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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

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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 *γ Tocotrienols* as a cholieterogenesis-inhabitory factor derived from barley (*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'-trienyl) -2H-1-benzopyran-6-o1] and didesmethyl Tocotrienols [3,4-dihydro-2-(4.8,12-trimethyltrdeca-3'(E),7'(E),11-trienyl)-2H-1-benzopyran-6-o1] (Qureshi *et al,* 2000). Cereals such as oat, rye and barley contain small amounts of Tocotrienols in them. γTocotrienols is the predominant form of Tocotrienols in oat(Areana satrica L) and barley (56 and 40 mg/kg of dry weight,respectively): β Tocotrienol is the major form of Tocotrienol found hulled and dehulled wheats (from 33 to 43 mg/kg of dry weight). Steamng and flaking of dehulled oat groats results in moderate losses of Tocotrienols but not of Tocopherols.

γ-Tocopherols transport protein (TTP), responsible to carry α- Tocotrienols to vital organs has a poorer efficiency to transport Tocotrienols to tissues. Although TTP is known to bind to γ-Tocotrienols with 8.5 fold lower affinity than that for α-Tocotrienols. Recently it has been noted that oral supplementation of female mice with α-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 γ-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 *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 *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 oxidiative 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 4^0C 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. After mixing with a vortex mixer, the suspension was allowed to stand for 10 min at 22^0C. The insoluble LDLs were then sedimented by centrifugation at 1,500 rpm for 10 min at 22^0C. 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), HDL_2 and HDL_3 subtractions. According to this method 100 µl of reagent containing 1.0 g of dextran sulfate dissolved in 100 ml of 0.5 mM $MgCl_2$ solution was added to 1.0 ml of plasma, and vertexed-mixed for 3 sec. After an incubation period of 10 min at 22^0C, the mixture was centrifuged at 1500 rpm for 15 min at 22^0C 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 HDL_2 and HDL_3 subtractions. For HDL fraction, 100 µl of reagent containing 1.0 g of dextran sulfate dissolved in 100 ml of 1.5 mM $MgCl_2$ solution was added to 1.0 ml of HDL supernatant, and vertexes-mixed for 3 sec. Following an incubation of 10 min at 22^0C, the mixture was centrifuged at 1500 rpm for 20 min at 22^0C and the supernatant containing HDL_3 fraction was removed, whereas the HDL_2 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* Cu^{++}-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* Cu^{++}-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 $CuSO_4$ to a final concentration of 2.5 µM and incubated at 37^0C. 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 4^0C 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 2.52×10^4 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, 4, 6-tripyridyl-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 30^0C. Incubation was done for 5 min at 30^0C 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 90^0 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^{-12} to 10^{-14}) 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 30^0C. 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 pyrrophosphate 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 30^0C and the reaction was initiated by the addition of NADH (780 µM), by incubating the samples for different time intervals at 30^0C. 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 ± 9.21kg , 28 ± 2.32 year, 166.22 ± 3.11 cm, 8 and 4 respectively .

Average Value of TC, VLDL-C, LDL-C, LDL-Apo-B100, HDL-C, HDL_2-C, HDL_3-C and Non HDL-C in Normallipidemic subjects.
As shown in Table 2, the average values of TC, VLDL-C, LDL-C, LDL-Apo-B100, HDL-C, HDL_2-C ,HDL_3-C and Non HDL-C in normal lipidemic subjects were 1.225
± 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 Normallipidemic Subjects.
As shown in Tabel 3, the average value of MDA, Catalase, SOD and Total Antioxidant Power of *Tocopherol* and *Tocorienols* 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 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-
As shown in Table 4., the average ratio values of TC/LDL-C, LDL-C/TC, HDL_2-C / HDL_3-C and HDL_3-C / HDL_2-C in normal lipidemic subjects were 4.056 , 0.246 , 0.042 and 23.51 respectively. The values of TC, LDL-C, HDL_2-C and HDL_3-C are given in Table2.

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 demonstrates that *Tocopherol* is more potent (≥68.25%) natural antioxidant than *Tocotrienols.*

Total Free Radical Scavenging Property of Plasma Isolated From Normal Subjecst 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* (10-20µg/ml), the absorbance at 593 nm slightly increased (35.48-47.22%) proportionally . Range of absorbance in *Tocopherol* (0.840-1.107) and *Tocotrienols* (0.842-0.952). On the other hand, as seen in Fig.3. and 4, the total free radical scavenging property of *Tocopherol* and *Tocotrienols* (20 µg) in plasma of normal subjects were 1785.05 µmol/ml and 1535.1 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 (20-100µg/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 (10-20µg/ml), the absorbance at 593 nm significantly increased (11.75%) 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 37ºC) Isolated from Normallipidemic Subjects in Absence or Presence of *Tocopherol* and *Tocotrienols
As shown in Table5., in absence of *Tocopherol* or *Tocotriebols*, 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 0.25mM $CuSO_4$) 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 0.25mM $CuSO_4$, 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 normallipidemic 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 normallipidemic 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 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 desmutase 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, LDL-Apo-B100, HDL-C, HDL_2-C ,HDL_3-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, HDL_2-C / HDL_3-C and HDL_3-C / HDL_2-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 ,O ,OH or O^2 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-tripyridyltriazine 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

respectively and hence this experiment suggests that *Tocopherol* is more potent (≥14%) natural antioxidant than *Tocotrienols in vitro* conditions.

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). 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. Recently, Bloomer [33] has shown that young novice smokers (pack-year history of 3±2) have a lower plasma antioxidant capacity and exhibited a greater degree of lipid peroxidation compared to nonsmokers. Our results indicate anti-oxidative protection of LDL by *Tocopherol* and *Tocotrienols* 135% and 115% inhibition, respectively. 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 0.25mM $CuSO_4$) from 213.50 µM/ml 290.07µM/ml i.e. increase by 1.3 folds. But in the presence of *Tocopherol* and *Tocotrienols* (100µg/ml) the increase in LDL oxidation decreased by 1.4 and 1.0 folds respectively. Thus this experiment implies that *Tocopherol* is more potent natural antioxidant than *Tocotrienols* in preventing the *in vitro* copper mediated oxidative modification of LDL. Based on these results, it seems possible that oxygen radicals formed over and above the detoxifying capacity of plasma can be prevented by treatment with these drugs and hence block the peroxidative breakdown of phospholipid fatty acids and accumulation of MDA and therefore membrane damage.

The levels of reactive oxygen species (ROS) are controlled by antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx), glutathione reductase (Gred) and non-enzymatic scavengers such as glutathione (GSH). An impaired radical scavenger function has been linked to decreased activity of enzymatic and non-enzymatic scavengers 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 [35], low levels of ROS are vital for proper cell functioning, while excessive *in vivo* generation of these products can adversely affect cell functioning [36-37]. 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 [38-39]. Increased MDA production in plasma is known to cause a decrease in the membrane fluidity of the membrane lipid bilayer and increased osmotic stability of cells [40]. Our results demonstrate that MDA formation in lysate isolated from normal lipidemic subjects decreased to 9.1% and 73% in presence of *Tocopherol* and *Tocotrienols* (100µg/ml).

The major intracellular antioxidant enzyme, SOD, specifically converts superoxide radicals to hydrogen peroxide, [41]and CAT as well as Gpx detoxifies hydrogen peroxide to water [42]. According to our data, the specific activity of Catalase in lysate isolated from normal lipidemic subjects decreased by 90.74%, 63%, 94% and 93.70% in presence of *Tocopherol* and *Tocotrienols* (50µg/ml and 100µg/ml each) respectively. Also, the specific activity of Superoxide dismutase in lysate isolated from normal lipidemic subjects decreased by 27%, 13.4%, 38% and 24% in presence of *Tocotrienols* and *Tocopherol* (50µg/ml and 100µg/ml each) respectively. These *in vitro* results indicate, our extracts inhibit the enzymatic activity (13.4% - 94%).

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. Reduced glutathione and H_2O_2 are twin substrates for Gpx. Reduced glutathione is formed from its oxidized form, GSSG by the enzyme Gred, which requires NADPH as a cofactor (Meister and Anderson, 1983; Meister, 1992) 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.

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.

Table1. Average Value of Age, Weight, Height, Male, Female of Normal Subjects

S.NO.	PERAMETER	NORMAL(n=12)
1.	Age	28±2.32year
2.	Body weight	60.22±9.21kg
3.	Height	166.22±3.11cm
4.	Male	8
5.	Female	4

Table 2. Average Value of TC, VLDL-C, LDL-C, LDL-Apo B100, HDL-C, HDL_2-C, HDL_3-C and Non HDL-C in Normal lipidemic Subjects

S.No	PERAMETERS	NORMAL VALUES (µg/ml)
1.	Total Cholesterol (TC)	1.225±0.025
2.	Very low density lipoprotein-cholesterol (VLDL-C)	0.122±0.015*
3.	Low density lipoprotein-cholesterol (LDL-C)	0.302±0.060
4.	Low density lipoprotein- Apo B100 (LDL-Apo B100)	6.25±0.230
5.	High Density Lipoprotein Cholesterol (HDL-3)	0.0906±0.0101
6.	High Density $Lipoprotein_2$ Cholesterol (HDL_2-C)	0.0271±0.0050*
7.	High Density $Lipoprotein_3$ Cholesterol (HDL_3-C)	0.635±0.230*
8.	Non- High Density Lipoprotein-Cholesterol (Non-HDL-Cholesterol)	1.1344±0.0500*

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

Table3. Average Value of MDA, Catalase, SOD and Total Antioxidant Power of *Tocopherol* and *Tocotrienols* in Normallipidemic Subjects.

S.No.	PARAMETERS	NORMAL VALUE
9.	Measurement of Malon dialdehyde in Plasma	1.73±0.033 µM/dl
10.	Catalase	0.27±0.04 unit/mg/min
11.	Super Oxide Dismutase	0.289±0.33 unit/mg/min
12.	Total Antioxidant Power of *Tocopherol*	2276.85µM/ml
13.	Total Antioxidant Power of *Tocotrienols*	466.01µM/ml

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

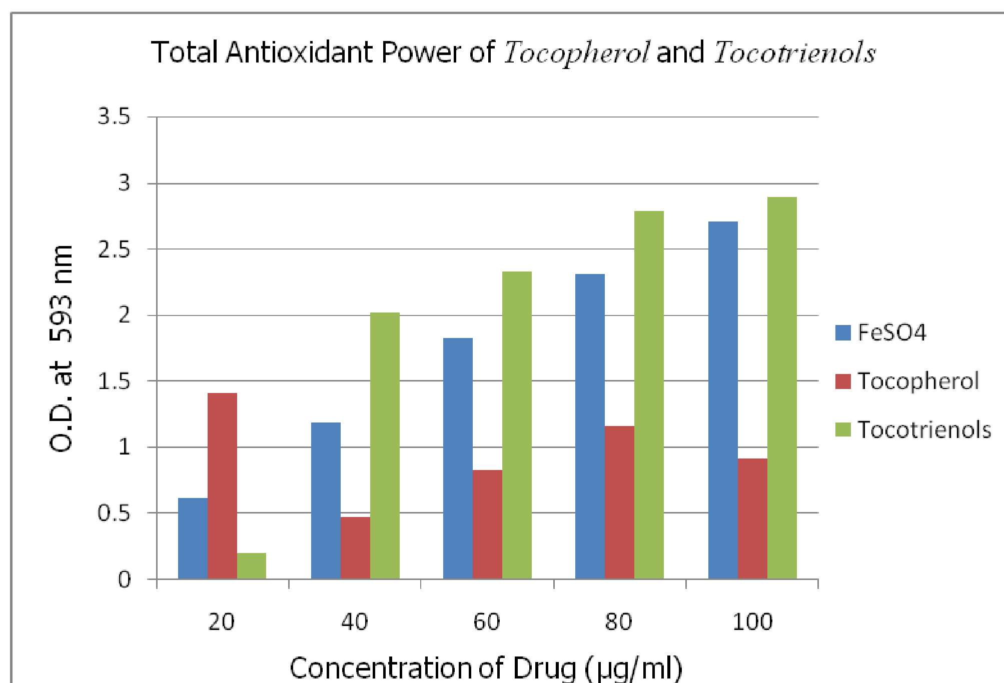


Fig.2. Total antioxidant Power of *Tocopherol* and *Tocotrienols*.

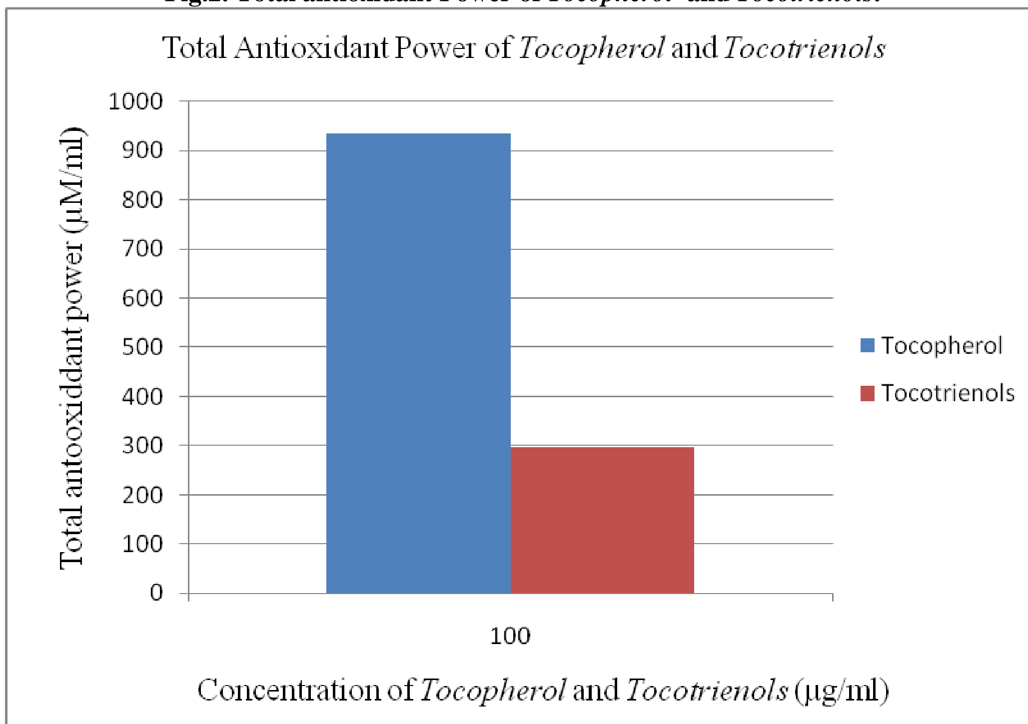


Fig.3. Total Free Radical Scavenging Property of Plasma Isolated From Normal Subjects In Absence & Presence of *Tocopherol*

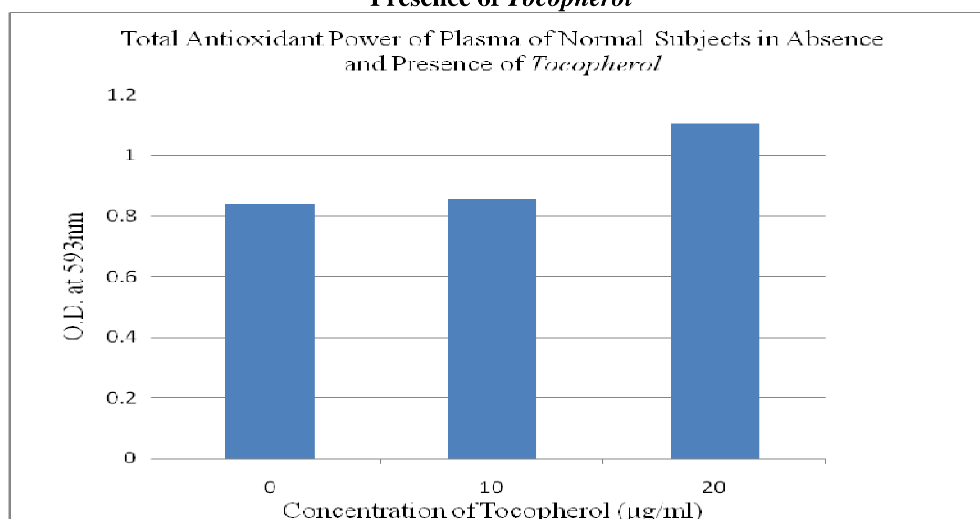


Table4. Average Ratio Value of TC/LDL-C, LDL-C/TC, HDL_2-C / HDL_3-C and HDL_3-C / HDL_2-C in Normallipidemic Subjects

S.No.	PARAMETERS	NORMAL VALUES
1.	TC / LDL-C	4.056
2.	LDL-C / TC	0.246
3.	HDL_2-C / HDL_3-C	0.042
4.	HDL_3-C / HDL_2-C	23.512

The values of TC, LDL-C, HDL_2-C and HDL_3-C are given in Table2.

Fig.4. Total Free Radical Scavenging Property of Plasma Isolated From Normal Subjects In Absence & Presence of *Tocotrienols*

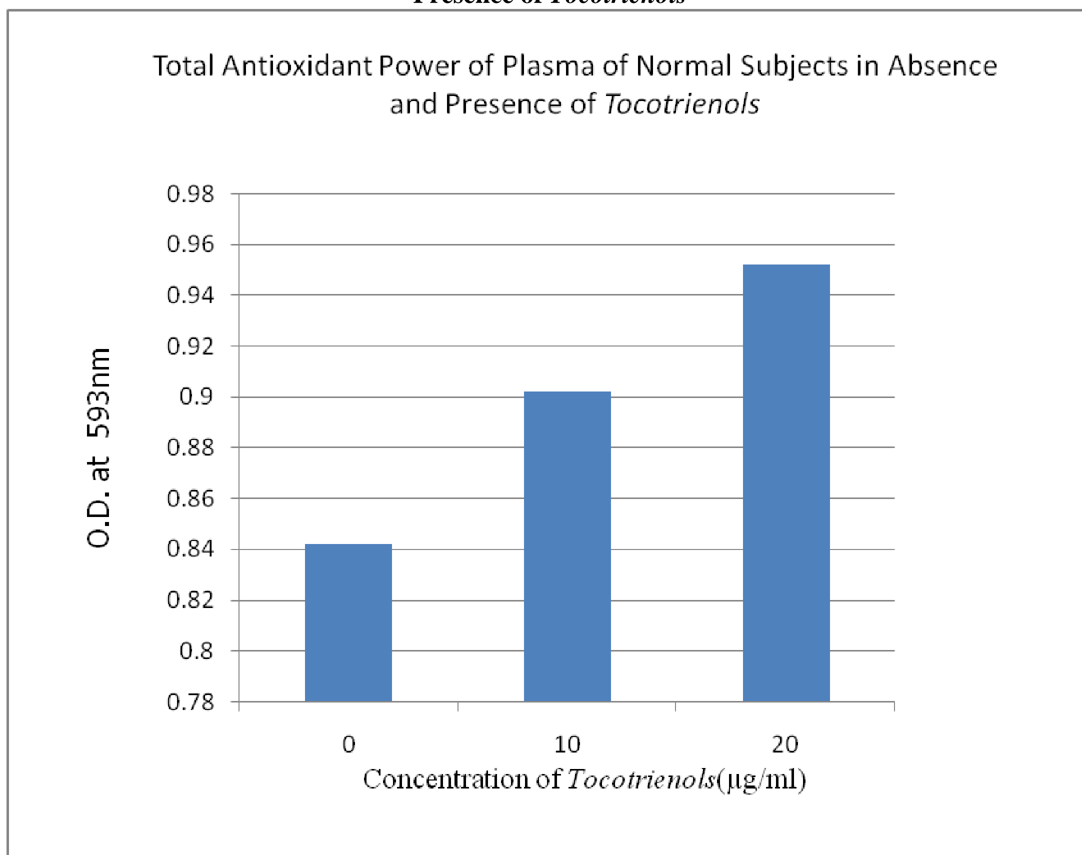


Fig.5. Total Free Radical Scavenging Property of Plasma Isolated From Normal Subjects at different Concentrations

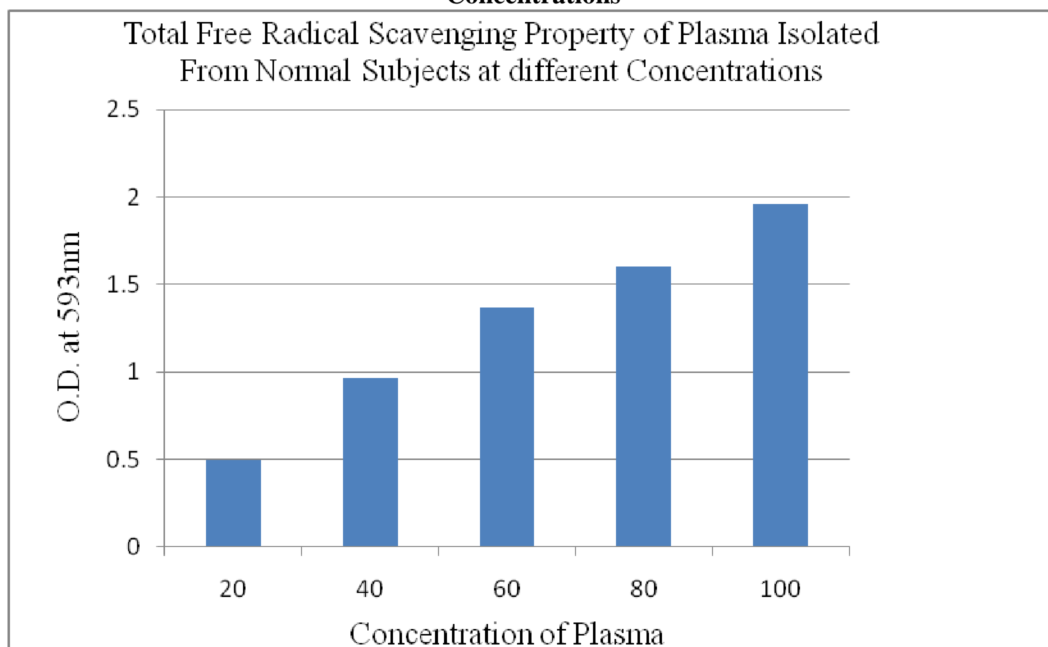


Fig.6. Total Free Radical Scavenging Property of LDL Suspension Isolated From Normal Subjects-

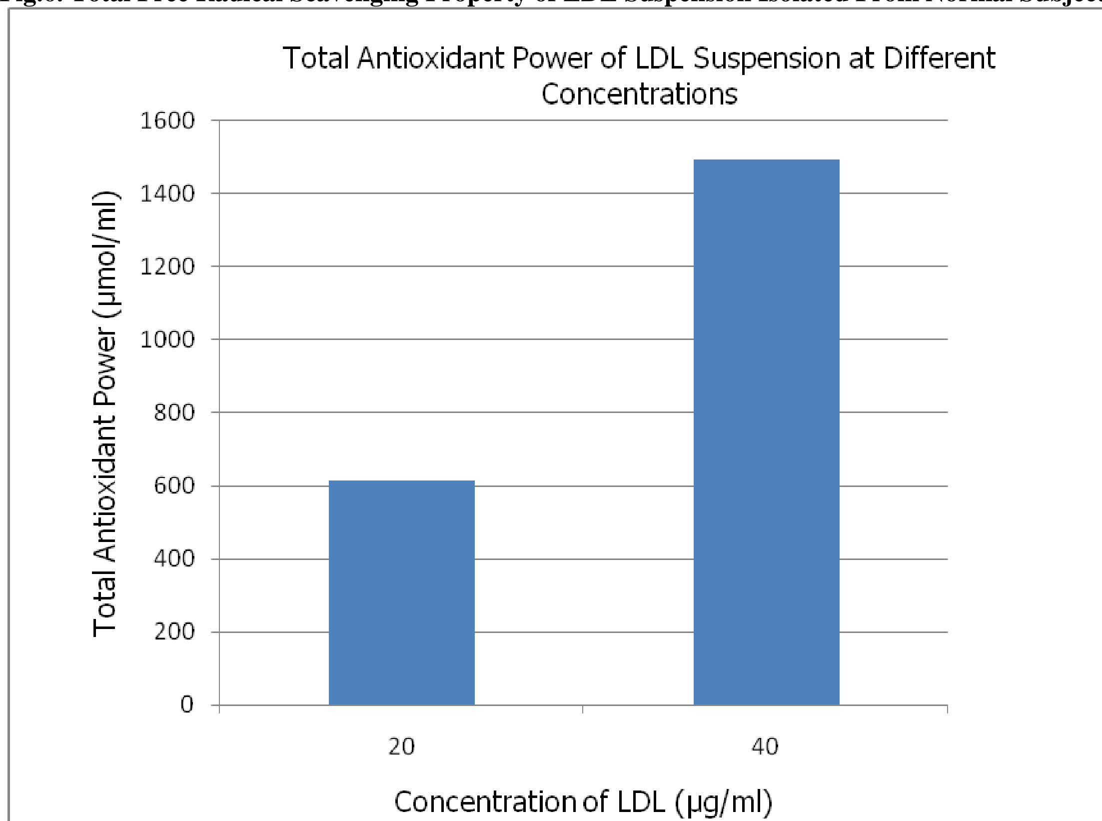


Table5. In vitro Copper Mediated Oxidative Modification of LDL (at 37°C) Isolated from Normal lipidemic Subjects in Absence or Presence of *Tocopherol* and *Tocotrienols*

LDL Incubation at 37^0 C	Conjugated diene Formation of LDL Isolated from Plasma (µM/ml)	Increase or Decrease in folds
0 minute **60 minutes + $CuSO_4$ (0.25 mM)**	213.50 (Basal) 290.07 (Maximal) (+26.39%)*	Increase by 1.357
0 minute **60 minutes + $CuSO_4$ (0.25 mM) + *Tocopherol* (100µg/ml)**	87.72 (Basal) (-143.38%)** 123.192(Maximal) (-135.46)+ (+28.79)*	Decrease by 1.40
0 minute **60 minutes + $CuSO_4$ (0.25 mM)+ *Tocotrienols* (100µg/ml)**	147.61(Basal) (-44.63%)** 134.92(Maximal) (-114.99)+ (-8.59)*	Decrease by 1.09

*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 $CuSO_4$.**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

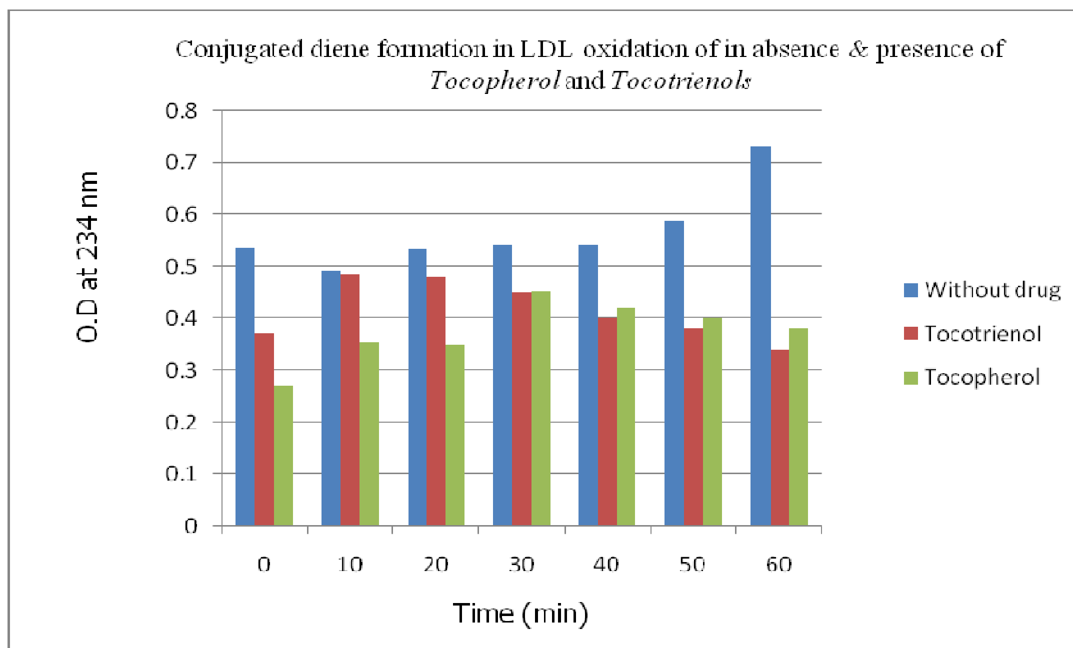


Fig.8. Percent inhibition of LDL oxidation by *Tocopherol* and *Tocotrienols*

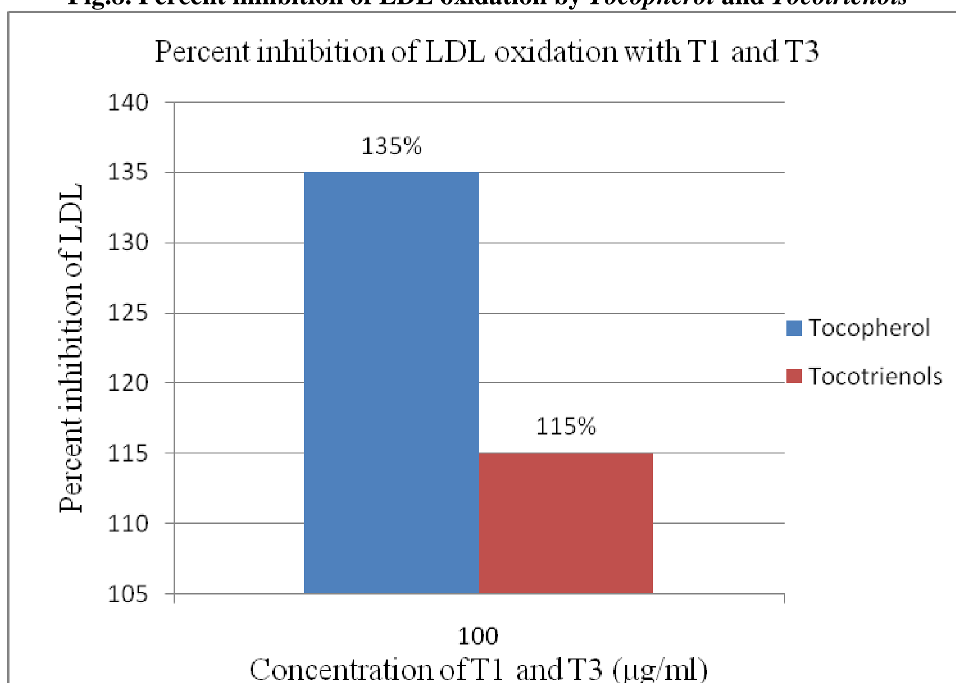


Fig.9. Antioxidant Impact of *Tocopherol and Tocotrienols* on Malon dialdehyde Contents in lysate Isolated from Normal Subjects.

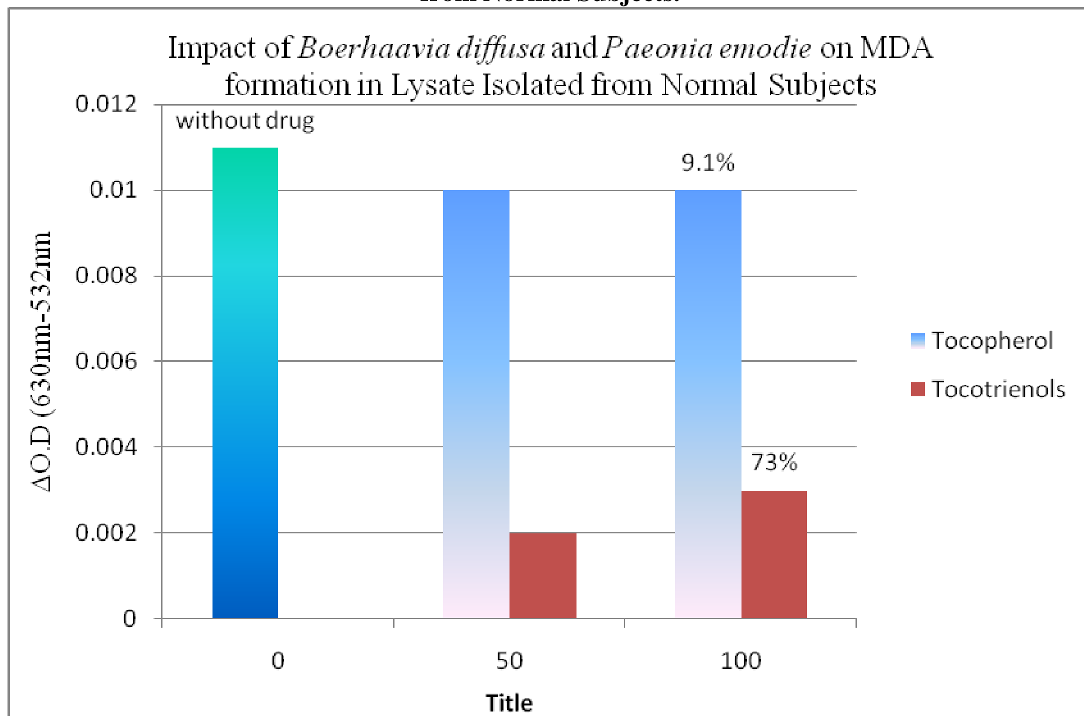


Fig.10. Impact of *Tocopherol* and *Tocotrienols* on the Activity of Catalase in Lysate Isolated from Normal Subjects

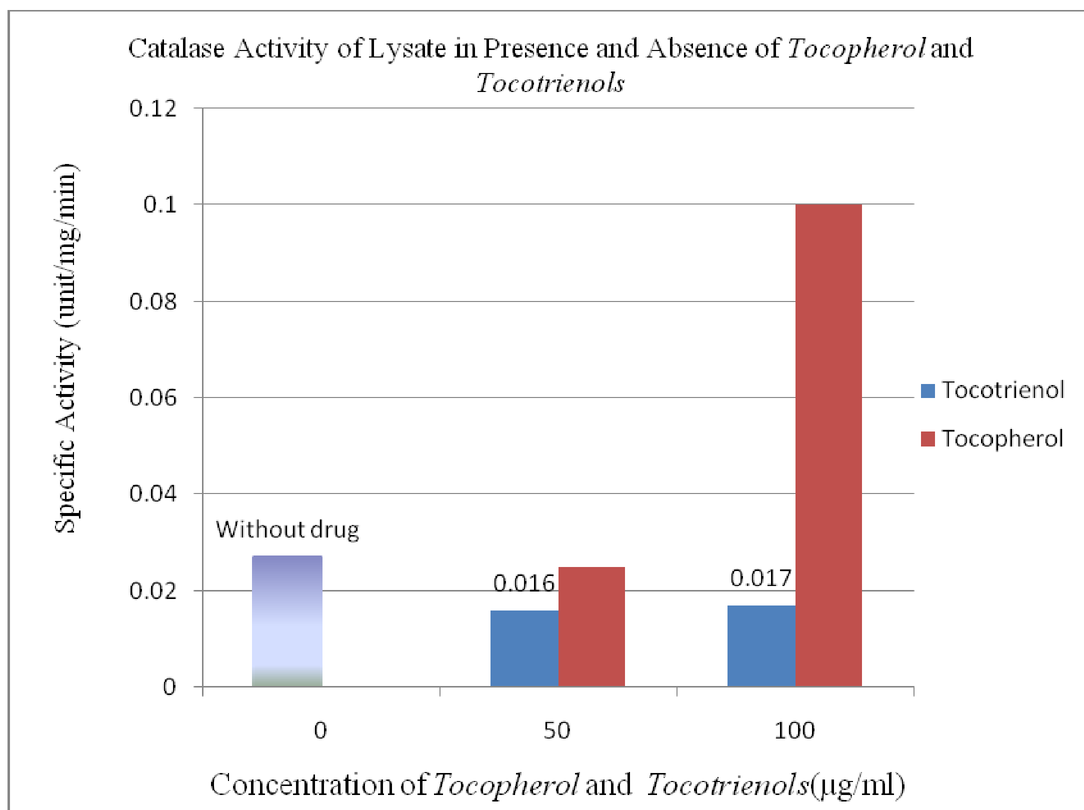
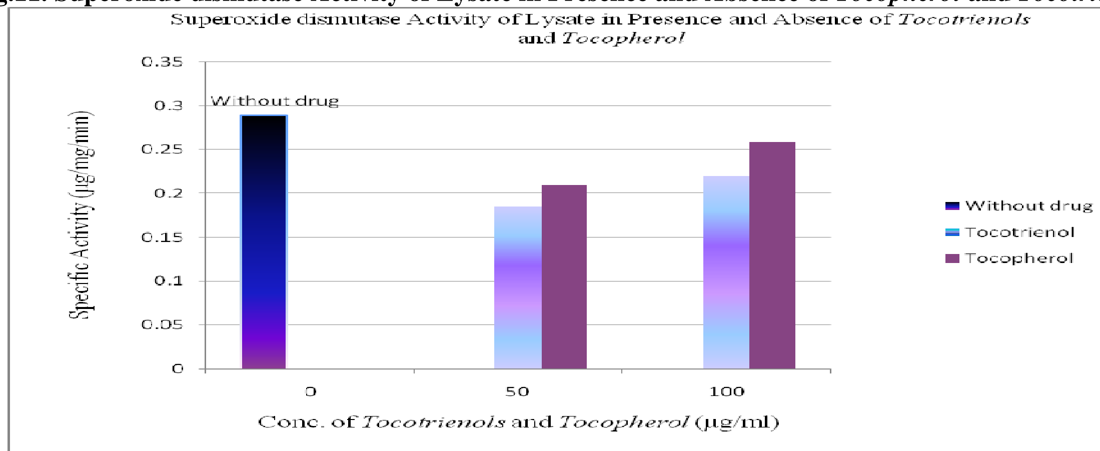


Fig.11. Superoxide dismutase Activity of Lysate in Presence and Absence of *Tocopherol* and *Tocotrienols*



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 rheumatid 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

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REFERENCES

[1] Stephens SG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ, and Brown MJ, *Lancet* . **1996**. 347; 781.
[2] WHO, **2004**. *The World Health Report*. Geneva, Switzerland.
[3] Reddy K S, and Yusuf S, Circulation. 1998. 97; 596-601.
[4] Leeder S, Raymond S, Greenberg H, Liu H, and Esson K. A race against time. **2004**. New York: Columbia University.
[5] Murray CJL, and Lopez AD, **1996**. Harvard School of Public Health. Boston.Vol.1
[6] Reddy KS, **2004**. *N. Engl. J. Med.* 350 ;2438-2440.
[7] Qureshi AA, Mo H, Packer L, and Peterson D M, **2000.** *J. Agri. and food chem.* **48**; 3130-3140.
[8] Diaz MN, Frei B, Vita JA, and Keaney JF, **1997**. *Heart Disease*. 337: 408-416.
[9] Haberland ME, Fong D, and Cheng L, **1988.** *Science*. **241**; 215-218.

[10] Church D and Pryor WA, **1985.** *Environ. Health Perspect*. **64**; 111-126.
[11] Yokode M, Kita T, Arai H, Kawai C, Narumiya S, and Fujiwara M, **1988.** *Proc Natl Acad Sci.* **85** ;2344-2348.
[12] Santanam N, Sanchez R, Hendler S, and Parthasarathy S, **1997.** *FEBS Lett.* **414**; 549-551.
[13] Chen C and Loo G, **1995.** *Atherosclerosis.* **112**; 177-185.
[14] Scheffler E, Huber L, Fruhbis J, Schulz I, Ziegler R, and Dresel HA, **1990.** *Atherosclerosis.* **82**; 261-265.
[15] Gouaz V, Dousset N and Dousset JC, **1998.** *Clin Chim Acta.* **277**: 25-37.
[16] Lakshmi S, and Rajagopal G, **1998**. *Biomedicines*. 18; 37-39.
[17] Wieland H, and Seidel D, **1989**. *Journal of Lipid Research*. 24; 904-909.
[18] Patsch W, Brown SA, Morrisett J D, Gotto JrA M and Patsch JR, 1989. *Clin. Chem.* 35; 265-270.
[19] 19. Annino JS, and Giese RW, **1976**. In Clinical Chemistry. Principles and Procedures, IV Ed. Little, Brown and Company, Boston. 33; 268-275.
[20] Esterbauer H, Gebicki J, Puhl H, and Jugens G, **1992**. *Free Radic. Bio. Med*. 13; 341-390.
[21] Esterbauer H, Striegel G, Puhl H, Oberreither S, Rotheneder M, El Saadani M, and Jurgens G, **1989**. *Ann. N. Y. Acad. Sci.* 570; 254-267.
[22] Benzie I F F, and Strain J J, **1996**. *Analytical Biochem*. 239; 70-76.
[23] Liu TZ, Chin N, Kiser MD, and Bigler W N, **1982**. *Clinical Chemistry*. 28; 2225-2228.
[24] Sinha AK, **1972**. *Anal. Biochem*. 47; 389-394.
[25] Kakkar P, Das B, and Viswanathan PN, **1984**. *Indian J. Biochem. Biophys*. 21; 130-132.
[26] Bradford MM, **1976**. *Anal. Biochem*. 72; 248-254.
[27] Al-Mamari A, **2009**. *Oman Medical Journal.* 24; 173-178
[28] Drexel H, Franz W, Amann RK, Neuenschwander C, Luethy A, Khan S, and Follath F, **1992**. *Am. J. Cardiol*. 70; 436-440.
[29] Chait A, Brazg RL, Tribble DL, and Krauss RM, **1993**. *Am. J. Med*. *94*; 350-356.
[30] Khan A, Ishaq F, Chandel SA, and Chhettri S, **2012**. *Asian Journal of Clinical Nutrition*. 1-12.
[31] Berliner JA, and Heinecke JW, **1996**. *Free Radic. Biol. Med*. 20; 707-727.
[32] Steinberg D, **1997**. *J Biol Chem*. 272; 20963-20966.
[33] Frei B, **1995**. *Food Sci. Nutr*. 35; 83-98.
[34] Suarna C, Dean RT, Stocker R, *et al*. **1995**. *Arterioscler Thromb Vasc Biol*. 15; 1616-1624.
[35] Bloomer R, **2007**. *Nutrition Journal*. 6; 1-6.
[36] McCord JM, **1993**. *Clin. Biochem*. 26; 351-7.
[37] Gregorevic P, Lynch GS, and Williams DA, **2001**. *Eur J Appl Physiol*. 86; 24-7.
[38] Fattman CL, Schaefer LM, and Oury TD, **2003**. *Free Radic Biol Med*. 35; 236-56.
[39] Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, and Grandjean P, **1997**. *Clin Chem*. 43; 1209-14.
[40] Draper HH, and Hadley M, **1990**. *Methods Enzymol.* 186; 421-31.
[41] Raccah D, Fabreguets C, Azulay JP, and Vague P, **1996**. *Diabetes Care.* 19; 564-568.
[42] Andersen HR, Nielsen JB, Nielsen F, and Grandjean P, **1997**. *Clin Chem*. 43; 562-8.
[43] Inoue M, **1994**. The liver: biology and pathobiology. Raven Press: New York, USA. 443-59.
[44] Khan Amir, **2011**. *Journal of Pharmacy Research.* 4(8); 2519-2522.
[45] Chauhan S K, Thapliyal RP, Ojha S K, Rai Himanshu, Singh Puneet, Singh Manendra and Khan Amir, **2011**. *Journal of Pharmacy Research*. 4(2); 446-448.
[46] Chauhan S K, Thapliyal RP, Ojha S K, Rai Himanshu, Singh Puneet, Singh Manendra and Khan Amir, **2011**. *International Journal of Chemical and Analytical Science.* 2(8); 103-107.
[47] Khan Amir, Chauhan S K, Singh R N, and Thapliyal R P, **2011**. *International Journal of Chemical and Analytical Science.* 2(12); 1260-1264.
[48] Khan Amir, Malhotra Deepti, Chandel Abhay S, Chhetri Samir, Fouzia Ishaq, Rathore N S, **2011**. *Pharma Science Monitor*. 1168-1181.
[49] Khan Amir, Chandel Abhay Singh, Ishaq Fouzia, Chhetri Samir, Rawat Seema, Malhotra Deepti, Khan Salman and Rathore Nagendra Singh, **2011**. *Der Pharmacia Sinica.*2-2; 341-354.
[50] Abhay Singh Chandel, Amir Khan, Fouzia Ishaq, Samir Chhetri, Seema Rawat and Deepti Malhotra, **2011**. *Der Chemica Sinica.* 2-2; 211-222.
[51] Khan Amir, Chandel A S, Ishaq F, Rawat S, Chettri S, and Malhotra D, **2011**. *Recent Research in Science & Technology.* 3(11); 13-21.
[52] Chhetri Samir, Khan Amir, Rathore N S, Ishaq Fouzia, Chandel Abhay S, and Malhotra Deepti, **2011**. *J of chemical and pharmaceutical research.* 3(3); 52-63.
[53] Khan M Salman, Khan Amir and Iqbal Jouhar, **2011**. *International Journal of Pharmacy and PharmaceuticalSciences.* 3-3; 277-284.
[54] Khan Amir, and Malhotra Deepti, **2011**. *European Journal of Experimental Biology*. 1 (1); 1-9
[55] Amir Khan et al., **2012**. *Drug Invention Today*. 4(1); 314-320