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Full Length Research Paper

Insulin resistance and oxidative stress in diabetic rats treated with flaxseed oil

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Hyperglycemia is a metabolic disorder that results in excessive production of free radicals which leads to severe oxidative damage of cell components like lipids, proteins and DNA. Supplementation with flaxseed oil has been reported to reduce oxidative stress. Eighty male albino rats were used in this study, and were divided randomly into four groups: control, flaxseed, diabetic and treated groups. Blood glucose and plasma insulin were measured, and insulin resistance was calculated. Membrane cholesterol, triglycerides and total lipids were estimated. Also, membrane advanced oxidation protein products (AOPP), malondialdehyde (MDA) and superoxide dismutase (SOD) were determined. Urinary 8-hydroxyguanosine was estimated by high performance liquid chromatography (HPLC). The data showed that flaxseed oil significantly decreased fasting blood glucose which may be related to significant decrease in insulin resistance. Also, there was a decrease in oxidative stress parameters indicating the beneficial effect of flaxseed oil in scavenging free radicals. Positive correlation was found between insulin resistance and oxidative stress parameters (MDA and 8-OHdG). We concluded that, flaxseed oil administration has a beneficial effect on decreasing insulin resistance in diabetic rats through the scavenging of free radicals and increasing antioxidant enzymes.

Key words: Cell membrane, flaxseed oil, oxidative stress, high performance liquid chromatography (HPLC), diabetes mellitus.

INTRODUCTION

Diabetes mellitus, the high incidence of microvascular and atherosclerotic disorders have been associated with abnormalities of erythrocyte composition, rheological function and increased oxidative stress (Shinde et al., 2010) which is generally attributed to the formation of the highly reactive hydroxyl radical ($OH^{\cdot}$) (Mckillop and Schrum, 2005).

Erythrocytes are perhaps the cells most exposed to peroxidation damage by free radicals (Shinde et al., 2010), due to the presence of fatty acids content in their membranes and high cellular concentrations of oxygen and hemoglobin (Sadrazadeh et al., 1984). Erythrocyte damage includes changes in membrane protein and lipid structure, which in turn induced alterations in its functions (Singh and Rajini, 2008), included the action of insulin and its receptors (Zeghari et al., 2000).

Hyperlipidemia has also been reported as one of the causative factors for increased lipid peroxidation in diabetes (Kesavulu et al., 2002), thus, the free radicals attack the polyunsaturated fatty acids in membranes to produce lipid peroxides (Saravanan et al., 2006) .

Advanced oxidation protein products (AOPP) is a reliable marker to estimate the degree of oxidant-mediated protein damage (Witko-Sarsat et al., 1996), and a potentially mutagenic DNA base, 8-hydroxyguanosine (8-OH-guanine or 8-oxo guanine) is repaired, released from the cell, and eventually excreted via the urine as the base (8-OH-guanine) or the nucleoside, 8-hydroxyl-2-deoxyguanosine

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(8–OH–dG, 8–oxo–dG). The urinary content of 8–OH–dG represents an average rate of oxidative damage to guanine in the form of the free nucleotide (dGTP) and in DNA (Svoboda et al., 2005).

Antioxidants have been reported to attenuate inflammatory response, insulin resistance, and diabetes development (Chun et al., 2008). One promising antioxidant is flaxseed. The active ingredient of flaxseed (lignan, secoisolariciresinol diglucoside (SDG)) has significant antioxidant effects by inhibiting DNA scissions and lipid peroxidation and decreasing ROS (Lee et al., 2008). In addition, Maddock et al. (2006) indicated that flaxseed is the best plant that contain high amount of polyunsaturated fatty acids, especially omega-3 (Harding et al., 2004), which are the key functional components (Larsson, 2004).

This study was conducted to investigate the role of flaxseed oil in decreasing insulin resistance in context of oxidative stress in streptozotocin (STZ) induced diabetic rats.

MATERIALS AND METHODS

Chemicals

STZ and 8-hydroxyguanosine standard (high performance liquid chromatography (HPLC) grade) were purchased from Sigma Chemicals Co. (Munih, Germany). Acetonitrile, methanol, ethanol, N-hexane, 2-propanol and phosphoric acid (HPLC grade) were purchased from ALDRICH, Germany.

Experimental animals

Male albino rats weighting 180 to 200 g were obtained from the animal house of National Research Center, Giza, Egypt. The guidelines of the ethical care and treatment of the animals followed the regulations of the ethical committee of the National Research Centre (NRC).

Induction of diabetes mellitus

STZ was dissolved in 50 mM sodium citrate (pH 4.5) solution containing 150 mM NaCl. The solution (6.0 mg/0.5 ml/100 g body weight (bw)) was subcutaneously administrated in rats, fasting blood sugar was estimated after 3 days to confirm the development of diabetes mellitus (Uchiyama and Yamaguchi, 2003).

Experimental design

Eighty male albino rats were housed in individual suspended stainless steel cages in a controlled environment (22 to 25°C) and 12 h light/12 h dark with food and water freely available, and were divided randomly into four groups, 20 rats in each group as follow:

1. Group I (control group): healthy rats received 1.2 ml corn oil/kg bw/day orally.
2. Group II (flaxseed oil group): healthy rats received 1.2 ml flaxseed oil/kg bw/day orally.
3. Group III (diabetic group): diabetic rats received 1.2 ml corn oil/kg bw/day orally.
4. Group IV (treated group): diabetic rats received 1.2 ml flaxseed oil/kg bw/day orally (Taylor et al., 2010).

After the experimental period (8 weeks), animals were kept in a metabolic individual cages for collection of 24 h urine samples, they were kept fasting for 12 h before blood sampling; blood was withdrawn from the retro-orbital venous plexus of the eye using capillary tubes and was collected in tubes containing sodium fluoride for blood glucose and heparinized tubes for other biochemical parameters.

Blood was centrifuged at 2000 rpm for 10 min using cooling centrifuge. Plasma was separated and immediately frozen. Packed red blood cells (RBCs) were used for extraction of erythrocyte membrane. The following parameters were estimated.

Blood glucose and plasma insulin

1. Fasting blood sugar was determined using enzymatic colorimetric method, Centronic, Germany, according to Trinder (1969).
2. Plasma insulin level was estimated by enzyme-linked immunosorbent assay (ELISA) kit according to Yalow and Bauman (1983).
3. Insulin resistance was calculated from the following equation according to Mathews et al. (1985):

Insulin resistance = Fasting glucose (mg/dl) × fasting insulin (µIU/ml)/405

Erythrocyte membrane lipids

Total lipids in the red blood cells membrane were extracted by chloroform: methanol method (DeGier and Van Deenen, 1964) modified from the method of Bligh and Dyer (1959). Erythrocyte membrane total lipids were determined according to Chabrol and Charonnat (1973). Membrane total cholesterol and triglycerides were estimated according to Richmond (1973) and Fossati (1982), respectively using enzymatic colorimetric method Centronic, Germany kit.

Ghost preparation

The method used for erythrocyte ghost preparation was based on the washing of backed RBCs by isotonic phosphate buffer and hemolysis of RBCs for removal of hemoglobin by hypotonic phosphate buffer (pH was adjusted at 7.4) (Post et al., 1960; Dodge et al., 1962).

AOPP as a marker of protein oxidation in erythrocyte membrane was estimated by ELISA kit according to Deschamps-Latscha et al. (2005). Membrane superoxide dismutase (SOD) as antioxidant enzyme was evaluated according to the method described by Nishikimi et al. (1972). Also, membrane malonedialdehyde (MDA) was measured according to the method of Uchiyamara and Mihara (1978).

Estimation of urinary 8-hydroxyguanosine by HPLC

Protocol for urinary 8-hydroxyguanosine (8-OHdG) analysis was modified from the method described by Kim et al. (2001). 8-OHdG was extracted from 1 ml urine. The eluents were dried under ultrapure N2 stream and were reconstituted in 5 ml deionized water. 20 µl from each sample and also from the different concentrations of the standard were injected in HPLC, and the concentration of urinary 8-OHdG was calculated from the standard curve and then was divided on the urinary creatinine. Urinary creatinine was estimated by kinetic method as described by Larsen (1972).

HPLC condition

HPLC column C18 (260 × 4.6, particle size 5 µl) using mobile phase acetonitrile/methanol/phosphate buffer (25/10/965) v/v. Phosphate buffer was prepared by dissolving 8.8 g of potassium dihydrogen phosphate in 1000 ml deionized water and pH was adjusted at 3.5. The buffer was then filtered 2 times before being used at a flow rate of 1 ml/min using electrochemical detector with cell potential of 600 mV (El-Khayat et al., 2010).

Statistical analysis

Statistical analysis of the results was carried out using the standard computer program Statistical Package for Social Sciences (SPSS) (V 12.0, Echosoft Corporation USA, 1998). Normally distributed results were compared using student's test. Differences among groups were evaluated using one way analysis of variance (ANOVA). Pearson's correlation test was done. Results were expressed as mean ± standard error (SE). P values less than 0.05 were considered to be significant.

RESULTS

In this study, it was found that, the mean values of fasting blood sugar and plasma insulin levels did not changed in flaxseed oil group as compared to the control group indicating the safety of this oil, while in diabetic group, the mean value of fasting blood sugar increased significantly as compared to the control group. On the other hand, flaxseed oil in the treated group significantly decreased both fasting blood sugar and insulin resistance, while plasma insulin level was insignificantly changed as compared to the diabetic group (Table 1).

The mean value levels of erythrocyte total lipids were significantly increased in diabetic group as compared to control group which may be related to the concomitant increase in both total cholesterol and triglycerides, while these values were significantly decreased by flaxseed oil in treated group (Table 2).

AOPP, MDA and 8-hydroxyguanozine were significantly increased in diabetic group, while SOD was significantly decreased as compared to control indicating the increase of oxidative stress, although, these values were improved in the treated group (Table 3 and Figures 1 to 4).

Positive correlation was observed in this study between oxidative stress parameters (MDA and 8-OHdG) and insulin resistance concomitant with a negative correlation between SOD and insulin resistance (Table 4).

DISCUSSION

In the current study, STZ elevated fasting blood sugar, although, this value was significantly decreased in flaxseed oil treated group as compared to diabetic one, this reduction of blood glucose may be due to the significant decrease of insulin resistance and also the slightly increase of insulin level which appeared in this study. In agreement, Rhee and Brunt (2011) indicated that flaxseed supplementation significantly reduced fasting plasma glucose concentration as compared to the baseline; however, it did not significantly change fasting plasma insulin concentration.

Diabetes mellitus is usually associated with an increase in plasma lipids levels, the risk factor for coronary heart disease (Al-Shamaony et al., 1994). Elevation of plasma membrane lipids, total cholesterol and triglycerides levels in streptozotocin diabetic rats observed in the present study may be as a result of increased breakdown of lipids and mobilization of free fatty acids from the peripheral stores, in contrast, flaxseed oil significantly decreased erythrocyte membrane total lipids, cholesterol and triglycerides. This result was confirmed by Makni et al. (2008) who found a beneficial effect of diet rich with nutritional products using flaxseed on hypercholesterolemic status in adult rats. The hypocholesterolemic effect of flaxseed may be due to the fact that, flaxseed contains bioactive nutrients with high levels of essential oils like n-3 fatty acids, including alpha-linolenic acid (ALA), which are reported to lower serum cholesterol and triglycerides levels (Hasler et al., 2002).

Hussein et al. (2011) indicated that triglycerides might influence the binding of insulin to its receptor or interfere with early post binding steps. Thus, high level of triglycerides leads to a resistance to the antilipolytic effect of insulin; therefore, a reduction in triglycerides levels might improve insulin sensitivity.

Increased oxidative stress could be one of the common pathogenic factors of diabetic complications (Araki and Nishikawa, 2010). Oxidative damage is generally attributed to the formation of highly reactive OH which leads to severe oxidative damage of the cell's components like lipids, proteins and DNA (Mckillop and Schrum, 2005).

In this study, the lipid peroxidation marker (MDA) was elevated in erythrocyte membranes of diabetic rats as reported earlier (Murugan and Pari, 2007). The increase in lipid peroxidation might be a reflection in enzymatic and non enzymatic antioxidants of defense systems. Erythrocyte membrane SOD was significantly decreased in diabetic group. In agreement, Feillet-Coudary et al. (1999) reported that the activity of SOD is low in diabetes mellitus. A decrease in SOD can lead to an excess availability of superoxide anion and hydrogen peroxide in biological system, which in turn generates OH, resulting in initiation and propagation of lipid peroxidation (Kumuhekar and Katyane, 1992). The result of increased activity of SOD in treated group in this study suggested that flaxseed oil contains a free radical scavenging activity, which could exert a beneficial effect against pathological alterations caused by the presence of O_2^- and OH^-. The increased activity of SOD accelerates dismutation of O_2^- to hydrogen peroxide, which is removed by catalase (CAT) (Murugan and Pari, 2007). This action could involve mechanisms related to scavenging activity of flaxseed oil.

In the same line, the elevation of AOPP in diabetic rats

Table 1. Blood glucose, insulin and insulin resistance levels in different studied groups.

Group	Parameter		
	Glucose (mg/dl)	Insulin (µlU/ml)	Insulin resistance ($mgdl^{-1}$, $\mu lU\ ml^{-1}$)
Control (Mean ± SE)	79.87 ± 4.1[b]	11.1 ± 0.9	2.19 ± 0.1[b]
Flaxseed (Mean ± SE)	79.62 ± 2.7[b]	11.5 ± 1.6	2.26 ± 0.1[b]
Diabetic (Mean ± SE)	243.87 ± 9.3[a]	8.8 ± 1.0	5.30 ± 0.2[a]
Treated (Mean ± SE)	177.50 ± 5.1[a,b]	10.3 ± 2.0	3.63 ± 0.1[a,b]

Significant P value < 0.05. [a]Significant difference compared to control group; [b]Significant difference compared to diabetic group. Number of cases = 20.

Table 2. Membrane lipid profile and total proteins in different studied groups.

Group	Parameter		
	Total lipids (mg/ml RBCs)	Cholesterol (mg/ml RBCs)	Triglycerides (mg/ml RBCs)
Control (Mean ± SE)	4.6 ± 0.1[b]	1.9 ± 0.1[b]	0.23 ± 0.02[b]
Flaxseed (Mean ± SE)	4.4 ± 0.1[b]	1.7 ± 0.3[b]	0.18 ± 0.01[b]
Diabetic (Mean ± SE)	6.9 ± 0.1[a]	2.9 ± 0.1[a]	0.31 ± 0.02[a]
Treated (Mean ± SE)	5.2 ± 0.2[a,b]	2.3 ± 0.2[b]	0.24 ± 0.02[b]

Significant P value < 0.05. [a]Significant difference compared to control group; [b]Significant difference compared to diabetic group. Number of cases = 20.

Table 3. Oxidant/antioxidant parameters in different studied groups.

Group	Parameter			
	AOPP (ng/ml RBCs)	MDA (nmol/ml RBCs)	8-OHdG (ng/mg creatinine)	SOD (U/ml RBCs)
Control (Mean ± SE)	16.2 ± 0.8[b]	0.87 ± 0.18[b]	5.0 ± 0.2[b]	486.5 ± 38[b]
Flaxseed (Mean ± SE)	16.0 ± 0.5[b]	0.98 ± 0.20[b]	5.6 ± 0.7[b]	488.8 ± 22[b]
Diabetic (Mean ± SE)	20.0 ± 1.6[a]	3.76 ± 0.24[a]	12.0 ± 0.3[a]	254.0 ± 22[a]
Treated (Mean ± SE)	18.2 ± 1.2	2.42 ± 0.19[a,b]	6.6 ± 0.4[a,b]	382.3 ± 35[a,b]

Significant P value < 0.05. [a]Significant difference compared to control group; [b]Significant difference compared to diabetic group. Number of cases = 20.

Table 4. Correlation between oxidative stress parameters and blood glucose and insulin resistance.

Parameter	AOPP	MDA	8-OHdG	SOD
Glucose				
r	0.472**	0.865**	0.812**	0.734**
P	0.006	0.000	0.000	0.000
Insulin resistance				
r	0.275	0.716**	0.734**	- 0.617**
P	0.127	0.000	0.000	0.000

**Significant correlation (P < 0.05).

of the present study may be due to the generation of reactive oxygen species, this result is in agreement with Abou-Seif and Youssef (2004) who reported that AOPP are formed during oxidative stress by the action of chloramines produced by myeloperoxidase in activated neutrophils and is accumulated in biological systems and thus causing damage to biological membranes.

In this study, we found a significant increase of 8-OHdG in diabetic group, this result was in agreement with Araki and Nishikawa (2010) who found a positive correlation

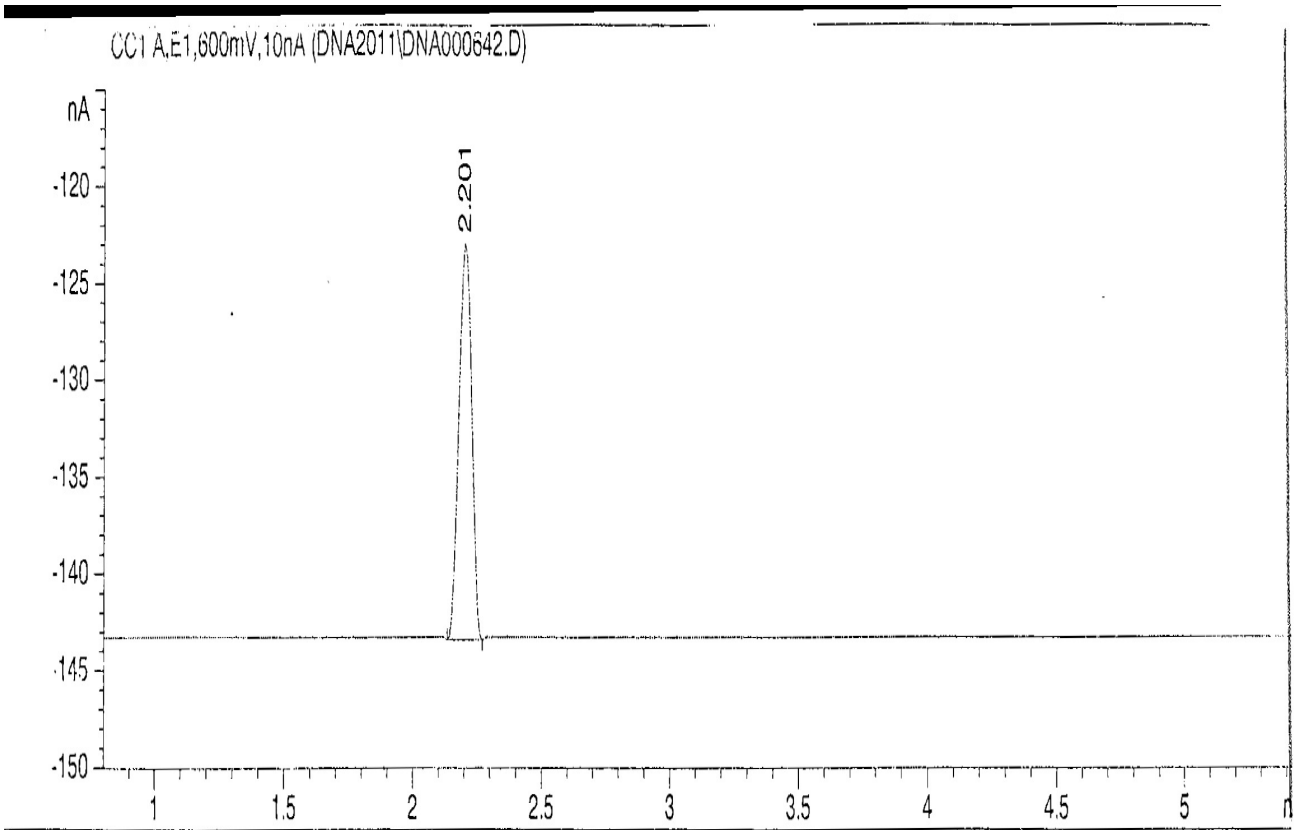

Figure 1. HPLC chromatogram showing urinary 8-OHdG levels in control group.

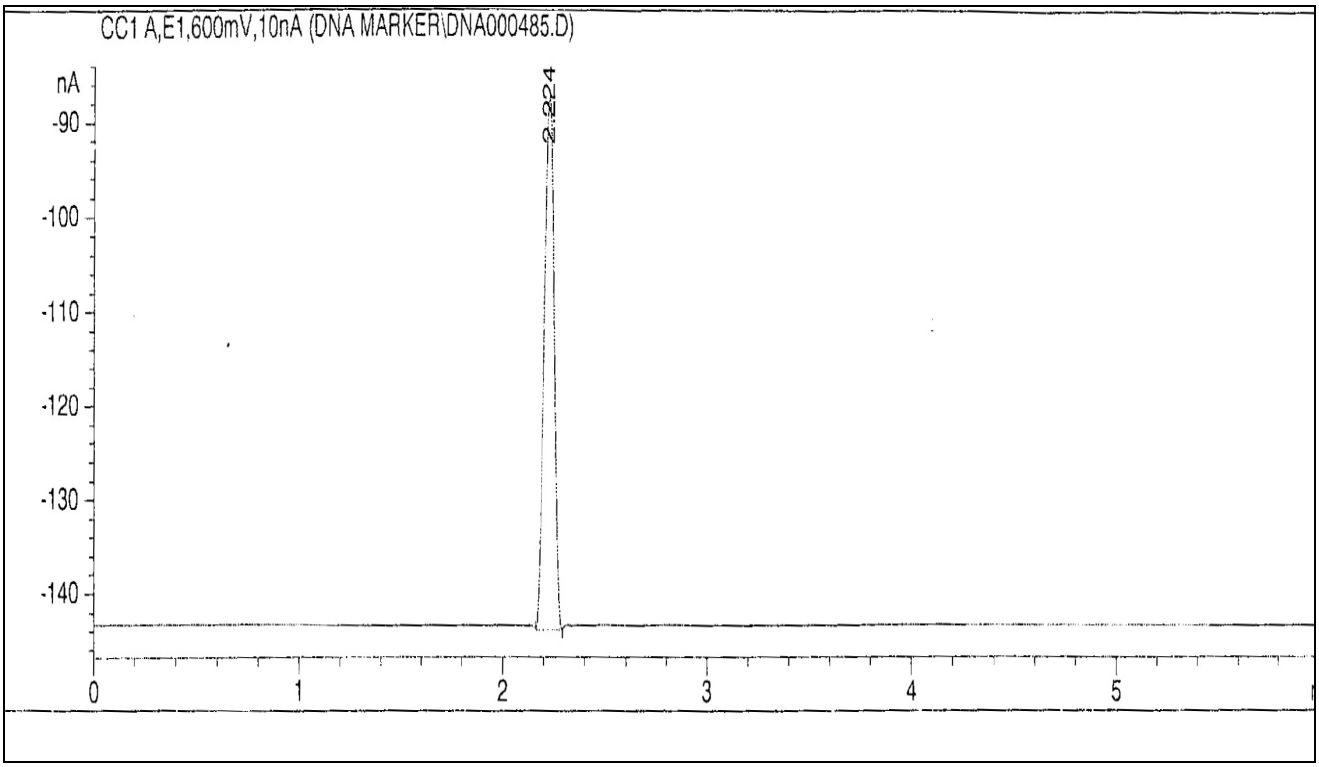

Figure 2. HPLC chromatogram showing urinary 8-OHdG levels in flaxseed oil group.

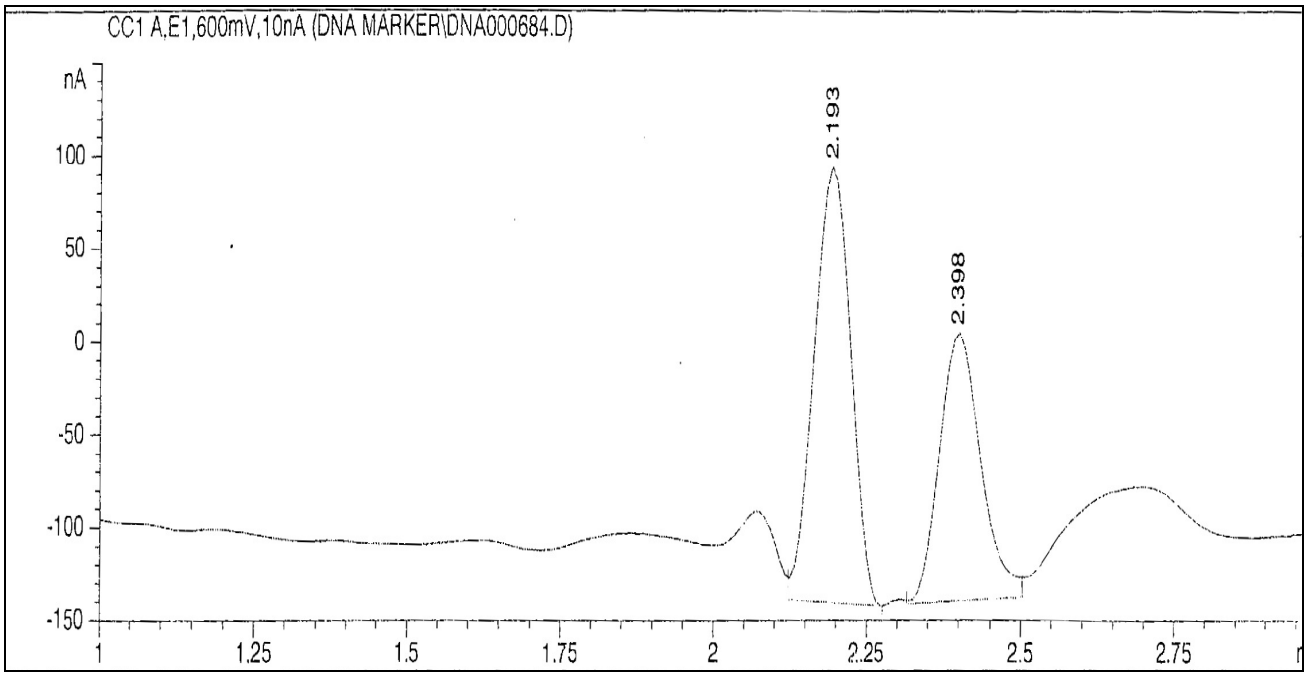

Figure 3. HPLC chromatogram showing urinary 8-OHdG levels in diabetic group.

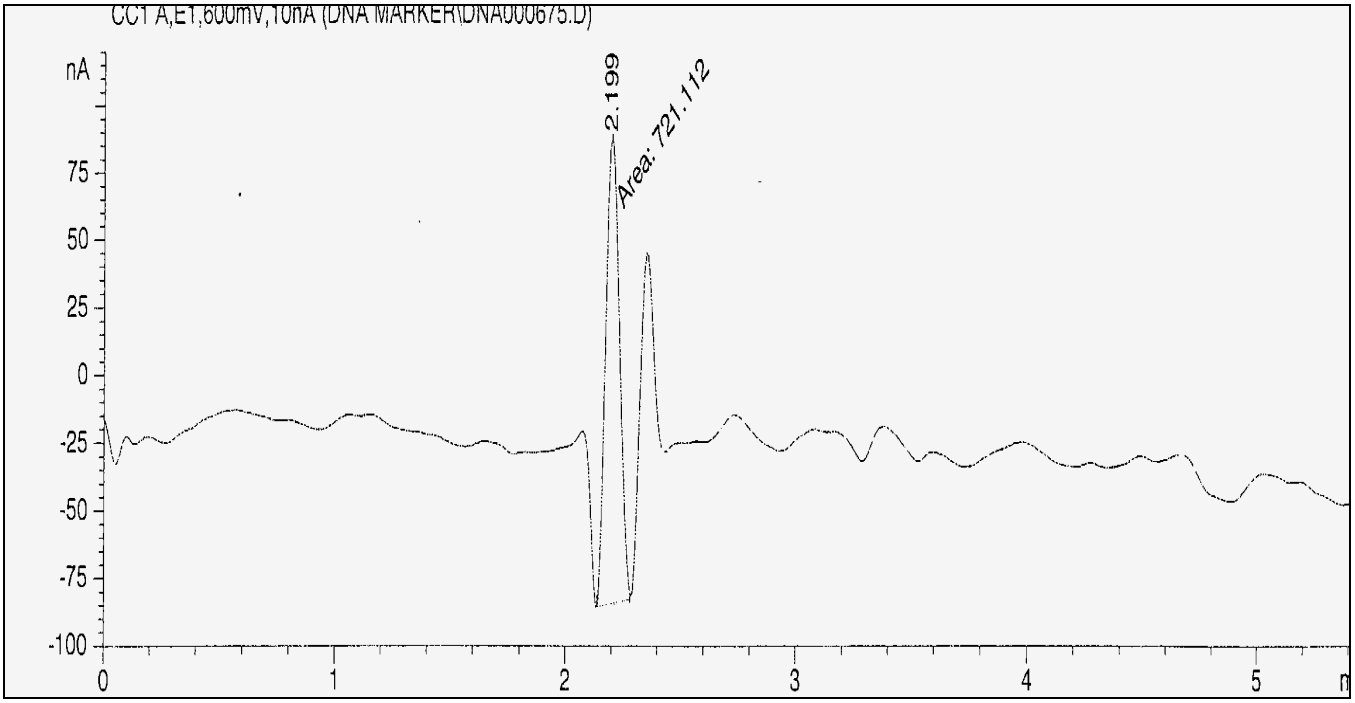

Figure 4. HPLC chromatogram showing urinary 8-OHdG levels in treated group.

correlation between HbA1C and urinary excretion of 8-OHdG which reflects mitochondrial oxidative damage. Flaxseed oil significantly decreased oxidative stress parameters in this study. In agreement, Lee et al. (2008) indicated that the active ingredient of flaxseed has significant antioxidant effects by inhibiting DNA scissions, lipid peroxidation and decreasing reactive oxygen species. Moreover, flaxseed oil contains high amount of unsaturated fatty acids, especially omega-3 (Maddock et al., 2006) which upregulate gene expression of antioxidants

enzymes and downregulate gene associated with production of reactive oxygen species (Harding et al., 2004).

In this study, there is a positive correlation between insulin resistance and oxidative stress parameters (MDA and 8-OHdG) concomitant with a negative correlation between insulin resistance and antioxidant enzyme (SOD). This result is in agreement with Park et al. (2009) who observed positive associations between oxidative stress markers and insulin resistance.

It was suggested that, increased oxidative stress may suppress insulin receptor activation or decrease the translocation of glucose transporter-4 (GLUT-4) on the cell membrane (Rudich et al., 1998). Antioxidants increase glucose disposal via increased translocation of GLUT-4 on the cell membrane and increase basal glucose uptake via redistribution of glucose transporter-1 (GLUT-1) (Estrada et al., 1996).

This study concludes that, flaxseed oil administration has a beneficial effect on decreasing insulin resistance in diabetic rats through the scavenging of free radicals and increase in superoxide dismutase.

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