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Antioxidant impacts of tocotrienols on copper-mediated *in-vitro* oxidative modification of low density lipoprotein in normal and type II diabetic patients

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ABSTRACT

Cardiovascular diseases (CVD) are the main cause of disability and premature dead worldwide. Epidemiological studies have suggested a link between atherosclerosis and diabetes. Atherosclerosis is a multifaceted diseases process with several different well defined risks factors, such as hypercholesterolemia, hypertension and diabetes. In this study we investigated the efficacy of antioxidant agent , tocotrienols (T3) by analyzing all the parameters in plasma, TC, VLDL-C, LDL-C, HDL-C, HDL2-C, HDL3-C, LDL-P, HDL-P, VLDL-P, TBARS, MDA and in vitro oxidizability of LDL in absence and presence of tocotrienols on normal and Type II diabetic patients. In this study, The in-vitro impact of tocotrienols on Total Antioxidant Power of the plasma 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 tocotrienols in Normal & Diabetic patients showed decrease for the Diabetic values. Similarly TAP for plasma without tocotrienols in Normal & Diabetic patients shows increase in value compared to individuals control condition as well as between them. Thus, it clearly denotes that tocotrienol is capable of showing the antioxidant properly to give promising results. On the other hand, Tocotrienols treatment significantly blocked the increase in plasma Conjugated Diene formation and MDA to a level close to corresponding normal values. In conclusion, Tocotrienols mediated multiple therapeutic benefits described in this study supports that daily intake of T3 as dietary supplement by DM maybe useful in the prevention and treatment of DM including hyperlipidemia and atherosclerosis. In addition, daily intake of dietary tocotrienols will be efficacious and cost efective and a good source of Vitamin E.

Key Words: dietary tocotrienols, Conjugated Diene, MDA, LDL oxidation, Diabetic Mellitus

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INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by high blood sugar mainly glucose level that results from defects in insulin secretion, or action or both. Diabetes is associated with symptoms of polyurea, polydipsia, poltphagia, hyperglycemia, fatigue etc. there are two types of DM: Type I and Type II. Type I also known as insulin dependent DM (IDDM) occurs in non obese people before 30 years of age due to failure of beta cells to respond to normal stimuli being an auto immune disease. Type II also known as Non Insulin Dependent DM

(NIDDM) occurs after 40 years of age and obesity is the major factor. Diabetes is a major risk factor for development of atherosclerosis and is associated with coronary and peripheral vascular diseases [1]. Several studies have established that in both men and women DM is a major independent risk factor for cardiovascular disease (CVD) [34-36]. Hyperglycemia is the most important factor in the onset and progress of diabetic complications mainly by producing oxidative stress [37]. Altered cellular metabolism caused by hyperglycemia play an important role in increasing the risk of cardiovascular, renal, ophthalmic and neurological complications of DM [38]. Although blood glucose is known to be highly predictive of microvascular disease, the contribution of all the measured risk factors can explain no more than 25 % of the excess macrovascular coronary heart disease (CHD) associated with diabetes

Tocotrienols (T3) are the primary source of Vitamin E in the seed endosperm of monocots including agronomically important cereal grains such as wheat, rice and barley. Palm oil contains significant quantities of tocotrienol [2]. Tocotrienol was first reported by Pennock and Whittle in 1964 isolated from rubber. The identification of γ-tocotrienol as a choliesterogenesis inhibitory factor derived from barley represents a landmark early discovery highlighting the unique significance of tocotrienols in health and diseases.In this study we investigated the efficacy of antioxidant agent , tocotrienols by analyzing all the parameters in plasma, TC, VLDL-C, LDL-C, HDL-C, HDL2-C, HDL3-C, LDL-P, HDL-P, VLDL-P,TBARS,MDA and *invitro* oxidizability of LDL in absence and presence of tocotrienols on normal and Type II diabetic patients.

MATERIALS 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 was supplied as a gift from CAROTECH BHD, Chemor, Malaysia.

Estimation: Fractionation of Plasma lipoprotein such as LDL [3], HDL and its fractions-HDL2, HDL3 [4], Plasma FRAP [5], determination of triglyceride and total cholesterol in liver homogenate [6], activities of antioxidant enzymes such as Catalase [7], Superoxide dismutase [8], Glutathione peroxidase [9] and Glutathione reductase [10] in liver homogenate were measured by following known procedures.

Experimental Design: The research was carried out at the Department of Biotechnology and Pharmaceutical Chemistry, UCST, Dehradun. Diabetic patients and Normal control subjects were recruited from Dr. Chhabra Pathology Lab, Dehradun. Informed consent from study enrollment was obtained from each of the study subject. All the subjects where ethnically 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 sub fractions, HDL2-C and HDL3-C.

Collection of Blood and Plasma: At the end of the experiment treatment, overnight fasted Diabetic patient's blood drawn in each group and 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, liquated and either stored at 4o C or frozen at -20o C for use in other experiments.

Statistical evaluation: This was done by employing two-tailed Student t-test as described by Bennet and Franklin [11]. P value less than 0.02 were considered significant.

RESULTS AND 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.No significant comparison can be made in these parameters for diabetic patients when compared to their corresponding values of normal subject.

TABLE 1: Measurement of physiological parameter of normal & diabetes type ii individuals

Parameters	Normal	Diabetes mellitus
Number	12	10
Male	7	6
Female	5	4
Age (yrs)	25±1.64*	51±3.27*
Weight (kg)	54.55±2.15*	65.55±3.25*
Height (cm)	167.66±1.19*	169.63±1.17*
DM history	-	15±2.301*
MDA (25µl)	0.4281±0.031*	0.5824±0.047*

**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. The results depicted in Fig.2 indicate values of lipoprotein which indicates increase in values of LDL-P and VLDL-P while decrease in value of HDL-P for diabetic patients in comparison 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 [12]. In another animal trial blood glucose level increased to a much higher folds for diabetic mice than nondiabetic mice [13]. Therefore, tocotrienols may exert their cholesterol lowering effect in inflammation /infection induced hyperlipidemic rats in a similar manner as previously reported for hyperlipidemic animals [14, 15] and humans[16, 17].

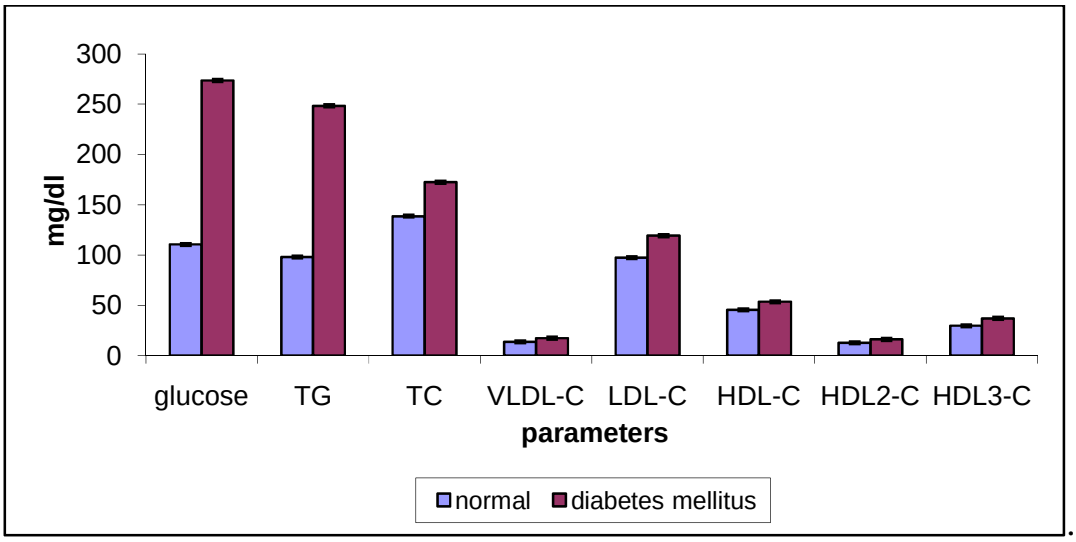

Figure 1. Measurement of glucose and lipid profile of Normal and Diabetic mellitus,
**Values are means ±S.D. from all groups of subject, TG, triglycerides; TC, total cholesterol; VLDLC,Very Low Density Lipoprotein; HDL-C, High Density Lipoprotein; and its subfractions HDL2-C, HDL3-C; LDL-C, Low Density Lipoprotein; + (increase) -(decrease).*

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 [14,18]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 [14], 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 [19]. 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* [19]. 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 [19]. 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 [20]. 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 [21].

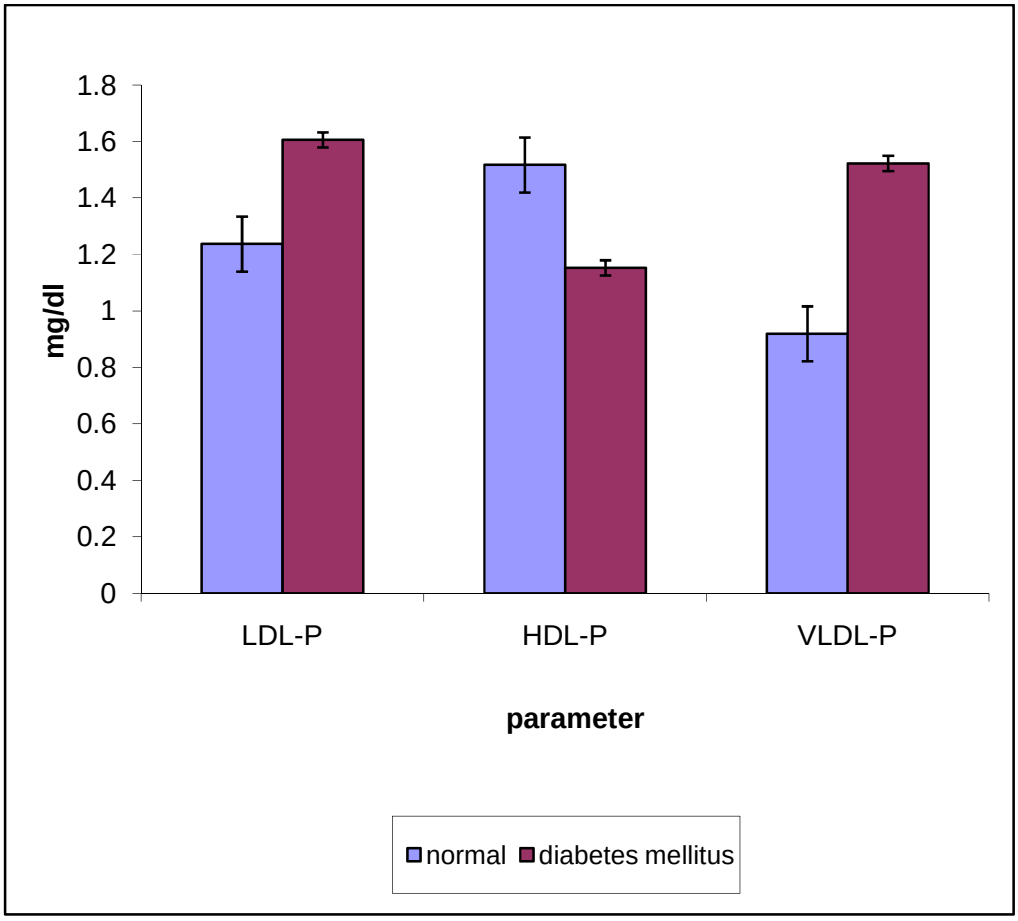

Figure 2. Measurement of lipoproteins of Normal and Diabetic mellitus,
**Values are means ±S.D. from all groups of subject, VLDL-P, Very Low Density Lipoprotein; HDL-P, High Density Lipoprotein; LDL-P, Low Density Lipoprotein; + (increase) -(decrease).*

TABLE 2: The ratio of LDL-C/HDL-C, LDL-C/TC, HDL-C/TC AND HDL_2-C/HDL_3-C in normal and type ii diabetic patients

Parameters	Normal (mg/dl)	Diabetes mellitus (mg/dl)
LDL-C/HDL-C	2.7192±0.37*	3.2382±0.28*
LDL-C/TC	0.6304±0.027*	0.72714±0.084*
HDL-C/TC	0.2319±0.011*	0.2246±0.019*
HDL_2-C/HDL_3-C	0.444±0.017*	1.2065±0.022*

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

Measurement of Total Antioxidant Power (TAP) in Tocotrienol: Fig.3 depicts increase in total antioxidant power due to tocotrienol with increase in concentration.

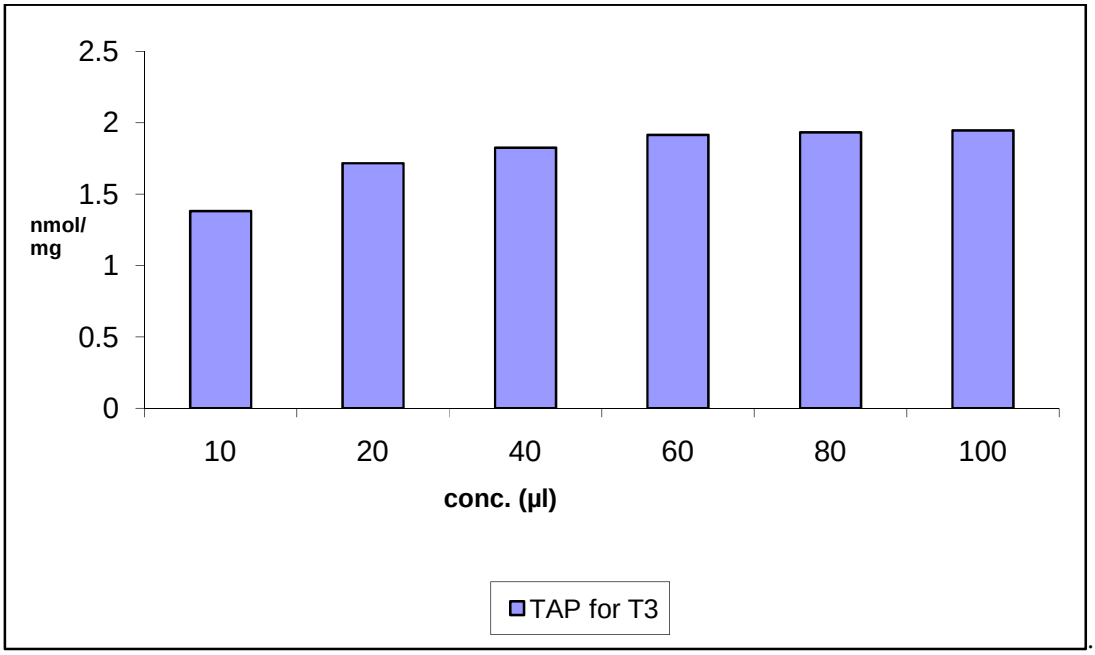

Figure 3. Measurement of Total Antioxidant Power (TAP) in Tocotrienols (T3).

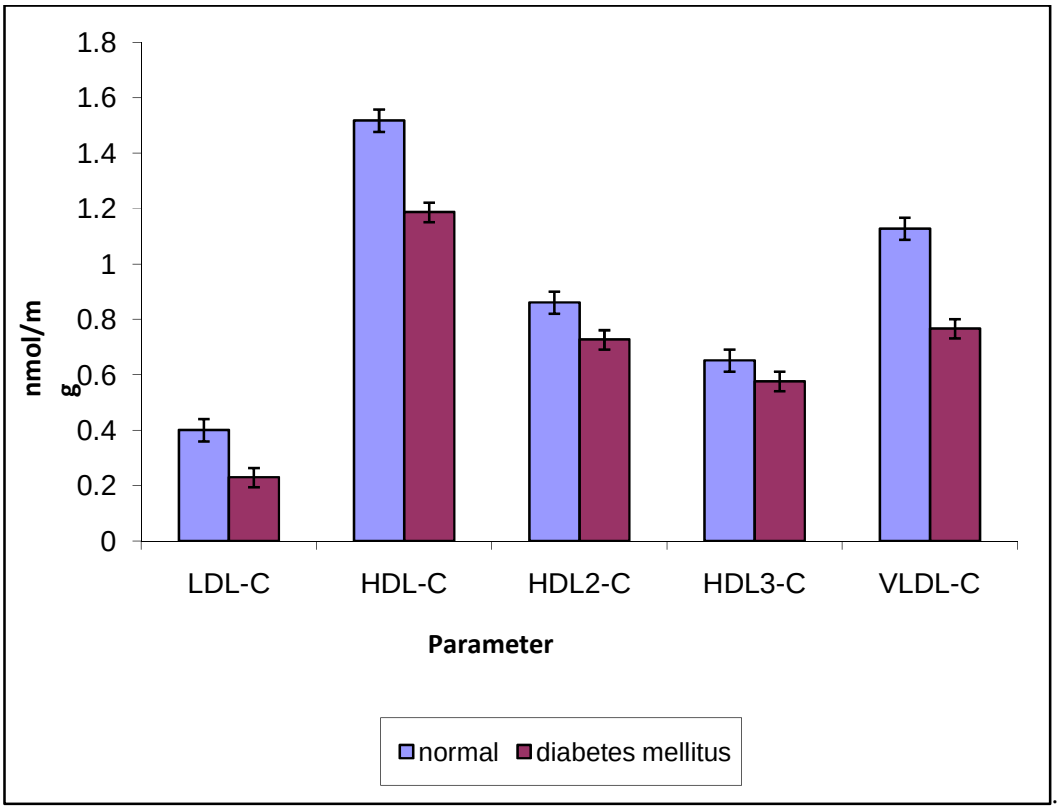

Figure 4. Measurement of FRAP of Normal & Diabetes Mellitus Type II,
**Values are means ± S.D. from all groups of subject , FRAP, Ferric Reducing Activity of Plasma; TAP, Total Antioxidant Power; VLDL-C, Very Low Density Lipid C; HDL-C, High Density LipidC; LDL-C, Low Density Lipoprotein; - (decrease).*

Measurement of FRAP of Normal and Diabetes Mellitus Type II Patients: The results shown in Fig. 4 for the measurement of FRAP for LDL-C, HDL-C, HDL2-C, HDL3-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 [22]. 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 [23].

***In-vitro* impacts of *Tocotrienol* on TAP in Plasma:** The *in vitro* impact of Tocotrienol on Total antioxidant power of the plasma as seen in Fig.5 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 Tocotrienol, in Normal & Diabetic patients showed decrease for the diabetic values. Similarly TAP for plasma without Tocotrienol in Normal & Diabetic patients shows increase in value compared to individuals control condition as well as between them. Thus, it clearly denotes that Tocotrienol is capable of showing the antioxidant properly to give promising results.

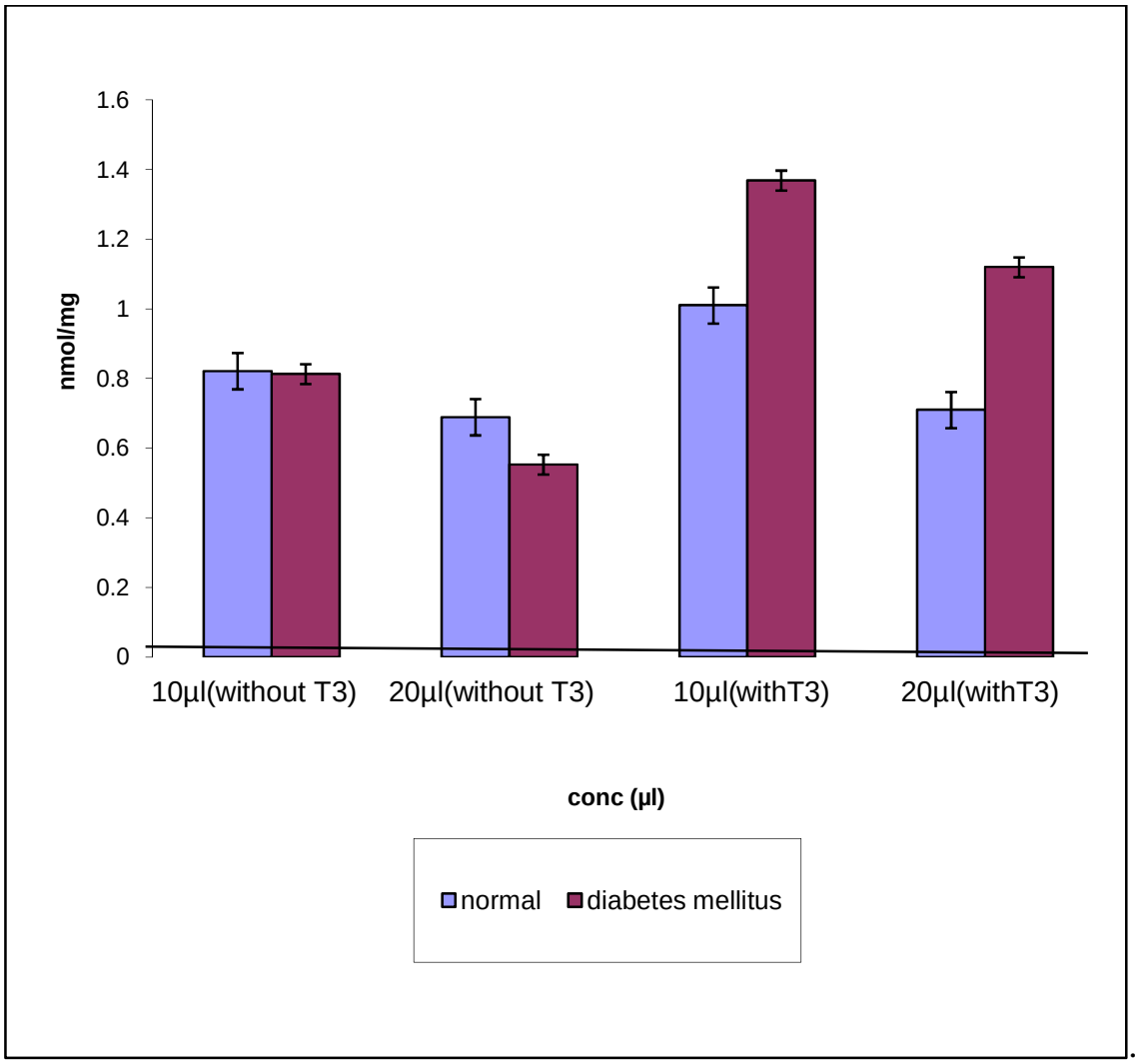

Figure 5. *In vitro* antioxidant effect of tocotrienols on total antioxidant status,
**Values are means ±S.D. from all groups of subject.*

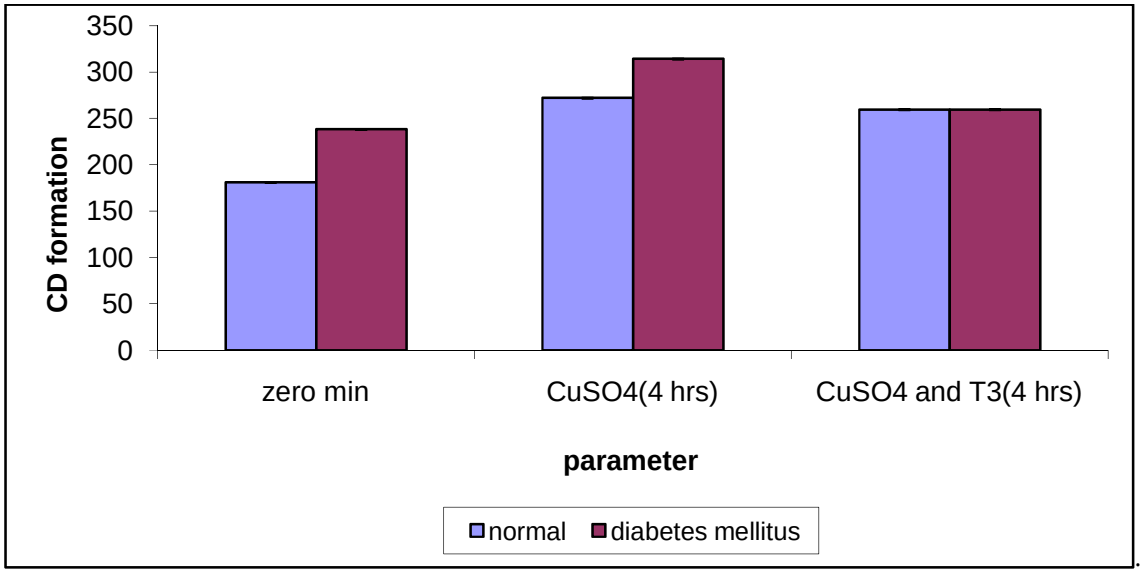

Figure 6. Copper mediated *In vitro* antioxidant impact of tocotrienols on Conjugated Diene (CD) formation in LDL.

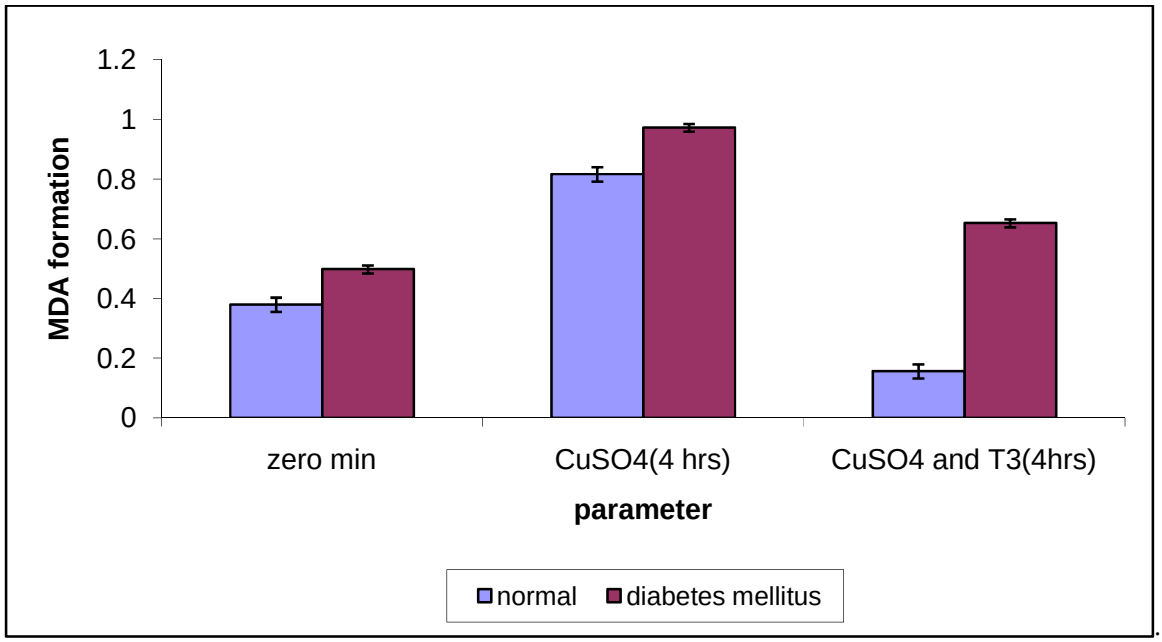

Figure 7. Copper mediated *In vitro* antioxidant impact of tocotrienols on Malondialdehyde (MDA) formation in LDL.

***In vitro* antioxidant impact on Conjugated Diene (CD) and Malondialdehyde (MDA) formation in LDL:** The *in-vitro* impact of tocotrienols on Total Antioxidant Power of the plasma as seen in Fig.6, Fig.7 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 tocotrienols in Normal & Diabetic patients showed decrease for the Diabetic values. Similarly TAP for plasma without tocotrienol in Normal & Diabetic patients shows increase in value compared to individuals control condition as well as between them. Thus, it clearly denotes that tocotrienol is capable of showing the antioxidant properly to give promising results. The results were supported by similar experimental trial [24-33].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 tocotrienols. Tocotrienols treatment significantly restored the total antioxidants level and blocked the increase in plasma CD and MDA to a level close to corresponding normal values.

CONCLUSION

Tocotrienols (T3) mediated multiple therapeutic benefits described in this study supports that daily intake of T3 as dietary supplement by DM maybe useful in the prevention and treatment of DM including hyperlipidemia and atherosclerosis. In addition, daily intake of dietary tocotrienols will be efficacious and cost efective and a good source of Vit. E.

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