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IN VITRO -IN VIVO EVALUATION OF CARDIOPROTECTIVE EFFECT OF THE LEAF EXTRACT OF *COLEBROOKEA OPPOSITIFOLIA SM.*

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ABSTRACT

Cardiovascular diseases (CVDs) are now the most common cause of death worldwide. CVDs accounts for ~30% of death worldwide, including nearly 40% in high-income countries and 28% in low and middle-income countries. The risk factors for heart diseases are related to family history, sex, increased lipid levels, diabetes mellitus, hypertension, obesity and cigarette smoking. There are overwhelming evidences indicating hypercholesterolemia and other lipid abnormalities as major risk factors in the development of atherosclerosis and coronary heart diseases. The plant kingdom represents an enormous reservoir of biologically active molecules and so far only a few plants with medicinal activity have been evaluated. Nearly 50% of drugs used in medicine are of plant origin. The objective of the present study was to carry out the phytochemical screening and to evaluate the cardio-protective activity of leaf of ***Colebrookea oppositifolia Sm.*** in laboratory animals. The anti neoplastic agent Doxorubicin was chosen for inducing cardio-toxicity in rats. The present

study showed for the first time that the leaves of ***Colebrookea oppositifolia Sm.*** are particularly useful agents, as they could enhance myocardial and endogenous blood antioxidant levels without producing any cytotoxic effects. Therefore, the protection against myocardial injury in the treated rats is attributed to enhanced endogenous antioxidant activity.

Keywords: Thiobarbituric acid reactive substances [TBARS], reduced glutathione [GSH], Superoxide dismutase [SOD], Doxorubicin, Methanol extract of *Colebrookea oppositifolia* (MECO).

INTRODUCTION:

Cardiovascular diseases incur a greater economical constraint than any other illness especially in the developing countries. In developed countries, coronary vascular diseases now constitute the principal cause of human mortality. Not surprisingly, therefore, this is an intensive research, not entirely devoted to treatment, but also to the prevention of these diseases[1]. The risk factors for heart diseases are related to family history- sex, increased lipid levels, diabetes mellitus, hypertension, obesity and cigarette smoking. There are overwhelming evidences indicating hypercholesterolemia and other lipid abnormalities as major risk factors in the development of atherosclerosis and coronary heart diseases. Therefore, cardiovascular diseases have become a very common problem in the affluent societies related to their life style[2]. The plant kingdom represents an enormous reservoir of biologically active molecules and so far only a few plants with medicinal activity have been evaluated. Nearly 50% of drugs used in medicine are of plant origin[3]. The biosynthesis of secondary metabolites although controlled genetically, is affected strongly by environmental factors. As a result, there are fluctuations in the concentration and quantities of the secondary metabolites such as alkaloids, glycosides, volatile oils and steroids. The secondary metabolites are separated by the development of the efficient separation techniques like chromatography as well as sensitive instrumental analysis methods such as UV, IR, NMR, ESR, ORD and Mass spectroscopy.

MATERIALS AND METHODS:

The leaves of *Colebrookea oppositifolia* were collected from Majhitar, East Sikkim. The plant material was identified, confirmed and authenticated by the Botanical Survey of India, Gangtok, E.

Sikkim, India. All chemicals used in the present study were of analytical and laboratory grade. All the biochemical parameters were estimated using standard kits (Direct HDL Cholesterol Test Kit Selective Detergent Method – Accelerated, Triglycerides Test Kit Enzymatic GPO-Trinder Method, AST (GOT) Modified UV (IFCC), Kinetic Assay, Alkaline phosphatase (ALP) pNPP-AMP(IFCC), Kinetic Assay, Span Diagnostics Ltd., Surat) with the help of Auto analyzer (Merck specialities private limited, Navi Mumbai). Moisture Content was determined by taking two gm of the air dried crude drug, accurately weighed in a tarred watch glass. The drug was kept in hot air oven at 105^0 C and dried for a period until constant weight was obtained[4]. The data is presented in table no.1.

Fluorescence Analysis was performed using the powdered drug which was examined under U.V. and ordinary light after treatment with different reagents. About 10 gms of the powdered drug was taken in a Petridish and treated with different reagents viz., methanol, methanolic sodium hydroxide, 50% sulphuric acid, 50% nitric acid, 5% potassium hydroxide, 1N Hydrochloric acid, 1N methanolic sodium hydroxide. These were observed under different wavelengths i.e., visible range and ultraviolet range (254 nm and 365 nm) using UV –Visible spectrophotometer (Pharma Spec 1700 (Shimadzu), Japan). Various color radiations emitted were observed and presented in table no.2.

Extraction was carried out after washing the leaves thoroughly with water to remove any unwanted matters. This was further dried in shade. After complete drying, it was powdered and passed through sieve no 60 and stored in air tight containers. About 750 gm of the air-dried powdered plant leaves was extracted in the Soxhlet apparatus with different solvents, starting from petroleum ether (60-80°C) followed by methanol and chloroform water. Each time before extracting with the next solvent, the powdered material was air dried below 50°C. Each extract was concentrated by distilling off the solvent and evaporating to dryness on water bath. It was weighed and the percentages of different extractive values were calculated in terms of air-dried weight of plant/ drug material. The color, consistency and extractive values were noted. Extractive values of the crude drug was determined and presented in table no.3.

The plant extracts were subjected to preliminary screening for the detection of various plant constituents present. For fluorescence studies, drug was sieved through 60 mesh and observations were

made. Physical constants were determined following Indian Pharmacopoeia, and the phytochemical tests were carried out following Kokate CK, *et al* (1995) [5]. The data obtained is tabulated in table no.4.

ANTIOXIDANT ASSAY (*In-Vitro*):

Tissue sample preparation for Lipid Peroxidation assay:

Goat liver was collected from slaughter house; liver lobes were washed with 0.9% sodium chloride solution (to remove excess blood). The lobes were dried by blotting papers and were cut into small pieces with a heavy-duty blade. From that 1g tissue were then homogenized with 10ml of cold phosphate buffer (pH-7.4) to get 10% homogenate in glass-Teflon homogenizing tubes and filtered to get clear solution. The solution was centrifuged at 3000 g at 4°C for 10 min. The supernatant was diluted with phosphate buffer up to final concentration of protein became 0.8-1.5 mg/0.1ml. That solution was taken for lipid peroxidation assay.

Lipid peroxidation assay [6]

The extent of lipid peroxidation in goat liver homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS) by using standard method with minor modifications with the help of spectrophotometer. Protein concentration was measured by using standard method of Lowery. The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls as per the following formula:

$$\% \text{ Inhibition} = \frac{(Control\ Absorbance - Test\ Absorbance)}{Control\ Absorbance} \times 100$$

The TBARS concentration was calculated by using the following formula (molar extinction coefficient of TBARS) and expressed as nM/mg of tissue.

$$\text{nM of TBARS/mg of tissue} = \frac{OD \times Volume\ of\ homogenate \times 100 \times 10^8}{(1.56 \times 10^5) \times Volume\ of\ extract\ taken}$$

PHARMACOLOGICAL EVALUATION:

Cardio protective activity (*in-vivo*):

Male albino rats, weighing between 150 to 200 g was included in the study. Rats were housed in the departmental animal house at an ambient temperature of 25°C, under a 12 hour dark -12 hour light, cycle, for the whole period of the study. Experiments were carried out according to the guidelines of the institutional animal ethics committee (IAEC) of the institution. The leaves were air dried under shade and pulverized. A suspension of the leaves in 5% Tween 80 (Vehicle) was made daily. Experiments were carried out on male albino Wistar rats [120-150gms body weight] according to the guidelines of the

IAEC. The rats were divided into 7 groups (6 in each group) and fed with the suspension of dried pulverized *Colebrookea oppositifolia* leaves extract of three doses (250 mg/kg, 500 mg/kg) by oral gavage once a day for 4 weeks (6 days/week), along with standard rat chow and water-*ad-libitum*[7]. At the end of the treatment period, group III, IV V, VI and VII were administered Doxorubicin 15 mg/kg intraperitoneally, for two consecutive days to induce oxidative stress injury. After 48 hours of the first dose of DOX the rats were sacrificed, hearts and blood samples were collected and immediately frozen in liquid nitrogen for biochemical estimation. Effect of doxorubicin, MECO and WECO on heart weight, body weight, liver weight and liver weight to body weight ratio and heart weight to body weight ratio is presented in table no.8. Serum Parameters like cholesterol, alkaline phosphatase, aspartate aminotransferase were determined as per methods mentioned in respective kits[8]. Determination of lactate dehydrogenase was performed by DGKC method, determination of HDL-Cholesterol by accelerated selective detergent method [9], and determination of Triglycerides by GPO- Trinder method. The results of the above parameters are presented in table no. 9. Protein estimation for the tissue samples were done by the method of Bradford (1976).[11] Myocardial GSH was estimated by the method of Ellman et al, (1959) [9] and shown in fig no. 1. Catalase level was estimated by the method described by Aebi et al. (1974) and shown in fig no.2. TBARS level in the myocardium was determined by the method described by Ohkawa et al (1997)[7] and shown in fig no.3, SOD levels in the hearts were determined by McCord and Firdovich method (1969) as modified by Kakkar et al, (1984)[12] and shown in fig no.4.The aforesaid biochemical parameters were studied in heart tissue and blood and the results obtained are shown in table no.7.

RESULTS AND DISCUSSION:

Table 1: Moisture Content

Fresh weight (gm)	Dry weight (gm)	Loss on drying (gm)	Moisture content (%)
3	2.75	0.25	8.33

Table 2: Fluorescence Analysis

Treatment of powder	Visible rays	Ultra -violet light	
		short wave (254 nm)	long wave(365 nm)
Powder as such	Brown	Green	Black
Powder + 50% H_2SO_4	Dark brown	Dark green	Black
Powder + 50% HNO_3	Dark brown	Dark green	Black
Powder + 5% KOH	Dark brown	Dark green	Black
Powder + Methanol	Brown	Dark green	Black
Powder + 1N HCl	Brown	Dark green	Black
Powder + 1N Methanolic NaOH	Brown	Dark green	Black

Table 3: Successive solvent extractive values and nature of extracts

Sl. No.	Solvent	Color	Consistency	Extractive value(%w/w)
1	Pet-ether	Yellow	Sticky	4.4%
2	Methanol	Dark brown	Sticky	5.12%
3	Chloroform water	Dark brown	Semi solid	12.8%

Table 4: Qualitative phytochemical Evaluation of Colebrookea oppositifolia

Test parameter	Petroleum ether	Methanol	Chloroform water
Alkaloid	-	+	-
Carbohydrate	+	-	+
Glycoside	-	+	-
Saponins	-	+	-
Proteins and Amino acids	-	+	–
Phytosterols	+	+	+
Fixed oils and Fats	-	+	-
Phenolic compounds and flavonoids	-	+	+
Terpenoids	-	+	+

(+) – Present, (-) – absent

Table.5- Effect of water extract of *Colebrookea oppositifolia* leaf on ferrous sulphate induced lipid peroxidation in goat liver homogenate.

Dwash Number	Treatment	% Inhibition	IC_{50} value and confidence interval	TBARS (nM/mg of tissue)
1	Control	–	–	–
2	Vitamin C (5mM)	83.21 ± 1.35	0.531±0.85	0.243
	Concentration of Water extract (µg/ml)			
3	200	20.12 ± 2.47	507.59 ± 3.72	0.857
4	400	39.16 ± 2.52		0.717
5	600	58.79 ± 2.10		0.583
6	800	78.07 ±2.64		0.406

Values were Mean ± S.E.M.; n=3

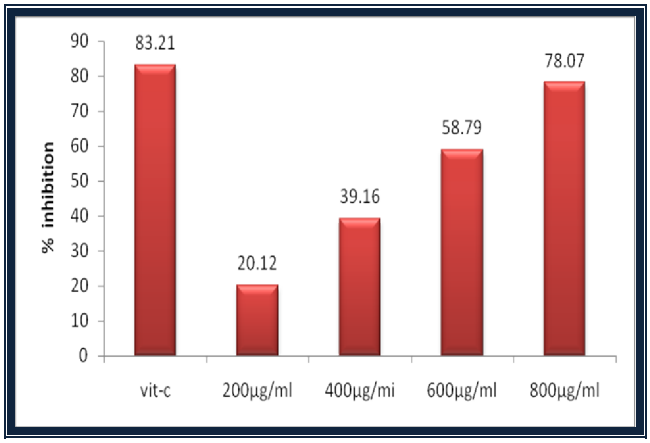

Fig: 1: Percentage of inhibition of lipid peroxidation by different concentrations of water extract of *Colebrookea oppositifolia* leaf, Vitamin C was used as standard

Catalase Assay (CAT):

Catalase activity was measured based on the ability of the enzyme to break down H_2O_2. 10 µl of sample was taken in test tube containing 3.0ml of H_2O_2 in phosphate buffer (M/15 phosphate buffer; pH-7.0). Time required for 0.05 optical density changes was observed at 240 nm against a blank containing the enzyme source in H_2O_2 free phosphate buffer (0.16ml H_2O_2 was 30% w/v was diluted to 100ml of phosphate buffer). The absorbance was noted at 240 nm after the addition of enzyme; Δt was noted till OD was 0.45. If Δt was longer than 60 seconds, the procedure was repeated with more concentrated enzyme sample. Reading was taken at every 5 second interval. A unit catalase activity was the amount of enzyme that liberates half the peroxide oxygen from H_2O_2 solution of any concentration in 100 seconds at 25^0C which was determined by CAT activity expression:

Moles of H_2O_2 consumed/min (units/mg) =

$$\frac{2.3}{\Delta t} \times \ln\left(\frac{E_{initial}}{E_{final}}\right) \times 1.63 \times 10^{-3}$$

Where E= optical density at 240nm,

Δt = time required for a decrease in the absorbance.

Table: 6. Effect of water extract of *Colebrookea oppositifolia* leaf on catalase assay in goat liver homogenate.

Dwash number	Treatment	Initial OD	Final OD	Time (min)	µM of H_2O_2consumed /min/mg tissue
1	Control	0.772± 0.0021	0.752 ± 0.0025	0.3	65.07 ± 0.57
2	Vitamin C (5mM)	0.847 ± 0.0016	0.794 ± 0.0012	0.3	163.83± 0.455
	Concentration of Water extract (µg/ml)				
3	200	0.832 ± 0.0022	0.814 ± 0.0023	0.42	66.02 ± 0.126
4	400	0.853 ± 0.0023	0.828 ± 0.0015	0.3	89.79 ± 0.034
5	600	0.850 ± 0.0023	0.821 ± 0.0012	0.42	104.73 ± 0.410
6	800	0.859 ± 0.0029	0.818 ± 0.0031	0.3	147.64 ± 0.868

Values were Mean ± S.E.M.; n=3 in each group.

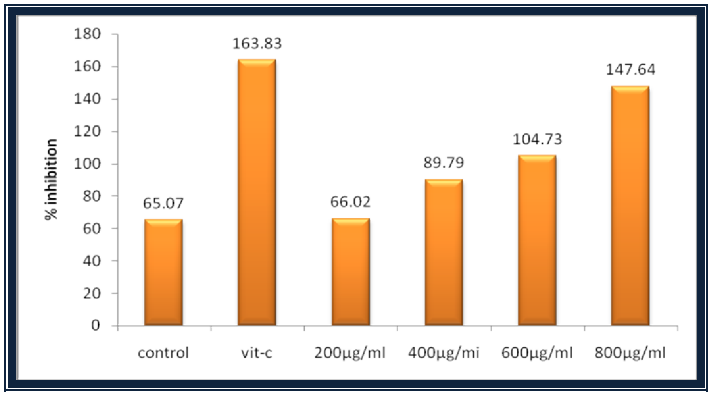

Fig: 2. Percentage of inhibition of lipid peroxidation by different concentrations of water extract of *Colebrookea oppositifolia* leaf, Vitamin C was used as standard.

Reduced glutathione assay (GSH):

Liver tissue was collected from slaughter house, washed in normal saline and soaked in filter paper. From that 1 g tissues were then homogenized in 10 ml of 0.15 M tris buffer (pH-7.4) and centrifuged at 3000 g at 4° C for 30 min. The supernatant was collected was taken for glutathione assay. Reduced glutathione (GSH) activity was assayed according to the method of Ellman. Reduced Glutathione was estimated spectrophotometrically at 412 nm by determination of DTNB (Dithiobis-(2-nitrobenzoic acid)) reduced by SH-groups against a reagent blank. Different concentrations (10-50µg) of standard glutathione were taken and processed as above for standard curve. The amount of reduced glutathione was expressed as µg of GSH/mg of wet tissue.

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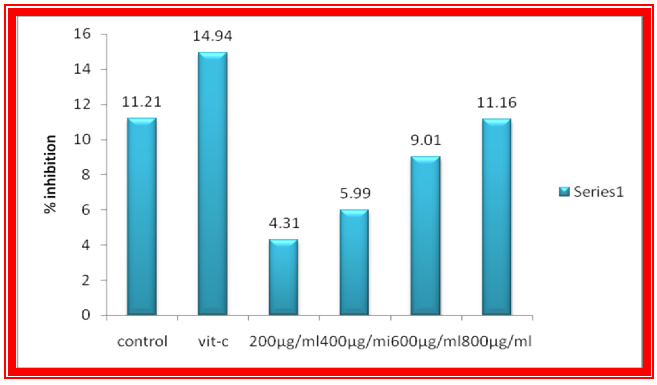

Fig: 3: Percentage of inhibition of lipid peroxidation by different concentrations of water extract of *Colebrookea oppositifolia* leaf, Vitamin C was used as standard.

Table: 7. Effect of water extract of *Colebrookea oppositifolia* leaf on glutathione assay in goat liver homogenate.

Dwash number	Treatment	Absorbance	Reduced glutathione (µg GSH/mg of wet tissue)
1	Control	0.6950 ± 0.0584	11.21 ± 0.9569
2	Vitamin C (5mM)	0.9223 ± 0.0360	14.94 ± 0.5897
	Concentration of Water extract (µg/ml)		
3	200	0.2662 ± 0.0110	4.31 ± 0.2818
4	400	0.3103 ± 0.0042	5.99 ± 0.1420
5	600	0.4530 ± 0.0199	9.01 ± 0.3317
6	800	0.5703 ± 0.0130	11.16 ± 0.2231

Values were Mean ± S.E.M.; n=3 in each group.

Table: 8- Effect of extract of *Colebrookea oppositifolia* on heart weight, body weight, liver weight, ratio of heart weight to body weight and ratio of liver weight to body weight in doxorubicin induced Cardiotoxicity (Values were mean ± SE from 6 rats)

Treatment	Body weight(g)	Heart weight(g)	Liver weight (g)	Heart wt/body wt ratio(X 10^{-3})	Liver wt/body wt ratio(X 10^{-3})
Control	190.41±11.23	0.6236±0.01541	3.934±0.0498	3.02	2.06
Standard (vita-c)	180.38±4.67	0.7291±0.00867*	3.697±0.0894*	4.04	2.04
DOX	159.20±5.270	0.9328±0.01132	5.697±0.088	5.85	3.57
Meco250mg/kg+ DOX	159.66±8.659*	0.5405±0.0103*	4.735±0.0513**	3.38	2.96
Meco500+ DOX	184.83±4.567	0.523±0.0111	3.715±0.0342	2.82	2.009
Weco250+ DOX	145.32±3.714*	0.479±0.0739**S	4.142±0.543**	3.29	2.85
Meco500+ DOX	190.83±3.964	0.774±0.0088**	4.992±0.104*	4.05	2.61
Degree of freedom	40	40	41	-	-
F value	7.557	6.235	6.586	-	-

P value; compared with (*) =p<0.05, (**) =p<0.01 Values were obtained by one way ANOVA followed by Dunnett multiple comparison test

Table: 9- Effect of MECO and WECO on lipid profile in doxorubicin induced Cardiotoxicity in rats (values were expressed in mg/dl were mean±SEM from n=6 rats)

Treatment mg/kg	Cholesterol	HDL	LDL	AST	TGA	ALP
Control	60.27±0.005	27.11±4.28	29.647±2.38	73.61±29.5	109.3±14.3	30.2±0.55
Standard	58.71±1.199**	46.31±1.32**	20.536±2.45**	174.2±21.4**	103.3±15.78**	42.87±1.99**
DOX	111.37±1.947	32.78±2.33	80.38±1.65	170±20.32	233.2±14.40	46.44±2.00
Meco250+Dox	64.30±7.630*	33.32±1.65**	55.27±2.35	171.43±23.10	174.00±13.00*	43.56±1.55***
Meco500+Dox	75.53±2.679*	35.72±3.21*	58.32±1.23*	175.22±17.23*	185.20±16.00	44.14±0.46*
Weco250+Dox	63.87±1.793**	34.41±1.22***	54.35±1.20*	168.51±19.20**	165.35±10.33**	38.2±0.65***
Meco500+Dox	79.7±0.9790**	36.78±1.44*	62.51±1.8*	172.23±15.33*	180.22±12.10*	38.7±0.83*
Degree of freedom	40	41	40	40	41	41
F value	74.761	73.622	74.051	74.629	73.351	73.428

Values were mean ± SEM (N=6). Values in the same row with different alphabet superscripts were significantly different at (*) < 0.05, (**) < 0.01, (***) < 0.001. Values were obtained by one way ANOVA followed by Dunnett multiple comparison test.

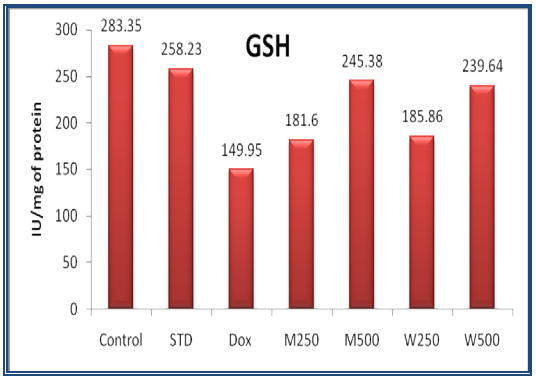

Fig: 4: Estimation of GSH in rat Heart

Table: 10- Effect of MECO and WECO on TBARS, GSH, SOD and CAT in rat heart

Treatment mg/kg	GSH IU/mg protein	CATALASE IU/mg protein	TBARS IU/mg protein	SOD IU/mg protein
Control	283.35±0.95	80.31±0.5	18.7±1.06	4.91±0.13
Standard(vit-e)	258.23±0.66**	78.95±0.31**	19.29±1.09**	4.66±0.32**
Doxorubicin	149.95±0.62	36.36±0.69	49.26±1.14	1.27±0.11
Meco250 + DOX	181.6±0.94*	54.97±0.69*	34.65±1.16*	3.16±0.34**
Meco500+ DOX	245.38±0.89**	73.58±0.73**	29.3±1.12**	4.24±0.20**
Weco250+ DOX	185.86±0.99*	58.32±0.99*	37.73±1.30*	3.62±0.41**
Weco500+ DOX	239.64±1.99**	75.52±.89**	21.52±1.21**	4.10±0.51**
Degree of freedom	41	41	41	41
F value	11.802	10.438	11.362	11.377

Values were mean ± SEM (N=6). Values in the same column with different alphabet superscripts were significantly different at (*) p< 0.05, (**) p< 0.01. Values were obtained by one way ANOVA followed by Dunnett multiple comparison test.

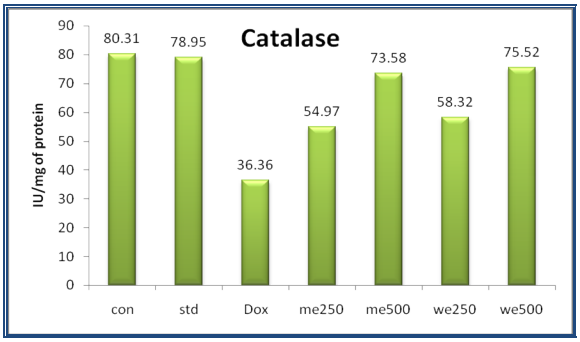

Fig: 5: Estimation of Catalase in rat Heart

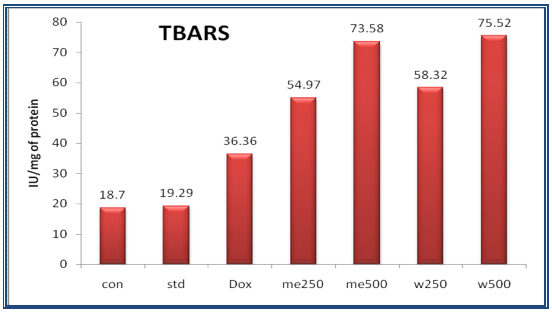

Fig: 6: Estimation of TBARS in rat Heart.

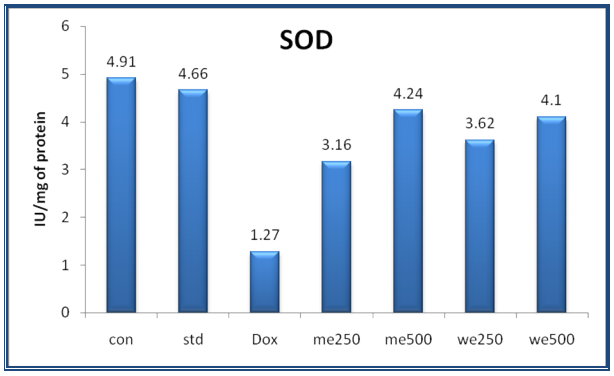

Fig: 7: Estimation of SOD in rat Heart

DISCUSSION:

For evaluating the cardioprotective activity of the plant extracts, *in-vivo* studies were performed by using a novel model of doxorubicin induced cadiotoxicity in rats. During the study of cardioprotective effects of *Colebrookea oppositifolia,* various parameters were tested. Chronic administration of doxorubicin induced cardiac toxicity and cardioprotective effects were established by significant change in cardiac biomarker enzymes and endogenous antioxidants.

General conditions of all groups of animals were recorded throughout the study. No significant changes were observed in all the groups except the control groups. Effect of doxorubicin on heart weight, body weight and liver weight, ratio of heart weight to body weight and liver weight to body weight were studied and it was found that weight of heart and the ratio of heart weight to body weight were significantly increased in the doxorubicin treated Group compared to the normal control Group whereas in treatment Groups this ratio was decreased.

Animals treated with doxorubicin produced significant increase in the levels of cholesterol, triglycerides, and LDL compared to control group and there was very slight difference in HDL level compared to control Group. Group 4, 5, 6 and 7 produced significant decrease in the levels of cholesterol, triglycerides and LDL but significant increase in the level of HDL as compared to Group 3.

The Study of lipid peroxidation and antioxidant enzymes showed the malondialdehyde level was increased; GSH, SOD and CAT levels were significantly decreased in doxorubicin treated group as compared to Control Group. Groups 4, 5, 6 & 7 produced significant decrease in the level of MDA

and increase in the status of antioxidant enzyme.

CONCLUSION:

It is concluded that the oral administration of the *Colebrookea oppositifolia* extract in rats, augments myocardial endogenous antioxidants, without causing any cellular injury. This offered protection against oxidative stress produced by doxorubicin. This study reveals an important and highly significant activity of the *Colebrookea oppositifolia,* in the treatment of heart diseases.

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