Impact of Swertia chirata extract on prevention of Cu^{2+} LDL oxidation and malondialdehyde formation

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Abstract: *Diabetes is a multisystem disorder marked by elevated blood sugar level. It has been estimated that the global burden of type 2 diabetes mellitus (T2DM) for 2030 is projected to increase to 438 million ; a 65 % increase. During diabetes, persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species (ROS) as by-products of normal cellular metabolism. The chronic hyperglycaemia, insulin resistance and abnormal lipoprotein profiles found in diabetes may contribute to a decrease of bioavability of vascular nitric oxide (NO), impairing endothelium- dependent vasodilatation documented in and in humans with diabetes. Oxidized LDL may contribute to the progression of atherosclerosis by enhancing endothelial injury.Extracts of chirata reduced LDL oxidation by about 40% which is very close to the efficacy of Atorvastatin (reduction by 51.72%).The management of diabetes nephropathy is extremely expensive and frustrating. Therefore, prevention is better. Sources of antioxidants, especially antioxidant vitamins are available and affordable in most environments. In present investigation aqueous and ethanolic Swertia chirata, increased total antioxidant power of normal plasma by 1.484 and 1.84 fold,and in diabetics it increased by 1.25 fold and 2.19 fold respectively. Extracts of chirata are also very effective in reducing MDA formation in plasma,also these are free of side effects and very cost effective.*

I. Introduction

Diabetes is a major health problem globally and is one of the top five leading causes of death in most developed countries. According to Evidences , it could reach epidemic proportions particularly in developing and newly industrialized countries. The countries covered in American Diabetic Assosiation are as follows: United States (US), China, India, Brazil, Russia, Germany, Pakistan, Mexico, Egypt and Japan. It has been estimated that the global burden of type 2 diabetes mellitus (T2DM) for 2030 is projected to increase to 438 million ; a 65 % increase [1].

It is a multisystem disorder marked by hyperglycemia, it also includes cardiovascular disease, renal failure, peripheral neuropathy, and retinopathy which may lead to blindness). In case of diabetes, persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species (ROS), for all tissues from glucose auto-oxidation and protein glycosylation. When the level of reactive oxygen and nitrogen species (ROS/RNS) increases, antioxidant defense is lowered and there is alterations of enzymatic pathways in humans with poorly controlled diabetes mellitus, it can contribute to endothelial, vascular and neurovascular dysfunction [2].

Lipoproteins consist of a hydrophobic core of TG and cholesterol esters (CE) surrounded by a hydrophilic surface of free cholesterol (FC),phospholipids and apolipoproteins. Plasma lipoproteins are typically classified into five major subclasses on the basis of their densities:chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) [3].

VLDL particles are synthesized in the liver. VLDL consists mainly of TG, and also contains some cholesterol, one apoB-100 molecule, apoCs and apoE. HDL is synthesized primarily in the liver and small intestine. HDL precursor is (nascent) discoidal particle that comprises phospholipid, cholesterol, apoE and apoA, but is devoid of cholesterol ester . HDL has various potentially antiatherogenic properties, including reverse transport of cholesterol from cells of the arterial wall to the liver , inhibition of LDL oxidation by HDL-bound PON1[4]. Low density lipoprotein (LDL) includes a class of proteins which carry cholesterol in the blood and make it available for the use by the cells. LDL is one of the five major groups of lipoproteins (VLDL, IDL, LDL, HDL and chylomicrons) which are classified according to their size and density. LDL is called **low-density lipoprotein** because LDL particles tend to be less dense than other kinds of cholesterol particles. Each LDL particle contains a single **apolipoprotien B-100 molecule**, which circulates the fatty acid keeping them soluble in aqueous environment.When cell requires cholesterol,it synthesizes LDL receptors and is inserted into plasma membrane. LDL particles in bloodstream after associating with clathrin- coated pits form vesicles and binds to these **extracellular LDL receptors** that are endocytosed. Optimal level of LDL in humans:For good health LDL level should be low.

The treatment of diabetes with synthetic drugs costs high and have a lot of side effects so, it is necessary to develop traditional and alternative medicine.

Swertia chirata

Swertia chirata (Family: Gentianaceae) is a valuable herb which is commonly available in India, Nepal and China. The plant is found at an altitude of 1200–3000 and available throughout the year. It comprises 170 species which are closely related to each other. Chirayata, also known as Indian gentian is a robust annual herb which grows upto about 1.5 meters in height.It is generally consumed by the older people and/or people with type 2 diabetes mellitus as it is useful for lowering the blood glucose level. Ethanolic extract possesses antidiabetic activity and have significant effect on cholesterol and triglyceride level.

The objective of my project is the study the diabetic complications and antioxidant impact of Swertia chirata and Atorvastatin extract on copper mediated oxidative kinetics of LDL in dyslipidemic subjects.

In my present investigations I have used extract of herbal extract of Swertia chirata to assess its antioxidant power and analyse their impact on prevention of LDL oxidation and malondialdehyde formation . On its contrary, drug Atorvastatin have been also used in comparision to natural extract of S.chirata.

II. Materials And Methods

Chemicals

2, 4, 6-Tripyridyl-s-Triazine, β- Mercaptoethanol, Butylated Hydroxyl Toluene, Ferric Chloride, Heparin, Tris Hydrochloride, Magnesium Chloride, Sodium Citrate, Sodium pyrophosphate, Sulphuric Acid , Malondialdehyde,Tricholoro Acetic Acid, Trisodium Citrate(HiMedia Laboratories Pvt. Ltd., India). Dextran Sulfate, Phenyl Acetate,Thiobarbituric Acid(Sigma-Aldrich Inc., USA). Sodium Dodecyl Sulfate(Bio-Rad Laboratories, USA) Hydrogen Peroxide(RFCL Limited, India) Ethylene diamine tetraacetate(Merck Limited, India) Pyrogallol (Ranbaxy Limited, India). Coommassie Brilliant Blue G 250(Ployscience Inc., USA).All other chemicals and reagents used in this study were of analytical grade .
Fresh Stems of S. chirata were collected from area of Dhanbad (Jharkhand).

Estimation

Determination of plasma cholesterol,Measurement of in vitro Cu++-mediated oxidation of LDL in the absence or presence of S. chirata ,Measurement of plasma "total antioxidant power" (FRAP),Determination of Malondialdehyde in erythrocytes of normal and diabetic patients.

Protein estimation

The protein was determined by the method of Bradford (1976), 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.

Cholesterol estimatation

Total cholesterol in plasma, LDL subfractions was determined as described by Annino and Giese (1976), 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.

III. Results

Table no.1 Average value of Age,Weight,Height,Male Female of Normal and DiabetiC subject.

S.NO.	PARAMETER	NORMAL(49)	DIABETIC(65)
1.	Age	32±1.53 year	55±3.82 year
2.	Body weight	59.42±8.29	64±9.32
4.	Male	30	43
5.	Female	19	22
6.	Drug taken by patient	-	Insulin,Metformin,Glycate

Table no. 2 Average value of TC,VLDL-C,LDL-C,LDL-Apo-B100,HDL-C,HDL2-C,HDL3-C in normallipidmic and Diabetic subject.

S.NO	PARAMETERS	NORMAL VALUE (μg/ml)	DAIBETIC VALUE (μg/ml)
1.	Total cholesterol in plasma	42.80 ± 2.39	114.66 ± 2.24
2	Low Density Lipoprotien – Cholesterol (LDL- C)	14.9 ± 2.09	60.89 ± 0.53
3.	Total protein in plasma	1.212 ±0 .066	1.190 ± 0.015
4.	Total protein in LDL	1.179 ± 0.348	1.046 ± 0.28
5.	High Density Lipoprotein cholesterol (HDL)	5.36 ± 0.46	7.025 ± 0.63
6.	High Density Lipoprotein2 cholesterol(HDL_2-C)	1.816 ± 0.34	1.514 ± 0.51
7.	High Density Lipoprotein3 Cholesterol(HDL_3-C)	3.172 ± 0.53	4.33 ± 0.247
8.	High Density Lipoprotein-protein(HDL)	0.301 ± 0.111	0.332 ± 0.0016
9.	High density Lipoprotien – protein(HDL_2-C)	0.164 ± 0.00014	0.183 ± 0.036
10.	High Density Lipoprotien – protein(HDL_3 –C)	0.601 ± 0.018	0.304 ± 0.0007
11.	Non- HDL - cholesterol	37.44 ± 1.93	107.63 ± 1.61 *

*Indirectly calculated values
All values are mean ± S.D from pooled serum of normal subjects (n=45)

Table no.3 Average Ratio Value Of TC/LDL-C,LDL-C/TC,HDL_2-C/HDL_2-C and HDL_3-C/HDL_2-C in Normallipidemic and Hyperllipidemic Subject.

S.NO.	PARAMETER	NORMAL VALUE	DAIBETIC VALUE
1.	TC/LDL-C	1.15	1.055
2.	LDL-C/TC	0.866	0.950
3.	HDL_2-C/HDL_3-C	0.233	0.602
4.	HDL_3-C/HDL_2-C	3.66	1.66

Average Value of Age, Weight , Height ,Male ,Female of Normal and diabetic subjects
   The average values of body weight,age,male and female of normal subjects(n=45) were 59.42 ± 8.39kg, 32 ± 1.86years, 26 and 19 resepectively and of diabetic subjects (n=60) were 64 ± 9.41kg, 58± 3.56,29 and 31 respectively as shown in table1.

Average value of TC, Total protein, LDL-C, HDL-3, HDL_2-C, HDL_3-C , HDL protein and non – HDL cholesterol in normal and diabetic subject
   As shown in table2, the average values of TC, LDL-C,total protein in plasma and LDL, HDL-C, HDL2-C ,HDL3-C ,HDL 3 protien,HDL_2C protein,HDL_3 C protein, Non HDL-C in normal lipidemic subjects were 42.80 ± 2.39 μg/ml, 14.9 ± 2.09 μg/ml, 1.212 ± 0.066 μg/ml, 1.179 ± 0.348μg/ml, 5.36 ± 0.46 μg/ml, 1.186 ± 0.34 μg/ml , 3.172 ± 0.53 μg/ml ,0.301 ± 0.111μg/ml,0.164 ± 0.00014μg/ml,0.601 ± 0.018μg/ml and 37.44 ± 1.93μg/ml respectively.
   The average values of TC, LDL-C,total protein in plasma and LDL, HDL-C, HDL2-C ,HDL3-C ,HDL 3 protien,HDL_2C protein,HDL_3 C protein, Non HDL-C in hyperipidemic subjects were 114.66 ± 2.24μg/ml, 10.89 ± 0.53μg/ml,1.190 ± 0.015μg/ml, 1.046 ± 0.28μg/ml, 7.025 ± 0.63μg/ml, 1.514 ± 0.51μg/ml, 4.33 ± 0.247 μg/ml, 0.332 ± 0.0016 μg/ml, 0.183 ± 0.036 μg/ml , 0.304 ± 0.0007 μg/ml, 107.63 ± 1.61 μg/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
   As shown in table 4, the 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 were 1.15, 0.866, 0.233 ,3.66 and of dyslipidemic subjects were 1.055,0.950,0.602,1.66 respectively.

In vitro copper mediated oxidative modification of LDL (at 37°C) isolated from normallipidemic and hyperlipidemic subjects

Cu^{2+} mediated oxidation of LDL in the presence or absence of glucose
   In absence of glucose ,0.24 fold(+24.20%) increase in oxidative modification of LDL was observed from basal value (285.11μM/ml) to maximal value (376.19 μM/ml)at 120 min. after adding 2.5 mM $CuSO_4$.But in case of LDL+glucose, this increase was of 40%(285.11 μM/ml - 475.59 μM/ml).
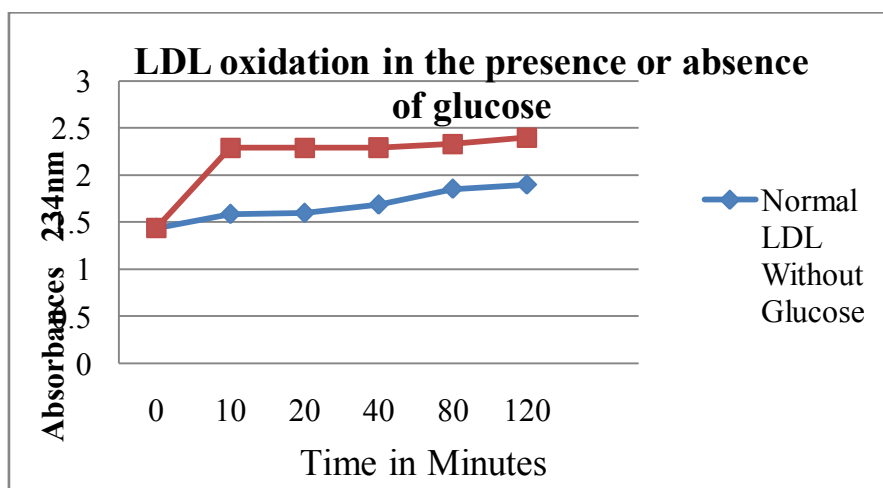
Cu^{2+} mediated oxidation of LDL in the presence or absence of
Ethanolic chirata in normal and diabetic subjects

In absence of Ethanolic Chirata (E.Ch) ,0.34 fold(+33.92%) increase in oxidative modification of LDL was observed from basal value (285.11μM/ml) to maximal value (432.73 μM/ml)at 200 min. after adding 2.5 mM $CuSO_4$.But in presence of E.Ch this increase was only 15%(285.11 μM/ml – 423.01μM/ml), in normallipidemic subjects. In absence of E.Ch ,0.145 fold(+14.5%) increase in oxidative modification of LDL was observed from basal value (510.12μM/ml) to maximal value (597.22μM/ml)at 200 min. after adding 2.5 mM $CuSO_4$.But in presence of E.Ch this increase was significantly reduced to 3.31%(510.12 μM/ml – 527.57μM/ml), in hyperlipidemic subjects.

Cu^{2+} mediated oxidation of LDL in the presence or absence of aqueous chirata in normal and diabetic subjects

In absence of Aqueous Chirata(Aq. Ch.) ,0.34fold(+34.11%) increase in oxidative modification of LDL was observed from basal value (285.11μM/ml) to maximal value (432.32 μM/ml)at 200 min. after adding 2.5 mM $CuSO_4$.But in presence of Aq.Ch this increase was only 30.61%(285.11 μM/ml – 434.32μM/ml), in normallipidemic subjects. in absence of Aq.Ch,0.255 fold(+25.5%) increase in oxidative modification of LDL was observed from basal value (510.12μM/ml) to maximal value (597.22μM/ml)at 200 min. after adding 2.5 mM $CuSO_4$.But in presence of Aq.Ch this increase was significantly reduced to 6.9%(510.12 μM/ml – 477.77μM/ml), in hyperlipidemic subjects.

Thiobarbituric acid reactive substances (TBARS)
Formation of malondialdehyde in plasma of normal and diabetic patients in the presence or absence of Swertia chirata extract and vitamin C

Baseline MDA formation at 535 nm, in normal and diabetic subjects without extracts were 265.87 ± 0.552 nM/mg/ml and 341.26 ± 0.611 nM/mg/ml respectively. After addition of extracts , baseline MDA formation in normal subjects were significantly reduced . Reduction in baseline value MDA after addition of E.C leaves, Aq.C leaves, E.Ch, Aq. Ch, E.C bark, Vitamin C, Atorvastatin in normal LDL were 41.9% ,60.5%,53.57% and 67.8% respectively,and reduction in baseline value after addition of E.Chirata, Aq. Chirata and Atorvastatin in diabetic subjects were 40.65%,55.43%,49.27% and 69.81% respectively.

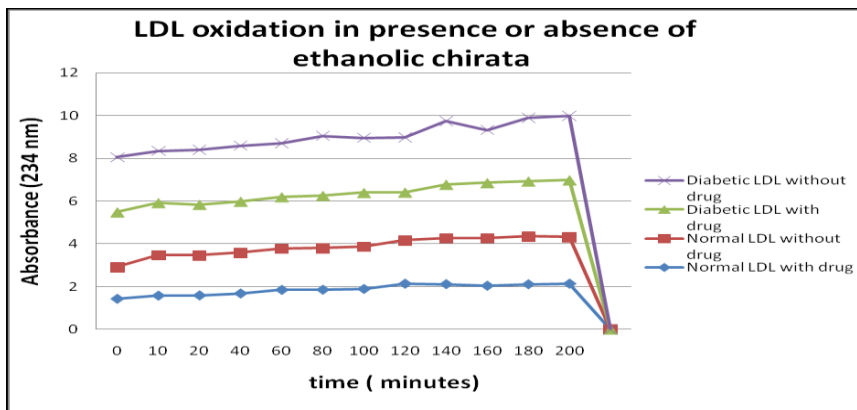**Fig 1: Cu ++ mediated LDL oxidation in the presence or absence of glucose**

Fig 2: Cu^{2+} mediated oxidation of LDL in the presence or absence of Ethanolic chirata in normal and diabetic subjects
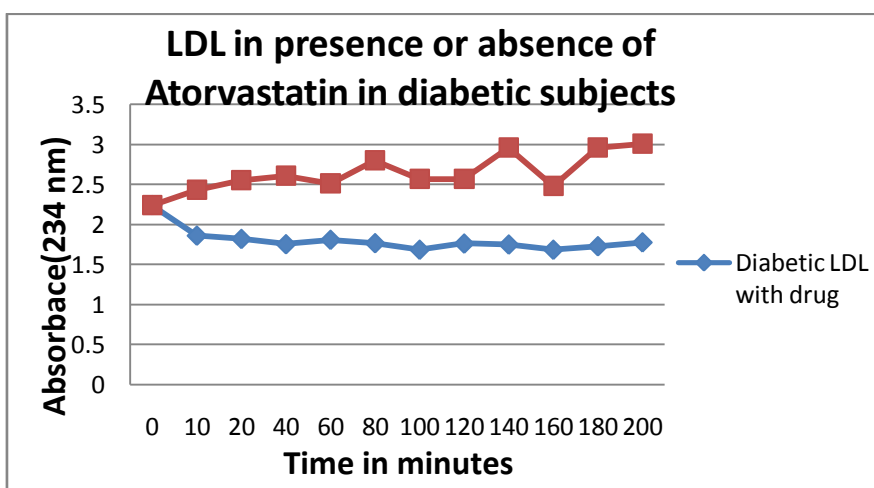

Fig3:Cu^{2+} mediated oxidation of LDL in the presence or absence of Atorvastatin in diabetic subjects Thiobarbituric acid reactive substances (TBARS).
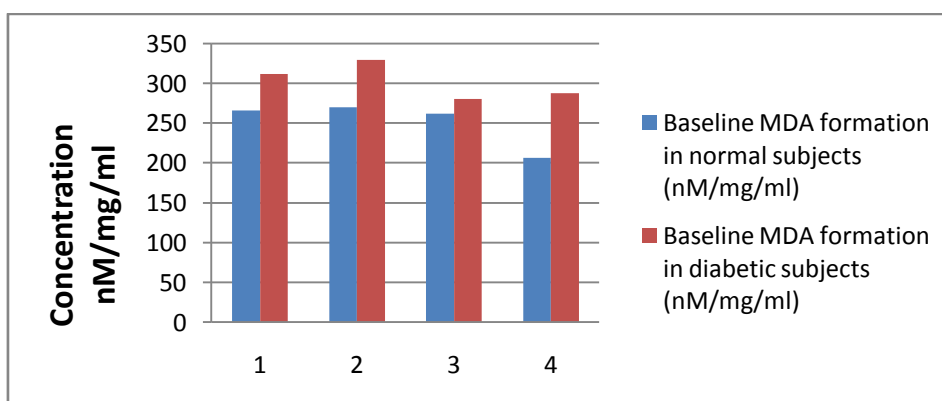

Fig 4:Formation of malondialdehyde in plasma of normal and diabetic patients in the presence or absence of Swertia chirata extract and vitamin C.

1= without drug , 2= ethanolic chirata , 3= Aq. chirata, 4= Atorvastatin .

IV. Discussion

Diabetes is a multisystem disorder marked by elevated blood sugar level. During diabetes, persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species (ROS), for all tissues from glucose auto-oxidation and protein glycosylation. Free radicals are generated as by-products of normal cellular metabolism; however, several conditions are known to disturb the balance between ROS production and cellular defense mechanisms [5].

In my baseline study of various physiochemical parameters, the average values of physical parameters like, of body weight, age, male and female of normal subjects (n=45) were 59.42 ± 8.39kg, 32 ± 1.86years, 26 and 19 resepectively, and of diabetic subjects (n=60) were 64 ± 9.41kg, 58 ± 3.56, 29 and 31 respectively.

Statistical evaluation of the lipid profile among the normal and diabetic patients were compared . According to Murray et al.(1990), and the normal range for total cholesterol should be 150-200 mg dl^{-1}. In the present study, the results showed that the lipid and the lipoprotein profiles of the diabetics were higher than that of the controls and they were comparable with the findings of Idogun et al., and Albrki et al.

The average values of TC, LDL-C,total protein in plasma and LDL, HDL-C, HDL_2-C ,HDL_3-C ,HDL-C protien,HDL_2C protein,HDL_3 C protein, Non HDL-C in normal lipidemic subjects were 42.80 ± 2.39 μg/ml, 14.9 ± 2.09 μg/ml, 1.212 ± 0.066 μg/ml, 1.179 ± 0.348μg/ml, 5.36 ± 0.46 μg/ml, 1.186 ± 0.34 μg/ml , 3.172 ± 0.53 μg/ml ,0.301 ± 0.111μg/ml,0.164 ± 0.00014μg/ml,0.601 ± 0.018μg/ml and 37.44 ± 1.93μg/ml respectively. The average values of TC, LDL-C, total protein in plasma and LDL, HDL-C, HDL_2-C ,HDL_3-C ,HDL protien, HDL_2C protein, HDL_3C protein, Non HDL-C in hyperipidemic subjects were 114.66 ± 2.24μg/ml, 10.89 ± 0.53μg/ml,1.190 ± 0.015μg/ml, 1.046 ± 0.28μg/ml, 7.025 ± 0.63μg/ml, 1.514 ± 0.51μg/ml, 4.33 ± 0.247 μg/ml, 0.332 ± 0.0016 μg/ml, 0.183 ± 0.036 μg/ml , 0.304 ± 0.0007 μg/ml, 107.63 ± 1.61 μg/ml respectively. The 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 were 1.15, 0.866, 0.233 ,3.66 and of dyslipidemic subjects were 1.055,0.950,0.602,1.66 respectively.

Dyslipidaemia was observed in the diabetic population, but that HDL-C was not significantly decreased. Lipid disorders are very common in both insulin dependent and non-insulin dependent diabetic mellitus **.**

The chronic hyperglycaemia, insulin resistance and abnormal lipoprotein profiles found in diabetes may contribute to a decrease of bioavability of vascular nitric oxide (NO), impairing endothelium- dependent vasodilatation documented in and in humans with diabetes **[5].** NO possesses a variety of antiatherogenic properties, and loss of these protective mechanisms may lead to an increase in susceptibility to vascular disease. In vitro cell mediated oxidative processes usually require the presence of transition metal ions to oxidize the LDL. Oxidized LDL may contribute to the progression of atherosclerosis by enhancing endothelial injury by inducing foam cell generation and smooth muscle proliferation,it also initiate endothelial inflammation leading to atherosclerosis and CVD.Modificatons take place either in plasma or in the inner layer of the artery**[6].**

In my present investigation it was found that in absence of glucose, 0.24 fold (+24.20%) increase in oxidative modification of LDL was observed from basal value (285.11μM/ml) to maximal value (376.19 μM/ml)at 120 min after adding 2.5 mM $CuSO_4$.But in case of LDL+glucose, this increase was of 40%(285.11 μM/ml - 475.59 μM/ml) which suggests that elevated glucose level promote LDL oxidation in normal plasma.

In vitro treatment was given to both normallipidemic and dyslipidemic subjects with natural herbal **Aq**. and **ethanolic** extracts of Swertia chirata and significant decrease in LDL oxidation was observed. In case of **E.Ch.** LDL oxidation was reduced by 48.92% in normallipidemic subjects and dyslipidemic subjects it was reduced by 41.19%.When in vitro treatment was given with **Aq.Ch.** LDL oxidation was decreased by 44% in normallipidemic subjects and in dyslipidemic subjects LDL oxidation was reduced greatly by 32.4%. Very potent drug, **Atorvastatin** of statin family was also used for inhibition LDL oxidation.Atorvastatin reduced LDL oxidation drastically by 51.72% in diabetic patients. FRAP is a novel method for assessing "antioxidant power" in which Ferric ion is reduced to ferrous ion at low pH and lead to formation of coloured ferrous tripyridyltriazine complex(Benzien and Strain ,1996).

Total antioxidant power of normal plasma, without any extract increased from 277.88 μM/ml to 431.003 μM/ml(0.35 fold increase) with time (from 0' to 5') whereas in diabetic patients it decreased from 185.78 μM/ml to 142.62 μM/ml (0.23fold decrease) with time (from 0' to 5') (Bisht and Sisodia,2011). In case of Aq. and ethanolic Swertia chirata, total antioxidant power of normal plasma increased by 1.484 and 1.84 fold,and in diabetics it increased 1.25 fold and 2.19 fold.

Baseline MDA formation at 535 nm, in normal and diabetic subjects without extracts were 265.87 ± 0.552 nM/mg/ml and 341.26 ± 0.611 nM/mg/ml respectively. After addition of extracts , baseline MDA formation in normal subjects were significantly reduced . Reduction in baseline value MDA after addition of E.C leaves, Aq.C leaves, E.Ch, Aq. Ch, E.C bark, Vitamin C, Atorvastatin in normal LDL were 41.9% ,60.5%,53.57% and 67.8% respectively,and reduction in baseline value after addition of E.Chirata, Aq. Chirata and Atorvastatin in diabetic subjects were 40.65%,55.43%,49.27% and 69.81% respectively.

Acknowledgments

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