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Therapeutic Impacts of Tocotrienols and *Boerhaavia diffusa* on Cholesterol Dynamics, Lipid Hydroperoxidation and Antioxidant status on Hyperlipidemic Rats: Induced by Oxidized Cholesterol

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Abstract

Cholesterol, the prominent member of steroid family, present in tissues and in plasma either as free cholesterol or as a storage form combined with a long chain fatty acid as cholesteryl ester. High blood cholesterol results in Atherosclerosis, which is characterized by presence of atheromas. In this we investigated the efficacy of antioxidant and hypolipidemic agents Tocotrienols and *Boerhaavia diffusa* by analyzing all the parameters in plasma lipoprotein lipids, TL, TC, TG, VLDL-C, LDL-C, non-HDL-C, MDA and *in-vitro* oxidizability of LDL, as investigated in oxidized cholesterol feeded rats treated with and without Tocotrienols and *Boerhaavia diffusa*. All the plasma lipids parameters, TL, TC, TG, VLDL-C, LDL-C, non-HDL-C and MDA levels were significantly increased in hyperlipidemic control rats. After 4 weeks administration of Tocotrienols and *Boerhaavia diffusa* significantly reduced the overall oxidative burden and effectively ameliorated the above altered parameters.

Key Words: Oxidized Cholesterol, Atherosclerosis, Hypolipidemic, Antioxidant, Tocotrienols

Introduction

Coronary heart disease (CHD) is the main cause of death in Western countries and Asia. Among CHDs, ischemic heart disease (IHD) leads to the highest mortality rate. The number of heart patients suffering from IHD worldwide is gradually increasing [1]. Several epidemiological studies have demonstrated the relationship between plasma cholesterol levels and the development of IHD. Cholesterol, the most prominent member of steroid family, is an important component of eukaryotic membranes [2]. Cholesterol is present in tissues and in plasma either as free cholesterol or as a storage form, combined with a long-chain fatty acid as cholesteryl ester. In plasma, both forms are transported in lipoproteins. It is synthesized in many tissues from acetyl-CoA and is the precursor of all other steroids in the body such as corticosteroids, sex hormones, bile acids, and vitamin D. Lipids are transported through plasma compartment in lipoproteins, which are complex water soluble molecules consisting of a core of cholesteryl esters and TG covered by a surface monolayer of phospholipids, free cholesterol and apolipoproteins. In the last two decades, there have been major advances in our understanding of the role of plasma lipoproteins, apolipoproteins, lipolytic enzymes, and lipoprotein receptors in cholesterol and lipoprotein metabolism. This new information has provided major insights into the role of cholesterol and lipoproteins in the pathogenesis of premature atherosclerosis. There are six major classes of human plasma lipoproteins, these include chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), high density lipoprotein (HDL) and lipoprotein (a) [Lp(a)] [3]. Nuclear magnetic resonance subdivides the lipoproteins in plasma into the following subclasses: (1) for VLDL, V6-V1 with V6 the largest and V1 the smallest; (2) IDL; (3) for LDL, L3-L1 with L1 being the smallest and dense [4]. HDL can be further separated by hydrated density into HDL_2a, HDL_2b, HDL_3a, HDL_3b and HDL_3c, [5]. These lipoproteins are distinguished on the basis of their lipid content, ultracentrifugation size, electrophoretic mobility and surface proteins. Fifteen major human plasma apolipoproteins have been identified and their gene and protein structures determined [6]. Atherosclerosis is a progressive disease and results due to the deposition of intracellular lipids in the smooth muscle cells of the inner arterial wall. These lesions narrow and eventually block the arteries due to the formation of fibrous, calcified plaques. The rough arterial wall promotes the formation of blood clots, which may also block the artery. Due to the blocking of arteries, blood flow stops and causes the death of the deprived tissues. The stoppage of blood flow is known as an infraction. The cholesterol lowering effect of tocotrienols was attributed mainly to their down regulation of HMG-CoA reductase-the rate-limiting enzyme of the cholesterol biosynthetic pathway. *Boerhaavia diffusa* is a medicinal plant widely used in the Ayurvedic medicine [7]. The

plant was named in honour of Hermann Boerhaave, a famous Dutch physician of the 18th century [8]. *Boerhaavia diffusa* (Spreading Hogweed in English) belonging to the family of the Nyctaginaceae, is mainly diffused perennial herbaceous creeping weed of India (known also under its traditional name as punarnava). The first pharmacological studies have demonstrated that the root of punarnava exhibits a wide range of properties: anti-inflammatory [9], diuretic, laxative [8], anti-urethritis febrifuge, antileprotic, anti-ashmatic, antiscabby and antistress activites. An aqueous extract of thinner roots of *B. diffusa* at a dose of 2 mg/kg exhibited the remarkable protection of various enzymes such as serum glutanic-ocaloacetic transaminase, serum glutanic-pyruvic transaminase, and bilirubin in serum against hepatic injury in rats [10]. As discussed above, hypercholestolamia is a major risk factor for the development of artherosclerosis and is associated with coronary and peripheral vascular disease. In this study we investigated the efficacy of antioxidant agent tocotrienols and *Boerhavvia diffusa* by analyzing all the parameters in plasma, TC, VLDL-C, LDL-C, HDL-C, TBAR, MDA, Hepatic TG, TC and antioxidant enzymes (CAT, SOD, GPx and GRED) as well as *in vitro* oxidizability of LDL.

Material and Methods

Chemicals: 1-Chloro 2, 4-Dinitrobenzene was purchased from Central drug house, Pvt. Ltd. (India). All other chemicals used for this study were of analytical grade and obtained from HIMEDIA (India), Sisco (India), Ashirwad (India), Sigma-Aldrich (USA), Miles (USA), Acros (USA) and Tocotrienols drug as well as RBD palm olein were supplied as a gift from CAROTECH BHD, Chemor, Malaysia.

Estimation: Plasma triglyceride [11], Plasma Cholesterol, LDL and HDL [12], Plasma VLDL-C [13], Fractionation of Plasma lipoprotein such as LDL [14], HDL and its fractions-HDL_2, HDL_3 [15], Plasma FRAP [16], *ex vivo* and *in vitro* Cu^{++}-mediated LDL oxidation [17, 18] were measured by following known procedures.

Plant Material:-The samples of the roots of *Boerhaavia diffusa* (Nyctaginaceae), collected in Aligarh (UP) were kindly provided by Prof. Siddiqui (Taxonomist), Department of Botany, Aligarh Muslim University, Aligarh, India and the voucher specimen (AMUBT8977) has been preserved in our research laboratory (Dept. of Biochemistry, J.N. Medical College, AMU, Aligarh) for future reference in research.

Preparation of Plant Extract: 250 gm of powder dried roots of *Boerhaavia diffusa* were taken in the round bottom flask of Soxhlet apparatus then 1 litre of ethanol was added. Refluxing was carried out for 48 hours. Further solvent was evaporated and its extract was calculated per gm of dried material. Pourslin chip are add in the refluxing process to avoid bumping.

Experimental Design: The experimental study was approved by the Dolphin Institute of Biomedical and Natural Science, Dehradun, Uttarakhand, where the study was conducted. The rats were given pelleted rat chow. Maintenance and treatment of all the animals was done in accordance with the principles of Institutional Animal Ethics Committee constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Healthy male albino rats, weighing about 150-180 g were purchased from Indian Veterinary Research Institute (IVRI), Bareilly (India), were maintained to animal house environmental condition prior to the experiment. For the present study, animals were divided into following 4 groups:

Normal Control (NC); six rats were given 1.0 ml saline/rat/day through gastric intubation for 4 weeks, Hyperlipidemic control rats (HC); six rats in this group were administered 1.0 mg oxidized cholesterol/rat/day through gastric intubation for 4 weeks, Hyperlipidemic Tocotrienols Treated Rats (H-T_3T); six rats in this group were given 6.0 mg Tocotrienols/rat/day through gastric intubation for 4 weeks and Hyperlipidemic *Boerhaavia diffusa* Treated Rats (H-BT); six rats in this group were given 1.0 *Boerhaavia diffusa* mg/rat/day through gastric intubation for 4 weeks

Collection of Blood and Plasma: For the estimation of different parameters, overnight fasted rats in each group were anaesthetized and blood drawn from cardiac puncture, and were collected in heparinised tube. Plasma was separated from blood by centrifugation at 2500 rpm for 30 min.

Statistical evaluation: This was done by employing two-tailed Student t-test as described by Bennet and Franklin [19]. P value less than 0.02 were considered significant.

Results

Impacts of Tocotrienols and Boerhaavia diffusa on average body weight in each group of rats: As seen in Table 1, depicts that the average body weight (g) of hyperlipidemic control rats (H-C), Tocotrienols treated (H-T_3T) and *Boerhaavia diffusa* treated (H-BT) rats was 180, 179 and 182 (g), respectively, whereas for normal control (H-C) rats the average body weight was 150 g, whereas, the average body weight of H-C, H-T_3T and H-BT rats showed a significant gain of 39%, 29% and 23% respectively after 4 weeks of treatment. These results demonstrate that in hyperlipidemic Tocotrienols treated rats the gain in body weight after 4 weeks was significantly higher than rats in H-C group.

Table 1: Impacts of Tocotrienols and *B. diffusa* on average body weight in each group of rats after 4 weeks of treatment. *Values are mean ± SD from 6 rats in each group, N-C, normal control; H-C, hyperlipidemic control; H-T_3T, fed 6 mg Tocotrienol/rat/day and H-BT, given 1mg B.D/rat/day for 4 weeks. Significantly different from N-C at [a]p<0.001. Significantly different from H-C at [a]p<0.001.

Average body weight/rat (g)		
Group	Before treatment	After Treatment
N-C	150.23±1.23*	210.11±3.12 (+39.85%)[a]
H-C	180.22±6.21*	212.63±1.42 (+17.98%)[a]
H-T_3T	179.11±3.22*	232.33±6.11 (+29.71%)[a]
H-BT	182.13±4.11*	224.11±3.61 (+23.04%)[a]

Impacts of Tocotrienols and B. diffusa on Plasma Lipids, Plasma Lipoprotein Lipids, and Lipid Peroxidation Status in Plasma in Hyperlipidemic Rats Treated for 4 Weeks:

Effect on plasma lipids: As seen in Fig. 1, all the plasma lipids parameters were significantly increased in hyperlipidemic control (H-C) rats, when compared to N-C values. Total lipids (TL), triglycerides (TG), free fatty acids (FFA) and total cholesterol (TC) significantly increased from 340, 53, 132 and 86 mg/dl in N-C to 523, 112,149 and 151 mg/dl, respectively, in H-C group. After 4 weeks of Tocotrienols treatment, levels of TL, TG, FFA and TC were significantly decreased by 10%, 41%, 10% and 28%, respectively, when compared to corresponding H-C values. Whereas, in *Boerhaavia diffusa* treated rats, TL, TG, FFA and TC levels were significantly reduced by 8%, 42%, 12% and 34% respectively, in comparison to corresponding values in H-C group. These results demonstrate that 4-week treatment of hyperlipidemic rats with 6 mg Tocotrienols and 1 mg *Boerhaavia diffusa* mediated a similar and significant reduction in above lipid parameters.

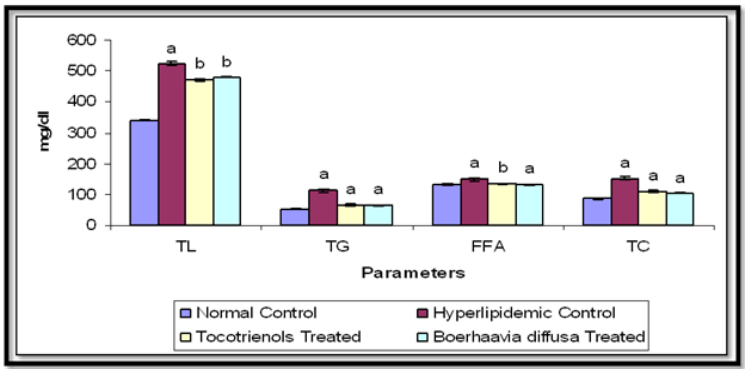

Fig. 1: Impacts of Tocotrienols and *Boerhaavia diffusa* on Plasma Total Lipid, Triglycerides, Free Fatty Acid and Total Cholesterol in Oxidized Cholesterol Feeded Rats after 4 weeks of treatment, *Values are mean (mg/dl) ± SD from pooled plasma of 6 rats in each group, N-C, normal control; H-C, hyperlipidemic control; H-T_3T, fed 6 mg Tocotrienol/rat/day and H-BT, given 1mg B.D/rat/day for 4 weeks, Significantly different from N-C at [a]p < 0.001, Significantly different from H-C at [a]p<0.001 and [b]p<0.05.

Effects on plasma lipoprotein lipids on the ratios of LDL-C/HDL-C and HDL-C/TC: As seen in Fig.2, plasma VLDL-C, LDL-C and non-HDL-C levels were significantly increased from 13, 54 and 63 mg/dl in N-C to 21 mg/dl (88%), 115 mg/dl (113%) and 133 mg/dl (113%) respectively, in H-C. After 4 weeks of Tocotrienols and *Boerhaavia diffusa* treatment, both VLDL-C, LDL-C and non-HDL-C levels showed a significant reduction 41%, 44% and 43%, respectively, in H-T_3T, whereas, in H-BT, VLDL-C, LDL-C and non-HDL-C were significantly reduced of 41%, 48% and 46%, respectively, in comparison to corresponding values in H-C rats. HDL-C, HDL_2-C and HDL_3-C levels were decreased from 21, 7 and 14 mg/dl in N-C to 18 mg/dl (14%), 5 mg/dl (30%) and 13 mg/dl (10%), respectively, in H-C values. After 4 weeks of Tocotrienols treatment (H-T_3T) HDL-C, HDL_2-C and HDL_3-C levels showed a significant increase of 72%, 160% and 42%, respectively, when compared to corresponding values in H-C, whereas, in H-BT, HDL-C, HDL_2-C and HDL_3-C levels were increased by 55%, 120% and 37%, respectively. These results demonstrate that both Tocotrienols and *Boerhaavia diffusa* are equally effective in reducing VLDL-C and LDL-C levels. On the other hand, in comparison to H-C values, treatment of hyperlipidemic rats with Tocotrienols mediated a significantly higher increase in HDL-C, HDL_2-C and HDL_3-C concentration than the increase seen in Lovastatin treated rats. On the other hand, as shown in Table 2, LDL-C/HDL-C and HDL-C/TC ratios were calculated from the data presented in Fig. 1 and 2. LDL-C/HDL-C ratio was significantly increased from 3.83 in N-C to 8.19 (113%) in H-C group, when compared to ratio in N-C. After 4 weeks of treatment, the increase in LDL-C/HDL-C ratio was significantly prevented and decreased to 3.50 and 3.55 in H-T3T and H-BT, respectively and HDL-C/TC ratio was significantly decreased from 0.343 in N-C to 0.212 (38%) in H-C group. Tocotrienols and *Boerhaavia diffusa* treatment to these rats significantly prevented the increase in HDL-C/TC ratios and fully restored them near to

the N-C. In addition, the ratios related to HDL-C in Tocotrienols and *Boerhaavia diffusa* treated rats were positively modulated and restored similar to normal control value, indicating normalization of cholesterol levels associated with the above lipoproteins.

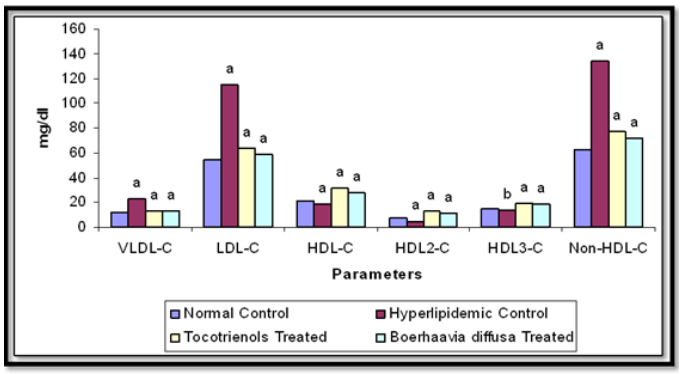

Fig. 2: Impacts of Tocotrienols and *Boerhaavia diffusa* on Plasma VLDL-C, LDL-C, HDL-C, HDL_2-C and HDL_3-C sub-fractions and Non-HDL-C in Cholesterol feeded rats after 4 weeks of treatment, Values are mean (mg/dl) ± SD from pooled plasma of 6 rats in each group, N-C, normal control; H-C, hyperlipidemic control; H-T_3T, fed 6 mg Tocotrienol/rat/day and H-BT, given 1mg B.D/rat/day for 4 weeks, Significantly different from N-C at $^{a}p<0.001$ and $^{b}p<0.02$, Significantly different from H-C at $^{a}p<0.001$.

Table 2: Effects of Tocotrienols and *Boerhaavia diffusa* on the Ratio of LDL-C/HDL-C and HDL-C/TC in Cholesterol feeded rats after 4 weeks of treatment, †For the calculation of ratios, data is taken from Fig. 1 and 2. *Values are mean ± SD from pooled plasma of 6 rats in each group, N-C, normal control; H-C, hyperlipidemic control; H-T_3T, fed 6 mg Tocotrienol/rat/day and H-BT, given 1mg *Boerhaavia diffusa*/rat/day for 4 weeks. Significantly different from N-C at $^{a}p<0.001$ and $^{b}p<0.01$. Significantly different from H-C at $^{a}p<0.001$, $^{b}p<0.05$

Ratio†	Group			
	N-C	H-C	H-T3T	H-BT
LDL-C/HDL-C	3.83±0.014*	8.19±0.061* (+113.83 %)[a]	3.50±0.816* (-56.26 %)[a]	3.55±0.019* (-56.65 %)[a]
HDL-C/TC	0.343±0.060	0.212±0.003 (-38.19 %)[b]	0.386±0.061 (+82.07 %)[b]	0.356±0.088 (+67.92 %)[b]

Lipid Lowering Effect on liver triglycerides and total cholesterol: As seen in Table 3, hepatic levels of triglyceride (TG) and total cholesterol (TC) were significantly increased in hyperlipidemic rats (H-C) by 29 % and 124 % respectively, when compared to corresponding values in H-C. Feeding of Tocotrienols and *Boerhaavia diffusa* to hyperlipidemic rats for 4 weeks was associated with a significant decline in liver TG and TC levels by 24 % and 40 % respectively, in H-T_3T, whereas, in H-BT group, TG, TC and FFA levels were reduced by 7 % and 42 % respectively, when compared to corresponding values in S-C group. These results demonstrate that similar to plasma TG and TC levels in liver was significantly increased in hyperlipidemic rats. In addition, feeding of Tocotrienols and *Boerhaavia diffusa* to hyperlipidemic rats resulted in a significant decline of TG and TC to a level similar to corresponding values in H-C. The combined results demonstrate that levels of TG, TC in plasma and liver lipids were significantly increased in hyperlipidemic rats. Treatment of these stressed rats with 6 mg Tocotrienols /rat/day or 1 mg *Boerhaavia diffusa*/rat/day mediated a significantly decline in the above lipid parameters, similar to corresponding values in H-C rats.

Table 3: Effects of Tocotrienols and *Boerhaavia diffusa* on Liver Triglycerides (TG) and Total cholesterol (TC) contents in cholesterol feeded rats after 4 weeks of treatment. *Values are mean ± SD from homogenate of pooled liver 6 rats in each group, N-C, normal control; H-C, Hyperlipidemic control; H-T_3T, fed 6 mg Tocotrienols/rat/day and H-BT, given 1mg *Boerhaavia diffusa*/rat/day for 4 weeks. Significantly different from N-C at $^{a}p<0.001$, Significantly different from H-C at $^{a}p<0.001$.

Group	Liver Homogenate	
	Triglycerides (mg /100mg protein)	Total cholesterol (mg /100mg protein)
N-C	0.634±0.003*	2.19±0.042
H-C	0.821±0.081* (+29.34%)[a]	4.92±0.031 (+124.65%)[a]
H-T_3T	0.630±0.151* (-23.26%)[a]	2.98±0.139 (-39.43%)[a]
H-BT	0.768±0.139* (-6.45%)[a]	2.83±0.190 (-42.47%)[a]

Impacts on plasma total antioxidants and lipid peroxidation products: As seen in Fig. 3, depicts the antioxidant impacts of Tocotrienols and *Boerhaavia diffusa* on plasma concentrations of total antioxidants, conjugated diene (CD), lipid hydroperoxide (LHPO) and malondialdehyde (MDA) in hyperlipidemic (H-C) rats. In H-C rats, plasma total antioxidants level was reduced from a control value of 43 to 30 (30%) μmole/dl. Treatment of H-C rats with Tocotrienols and *Boerhaavia diffusa* for 4 weeks resulted in a significant increase of total antioxidants levels by 31 % and 20 %, when compared to H-C value. The oxidative stress induced in H-C rats significantly enhanced plasma lipid peroxidation products, such as conjugated diene, lipid hydroperoxide and MDA. Formation of conjugated diene, lipid hydroperoxide and MDA in plasma was increased from 8.32, 1.52 and 2.83 in N-C to 13.38 (60 %), 1.95 (59 %) and 3.23 (107 %) μmole/dl, respectively, in H-C. After Tocotrienols treatment, in H-T_3T, a significant decrease of 14 %, 17 % and 32 % was seen in the formation of conjugated diene, lipid hydroperoxide and MDA, respectively, when compared to corresponding values in H-C rats. Similarly in H-BT, conjugated diene, lipid hydroperoxide and MDA in plasma were also significantly decreased by 8 %, 13 % and 29 %, respectively, when compared to corresponding values in H-C rats. These results demonstrate that in H-C rats, due to increase in oxidative stress, total antioxidants level was decreased, whereas, concentration of plasma conjugated diene, lipid hydroperoxide and MDA were significantly increased. Tocotrienols or *Boerhaavia diffusa* treatment significantly restored the total antioxidants level and blocked the increase in plasma conjugated diene, lipid hydroperoxide and MDA to a level close to corresponding normal values.

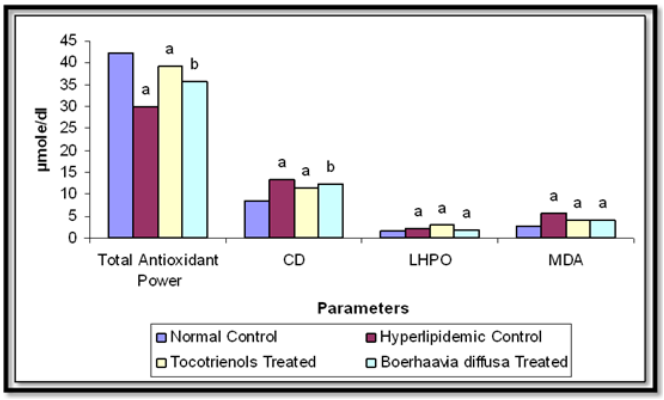

Fig. 3: Antioxidant impacts of tocotrienols and *Boerhaavia diffusa* on plasma Total antioxidants, Conjugated diene, Lipid hydroperoxide and Malondialdehyde contents in cholesterol feeded rats after 4 weeks of treatment. *Values are mean (μmole/dl) ± SD from pooled plasma of 6 rats in each group, N-C, normal control; H-C, hyperlipidemic control; H-T_3T, fed 6 mg Tocotrienol/rat/day and H-BT, given 1mg B.D/rat/day for 4 weeks. Significantly different from N-C at $^{a}p<0.001$. Significantly different from H-C at $^{a}p<0.001$ and $^{b}p<0.05$.

Impacts on the regulation of hepatic Catalase and Superoxide dismutase, Glutathione peroxidase and Glutathione reductase activities: As seen in Table 4, Catalase activity in liver was significantly decreased from a value of 3.22 unit in N-C to 2.12 (35 %) in H-C, respectively. Administration of Tocotrienols to hyperlipidemic rats (H-T_3T) resulted in a significant increase in liver catalase activities by 3.10 (46 %) unit, respectively. In *Boerhaavia diffusa* treated (H-BT) group, liver catalase activity was significantly increased by 43 %. However, in comparison to corresponding tissue values of normal control rats (N-C), the decline in hepatic SOD activity of hyperlipidemic rats was 17 %. Treatment of Tocotrienols and *Boerhaavia diffusa* hyperlipidemic rats resulted in a significant increase in hepatic SOD activity by 30 % and 27 %, respectively from normal value. In hyperlipidemic rats, Gpx activity in liver was significantly increased from a value of 52 unit in N-C to 65 (23 %), respectively, in H-C rats. As evident, after 4 weeks of treatment with Tocotrienols, Gpx activity in liver was significantly decreased by 25 %. In H-BT rats, the Gpx activity in liver was decreased by 24 % respectively, when compared to corresponding tissue values in H-C group. On the other hand, in hyperlipidemic rats, the enzymatic activities of hepatic Gred were decreased significantly by 23 % when compared to corresponding values of H-C rats. Feeding of Tocotrienols and *Boerhaavia diffusa* to hyperlipidemic rats significantly blocked the decrease in hepatic Gred activities and increased them to a similar value of 15 % respectively. Administration of Tocotrienols and *Boerhaavia diffusa* to hyperlipidemic rats significantly prevented the decrease in Gred activity and increased to a level, which is similar to normal value. In summary, hepatic catalase, SOD, Gpx and Gred enzymes, which constitute a mutually supportive team of defense against ROS, are significantly decreased in hyperlipidemic rats. However, feeding of Tocotrienols and *Boerhaavia diffusa* substantially quenches these free radicals (ROS), thus positively normalizing the above enzyme levels.

Table 4: Impacts of Tocotrienols and *Boerhaavia diffusa* on liver catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase activities in cholesterol feeded rats after 4 weeks of treatment. †One unit (U/mg protein) of enzyme activity is defined as the µmoles of H_2O_2 decomposed/min/mg protein. ‡One unit (U/mg protein) of enzyme activity is defined as the amount of enzyme required to inhibit O.D. at 560 nm of chromogen production by 50 % in one minute. ⁺One unit (U/ mg protein) of enzyme activity is defined as nmole oxidized glutathione formed/min/mg homogenate protein. ‡One unit (U/ mg protein) of enzyme activity is defined as nmole NADPH oxidized/min/mg PMS protein. *Values are mean ± SD from homogenate or PMS fraction of pooled liver of 6 rats in each group, N-C, normal control; H-C, Hyperlipidemic control; H-T_3T, fed 6 mg Tocotrienol/rat/day and H-BT, given 1mg B.D/rat/day for 4 weeks. Significantly different from N-C at [a]p<0.001. Significantly different from H-C at [a]p<0.001

Group	Catalase†	Superoxide dismutase††	Glutathione peroxidase⁺	Glutathione reductase‡
N-C	3.22±0.132*	0.611±0.004	52.32±0.101*	10.68±0.215
H-C	2.12±0.631* (-34.16 %)[a]	0.505±0.210 (-17.34 %)[a]	65.13±1.15* (+24.48%)[a]	8.32±0.219 (-22.09%)[a]
H-T_3T	3.10±0.261* (+46.22 %)[a]	0.658±0.134 (+27.32 %)[a]	49.33±1.89* (-24.25%)[a]	9.49±0.161 (+14.06.76%)[a]
H-BT	3.02±0.448* (+42.45 %)[a]	0.643±0.521 (+27.32 %)[a]	50.13±1.24* (-23.03%)[a]	9.62±0.639 (+15.62%)[a]

Antioxidant effects on basal levels of conjugated diene formation and lag phase in LDL: As depicted in Table 5, the *ex vivo* base line diene conjugation (BDC) levels of LDL in hyperlipidemic rats was increased by 48 % respectively, in comparison to the corresponding N-C values. Feeding of Tocotrienols to hyperlipidemic rats partially blocked the *in vivo* oxidation of LDL and reduced their BDC levels by 19 % respectively. Similarly, after *Boerhaavia diffusa* treatment (H-BT), BDC levels in was reduced by 19 % respectively, in comparison to the corresponding H-C values. As expected, the lag phase time of LDL oxidation was reduced from 88 min in N-C to 56 min in H-C. Treatment of hyperlipidemic rats with Tocotrienols *Boerhaavia diffusa* restored the lag phase time of LDL oxidation to 79 min and 68 min, respectively.

Table 5: *Ex- vivo* and Copper-mediated *in vitro* oxidation of LDL in cholesterol feeded rats after 4 weeks of Tocotrienols and *Boerhaavia diffusa* treatment. *The conjugated diene (CD) values are expressed as nmole malondialdehyde equivalents/mg protein. Basal conjugated diene represent the status of oxidized LDL *in vivo*. **The lag phase is defined as the interval between the intercept of the tangent of the slope of the curve with the time expressed in minutes. ⁺Values are obtained from LDL, isolated from pooled plasma of 6 rats in each group, N-C, normal control; H-C, Hyperlipidemic control; H-T_3T, fed 6 mg Tocotrienol/rat/day and H-BT, given 1mg B.D/rat/day for 4 weeks. †Percent increase with respect to basal value in N-C, ††Percent decrease with respect to basal value in H-C, ¶Percent decrease with respect to lag phase value in N-C, §Percent increase with respect to lag phase value in H-C.

	LDL OXIDATION	
Group	Basal value of CD*	Lag phase**
N-C	170.22⁺	88
H-C	251.82⁺ (+47.93%) †	56 (- 36.36%) ¶
H-T_3T	202.61⁺ (-19.54%) ††	79 (+41.07%) §
H-BT	205.27⁺ (-18.48%) ††	68 (+21.42%) §

Discussion

Results from the present study, indicates that hypercholestrolemic rats experience an exaggerated oxidative stress when compared with hypocholesterolemic rats. The oxidized cholesterol induced extensive proatherogenic changes, that occurred in rats, were reflected on a variety of parameters, such as, plasma and lipoprotein lipids including cholesterol and apoB content of LDL and its subfractions, plasma lipid peroxidation products including ex vivo and in vitro oxidizability of LDL, plasma total antioxidants and HDL-associated activities, MDA release and antioxidant enzymes in erythrocytes. Supplementation of rats with dietary Tocotrienols (Tocomin) and *Boerhaavia diffusa* (B.D) for 4 weeks significantly reduced the overall oxidative burden and effectively ameliorated the above altered parameters, thus, indicating a strong hypolipidemic/antiatherogenic and antioxidant effect of Tocomin and *Boerhaavia diffusa*. Four-week Tocomin and *Boerhaavia diffusa* treatment of these hypercholestrolemic rats caused a significant reduction in plasma LDL oxidation, which were reversed to 45% and 50% of respective control values of hypochlolesterolemic rats. These results indicate a strong protective effect of dietary tocotrienols and *Boerhaavia diffusa*, which may help lower the risk of myocardial infarction in hypercholestrolemic rats. Our results indicate a modest and significant increase in plasma total lipid, TG, TC and free fatty acids (FFA) in hypercholestrolemic rats. The increase in plasma TG levels is apparently due to an increase in VLDL which can be the result of either increased VLDL production or decreased VLDL clearance. It is possible that massive free radical load in hypercholestrolemic rats may stimulate VLDL production by increasing adipose tissue lipolysis, increasing hepatic de novo fatty acid synthesis, and decreasing hepatic fatty acid oxidation, all of which provide fatty acid substrate for esterification into TG and assembly into VLDL particles in the liver as well as increase in plasma FFA. Tocomin and

Boerhaavia diffusa effectively blocked the increase in the above lipid parameters and reversed them to a level similar to their respective control values of hypocholesterolemic rats. Therefore, tocotrienols may exert their cholesterol lowering effect in hyperlipidemic rats in a similar manner as previously reported for hyperlipidemic animals [20, 21] and humans [22, 23]. Mechanism wise, as previously shown in HepG2 cells, as well as in normolipidemic and hyperlipidemic rats, tocotrienols reduce cholesterol synthesis by suppressing HMG-CoA reductase activity, which in turn is reduced by a decline in its protein mass [20, 24]. The decline in protein mass may be achieved by inhibition of HMG-CoA reductase synthesis and/or enhanced degradation. Consistent with *in vivo* results in rats [20], γ-tocotrienol has been shown to mediate the suppression of enzymatic activity and protein mass of HMG-CoA reductase in HepG2 cells through decreased synthesis (57 % of control) and enhanced degradation (2.4-fold versus control) of the enzyme [24]. In addition, γ-tocotrienol was shown to upregulate LDL receptor in mammalian cells and may be implicated in part for the reduction of apoB-lipoprotein *in vivo* [24]. Thus, tocotrienols reduce cholesterol formation in mammalian cells by suppressing HMG-CoA reductase activity through two actions: decreasing the efficiency of translation of HMG-CoA reductase mRNA and increasing the controlled degradation of HMG-CoA reductase protein, post-transcriptionally [24]. In addition, another report indicates that γ-tocotrienol influences apoB secretion by both cotranslational and posttranslational processes involving a decreased rate of apoB translocation and accelerated degradation of apoB in HepG2 cells. This activity correlated with a decrease in free and esterified cholesterol [25]. Taken together, the information indicates an association between the suppression of hepatic cholesterol synthesis and apoB secretion, and the observed lowering of apoB and LDL-C levels in animal and human models [26]. Oxidative modification of lipoproteins is believed to play a central role in the pathogenesis of atherosclerosis [27]. Because plasma contains several antioxidants [28] and lipoproteins with oxidative damage have been isolated from atherosclerotic lesions [27], lipoprotein oxidation generally is considered to occur in the vessel wall. Although lipid oxidation in the vessel wall is thought to occur as a result of a local deficiency of endogenous antioxidants or an excess of free metal ions, only limited data support these hypotheses. Research has shown that human atherosclerotic plaques contain massive amounts of lipid peroxidation products, despite the presence of large quantities of α-tocopherol (vitamin E) and ascorbate [29]. Therefore, it is unclear whether oxidized lipoproteins originate in the arterial wall or are produced in the circulation and then enter the intimal space. Our data show that due to sustained high cholesterol diet in hypercholestrolemic rats, oxidation of lipid/lipoprotein particles is considerably enhanced. Conjugated diene (which measure the initial phase of lipid peroxidation), lipid hydroperoxide (intermediate product of lipid peroxidation) and MDA (which measure the degradation phase of lipid peroxidation) in plasma are significantly increased in hypercholestrolemic rats. The increase in plasma lipid peroxidation products is associated with a significant decline in plasma total antioxidants. The former suggests increased production of oxidants while later indicates diminished antioxidant defense. Both the changes indicate an existence of profound oxidative stress. Our results indicate a significant decrease in plasma lipid peroxidation products with a concomitant and significant increase in plasma total antioxidants in Tocomin and *Boerhaavia diffusa* treated hypercholestrolemic rats. In this context, our data provide suggestive evidence for the increased CVD risk observed in hypercholestrolemic rats. In addition, daily intake of tocotrienols as a dietary supplement will be useful in the prevention and treatment of hypercholesterolemia induced dyslipidemia and atherosclerosis. The levels of reactive oxygen species (ROS) are controlled by antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx), glutathione reductase (Gred). An impaired radical scavenger function has been linked to decreased activity of enzymatic of free radicals. These enzymes are important in defending the body against free radicals as well as toxic substances by converting them to a form that can be readily excreted. Therefore, any changes in these enzymes could be potentially detrimental to the host by altering these defense mechanisms. Normal cellular metabolism involves the production of ROS, low levels of ROS are vital for proper cell functioning, while excessive *in vivo* generation of these products can adversely affect cell functioning. Malondialdehyde (MDA) is one of the final products of lipid peroxidation in human cells, and an increase in ROS causes over production of MDA, which is considered a surrogate marker of oxidative stress. Increased MDA production in erythrocytes is known to cause a decrease in the membrane fluidity of the membrane lipid bilayer and increased osmotic stability of cells. The major intracellular antioxidant enzyme, SOD, specifically converts superoxide radicals to hydrogen peroxide, CAT as well as Gpx detoxifies hydrogen peroxide to water [30]. Glutathione peroxidase protects against free radical injury by reducing the peroxide concentration via a glutathione dependent reduction process, thereby reducing the amount of peroxides available to produce cellular damage. Reduced glutathione is a major intracellular non-protein sulfhydryl compound. It has many biological functions, including maintenance of membrane protein and lipoprotein SH groups in the reduced form, the oxidation of which can otherwise cause altered cellular structure and function. Glutathione cycle operates in the erythrocytes for the disposal of H_2O_2 generated in the cell supplementing the function of CAT. Glutathione and H_2O_2 are twin substrates for Gpx. Glutathione is formed from its oxidized form, GSSG by the enzyme Gred, which requires NADPH as a cofactor [31] therefore, as the balance between ROS production and antioxidant defenses is lost, the resultant oxidative stress through a series of events deregulates the cellular functions leading to various pathological conditions. An antioxidant compound might contribute partial or total alleviation of such damage. Treatment of hypercholestrolemic rats with dietary tocotrienols and *Boerhaavia diffusa* for 4 weeks significantly improved the integrity of erythrocytes membrane as shown by improved protection against lipid peroxidation as well as reversal of SOD, CAT, Gpx and Gred activities to near normal levels. Similar to our results, a reversal in the erythrocytes SOD and GPx activities has previously been reported in healthy cholesterolemic rats supplemented with 200 mg of TRF for 4 weeks. It is interesting to mention that in general, the results obtained from the

oxidized cholesterol feeded hypercholestrolemic rats treated with dietary tocotrienols and *Boerhaavia diffusa* exhibited great similarities with the results in supplementation for 4 weeks. In addition, in this rat model, the hypochlolesterolemic and antioxidative properties of *Boerhaavia diffusa* are also comparable to Tocomin, used in animal model. First, we have summarized the similarities between the common parameters investigated in both the agents. These reports clearly indicate that daily intake of routine dietary antioxidant vitamins or their heavy dose supplements by hypercholestrolemic rats will not sufficiently alleviate the enormous *in vivo* oxidative stress/damage induced by oxidized cholesterol. However, since, dietary tocotrienols and *Boerhaavia diffusa*, because of their potent hypochlolesterolemic/anti-atherogenic and antioxidant actions, were able to substantially ameliorate/normalize all the altered parameters including atheroprotective function of HDL described in the thesis, we initially recommend daily supplementation of hypercholestrolemic patients with dietary tocotrienols (Tocomin). In conclusion, based on Tocomin and *Boerhaavia diffusa* mediated multiple therapeutic benefits, described in the present study, daily intake of tocotrienols as a dietary supplement and *Boerhaavia diffusa,* as herbal supplementation may be useful in the prevention and treatment of high cholesterol mediated atherosclerosis. In addition, daily use of dietary tocotrienols will be efficacious, cost effective, and good source of vitamin E.

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