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EFFECT OF RUBIA CORDIFOLIA ALONE OR AS AN ADJUVANT WITH ATORVASTATIN IN EXPERIMENTAL HYPERLIPIDEMIA AND ATHEROSCLEROSIS

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ABSTRACT

The current study was aimed to assess whether the ethanolic extract of *Rubia cordifolia* alone or as an adjunct with atorvastatin, would effectively prevent and/or cure experimentally induced-hyperlipidemia and atherosclerosis in rats. In this study, hyperlipidemia and atherosclerosis in male Wistar rat was induced by administering laboratory diet enriched with cholesterol (1%) and methionine (2 g/kg, body weight) for the duration of 60 days. Treatment schedule was divided into three parts preventive (2-months), curative (2-months) and combination (1-month). Effect of Ethanolic extract of *Rubia cordifolia* and atorvastatin alone and in combination was evaluated. Blood pressure was recorded at the end of the study. Markers of hyperlipidemia and atherosclerosis (lipid profile, reduced glutathione, total anti-oxidant capacity, C-reactive protein, HMGCo-A reductase, nitric oxide and Na^+K^+ATPase) were estimated in serum, aorta and liver. Ethanolic extract of *Rubia cordifolia* was found to possess anti-dyslipidemic, antioxidant, anti-inflammatory, membrane protecting and vasoprotective activity. The ethanolic extract of *Rubia cordifolia* when used as an adjunct with atorvastatin therapy, reduced the duration of atorvastatin therapy to one month. From the study it was concluded that Ethanolic extract of *Rubia cordifolia* was effective in preventing/treating cholesterol and methionine-induced hypelipidemia and atherosclerosis. Combing the ethanolic extract of *Rubia cordifolia* to atorvastatin treatment may reduce the duration of atorvastation therapy, clinically.

KEYWORDS: Atherosclerosis, Cholesterol, Methionine, Homocysteine, Atorvastatin, *Rubia cordifolia.*

INTRODUCTION

Hyperlipidemia is a disorder of lipoprotein metabolism manifested as hypercholesterolemia, hypertriglyceridemia, or a combination, with elevated plasma apolipoprotein B. Hyperlipidemia is a risk factor for atherosclerosis, gall stone, pancreatitis and xanthomas, whereas atherosclerosis is a risk factor for coronary artery disease (CAD), myocardial infarction (MI), hypertension and cerebrovascular accidents. CAD could be considered as the most common cause of death globally, including India, by 2020.[1] Both the prevalence and mortality due to CAD has been increasing in India over the past few decades. Till 1990's there has been 8 % increase in CAD's prevalence rate since 1960's in urban areas whereas in rural areas, this increase has been found to be 2 %.[2] Conventional risk factors *viz*. age, male sex, family history, diabetes mellitus, dyslipidemia, hypertension, obesity, and tobacco smoking are responsible for the increasing prevalence of CAD.

Atherosclerosis, being one of the major complications of CAD, is a complex multifactorial process of chronic low level inflammatory disorder resulting from excessive inflammatory response to various forms of injurious stimuli to the artery wall.[3] High plasma cholesterol and/or homocysteine (hcy) are amongst the major causal factors for the development of hyperlipidemia, which inturn may lead to atherosclerosis.[4, 5]

A positive association between plasma hcy concentration and risk for cardiovascular disorders has been documented in several epidemiological studies.[6,7] Hyperhomocysteinemia (Hhcy) can be detected in 30% patients with CAD.[8] This is in agreement with hcy theory of atherosclerosis proposed by McCully and Wilson, which states that the Hhcy might be the reason for the presence of extensive arterial thrombosis and atherosclerosis.[9]

The atheromatous plaques formed due to Hhcy and/or hypercholesterolemia blocks the arteries. Thus, it is possible that the combination of high dietary cholesterol with methionine would lead to dyslipidemia and atherosclerosis development. In the present study, we tested the hypothesis that the combination of high dietary cholesterol and methionine would exacerbate the development of atherosclerosis and endothelial dysfunction.

A wide variety of allopathic drug treatments are available for the treatment of atherosclerosis like, statins, fibrates, bile acid binding agents and in case of associated hypertension, angiotensin converting enzyme inhibitors (ACE inhibitors). Of all the available allopathic drugs, statins are the most potent hypolipidemic drugs.[10]

Atorvastatin is a selective, competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), the rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, a precursor of sterols, including cholesterol. It has shown to regress atheroma (ASTEROID trial), improve endothelial function, modulate inflammatory responses, maintain plaque stability, and prevents thrombus formation.[10,11]

Generally, statins are well tolerated drugs but have few major limitations like gastrointestinal disturbances, severe myositis, angioedema, myopathy with elevation of creatinine kinase, liver transaminase enzymes concenteration and rhabdomyolysis.[12] In addition to these, the therapy is required for a long duration which increases the cost of treatment.

The demand of herbal medicines has steadily increased over the past decade because of the limitations of modern medicines; general chemophobia to chemical based therapies; and perceptions that plant based drugs are less toxic and therefore, are safer alternatives. Despite considerable progress in the management of atherosclerosis by synthetic drugs, the search for indigenous cardioprotective agents still continues. There are wide varieties of herbal drugs available to treat atherosclerosis like Triphla (*Terminalia chebula, Embelica officinalis, Terminalia belerica*); *Terminalia arjuna*; Garlic (*Allium sativum*); Green tea (*Camellia sinensis*).[13-15]

Owing to multi-factorial etiology the interest has been focused to search for the herbal drugs capable of combating at multiple steps linked to atherosclerosis development. Plants as a whole and/or their extracts alone or as an adjunct to allopathic preparations, having hypolipidemic, antioxidant and anti-inflammatory action, can prevent, reduce or cure the factors responsible for the formation and/or progression of dyslipidemia and atherosclerosis.[16] These drugs may enhance the efficacy and lower the side effects and duration of treatment of allopathic preparations.

Rubia cordifolia (RC) is a well known medicinal plant and is commonly known as Indian madder (Manjishtha), belonging to the family Rubiaceae. It is used for treatment of various

ailments in Ayurvedic system of medicine.[17] Rubiadin (anthraquinone glycoside), isolated from the roots of *Rubia cordifolia* was found to have potent antioxidant property. In addition, rubiadin has been found to inhibit lipid peroxidation[18] and has been reported for anti-inflammatory,[19] immunomodulatory,[20] anticonvulsant and anxiolytic,[21] anti-platelet activation[22] and anti-tumor activities[23]. The ethanolic extract has been reported to possess antioxidant, hepatoprotective,[17] anti-inflammatory,[19] hypoglycemic activities.[24] The chloroform extract too has antioxidant property.[25]

To the best of our knowledge there is no scientific report on its anti-hypercholesterolemic and anti-atherosclerotic activity. RC is well known to possess antioxidant, anti-inflammatory and hypoglycemic effect, no scientific data have been published, so far, supporting anti-hypercholesterolemic and anti-atherosclerotic use. Thus, this study was aimed to assess whether the RC extracts alone or as an adjunct in combination with atorvastatin (ATR), would effectively prevent and/or cure experimentally induced-hyperlipidemia and atherosclerosis in rats with reference to histology of rat aorta and biochemical markers in serum and aorta.

MATERIALS AND METHODS

Animals

Healthy male Wistar albino rats, weighing between 200–250 g, were procured from the Central Animal House, Jamia Hamdard (Hamdard University), New Delhi. Prior to the commencement of the experiment, the animals were allowed to acclimate for 7 days; food and water were available *ad libitum*. The rats were kept in a room maintained at 23 ± 2°C and 55± 15% humidity with a 12 hr light-dark cycle. All animal procedures were approved by the ethics committee at our institution (Project no. 470, year: 2008) and performed in compliance with institutional guidelines for the care and handling of experimental animals.

Chemicals

Plant material and Extraction

Fresh RC roots were purchased from local market and botanical authentification was carried out by Dr. Mohammad Ali, Department of Pharmacognosy, Faculty of Pharmacy, Hamdard University, New Delhi, India (voucher No. 2008/21). Roots were finely powdered and were exhaustively extracted with ethanol (70%) in a Soxhlet extractor to obtain the ethanolic extract. Extract was concentrated under vacuum. The dried ethanolic extract of RC was dissolved in distilled water and administered orally in the doses of 100 mg/kg and 200 mg/kg.

The phytochemical studies showed the presence of sterols, anthraquinone, glycosides and saponins.

Chemicals

Sodium nitroprusside, 1-2-dithio-bis-nitrobenzoic acid (DTNB), and thiobarbituric acid were obtained from Sigma USA. Cholesterol and methionine powder were obtained from (CDH, India). Enzyme kits for serum lipid profile (Span Diagnostics India). Atorvastain obtained as gift sample from Ranbaxy Ltd. (India). All other reagents used in the study were of analytical grades. Nitric oxide kit purchased from Intron Biotchnology.

Establishment of model for hyperlipidemia and atherosclerosis

To develop the model for hyperlipidemia and atherosclerosis rat were given the diet supplemented with cholesterol (1%) and methionine (2 g/kg), [C+M] diet for two months. For this, the crushed pellet diet, cholesterol (1% w/w) and methionine (2 g/kg, body weight) powder was mixed; the pellets were reconstituted with water and dried properly to avoid any fungal contamination.

Experimental Design

The whole study was divided into three parts namely preventive, curative and combination. Each study consisted of various groups and each group had eight rats. Each treatment was given orally.

Preventive study

This study consisted of five groups: Group 1 (control): given normal rat diet along with normal saline, Group 2 (toxic) : given [C+M] diet, Group 3 (toxic + ERC100): given ethanolic extract of RC (ERC) (100 mg/kg) along with [C+M] diet, Group 4 (toxic + ERC200): given ERC (200 mg/kg) along with [C+M] diet, Group 5 (toxic + ATR): given ATR (10 mg/kg) along with [C+M] diet. Each treatment was given orally for 60 days.

Curative study

The study was divided into three groups. In this, (C+M diet) was given for first 60 days then from the 61st day onwards all the treatments were initiated orally for the next 60 days. Group 1 (toxic + ERC100): given [C+M] diet and ERC (100 mg/kg), Group 2 (toxic + ERC200): given [C+M diet] and ERC (200 mg/kg) and Group 3: (toxic + ATR): given [C+M] diet and ATR (10 mg/kg).

Combination study

The study was divided into three groups. In this, [C+M] diet was given for first 60 days and from the 61st day onwards the drugs were administered as follows, for the next 30 days. Group 1 (toxic + ERC100 + ATR): given [C+M] diet; ERC (100 mg/kg) and ATR (10 mg/kg), Group 2 (toxic + ERC200 + ATR): given [C+M] diet; ERC (200 mg/kg) and ATR (10 mg/kg) and Group 3 (toxic + ATR): given [C+M] diet and ATR (10 mg/kg).

The rats were fasted overnight prior to collection of blood samples from tail vein for biochemical estimations. Immediately after blood collection, the rats were euthanized for collection of aorta for biochemical estimations and histopathological analysis. Serum samples were separated by centrifugation for 10 min at 3000 rpm and were stored at -20°C until analysis was carried out.

METHODOLOGY

Blood Pressure

Blood Pressure (BP) was recorded by using the non-invasive method using Biopac instrument. BP was recorded as the mean of two observations made on 59th and 60th day in prophylactic groups, 119th and 120th day in therapeutic groups and 89th and 90th day in combination groups.

Biochemical determinations

Lipid profile

Serum total cholesterol (TC), triglycerides (TG), and high density lipoprotein (HDL-C) were determined by commercially available spectrophotometric assay kits (Span diagnostics, India). Low density lipoprotein (LDL-C) was calculated as TC - HDL-C - VLDL-C; using Friedewald formula[26]. VLDL-cholesterol was measured: 0.5x total triglyceride concentration.

Activity of HMG-CoA reductase (HMGR)

The activity of HMG-CoA reductase (HMGR) in rats was determined by the method of Venugopala and Ramakrishanan[27]. Briefly, the ratio of absorbance of 3-hydroxy-3-methylglutaryl CoA and mevalonate was measured in a UV spectrophotometer at 540 nm after treating the 10% liver homogenate (i.e. 1 g of liver/10 ml of saline arsenate sol.) with hydroxylamine hydrochloride reagent (in water) and alkali hydroxylamine hydrochloride

reagent (in sodium hydroxide), respectively followed by the addition of ferric chloride. The ratio of HMG-CoA/Mevalonate was taken as an index of HMG-CoA reductase activity.

Estimation of serum reduced glutathione and Total antioxidant capacity

Reduced glutathione was determined by the method of described by Jollow and co-workers.[28] Total antioxidant capacity (TAC) in serum of rats was assessed by method of Koracevic et al.[29] Briefly, the assay measured the capacity of the serum/plasma to inhibit the production of thiobarbituric acid reactive substances from sodium benzoate under the influence of the free oxygen radicals derived from Fenton's reaction. A solution of 1 mmol/L uric acid was used as standard.

Measurement of Serum Nitric Oxide

Nitric oxide (NO) released in the serum of methionine and cholesterol treated rats was determined by using the commercially available kits (Intron bio), measuring accumulation of nitrates and nitrites, as described by Tracey et al.[30]

Serum Hcy levels

Serum hcy levels were measured by using commercially available kit based on the principle of fluorescence polarization immunoassay (FPIA) method on the AxSYM System analyzer (Abbott Laboratories, Abbott Park, IL).

Na^+K^+ATPase

Na^+K^+ATPase activity was assayed aorta in a medium containing 20 mM Tris-HCI, pH 7.4, 140 mM NaCl, 14 mM KCl, 3 mM MgCl, 3 mM ATP, and 0.2 mM EDTA in a final volume of 0.5 ml, incubations were carried out at 37°C. The reaction was initiated by adding ATP. Inorganic phosphate released was determined by the method of Fiske and SubbaRow[31] and protein according to Lowry's method.[32] One unit of enzyme activity is defined as micromoles of Pi liberated per minute per mg of protein.

C -reactive protein

To analyse the effect of homocysteine and cholesterol on cardiovascular markers the serum level of C-reactive protein (CRP) was analysed by turbidimetric test (CRP-TURBI) according to the kit manufacturer's instructions.

Histopathological analysis

At the end of the experiment, aortic tissues from all the groups were subjected to histopathological studies. The tissues were fixed in formalin (10%), processed following routine method and embedded in paraffin wax. Paraffin section (5µm) were cut on glass slides and stained with hematoxylin and eosin after dewaxing and examined under light microscope.

Statistical analysis

The data are presented as Mean±S.E.M. Statistical analysis was done by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests, for analysis between the groups. The value was considered significant at $P<0.05$.

RESULTS

Effect of ERC, ATR alone and in combination with ATR on Blood pressure

Blood pressure (BP) recorded in the various groups has been shown in Table 1. In the toxic control rats (rats fed with [C+M] diet), mean arterial BP was found to be significantly elevated as compared to normal rats ($p<0.001$). Treatment with ERC extract dose dependently prevented the rise mean arterial pressure. Hypercholesterolemic rats treated with ERC showed similar response in reducing the elevated BP as observed in preventive treatment group. Combinational groups of ERC and ATR almost completely reverted the rise in BP ($p<0.001$). ATR treatment whether given for preventive (60 days)/curative (30 and 60 days) significantly reduced the BP as compared to rats fed with normal saline and [C+M] diet, respectively ($p<0.001$).

Table 1 Effect of ERC, ATR alone and in combination with ATR on mean BP (mmHg)

Treatment groups			Preventive treatment			Curative treatment			Combination treatment		
	Control	Toxic	Toxic + ERC100	Toxic + ERC200	Toxic + ATR	Toxic + ERC 100	Toxic + ERC 200	Toxic + ATR	Toxic + ERC 100 + ATR	Toxic + ERC 200 + ATR	Toxic + ATR
	102.4±4.48	148.6±5.09[a]	125.2±0.49[b]	105.4±2.13[b]	106.1±1.43[b]	128.3±2.26[b]	107.6 ±2.69[b]	97.3±1.72[b]	116.8 ± 2.68[b]	105.3 ± 2.71[b]	114.7 ± 2.72[b]

Results are expressed as Mean ± SEM.; Toxic, administered with [C+M] diet; ATR, Atorvastatin (10 mg/kg); Ethanolic extract of *Rubia cordifolia* (100 mg/kg), (ERC 100); Ethanolic extract of *Rubia cordifolia* (200 mg/kg), (ERC 200); BP, Blood pressure (mm of mercury); [a]p<0.001 versus control; [b]p<0.001 versus toxic

1.1 Effect of ERC, ATR alone and in combination with ATR on serum lipid profile

The differences in the serum lipid profile between the groups are shown in Table 2. Two months with [C+M] diet developed significant dyslipidemia in rats (↑TC, ↑TG, ↑VLDL-C, ↑LDL-C and ↓HDL-C), as compared to the normal saline treated rats (p<0.001). In the rats fed with [C+M] diet along with ERC (100 mg/kg and 200 mg/kg), it was observed that the ERC extracts ameliorated the markers of lipid profile (↓TC, ↓TG, ↓VLDL-C, ↓LDL-C, ↑HDL-C) in a dose dependent manner in both preventive and curative treatment, though the improvement was more marked with the preventive treatment. When ERC was administered along with ATR for one month post treatment, improvement in lipid profile was found to be statistically significant. Administration of standard drug, ATR along with [C+M] diet significantly prevented the progression of dyslipidemia in both preventive and curative treatment (p<0.001). One month post treatment with ATR too produced the beneficial effects though the degree of response was not as prominent as with the two month treatment.

Table 2 *Effect of ERC, ATR alone and in combination with ATR on serum lipid profile*

Parameters	Treatment groups										
	Control	Toxic	Preventive treatment			Curative treatment			Combination treatment		
			Toxic + ERC 100	Toxic + ERC 200	Toxic + ATR	Toxic+ ERC100	Toxic +ERC 200	Toxic+ATR	Toxic+ ERC100 +ATR	Toxic+ ERC200 +ATR	Toxic +ATR
TC (mg/dl)	98.38±3.28	217.53±10.5[a]	192.40±3.14[c]	150.03±2.52[d]	131.86±3.18[d]	192.50±3.50[b]	189.31±2.82[c]	130.11±1.96[d]	152.55±3.75[d]	131.53±2.14[d]	191.15±2.49[c]
TG (mg/dl)	86.89±2.09	166.89±1.81[a]	151.37±2.4[c]	135.22±1.60[d]	103.70±3.34[d]	157.14±1.93[b]	154.67±2.07[c]	118.30±2.56[d]	139.75±1.05[d]	118.70±3.76[d]	153.85±1.22[b]
HDL-C	35.74±1.9	18.98±1.10[a]	29.55±1.19[b]	33.64±2.41[d]	32.32±1.84[d]	27.02±0.86[b]	36.26±1.36[c]	35.56±1.14[d]	27.73±0.84[c]	30.80±1.59[d]	28.52±1.64[c]

(mg/dl)											
LDL-C (mg/dl)	45.26±4.85	165.1±8.51[a]	132.5±3.63[d]	89.34±4.37[d]	78.81±3.37[d]	134.1±3.73[c]	122.1±2.85[d]	70.89±2.28[d]	96.87±3.46[d]	76.99±3.19[d]	131.9±3.34[d]
VLDL-C (mg/dl)	17.38±0.42	33.38±0.36[a]	30.27±0.48[c]	27.04±0.32[d]	20.74±0.67[d]	31.43±0.39 [b]	30.93±0.41[c]	23.66±0.52[d]	27.95±0.21[d]	23.74±0.75[d]	30.77±0.24[b]

Results are expressed as Mean ± SEM.; Toxic, administered with [C+M] diet; ATR, Atorvastatin (10 mg/kg); Ethanolic extract of *Rubia cordifolia* (100 mg/kg), (ERC 100); Ethanolic extract of *Rubia cordifolia* (200 mg/kg), (ERC 200); TC, Total cholesterol; TG, Triglycerides; HDL-C, Highdensity lipoprotein cholesterol; LDL-C, Lowdensity lipoprotein cholesterol; VLDL-C, Very lowdensity lipoprotein cholesterol; [a]p<0.001 versus control; [b]p<0.05 versus toxic; [c]p<0.01 versus toxic, [d]p<0.001 versus toxic

Effect of RC, ATR alone and in combination with ATR on HMGR

Rats fed with [C+M] diet showed significant decrease in the ratio of absorbance of HMG Co-A/Mevalonate indicating marked increase in HMGR activity ($p<0.001$) as shown in Table 3. ERC at 100 mg/kg treatment along with [C+M] diet did not modulate the HMGR activity ($p>0.05$). ERC at 200 mg/kg significantly decrease the HMGR activity in preventive treatment ($p<0.001$). In curative treatment group ERC (200 mg/kg) significantly decrease the HMGR activity. Combing ERC with ATR produced significant increase in the ratio of absorbance of HMG Co-A/Mevalonate in one month curative treatment. ATR treatment whether given for preventive or curative treatment (60 days), significantly decrease the HMGR activity ($p<0.001$). Post treatment with ATR for 30 days produced only mild decrease in HMGR activity ($p<0.01$).

Effect of RC, ATR alone and in combination with ATR on reduced glutathione and TAC

Table 3 shows the effect of RC, ATR on serum anti-oxidant profile. In the serum samples from rats administered with [C+M] diet and from rats administered with normal saline, there was a significant modulation in anti-oxidant levels ($p<0.001$), signifying the induction of oxidative stress by cholesterol and homocysteine. Treatment with ERC along with [C+M] diet showed significant increase in reduced glutathione contents in dose dependent manner, in comparison to the levels observed in hypercholesterolemic rats. The serum TAC levels of concomitant treatment groups of [C+M] diet with the higher dose of ERC (200 mg/kg) were at par with vehicle control group. On the other hand, lower doses of ERC administered along with [C+M] diet showed a slightly but significant elevation in TAC levels ($p<0.01$). The same trend was noticed in a dose-dependent manner with ERC treated animals in the post treatment groups. The pro-oxidation potential of cholesterol and hcy was markedly

reduced ($p<0.001$ for TAC and GSH) with ATR treatment in both one month and two month treatment (preventive/curative). Combining the ERC with ATR produced additive effects in combating the oxidative stress ($p<0.001$ for TBARS and TAC).

Table 3 Effect of ERC, ATR alone and in combination with ATR on serum reduced glutathione, TAC, NO, CRP, Hcy and liver HMGR and Aortic Na+K+ATPase

Parameters	Treatment groups											
	Control	Toxic	Preventive treatment			Curative treatment			Combination treatment			
			Toxic + ERC 100	Toxic + ERC 200	Toxic + ATR	Toxic + ERC 100	Toxic + ERC 200	Toxic + ATR	Toxic + ERC 100+ATR	Toxic + ERC 200+ATR	Toxic + ATR	
HMGR (Ratio of absorbance of HMG Co-A/Mevalonate)	1.26±0.002	0.99±0.003^{a}	0.99±0.003^{ns}	1.19±0.002^{d}	1.23±0.006^{d}	1.01±0.016^{ns}	1.05±0.013^{c}	1.25±0.008^{d}	1.07±0.003^{b}	1.25±0.009^{d}	1.09±0.023^{c}	
Reduced glutathinie (µM/L)	2.56±0.06	1.50±0.07^{a}	2.09±0.04^{c}	2.27±0.04^{d}	2.38±0.04^{d}	1.87±0.05^{c}	2.41±0.05^{d}	2.51±0.01^{d}	2.24±0.04^{d}	2.37±0.04^{d}	1.84±0.04^{c}	
TAC (mM/L)	1.31±0.01	0.68±0.02^{a}	0.83±0.03^{c}	1.37±0.02^{d}	1.30±0.02^{d}	0.80±0.01^{c}	0.80±0.01^{c}	1.24±0.02^{d}	1.07±0.02^{d}	1.19±0.02^{d}	0.96±0.06^{d}	
NO (nM/L)	2.20±0.06	0.95±0.03^{a}	1.04±0.03^{ns}	1.24±0.02^{c}	1.19±0.01^{b}	1.04±0.02^{ns}	1.14±0.02^{c}	1.85±0.04^{d}	1.16±0.02^{b}	1.38±0.07^{d}	1.21±0.03^{c}	
CRP (mg/dl)	0.41±0.01	1.15±0.03^{a}	1.01±0.02^{b}	0.98±0.03^{c}	0.37±0.03^{d}	1.11±0.03^{b}	1.02±0.02^{c}	0.36±0.01^{d}	1.01±0.02^{c}	0.50±0.02^{d}	1.00±0.02^{c}	
$Na^{+}K^{+}$ATPase (µmoles/min/mg protein)	7.70±0.11	5.62±0.06^{a}	5.75±0.15^{ns}	6.73±0.10^{c}	6.72±0.13^{c}	5.42±0.08^{ns}	6.43±0.19^{b}	7.28±0.213^{d}	6.54±0.17^{c}	8.25±0.28^{d}	6.36±0.06^{b}	
Hcy (µmole/L)	9.63 ± 0.872	34.71± 1.323^{a}	30.25 ± 1.315^{b}	14.77 ± 0.445^{d}	18.96 ± 1.002^{d}	29.23 ± 0.421^{b}	28.32 ± 1.321^{c}	19.18 ± 1.242^{d}	28.57 ± 1.011^{d}	14.56 ± 0.658^{d}	28.81 ± 0.996^{b}	

Results are expressed as Mean ± SEM.; Toxic, administered with [C+M] diet; ATR, Atorvastatin (10 mg/kg); Ethanolic extract of *Rubia cordifolia* (100 mg/kg), (ERC 100); Ethanolic extract of *Rubia cordifolia* (200 mg/kg), (ERC 200); HMGR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase; TAC: Total antioxidant capacity; NO: Nitric oxide; CRP: C-reactive protein; $^{a}p<0.001$ versus control; $^{b}p<0.05$ versus toxic; $^{c}p<0.01$ versus toxic, $^{d}p<0.001$ versus toxic, $^{ns}p>0.05$ versus toxic

Effect of ERC, ATR alone and in combination with ATR on serum hcy levels

The changes in serum hcy levels are shown in Table 3. The serum total hcy levels showed a significant increase in rats fed with [C+M] diet for 60 days compared with the serum hcy levels of normal saline treated rats ($p<0.001$). In preventive treatment groups, ERC demonstrated the significant decrease in the serum hcy levels in a dose dependent manner. Whereas in curative treatment groups, ERC (at both doses) showed reduction in serum hcy levels as compared to the rats fed with [C+M] diet. The ATR treatment given for one month to the hyperhomocysteinemic rats reduced the serum hcy levels, but mildly ($p<0.01$). However, the marked lowering effect on serum hcy levels obtained after ATR treatment (two months) as a preventive drug was at par with the effect obtained in curative treatment group. Groups treated with concomitant treatment of ATR and ERC showed significant diminution in serum hcy levels only after one month as compared to the individual drug treatment given for two months.

Effect of ERC, ATR alone and in combination with ATR on serum CRP levels

Table 3 shows CRP levels of serum samples from the treated and control groups of rats. The levels of CRP in group treated with [C+M] diet showed significant elevation as compared to saline fed rats ($p<0.001$). Preventive treatment with ERC, dose dependently exerted anti-inflammatory effect, as evident from the approximately restored serum CRP levels to normal. Similar lowering in CRP values was found in curative treatment groups of ERC as observed with preventive treatment groups. The group treated with ATR showed significant difference in serum CRP levels after 30 days ($p<0.01$) which improved further after 60 days ($p<0.001$) in comparison to rats fed with [C+M] diet. When the ERC was used as an adjunct to ATR treatment, the decline in serum CRP levels was more prominent than the individual treatment with ATR or ERC in 30 days post treatment.

Effect of ERC, ATR alone and in combination with ATR on aortic Na^+K^+ATPase activity

Table 3 depicts the effect of ERC and ATR on aortic Na^+K^+ATPase activity. Hypercholesterolemia and hyperhomocysteinemia caused a significant decrease in Na^+K^+ATPase activity in rats fed with [C+M] diet versus normal saline treated rats ($p<0.001$). The ratio of Na^+K^+ATPase activity in the hypercholesterolemic aorta relative to that in the non-hypercholesterolemic aorta region was significantly different between the preventive treatment with higher dose (200mg/kg) of ERC and [C+M] diet fed groups

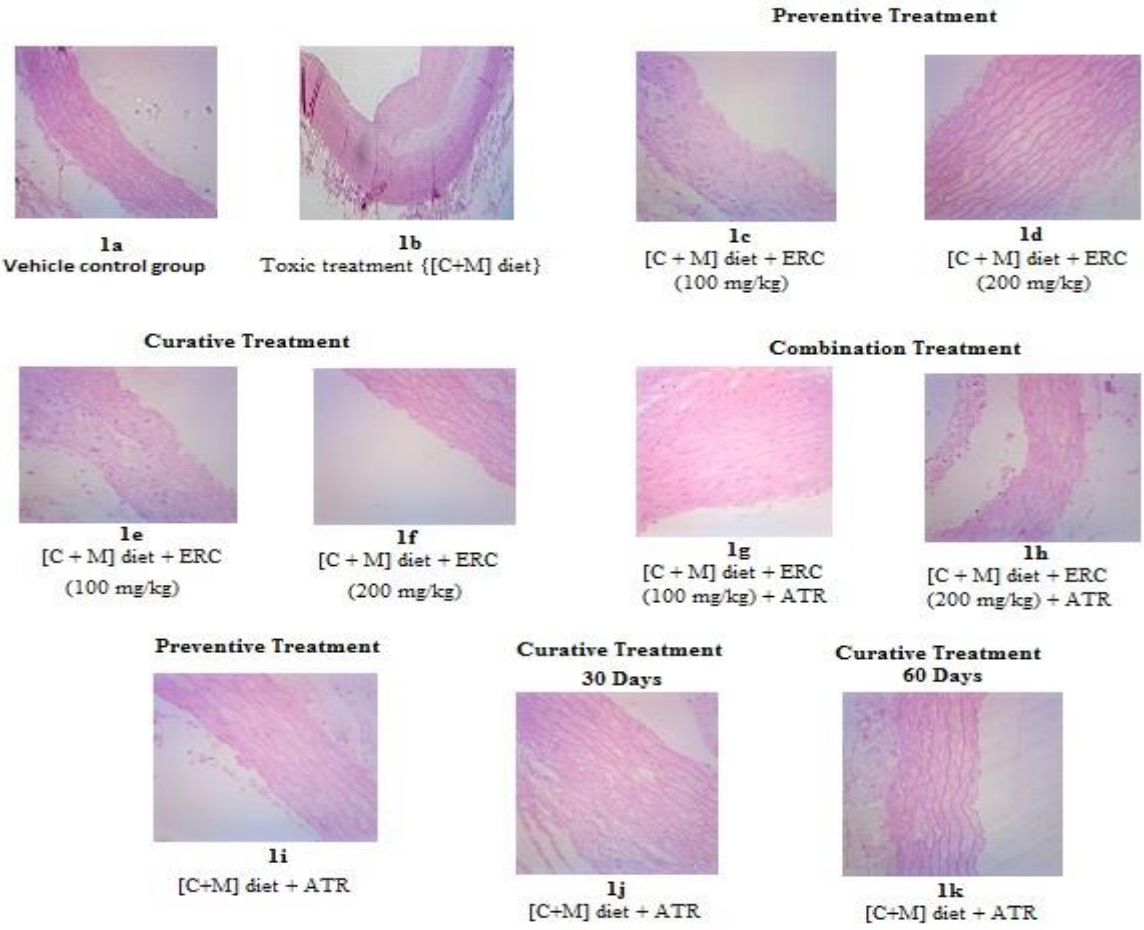
($p<0.01$). Post treatment with ERC at 200 mg/kg did cause a significant change ($p<0.01$) in Na^+K^+ATPase activity in comparison to rats fed with [C+M] diet. Curative treatment with ATR ($p<0.001$) was found to be better than the preventive treatment ($p<0.01$) in the rats. One month post treatment with ATR only produced mild response in elevating Na^+K^+ATPase activity in aorta whereas, one month post treatment with ERC as an adjunct to ATR showed a beneficial effect on Na^+K^+ATPase activity relative to the individual treatment (two months).

Effect of ERC, ATR alone and in combination with ATR on serum NO levels

The data for the changes in serum NO level is presented in Table 3. Hypercholesterolemia and hyperhomocysteinemia caused a significant decrease in serum NO levels in rats fed with [C+M] diet versus normal saline treated rats ($p<0.001$). No significant difference was observed in the concurrent treatment group of ERC (100 mg/kg) with [C+M] diet in comparison to the rats fed with [C+M] diet alone ($p>0.05$). Increasing the dose to 200 mg/kg of ERC significantly improved the serum NO levels ($p<0.01$). Curative treatment group of ERC (at both the doses) showed similar results as observed with the preventive treatment. The combination of ERC with ATR, dose dependently improved serum NO levels in one month treatment duration, suggesting that the both ERC and ATR are acting in an additive manner. ATR treatment to rats along with [C+M] diet produced mild elevation in serum NO levels in comparison to the serum NO levels of rats fed with [C+M] diet alone ($p<0.05$). In post treatment (30/60 days) with ATR the serum NO levels were found to be significantly elevated, relative to the rats fed with [C+M] diet ($p<0.001$).

Effect of ERC, ATR alone and in combination with ATR on histology of aorta (40 X) (Figure 1)

Aorta of healthy rats showed normal histology (Figure 1a). Aortas of rats administered with [C+M] diet showed marked deposition of cholesterol, profuse calcium deposition and endothelial damage (Figure 1b). ERC in a dose dependent manner improve the cholesterol deposition and endothelial damage in both preventive and curative treatment (Figure 1c-1f). Preventive treatment with ATR also demonstrated the normal architecture of aorta (Figure 1g). ERC when combined with ATR, showed normal architecture of aorta at both the doses in one month post treatment (Figure 1h and 1i). Curative treatment with ATR for 30 days showed endothelial disruption and mild smooth muscle proliferation (Figure 1j) which were get normalized on further treatment with ATR for the next 30 days (Figure1k).



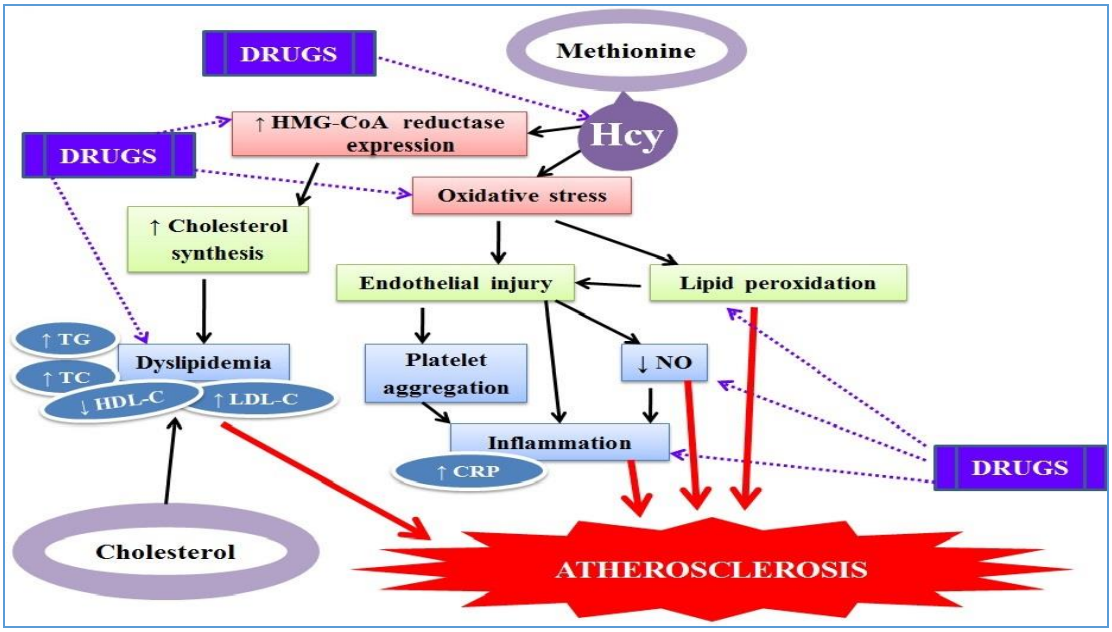


Figure 2 sums up the probable mechanisms leading to Hyperlipidemi and atherosclerosis development and probable path by which the drugs atorvastatin and ethanolic extract of *Rubia cordifolia*, individually and in combination with each other, were found to exhibit anti-hyperhomocysteinemic, lipid lowering, antioxidant, anti-

inflammatory and anti-hypertensive activity. CRP, C-reactive protein; HDL-C, highdensity lipoprotein cholesterol; HMGCoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDL-C, lowdensity lipoprotein cholesterol; NO, nitric oxide; TC, total cholesterol; TG, triglycerides.

DISCUSSION

The present study was designed with the aim to evaluate the efficacy of ERC in preventing or treating the hyperlipidemia and atherosclerosis (HL-AT). An attempt was also made to unravel whether the combination of ERC (as an adjunct) to the conventional therapy for HL-AT, ATR, will have any beneficial effects in combating the limitations (duration of treatment) observed with the ATR.

Both the cholesterol and methionine (the only dietary source of hcy) are usually ingested jointly through animal products such as eggs, meat, and milk and thus, their combination has been found to exacerbate the development of atherosclerosis and endothelial dysfunction as they both aggravate each other's toxic effects on endothelium.[33] Thus, we chose cholesterol (1 g cholesterol in 100 g diet; 1% w/w) and methionine (2 g/kg body weight) [C+M] to induce HL-AT. The study provided first experimental evidence of atherosclerosis development in rats, by feeding them with [C+M] diet for 60 days. This was evident by histopathological analysis of aorta (Figure 1b).

There is a lattice of the pathophysiological mechanisms of hcy-induced HL-AT that can be either independent or dependent on each other. The mechanisms are: Disruption of redox homeostasis by enhanced production of reactive oxygen species (peroxinitrite, oxycholesterols) which induces lipid peroxidation and vascular dysfunction; reduction of bioavailable nitric oxide which impairs endothelial function and enhances smooth muscle cell proliferation; and induction of inflammation and apoptosis.[34] In addition to the above mechanisms promoting atherosclerosis, cholesterol can also cause dyslipidemia which is characterized by increased TC, LDL-C, TG and decreased HDL-C levels.[35]

The Hhcy in the rats fed with [C+M] diet could be due to hypercholesterolemia[36] or the enhanced dietary intake in the form of methionine. The free radical generation owing to hcy accumulation can trigger a cascade of events contributing to pathological changes in aorta (as explained above). Lowering effect on serum hcy with ATR treatment was consistent with the study in hypercholesterolemic rabbits by Koladiya and co-workers.[37] In contrast with our

data, no significant reduction in hcy levels was observed with ATR therapy.[38, 39] The diminutive effect of hcy lowering effect of ERC and concomitant treatment of the ERC with ATR, in favor of their "cardioprotective" properties, could be due to their anti-hyperhomocysteinemic potential.

In our study, significant elevation of serum cholesterol levels were found in rats fed with [C+M] diet, along with corresponding increase in serum TG, LDL-C, VLDL-C and reduction in serum HDL-C levels in two months. This is consistent with the earlier studies reporting altered lipid profile in the rats fed with either cholesterol or methionine.[4, 40] ATR displayed its well-known cholesterol-lowering potential in our study as well, whether administered as a preventive agent to inhibit progression of atherosclerosis; or as a curative agent to treat hyperlipidemia/dyslipidemia in the hypercholestrolemic rats.

The phytochemical analysis of ERC revealed the presence of anthraquinone glycosides and saponins. The lipid lowering ability of the constituents (anthraquinone glycosides and saponins) is well proven.[41-43] Dose dependent amelioration in the lipid profile was observed in the groups treated with ERC. This finding is further supported by the study performed by Somani et al which showed significant decrease in serum TC and TG with daily administration of ERC for two weeks.[24] The concomitant treatment of ERC with ATR showed additive lipid lowering effect as these drugs might act at different steps involved in the cascade of atherosclerosis development.

Hcy induces the expression of HMGR which enhances the cholesterol synthesis.[44, 45] HMGR activity of the rats fed with [C+M] diet was found to be increased by ~22% in comparison to the activity found in normal rats. This enhanced HMGR activity might serve as an etiological factor to increase cholesterol levels in the rats, in addition to the cholesterol rich diet. The rats fed with ATR and ERC showed antihypercholesterolemic effect, which could be due to the reduction in the of HMGR activity. The preventive treatment with higher doses (200 mg/kg) of ERC markedly reduced the HMGR activity (~16%-20%). In the curative treatment groups, ATR showed 27% reduction in the HMGR activity as compared to [C+M] diet fed rats. One month treatment with ATR produced only ~10% reduction in HMGR activity, whereas co-administration of ATR with ERC (at higher doses) produced greater reduction in HMGR activity (~22%-27%). This further suggests that the combinational therapy might be advantageous with respect to the duration of therapy required.

The interference of cholesterol and hcy with the antioxidant defense systems creates an oxidative milieu. Both the cholesterol and hcy are well known pro-oxidants.[46-48] Their pro-oxidation potential was confirmed in our study as well. Our results showed a significant attenuation in the serum GSH levels of the rats fed with [C+M] diet as compared with controls. The reduction of this physiological free radical scavenger signifies perturbed anti-oxidant defense mechanisms. Compelling evidence point towards ATR's anti-oxidant property, one of its potent pleiotropic action.[39, 49] In our study the anti-oxidant potential of ATR was again proved. Reduction of oxidative stress by ATR might be dependent or independent of ATR's hypocholesterolemic effect.[50] The increased serum TAC and GSH levels observed in rats treated with ERC justified the antioxidant properties of the extracts. In support of this, earlier studies have shown the antioxidant properties of the RC.[17, 20, 50] Many of the biochemical effects of RC might be due to the presence of rubiadin (anthraquinone glycoside moiety). Its scavenging property is attributed to its free electron trapping capacity, metal chelation and subsequent inhibition of lipid peroxidation.[17, 51] The combination of ERC with ATR showed synergistic anti-oxidant potential.

C-reactive protein (CRP) is a biomarker of inflammatory conditions and independent risk factor for atherosclerosis.[52] It is evident from our studies that Hhcy in rats fed with [C+M] diet induces augmentation of serum CRP levels, consistent with epidemiological data collected by Akalin et al and Balogh et al.[53,54] Furthermore, elevated cholesterol levels have been shown to correlate with the increased CRP levels, which could be due to the oxidative stress or through induction of CRP secretions from adipocytes directly.[55] ATR has shown to attenuate serum CRP levels thereby arresting atherosclerosis progression. This is in line with the reports by Konduracka et al.[56] Aqueous extracts of RC have been reported to possess anti-inflammatory properties.[19] The CRP lowering effects of ERC can be attributed to rubiadin, an antioxidant moiety. Combing ERC with ATR produced superior CRP lowering effects as compared to individual drug treatment, given for a month. Thus, based on results, it can be inferred that their combination (ATR with ERC) can produce a superior effects in lowering serum CRP levels, in addition to reducing the treatment duration for one month.

Na^+K^+ATPase, a plasma membrane protein, is responsible for regulating the ionic homeostasis of sodium and potassium in tissues and cells. Rats fed with [C+M] diet had significantly reduced aortic Na^+K^+ATPase activity. These results suggest that hypercholesterolemia and/or Hhcy could be involved in the damaging effect on this

membrane protein. This reasoning is supported by reports stating that Na^+K^+ATPase enzyme is vulnerable to oxidative stress induced by hypercholesterolemia and/or Hhcy.[57, 58] ERC's anti-hyperhomocysteinemic potential has been found to improve the lowered Na^+K^+ATPase activity.

The plausible mechanisms responsible for decrease in serum NO levels in the group fed with [C+M] diet could be due to Hhcy-induced free radical generation, oxidative degradation of NO and dysregulation of endothelial NO synthase (eNOS)[59, 60]; and hypercholesterolemia-mediated inactivation of NO via free radicals generation.[61] This observation is consistent with our study as Hhcy was found to produce oxidative stress. In our study, ATR and only the higher doses of the ERC produced significant elevation in NO serum levels. ATR enhances endothelial NO production by stimulating and upregulating eNOS.[62] In our study, the antioxidant, anti-hyperhomocysteinemic and anti-hyperlipidemic effects of ATR and the ERC are in line with the modulation observed in serum NO levels.

Recent studies have indicated that the cholesterol or methionine-enriched diet induced elevation in BP.[40, 63] This observation was consistent with the observed alteration mean BP by feeding the rats with [C+M] diet. Elevated ROS levels observed in the rats fed with [C+M] diet support the notion that Hhcy and hypercholesterolemia influences ROS generation and/or damage the endothelial layer. The damage to the endothelium might have lowered NO production and ultimately, modulated the BP.[64] ATR decreased BP in preventive treatment groups and as a curative treatment to hyperlipidemic rats, in agreement with the findings of other investigators.[65, 66] ERC lowered the BP in a dose dependent manner in preventive and curative treatment. The BP lowering effect of the extract and ATR might be related to their ability to reduce ROS levels.

CONCLUSION

The present findings are preliminary but significant in terms of establishing the role of ethanolic extract of *Rubia cordifolia* in hypercholesterolemia and hyperhomocysteinemia (Figure 2). Anti-hypercholesterolemic and anti-hyperhomocysteinemic action of the extracts appear to be mediated via free radical scavenging, HMGR inhibition and vaso-protective. Combining the ethanolic extract of *Rubia cordifolia* with atorvastatin may reduce the duration of atorvastatin therapy. Further, their use as adjuncts in atorvastatin therapy may prove beneficial in terms of efficacy.

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