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IN VITRO ANTIOXIDATIVE EFFECT OF *BOERHAAVIA DIFFUSA* ON COPPER MEDIATED OXIDATIVE MODIFICATION OF LDL IN *TYPE-II* DIABETIC PATIENTS

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ABSTRACT

Type-II Diabetic Mellitus is characterized by reduced insulin sensitivity by insulin receptors in the cell membrane or insulin resistance combined with reduced insulin secretion due to defective responsiveness of body tissues to insulin. The purpose of this study was to estimate the effect of a methanolic extract of *Boerhaavia diffusa* on oxidative stress. Results through this research demonstrate that diabetic patients experience on exaggerated oxidative stress when compared with normal significant increase in plasma, TG, TC, VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C and non HDL-C levels. This may be due to markedly increased production of oxidant and significantly diminished antioxidant defence including a decline in total plasma antioxidant power. Thus the study depicts that daily intake of *Boerhaavia diffusa* extract by Diabetic Mellitus patients may be useful in the prevention and treatment of the Diabetes-induced hyperlipidemia and atherosclerosis. In addition, daily use of *Boerhaavia diffusa* will be efficacious and cost effective and good source of natural antioxidant.

Keywords: Diabetic Mellitus, Antioxidants, *Boerhaavia diffusa*, Total Antioxidant Power.

INTRODUCTION

Diabetes Mellitus (DM) often referred as simply diabetes is a condition in which the body does not produce enough, or properly respond to insulin, a hormone produced by the pancreas. Diabetes is presently estimated to affect more than 150 million patients worldwide, and this number is expected to double by 2025 [1].Asia is the major site of a rapidly emerging epidemic of diabetes. Among the 10 leading countries in terms of the number of people with diabetes mellitus in the year 2025, five are in Asia [2]. India, due to

its immense population size and high diabetes prevalence, will contribute 57 million [1].The American Diabetes Association reported in 2005 that there are 23.6 million children and adults in the United States 7.8% of population who have Diabetes. Diabetes Mellitus is classified into two types-Type I results from the body's failure to produce insulin. It is also termed as childhood onset Diabetes, juvenile Diabetes and insulin-dependent Diabetes Mellitus (IDDM). It is characterized by loss of the insulin producing beta cells of the islet of langerhans in the pancreas leading to insulin deficiency. Type II results from insulin resistance or reduced insulin sensitivity, combined with reduced insulin secretion which in some cases become absolute. The defective responsiveness of the body tissues to insulin almost certainly involves the insulin receptor in cell membrane. It is the most common type of Diabetes Mellitus [3]. Diabetes is characterized by many complications including hyperglycemia, diabetic ketosis, hyperlipidemia, polyuria, weight loss, polydipsia, polyphagia, lethargy, blurred vision, breath smelling of acetone, nausea, vomiting and abdominal pain. Diagnosis of Diabetes Mellitus is characterized by recurrent or persistent hyperglycemia [4] and demonstrating any one of the following- Fasting plasma glucose at or above 126 mg/dl., Plasma glucose at or above 200 mg/dl two hours after a 75 g oral glucose load as in glucose tolerance test, Symptoms of hyperglycemia and casual plasma glucose at or above 200 mg/dl. Besides the above screening tests a partial list includes-High blood pressure elevated cholesterol levels, coronary artery disease, past gestational diabetes, polycystic ovary syndrome, chronic pancreatitis, fatty liver, cystic fibrosis etc. People with confirmed diagnosis of Diabetes are tested routinely for complications. This includes yearly urine testing for micro-albuminuria and examination of the retina of the eye for retinopathy. *Boerhaavia diffusa* (spreading hogweed) belongs to the family of Nyctaginaceae, is a perennial herb of India with traditional name as Punarnava. It has a large root system bearing rootlets. The tap root is thick, fleshy and bitter in taste [5]. Its roots, leaves and aerial parts are used for the treatment of numerous disorders in Ayurvedic herbal medicine. the root is mainly used for the treatment of gonorrhea, inflammations, dyspepsia, anemia, liver, gall bladder and kidney disorders, cancer etc [6].The first pharmacological studies have demonstrated that the root of punarnava exhibits a wide range of properties : anti-inflammatory[7],diuretic[8], laxative[9], antiurthritis[10], anticonvulsant[11], antinematodal [12], immunosuppressive and

immunomodulatory properties are also under investigation and studied by various researchers[13], Pain-relieving Actions [14] antifibrinolytic[15], diuretic, hepatoprotective[16, 17], immunosuppressive, antihyperlipidemic[18], antiproliferative [13] and immunomodulatory[13] activities. The recent study carried out by [18] demonstrated that *B. diffusa* reduced the level of glucose in the blood increasing insulin release from bet cells of pancreas. The present study aims to estimate the effect of a methanolic extract of *Boerhaavia diffusa* on the intestinal motility, to evaluate a potential antioxidant effect of the methanolic extract of *Boerhaavia diffusa*, to estimate a genoprotective effect of the methanolic root extract of *Boerhaavia diffusa* for treatment of Diabetes Mellitus type II in human experiment.

MATERIAL AND METHODS

Chemicals: All the chemicals used in the study were of analytical grade and procured from standard suppliers like Himedia, Sigma Lab. Pvt. Etc. All the Glasswares used were of borosil company and plastic wares from MS Tarson and Himedia. Micropipette from Eppendorf company, India. Instruments used during study were Electronic balance, pH meter, centrifuge, spectrophotometer, incubatory rotatory shaker, soxhlet apparatus, autoclave, deep freezer, refrigerator, magnetic stirrer, hot air oven, water bath etc.

Estimation: Determination of Plasma Triglycerides [19] Fractionation of plasma lipoproteins such as LDL [20], HDL and its subtractions HDL_2 and HDL_3 [21]. Protein is to be estimated by the Bradford method [22] using bovine serum albumin as standard, Plasma FRAP [23] Ex *vivo* and *in vitro* Cu^{++} mediated susceptibility of isolated LDL[24, 25]

Experimental Design: The research was carried out at the Department of Biotechnology and Biomedical Science, DIBNS, Dehradun. Diabetic patients and Normal control subjects where recruited from Dr. Chhabra Pathology Lab, Dehradun. Informed consent from study enrollment was obtained from each of the study subject. All the subjects where ethically homogenous with similar nutritional habits free from alcohol consumption and were drinking maximum 3-2 cups of tea a day, had no vitamin intake 3 months before the initiation of the study. During recruitment the blood was drawn from the subjects in the morning after all night fasting, transferred into a heparinized glass tube. Plasma was collected by centrifugation and used for the analysis of Glucose, TG, TC, VDL-C, LDL-C, HDL-C and its subfractions, HDL_2-C and HDL_3-C.

Collection of Blood and Plasma: At the end of the experiment treatment, overnight fasted Diabetic patients in each group were anaesthetisized and blood drawn from cardiac puncture. The blood from each patient in a given group was collected in heparinized tubes, mixed gently by inversion 2-3 times and incubated at 4^{o} C for 2-3 hrs. Plasma was separated from the blood by centrifugation at 25000 rpm for 30 min, aliquoted and either stored at 4^{o} C or frozen at -20^{o} C for use in other experiments.

Statistical evaluation: This was done by employing two-tailed Student t-test as described by Bennet and Franklin [26]. P value less than 0.02 were considered significant.

RESULT & DISCUSSION

Measurement of Physiological parameter of Normal and Diabetes Type II individual: The results for the physiological parameters of age, height for male and females of either normal and Diabetes mellitus shown in Table 1, do not have any significant difference. This depicts that they do not have any significant role in deciphering the diabetic patients from the normal healthy persons. However, weight comparison between normal and diabetic patients did show decrease in value for the latter as depicted to be a symptoms for the disease. The result was in accordance to similar trials-hyperglycemias, Polyuria and Polydipsia accompanied by weight loss as seen in adult rats within 3 days of Streptozotocin induced diabetes on rats [27].

TABLE 1: MEASUREMENT OF PHYSIOLOGICAL PARAMETER OF NORMAL & DIABETES TYPE II INDIVIDUALS

Parameters	Normal	Diabetic mellitus
Numbers	12	10
Male	7	6
Female	5	4
Age (yrs)	21.25±1.64*	51±3.09*
Weight (kg)	69.50±0.89*	55.58±10.83*
Height (cm)	165.66±0.10*	167.63±0.17*
Diabetic history of Patients	-	15.4± 2.8 months
Treatment	-	Glycomate glycheck

*Values are means ±S.D. from all groups of subject.

Measurement of Glucose and lipid profile of Normal and Diabetic Patients: The results depicted in Fig. 1 indicate that the Glucose, TG, TC, VLDL-C, HDL-C, HDL2-C,

HDL3-C, non HDL-C were significantly increased from the normal control value, however HDL3-C showed a decrease in the value compared to normal. The percentage ratio for LDL-C/HDL-C, LDL-C/TC and TC/HDL-C as shown in Table 2, also demonstrated increased values. Similar results have been proven by [28]. In another animal trial blood glucose level increased to a much higher folds for diabetic mice than non-diabetic mice [29]. Therefore, tocotrienols may exert their cholesterol lowering effect in inflammation /infection induced hyperlipidemic rats in a similar manner as previously reported for hyperlipidemic animals [30, 31] and humans[32, 33]. 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 [30, 34]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 [30], 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 [34]. 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* [34]. 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 [34]. 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 [35]. 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 [36].

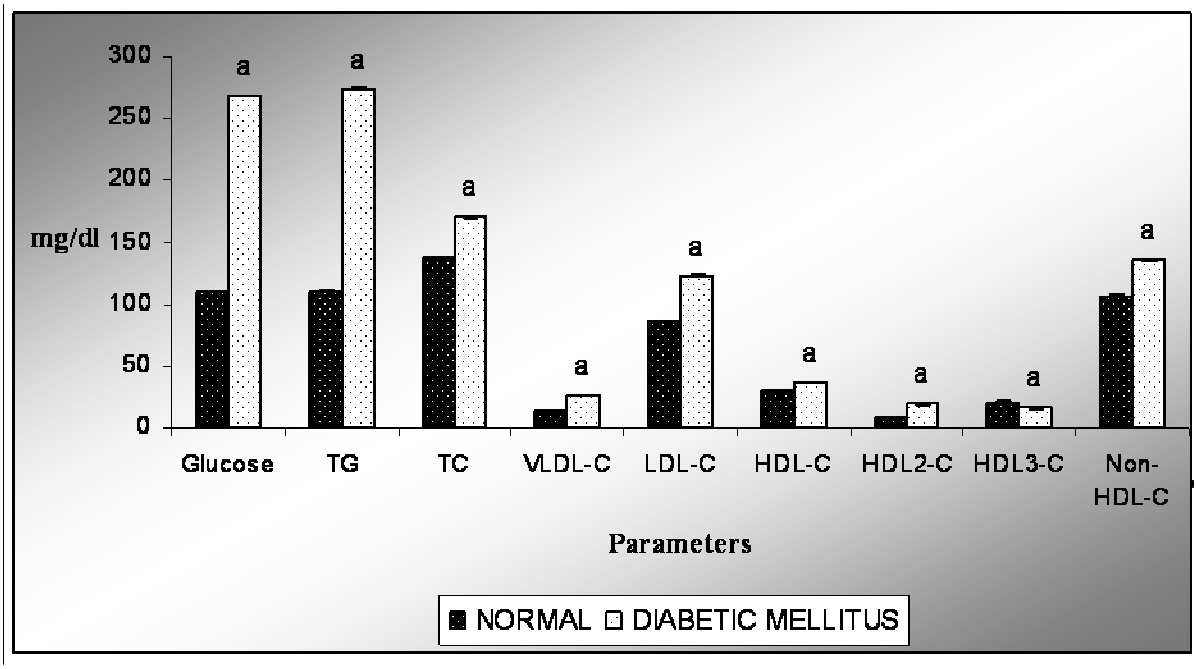

Figure 1

Measurement of glucose and lipid profile of Normal and Diabetic mellitus, *Values are means ±S.D. from all groups of subject, TG, triglycerides; TC, total cholesterol; VLDL-C, Very Low Density Lipoprotein; HDL-C, High Density Lipoprotein; and its subfractions HDL_2-C, HDL_3-C; LDL-C, Low Density Lipoprotein; + (increase) - (decrease). Significantly different from Normal Control at $^aP<0.001$

TABLE 2: THE RATIO OF LDL-C/HDL-C, LDL-C/TC AND TC/HDL-C IN NORMAL AND TYPE II DIABETIC PATIENTS

Parameters	Normal (mg/dl)	Diabetes Mellitus (mg/dl)
LDL-C/HDL-C	2.83±0.039*	3.36±0.12* (+18.6%)[a]
LDL-C/TC	0.63±0.029*	0.72±0.03* (+15.8%)[a]
TC/HDL-C	4.60±0.21*	4.64±0.23* (+0.94%)[e]

*Values are means ±S.D. from all groups of subject. Significantly different from Normal Control at $^aP<0.001$, ^eP is not significant.

Measurement of Total Antioxidant Power (TAP) in different *B. diffusa* extracts: The three *Boerhaavia diffusa* extracts were experimented for the total antioxidant power or FRAP (Ferric Reducing Ability of Plasma/Proteins) as shown in Fig. 2. Ethanol extract showed least TAP values, moderate values for Methanol extract while Aqueous extract showed maximum ferric reducing ability of proteins. The root and aerial parts of

chloroform and aqueous extract of *B. diffusa* was conducted to study the antidiabetic activity in rats to provide scientific evidence for its traditional usage in the control of Diabetes [37]. The results showed by the aqueous extract of roots exhibited more protection to serum parameters compared to ethanol extract [17]. Ethanol extract of B.diffusa has been widely studied for demonstration of immunosuppressive potential [13], antilymphoproferative activity [38].

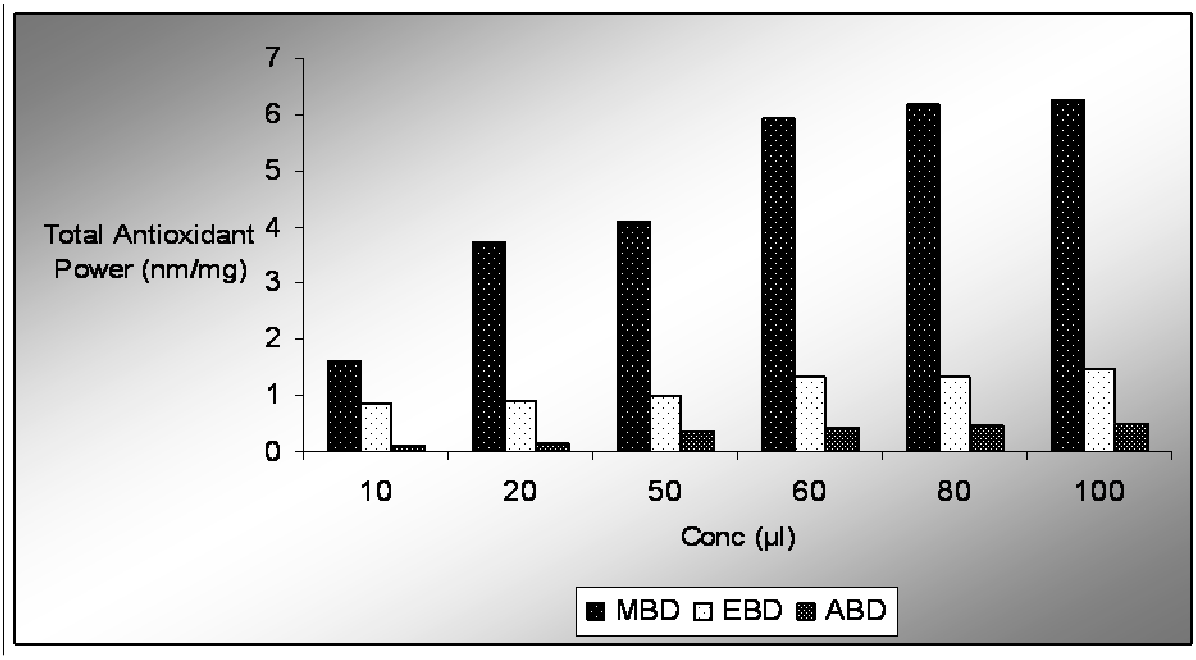

Figure 2
Measurement of Total Antioxidant Power (TAP) in different *Boerhaavia diffusa* extracts, TAP, Total Antioxidant Power; MBD, Methanol Extract of *Boerhaavia diffusa*; EBD, Ethanol Extract of *Boerhaavia diffusa*; ABD, Aqueous Extract of *Boerhaavia diffusa*. +, increase.

Measurement of FRAP of Normal and Diabetes Mellitus Type II Patients: The results shown in Fig. 3 for the measurement of FRAP for LDL-C, HDL-C, HDL_2-C, HDL_3-C, VLDL-C to compare between normal and diabetic individuals show significant decrease in Total Antioxidant Power for the latter. It signifies that the lipid content for diabetic patients have reduced total antioxidant power. Similar analysis for cholesterol and lipoprotein profile in Diabetic rats showed decrease in value compared to normal rats [39]. Glucose, Total cholesterol, Total triglycerides, HDL, LDL was studied in control and

Diabetic Mellitus type II humans while glucose level in blood increase the other clinical characteristics depicted significant decrease in respective values [40].

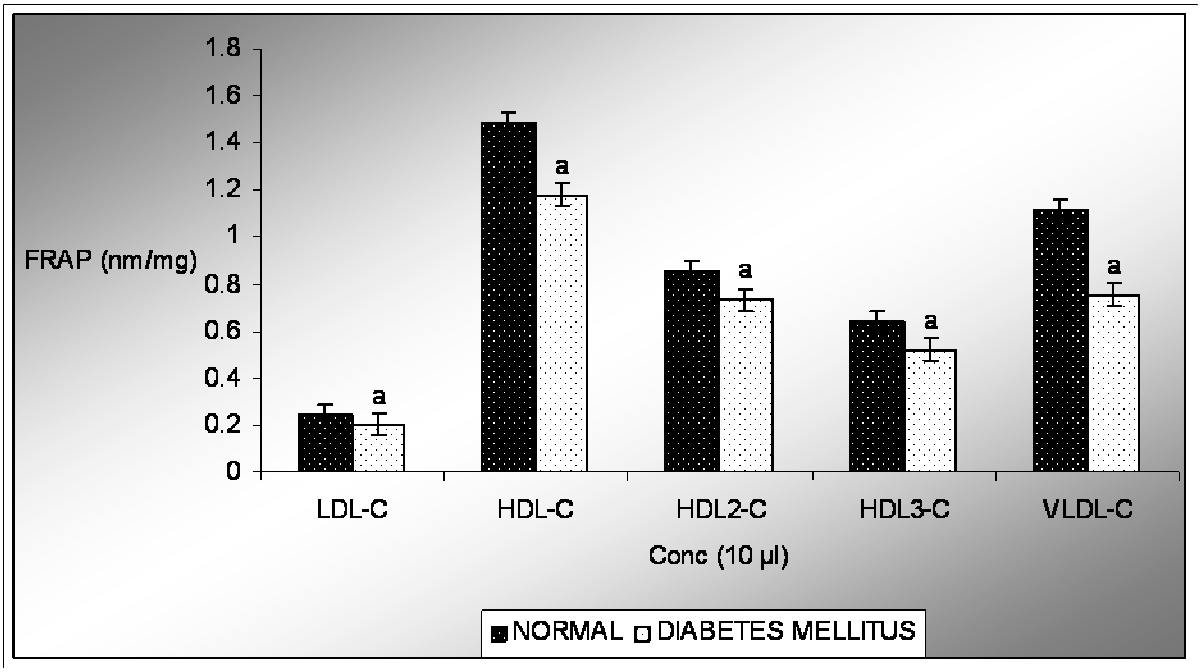

Figure 3

Measurement of FRAP of Normal & Diabetes Mellitus Type II, *Values are means ± S.D. from all groups of subject, FRAP, Ferric Reducing Activity of Plasma; TAP, Total Antioxidant Power; VLDL-C, Very Low Density Lipid C; HDL-C, High Density Lipid C; LDL-C, Low Density Lipoprotein; - (decrease). Significantly different from Normal Control at $^{a}P<0.001$

***In-vitro* impacts of *Boerhaavia diffusa* on TAP in Plasma:** The invitro impact of *Boerhaavia diffusa* on Total antioxidant power of the plasma as seen in Table 3 for the normal & diabetic individual before and after the treatment have shown results similar to the ones expected. On comparing the TAP for plasma without *Boerhaavia diffusa*, in Normal & Diabetic patients showed decrease for the diabetic values. Similarly TAP for plasma without *Boerhaavia diffusa* in Normal & Diabetic patients shows increase in value compared to individuals control condition as well as between them. Thus, it clearly denotes that *Boerhaavia diffusa* is capable of showing the antioxidant properly to give promising results. The results were supported by similar experimental trial on alloxan induced diabetic rats [41]. The results obtained showed similarity with the study done by Chude [42], and Baily [43].

TABLE 3: *IN-VITRO* IMPACTS OF *BOERHAAVIA DIFFUSA* ON TAP IN PLASMA

Conc. (µl)	TAP (nm/mg)			
	Normal		Diabetes mellitus type-II	
	Without BD	With BD	Without BD	With BD
10	0.839±0.055*	1.001±0.008* (+19.32%)[a]	0.807±0.108*	1.319±0.021* (+63.3%)[a]
20	0.699±0.046*	0.703±0.073* (+0.42%)[e]	0.573±0.046*	1.108±0.017* (+93.4%)[a]

*Values are means ± S.D. from all groups of subject, TAP, Total Antioxidant Power; BD, *Boerhaavia diffusa*. Significantly different from [a]P<0.001 and [e]P is not significant.

***In vitro* antioxidant impact on Conjugated Diene (CD) and Malondialdehyde (MDA) formation in LDL:** The *in-vitro* impact of *Boerhaavia diffusa* on Total Antioxidant Power of the plasma as seen in Table 4 for the normal & diabetic individual before and after the treatment have shown results similar to the ones expected. On comparing the TAP for plasma without *Boerhaavia diffusa* in Normal & Diabetic patients showed decrease for the Diabetic values. Similarly TAP for plasma without *Boerhaavia diffusa* in Normal & Diabetic patients shows increase in value compared to individuals control condition as well as between them. Thus, it clearly denotes that *Boerhaavia diffusa* is capable of showing the antioxidant properly to give promising results. The results were supported by similar experimental trial on alloxan induced diabetic rats [41]. *In vitro* antioxidant impact on conjugated diene and malondialdehyde of Normal & Diabetic patients is shown in the Table 4. Comparing CD & MDA formation between Normal & Diabetic patients shows increase in the case of Diabetic patients for both parameters comparing at each level of oxidation. However the general trend seen for all cases of normal observed for CD or MDA and Diabetic individual observed for CD or MDA after 4hrs for CuS04 addition shows significant increased value for all and after the addition of BD for the next 4 hrs, all the four cases showed decrease in CD or MDA formation compared to the case of no addition of *Boerhaavia diffusa.*

TABLE 4: IN-VITRO ANTIOXIDANT IMPACT ON CONJUGATED DIENE (CD) AND MALONDIALDEHYDE (MDA) FORMATION IN LDL

Conc. (µl)	Incubation time (32°C) in hrs	CD formation (nmole/mg protein)		MDA formation (nmole/mg protein)	
		Normal	DM	Normal	DM
10	0	180.21±0.003*	240.36±0.04* (+32.66%)	0.366±0.003*	436±0.002* (+26.10%)
10	CuS04 (4 hrs)	270.36±0003* (+50.02%)[a]	311.39±0.03* (+31.12%)[a]	786±0.003* (+109.12%)[a]	2032±0.002* (+96.79%)[a]
10	CuS04+ BD (4 hrs)	210.11±0.04* (-22.28%)[a]	270.15±0.03* (-13.18)[b]	0.168±0.01* (-74.11%)[a]	0.732±0.001* (-31.42%)[a]

*Values are means ± S.D. from all groups of subject, CD=Conjugated dienes, MDA=Malondialdehyde, + (increase), - (decrease). Significantly different from [a]P<0.001 and [b]P<0.05

CONCLUSION

Boerhaavia diffusa mediated multiple therapeutic benefits described in the present study that daily intake of *Boerhaavia diffusa* by Diabetes Mellitus patients maybe useful in the prevention and treatment of Diabetes-induced hyperlipidemia, Cardiovascular disease, Coronary heart disease and atherosclerosis. In addition, daily use of *Boerhaavia diffusa* will be efficacious and cost effective and good resource of natural antioxidant.

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