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Arginase Enzyme Inhibition and with Subsequent Atheroprotection of Butanol Fraction of *Rivea ornata* in Lipid Emulsion Induced Atherosclerosis in Rats

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ABSTRACT

Objectives: 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 current study was wanted to explore the atheroprotective effect of polyphenolic fraction of *Rivea ornata* by using lipid emulsion induced atherosclerosis in rat model. **Materials and Methods:** 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. **Results:** 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 HDL, paraoxonase, nitric oxide, tissue thiol levels, endogenous antioxidants and decreases TG, TC, VLDL, LDL myeloperoxidase, arginase, hydroxyproline, lipid peroxidation. And atheroprotection reflected in histopathology studies also. Lipid emulsion associated foam cells formation is inhibited by butanol fraction. **Conclusion:** This is all due to presence of gallic acid in polyphenol rich butanol fraction is responsible for the underlying mechanism of atheroprotection.

Key words: Butanol fraction, Cardiac rick factors, HPLC analysis, Inflammation markers, Lipid emulsion induced atherosclerosis, Lipid profiles.

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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 patents 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] Ischemic heart disease and stroke are consequence of atherosclerosis and are responsible for >80% total cardiovascular death (CVD). Cardiovascular diseases (CVDs) have now become the leading cause of mortality in India, a quarter of all mortality is attributable to CVD. As per WHO, 23.6 million people are expected to die from CVD by 2030. People with presence of one or more risk factors such as hypertension, diabetes, 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 involved in metabolism of L-arginine in to ornithine and urea, due to which decline 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] Basing on our preliminary studies of butanol fraction of *Rivea ornata* respective to phenolic and flavonoid content we planned for anti-atherosclerotic effect of *Rivea ornata*. *Rivea ornata* is a climber with cylindrical stem, broader leaves, white silky flowered and smooth surface sub globose brown-coloured fruits. Literature mentions its traditional importance in treating disorder of gallbladder, disease of heart, bronchitis, and fatigue. In the Konkan, its juice is used to treat piles.[8] In Sri Lanka 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]

MATERIALS AND METHODS

Collection and identification of the plant material

Fresh leaves collected from flowering plant of *Rivea ornata*, were collected from Tirumala hills during between 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, (A.P, India).

Extract preparation, phytochemical screening

1 kg of dried leaves powder was macerated with 3 litters of ethanol (99%) for 1 week. Filtrate of the extract was concentrating under reduced pressure using rota evaporator. Crude semisolid extract was dissolved in hot water and further fractionated with solvents from low polar to high polar such as n-hexane (3x200ml), ethyl acetate (3x200ml), butanol (3x200ml) and finally remaining aqueous fraction. Each fraction was evaporating to dryness using rota evaporate to get the concentrate form. Phytochemical investigation was determined in crude ethanol extract and its fractions as per established methods.[10]

Estimation of Total phenolic and flavonoid content

Phenolic and flavonoid contents of crude ethanol extracts and its fraction (n-hexane, ethyl acetate, butanol and aqueous fraction) was estimated by Folin- Ciocalteu reagent and aluminium chloride reagent. For phenolic content estimation, the (0.5 mL) diluted extracts and its fractions at concentration of 1mg/mL and varying concentration of gallic acid (20 µg,40 µg,60 µg,80 µg and 100 µg /mL) mixed with 5 mL of Folin-Ciocalteu's reagent (10% v/v) and shaken it well. Add 4 mL of Na_2CO_3 (7% w/v) solution after 5 min to the above solution. Incubate the solution for 90 min at room temperature and absorbance was determined at 750 nm. For flavonoid content, the 1 mL of extracts (crude ethanol extracts and its fractions-n-hexane, ethyl acetate, butanol and aqueous) and standard solutions of quercetin at concentration of 20, 40, 60, 80 and 100 µg /mL were mixed with 2.5 mL of freshly prepared $AlCl_3$ (10%w/v) after 5 min and add 1M NaOH of about 2 mL and total volume make up to 10 mL with DW. The whole solution was mixed up and absorbance was measured against blank at 510 nm after 30 mins incubation. The contents were expressed as milligrams of gallic acid equivalents (GAE) and Quercetin equivalents (QE) per g dry weight of extract.[11]

Column chromatography of butanol fraction

The butanol fraction was subjected to Silica gel column chromatography for the separation of polyphenolic compounds. Initially methanol solubilized butanol fraction (1.5g) was mixed with silica gel in motor by trituration with pestle. Fraction coated silica gel placed on the top of silica gel (60-120 mesh) packed chromatography column (75cm×3.5cm). The column was serially eluted with hexane, hexane and ethyl acetate (1:1 ration) and ethyl acetate. Total 40 sub-fractions of 150 mL were collected and concentrated using rotary evaporator at 45°C under reduced pressure, then mixed on the basis of their TLC matching with standard flavonoid (quercetin).[12]

Identification of Flavonoids by TLC

n-Butanol fraction was tested in TLC for presence of flavonoids. The TLC pre-coated silica gel 60 GF_{254} plates were developed using a flat bottom chamber which was pre-saturated with the mobile phase for 30 min. In the TLC method, n-hexane and ethyl acetate (1:1) was used as optimized mobile phase in order to effective separation. After development of TLC chromatogram of butanol extract and standard flavonoid, plates had been removed and dried, the spots were visualized by kept in and iodine chamber. Fraction which are matched with standard are pooled together and further they are analyzed by HPLC for polyphenolic profiling.[13]

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: C_{18} 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)

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

Animals

Male wistar rats (160-200 g body weight) were used for this study. They were housed at ambient temperature (24 ± 2°C), relative humidity (45 ± 5%) and 12h/12h light dark cycle. Animals had free access to pellet and drinking water was given *ad libitum*. The Institutional Animal Ethics Committee (*878/ac/05/CPCSEA/005/2016*) approved the experimental protocol at Department of Pharmacology, Raghavendra Institute of Pharmaceutical Education and Research, Anantapuramu, Andhra Pradesh, India in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, New Delhi.

Acute toxicity studies

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

Lipid emulsion induced atherosclerosis model

To induce atherosclerosis in wistar rats, feed orally with freshly prepared lipid emulsion daily for the period of 12 weeks continuously at the dose of 1.5ml/kg.[16]

Composition of lipid emulsion

Content	Quantity
Cholesterol	45mg/mL
Lard	15mg/mL
Sodium cholate	7.5mg/mL
Propylthiouracil	3mg/mL
Sugar	75mg/mL
Vitamin D_3	100000 IU/kg

Experimental design

A total of 30 male wistar rats are allocated in to five groups and six animals in each. All the animals were treated 12 weeks' period.

Groups	Type of treatment	Diet provided daily
I	Normal control	Normal pellet diet
II	Vehicle control, 22ml/kg, oral	Lipid emulsion
III	Standard – Atorvastatin,10 mg/kg oral	Lipid emulsion
IV	Butanol fraction of *Rivea ornata*, 200 mg/kg oral (BFRO)	Lipid emulsion
V	Butanol fraction of *Rivea ornata*, 400 mg/kg oral (BFRO)	Lipid emulsion

Estimation of serum parameters

Blood samples collected via puncturing the retrorbitol plexus under anaesthesia and allowed to clot for 10 mins at room temperature. It was centrifuged at 3000rpm for 15 min. Separated serum was stored at 4°C until used. This serum was used for the estimation of following biochemical parameter such as triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), very low-density lipoprotein (VLDL), low density lipoprotein (LDL), lactate dehydrogenase (LDH), creatin kinase-MB (MB) and c-reactive protein (CRP).[17]

Paraoxonase-1 (PON-1) activity

The arylesterase activity in the serum was determined by the procedure described by Gan et al., (1991). Briefly, the reaction was carried out in a 1ml spectrophotometric cuvette. The reaction mixture contained Tris-HCl buffer pH 8, 200 mM $CaCl_2$, and 2 mM phenyl acetate as substrate. The reaction was initiated by the addition of 7 ml of serum. The change in optical density at 270 nm was recorded for every 20 sec up to 1 min Blanks without the serum were used to subtract spontaneous hydrolysis of phenyl acetate. One unit of enzymes activity was defined as that amount of enzyme that release 1 m mol of phenol per min under the assay conditions. Molar extinction coefficient of 1310M^{-1} cm^{-1} was used to calculate the product formed. The results are expressed as units per ml serum.[18]

Estimation of tissue parameters

The animals were euthanasiously sacrificed after blood collection; thoracic aorta was quickly transferred to chilled ice-cold phosphate buffered saline (10mM, pH 7.4). The tissues were then minced and homogenized to a concentration of 10% w/v. The homogenate was centrifuged at 7,000 rpm at 0°C for 25 min using Remi C-24 high speed cooling centrifuge. The tissue homogenate used for estimation of markers of oxidative stress such as reduced glutathione, catalase, superoxide dismutase and products of lipid peroxidation.[19]

Determination of total thiol levels

Total thiol levels were determined via a spectrophotometric method using Ellman's reagent. DTNB (2,2'-dinitro5,5'-dithiodibenzoic acid) reacts with the SH group found in thiol molecules.

The product formed is yellow in huewith a peak absorbance at 412nm. Briefly, 50 µl of supernatant was added to 1 ml Tris-EDTA (ethylenediaminetetraacetic acid) buff er (pH=8.6) and the absorbance was read at 412 nm against Tris EDTA buffer alone (A1). Then, 20 µl of 10 mM solution of DTNB (10 mM in methanol) was mixed with heart or aorta homogenate and the absorbance was read again (A2). The absorbance of DTNB reagent was also read as blank (B). Total thiol was expressed as mM per gram of tissue.[20]

Measurement of arginase activity

Aorta tissue homogenate (50 mL) was added into 75 mL of Tris HCl (50 mmol/L, pH 7.5) containing 10 mmol/L $MnCl_2$. Heating the lysate at 55-60°C for 10 min activated arginase. The hydrolysis reaction of L-arginine by arginase was performed by incubating the mixture containing activated arginase with 50 µL of L-arginine (0.5 mol/L, pH 9.7) at 37°C for 1 hr and was stopped by adding 400 mL of the acid solution mixture (H_2SO_4:H_3PO_4: H_2O=1:3:7). For calorimetric determination of urea, α-isonitrosopropiophenone (25 µL, 9% in absolute ethanol) was then added and the mixture was heated at 100°C for 45 min. After placing the sample in the dark for 10 min at room temperature, the urea concentration was determined spectrophotometrically, with the absorbance at 550 nm being measured with a microplate reader. The amount of urea produced, after normalization with protein, was used as an index for arginase activity.[21]

Estimation of nitric oxide

Nitric oxide (NO) concentration in the samples was assayed by the colorimetric method by using Griess reagent. Equal volume of supernatant was rapidly mixed with Griess reagent (v/v) and kept in the dark for 20 min. The absorbance was measured at 550 nm and the quantification was calculated from a standard curve pre-established with different concentration of sodium nitrite.[22]

Estimation of myeloperoxidase in cardiac tissue

The myeloperoxidase (MPO) activity was assessed as previously described by method. 10% w/v cardiac tissue homogenate was prepared by homogenized buffer (0.5% of hexadecyl trimethyl ammonium bromide in 50 mM phosphate buffer, pH 6.0). Then the homogenate undergone three cycles of freezing and thawing and followed by centrifugation at 4°C for 20 min. Supernatant (0.1ml) of the homogenate was mixed with 2.9 ml of reaction agent (combination of 50 mM phosphate buffer (4.9 ml; pH 6.0), 0.167 mg/ml of O-dianisidine hydrochloride and hydrogen peroxide (0.0005%). With UV spectrophotometer change in the absorbance (5 min) was recorded at 460 nm. The MPO activity was calculated using the following formula. MPO activity (U/g) = X / weight of the piece of tissue taken, where X = 10 × change in absorbance per min/volume of supernatant taken in the final concentration.[23]

Estimation of Hydroxyproline Content in cardiac tissue

A colorimetric assay as described by Reddy and Enwemeka was used to estimate hydroxyproline in cardiac tissue sample. Briefly, 100 mg dry tissue sample was digested in 2ml of HCl 6 M by keeping at boiling water bath for 3h. Tissue hydrolysate was added NaOH to adjust the pH to 6.5 - 7.0. Then, 1.0 mL of chloramine T solution (0.05 M/L) was added to tissue hydrolysate solutions (1 mL) and allowed the mixture aside for 20 min at room temperature. To this, added 1 mL of 20% p-dimethyl benzaldehyde (Ehrlichs reagent) solution, again the solution was placed at 60°C for 20 min. Absorbance of final solution was read at 550 nm.[24]

Histopathology

Immediately after scarification, hearts were removed and placed in 10% buffered formalin, embedded in paraffin, cut to 5µm section for slides and stained with hematoxylin and eosin. The slides were examined with a Magnus microscope. The sections were observed under 10X and 40X magnifications

Statistical analysis

All the data are expressed as mean ± SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test as appropriate using computer based fitting program (Prism, Graph pad). Differences were considered to be statistically significant when $P < 0.05$

RESULTS

Result of qualitative analysis and its contents of (phenolic and flavonoid) in various extracts/fractions of *Rivea ornata*

From the observation of qualitative test, we observed that butanol fraction gets positive evidence of flavonoids by all flavonoid assessment tests. Phenolic and flavonoid content were calculated from calibration curve of gallic acid and quercetin respectively. Butanol fraction has highest contents (82.4 ± 4.61mg of GAE/g, 105 ± 4.09mg of quercetin/g) compared to crude ethanol and its other fractions such as n-hexane, ethyl acetate and aqueous fraction. Results are shown in Table 1.

Test 1. Shinoda test (Deep red colour); Test 2. Lead acetate test (Yellow colour ppt); Test 3. Pews tests (Red colour to cherry red colour); Test 4. NaOH (Yellow colour); TPC (Total phenolic content -mg of gallic acid equivalent/g); TFC (Total flavonoid content -mg of quercetin equivalent/g). All values are expressed as the mean ± SD of three replicate. Statistical values were consider significant when p value lower than 0.05.

HPLC Profile of collected fractions

TLC matched butanol fractions were observed peaks identical with retention time of standard phenolic compounds in the HLPC chromatogram. The retention time of fractions were matched with gallic acid retention time, it is known phenolic compound having broad biological activity. This data supports the presence of gallic acid type polyphenolic compounds. Results are shown in Figure 1 and 2.

Table 1: Qualitative results of flavonoids in *Rivea ornata* plant extracts.

Extract	Test 1	Test 2	Test 3	Test 4	TPC	TFC
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**

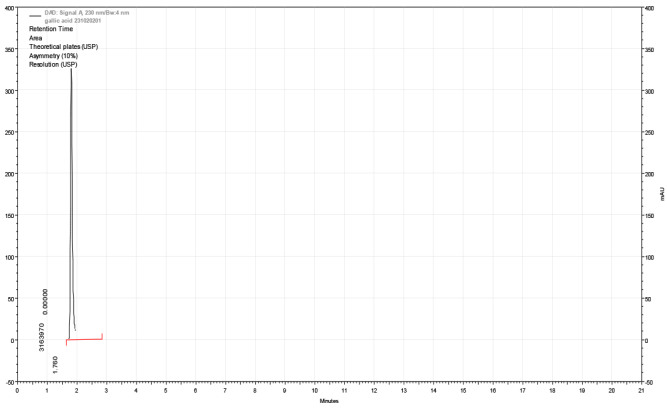

Figure 1: HPLC chromatogram of Gallic acid (standard Phenolic compound).

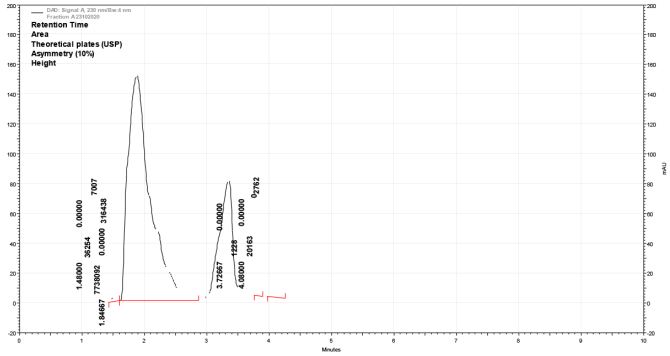

Figure 2: HPLC chromatogram of butanol fraction.

Table 2: 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

Effect of biactive butanol fraction on physiological changes

After 12 weeks of lipid emulsion administration there is 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) is efficiently (** p<0.01) control the weight gain induced by lipid emulsion. But body weight changes are not significant in lower dose of test fraction. Lipid emulsion alone has increase the atherogenic index significantly (*** p<0.001) compared to normal pellet diet. Atorvastatin and higher dose of butanol fraction reduces atherogenic index significantly (*** p<0.001 and ** p<0.01)). And also exhibit higher percentage of atheroprotection from higher dose of butanol fraction. Results are represented in Table 2.

The data represented as mean ±SEM for six rats, Values were considered significant when p value less than 0.05. Significant values were represented as follows such as * p<0.05, ** p<0.01, *** p<0. 001. Statistical values of all groups were compared with group II.

Effect of butanol fraction on serum lipid parameters

Serum lipid profiles of animals of all groups were determinate every 21 days up to 84 days. Base line lipid profiles of all groups of animals were not significant at 0 week. Lipid emulsion alone start producing hyperlipidaemic state after 21 days and it is continued up to 84 days compared to control animals received normal pellet diet. It is evident by significant (*** p<0.001) increase in bad lipids such as TG, TC, LDL, VLDL and decrease in good lipid such as HDL was observed with lipid emulsion. Chronic administration of butanol fraction start showing antihyperlipidemic effect significantly (* p<0.05) after 21 days especially at dose of 400 mg/kg but not lower dose and continued up to 84 days. It is evident by significant decrease in TG, TC, LDL, VLDL and decrease in HDL. Results are shown in Table 3.

Table 3: Lipid profile changes induced by lipid emulsion and its effects by butanol fraction of *Rivea ornate*.

Test	Day	Group I	Group II	Group III	Group IV	Group V
TC (mg/dL)	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 (mg/dL)	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 (mg/dL)	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 (mg/dL)	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 (mg/dL)	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**

The data represented as mean ±SEM for six rats, Values were considered significant when *p* value less than 0.05. Significant values were represented as follows such as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistical values of all groups were compared with group II.

Effect of butanol fraction on serum and tissue biochemical parameters

Atherosclerosis markers from all groups of animals 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 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 decreases C-reactive protein (** $p<0.01$), myeloperoxidase (* $p<0.05$) and hydroxyproline(** $p<0.01$) and increases paraoxonase (* $p<0.05$) compared to lipid emulsion received animals. 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 decreases significantly (*** $p<0.001$) serum nitric oxide and aorta homogenate arginase and tissue thiols levels compared to normal pellet diet. But butanol fraction of *Rivea ornata* significantly increases the serum nitric oxide (* $p<0.05$) and aorta homogenate arginase (** $p<0.01$) and tissue thiols (** $p<0.01$) levels. Significantly ($P < 0.001$) decreased in the aorta tissue levels of catalase (CAT), superoxide dismutase (SOD), and reduced glutathione (GSH) and increased in the levels of Malondialdehyde (MDA) in lipid emulsion received animals compared to normal pellet diet received rats. 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 decrease the levels of MDA (* $P < 0.05$) in the aorta tissue compared with lipid emulsion received rats. Results are shown in Table 4.

The data represented as mean ±SEM for six rats, Values were considered significant when p value less than 0.05. Significant values were represented as follows such as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistical values of all groups were compared with group II.

Histopathology

In normal pellet diet received aorta looking normal histoarchitecture. Lipid emulsion received rats observed with more foam cell in the tunica media and with damaged endothelium. Atorvastatin treated animals observed with less foam and intact endothelium. Butanol fraction at 200 mg/kg still observed with more foam cells. But at the dose of 400 mg/kg observed few very few foam cells and observed better protection. Histopathological changes are noted in Figure 3.

DISCUSSION

Hypercholesterolemia, inflammation and oxidative stress are the key factors involved in initiation and progression of atherosclerosis by endothelial dysfunction. Along with, reduction of availability of vasoprotective nitric oxide due to enhanced activity of arginase contribute some extent.[25] So, complexity in etiology of atherosclerosis emphasis us to use such a drug 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

Table 4: Effect of butanol fraction of *Rivea ornata* on serum and tissue parameters.

Test group	I	II	III	IV	V
CRP (mg/L)	1.80±0.19	5.83±0.20***	3.15±0.34***	5.59±0.19	4.30±0.42**
Paraoxonase-1(U/mL)	166±4.40	107±6.44***	140±5.86**	107±6.74	133±2.71*
Myeloperoxidase(U/g)	1.68±0.230	6.60±0.225***	2.60±0.215***	6.31±0.441	5.04±0.351*
Hydroxyproline (µg/mg protein)	17.9±1.09	57.3±2.06***	37.7±1.62***	45.7±3.66*	42.6±2.92**
Nitric oxide **(µmol/L)**	51.6±2.59	13.7±3.30***	41.1±1.43***	14.9±4.34 NS	27.4±2.74 *
Arginase (mg Urea/min/mg protein)	0.27± 0.05	1.56 ± 0.19***	0.56± 0.03***	1.01± 0.133*	0.93± 0.07**
Total thiol (µmol/mg protein)	5.39±0.479	2.22±0.208***	4.11±0.253**	3.72±0.363*	4.05±0.311**
SOD	135±3.96	50.4±3.16***	104±4.68***	74.9±7.75*	85.7±5.27***
CAT	208± 7.89	116±2.82***	175±5.30***	128±6.42ns	152±5.41**
GSH	72.1±3.61	32.6±2.86***	61.6±2.71***	41.1±2.96ns	46.3±2.15*
MDA	37.4±4.49	78.3±4.09***	49.2±2.25***	64.6±2.07ns	62.1±3.14*

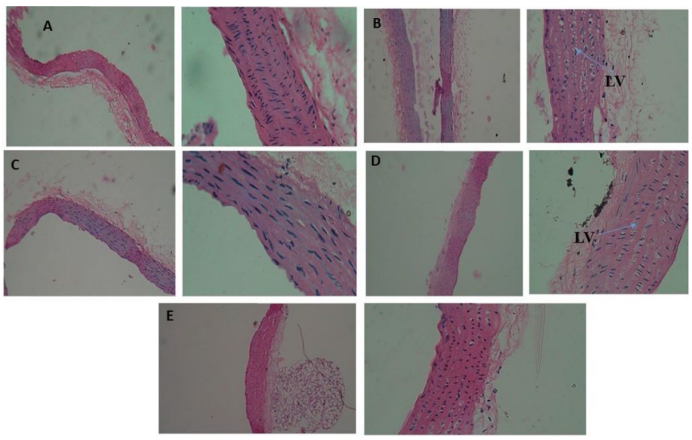

Figure 3: Histopathology sections of thoracic aorta: A. Normal rats; B. Lipid emulsion; C. Atorvastatin; D. Lower dose of *Rivea ornata* (200 mg/kg); E. Upper dose of *Rivea ornata* (400 mg/kg). LV= Lipid vacuoles.

atherosclerosis and associated ischemic heart diseases like angina pectoris and myocardial infraction would be treated much better than current therapy. It also reported earlier from various epidemiological and clinical trials.[26] Based on the evidence we carry out the antiatherosclerotic effect of butanol fraction of *Rivea ornata* in lipid emulsion induced atherosclerosis in rat. Basically, polyphenols rich extracts have been proved with many pharmacological effects related to atherosclerosis.[27] So, we extracted leaves of *Rivea ornata* with ethanol and latter fractionation were performed with solvents from nor polar to polar to get the polyphenol rich fraction. We found butanol extract was demonstrated positive for the polyphenols such as tannins, flavonoids 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 able to attract the polar polyphenolic substances from plant materials so it is considered as sources of bioactive polyphenolic rich extracts.[28] Polyphenolic compounds are exhibit antoxidant activity by donating its hydroxyl groups to unstable free radicals, made it to stable that's why they protect the free radical induced oxidative stress, is known to common thing to cause LDL oxidation. LDL oxidation is the preliminary reaction before deposit in the vascular endothelium.[29] Gallic acid is the major phenolic compound identified in the butanol extract by HPLC analysis based on matching of retention time of fraction and its standard gallic acid.[30] For the *in vivo* atheroprotection activity we used lipid emulsion along with vitamin D to induce atherosclerosis in rats, as is most simple method with short time as well as to cause aorta calcification as like human atherosclerosis. Having obesity and hyperlipidaemia are known risk factors for atherosclerosis and these conditions were significantly established by administering the high fat lipid emulsion for the period of 12 weeks.[31] Which were reflected as significant gain in the body weight and increase in the plasma levels of bad lipids such as TG, TC, LDL, VLDL and decrease in good lipids such as HDL compared to rats received balanced lipid containing pellet diet. Chronic administration of butanol fraction for 12 weeks exhibit dose dependently alleviate effect on the gain in the body weight and serum bad lipids such as TG, TC, LDL, VLDL and increase in good lipids such as HDL. This antihyperlipidemic effects of butanol fraction might be able to prevent the development of atherosclerosis.[32] This effect also reflected on the atherogenic index, higher atherogenic index lower protection from atherosclerosis. Atherogenic index is the indicator of risk of coronary artery diseases. Antihyperlipidemic effect of butanol fraction is able to lower the atherogenic index and improved percentage protection from hyperlipemia induced cardiac risk dose dependently.[33] Paraoxonase-1 is an anti-inflammatory and antioxidant protein associated with HDL prevent 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 cause significant decrease the atheroprotective paraoxonase-1due to which promote the oxidation of LDL and promote the endothelial inflammation. Butanol fraction dose dependently increases paraoxonase-1 level means is able to protect the oxidation of LDL. Inflammatory reaction associated with oxidized LDL promote the recruitment and activation of neutrophils release myeloperoxidase further propagates the oxidative damage in the vascular endothelium. We observed lipid emulsion induced vascular inflammation is indicated by significant increase in myeloperoxidase in serum of rat under hyperlipemia and it is decrease by butanol extract.[34] Hypercholesterolemia associated with lipid emulsion causes 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 that

is arginine. Hypercholesterolemia induced arginase activity impair 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.[35] Hypercholesterolemia Vascular stiffness is the ultimate outcome of athresclerosis due to deposition of calcium and collagen synthesis. Excessive collagen synthesis is consequence of vascular inflammation by hypercholesterolemia. High hydroxyproline in the vascular tissue is the index of collagen deposition. Lipid emulsion causes high vascular hydroxyproline indicate promotion of vascular stiffness.[36] Butanol fraction ameliorate the deposition of collagen dose dependently. 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. Which indicate the antioxidant capacity of butanol fraction might have positive impact on oxidative damage of vascular tissue.[37] Butanol fraction atheroprotection also observed in histopathology of rat aorta. Lipid emulsion administration promote the lipid deposition and wide gaps in the muscle layer. Whereas butanol fraction administration dose dependently antagonises the lipid disposition and gaps in the aorta. Which might be prevent the accumulation of fat in the vascular tissue by butanol fraction.[38]

CONCLUSION

By the observation of results of current study polyphenolic rich butanol fraction have antiatherosclerosis effect by balancing inflammation and oxidative stress by suppression of lipid emulsion induced inflammation markers and strengthen endogenous antioxidant system. Additionally, avail the availability of vasodilatory nitric oxide by inhibition of arginase helps further.

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CONFLICT OF INTEREST

The Authors Declare no conflict of interest.

ABBREVIATIONS

HPLC: High Performance Liquid Chromatography; **WHO:** World Health Organization; **Na_2CO_3:** Sodium carbonate; **$AlCl_3$:** Aluminium chloride; **NaOH:** Sodium hydroxide **DW:** Distilled water; **TLC:** *Thin Layer chromatography;* **LD_{50}:** Lethal dose 50; **ANOVA:** Analysis of variance; **$CaCl_2$:** Calcium chloride; **$MnCl_2$:** Manganese chloride; **H_2SO_4:** Sulfuric Acid; **H_3PO_4:** Phosphoric acid; **H_2O:** Water**; OECD:** Organisation for Economic Co-operation and Development; **TPC:** Total phenolic content; **GAE:** Gallic acid equivalent; **TFC:** Total flavonoid content; **eNOS:** Endothelial nitric oxide synthase.

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