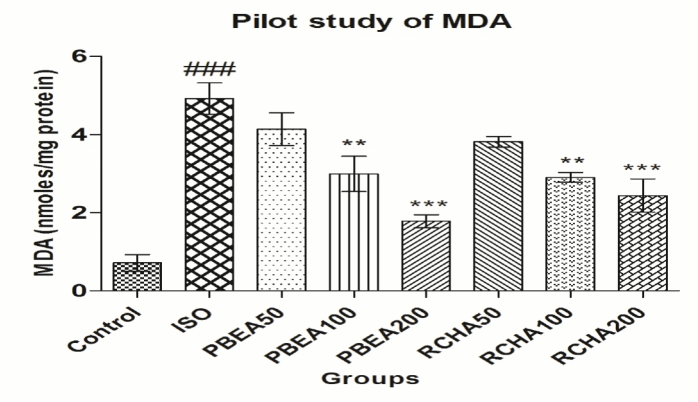


Figure 6.45 Effect of PBEA and RCHA on MDA in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (**P<0.01 and ***P<0.001).

6.5.2.1.4 Effect of PBEA and RCHA on glutathione in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) decreased in GSH level (8.38±1.55) as compared to control group (27.64±2.93). PBEA control group (PBEA100 and PBEA200) significantly (P<0.05 and P<0.001) increased GSH level (19.05±0.56 and 25.88±2.54) and RCHA control group (RCHA100 and RCHA200) significantly (P<0.05 and P<0.01) increased GSH level (17.30±1.28 and 22.08±3.55) as ISO control groups. Moreover, another treatment group (PBEA50 and RCHA50) insignificantly increased GSH level as compare to ISO control groups.(Figure 6.46)

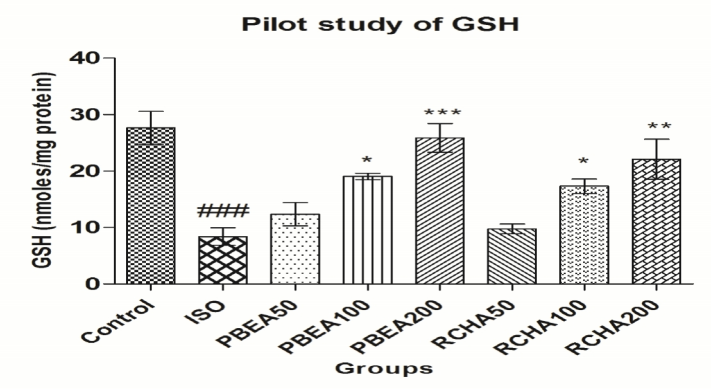


Figure 6.46 Effect of PBEA and RCHA on GSH in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (*P<0.05, **P<0.01 and ***P<0.001).

6.5.2.2 Drug efficacy Study

ISO control group at (0 and 2 h) insignificantly increased (9.55±0.40 and 11.0±0.37) calcium level as compared to control group (9.91±0.33 and 9.95±0.33). ISO control group at (4, 6, 8, 12, 24, 28, 30, 36 and 48 h) significantly increased in calcium level (12.65±0.40, 12.80±0.43, 12.91±0.40, 13.21±0.40, 13.55±0.42, 14.32±0.37, 14.52±0.40, 14.62±0.42, and 14.73±0.43) as compared to control group (9.94±0.34, 9.89±0.36, 9.85±0.33, 9.94±0.34, 9.91±0.34, 9.90±0.34, 9.96±0.34, 9.94±0.33 and 9.94±0.33). Moreover, 2-APB control group at (4, 6, 8, 12, 24, 28, 30, 36 and 48 h) significantly (P<0.001) decreased calcium level (10.11±0.33, 10.31±0.28, 10.43±0.29, 10.70±0.32, 10.81±0.32, 11.13±0.31, 11.25±0.31, 11.37±0.29 and 11.52±0.27) as compared to ISO control groups. 2-APB control group at (0 and 2 h) insignificantly decreased (9.73±0.30 and 9.72±0.32) calcium level as compared to ISO control group (9.55±0.40 and 11.0±0.37). (Figure 6.47)

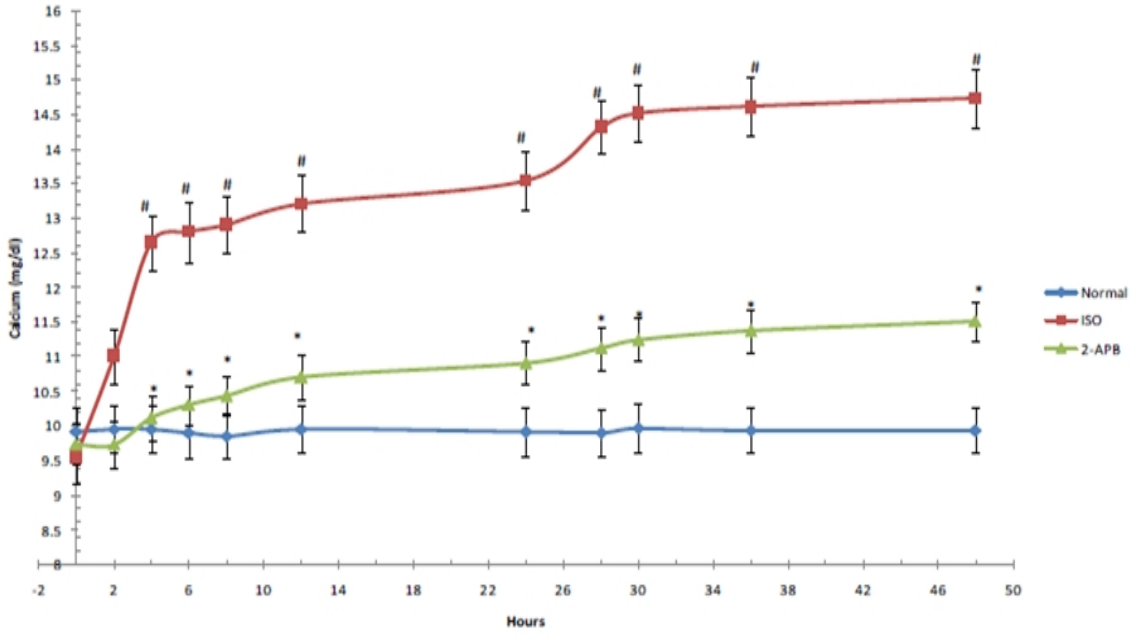


Figure 6.47 Effect of 2-APB on calcium level at different time point in ISO induced global ischemia.

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

6.5.2.3 Main studies

6.5.2.3.1 Effect of drugs on blood pressure in ISO induced global ischemia in rats.

ISO control group significantly (P<0.05 and P<0.001) decreased in SBP, DBP and MBP (99.17±4.50, 69.0±3.00 and 84.08±2.70) as compared to control group (123.5±2.96, 88.33±3.02 and 105.9±2.61). Treatment control group (Eugenol and PBEA) significantly (P<0.05 and P<0.01) increased SBP and DBP, respectively (110.8±2.56 and 85.17±2.82) as compare to ISO control groups. Moreover, Treatment control group (2-APB, Eugenol and PBEA) significantly (P<0.05 and P<0.01) increased MBP (92.9±1.46, 95.2±2.54 and 97.0±2.13) as compare to ISO control groups. Another treatment group didn't show significantly increased SBP, DBP and MBP. (Figure 6.48)

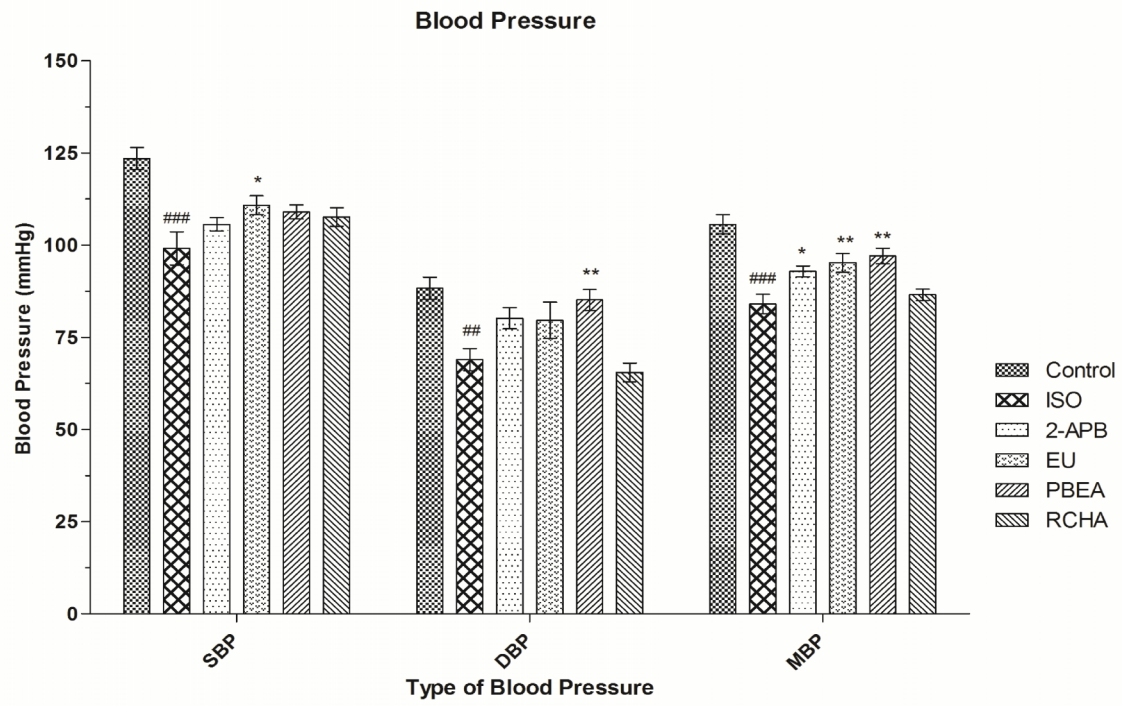


Figure 6.48 Effect of drugs on blood pressure in ISO induced global ischemia.

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 control vs. ISO control (##P<0.01 and ###P<0.001); ISO control vs. Treatment groups (*P<0.05 and **P<0.01).

6.5.2.3.2 Effect of drugs on electrocardiography in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in heart rate as compared to control group. Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.01 and P<0.001) decreased heart rate as compare to ISO control groups.(Table 6.27)

Moreover, ISO control group significantly (P<0.001) elevation in ST-segment as compared to control group. (Figure 6.49) Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.01) decreased ST-segment as compare to ISO control groups. (Table 6.27)

In addition, ISO control group significantly (P< 0.05 and P<0.001) decreased in P wave, QRS complex and R-R intervals as compared to control group. Treatment control group

(2-APB, Eugenol, PBEA and RCHA) significantly ($P<0.05$, $P<0.01$ and $P<0.001$) increased P wave, QRS complex and R-R intervals as compare to ISO control groups. Moreover, ISO control group significantly ($P<0.001$) increased in QT intervals as compared to control group. Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly ($P<0.05$, $P<0.01$ and $P<0.001$) decreased QT intervals as compare to ISO control groups. (Table 6.27)

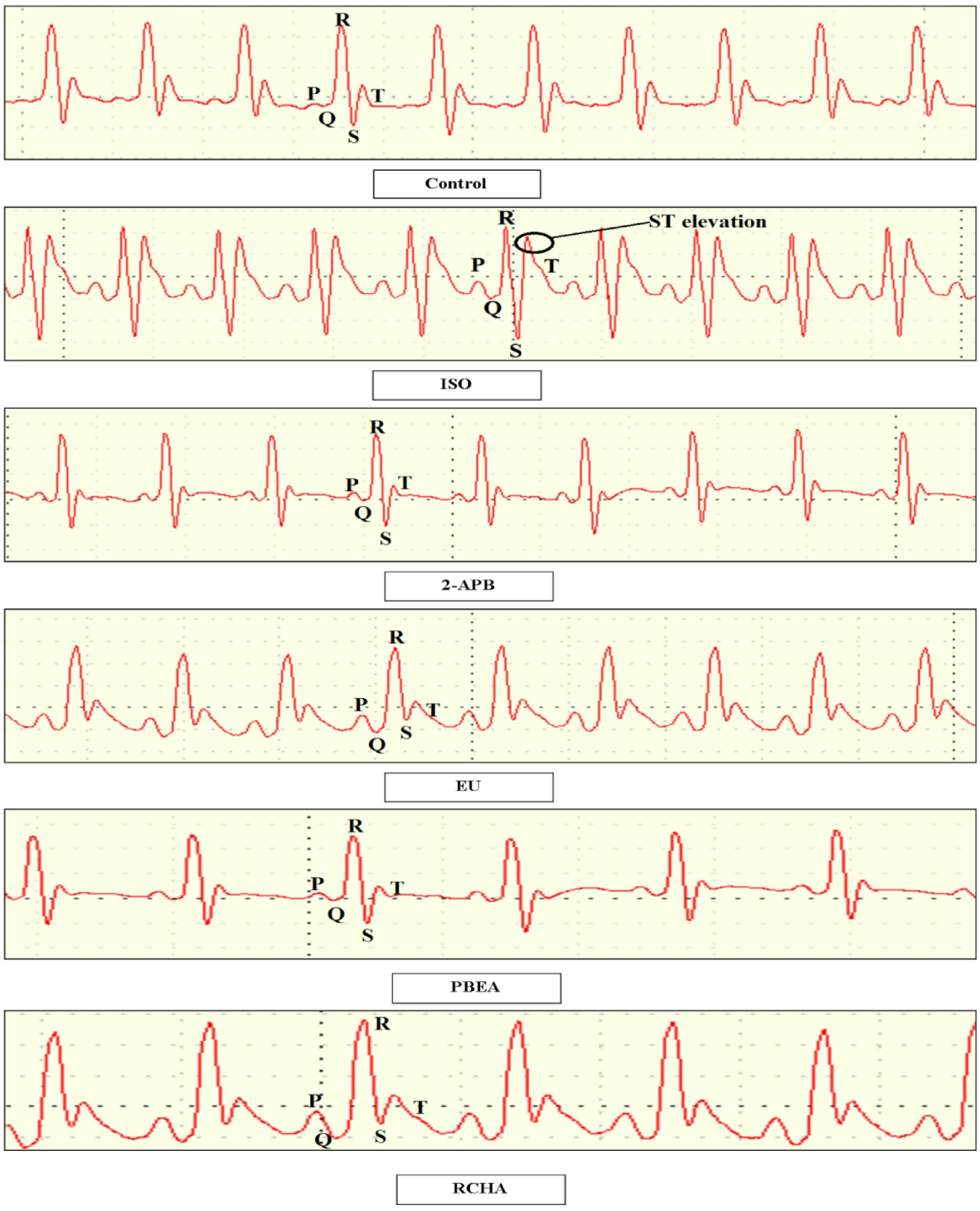


Figure 6.49 Electro cardiographic pattern of different drug on ISO induced global ischemia.

Table 6.27 Effect of drugs on electrocardiography in ISO induced global ischemia.

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**

Each group 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 control vs. ISO control (#P<0.05 and ###P<0.001); ISO control vs. Treatment groups (*P<0.05 and **P<0.01 and ***P<0.001).

6.5.2.3.3 Effect of drugs on hemodynamic parameters in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) decreased coronary flow as compared to control group. Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.001) increased coronary flow as compare to ISO control groups. Moreover, ISO control group significantly (P<0.001) increased in LVEDP as compared to control group. Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05, P<0.01 and P<0.001) decreased LVEDP as compare to ISO control groups. (Table 6.28)

Table 6.28 Effect of drugs on hemodynamic parameters in ISO induced global ischemia.

Groups	Coronary flow (ml)	LVEDP (mmHg)
Control	19.15 ± 0.10	6.26 ± 0.27
ISO	7.33 ± 0.84$^{###}$	15.14 ± 0.77$^{###}$
2-APB	16.41 ± 0.49***	10.88 ± 0.51***
EU	14.28 ± 0.64***	12.03 ± 0.83**
PBEA	15.86 ± 0.44***	9.86 ± 0.63***
RCHA	14.96 ± 0.54***	12.6 ± 0.31*

Each group represents Mean ± SEM of 3 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were compared with control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (*P<0.05 and **P<0.01 and ***P<0.001).

ISO control group significantly (P<0.001) decreased dp/dt_{max} and dp/dt_{min} (1752.67 ± 157.28 and 1032 ± 130.43) as compared to control (3048.57 ± 196.46 and 2273.5 ± 178.66). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05, P<0.01 and P<0.001) increased dp/dt_{max} (2801.83 ± 134.43, 2595.83 ± 235.35, 2746 ± 140.74 and 2484.5 ± 154.27) as compare to ISO control groups. Moreover, Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05 and

P<0.01) increased dp/dt$_{min}$ (1745.17 ± 142.70, 1642.67 ± 150.85, 1847 ± 169.14 and 1683.33 ± 133.40) as compare to ISO control groups. (Figure 6.50)

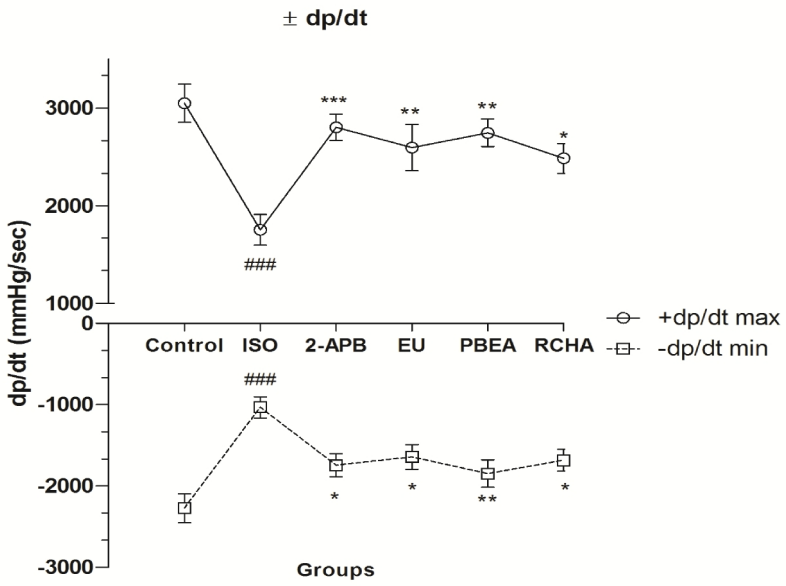


Figure 6.50 Effect of drugs on dp/dt in ISO induced global ischemia.

Each point represents Mean ± SEM of 3 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were compared with control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (*P<0.05, **P<0.01 and ***P<0.001).

6.5.2.3.4 Effect of drugs on heart weight to body weight ratio in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in heart weight/body weight ratio (0.37±0.01) as compared to control group (0.28±0.005). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05 and P<0.01) decreased heart weight/body weight ratio (0.31±0.01, 0.30±0.01, 0.30±0.56 and 0.30±0.017) as compare to ISO control groups. (Figure 6.51)

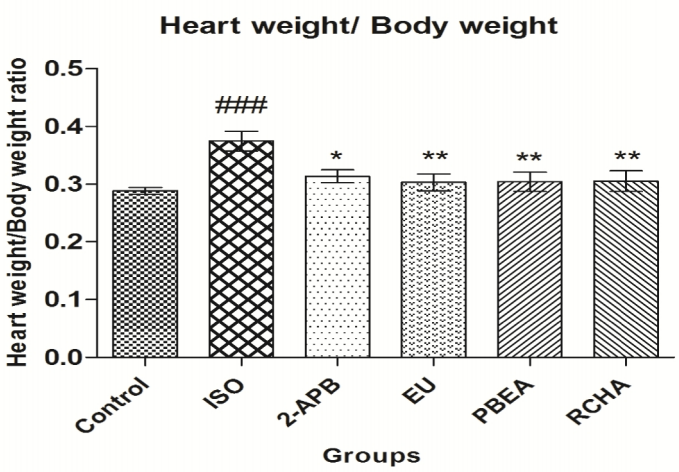


Figure 6.51 Effect of drugs on heart weight/ body weight ratio in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (*P<0.05 and **P<0.01).

6.5.2.3.5 Effect of drugs on CK-MB in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in CK-MB activity (246.3±14.31) as compared to control group (103.0±8.32). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.01 and P<0.001) decreased CK-MB activity (167.8±25.10, 133.3±7.52, 146.3±9.24 and 168.9±19.67) as compare to ISO control groups.(Figure 6.52)

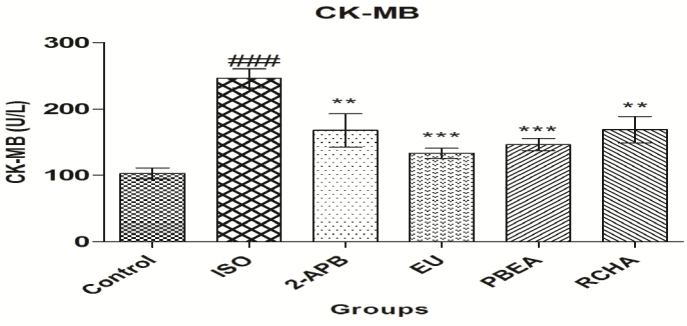


Figure 6.52 Effect of drugs on CK-MB in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (**P<0.01 and ***P<0.001).

6.5.2.3.6 Effect of drugs on LDH in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in LDH activity (1260±82.15) as compared to control group (490.7±36.13). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.01 and P<0.001) decreased LDH activity (864.4±36.17, 781.0±68.39, 835.8±62.04 and 961.0±45.05) as compared to ISO control groups. (Figure 6.53)

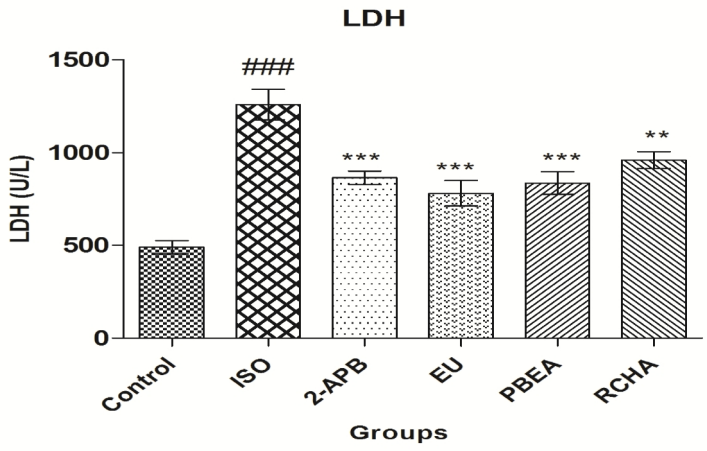


Figure 6.53 Effect of drugs on LDH in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (**P<0.01 and ***P<0.001).

6.5.2.3.7 Effect of drugs on serum calcium in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in calcium level (13.49±0.54) as compared to control group (9.76±0.38). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.001) decreased calcium level (10.46±0.24,

10.20±0.22, 10.29±0.34 and 11.02±0.46) as compared to ISO control groups. (Figure 6.54)

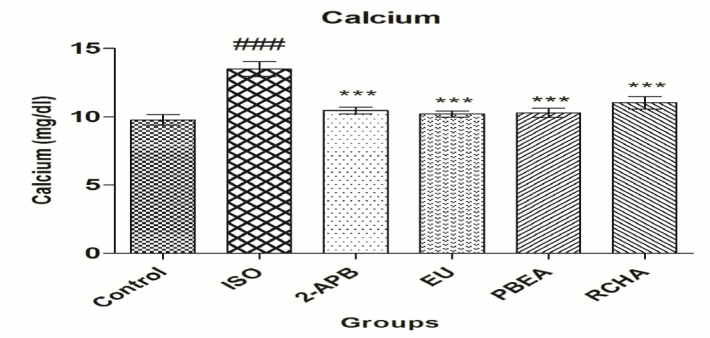


Figure 6.54 Effect of drugs on serum calcium level in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (***P<0.001).

6.5.2.3.8 Effect of drugs on heart calcium in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in calcium level (14.22±0.57) as compared to control group (9.51±0.28). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.01 and P<0.001) decreased calcium level (11.17±0.29, 11.61±0.56, 11.46±0.60 and 11.45±0.50) as compared to ISO control groups. (Figure 6.55)

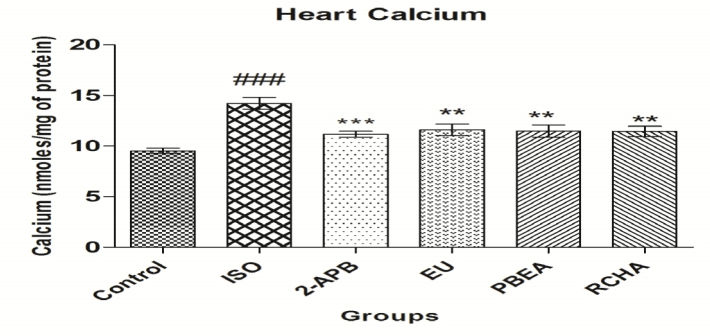


Figure 6.55 Effect of drugs on heart calcium level in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (**P<0.01 and ***P<0.001).

6.5.2.3.9 Effect of drugs on potassium in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) decreased in potassium level (3.73±0.66) as compared to control group (9.54±0.75). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.01 and P<0.001) decreased potassium level (9.79±0.60, 9.05±0.77, 9.16±0.63 and 7.86±1.23) as compared to ISO control groups. (Figure 6.56)

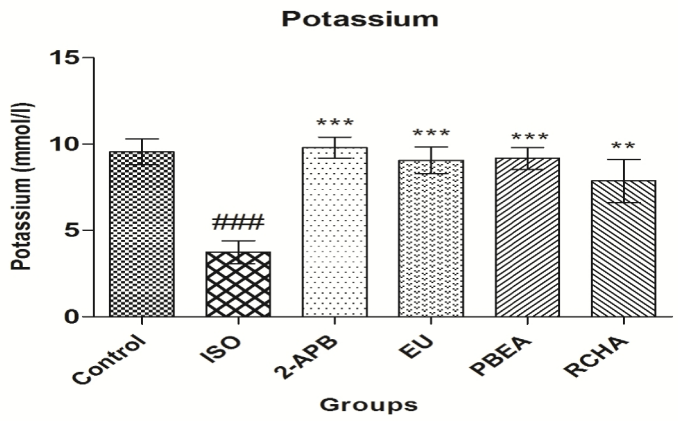


Figure 6.56 Effect of drugs on potassium level in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (**P<0.01 and ***P<0.001).

6.5.2.3.10 Effect of drugs on sodium in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in sodium level (196.9±5.65) as compared to control group (148.4±4.46). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.001) decreased sodium level (148.4±8.38, 132.1±7.90, 137.3±8.61 and 141.0±9.72) as compared to ISO control groups. (Figure 6.57)

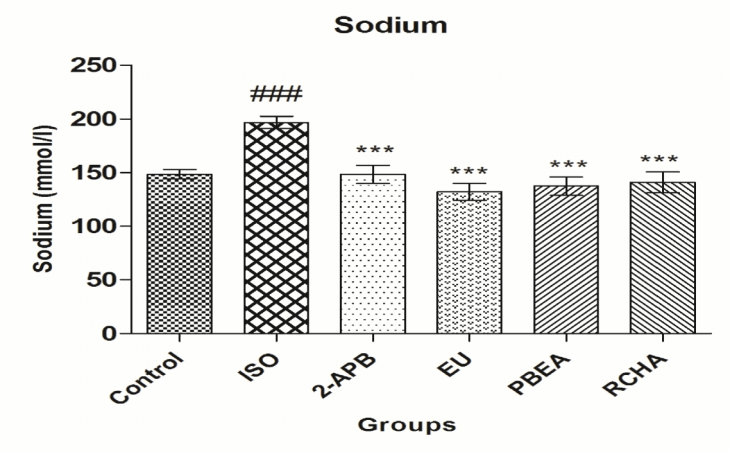


Figure 6.57 Effect of drugs on sodium level in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (***P<0.001).

6.5.2.3.11 Effect of drugs on TNF-α in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in TNF-α level (1291±48.13) as compared to control group (424.2±38.05). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.001) decreased TNF-α level (635.0±47.92, 695.2±21.71, 713.7±18.28 and 569.7±37.87) as compared to ISO control groups. (Figure 6.58)

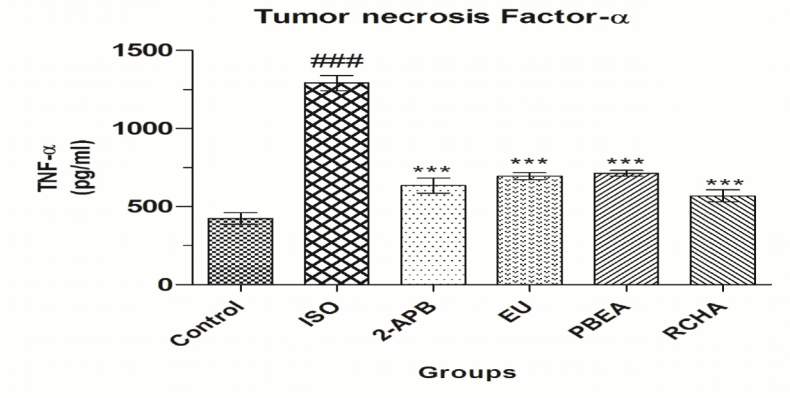


Figure 6.58 Effect of drugs on TNF-α level in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (***P<0.001).

6.5.2.3.12 Effect of drugs on IL-6 in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in IL-6 level (272.7±19.23) as compared to control group (91.50±11.43). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.001) decreased IL-6 level (205.2±24.34, 169.2±17.64, 191.0±9.74 and 159.2±8.69) as compared to ISO control groups. (Figure 6.59)

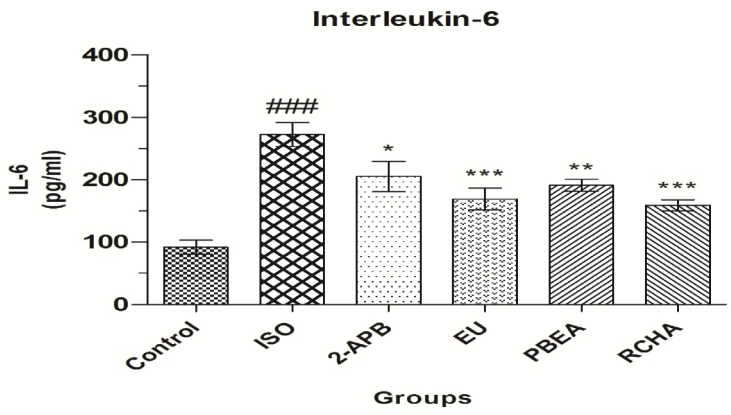


Figure 6.59 Effect of drugs on IL-6 level in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (***P<0.001).

6.5.2.3.13 Effect of drugs on antioxidant enzymes in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased MDA level as compared to control group. Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.01 and P<0.001) decreased MDA level as compared to ISO control groups. (Table 6.29)

Moreover, ISO control group significantly ($P<0.001$) decreased SOD, Catalase and GSH level, GPx and GST activity as compared to control group. Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly ($P<0.05$, $P<0.01$ and $P<0.001$) increased SOD, Catalase and GSH level, GPx and GST activity as compared to ISO control groups. (Table 6.29)

Table 6.29 Effect of drugs on antioxidant enzymes in ISO induced global ischemia.

Groups	MDA (nmoles/mg proteins)	GSH (nmoles/mg proteins)	Gpx (U/mg proteins)	GST (nmoles of CDNB congumed/ 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**

Each group 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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (*P<0.05, **P<0.01 and ***P<0.001).

6.5.2.3.14 Effect of drugs on Na^+/K^+ ATPase in ISO induced global ischemia in rats.

ISO control group significantly (P<0.01) decreased Na^+/K^+ ATPase activity (2.33±0.25) as compared to control group (5.16±0.88). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05 and P<0.01) increased Na^+/K^+ ATPase activity (4.50±0.51, 4.31±0.37, 4.98±0.22 and 4.67±0.55) as compared to ISO control groups. (Figure 6.60)

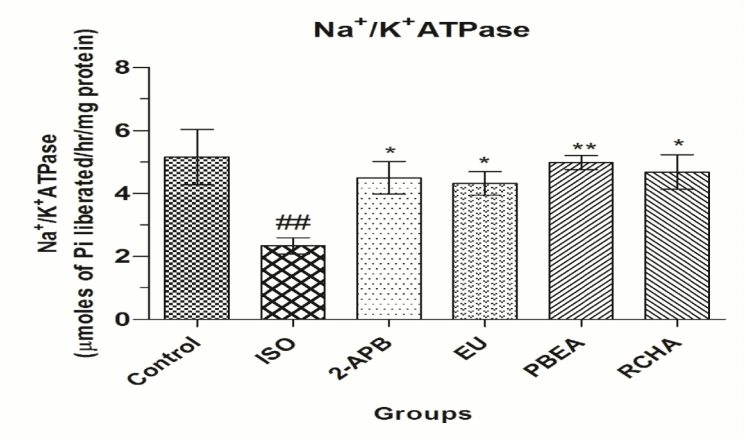


Figure 6.60 Effect of drugs on Na^+/K^+ ATPase activity in ISO induced global ischemia.

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 control vs. ISO control (##P<0.01); ISO control vs. Treatment groups (*P<0.05 and **P<0.01).

6.5.2.3.15 Effect of drugs on Ca^{++} ATPase in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased Ca^{++}ATPase activity (4.64±0.58) as compared to control group (2.64±0.30). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.01 and P<0.001) decreased Ca^{++}ATPase activity (2.62±0.08, 3.04±0.30, 2.92±0.11 and 2.74±0.35) as compared to ISO control groups. (Figure 6.61)

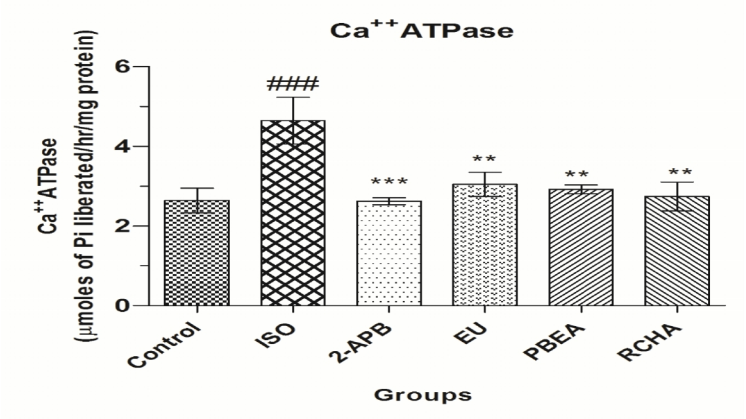


Figure 6.61 Effect of drugs on Ca^{++} ATPase activity in ISO induced global ischemia.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (**P<0.01 and ***P<0.001).

6.5.2.3.16 Effect of drugs on Mg^{++} ATPase in ISO induced global ischemia in rats.

ISO control group significantly (P<0.01) decreased Mg^{++}ATPase activity (1.36±0.17) as compared to control group (2.91±0.30). PBEA control group significantly (P<0.05) increased Mg^{++}ATPase activity (2.43±0.30) as compared to ISO control groups. Moreover, another treatment control groups insignificantly increased Mg^{++}ATPase activity. (Figure 6.62)

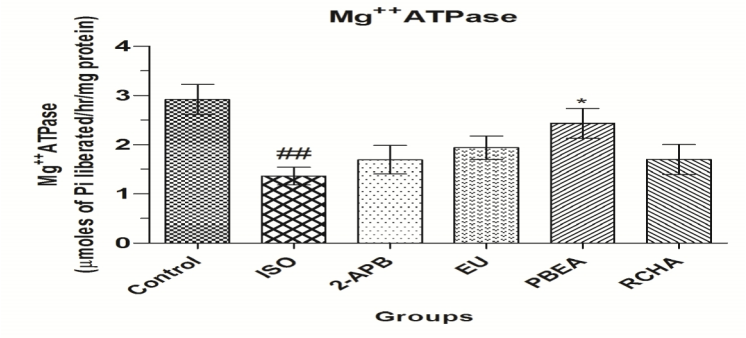


Figure 6.62 Effect of drugs on Mg^{++} ATPase activity in ISO induced global ischemia.

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 control vs. ISO control (##P<0.01); ISO control vs. Treatment groups (*P<0.05).

6.5.2.3.17 Effect of drugs on nitrite in ISO induced global ischemia in rats.

ISO control group significantly (P<0.01) increased nitrite level (12.63±1.90) as compared to control group (6.53±1.05). PBEA control group significantly (P<0.05) decreased nitrite level (7.69±0.54) as compared to ISO control groups. Moreover, another treatment control groups insignificantly decreased nitrite level. (Figure 6.63)

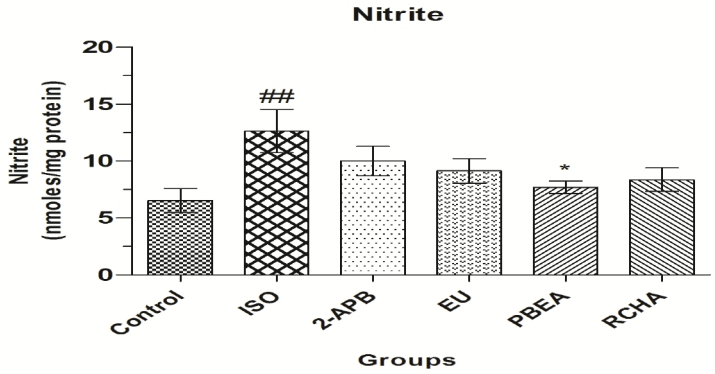


Figure 6.63 Effect of drugs on nitrite level in ISO induced global ischemia.

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 control vs. ISO control (##P<0.01); ISO control vs. Treatment groups (*P<0.05).

6.5.2.3.18 Effect of drugs on MPO in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased in MPO level (272.7±19.23) as compared to control group (91.50±11.43). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.001) decreased MPO level (205.2±24.34, 169.2±17.64, 191.0±9.74 and 159.2±8.69) as compared to ISO control groups. (Figure 6.64)

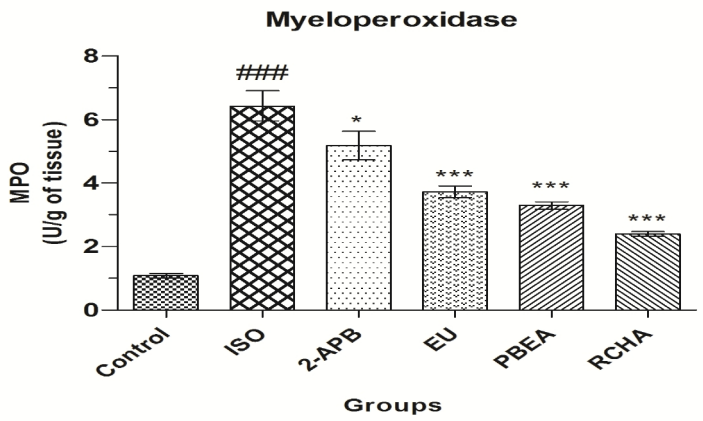
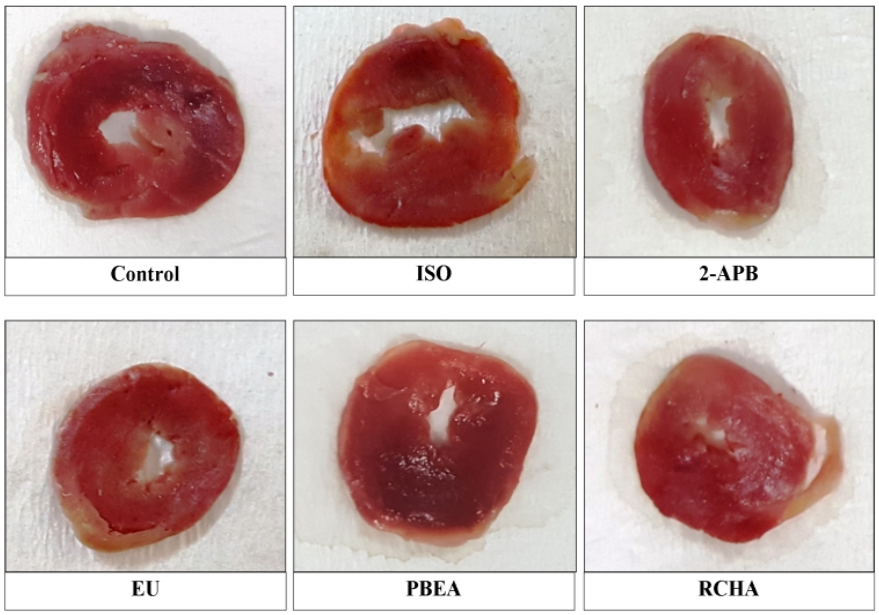


Figure 6.64 Effect of drugs on MPO level in ISO induced global ischemia.

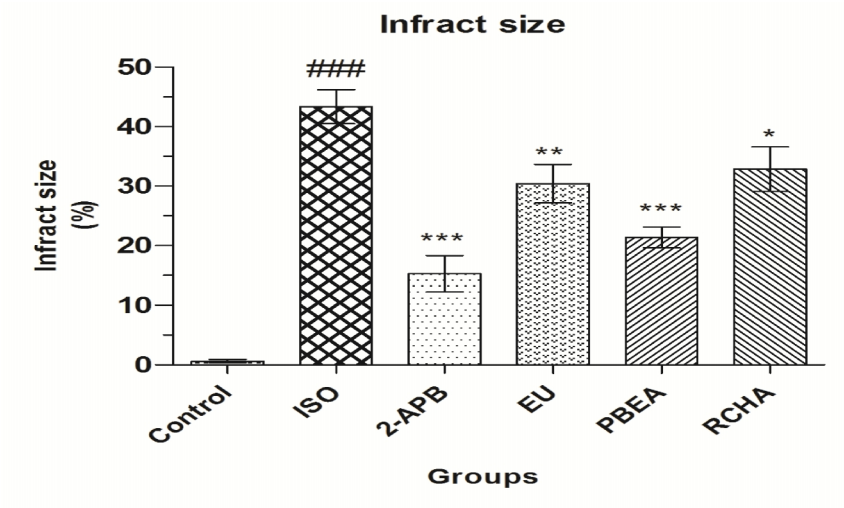
Each bar represents Mean ± SEM of 6 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were compared with control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (*P<0.05 and ***P<0.001).

6.5.2.3.19 Effect of drugs on infracts size in ISO induced global ischemia in rats.

ISO control group significantly (P<0.001) increased infract area (43.35±2.84) shown by yellowish color as compared to control group (0.61±0.26). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05, P<0.01 and P<0.001) decreased infract area and staining (15.28±3.03, 30.40±3.22, 21.36±1.74 and 32.85±3.71) as compared to ISO control groups. (Figure 6.65)



(a)



(b)

Figure 6.65 Effect of drugs on infract size in ISO induced global ischemia.

(a) Microscopy of heart slices (b) % infracts size of heart slices.

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 control vs. ISO control (###P<0.001); ISO control vs. Treatment groups (*P<0.05, **P<0.01 and ***P<0.001).

6.5.2.3.20 Effect of drugs on histopathology in ISO induced global ischemia in rats.

The histopathological photographs of heart tissues of control and experimental rats are illustrated in Figure 6.66. Histopathological examination of myocardial tissue obtained from control rats exhibited clear integrity of myocardial membrane. Control and baseline treated rats showed normal cardiac fibers without any infarction and infiltration of inflammatory cells was not seen in this group. Histopathological findings confirmed the induction of global ischemia by isoproterenol. Heart tissues from isoproterenol injected rats engendered subendocardial necrosis along with entrapment of leukocytes, increased myocardial microvascular permeability, myocardial edema and myofibrillar vacuolization as well as massive myofibers disruption and atomization, (Figure 6.66) as compared to control group. Pretreatment of 2-APB, Eugenol, PBEA and RCHA in isoproterenol injected rats depicted decreased degree infiltration of inflammatory cells and all the treatment groups except Eugenol, the morphology of cardiac muscle fibers were relatively well preserved with no evidence of focal necrosis.

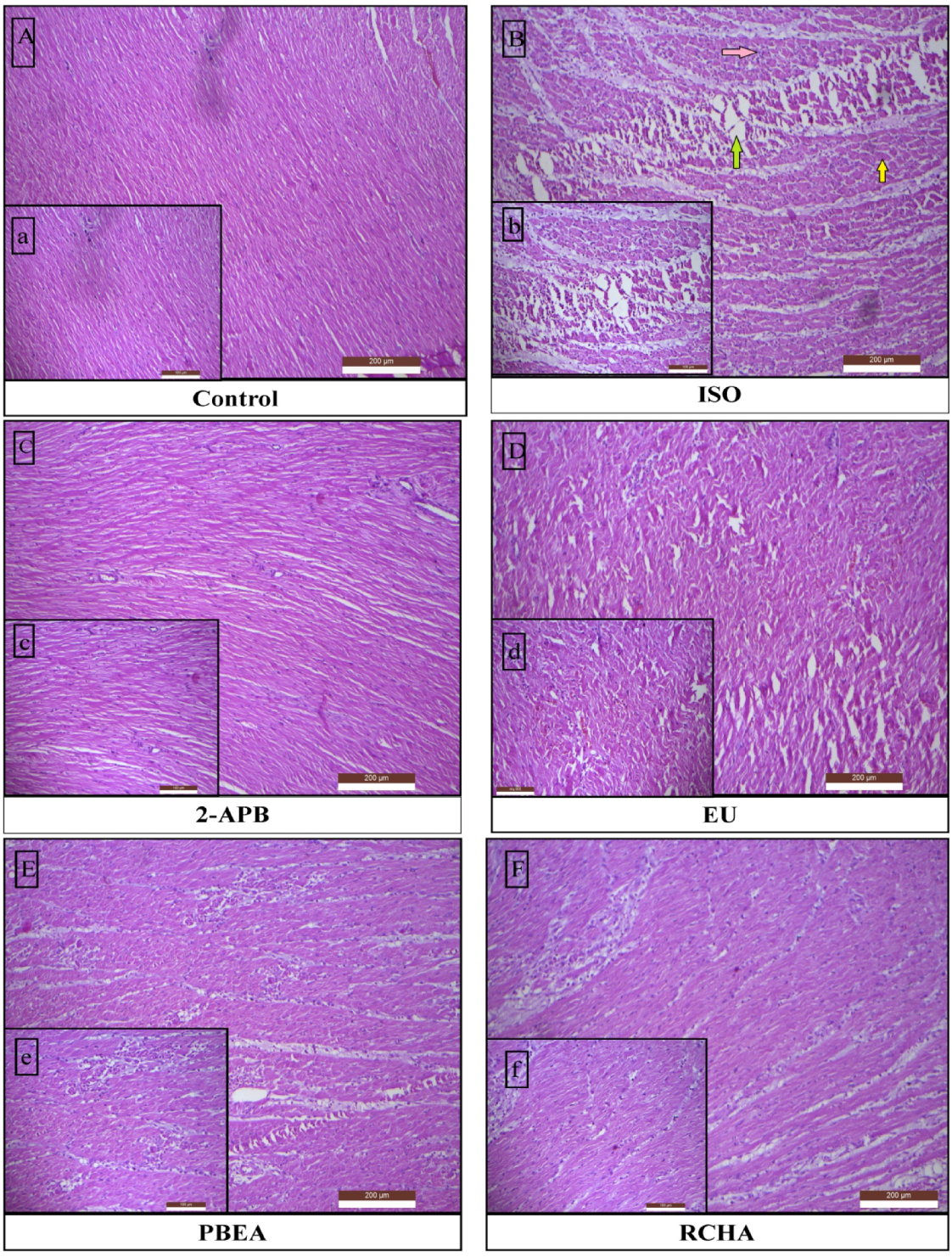


Figure 6.66 Effect of drugs on histopathology of heart tissue in ISO induced global ischemia. A) Normal Heart: Normal cardiac fibers and no leukocytes infiltration. B) ISO treated Heart: Leukocytes infiltration, Myocardial edema, Myocardial vacuolization and cardiac fiber disarrangement along with necrosis. C) 2-APB treated Heart: No leukocyte

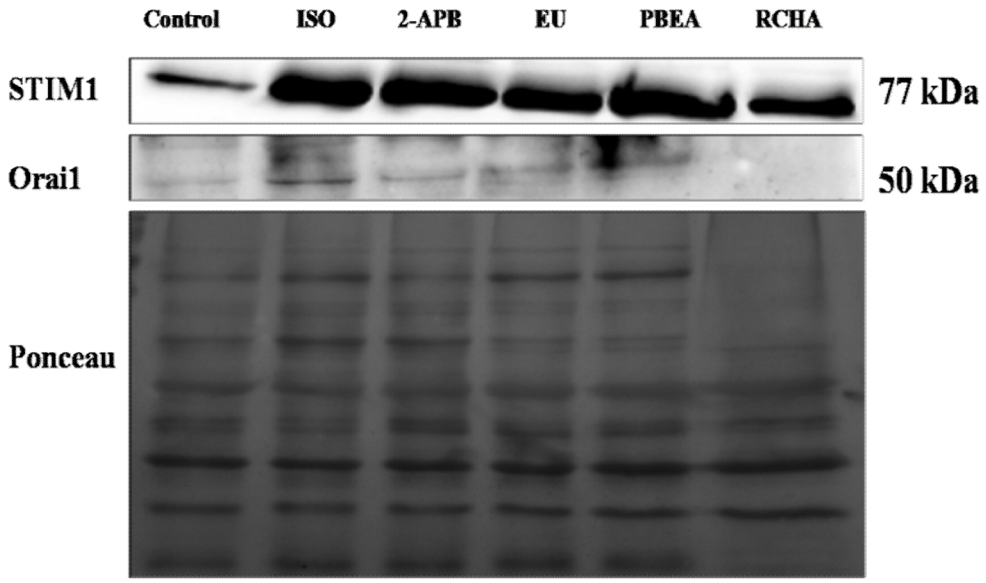
infiltration and myocardial vacuolization. D) Eugenol treated Heart: No leukocyte infiltration but myocardial vacuolization. E) PBEA treated Heart: Less leukocyte infiltration and myocardial edema. F) RCHA treated Heart: Less leukocyte infiltration and No myocardial edema and vacuolization.

Leukocytes infiltration **Myocardial vacuolization** **Myocardial edema**

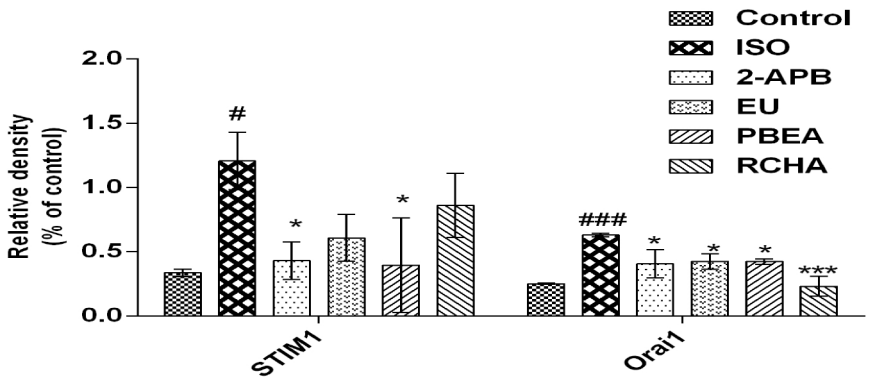
Magnification 10X and 20X with 200µm Scale.

6.5.2.3.21 Effect of drugs on STIM1 and Orai1 expression in ISO induced global ischemia in rats.

ISO administration in rats significantly ($P<0.05$ and $P<0.001$) elevated STIM1 and Orai1 expression in heart as compare to control group which is an indication of SOCE activation. However, pretreatment group (2-APB and PBEA) significantly ($P<0.05$) decreased STIM1 expression in heart and another pretreatment group (Eugenol and RCHA) didn't reverse the STIM1 expression in heart. Moreover, pretreatment group (2-APB, Eugenol, PBEA and RCHA) significantly ($P<0.05$ and $P<0.001$) decreased Orai1 expression in heart. (Figure 6.67)



(a)



(b)

Figure 6.67 Effect of drugs on STIM1 and Orai1 expression in ISO induced global ischemia. Immunoblotting assay: (a) Western blot analysis for STIM1 and Orai1 proteins expression of heart in ISO induced global ischemia. (b) Densitometry plot for STIM1 and Orai1 proteins.

Each bar represents Mean ± SD of 3 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were compared with control vs. ISO control (#-P<0.05 and ###P<0.001); ISO control vs. Treatment groups (*P<0.05 and ***P<0.001).

6.5.3 Coronary artery ligation induced reperfusion injury.

6.5.3.1 Effect of drugs on CK-MB in LAD induced reperfusion injury in rats.

LAD control group significantly (P<0.001) increased CK-MB level (294.7±19.7) as compared to control group (82.90±4.7). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly (P<0.05, P<0.01 and P<0.001) decreased CK-MB level (179.1±25.35, 174.4±20.01, 151.1±6.92 and 181.8±51.30) as compared to LAD control groups. (Figure 6.73)

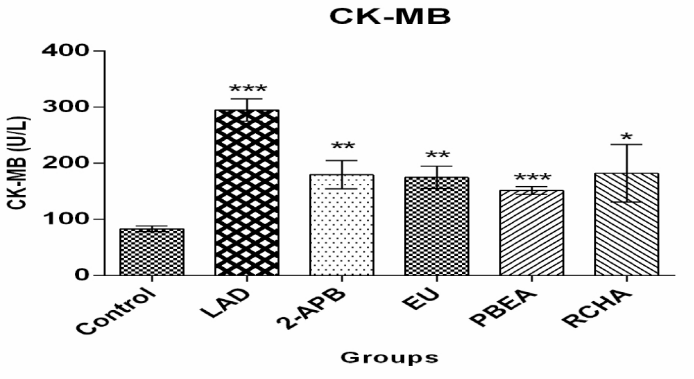


Figure 6.68 Effect of drugs on CK-MB level in LAD induced cardiotoxicity.

Each bar represents Mean ± SEM of 4-5 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were compared with control vs. LAD control (###P<0.001); LAD control vs. Treatment groups (*P<0.05, **P<0.01 and ***P<0.001).

6.5.3.2 Effect of drugs on STIM1 and Orai1 in LAD induced reperfusion injury in rats.

LAD performed rats significantly (P<0.01 and P<0.001) elevated STIM1 and Orai1 expression in heart as compare to control group which is an indication of SOCE activation. However, Pre-co-treatment group (2-APB and RCHA) significantly (P<0.01) decreased STIM1 expression in heart and another pre-co-treatment group (Eugenol and PBEA) didn't reverse the STIM1 expression in heart. Moreover, Pre-co-treatment group (2-APB, PBEA and RCHA) significantly (P<0.05, P<0.01 and P<0.001) decreased Orai1 expression in heart and another pre-co-treatment group (Eugenol) didn't reverse the Orai1 expression in heart. (Figure 6.69)

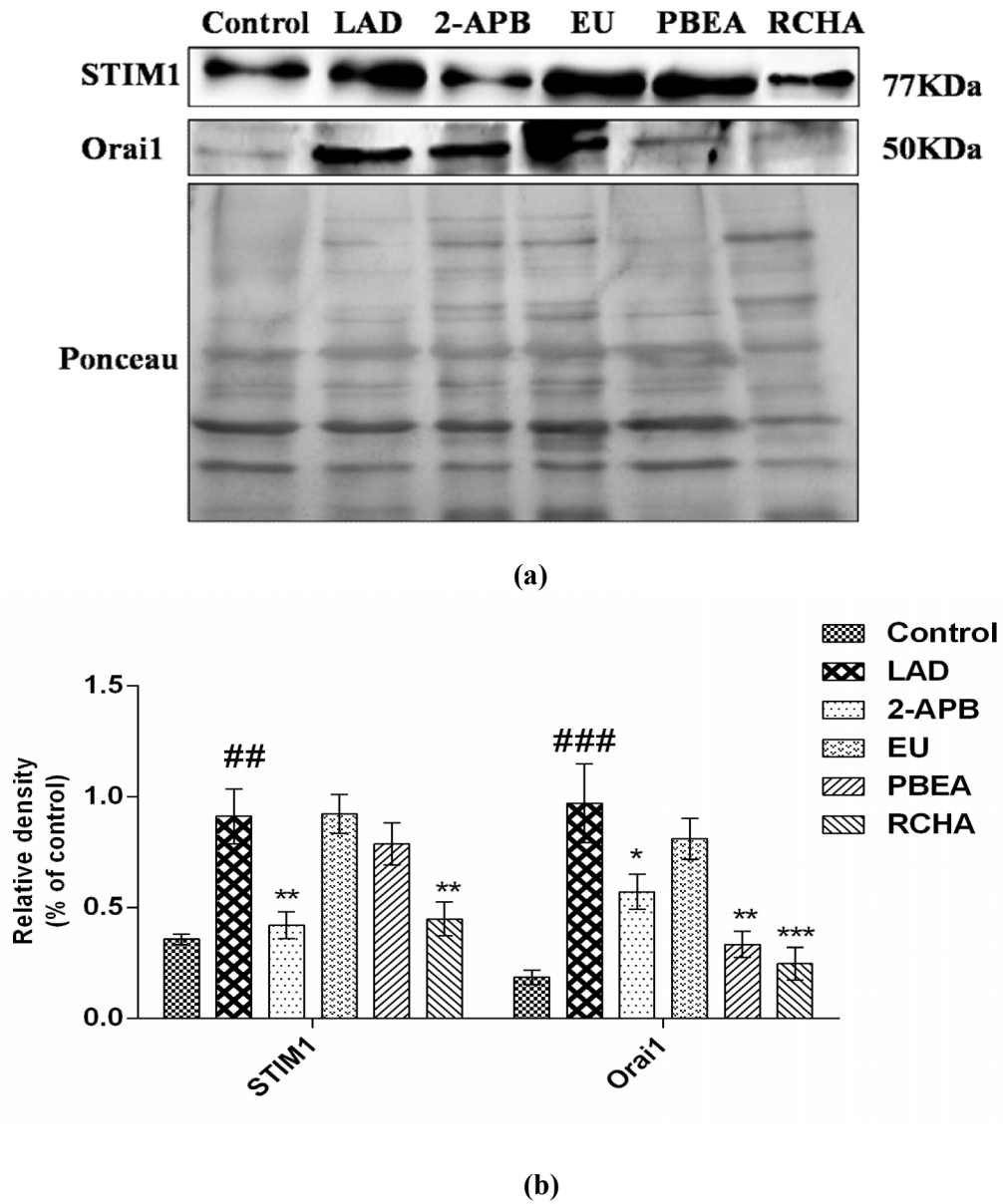


Figure 6.69 Effect of drugs on STIM1 and Orai1 expression in LAD induced reperfusion injury.

Immunoblotting assay: (a) Western blot analysis for STIM1 and Orai1 proteins expression of heart in LAD induced reperfusion injury. (b) Densitometry plot for STIM1 and Orai1 proteins.

Each bar represents Mean ± SD of 3 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were compared with control vs. LAD control (#P<0.05 and ##-P<0.01); LAD contrl vs. Treatment groups (*P<0.05).

6.6 Saftey study of drugs

6.6.1 Cellular toxicity of drugs

Treatment with PBEA (0.1, 1, 10 and 25 µg/ml) showed cell survival to 108.20 ± 0.67%, 111.63 ± 1.88% , 111.5± 2.09% and 101.33 ± 3.86% , respectively. Moreover, PBEA at the dose of 50, 100 and 500 µg/ml showed cell death.

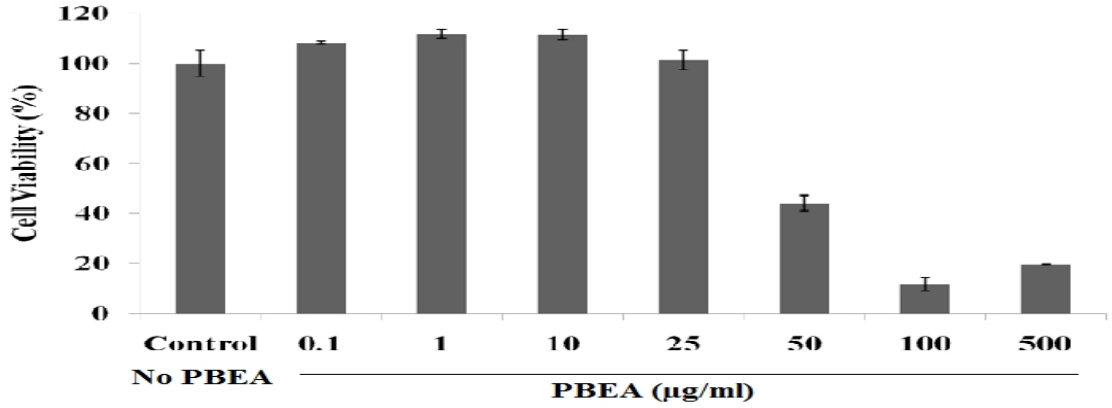


Figure 6.70 Cell Cytotoxicity of PBEA.

Treatment with RCHA (1, 10, 50, 100,200, 300, 400, 600, 800 and 1000 ng/ml) showed cell survival to 99.92 ± 3.16%, 92.24 ± 3.74% , 104.13± 4.28%, 104.04 ± 0.76%, 104.24 ± 3.19%, 105.19 ± 0.74%, 104.64 ± 0.36%, 93.32 ± 9.67%, 103.19 ± 1.63% and 96.58 ± 0.54%, respectively. Moreover, RCHA at the dose of 2, 3, 6, 12 and 24 µg/ml showed gradually increased cell death.

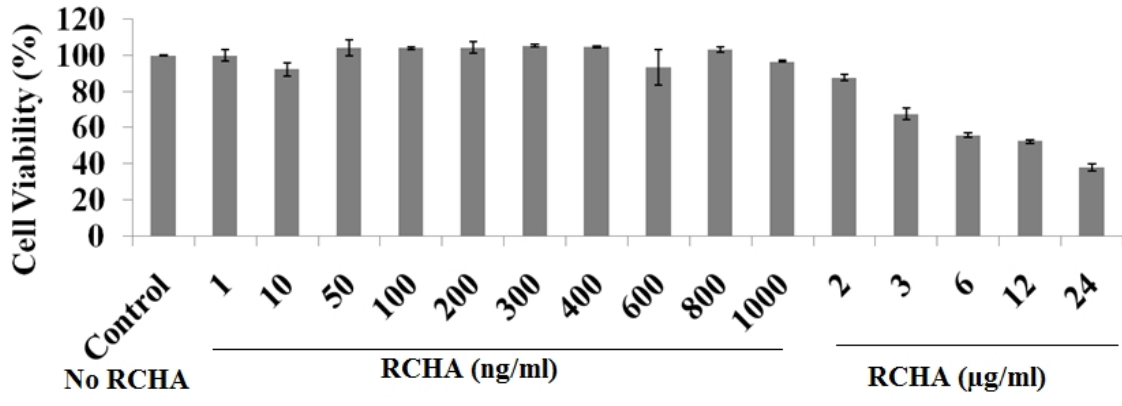


Figure 6.71Cell Cytotoxicity of RCHA.

Treatment with 2-APB (25, 50, 75, 100 and 150 µM) showed cell survival to 112.53 ± 1.51%, 117.85 ± 2.98%, 122.1± 1.00%, 118.09 ± 4.01% and 127.26 ± 5.59%, respectively. Moreover, 2-APB at the dose of 300and 500 µM showed cell death.

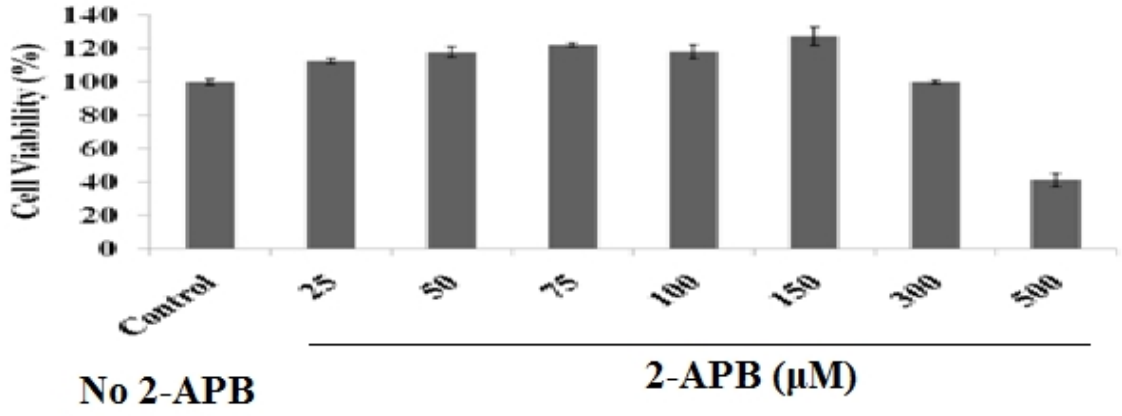


Figure 6.72 Cell Cytotoxicity of 2-APB.

Treatment with Eugenol (25, 50, 75, 100 and 150 µM) showed cell survival to 104.74 ± 2.33%, 107.69 ± 8.04%, 107.28 ± 7.75%, 105.19 ± 7.01% and 103.0 ± 4.46%, respectively. Moreover, Eugenol at the dose of 300and 500 µM showed cell death.

6.6.2 Doxorubicin induced Cardiotoxicity.

6.6.2.1 Effect of drugs on lactate dehydrogenase in DOXO induced cardio toxicity in rats.

DOXO control group significantly ($P<0.001$) increased LDH level (1439±162.5) as compared to control group (365.9±28.98). Treatment control group (2-APB, Eugenol, PBEA and RCHA) significantly ($P<0.05$ and $P<0.001$) decreased LDH level (581.4±51.26, 622.9±23.67, 730.3±89.85 and 1012±81.22) as compared to DOXO control groups. (Figure 6.73)

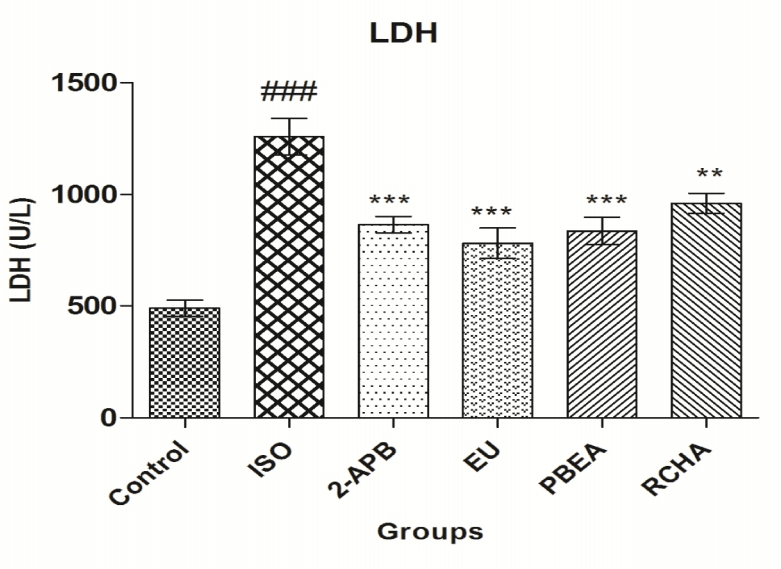
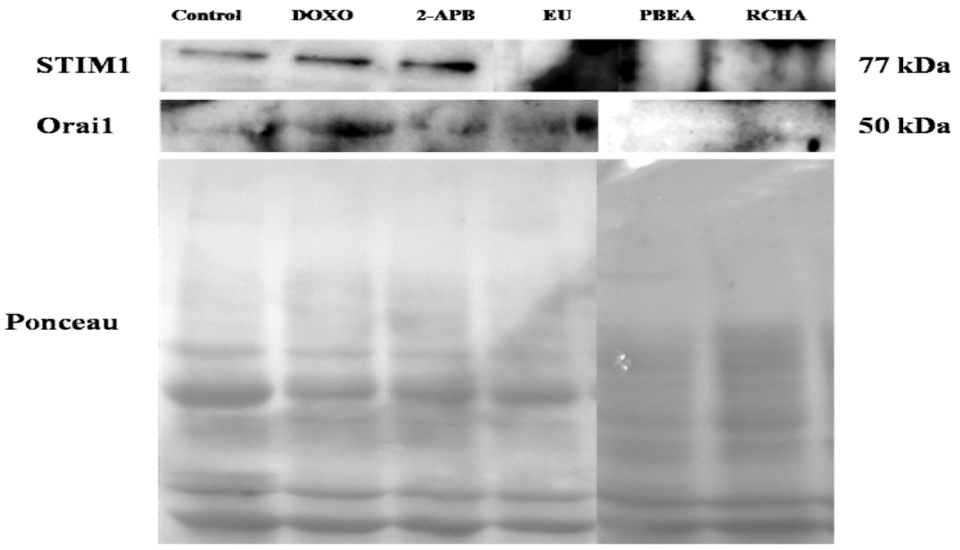


Figure 6.73 Effect of drugs on LDH level in DOXO induced cardiotoxicity.

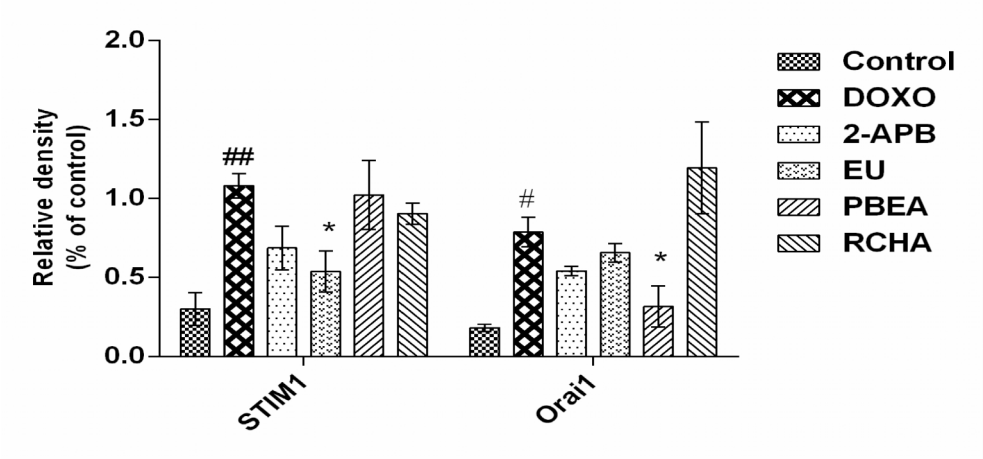
Each bar represents Mean ± SEM of 6 animals. Values are statistically evaluated using one way ANOVA analysis followed by dunnett's post hoc test. Significant values were compared with control vs. DOXO control (###P<0.001); DOXO control vs. Treatment groups (*P<0.05 and ***P<0.001).

6.6.2.2 Effect of drugs on STIM1 and Orai1 in DOXO induced cardio toxicity in rats.

DOXO administration in rats significantly (P<0.05 and P<0.01) elevated STIM1 and Orai1 expression in heart as compare to control group which is an indication of SOCE activation. However, pretreatment group (Eugenol) significantly (P<0.05) decreased STIM1 expression in heart and another pretreatment group (2-APB, PBEA and RCHA) didn't reverse the STIM1 expression in heart. Moreover, pretreatment group (PBEA) significantly (P<0.05) decreased Orai1 expression in heart and another pretreatment group (2-APB, Eugenol and RCHA) didn't reverse the Orai1 expression in heart. (Figure 6.74)



(a)



(b)

Figure 6.74 Effect of drugs on STIM1 and Orai1 expression in DOXO induced cardiotoxicity.

Immunoblotting assay: (a) Western blot analysis for STIM1 and Orai1 proteins expression of heart in DOXO induced cardiotoxicity. (b) Densitometry plot for STIM1 and Orai1 proteins.

Each bar represents Mean ± SD of 3 animals. Values are statistically evaluated using one tailed unpaired t- test. Significant values were compared with control vs. DOXO control (#P<0.05 and ##-P<0.01); DOXO control vs. Treatment groups (*P<0.05).

6.6.3 Acute 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, starting dose 300 mg/kg and the dose of 2000 mg/kg (limit test – OECD, 2008a) of PBEA did not cause the death of any animal. The 300 mg/kg female rats exposed presented no behavioral changes but 2000 mg/kg female rats showed hyperactivity and chromodacryorrhea during the treatment period, as well as no changes in water and food consumption and ponderal evolution, in relation to the control group (Table 6.30). No abnormality was found in the organs at necropsy. Thus, the PBEA tested falls in class 5 (a substance with oral lethal dose (LD50) higher than 2000 mg/kg), hence considered of low toxicity. In this study, it was observed that the lethal oral toxicity of this PBEA was estimated to be higher than 2000 mg/ kg, classified as category 5 according to OECD Guide 423, indicating a certain safety margin associated with the use of PBEA as therapeutic agent.

After the acute toxicity test, starting dose 300 mg/kg of RCHA did not cause the death of any animal but the dose of 2000 mg/kg cause the death of 2 animals. The 300 mg/kg female rats exposed presented no behavioral changes but 2000 mg/kg female rats showed bradycardia and lethargy during the treatment period, as well as no changes in water and food consumption and ponderal evolution, in relation to the control group (Table 6.30). No abnormality was found in the organs at 300mg/kg rats but abnormality was found in heart and kidney at 2000 mg/kg during necropsy. Thus, the RCHA tested falls in class 4 (a substance with oral lethal dose (LD_{50}) higher than 300-2000 mg/kg), hence considered of low toxicity (OECD, 2008a). In this study, it was observed that the lethal oral toxicity of this RCHA was estimated to be higher than 300-2000 mg/kg and LD_{50} cutoff value was 1000 mg/kg, classified as category 4 according to OECD Guide 423, indicating an indefinite safety margin associated with the use of RCHA as therapeutic agent.

Table 6.30 Body weight gain and food and water consumption of rats treated orally with PBEA and RCHA.

Parameters (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

Each group represents Mean ± SEM of 6 animals. Values are statistically evaluated using two tailed unpaired t-test analysis. Values were compared with 300mg/kg vs. 2000mg/kg.

6.6.4 Sub-acute toxicity study of PBEA by OECD 407

In the present study, after sub-acute 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 female and male groups treated with PBEA, at all doses, decreased as compared to the control group. Although, the weight gain values (Table 6.31) did not differ among groups. Similarly, in the present study, the relative weights of all organs examined did not vary significantly among groups (Table 6.32), supporting the hypothesis of low toxicity of the PBEA after sub-acute exposure.

Table 6.31 Body weight gain and food and water consumption of rats treated orally with PBEA.

Each group represents Mean ± SEM of 5 animals. Values are statistically evaluated using one way ANOVA analysis followed by bonferroni's multiple comparison post hoc test. Values were compared with Control vs. Treatment control; S-control vs. Satellite (*P<0.05 **P<0.01 and ***P<0.001).

Parameters (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
Parameters (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 6.32 Relative organ weight (g/100 g of body weight) of rats treated orally with PBEA.

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 ± 0.014
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

Each group represents Mean ± SEM of 5 animals. Values are statistically evaluated using one way ANOVA analysis followed by bonferroni's multiple comparison post hoc test. Values were compared with Control vs. Treatment control; S-control vs. Satellite ($P>0.05$).

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed and under certain circumstances may provide useful information. Some enzymes and proteins can be used as indicative of hepatocellular effects (such as ALT, AST, gamma-glutamyl transferase, bilirubin), and others as biomarkers of nephron functional injury (creatinine, blood urea nitrogen). In this study, there was no statistical difference in liver or renal parameters (ALT, AST, Total protein, albumin, globulin, A/G Ratio, bilirubin levels, Total cholesterol, creatinine, sodium, potassium and blood urea nitrogen) between the treated and control groups (Table 6.33 and Table 6.34). One parameter in female group (calcium) was statistically different when compared to the control group. However, this increase has no clinical significance. According to Giknis and Clifford (2006), the values found in this study are within the normal range for healthy rats at this age, indicating the absence of liver or renal toxicity.

Table 6.33 Biochemical parameters of female rats treated orally with PBEA.

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

Each group represents Mean ± SEM of 5 animals. Values are statistically evaluated using one way ANOVA analysis followed by bonferroni's multiple comparison post hoc test. Values were compared with Control vs. Treatment control; S-control vs. Satellite (*P<0.05) (P>0.05).

Table 6.34 Biochemical parameters of male rats treated orally with PBEA.

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

Each group represents Mean ± SEM of 5 animals. Values are statistically evaluated using one way ANOVA analysis followed by bonferroni's multiple comparison post hoc test. Values were compared with Control vs. Treatment control; S-control vs. Satellite ($P>0.05$).

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 one parameter observed in female group (WBC) showed a statistical difference in the groups treated with PBEA (800mg/kg), the other hematological parameters were similar among groups (Table 6.35). Similarly as in the biochemical analysis, 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.

Table 6.35 Hematological parameters of rats treated orally with PBEA.

Hematological Parameters (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 Parameters (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 /µL)	809.60 ± 60.04	865.40 ± 66.49	910.60 ± 20.49	861.80 ± 38.30	860.4 ± 34.00	903.4 ± 23.24

Each group represents Mean ± SEM of 5 animals. Values are statistically evaluated using one way ANOVA analysis followed by bonferroni's multiple comparison post hoc test. Values were compared with Control vs. Treatment control; S-control vs. Satellite (**P<0.01) (P>0.05).

The assessment of pathological changes in the organs of treated animals, both macro and microscopically, is the basis of a safety assessment. In this study, the macroscopic analysis, the PBEA, at all doses tested, produced no changes in the treated animals, vital and reproductive organs in the qualitative analysis. Similarly, in the histopathological analyses there were no findings suggestive of toxic effects (Figure 6.75 and Figure 6.76). These results proved to be consistent with biochemical analyses, confirming the safety of using the PBEA.

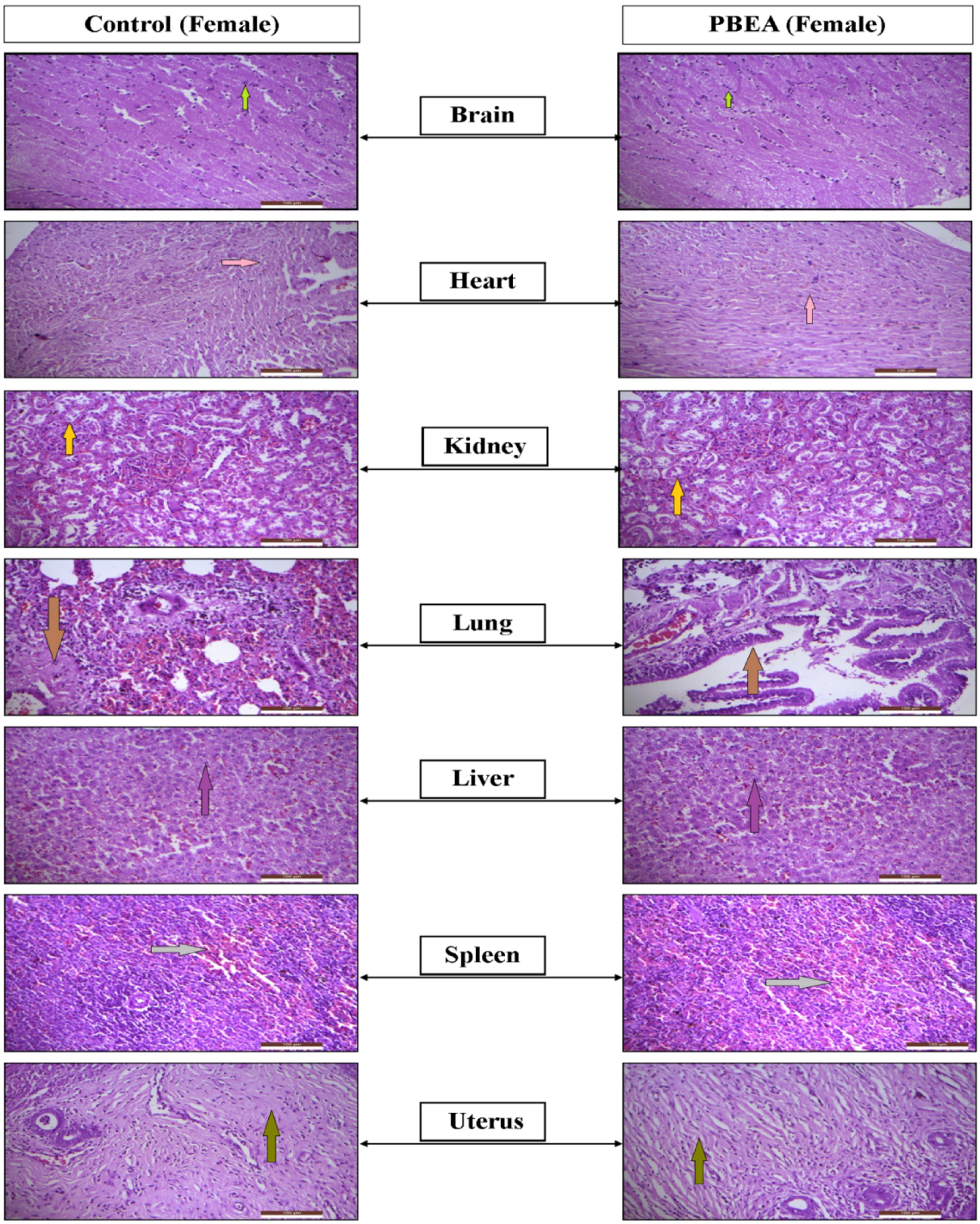


Figure 6.75 Histopathological analysis of female organs treated with PBEA in the sub-acute toxicity. A) Brain: Neurogial cells. B) Heart: Cardiac Muscle. C) Kidney: Tubular cells. D) Lungs: Epithelial layer around bronchioles. E) Liver: Hepatocytes. F) Spleen: Red and white pulp. G) Uterus: Myometrium. (H&E ×20).

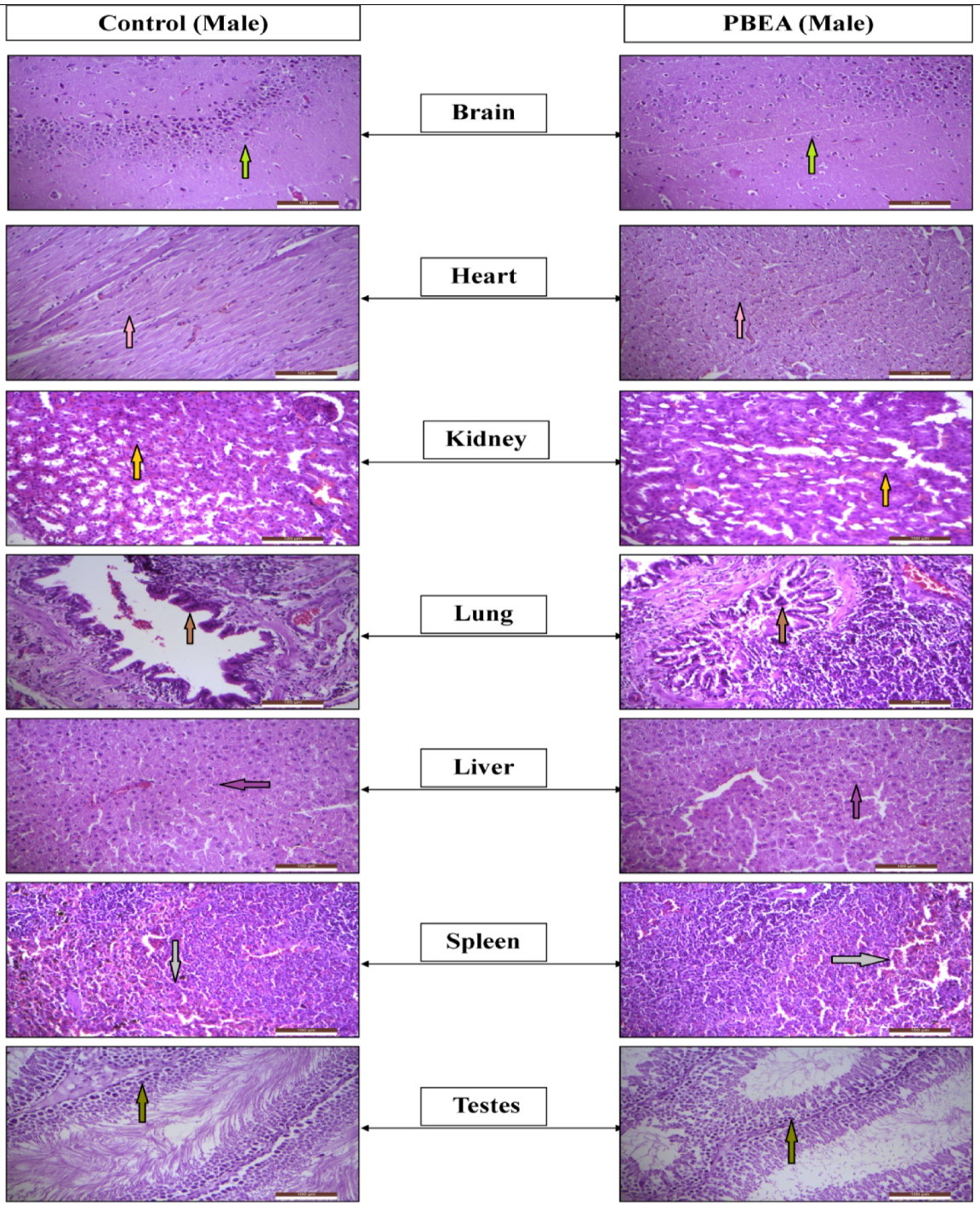


Figure 6.76 Histopathological analysis of male organs treated with PBEA in the sub-acute toxicity. A) Brain: Neurogial cells. B) Heart: Cardiac Muscle. C) Kidney: Tubular cells. D) Lungs: Epithelial layer around bronchioles. E) Liver: Hepatocytes. F) Spleen: Red and white pulp. G) Testes: Spermatocytes. (H&E ×20).