JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2022/27855
RESEARCH ARTICLE

Butanol Fraction of *Rivea ornata* Attenuate Endothelial Dysfunction in Rats via Modulation of Cardiovascular Risk Factors

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Abstract

Atherosclerosis is caused by vascular inflammation and oxidative stress. Pro-atherogenic effect of hypercholesterolemia caused by impairment of nitric oxide generation due to activated arginase. The study takes up to find the atheroprotective role of polyphenolic fraction of *Rivea ornata* leaves by using lipid emulsion induced atherosclerosis in rat model. The study carried out by studying atherogenic markers in the serum (lipid profiles, C-reactive protein), vascular tissue (myeloperoxidase, arginase, hydroxyproline, lipid peroxidation) and atheroprotective factors in the serum (paraoxonase, nitric oxide), and in the vascular tissue (thiol levels, endogenous antioxidants) after feeding the rats with lipid emulsion for 12 weeks. Treatment of polyphenolic rich butanol fraction is able to correct the imbalance of atherogenic and antiatherogenic factors induced by lipid emulsion feeding. Butanol fraction at the dose of 400 mg/kg significantly increases high density lipoprotein, paraoxonase, nitric oxide, tissue thiol levels, endogenous antioxidants and decreases total triglycerides, total cholesterol, very low-density lipoprotein, low density lipoprotein, myeloperoxidase, arginase, hydroxyproline, lipid peroxidation. The atheroprotection reflected in histopathology studies also. Lipid emulsion associated foam cells formation is inhibited by butanol fraction. These all are due to the presence of gallic acid in polyphenol rich butanol fraction is responsible for the underlying mechanism of atheroprotection.

Keywords: Butanol Fraction, Cardiac Rick Factors, High Performance Liquid Chromatography Analysis, Inflammation Markers, Lipid Emulsion Induced Atherosclerosis, Lipid Profiles

1. Introduction

Atherosclerosis is characterised by deposition of atherosclerotic plaques, composed of cholesterol, calcium, fibrotic tissue in the lumen of medium and large sized arteries leads to reduction of blood flow at sufficient level[1]. The patients don't exhibit any sign of ischemia even lumen obstruction reached to 70 %, after that patient getting experience the symptoms of angina and further myocardial infraction[2]. Among all cardiovascular deaths, deaths from coronary obstruction are placed at top position, are consequence of coronary atherosclerosis. As per WHO, 23.6 million people are expected to die from Cardio Vascular Diseases (CVD) by 2030. People with the presence of one or more co-morbidities like chronic hypertension,

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Article Received on: 28.05.2021 Revised on: 12.10.2021 Accepted on: 17.01.2022

hyperlipidaemia, obesity are prone for development of atherosclerosis[3]. Molecular bases for atherosclerosis include series of events includes endothelial damage, deficiency of vasodilator nitric oxide, intraluminal deposition of oxidized LDL, infiltration of monocytes, secretion of inflammatory cytokines, deposition of lipid loaded macrophage causes of blocking of lumen of arterial wall partially or completely[4]. So many researchers are trying to find the potential targets which influence the progress of atherosclerosis. One of such identified targets is arginase pathway. Arginase make the L-arginine in to inactive products, due to which it declines the levels of arginine, is a precursor of nitric oxide in the vascular endothelial cells. As a result, causes vascular endothelial dysfunction, is the step of initiation of atherosclerosis. In one recent study arginase knockout rats observed improved nitric oxide signalling and also vascular endothelial function[5]. Apart from this, various clinical and non-clinical studies proven that arginase pathway is involved in endothelial dysfunction and as associated problems such as atherosclerosis, hypoxia, diabetes and hypertension[6]. Arginase enzyme inhibitors are attractive targets for treatment of atherosclerosis, in parallel polyphenolic compounds are known reported as arginase enzyme inhibitors[7]. Based on our preliminary studies of butanol fraction of *Rivea ornata* respective to phenolic and flavonoid content we planned for studying anti-atherosclerotic effect. Literature supports the polyphenolic compounds being a one of the known arginase inhibitors. *Rivea ornata* is a climber with cylindrical stem, broader leaves, white silky flowered and smooth surface sub globose brown-coloured fruits. Based on the traditional claim reported in literature for cardiovascular disorders and its antioxidant activity we chosen this plant to explore the anti-atherosclerotic activity. In addition, it is used in disorder of gallbladder, bronchitis, and fatigue. In Konkan, its juice is used to treat piles[8]. In Srilanka fresh leaves used in diabetes, bergenin is a polyphenolic compound identified in that plant, aerial parts of this plant have anti-inflammatory effect, reported as analgesic and antipyretic and also is a one of the components of *sidda* formulation named *Maavilingapattai chooranam* used as hepatoprotective agent[9].

2. Materials and Methods

2.1 Collection and Identification of the Plant Material

Leaves of *Rivea ornata* were collected from Tirumala hills during December to January and dried under shade. The plant was authenticated by Prof. K. Madhava Chetty, Taxonomist, and a voucher specimen (voucher number: 0913) has been stored at S.V University (Botany department), Tirupati, (Andra Pradesh, India).

2.2 Extract Preparation, Phytochemical Screening

1 kg of dried leaves powder was macerated with 3 litres of ethanol (99 %) for 1 week. The resulting crude solvent was evaporated to get the semisolid extract and it was dissolved in hot water. It was further proceeded with solvent/solvent fractionation. All the resulting solvents fractions (n-hexane, ethyl acetate, butanol and remaining aqueous) were evaporated to yield a concentrated form of about 18.6 %. Phytochemical investigation was determined in crude ethanol extract and its fractions as per established methods[10].

2.3 Estimation of Total Phenolic and Flavonoid Content

For the estimation of phenolic content, 0.5 ml of diluted extracts and its fractions at concentration of 1mg/ml and varying concentration of gallic acid (20 to 100 µg /mL) were added with Folin-Ciocalteu's reagent (5 ml of 10 % v/v) and shaken well. 4 ml of Na_2CO_3 (7% w/v) solution was added after 5 min to the above solution. It is further incubated (90 min) and absorbance of the coloured is solution was noted at 750 nm. For flavonoid content, the 1 mL of extracts/fractions and standard solutions of quercetin (20 to 100 µg /ml) were mixed with freshly prepared $AlCl_3$ (2.5 ml of 10 %w/v) and then with 1 M NaOH (2 ml) and total volume make up to 10 ml with DW. The final-coloured solution was marked at 510 nm after 30 min incubation. The contents were reported as gallic acid and quercetin equivalents (mg/g of weight of extract)[11].

2.4 Column Chromatography of Butanol Fraction

The butanol fraction was processed for column chromatography to separate the polyphenolic compounds. Initially methanol solubilized butanol fraction (1.5 g) was mixed with silica gel in motor by trituration with pestle. Fraction coated silica gel was placed on the top of silica gel (60-120 mesh) packed chromatography column (75 cm×3.5 cm). The column was serially eluted with hexane, hexane and ethyl acetate (1:1) and ethyl acetate. Total 40 sub-fractions (each of 150 ml) were collected and then mixed on the basis of their TLC matching with standard flavonoid (quercetin)[12].

2.5 Identification of Flavonoids by TLC

n-Butanol fraction was tested by TLC for the presence of flavonoids. The TLC pre-coated silica gel 60 GF254 plates were developed using a flat bottom chamber which was pre-saturated with the mobile phase for 30 min. Mixture of n-hexane and ethyl acetate (1:1) was used as optimized mobile phase for the effective separation. After development of TLC chromatogram of butanol extract and standard flavonoid, plates have been removed and dried, the spots were visualized by kept in iodine chamber. Fraction which are matched with standard were pooled together and further analysed by HPLC for polyphenolic profiling since butanol fraction was known to be rich in polyphenols and proved by preliminary analysis for polyphenolic content and TLC profiling. To confirm the purpose, the butanol fraction was analysed by HPLC to identify the polyphenolic compounds[13].

2.6 HPLC Analysis of Butanol Fraction

HPLC analysis of polyphenolic compounds of butanol fraction was determined based on retention time of eluted compounds.

HPLC chromatography conditions

System: Agilent LC1200

Software: EZ Chrome Elite

Column: C18 Normal-phase analytical column (250 × 4.6 mm)

Solvent system: Binary gradient mode, (Acetonitrile: water -65:35)

Pump: Pneumatic pump

Injection volume: Injection volume 20 µL

Solvent flow rate: Total flow 1 mL/min, column oven temperature was 25 °C

Detection wavelength: 230 nm.

Detector: Diode Array Detector (DAD)

Polyphenolics matched TLC butanol fraction was dissolved in methanol and further filtered with millipore filters before injection. Standard polyphenolic compounds were processed as same as that of butanol fraction. Standards used in this experiment were quercetin, gallic acid, naringin, ellagic acid for qualitative evaluation of phenolic compounds[14].

2.7 Animals

Animal were procured and experiments were conducted as per the protocol approved by The Institutional Animal Ethics Committee (*878/ac/05/CPCSEA/005/2016*) of Raghavendra Institute of Pharmaceutical Education and Research, Anantapuramu, Andhra Pradesh, India.

2.8 Determination of LD_{50}

LD_{50} was determined as per specification of OECD, 423 protocol. No toxic symptoms or mortality was observed until 14 days period with dosing of butanol fraction of *Rivea ornata* (5,50,300 and 2000 mg/kg bw). Hence, further experimental dose was selected as one tenth and one fifth of the LD_{50} dose that is 200 mg/kg and 400 mg/kg[15].

2.9 Lipid Emulsion Induced Atherosclerosis Model

Wistar rats were fed orally with freshly prepared lipid emulsion daily for the period of 12 weeks continuously at the dose of 1.5 ml/kg to induce atherosclerosis[16].

2.9.1 Composition of Lipid Emulsion

Content	Quantity
Cholesterol	45 mg/mL
Lard	15 mg/mL
Sodium cholate	7.5 mg/mL
Propylthiouracil	3 mg/mL
Sugar	75 mg/mL
Vitamin D3	100000 IU/kg

Groups	Type of treatment	Diet provided daily
I	Normal control	Normal pellet diet
II	Vehicle control, given orally distilled water (2 ml/kg)	Lipid emulsion
III	Standard – Atorvastatin,10 mg/kg oral (refence)	Lipid emulsion
IV	Butanol fraction of *Rivea ornata* (BFRO), 200 mg/kg	Lipid emulsion
V	Butanol fraction of *Rivea ornata* (BFRO), 400 mg/kg	Lipid emulsion

2.10 Experimental Design

A total of 30 albino male wistar rats were allocated in to five groups containing six animals each. All the animals were treated for a period of 12 weeks.

Vehicle control animals received carboxy methyl cellulose (1 %w/v) in distilled water. The reference drug, atorvastatin and butanol fraction were orally administered after suspending in distilled water with carboxy methyl cellulose (1 %w/v).

2.10.1 Estimation of Serum Parameters

Blood samples were collected via puncturing the retrorbitol plexus under anaesthesia. The separated serum was used for the estimation of following biochemical parameter such as blood lipid levels (TG, TC, HDL, VLDL, LDL) and other markers (LDH, CKMB and CRP)[17].

2.10.2 Paraoxonase-1 (PON-1) Activity

Paraoxonase-1 activity was estimated by established colorimetric methods. The enzyme activity was started by addition of serum (10 µl) to 3 ml of substrate solution (100 mM Tris-Hcl with pH 8, 2 mM $CaCl_2$, and 2 mM phenyl acetate). The absorbance of final products was read at 270 nm for every 20 sec up to 1 min against blank without the serum.The amount of product formed was determined from established molar extinction coefficient (1310 M^{-1} cm^{-1}) and the results were expressed as units per ml serum[18].

2.10.3 Fecal Cholesterol Excretion Study

Fecal matter was collected during 24 hours, dried and powdered which is used for the estimation of cholesterol by using ERBA kits[19].

2.10.4 Estimation of Tissue Parameters

All the animals from every group were subjected to painless death and freshly isolated thoracic aorta was homogenized to get 10 % w/v homogenate in ice-cold phosphate buffered saline (10 mM, TpH 7.4). This tissue homogenate was used for the estimation of HMG CoA reductase activity[20], reduced form of glutathione, catalase, superoxide dismutase, products of lipid peroxidation[21].

2.10.5 Determination of Total Thiol Levels

Total thiols in the tissue homogenate were determined using Ellman's color reagent. The reagent solution was prepared by mixing 1 ml of supernatant of tissue homogenate, 1 ml of Tris-EDTA (pH=8.6) and 1 ml of DTNB (10 mM). The reagent solution incubates at room temperature for 30 min and read the absorbance at 412 nm. The quantity of tissue thiols was noted as µmol/mg protein[22].

2.10.6 Measurement of Arginase Activity

The reaction reagent was prepared by adding 1 ml of aorta tissue homogenate, 1.25 ml of $MnCl_2$ (10 mmol/L) in Tris Hcl (50 mmol/L, pH 7.5) and heated for 10 min at 55-60 °C to activate the arginase enzyme. To the above reaction reagent, 1 ml of L-arginine (0.5 mol/L, pH 9.7) was added and incubated for 1 hour at room temperature to undergo hydrolysis of arginine. The reaction stopped by adding 8 mL of acid solution containing H_2SO_4:H_3PO_4:H_2O (1:3:7). The amount of urea formed was determined spectrophotometrically at 550 nm after adding 1 ml of color reagent (α-isonitrosopropiophenone)[23].

2.10.7 Estimation of Nitric Oxide

Nitric oxide (NO) in the serum samples was quantified by using Griess reagent. 200 µl of serum was mixed with 200 µl of Griess reagent (v/v). The reaction started by incubating the solution for 30 min in dark place. The resulting coloured solution was read at 550 nm. The

amount of nitric oxide was determined from standard curve of sodium nitrite[24].

2.10.8 Estimation of Myeloperoxidase in Rat Aorta

The isolated rat aorta was homogenized with 0.5 % of hexadecyl trimethyl ammonium bromide in phosphate buffer (50 mM, pH 6.0) and prepared 10 % w/v homogenate. After three cycles of freezing and thawing of homogenate, 1 ml of tissue supernatant was mixed with 4.9 ml of reaction reagent containing O-dianisidine hydrochloride and hydrogen peroxide (0.0005 %). The absorbance of the above solution was read at 460 nm. MPO activity was calculated from established formula[25].

2.10.9 Estimation of Hydroxyproline Content in Aorta

A colorimetric assay as described by Reddy and Enwemeka was used to estimate hydroxyproline in rat aorta tissue. Briefly, 100 mg dry tissue sample was digested in 2 ml of HCl by keeping at boiling water bath for 3 h. The pH of tissue hydrolysate was adjusted to 7.0 with NaOH and 1.0 mL of chloramine T solution was added. To this, 1 mL of Ehrlich's reagent solution was added and then heated for 20 minutes at 60 °C. The absorbance of the final solution was read at 550 nm[26].

2.10.10 Histopathology

Immediately after scarification, isolated aorta was processed for histopathology by using haematoxylin and eosin staining. The stained sections were observed under 100 X and 200 X.

2.11 Statistical Analysis

The data generated in the study were reported as mean ± SEM and were analysed by one-way ANOVA followed by Bonferroni test using graph pad prism software version 8 and significant value of $P < 0.05$ were considered.

3. Results

3.1 Result of Qualitative Analysis of Flavonoid in Various Extracts/ Fractions of *Rivea ornata*

From the observation of qualitative test, the ethanol extract and butanol fraction resulted positive for flavonoids through flavonoid assessment tests but flavonoid and phenolic contents were high in butanol fraction rather than ethanol extract. It might be due to attainment of high concentration of polyphenols in butanol solvent while solvent / solvent fractionation (Table 1).

3.2 Total Phenolic and Flavonoid Content

Total phenolic and flavanoid contents were determined from known standard concentration and absorbance curves. Butanol fraction had highest contents (82.4 ± 4.61 mg of GAE/g, 105 ± 4.09 mg of quercetin/g) compared to crude ethanol and its other fractions (n-hexane, ethyl acetate and aqueous fraction). From preliminary analysis, it was evident that butanol fraction contained high phenolic and flavonoid contents. Henceforth the butanol fraction was take up for screening of antiatherosclerosis activity, as polyphenolics were reported in the literatures (Table 2 and Figure 1).

Table 1. Qualitative results of flavonoids in *Rivea ornata* leaves ethanol extract/its fractions

Extract/ Fraction	Shinoda test (Deep red colour)	Lead acetate test (Yellow colour ppt)	Pews tests (Red colour to cherry red colour)	NaOH (Yellow colour)
Ethanol	+	+	+	+
n-Hexane	-	-	-	-
Ethyl acetate	-	+	+	+
Butanol	+	+	+	+
Aqueous	-	-	-	-

Table 2. Total phenolic and flavonoid contents of *Rivea ornata* extract and its fraction

Extract	TPC (mg of gallic acid equivalent/g)	TFC (mg of quercetin equivalent/g)
Crude ethanol extract	56.9 ± 4.30	55.1±2.79
n-Hexane fraction	6.39 ± 1.38***	16.7 ± 0.351***
Ethyl acetate fraction	64.2 ± 1.56	77.5 ± 6.20*
Butanol fraction	82.4 ± 4.61**	105 ± 4.09***
Aqueous fraction	18.1 ± 3.52 ***	27.7 ± 4.10**

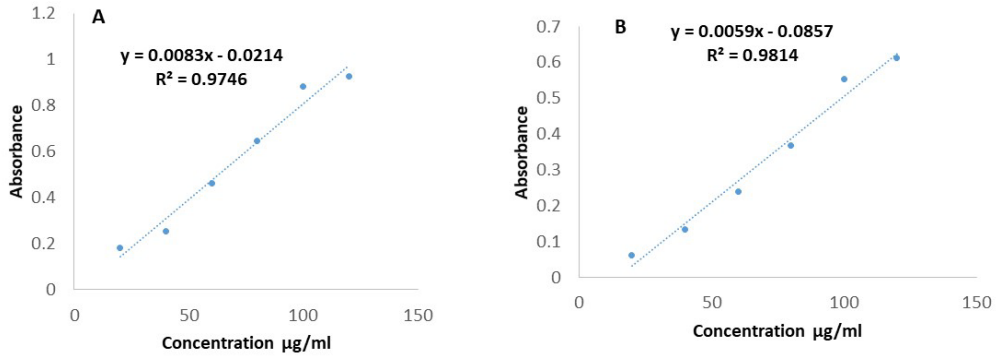

Figure 1. Regression line of gallic acid and quercetin

Data generated represented as mean ± SD and significant changes were considered when p value less than 0.05.

3.3 HPLC Profile of Collected Fractions

HPLC chromatogram was observed with one major peak and three smaller peaks. The retention time of major peak of butanol fraction was matched with gallic acid (phenolic compound marker). The retention time of butanol fraction and gallic acid are as follows: 1.76 (gallic acid) and 1.84 (butanol fraction). This data supported the presence of gallic acid, a polyphenolic compound in our butanol fraction. So it was found that gallic acid was the major component of butanol fraction of *Rivea ornata* leaf extract. It was a known phenolic compound with broad biological activity (Figures 2 and 3).

3.4 Effect of Biactive Butanol Fraction on Physiological Changes

After 12 weeks of lipid emulsion administration there was significant (*** p<0.001) increase in body weights of group II (Lipid emulsion group) animals compared to normal diet received animals. Whereas butanol fraction of *Rivea ornata* at the higher dose (400 mg/kg) was efficiently (**p<0.01) controlling the weight gain induced by lipid emulsion. But body weight changes were not significant in lower dose of test fraction. Lipid emulsion alone had increased the atherogenic index significantly (*** p<0.001) compared to normal pellet diet. Atorvastatin and higher dose of butanol fraction reduced the atherogenic index significantly (*** p<0.001 & ** p<0.01) and also exhibited higher percentage of atheroprotection (Table 3).

The data represented as mean ± SEM for six rats, statistical data accepted as significant when p value smaller than 0.05. P value of group II was compared with all groups and significant changes were represented as * (p<0.05), **(p<0.01) and *** (p<0. 001).

3. 5 Effect of Butanol Fraction on Serum Lipid Parameters

Serum lipid profiles of animals from all groups were determined for every 21 days up to 84 days. Baseline lipid profiles of all groups of animals were not significant at 0 week. Lipid emulsion alone started producing hyperlipidemia after 21 days and it was continued upto 84 days compared to control animals received normal pellet diet. It was evident by a significant (*** p<0.001) increase in bad lipids such as TG, TC, LDL, VLDL and decrease in good lipid such as HDL. Chronic administration of butanol fraction started showing antihyperlipidemic effect significantly (* p<0.05) after 21 days especially at the dose of 400 mg/kg but not lower dose and continued upto 84 days. It was evident by a significant decrease in TG, TC, LDL, VLDL and HDL (Table 4).

The data represented as mean ± SEM for six rats, statistical data accepted as significant when p value smaller than 0.05. P value of group II was compared with all groups and significant changes were represented as * (p<0.05), **(p<0.01) and *** (p<0. 001).

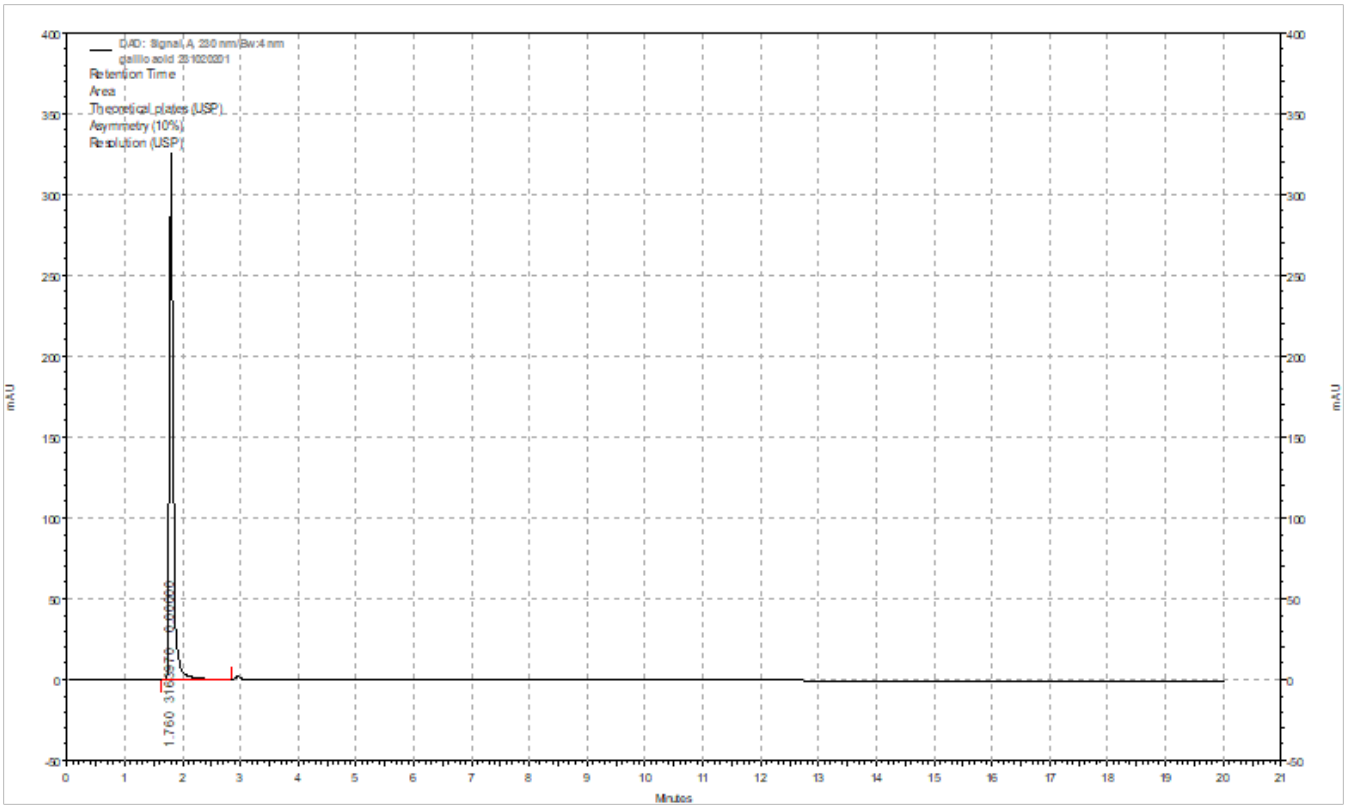

Figure 2. HPLC chromatogram of gallic acid (standard Phenolic compound).

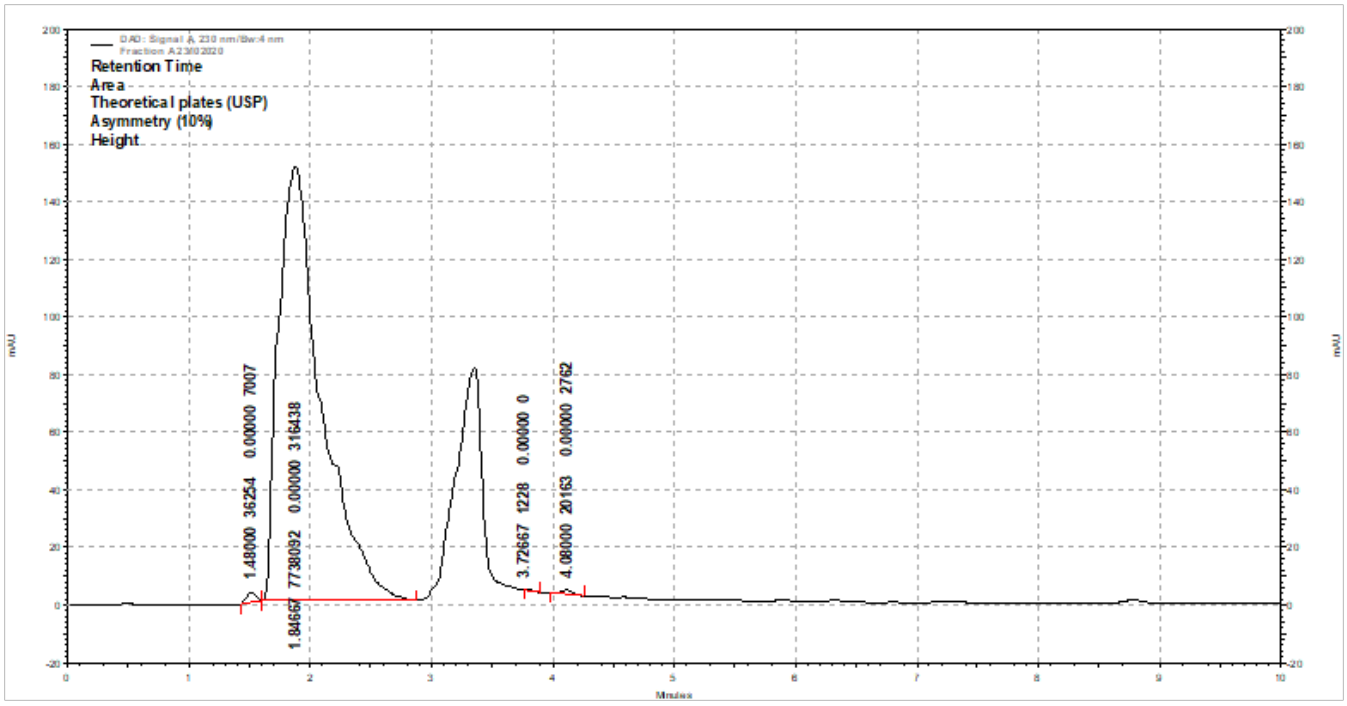

Figure 3. HPLC chromatogram of butanol fraction.

Table 3. Observation of body weight changes, atherogenic index and percentage protection

Group	Initial body weight (g)	Final body weight (g)	Body weight difference (g)	Atherogenic index	% Protection
I	198 ± 3.58	310 ± 10.7	153 ± 3.58	2.70 ± 0.127	
II	203± 10.3	435 ± 9.05	252 ± 9.05***	22.9 ± 1.53***	
III	230 ± 10.5	444 ± 14.0	214 ± 4.50**	4.77 ± 0.487***	79.2±1.48
IV	198 ± 6.18	428 ± 9.27	230 ± 5.83 NS	21.2 ± 2.91	16.9±6.20
V	210 ± 6.92	423 ± 9.41	213 ± 5.97**	12.7 ± 1.11**	43.9±4.69

Table 4. Lipid profile changes induced by lipid emulsion and effects of butanol fraction of *Rivea ornata* on lipid profiles

Test	Day	I	II	III	IV	V
TC	0	41.3 ± 2.50	42.8 ± 2.12	43.0 ± 2.26	47.0 ± 2.05	46.3 ± 3.36
	21	47.8 ± 2.84	80.1 ± 5.87***	58.3 ± 0.910**	90.7 ± 2.45	70.2 ± 4.47
	42	56.3 ± 2.35	124 ± 8.65***	69.8 ± 2.26 ***	110 ± 3.37	98.8 ± 4.96*
	63	65.3 ± 1.57	157 ± 7.45***	76.9 ± 1.71 ***	166 ± 5.36	135 ± 3.78*
	84	71.6 ± 2.29	177 ± 8.71***	107 ± 7.46***	176 ± 5.42	146 ± 6.92*
TG	0	38.3 ± 2.09	40.0 ± 2.35	39.8 ± 1.71	42.1 ± 1.68	43.7 ± 1.56
	21	40.1 ± 2.10	56.6 ± 2.42***	53.2 ± 2.91	61.4 ± 2.32	53.6 ± 2.00
	42	41.1 ± 2.10	71.0 ± 2.21***	49.6 ± 2.99***	75.3 ± 2.62	59.2 ± 1.80*
	63	40.7 ± 1.83	88.5 ± 1.93***	69.7 ± 3.14***	85.4 ± 2.35	76.9 ± 1.69*
	84	41.8 ± 2.04	106 ± 3.65***	75.9 ± 1.77***	99.4 ± 4.81	89.9 ± 1.42**
LDL	0	15.7 ± 2.59	16.9 ± 2.50	16.8 ± 2.34	20.7 ± 1.99	20.3 ± 2.91
	21	21.0 ± 2.95	54.9 ± 6.23**	30.4 ± 1.68**	63.7 ± 2.64	44.0 ± 4.61
	42	29.3 ± 2.60	96.9 ± 8.57***	42.8 ± 2.30***	82.8 ± 3.43	70.9 ± 4.94*
	63	37.6 ± 1.62	128 ± 7.37***	44.6 ± 1.53***	136 ± 5.33	105 ± 4.60*
	84	43.8 ± 2.32	148 ± 8.58***	73.2 ± 7.71***	148 ± 5.34	118 ± 7.12*
VLDL	0	7.66 ± 0.419	8.00 ± 0.470	7.96 ± 0.342	8.41 ± 0.337	8.74 ± 0.312
	21	8.02 ± 0.419	11.3 ± 0.483***	10.6 ± 0.582	12.3 ± 0.465	10.7 ± 0.400
	42	8.21 ± 0.419	14.2 ± 0.443***	9.93 ± 0.599***	15.1 ± 0.524	11.8 ± 0.361*
	63	8.13 ± 0.366	17.7 ± 0.386***	13.9 ± 0.629***	17.1 ± 0.469	15.4 ± 0.337*
	84	8.36 ± 0.409	21.2 ± 0.730***	15.2 ± 0.355***	19.9 ± 0.956	18.0 ± 0.283**
HDL	0	18.0 ± 0.401	17.9 ± 0.522	18.2 ± 0.741	17.9 ± 0.463	17.3 ± 0.432
	21	18.8 ± 0.233	13.9 ± 0.567***	17.3 ± 0.476***	14.7 ± 0.733	15.5 ± 0.446
	42	18.9 ± 0.284	13.2 ± 0.700***	17.1 ± 0.547***	12.2 ± 0.683	16.1 ± 0.481*
	63	19.6 ± 0.408	10.8 ± 0.441***	18.3 ± 0.436***	13.0 ± 0.747	14.7 ± 0.849**
	84	19.4 ± 0.423	7.46 ± 0.343***	18.7 ± 0.332***	8.43 ± 0.811	10.9 ± 0.586**

Table 5. Atherogenic markers changes in different groups of animals

Group	CRP (mg/L)	Paraoxonase-1(U/mL)	Myeloperoxidase(U/g)	Hydroxyproline (µg/mg protein)
I	1.80 ± 0.19	166 ± 4.40	1.68 ± 0.230	17.9 ± 1.09
II	5.83 ± 0.20***	107 ± 6.44***	6.60 ± 0.225***	57.3 ± 2.06***
III	3.15 ± 0.34***	140 ± 5.86**	2.60 ± 0.215***	37.7 ± 1.62***
IV	5.59 ± 0.19	107 ± 6.74	6.31 ± 0.441	45.7 ± 3.66*
V	4.30 ± 0.42**	133 ± 2.71*	5.04 ± 0.351*	42.6 ± 2.92**

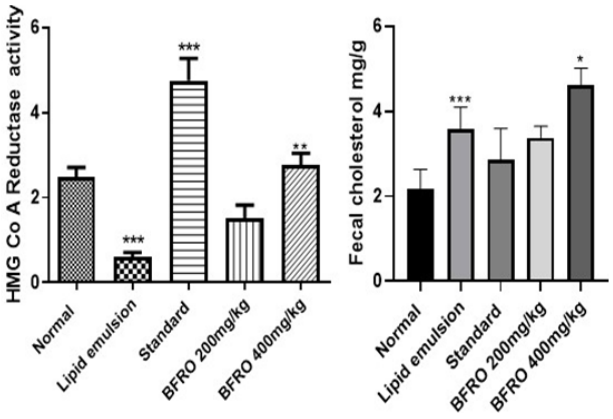

Figure 4. Effect of butanol fraction of *Rivea ornataon* HMG CoA reductase and fecal cholesterol content.

compared to lipid emulsion received animals. Increase in fecal cholesterol excretion by butanol fraction accounted its cholesterol absorption inhibitor activity which aids in antihyperlipidemic activity of butanol fraction (Figure 4).

3.7 Effect of Butanol Fraction on Atherosclerosis Markers

Atherosclerosis markers from all groups of animals were determined in serum (C-reactive protein and paraoxonase) and homogenate of aorta (myeloperoxidase and hydroxyproline) on 85th day of protocol. Lipid emulsion has significantly (***p<0.001) increased C-reactive protein, myeloperoxidase and

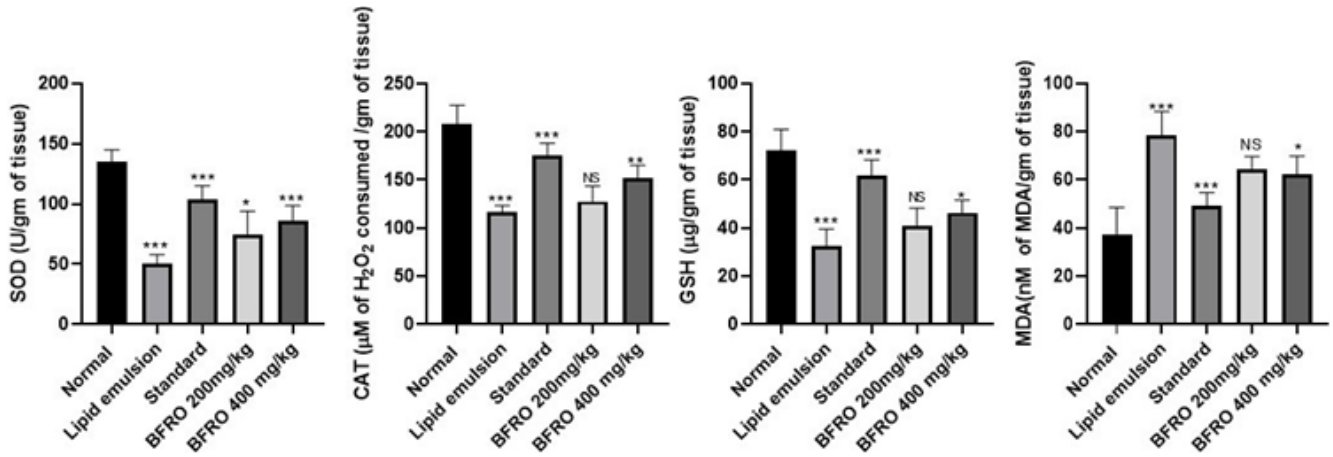

Figure 5. Effect of Butanol Fraction of *Rivea ornata* on Cardiac Tissue Antioxidants and Lipid Peroxidation.

3.6 Effect of Butanol Fraction on HMG CoA Reductase Activity and Fecal Cholesterol Excretion

The ratio of HMG CoA and mevalonate in hepatic tissue was considered to express the HMG CoA reductase activity. Lower the ratio, higher the activity and vice versa. There was significant lower ratio of HMG CoA and mevalonate in lipid emulsion received animals indicated higher enzyme activity and there was a significant increase in ratio of HMG CoA and mevalonate in butanol fraction of *Rivea ornata* received animals indicate the HMG CoA reductase inhibitory activity of butanol fraction. In other mechanistic study, there was a significant increase in fecal cholesterol excretion in butanol fraction received animals

hydroxyproline and decreased paraoxonase compared to animals received normal pellet diet. Lipid emulsion induced atherosclerosis markers significantly modulated by butanol fraction of *Rivea ornata* especially by upper dose (400 mg/kg). It decreased the C-reactive protein (**p<0.01), myeloperoxidase (*p<0.05) and hydroxyproline (**p<0.01) and increased paraoxonase (*p<0.05) compared to lipid emulsion received animals (Table 5).

The data represented as mean ± SEM for six rats, statistical data accepted as significant when p value smaller than 0.05. P value of group II was compared with all groups and significant changes were represented as * (p<0.05), **(p<0.01) and *** (p<0. 001).

Table 6. Effect of butanol fraction on vascular protective markers

Group	Nitric oxide (µmol/L)	Arginase (mg Urea/min/mg protein)	Total thiol levels (µmol/mg protein)
I	51.6±2.59	0.27± 0.05	5.39±0.479
II	13.7±3.30***	1.56 ± 0.19***	2.22±0.208***
III	41.1±1.43***	0.56± 0.03***	4.11±0.253**
IV	14.9±4.34 NS	1.01± 0.133*	3.72±0.363*
V	27.4±2.74 *	0.93± 0.07**	4.05±0.311**

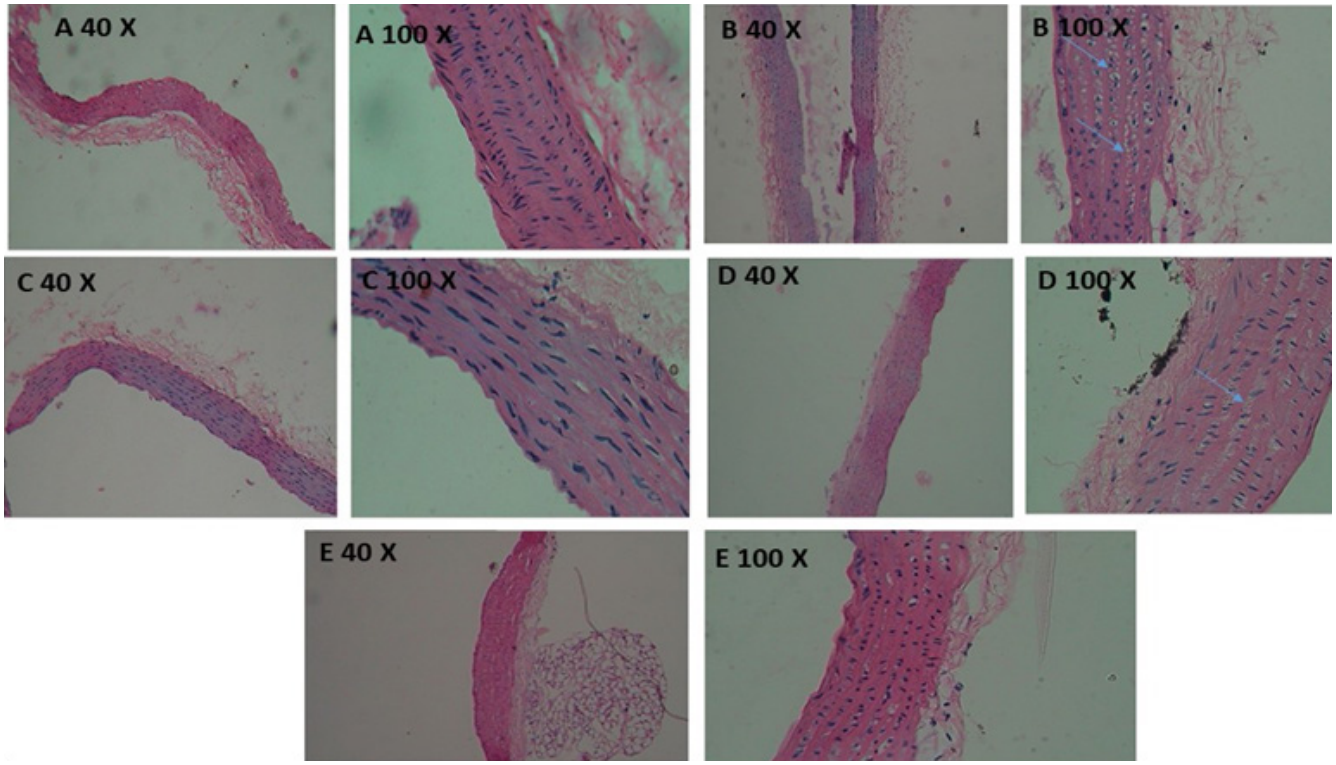

Figure 6. Histopathology sections of thoracic aorta: **A.** Normal rats **B.** Lipid emulsion **C.** Atorvastatin **D.** Lower dose of Rivea ornata (200 mg/kg) E. Upper dose of *Rivea ornata* (400 mg/kg). LV= Lipid vacuoles.

3.8 Effect of Butanol Fraction on Vascular Protective Markers

Vascular protective markers such as serum nitric oxide and aorta homogenate arginase and tissue thiols were determined at the end of 85th day of study in all groups of animals. Lipid emulsion decreased the serum nitric oxide, tissue thiols levels and increased tissue arginase levels significantly (*** p<0.001) compared to normal pellet diet. But butanol fraction of *Rivea ornata* at the dose of 400 mg/kg significantly increased the serum nitric oxide (* p<0.05), tissue thiols (** p<0.01) levels and decreased aorta homogenate arginase (** p<0.01) and (Table 6)

The data represented as mean ± SEM for six rats, statistical data accepted as significant when p value smaller than 0.05. P value of group II was compared with all groups and significant changes were represented as * (p<0.05), **(p<0.01) and *** (p<0. 001).

3.9 Effect of Butanol Fraction on Aorta Tissue Oxidative Stress Parameters

Significant ($P < 0.001$) decrease in aorta tissue levels of Catalase (CAT), Superoxide Dismutase (SOD), and reduced Glutathione (GSH) and increase in the levels of Malondialdehyde (MDA) in lipid emulsion received animals compared to normal pellet diet received rats was observed. Prior treatment with butanol fraction of *Rivea ornata* treatment for 84 days significantly (400 mg/kg) increased the levels of catalase (** $P < 0.01$), SOD (*** $P < 0.001$), GSH (* $P < 0.05$) and decreased the levels of MDA (* $P < 0.05$) in the aorta tissue compared with lipid emulsion received rats (Figure 5).

The data represented as mean ± SEM for six rats, statistical data accepted as significant when p value smaller than 0.05. P value of group II was compared with all groups and significant changes were represented as * ($p<0.05$), **($p<0.01$) and *** ($p<0. 001$).

4. Histopathology

The histoarchitecture of aorta looked normal in those who received normal pellet diet. Lipid emulsion received rats was observed with more foam cell in the tunica media and with damaged endothelium. Atorvastatin treated animals was observed with less foam and intact endothelium. Butanol fraction at 200 mg/kg was still observed with more foam cells. But at the dose of 400 mg/kg was observed with very few foam cells and observed better protection (Figure 6).

5. Discussion

Hypercholesterolemia, inflammation, oxidative stress and associated endothelial damage made the coronary artery more vulnerable to the development of atherosclerosis. The reduction of availability of vaso protective nitric oxide was observed due to enhanced activity of arginase[27]. So, complexity in etiology of atherosclerosis emphasized the use of drugs which have multiple effects such as antihyperlipidemic effect, anti-inflammatory and antioxidant with arginase enzyme inhibition may have considered as potential way to prevent and control the atherosclerosis and associated ischemic heart diseases like angina pectoris and myocardial infraction would be treated much better than current therapy. It was also reported in various epidemiological and clinical trial studies[28]. Basically, usage of polyphenols rich extracts had been reported to have a good therapeutic strategy to control the atherosclerosis in various preclinical studies[29]. Polyphenols had broad biological activities such as antihyperlipidemic activity, anti-oxidant activity, anti-inflammatory activity, arginase inhibition property that might be beneficial in the management of atherosclerosis. So, the extraction of *Rivea ornata* leaves were extracted with ethanol and solvent/solvent fractionation to get the polyphenol rich fraction. We found that butanol fraction showed positive evidence for the polyphenols such as tannins, flavonoids in preliminary phytochemical investigation and as well as observed with highest polyphenolic and flavonoid content than crude ethanol extract, other fractions including hexane, ethyl acetate and remining aqueous fractions. Butanol solvent was able to attract the polar polyphenolic substances from plant materials so its fraction is considered as sources of bioactive polyphenolic compounds[30]. Polyphenolic compounds exhibited antioxidant activity by donating its hydroxyl groups to unstable free radicals, making it stable to protect the free radical induced oxidative stress[31]. The major phenolic compound reported in butanol fraction was gallic acid which was analyzed by HPLC. The polyphenolic compound was proved to have strong antioxidant and antihyperlipidemic effect[32]. For the *in vivo* atheroprotection activity lipid emulsion along with vitamin D was used to induce atherosclerosis in rats since it was considered as simple method with short time as well as to cause aorta calcification like human atherosclerosis[33]. The obesity and hyperlipidaemia are the known risk factors for atherosclerosis and these conditions were significantly established by administering the high fat lipid emulsion for the period of 12 weeks[34]. Dyslipidemia is characterised by elevated levels of plasma lipids or lipoproteins such as TG, TC, LDL, VLDL and subsequent decrease in HDL are one of the major risk factor for development of cardiovascular diseases such as atherosclerosis and ischemic heart disease. The increased serum levels of bad lipids such as TG, TC, LDL, VLDL and decrease in good lipids such as HDL in diet induced hyperlipidemia has been reported[35]. The

reversal of hyperlipidemia associated with high fat diet by butanol fraction of *Rivea ornata* was observed week by week and maximum the antihyperlipidemic effect was observed at the end of 12 weeks. This improved serum lipid profiles could be due to the presence of gallic acid in butanol fraction of *Rivea ornata*. Similar observations were observed with gallic acid in earlear studies[36]. Gain in body weight directly related to engery intake and is reflected in the chronic lipid emulsion administered rats. Chronic administration of butanol fraction for 12 weeks exhibited dose dependently which alleviated gain in body weight[37]. Dysregulation of cholesterol homeostasis mechansims include excessive HMG CoA redusctase activity, cholesterol absorption from gut and impaired cholesterol clearance from liver contributed for hyperlipidemia and associated cardiovascular diseases[38]. The results of the study indicated antihyperlipidemic mechanism of butanol fraction as reflected by decrease of HMG CoA activity, increase in cholesterol content in the fecal matter and also increase in fecal bile acids. Overall effect of butanol fraction was hypothsised to inhbit the cholesterol sysnthesis, cholesterol absorption and enhance cholesterol excretion that are responsible for reduction of LDL cholesterol in the serum, which further prevented the development of atherosclerosis. Higher Atherogenic Index (AI) and lower percentage protection from atherosclerosis are strong and reliable indicators of the prediction of ischemic heart disease. Higher the AI value, higher is the risk and vice versa[39]. Significant decrease in AI and higher atheroprotection percentage suggest the protective capability of butanol fraction of *Rivea ornata* against atherosclerosis, ischemic heart diseases. Similar protection was reported after administration of extract containing gallic acid. Paraoxonase-1 is an anti-inflammatory and antioxidant protein associated with HDL that prevented the oxidative modification of LDL. Oxidized LDL deposited in the tunica medium and immediately recruited macrophages engulf this further form lipid loaded fatty plaques in the lumen of artery. Lipid emulsion administration caused significant decrease in the atheroprotective paraoxonase-1due to which the oxidation of LDL and the endothelial inflammation was promoted[40]. Butanol fraction dose dependently increased paraoxonase-1 level which had the ability to protect the oxidation of LDL. Inflammatory reaction associated with oxidized LDL promoted the recruitment and activation of neutrophils released myeloperoxidase which further propagated the oxidative damage in the vascular endothelium. The lipid emulsion induced vascular inflammation was observed which indicated by a significant increase in myeloperoxidase in the serum of rat under hyperlipemia and a decrease by butanol extract[41]. Hypercholesterolemia associated with lipid emulsion caused endothelial dysfunction by impairment of eNOS function. eNOS is known for production of nitric oxide. Impairment of nitric oxide by enhanced activity of arginase which metabolize the nitric oxide percussor like arginine. Hypercholesterolemia induced arginase activity impaired the nitric oxide bioavailability due to which cause initiate the lipid deposition in the intima tunica. But butanol extract treated rat observed with inhibition of arginase by which it restores the nitric oxide levels and prevents the vascular plaques development and vascular stiffness[42]. Hypercholesterolemia associated athresclerosis promoted the deposition of calcium and collagen which progressed to vascular stiffness. Excessive collagen synthesis was the consequence of vascular inflammation by hypercholesterolemia. High hydroxyproline in the vascular tissue was the index of collagen deposition. Lipid emulsion causes high vascular hydroxyproline indicate promotion of vascular stiffness[43]. Butanol fraction ameliorated the deposition of collagen dose dependently. This reflect the protective effect of butanol fraction on vascular stiffness along with its antihyperlipidemic effect. Oxygen derived toxic metabolites such as superoxide anion, hydroxyl radicle and hydrogen peroxide during hypercholesterolemic atherogenesis overwhelmed endogenous antioxidant molecules such as catalase, superoxide dismutase, reduced glutathione lead to oxidative damage Oxidative damage induced by hypercholesterolemia causes lipid peroxidation and oxidation of LDL. Both contribute to vascular damage. Antioxidant rich polyphenols containing butanol fraction significantly supresses the oxidative stress indicated by restoration of endogenous antioxidant such as catalase, superoxide dismutase, reduced glutathione and reduction of lipid peroxidation. The antioxidant capacity of butanol fraction might have positive impact on oxidative damage of vascular

tissue[44]. The atheroprotection of butanol fraction was also observed in histopathology of rat aorta. Lipid emulsion administration promoted the lipid deposition and wide gaps in the muscle layer whereas butanol fraction administration dose dependently antagonised the lipid disposition and gaps in the aorta. The accumulation of fat in the vascular tissue was reduced by butanol fraction[45].

6. Conclusion

From the results of the studies conducted, the polyphenolic rich butanol fraction of *Rivea ornata* possessed antiatherosclerosis effect by suppression of lipid emulsion induced hyperlipidemia, oxidative stress, vascular inflammation markers and vascular collagen deposition. The revelaed mechanism of antihyperlipidemic effect was HMG CoA reductase enxyme and dietary cholesterol absorption inhibition. Additionally, the availability of vasodilatory nitric oxide was availed by the inhibition of arginase that helped to protect the endothelial dysfunction.

7. Acknowledgement

Authors are thankful to the Principal, Correspondent for their constant support, encouragement and DST-FIST Facility of Raghavendra Institute of Pharmaceutical Education and Research, Anantapur, AP, India for the providing the proper facility to carry out this work.

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