Anti-atherogenic and Anti-oxidant effect of tocopherol and tocotrienols on Cu^{++} mediated low-density lipoprotein (LDL) oxidation in normallipidemic subjects

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ABSTRACT

Atherosclerosis is a multifaceted diseases process with several different well defined risk factors, such as hypercholesterolemia, hypertension and diabetes. Atherosclerosis is a multifaceted disease process with several different well defined risks factors, such as hypercholesterolemia, smoking, hypertension and diabetes. Oxidative damage to cholesterol component of the Low-Density Lipoprotein (LDL) leads to oxidized LDL by a series of consecutive events. This induces endothelial dysfunction which promotes inflammation during atherosclerosis. Oxidized LDL acts as a trigger to initiate endothelial inflammation leading to atherosclerosis and vascular thrombosis (heart attack and stroke). Modified LDLs are produced during chemical modification that LDLs undergo after synthesis. In this study, Tocopherol and Tocotrienols inhibits the oxidative modification of LDL in normallipidemic subjects by 135% and 115% respectively. It has also been experimentally proved that these extracts are effective in lowering the formation of Malondialdehyde contents in lysate isolated from normallipidemic subjects. In conclusion, based on in vitro study of Tocopherol and Tocotrienols on Total antioxidant power of plasma and LDL, LDL oxidation with copper sulphate and enzymatic activity described in the present study, administration of Tocopherol and Tocotrienols may be useful in the prevention and treatment of dyslipidemia/hyperlipidemia and atherosclerosis. In addition, daily use of dietary Tocotrienols will be efficacious, cost effective, and a good source of vitamin E.

Key Words: Dietary Tocotrienols, Tocopherol, Atherosclerosis, hyperlipidemia, Low-Density Lipoprotein, Malondialdehyde

INTRODUCTION

The body has the ability to produce endogenous antioxidants such as superoxide dismutase, catalase and glutathione peroxidase. Compounds, which have been widely tried from their antioxidant properties, are vitamins such as alpha-Tocopherol [1], and ascorbic acid. Vitamin C doses of between 500mg and 2000mg have produced controversial result. Despite these antioxidative and anti-atherogenic effects, the clinical results of vitamin E supplementation in human subjects varied [1]. The Cambridge Heart Antioxidant Study using alpha-Tocopherol showed that a higher dose of 800 IU daily did not have additional advantage over a dose of 400 IU daily [1]. Cardiovascular diseases (CVD), as a group, are the leading cause of death in the world. Tobacco use is a major known risk factor for CVD
and leads to a high burden of early death and disability. CVD is also the largest contributor to tobacco-related deaths, in terms of absolute numbers. In many countries, deaths due to CVD considerably outnumber cancer-related deaths.

CVD-related deaths, therefore, become the leading form of tobacco-related death in these countries. Cardiovascular diseases accounted for 16.7 million or 29.2% of the total global deaths in 2002, according to the World health report 2003. Around 80% of deaths due to CVD took place in low- and middle-income countries. By 2010, CVD will be the leading cause of death in developing countries [2]. The contribution of developing countries to the global burden of CVD, in terms of disability-adjusted life-years (DALYs) lost, was 2.8 times higher than that in developed countries [3]. India contributed to 17% of the worldwide CVD mortality in 1990 [3]. CVD-related deaths in India are expected to rise from about 3 million in 2000 to 4.8 million in 2020 [4]. By 2020, about 42% of the total deaths in India are projected to be due to cardiovascular causes [5]. During the period 2000-2030, about 35% of all deaths due to CVD in India are projected to occur in the age group of 35-64 years [4]. Tobacco, as a major cause of premature CVD, becomes especially relevant in this context. There is also increasing evidence that the lower-middle class and urban poor are becoming highly vulnerable to CVD as the epidemic advances in India [6]. The social gradient of tobacco consumption in India, which is characterized by higher consumption patterns among the poor, is also relevant to these social dimensions of CVD.

The identification of \( \gamma \) Tocotrienols as a cholieterogenesis-inhibitory factor derived from barley (\textit{Hordeum vulgare}\ L) represents a landmark early discovery highlighting the unique significance of Tocotrienols in health and disease [7]. Palm oil represents one of the most abundant natural sources of Tocotrienols. The distribution of vitamin E in palm oil is 30% Tocopherols and 70% Tocotrienols [7].

The oil palm (E guineensis) is native to many West African countries, where local populations have used its oil for culinary and other purposes. Palm oil is different from other plant and animal oils in that it contains 50% saturated fatty acids, 40% unsaturated fatty acids, and 10% polyunsaturated fatty acids. Because of its high saturated fat content, palm oil has not been very popular in the United States. Palm oil is free of trans-fatty acids and is rapidly gaining wider acceptance by the food industry in the country. Primary applications include bakery products, breakfast cereals, wafers and candies. Rice bran oil provides desmethyl Tocotrienols. These new Tocotrienols are known as desmethyl Tocotrienols [3,4 dihydro-2-methyl-2 (4.8.12-trimethyltrideca-3'(E), 11'-triethyl) -2H-1-benzopyran-6-o1] and didesmethyl Tocotrienols [3,4-dihydro-2-(4,8,12-trimethyltrideca-3'(E),7'(E),11'-triethyl) -2H-1-benzopyran-6-o1] (Qureshi et al, 2000 ). Cereals such as oat, rye and barley contain small amounts of Tocotrienols in them. \( \gamma \)Tocotrienols is the predominant form of Tocotrienols in oat (\textit{Araena satrica}\ L) and barley (56 and 40 mg/kg of dry weight, respectively): \( \beta \) Tocotrienol is the major form of Tocotrienol found in hulled and dehulled wheats (from 33 to 43 mg/kg of dry weight). Steaming and flaking of dehulled oat groats results in moderate losses of Tocotrienols but not of Tocopherols.

\( \gamma \)-Tocopherol transport protein (TTP), responsible to carry \( \alpha \)-Tocotrienols to vital organs has a poorer efficiency to transport Tocotrienols to tissues. Although TTP is known to bind to \( \gamma \)-Tocotrienols with 8.5 fold lower affinity than that for \( \alpha \)-Tocotrienols. Recently it has been noted that oral supplementation of female mice with \( \alpha \)-Tocotrienols restored fertility of TTP knock-out mice suggesting that Tocotrienols was successfully delivered to the relevant tissues and that Tocotrienols supported reproductive function under conditions of \( \gamma \)-tocotrienols deficiency.

Current findings support that oral Tocotrienols (Carotech Inc NI) not only reaches the brain but it does so in amounts sufficient to protect against stroke. Five generations of rats were studied over sixty weeks. Skin, adipose, heart, lungs, skeletal muscle brain, spinal cord, liver and blood were studied. Oral Tocotrienols was delivered to all vital organs. In some tissues, the level of Tocotrienols exceeded that of Tocopherol indicating the presence of an efficient Tocotrienols transport system \textit{in vivo}.

As already discussed, an imbalance between free radicals production and its scavengers in the body can lead to development of many number of diseases. Main cause of development of CVD and atherosclerosis is the oxidative modification of LDL. Oxidative modification of LDL may be a key early step in the pathogenesis of atherosclerosis [8]. Evidence of oxidized LDL has been obtained from arterial walls of animal models of atherosclerosis and of CHD patients [9]. Cigarette smoke contains numerous free radical species that can contribute to the oxidation of LDL \textit{in vitro} [10-12], although data are conflicting [13]. LDL isolated from smokers may be more susceptible to oxidation than LDL from nonsmokers [14-15]. The levels of reactive oxygen species (ROS) are controlled by antioxidant enzymes, SOD, Catalase, Gpx, Gred and non-enzymatic scavengers such as GSH.

In this study, it is tried to demonstrate that the normal human plasma has more chances of oxidative modification of LDL rather than those that are incubated with the drugs (Tocopherol and Tocotrienols) and thus it tells about the
medicinal value of these drugs for cure against CVD, atherosclerosis and certain other disorders. In this study we investigated the efficacy of antioxidant agent (drug) by analyzing all the parameters in plasma LDL, HDL, MDA and in-vitro oxidizability of LDL in absence or presence of extracts of Tocopherol and Tocotrienol.

**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 α-Tocopherol & Tocotrienols drug as well as RBD palm olein were supplied as a gift from CAROTECH BHD, Chemor, Malaysia.

**Collection of blood and packed erythrocytes**

Fresh Human blood sample was collected from the pathology laboratory of Sardar bhagwan Singh Instt., Balawala, Dehradun. Packed erythrocytes hemolysate was prepared as described by [16]. After the separation of plasma, the packed erythrocytes obtained were washed thrice with physiological saline and a portion of washed erythrocytes was lysed in hypotonic (10mM) sodium phosphate buffer, pH 7.4. A portion of the washed packed erythrocytes was stored at 4°C for future use.

**Fractionation of plasma lipoproteins**

The precipitation method described by Wieland and Seidel [17] was used for the isolation of plasma low density lipoprotein (LDL). The precipitation buffer consisted of 64 mM trisodium citrate adjusted to pH 5.05 with 5 N HCL, containing 50,000 IU/L heparin. Before precipitation of LDL, plasma samples and precipitation reagent were allowed to equilibrate to room temperature. One ml of plasma sample was added to 7.0 ml of heparin-citrate buffer containing 50,000 IU/L heparin. Before precipitation of LDL, plasma samples and precipitation reagent were allowed to equilibrate to room temperature. One ml of plasma sample was added to 7.0 ml of heparin-citrate buffer. After mixing with a vortex mixer, the suspension was allowed to stand for 10 min at 22°C. The insoluble LDLS were then sedimented by centrifugation at 1,500 rpm for 10 min at 22°C. The pellet was resuspended in 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9 % NaCl.

The dual precipitation method of Patsch et al. [18] was used for the isolation of high density lipoprotein (HDL), HDL₂ and HDL₃ subtractions. According to this method 100 µl of reagent containing 1.0 g of dextran sulfate dissolved in 100 ml of 0.5 mM MgCl₂ solution was added to 1.0 ml of plasma, and vertexes-mixed for 3 sec. After an incubation period of 10 min at 22°C, the mixture was centrifuged at 1500 rpm for 15 min at 22°C and the supernatant containing HDL was removed. The clear supernatant was used for the analysis of HDL-C as well as for the isolation of HDL₂ and HDL₃ subtractions. For HDL fraction, 100 µl of reagent containing 1.0 g of dextran sulfate dissolved in 100 ml of 1.5 mM MgCl₂ solution was added to 1.0 ml of HDL supernatant, and vertexes-mixed for 3 sec. Following an incubation of 10 min at 22°C, the mixture was centrifuged at 1500 rpm for 20 min at 22°C and the supernatant containing HDL₃ fraction was removed, whereas the HDL₂ precipitate was dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9 % NaCl.

**Determination of plasma cholesterol**

Total cholesterol in plasma, LDL subtractions was determined as described by Annino and Giese [19], with a minor modification. For the determination of cholesterol in plasma and lipoproteins, 0.1 volume of plasma was mixed with 1 volume of isopropanol, allowed to stand for 5 min and centrifuged at 3,000 rpm for 10 min. A suitable aliquot of isopropanol extract was used for cholesterol determination in a total volume of 0.75 ml. To each tube 0.25 ml of 7.03 mM ferric chloride dissolved in glacial acetic acid, was added, mixed instantly, followed by the addition of 0.8 ml of sulphuric acid with thorough mixing. After 5 min, the absorbance was read at 550 nm in a Beckman DU 640 spectrophotometer. The cholesterol content in the samples was determined by using a cholesterol standard.

**Measurement of in vitro Cu²⁺-mediated oxidation of LDL in the absence or presence of Boerhaavia diffusa and Paeonia emodie**

This was performed as described by Esterbauer et al. [20-21]. The in vitro Cu²⁺-mediated susceptibility of isolated LDL to oxidation was assessed by determining the formation of conjugated diene. Prior to oxidation studies LDL samples were dialyzed against 5 mM phosphate buffer saline (PBS), pH 7.4, for 24 h. The incubation mixture contained LDL (3 mg TC/dl) in the absence or presence of Tocopherol and Tocotrienols (1mg/ml respectively). At time zero, the absorbance of lipoprotein samples was taken at 234 nm. Then, lipoprotein samples were mixed with CuSO₄ to a final concentration of 2.5 µM and incubated at 37°C. In one series, at different time intervals of oxidation, 1.0 ml aliquots from LDL incubation mixture were taken out, mixed with 0.5 mM EDTA, pH 7.4, stored at 4°C and used for the assessment of conjugated dienes. The oxidation for LDL was carried out for 60 minutes. The formation of conjugated dienes in each aliquot was measured by monitoring absorbance at 234 nm in a Beckman DU 640 spectrophotometer. Conjugated dienes was calculated by using an extinction coefficient of 2.52 x 10³ M⁻¹ cm⁻¹ and expressed as µmole MDA equivalent per mg LDL protein.
Measurement of plasma “total antioxidant power” (FRAP)
The method of Benzie and Strain [22] was used for measuring the ferric reducing ability of plasma, the FRAP assay, which estimate the “total antioxidant power”, with minor modification. Ferric to ferrous ion reduction at low pH results in the formation of a colored ferrous-tripyridyl triazine complex. The assay was carried out in a total volume of 1.0 ml containing a suitable aliquot of plasma in 0.1 ml and 900 µl of freshly prepared FRAP reagent, prepared by mixing 10.0 ml of 22.78 mM sodium acetate buffer, pH 3.6, 1.0 ml of 20 mM ferric chloride and 1.0 ml of 10 mM 2, 6-triprydyl-4-s-triazine solution prepared in 40 mM HCl. Before starting the reaction, both FRAP reagent and plasma samples were pre-incubated for 5 min at 30°C. Incubation was done for 5 min at 30°C and absorbance was recorded at 593 nm against a reagent blank in spectrophotometer. Ferrous sulphate (1mM) was used as a standard for calculating the “total antioxidant power”.

Determination of Malondialdehyde in erythrocytes
MDA is the most abundant individual aldehyde resulting from lipid peroxidation and its determination by TBA is the most common method of estimating lipid peroxidation [23].

The suspended erythrocytes in the washed blood sample were incubated with Tocopherol and Tocotrienols (50-100 µg/ml separately). 0.2 ml of packed erythrocytes were suspended in 3 ml of PBS. Then 1 ml of this lysate was added to 1 ml of 10% TCA (for precipitation of protein) and centrifuged for 5 minutes at 3,500 rpm. 1ml of supernatant was added to 1 ml of 0.6% TBA in 0.05m NaOH and boiled for 20 minutes at temperature greater than 90°C. After cooling the tubes to room temperature the absorbance of each sample was read against a reagent blank at 532 nm in spectrophotometer. Malondialdehyde was used as a standard (10^{-13} to 10^{-10}) for the calculation of MDA concentration [23].

Activities of antioxidant enzymes

Determination of catalase activity in erythrocytes
Enzymatic activity of catalase in erythrocytes hemolysate and postmitochondrial supernatant (PMS) of liver, lung and kidney was carried out according to the procedure described by Sinha [24]. The reaction was carried out in a total volume of 1.0 ml containing 10 mM phosphate buffer, pH 7.0 and erythrocytes hemolysate equivalent to 22-36 µg hemoglobin 1060-1074 µg liver, 600-648 µg lung and 721-657 µg kidney of PMS protein. After a preincubation of 10 min at 30°C, the reaction was started by the addition of hydrogen peroxide resulting in a final concentration of 100 mM and the tubes were incubated for 5 or 10 min at 30°C. At the end of incubation, the reaction was terminated by the addition of 1.0 ml of potassium dichromate-acetic acid reagent, prepared by mixing 1 volume of 5 % aqueous solution of potassium dichromate with 3 volumes of glacial acetic acid. For zero time control, hydrogen peroxide was added after stopping the reaction. The tubes were heated in a boiling water bath for 15 min, cooled and the optical density was measured at 590 nm against a reagent blank in a Beckman DU 640 spectrophotometer. Liver, lung, kidney and erythrocytes catalase activity was calculated by using a standard hydrogen peroxide calibration curve.

Determination of superoxide dismutase activity in erythrocytes.
Enzymatic activity of superoxide dismutase (SOD) in erythrocytes hemolysate and PMS fraction of liver, lung and kidney was determined by the method as described by Kakkar et al.[25]. The assay mixture containing 52 mM sodium pyrophosphate buffer, pH 8.3, 300 µM nitroblue tetrazolium, 186 µM phenazine methosulphate and erythrocytes hemolysate equivalent to 29-50 µg hemoglobin 1060-1074 µg liver, 600-648 µg lung and 721-657 µg kidney PMS protein was preincubated for 5 min at 30°C and the reaction was initiated by the addition of NADH (780 µM), by incubating the samples for different time intervals at 30°C. The reaction was stopped by the addition of 0.5 ml of glacial acetic acid at 1 min time interval up to 4 min. For zero time control, to the above incubation mixture, 0.5 ml of glacial acetic acid was added prior to the addition of NADH. To each tube including reagent blank 2.0 ml of n-butanol was added, rigorously extracted and centrifuged at 3,000 rpm for 5 min, color intensity of the chromogen in the butanol extract was measured at 560 nm against a reagent blank in a Beckman DU 640 spectrophotometer. SOD activity was calculated in terms of an arbitrary unit, which is defined as the enzyme concentration required inhibiting the chromogen formation by 50 % in one min under the above assay conditions.

Protein estimation
The protein was determined by the method of Bradford [26], using bovine serum albumin as standard. Aliquots of LDL and HDL, were first precipitated with 10 % TCA. The protein pellets were dissolved in 0.5 N NaOH and suitable aliquots were used for protein determination.
RESULTS AND DISCUSSION

Average Value of Age, Weight, Height, Male, Female of Normal Subjects
As shown in Table 1, the average values of body weight, age, height, male and female of normal subjects (n=12) were 60.22 ± 9.21 kg, 28 ± 2.32 year, 166.22 ± 3.11 cm, 8 and 4 respectively.

As shown in Table 2, the average values of TC, VLDL-C, LDL-C, LDL-Apo-B100, HDL-C, HDL₃-C and Non HDL-C in normal lipidemic subjects were 1225 ± 0.025 µg/ml, 0.122 ± 0.015 µg/ml, 0.302 ± 0.060 µg/ml, 6.25 ± 1.25 µg/ml, 0.0906 ± 0.0101 µg/ml, 0.027 ± 0.005 µg/ml, 0.635 ± 0.053 µg/ml and 1.1344 ± 0.0500 µg/ml respectively.

Average Value of MDA, Catalase, SOD and Total Antioxidant Power of Tocopherol and Tocotrienols in Normal Lipidemic Subjects.
As shown in Table 3, the average value of MDA, Catalase, SOD and Total Antioxidant Power of Tocopherol and Tocotrienols in Normal Lipidemic Subjects were 1.73 ± 0.33 µmole/dl, 0.27 ± 0.04 unit/mg/min, 0.289 ± 0.33 unit/mg/min, 2276.85 ± 1.05 µM/ml and 466.01 ± 5.85 µM/ml respectively.

Average Value of TC/LDL-C, LDL-C/TC, HDL₃-C / HDL₂-C and HDL₂-C / HDL₃-C in Normal Lipidemic Subjects.
As shown in Table 4, the average ratio values of TC/LDL-C, LDL-C/TC, HDL₃-C / HDL₂-C and HDL₂-C / HDL₃-C in normal lipidemic subjects were 4.056, 0.246, 0.042 and 23.51 respectively. The values of TC, LDL-C, HDL-C, HDL and HDL₃-C are given in Table 2.

Total antioxidant Power of Tocopherol and Tocotrienols at different Concentrations
As shown in Fig. 1., with an increase in concentration of Tocopherol and Tocotrienols (20-100 µg/ml), the absorbance at 593 nm significantly increased (51.22%-77.80%) proportionally. Range of absorbance in Tocopherol 1.412-2.895 and Tocotrienols 0.204-0.919. On the other hand, as seen in Fig.2., the total free radical scavenging ability of Tocopherol and Tocotrienols (100 µg) were 933.63 µmol/ml and 296.37 µmol/ml respectively. These results demonstrate that Tocopherol is more potent (≥68.25%) natural antioxidant than Tocotrienols.

Total Free Radical Scavenging Property of Plasma Isolated From Normal Subject In Absence & Presence of Tocopherol.
As shown in Fig.3. and Fig.4., in plasma of normal subjects, with an increase in concentration of Tocopherol and Tocotrienols (10-20 µg/ml), the absorbance at 593 nm slightly increased (35.48-47.22%) proportionally. Range of absorbance in Tocopherol 1.412-2.895 and Tocotrienols 0.204-0.919. On the other hand, as seen in Fig.2., the total free radical scavenging ability of Tocopherol and Tocotrienols (100 µg) were 933.63 µmol/ml and 296.37 µmol/ml respectively. These results in vitro studies, demonstrates that Tocopherol is more potent (≥14%) natural antioxidant than Tocotrienols.

Total Free Radical Scavenging Property of Plasma Isolated From Normal Subjects at different Concentrations
As shown in Fig.5., with an increase in concentration of plasma (20-100 µg/ml), the absorbance at 593 nm significantly increased (74.62%) proportionally. On the other hand, as seen in Fig.5., the total free radical scavenging property of plasma of normal subjects were 798.18 µmol/ml at concentration of 20 µg/ml in absence of Tocopherol and Tocotrienols.

Total Free Radical Scavenging Property of LDL Suspension Isolated from Normal Subjects
As shown in Fig.6., with an increase in concentration of LDL suspension (10-20 µg/ml), the absorbance at 593 nm significantly increased (74.62%) proportionally. On the other hand, as seen in Fig.6., the total free radical scavenging property of LDL suspension of normal subjects increased from 612.75 µM/ml to 1494.78 µM/ml as the concentration of LDL suspension increased from 10-20 µg/ml.

In vitro Copper Mediated Oxidative Modification of LDL (at 37°C) Isolated from Normallipidemic Subjects in Absence or Presence of Tocopherol and Tocotrienols
As shown in Table 5., in absence of Tocopherol or Tocotrienols, a significant increase in oxidative modification of LDL was observed from basal value (at 0 minute before oxidation) to maximal value (at 60 minute after adding 0.25 mM CuSO₄) from 213.50 µM/ml to 290.07 µM/ml i.e. increase by 1.3 folds. Also, when the same sample was again subjected to oxidation with 0.25 mM CuSO₄, but this time in presence of Tocopherol and Tocotrienols.
separately (100 µg/ml each), a significant decrease from basal to maximal values were observed (87.72 µM/ml, 123.19 µM/ml and 147.61 µM/ml-134.92 µM/ml respectively) i.e., decrease in oxidative modification of LDL suspension isolated from normolipidemic subjects by Tocopherol and Tocotrienols by 1.4 and 1.0 folds respectively. This experiment suggests that at the concentration of 100 µg/ml, Tocopherol has a better antioxidant property in preventing the oxidative modification of LDL in normolipidemic subjects than Tocotrienols. On the other hand as shown in Fig.7., the absorbance of conjugated diene formed in the sample after oxidation without any plant extract at 234 nm increased from 0.538-0.731 between 0-60 minutes, whereas, in the presence of Tocopherol and Tocotrienols the absorbance in the same sample decreased from 0.272-0.382 and 0.372-0.340 respectively after oxidation from 0-60 minute. These results indicate a strong anti-oxidative protection of LDL by Tocopherol and Tocotrienols 135 % and 115 % inhibition, respectively (Fig.8).

Antioxidant Impact of Tocopherol on Malondialdehyde Contents in Lysate Isolated from Normal Subjects

As shown in Fig.9., in absence of Tocopherol and Tocotrienols, the change in absorbance (ΔO.D.) of lysate isolated from normal lipidemic subjects was 0.011. On the other hand, addition of Tocopherol and Tocotrienols (50µg/ml and 100µg/ml of each) lead to significant decrease in MDA formation (9.1% at concentration of 50µg/ml and 73% at 100µg/ml respectively). Thus, this demonstrates that Tocopherol is more potent than Tocotrienols but only at higher concentration.

Impact of Tocopherol and Tocotrienols on the Activity of Catalase in Lysate Isolated from Normal Subjects

As shown in Fig.10., the specific activity of Catalase enzyme in lysate obtained from normal lipidemic subjects in absence of any Drug was 0.027 unit/mg/min. On the other hand, after the addition of Tocopherol and Tocotrienols (50µg/ml and 100 µg/ml of each) The specific activity of enzyme decreased to 0.0025 unit/mg/min(90.74%) and 0.01 unit/mg/min(63%) (in 50µg/ml and 100 µg/ml of Tocopherol respectively) and 0.0016 unit/mg/min(94%) and 0.0017 unit/mg/min(93.7%) (in 50µg/ml and 100 µg/ml of Tocotrienols respectively).

Superoxide dismutase Activity of Lysate in Presence and Absence of Tocopherol and Tocotrienols

As shown in Fig.11., the Superoxide dismutase Activity from normal lipidemic subjects in absence of any Drug was 0.289 unit/mg/min. On the other hand, after the addition of Tocopherol and Tocotrienols (50µg/ml and 100 µg/ml each) The specific activity of enzyme decreased to 0.21 unit/mg/min (27%) and 0.25 unit/mg/min(13.5%) (in 50µg/ml and 100 µg/ml of Tocopherol respectively) and 0.18 unit/mg/min(38%) and 0.22 unit/mg/min(24%) (in 50µg/ml and 100 µg/ml of Tocotrienols respectively).

Atherosclerosis is a multifaceted diseases process with several different well defined risk factors, such as hypercholesterolemia, hypertension and diabetes. Atherosclerosis is a multifaceted disease process with several different well defined risks factors, such as hypercholesterolemia, smoking, hypertension and diabetes. Although some studies have suggested that specific infectious agents play a direct role in the vessel wall in the formation of atherosclerotic lesions2. Both infection and inflammation are accompanied by a systemic host response known as the acute-phase response (APR). Acute phase response represents a complex reaction of the host that is accompanied by alterations in lipid and lipoprotein metabolism that could be a mechanism for enhanced susceptibility to atherogenesis. In animal models lipopolysachharide (LPS) treatment rapidly increases serum triglyceride (TG), total cholesterol (TC) and LDL cholesterol levels, while exhibited a decrease in antiatherogenic high density lipoprotein (HDL) cholesterol levels1. Atherosclerosis and coronary heart disease have been considered as major health problem worldwide. Abnormalities in lipids and lipoprotein metabolism and impairment of endothelial function have been implicated as the main contributing factors in atherosclerosis and its progression [27].

The clinical presentations of atherosclerosis mainly involve the coronary and carotid arteries, which remain the leading causes of morbidity and mortality in both men and women of all racial groups with Coronary Heart Disease (CHD) the leading cause of death worldwide. Excessive dietary lipids and cholesterol are the major factor of relevance for the development of hypertriglyceridemia and hypercholesterolemia, two important cardiovascular risk factors. Abnormalities in lipid profiles, folate metabolism and other traditional risk factors (e.g., diabetes mellitus and hypertension) play a rather peripheral role and serve to amplify the atherosclerotic process initiated by persistence of infection and inflammation. Infection and inflammation are accompanied by cytokine induced alterations in lipid and lipoprotein metabolism. Of note, inflammatory cytokines are increased and play a pathogenic role in a variety of very common disorders, such as diabetes, obesity, metabolic syndrome, hypertension, chronic heart failure, chronic renal failure, and atherosclerosis. In our base line study of various physiochemical parameters, the average values of physical parameters such as body weight, age, height, number of male and female in normal subjects (n=12) were 60.22 ± 9.21kg, 28 ± 2.32 year, 166.22 ± 3.11 cm, 8 and 4 respectively.
The dyslipidemic profile of diabetics and affected individuals of other lipid abnormalities includes increased levels of plasma triglycerides (TG), total cholesterol (TC), very low density lipoprotein cholesterol (VLDL-C), low density lipoprotein cholesterol (LDL-C) and small dense (sd)-LDL-C, increased glycation of LDL and decreased plasma antiatherogenic high density lipoprotein (HDL) concentrations. Previous reports indicate that altered plasma lipoprotein profile in the excess atherosclerosis associated with DM may be most critical, because at any total cholesterol level, diabetics have 3- to 5-fold higher CAD mortality rates than do non-diabetic subjects. In addition, 80% of all type 2 diabetics will die of an atherosclerotic event. Our data shows that the average values of TC, VLDL-C, LDL-C, HDL-Apo-B100, HDL-C, LDL2-C HDL3-C and Non HDL-C in normal lipidemic subjects were 1.225µg/ml, 0.122µg/ml, 0.302µg/ml, 6.25µg/ml, 0.0906µg/ml, 0.0271µg/ml, 0.635µg/ml and 1.1344µg/ml respectively. It has previously been established that LDL-C/HDL-C and HDL-C/TC ratios are good predictors for the presence and severity of CAD [28]. The average ratio values of TC/LDL-C, LDL-C/TC, HDL2-C / HDL3-C and HDL2-C / HDL3-C in normal lipidemic subjects were 4.056, 0.246, 0.042 and 23.512 respectively.

Oxidative damage to cholesterol component of the Low-Density Lipoprotein (LDL) leads to oxidized LDL by a series of consecutive events. This induces endothelial dysfunction which promotes inflammation during atherosclerosis. Oxidised LDL acts as a trigger to initiate endothelial inflammation leading to atherosclerosis and vascular thrombosis (heart attack and stroke). Modified LDLs are produced during chemical modification that LDLs undergo after synthesis. Modifications take place either in the plasma or in the inner layer of the artery and pertain to either the lipid or the protein fraction induced by hydrolytic or proteolytic enzymes. O2 radicals or other non-enzymatic mechanisms, modifications concern the production of lipoprotein-autoantibody complexes [28-29, 43-51].

A simple, automated test measuring the ferric reducing ability of plasma, the FRAP assay, is presented as novel method for assessing “antioxidant power.” Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyrindyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form [22]. Our experiment shows that total antioxidant power of Tocopherol and Tocotrienols in normal lipidemic subjects were 2276.85µM/ml and 466.01µM/ml respectively demonstrating that Tocopherol is a better antioxidant than Tocotrienols in experimental conditions.

The body has the ability to produce endogenous hepatic antioxidants such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (Gpx) and Glutathione reductase (Gred). In the instance where there are excessive free radicals, the available tissue antioxidants may become depleted, leading to oxidative damage. CAT and SOD, which play an important role in scavenging the toxic intermediate products of incomplete lipid peroxidation. A decrease in the activity of these enzymes, can lead to the excessive availability of superoxides and peroxyl radicals, which in turn generate hydroxyl radicals, resulting in the initiation and propagation of more lipid peroxidation products. It is well known that glutathione (GSH) acts as a reducing agent and plays a vital role in detoxification. It provides antioxidant protection in the aqueous phase of cellular systems, its antioxidant activity is through the thiol group of its cysteine residue. Like ascorbic acid, another important water soluble antioxidant, GSH can directly reduce a number of ROS and is oxidized to GSSG in the process. Liver is viewed as a glutathione-generating site, which supplies the kidney and intestine with other constituents for glutathione resynthesis. Intra-hepatic glutathione is reported to afford protection against liver dysfunction by at least two ways: (i) as a substrate for glutathione peroxidase (Gpx), GSH serves to reduce large variety of hydroperoxides before they attack unsaturated lipids or convert already formed lipid hydroperoxide to the corresponding hydroxyl compounds; (ii) As a substrate of glutathione-S-transferase (GST), it enables the liver to detoxify foreign compounds or other metabolites and to excrete the products, preferably in to bile. Elevated lipid peroxidation products formed may generate a tissue antioxidant/oxidant imbalance that could represent a crucial link between cigarette smoke and atherosclerosis [29,43-51] (Khan et al.). Also, according to our data, the average value of MDA, Catalase and SOD in normallipidemic subjects were 1.73µmole/dl, 0.27unit/mg/min and 0.289unit/mg/min respectively.

Oxidative modification of lipoproteins is believed to play a central role in the pathogenesis of atherosclerosis [30-31]. Because plasma contains several antioxidants [32] and lipoproteins with oxidative damage have been isolated from atherosclerotic lesions [30-31] 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 hypothesis. 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 [33]. 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 shows that the total antioxidant property of Tocopherol and Tocotrienols (20µg) were 1785.05µmol/ml and 1535.1µmol/ml
Atherosclerosis. In addition, daily use of dietary Tocopherol and Tocotrienols may be useful in the prevention and treatment of dyslipidemia/hyperlipidemia and atherosclerosis. In addition, daily use of dietary Tocotrienols will be efficacious, cost effective, and a good source of vitamin E.

In conclusion, based on in vitro study of Tocopherol and Tocotrienols on Total antioxidant power of plasma and LDL, LDL oxidation with copper sulphate and enzymatic activity described in the present study, administration of Tocopherol and Tocotrienols may be useful in the prevention and treatment of dyslipidemia/hyperlipidemia and atherosclerosis. In addition, daily use of dietary Tocotrienols will be efficacious, cost effective, and a good source of vitamin E.
Table 1. Average Value of Age, Weight, Height, Male, Female of Normal Subjects

<table>
<thead>
<tr>
<th>S.NO.</th>
<th>PARAMETER</th>
<th>NORMAL(n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Age</td>
<td>28±2.32year</td>
</tr>
<tr>
<td>2.</td>
<td>Body weight</td>
<td>60.22±9.21kg</td>
</tr>
<tr>
<td>3.</td>
<td>Height</td>
<td>166.22±3.11cm</td>
</tr>
<tr>
<td>4.</td>
<td>Male</td>
<td>8</td>
</tr>
<tr>
<td>5.</td>
<td>Female</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2. Average Value of TC, VLDL-C, LDL-C, LDL-Apo B100, HDL-C, HDL₂-C, HDL₃-C and Non HDL-C in Normal lipidemic Subjects

<table>
<thead>
<tr>
<th>S.No</th>
<th>PARAMETERS</th>
<th>NORMAL VALUES (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total Cholesterol (TC)</td>
<td>1.225±0.025</td>
</tr>
<tr>
<td>2.</td>
<td>Very low density lipoprotein-cholesterol (VLDL-C)</td>
<td>0.122±0.015*</td>
</tr>
<tr>
<td>3.</td>
<td>Low density lipoprotein-cholesterol (LDL-C)</td>
<td>0.302±0.060</td>
</tr>
<tr>
<td>4.</td>
<td>Low density lipoprotein- Apo B100 (LDL-Apo B100)</td>
<td>6.25±0.230</td>
</tr>
<tr>
<td>5.</td>
<td>High Density Lipoprotein Cholesterol (HDL-3)</td>
<td>0.0906±0.0101</td>
</tr>
<tr>
<td>6.</td>
<td>High Density Lipoprotein Cholesterol (HDL₂-C)</td>
<td>0.027±0.0050*</td>
</tr>
<tr>
<td>7.</td>
<td>High Density Lipoprotein-Cholesterol (HDL₃-C)</td>
<td>0.635±0.230*</td>
</tr>
<tr>
<td>8.</td>
<td>Non- High Density Lipoprotein-Cholesterol (Non-HDL-Cholesterol)</td>
<td>1.1344±0.0500*</td>
</tr>
</tbody>
</table>

*Indirectly calculated values.
All values are mean ± S.D. from pooled serum of normal subjects (n=12).

Table 3. Average Value of MDA, Catalase, SOD and Total Antioxidant Power of Tocopherol and Tocotrienols in Normallipidemic Subjects.

<table>
<thead>
<tr>
<th>S.No</th>
<th>PARAMETERS</th>
<th>NORMAL VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.</td>
<td>Measurement of Malondialdehyde in Plasma</td>
<td>1.73±0.033 µM/dl</td>
</tr>
<tr>
<td>10.</td>
<td>Catalase</td>
<td>0.27±0.04 unit/mg/min</td>
</tr>
<tr>
<td>11.</td>
<td>Super Oxide Dismutase</td>
<td>0.289±0.33 unit/mg/min</td>
</tr>
<tr>
<td>12.</td>
<td>Total Antioxidant Power of Tocopherol</td>
<td>2276.85µM/ml</td>
</tr>
<tr>
<td>13.</td>
<td>Total Antioxidant Power of Tocotrienols</td>
<td>466.01µM/ml</td>
</tr>
</tbody>
</table>

All values are mean ± S.D. from pooled plasma of normal subjects (n=12).

Fig.1. Total antioxidant Power of Tocopherol and Tocotrienols at different Concentrations
Fig. 2. Total antioxidant Power of Tocopherol and Tocotrienols.

Fig. 3. Total Free Radical Scavenging Property of Plasma Isolated From Normal Subjects In Absence & Presence of Tocopherol

Table 4. Average Ratio Value of TC/LDL-C, LDL-C/TC, HDL$_2$-C / HDL$_3$-C and HDL$_3$-C / HDL$_2$-C in Normallipidemic Subjects

<table>
<thead>
<tr>
<th>S.No.</th>
<th>PARAMETERS</th>
<th>NORMAL VALUES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>TC / LDL-C</td>
<td>4.056</td>
</tr>
<tr>
<td>2.</td>
<td>LDL-C / TC</td>
<td>0.246</td>
</tr>
<tr>
<td>3.</td>
<td>HDL$_2$-C / HDL$_3$-C</td>
<td>0.042</td>
</tr>
<tr>
<td>4.</td>
<td>HDL$_3$-C / HDL$_2$-C</td>
<td>23.512</td>
</tr>
</tbody>
</table>

The values of TC, LDL-C, HDL$_2$-C and HDL$_3$-C are given in Table 2.
Fig. 4. Total Free Radical Scavenging Property of Plasma Isolated From Normal Subjects In Absence & Presence of Tocotrienols

![Graph showing Total Antioxidant Power of Plasma of Normal Subjects in Absence and Presence of Tocotrienols.](image)

Fig. 5. Total Free Radical Scavenging Property of Plasma Isolated From Normal Subjects at different Concentrations

![Graph showing Total Free Radical Scavenging Property of Plasma Isolated From Normal Subjects at different Concentrations.](image)
Table 5. In vitro Copper Mediated Oxidative Modification of LDL (at 37°C) Isolated from Normal lipidemic Subjects in Absence or Presence of Tocopherol and Tocotrienols

<table>
<thead>
<tr>
<th>LDL Incubation at 37°C</th>
<th>Conjugated diene Formation of LDL Isolated from Plasma (µM/ml)</th>
<th>Increase or Decrease in folds</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minute</td>
<td>213.50 (Basal)</td>
<td>Increase by 1.357</td>
</tr>
<tr>
<td>60 minutes + CuSO₄ (0.25 mM)</td>
<td>290.07 (Maximal) (+26.39%)*</td>
<td></td>
</tr>
<tr>
<td>0 minute</td>
<td>87.72 (Basal) (-143.38%)**</td>
<td>Decrease by 1.40</td>
</tr>
<tr>
<td>60 minutes + CuSO₄ (0.25 mM) + Tocopherol (100µg/ml)</td>
<td>123.192(Maximal) (-135.46%)+ (28.79)*</td>
<td></td>
</tr>
<tr>
<td>0 minute</td>
<td>147.61(Basal) (-44.63%)**</td>
<td>Decrease by 1.09</td>
</tr>
<tr>
<td>60 minutes + CuSO₄ (0.25 mM) + Tocotrienols (100µg/ml)</td>
<td>134.92(Maximal) (-114.99%)+ (8.59 )*</td>
<td></td>
</tr>
</tbody>
</table>

The conjugated diene values are expressed as µM/ml malondialdehyde equivalents/mg protein. Maximal in vitro oxidation of LDL (312.5 µg/ml) alone or in the presence of Tocopherol and Tocotrienols (100 µg/ml) was achieved after 60 minutes of incubation with CuSO₄. **Values are obtained from LDL isolated from pooled plasma of normal subjects. LDL oxidation (312.5µg/ml) was carried out in the absence or presence of Tocopherol and Tocotrienols (100 µg/ml) which was isolated from pooled plasma of normal subjects.

Decrease or increase in folds with respect to corresponding basal value.
*Percent increase or decrease with respect to basal value.
**Percent decrease with respect to basal value of without treatment.
+Percent decrease with respect to maximal value of without treated LDL oxidation.
Fig. 7. Antioxidant Impact of Tocotrienols and Tocopherol in in vitro copper mediated oxidative modification of LDL (at 37°C) Isolated from Normal Subjects

Fig. 8. Percent inhibition of LDL oxidation by Tocopherol and Tocotrienols
Fig. 9. Antioxidant Impact of *Tocopherol* and *Tocotrienols* on Malondialdehyde Contents in lysate Isolated from Normal Subjects.

Fig. 10. Impact of *Tocopherol* and *Tocotrienols* on the Activity of Catalase in Lysate Isolated from Normal Subjects.
CONCLUSION

Oxidative damage to cholesterol component of the Low-Density Lipoprotein (LDL) leads to oxidized LDL by a series of consecutive events. This induces endothelial dysfunction which promotes inflammation during atherosclerosis. Oxidized LDL acts as a trigger to initiate endothelial inflammation leading to atherosclerosis and vascular thrombosis (heart attack and stroke). Modified LDLs are produced during chemical modification that LDLs undergo after synthesis.

Tocopherol and Tocotrienols inhibits the oxidative modification of LDL in normallipidemic subjects by 135% and 115% respectively. It has also been experimentally proved that these extracts are effective in lowering the formation of Malondialdehyde contents in lysate isolated from normallipidemic subjects.

Though these extracts do not show considerable effects in enhancing the catalytic activity of cellular enzymes such as Catalase and Superoxide dismutase. At high concentrations, free radicals can cause structural damage to cells, proteins, nucleic acid, membranes and lipids, which exhibits a displacement towards the oxidative burst from phagocytes in which foreign components like proteins are denatured and destroyed. Many diseases such as rheumatoid arthritis, pulmonary abnormalities, cardiovascular diseases, reproductive disorders, infertility, retinopathy, diabetes, neurodegenerative diseases, nephropathy and cancer. One of the major cause of these diseases is the oxidative stress within the cells which arises due to an imbalance between the level of antioxidants produced and its scavengers. Therefore, Tocotrienols, being a natural antioxidant and cost effective agent, could be utilized in the prevention and treatment of diabetes linked hyperlipidemia without and with CHD and atherosclerosis.

In conclusion, our results indicate that the alleviation of inflammatory conditions and oxidative stress as well as inhibition of LDL oxidation are due to potent free radical scavenging properties of dietary Tocotrienols and, thus, can be used as a dietary supplements in the prevention and treatment of systemic inflammatory process which might induce atherosclerosis

Acknowledgements
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REFERENCES