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Lipid lowering and antioxidant activity of rohitukine from *Dysoxylum binectariferum* in hyperlipidemic rats

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ABSTRACT

The lipid lowering and antioxidant activity of rohitukine a pure compound isolated from the stem bark of the *Dysoxylum binectariferum* has been studied in triton and cholesterol fed hyperlipidemic rats (*in vivo*). Serum lipids were found to be lowered by rohitukine (at 200 mg/kg.) in triton WR-1339 induced hyperlipidemia in experimental animals.. Chronic feeding of this compound (at 100 mg/kg) in animals simultaneously fed with high fat diet (HFD) for 30 days caused lowering in the lipid and apoprotein levels of very low density (VLDL) and low density lipoproteins (LDL) and increased high density lipoprotein (HDL). Rohitukine activated lipolytic enzymes in plasma and liver lipids. The hypolipidemic activity of the rohitukine is mediated through increased faecal bile acid excretion and enhanced plasma lecithin-cholesterol acyl transferase activity. This molecule showed potent antioxidant and oxygen free radical scavenging activity (in *in vitro* studies).

KEYWORDS

Dysoxylum binectariferum;
Rohitukine;
Lipid lowering;
Antioxidant activities;
Triton model.

INTRODUCTION

Atherosclerosis and associated complications is now the major cause of myocardial morbidity and mortality worldwide. Elevated level of plasma concentration of cholesterol especially low density lipoprotein (LDL) and triglyceride along with free radicals oxidative stress are recognized as leading cause in the development of atherosclerosis and coronary heart diseases. Several drugs are being used in the treatment of dyslipidemia. Treatment of hyperlipidemia using statins has been used to decrease serum levels of cholesterol and triglyceride. Statin such as atrovastatin, lovastatin, fluvastatin, simvastatin, and pravastatin are HMG-CoA reductase inhibitirs which act by inhibiting cholesterol synthesis and up regulate LDL receptors in liver. However common side effects of statins are myositis, arthralgias, gastrointestinal upset and elevated liver function test. Thus there is a need of the therapeutic benefits of several antidislipidemic drugs while simultaneously reducing the severe side effects.

The involvement of hydroxyl free radicals (OH) has been found to be a major causative factor for peroxidative damage to lipoproteins which is responsible for inhibition and progression of atherosclerosis in hyperlipidemic subjects[1]. Hyperlipidemia may also induce other abnormalities like oxidation of fatty acids, leading to the formation of ketone bodies as well as masking liver and muscle resistance to insulin which initiates the progress of diabetes in patients[2]. Furthermore due to hyperglycemia, increases in nonenzymic glycosilation occurs, accompanied with glucose oxida-

tion and these reactions being catalized by Cu^{2+} and Fe^{2+},resulting in formation of O^{2-} and OH radicals which further accelerates the risk of cardiac diseases in dyslipidemic patients[3]. Oxidation is one of the destructive processes wherein it breaks down and damages various molecules. Oxygen via its transformation produces reactive oxygen species (ROS) such as superoxides hydroxyl radicals and hydrogen peroxide. They provide uncontrolled reactions[4]. Molecular oxygen is an essential component for all living organisms, free radicals attack and induce oxidative damage to various molecules including proteins, lipids, lipoproteins and DNA[5,6]. The body possesses several defense systems comprising enzymes and radical scavengers[4]. Some of them constitute the repair systems for molecules that are damaged by free radicals[6]. Antioxidants are compounds that act as inhibitors of the oxidation process and are found to inhibit oxidant chain reactions at small concentrations and there by eliminate the threat of pathological processes[4]. Phenolic compounds present in the medicinal plants have been reported to posses powerful antioxidant activity[1]. Flavonoids are a major class of phenolic compound present in the medicinal plants and are found to have a potential role in prevention of various diseases through their antioxidant activity[7].

Dysoxylum binacteriferum is found in the Western Ghats of India. Crude extracts of this plant were found to be highly effective against ovarian and breast cancer[8]. Few previous studies showed that the anticancer activity of of this plant. Since rohitukine[9]. was recently established to possess antiestrogenic effect in adult female rats[10]. Lipid lowering activity as well as antioxidant property of rohitukine was done according to methodology given in literature [11-16].

MATERIAL AND METHODS

Collection of the plant material

The stem bark of this plant was collected and identified by the Botany Division of the Institute from the Andaman coast of India. The voucher specimen (No. 8091) has been kept in the herbarium of the Institute.

Preparation of extract/fractions/compound

Air-dried powdered plant material (1.0 kg) was extracted with distilled ethanol, concentrated under reduced pressure and further fractionated into four fractions. From the chloroform fraction, a known alkaloid rohitukine[9] {5,7-dihydroxy-2-methyl-8- [4-(3-hydroxy-1-methyl)-piperidinyl]-4H-1-benzopyran-4-one)} was isolated.

Bioassays

Rats (Charles Foster strain, male adult, body weight 100 -150 g) were kept in a room with controlled temperature (25-26 °C), humidity (60-80%) and 12/12 hours light/dark cycle, (light on from 8.00 am to 8.00 pm) under hygienic conditions. Animals, which were acclimatized for one week before starting the experiment, had free access to the normal diet and water ad libitum.

The lipid lowering activity of *D. binacteriferum* extract was evaluated in tritan treated hyperlipidemic rats. The rats were divided into control, triton treated and triton plusextract treated groups containing six rats in each group. In the acute experiment tritan WR-1339 (Sigma Chemical Company, St. Louis, M O, USA) was administered (400mg/kg) by intraperitonial injection for 18 h. Rohitukine and Gemfibrozil (Cipla Ltd. Bombay, India) were macerated with 0.2% aqueous gum acacia suspension and fed orally Simultaneously with tritan in the chronic experiment. Hyperlipidemia was produced by feeding with high fat diet (Novo Dordisk, Denmark) once a day for 30 days. Drugs were administered orally (100 mg/kg.) simultaneously with cholesterol in drug treated groups. Control animals received same amount of normal saline or groundnut oil. At the end of experiments rats were fasted overnight and blood was withdrawn. The animals were killed and the liver was excised immediately.

Superoxide anions (O^{-2}) were generated enzymatically by Xanthine (160 mM), Xanthine oxidase (0.04 U) and nitro blue- tetrazolium (320μ M) in the absence or presence of rohitukine (100 and 200 μg /ml) in 100 m M phosphate buffer (pH 8.2) was sonicated well in phosphate buffer before use. The reaction mixture was incubated at 37^0 C and after 30 min the reaction was stopped by adding 0.5ml glacial acetic acid. The amount of formazone formed was measured spectrophotometrically. In another set of experiment effect of this compound on the generation of hydroxyl radicals (OH) was studied by non enzymic reactants. Briefly OH^- was gen-

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erated in a non- enzymic system comprising deoxyribose (2.8 mM) $FeSO_4$. $7H_2O$ (2.0 m M) sodium ascorbate (2.0 mM) and H_2O_2 (2.8 mM) in 50 mM KH_2PO_4 buffer, pH 7.4 to a final volume of 2.5 ml. The above reaction mixture, in the absence or presence of test sample (100 µg/ml and 200 µg/ml) was incubated at 37^0 C for 90 minutes. The rohitukine was also studied for inhibitory action against microsomal lipid peroxidation *in vitro* by non- enzymic inducer. Reference tubes and blank were also run simultaneously. Malondialdehyde (MDA) contents in both experimental and reference samples were estimated spectrophotometrically by thiobarbituric acid method as mentioned above. Allopurinol, Manitol, and α- tocopherol were used as standard drugs for superoxide hydroxylation and microsomal lipid peroxidation.

Plasma lecithin: cholesterol acyl transferase (LACT) activity[11]. and Post heparin lipolytic activity (PHLA) were assayed[12]. Serum was fractionated into very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) by polyanimic precipitation methods[13]. Serum as well as lipoproteins were analysed for their total cholesterol (TC), phospholipids (PL), triglyceride (TG) and protein by standard procedures reported earlier[14,15].

Liver was homogenised (10% w/v) in cold 100 mM phosphate buffer, pH 7.2 and used for the assay of total lipolytic activity of the lipid extract of each homogenate was used for the estimation of TC, PL, TG and protein. The rat faeces were collected from all groups throughout 30 days and processed for the estimation of cholic acid and de-oxy cholic acid[16]. Data were analyzed using student's t-test. Hyperlipidemic groups were compared with control and plant extract treated hyperlipidemic. $P<0.05$ was considered as significant.

TABLE 1 : Lipid lowering activity of rohitukine in triton treated hyperlipemic rats.

Experimental Schedule	Total Cholesterol [a]	Phospholipid [a]	Triglyceride [a]	Protein [b]
Contol	88.72±5.37	90.33±8.00	85.40±6.14	7.33±0.26
Triton treated	254.66±20.14***	220.44±16.14***	245.81±20.23***	15.18±1.10***
	(+2.87F)	(+2.87F)	(2.80F)	(2.07F)
Triton + Rohitukine	190.88±13.68***	168.27±***	188.37±14.23***	12.00±0.78**
	(-25)	(-23)	(-23)	(-21)
Triton+Gemfibrozil (standard Drug)	1.65±13.00***	140.10±10.17***	160.20±14.11***	11.08±0.66***
	(-35)	(-33)	(-35)	(-27)

Unit: a. mg/dl b. g/dL; Values are mean ± SD from 6 rats ***P< 0.001, **P< 0.01. Tritan group compared with control, triton and drug treated compared with triton.

TABLE 2 : Effect of rohitukine and Gemfibrozil on blood lipids and lipolytic enzymes in hyperlipidemic rats.

Parameters	Control	Cholesterol treated	Cholesterol and rohitukine treated	Cholesterol and Gemfibrozil treated
Serum				
Total Cholesterol [a]	86.66±5.48	258.11±20.62***	190.44±13.88***	170.84±13.69***
		(+2.97F)	(-26)	(-34)
Phospholipid [a]	83.47±6.00	239.22±19.39***	180.27±14.48***	166.66±10.82***
		(+2.86F)	(-25)	(-30)
Trigliyceride [a]	106.88±9.00	201.93±14.44***	148.21±5.59***	128.37±7.88***
		(+1.88F)	(-26)	(-36)
Protein [b]	6.38±0.17	12.27±1.00***	8.98±0.17***	8.00±0.47***
		(+2.85F)	(-27)	(-35)
VLDL				
Total Cholesterol [a]	8.32±0.40	32.40±2.20***	25.00±1.50***	21.37±1.62***
		(+3.89F)	(-23)	(-34)
Phospholipid [a]	15.00±0.48	31.24±2.00***	27.00±1.64***	20.14±2.00***
		(2.08F)	(-26)	(-35)

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Parameters	Control	Cholesterol treated	Cholesterol and rohitukine treated	Cholesterol and Gemfibrozil treated
Triglyceride [a]	40.37±2.82	90.87±6.82*** (+2.25F)	72.30±4.00*** (-23)	65.12±5.37*** (-28)
Apoprotein[b]	6.40±0.38	12.64±0.87*** (+1.97F)	9.40±0.38*** (-25)	9.00±0.27*** (-28)
LDL				
T Cholesterol [a]	13.44±0.62	63.27±5.12*** (+4.70F)	48.77±2.62*** (-23)	43.72±4.00*** (-38)
Phospholipid [a]	12.64±1.00	44.12±2.87*** (+3.49F)	34.00±2.12** (-22)	29.14±2.17*** (-33)
Triglyceride [a]	15.28±1.00	35.17±2.61*** (+2.30F)	25.38±2.00*** (-27)	24.88±1.62*** (-29)
Apoprotein[b]	17.00±1.14	30.27±1.88*** (+1.78F)	23.00±1.00*** (-24)	21.00±1.60*** (-30)
HDL				
T Cholesterol [a]	46.38±4.00	36.17±2.40*** (- 22)	43.37±2.88* (+17)	44.00±3.16* (+18)
Phospholipid [a]	39.00±3.00	29.38±2.17*** (- 25)	33.80±2.14* (+13)	34.66±2.81* (+15)
Triglyceride [a]	16.14±1.00	12.00±0.78*** (- 26)	15.17±1.00** (+21)	16.00±0.79*** (+25)
Apoprotein[b]	170.33±13.62	122.62±10.14*** (- 28)	141.24±12.44* (+13)	150.39±14.00* (+18)
Plasma LCAT activity[c]	70.30±4.84	35.69±2.44*** (- 49)	50.31±3.82*** (+29)	52.77±5.00*** (+32)
PHLA[d]	18.00±1.17	11.00±0.62*** (-38)	14.00±0.79** (+21)	14.48±1.01*** (+24)

Units: a. mg/dl serum, b. g/dL serum, c. n mol cholesterol released /h/l plasma, d. n mol free fatty acid formed /h/ml plasma; Values are mean ± SD from six animals; ***P<0.001, **P<0.01, *P<0.05; Cholesterol treated compared with control, cholesterol and drug treated with triton only.

TABLE 3 : Effect of rohitukine and Gemfibrozil on hepatic lipids and faecal bile acid excretion in hyperlipemic rats.

Parameters	Control	Cholesterol treated	Cholesterol and rohitukine treated	Cholesterol and Gemfibrozil treated
A Liver				
LPL activity[a]	132.22 ±10.60	73.30 ±5.69*** (-44)	83.66 ±8.00 (+12)	89.27 ±5.77* (+18)
Total cholesterol[b]	7.00 ±0.25	12.17 ±1.00*** (+1.73F)	9.11 ±0.37** (-25)	8.80 ±0.40*** (-28)
Phospholipid[b]	24.31 ±2.00	40.18 ±3.12*** (+1.65F)	28.66 ±1.60*** (-28)	26.92 ±2.00*** (-33)
Triglyceride[b]	11.23 ±0.77	16.68 ±1.10*** (+1.48F)	13.11 ±0.69** (-21)	12.00 ±1.00*** (-28)
Protein[b]	152.88 ±13.18	220.84 ±13.92*** (+1.44F)	178.80 ±14.42*** (-19)	165.50 ±14.00*** (-25)
B Faecal bile acids				
Cholic acid[c]	85.73 ±6.89	50.22 ±3.78*** (-41)	69.92 ±6.00*** (+28)	67.00 ±6.10*** (+25)
Deoxycholic acid[c]	55.77 ±5.00	25.10 ±2.00*** (-55)	38.80 ±3.00*** (+54)	44.89 ±3.12*** (+44)

Unit: a. n mole free fatty acid formed/h/mg protein, b. mg/g, c. µg/g; Values are mean ±SD of six animals; ***P<0.001, **P<0.01, *P<0.05. Cholesterol treated group compared with control and cholesterol plus drug treated group compared with cholesterol treated.

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TABLE 4 : Effect of rohitukine on the generation of superoxide anions, hydroxyl radicals and lipid peroxidation in microsomes.

Test compound	Conc of compounds (µg/ml)	superoxide anions[a] (O_2^-)	hydroxyl radicals[b](OH•)	Microsomal lipid peroxidation[b]
	Control	180.24±13.79	94.90±6.82	98.28±7.00
Rohitukine	100	144.39 ±10.30***	70.18 ±5.00***	80.20±5.72***
		(-20)	(-26)	(-18)
	200	114.30±8.30***	60.27±3.87***	70.33±2.88***
		(-37)	(-36)	(-28)
Standard drug	200	85.20±6.14***	45.57±2.89***	53.33±3.14***
		(-53)	(-52)	(-46)

Units: a. n mol formazone formed/min. b. n mol MDA formed/h/mg protein; Each value is mean ± SD of six values ***P< 0.001, **P< 0.01. Experimental data compared with control experiment.

RESULTS

Effect of rohitukine in triton induced hyperlipidaemia

The acute administration of triton WR-1339 caused a marked increase in the serum levels of TC (+ 2.87 F), PL (+2.44 F), TG (+2.87 F) and protein (+2.07 F). Treatment with these extract caused reversal in these levels of TC (-25%), PL (-23%), TG (-23%) and protein (-21%). The lipid lowering activity of rohitukine in the hyperlipidemic rats was comparable to that of Gemfibrozil.

Effect of rohitukine on lipid composition in serum lipoproteins and liver

The data showed that the administration of HDF in rats increased their serum levels of TC, PL and TG by 2.97, 2.86 and 1.88 fold respectively. Feeding with these extract and Gemfibrozil reversed the levels of these serum lipids (26, 25 and 27%) in cholesterol and extract treated animals. The analysis of hyperlipidemic serum showed a marked increase in the levels of lipids and apoproteins constituting β-lipoproteins and these effects were pronounced for VLDL-TG (-23%) and LDL-TC (-22%). Treatment with these extract and Gemfibrozil significantly reduced these levels of VLDL lipids (-28%) as well as LDL-TC (-38%), PL (-33%), TG (-29%) and apo-LDL (-30%) respectively in hyperlipidemic rats. At the same time the decreased levels of HDL-lipids and apo-HDL in these animals were partially recovered. The increased levels of TC, PL, TG and protein (1.73, 1.65, 1.48 and 1.44 F) in liver of cholesterol fed rats were observed to be lowered by their treatment with this compound.

Effect on lipolytic enzymes

HDF feeding caused the inhibition of plasma LCAT (-49%) and PHLA (-38%) respectively and total lipolytic activity (-44%) in liver. Treatment with these extract and Gemfibrozil partially reactivated these lipolytic activities in plasma and liver of hyperlipidemic rats.

Effects on faecal excretion of bile acids

Feeding with HDF caused a significant decrease in faecal excretion of cholic acid (-41 %) and deoxycholic acid (-55%) and these levels were shown to be recovered by the treatment with rohitukine (+28% and +54%) and gemfibrozil (+25 and +44%) in HDF and extract fed animals.

Antioxidant activity of rohitukine

The antioxidant activity of this compound was evaluated by generating free radicals [superoxide ions ($O2^-$), hydroxyl radicals (OH•), microsomal lipid peroxidation] *in vitro* with absence and presence of this compound. The scavenging potential of the rohitukine and the standard drug allopurinol at 200 µg/ml against formation of superoxide ions ($O2^-$) shown in TABLE 4. This compound showed potent inhibition of antioxidant activity at 100 µg /ml and 200 µg /ml by -20% and -37% respectively. Manitol was taken as standard drug in order to compare the hydroxyl radical (OH•) scavenging potential of this compound, manitol showed -53% hydroxyl radicals scavenging activity at 200 /µg /ml., rohitukine showed -37 % activity at 200µg /ml concentration and -20 % activity at 100 µg /ml respectively.

The microsomal lipid peroxidation scavenging activities of the rohitukine was studied. ά-tocopherol was taken as standard drug which showed -46% activity at 200µg/ml concentration where as this compound exhibited potential inhibition -28% at the same concentration.

DISCUSSION

Rohitukine from *D. binacteriferum* and Gemfibrozil both cause a significant decrease in the serum level of lipids in triton induced hyperlipidemic rats and this model has been successfully used for the evaluation of lipid lowering activity of rohitukine in the rats[17,18]. The present investigation with HDF fed hyperlipidemic animals shows that rohitukine could increase the level of HDL by increasing the activity of LCAT, which play a key role in lipoprotein metabolism. The increase of the receptor mediated catabolism of LDL as well as the lipolytic activity in liver and the level of blood HDL-TC followed by the decrease of B-lipoprotein lipids and the suppression of hepatic lipids by these extract are of great utility for regressing atherosclerosis[19]. The stimulation of LDL catabolism by these extract in hyperlipidemic animals may be due to a significant decrease in the level of serum and tissue lipids. The compound may also enhance the synthesis of LDL apoprotein (Apo B) as well as receptor protein to accelerate the turnover of cholesterol. Increased synthesis of receptor protein decreased the rate of hepatic lipid synthesis and inhibition of oxidative modification in LDL may regulate the cholesterol level in the body.

The superoxide anions, hydroxyl radicals, and hydrogen peroxide are continuously generated inside the human body as a consequence of exposure to exogenous chemicals and/ or a number of endogenous metabolic processes involving redox enzymes and bioenergetics electron transfer. Owing to the ROS overproduction and/ or inadequate antioxidant defense, there is upsurge of ROS and this culminates in oxidative stress. It is quite interesting to note that plants have good antioxidant ability and are safer then the synthetic antioxidants. The antioxidant activity can be attributed to various mechanism like prevention of chain initiation, binding of transition metal ion catalyst, decomposition of peroxides, reductive capacity and radical scavenging activity.

Hyperlipidemia is one of the important risk factors involved in the development of cardiovascular diseases. Atherosclerosis and congestive heart diseases are strongly associated with disorders of lipid metabolism and plasma lipoproteins. Triton WR-1339 treated rats are considered to be an usefull acute hyperlipidemic model associated with inactive lipoprotein lipase[20]. Triton WR-1339 act as a surfactant to block the uptake of lipoprotein from the circulation by extra hepatic tissues resulting in an increase in the level of circulatory lipoproteins[21]. This extract was found to be enriched in flavonoids and it is reported that flavonoids are found to be inhibit HMG- Co A reductase activity.

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