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Olive Leaves Extract Restored the antioxidant Perturbations in Red Blood Cells Hemolysate in Streptozotocin Induced Diabetic Rats

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***Abstract*—**Oxidative stress and overwhelming free radicals associated with diabetes mellitus are likely to be linked with development of certain complication such as retinopathy, nephropathy and neuropathy. Treatment of diabetic subjects with antioxidant may be of advantage in attenuating these complications. Olive leaf (Oleaeuropaea), has been endowed with many beneficial and health promoting properties mostly linked to its antioxidant activity. This study aimed to evaluate the significance of supplementation of Olive leaves extract (OLE) in reducing oxidative stress, hyperglycemia and hyperlipidemia in Sterptozotocin (STZ)-induced diabetic rats. After induction of diabetes, a significant rise in plasma glucose, lipid profiles except High density lipoprotein-cholestrol (HDLc), malondialdehyde (MDA) and significant decrease of plasma insulin, HDLc and Plasma reduced glutathione GSH as well as alteration in enzymatic antioxidants was observed in all diabetic animals. During treatment of diabetic rats with 0.5g/kg body weight of Olive leaves extract (OLE) the levels of plasma (MDA) ,(GSH), insulin, lipid profiles along with blood glucose and erythrocyte enzymatic antioxidant enzymes were significantly restored to establish values that were not different from normal control rats. Untreated diabetic rats on the other hand demonstrated persistent alterations in the oxidative stress marker (MDA), blood glucose, insulin, lipid profiles and the antioxidant parameters. These results demonstrate that OLE may be of advantage in inhibiting hyperglycemia, hyperlipidemia and oxidative stress induced by diabetes and suggest that administration of OLE may be helpful in the prevention or at least reduced of diabetic complications associated with oxidative stress.

***Keywords*—**Diabetes mellitus, olive leaves, oxidative stress, red blood cells

I. Introduction

DIABETES mellitus (DM) is a chronic metabolic disease with the highest rates of prevalence and mortality worldwide that is caused by an absolute or relative lack of insulin and or reduced insulin activity [1]. It is characterized by hyperglycemia and long-term complications affecting the eyes, kidneys, nerves, and blood vessels. Although the leading mechanism of diabetic complications remains unclear, much attention has been paid to the role of oxidative stress. It has been suggested that oxidative stress may contribute to the pathogenesis of different diabetic complications [2]. Furthermore, with diabetes, several features appear including an increase in lipid peroxidation [3], alteration of the glutathione redox state, a decrease in the content of individual natural antioxidants, and a reduction in the antioxidant enzyme activities.

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These changes suggest an oxidative stress caused by hyperglycemia[4]. Many defense mechanisms are involved in diabetes-induced oxidative damage. Among these mechanisms, antioxidants play the role of a free-radical scavenger [5]. Nowadays, herbal drugs are gaining popularity in the treatment of diabetes and its complications. As a new strategy for alleviating the oxidative damage in diabetes, a growing interest has been noticed in the usage of natural antioxidants. It has been suggested that many of the negative effects of oxidative stress are diminished upon supplementation with certain dietary antioxidants such as vitamins and other nonnutrient antioxidants such as flavonoids and polyphenols [6]. Among natural antioxidants, the olive tree has been widely accepted as one of the species with the highest antioxidant activity via its oil, fruits, and leaves [7]. It is well known that the activity of the olive tree byproduct extracts in medicine and food industry is due to the presence of some important antioxidant and phenolic components to prevent oxidative degradations [8]. The olive tree has long been recognized as having antioxidant molecules, such as oleuropein, hydroxytyrosol, oleuropein aglycone, and tyrosol [9]. Furthermore, olive leaves are considered as a cheap raw material which can be used as a useful source of high-added value products [10]. The main phenolic compounds in olive leaves are the glycosylated forms of oleuropein and ligstroside [11]. The main active component in olive leaf extract is oleuropein, a natural product of the secoiridoid group. Several studies have shown that oleuropein possesses a wide range of pharmacologic and health promoting properties including antiarrhythmic, spasmolytic, immune-stimulant, cardioprotective, hypotensive, antiinflammatory, antioxidant, and anti- thrombic effects [12]. Many of these properties have been described as resulting from the antioxidant character of oleuropein [13]. Previously, oleuropein was reported to have an antihyperglycaemic effect on diabetic rats [14]. However, as regards the antioxidant properties of oleuropein, its mechanism in attenuating hyperglycaemia is still not well recognized. Upon hydrolysis, oleuropein can produce elenolic acid, hydroxytyrosol, tyrosol, and glucose [15]. However, particular attention has been paid to hydroxytyrosol [16], which occurs naturally in olive byproducts. This o-diphenol, like the majority of the olive phenols such as tyrosol, has been proven to be a potent scavenger of superoxide anion and hydroxyl radical [17]-[18]. It is endowed with significant antithrombotic, antiatherogenic, and anti-inflammatory activities [19].

This study aimed to evaluate the effect of olive leaves extract on oxidative stress and enzymatic antioxidants in streptozotocin-induced diabetic rats. Furthermore, most of the

reported antioxidant charcteristics of OLE are drawn from in vitro investigations [11], and even those who involved animals or human subjects the antioxidant activity of OLE was demonstrated in a condition at which there is no established oxidative challenge [20]. The results obtained from this study may provide further information on the antioxidative effect of OLE in an animal model of oxidative stress.

II. Materials and Methods

A. Chemicals

STZ and all reagents used for the determination of oxidative indices were purchased from Sigma chemicals (St Louis, Mo, USA). Other reagents of analytical grade were obtained from normal commercial sources.

B. Plant authentication and extract preparation

Olive leaves were collected from olive farms and were scientifically approved in Animal Production Department, Faculty of Agriculture, Damanhur University. The leaves were cleaned, shed dried at room temperature then ground with a blender. Dried and ground leaves were submitted to extraction with ethanol in Soxhlet apparatus for 72 hours. After extraction, the solvents were filtered then evaporated. The olive leaves extract was suspended in distilled water and administered orally (0.5 g/kg body weight[21].

C. Animals

Forty male wistar albino rats (100-150 g) obtained from Department of Animal Science, Faculty of Science, Tanta University. were used for the study. They were kept in rat cages in well ventilated house, temperature of 27 – 30 ^{o}C, 12 h natural light and 12 h darkness, with free access to tap water and dry rat pellet. They were allowed to acclimatize for 15 days prior to the experiment. All animals received humane care in compliance with the institution's guideline and criteria for humane care as outlined in the National Institute of Health Guidelines for the Care and Use of Laboratory Animals [22]. Treatment of the animals was in accordance with the Principles of Laboratory Animal Care. Rats were divided into four equal groups of 10 rats each. The first group used as control group and this received 1.0 ml of physiological saline orally daily. The second group was injected Intra peritoneal (i.p) by Streptozotocin, (STZ) at a single dosage of 45 mg/kg b. wt. dissolved in citrate buffer (pH 4.5) [23]. The third group was gastro-gavaged with 0.5 g/kg body weight [21] of OLE orally on daily basis. The fourth group was injected i.p by Streptozotocin, (STZ) at a single dosage of 45 mg/kg b. wt. and gastro-gavaged with 0.5 g/kg of OLE orally on daily basis after 3 days of STZ injection (after induction of experimental diabetes). The experimental period was extended to thirty day.

D. Samples

At the end of the experimental period, fasted control and other thee groups were anesthetized under diethyl ether, the heparinized venous blood was collected from orbital venous sinus and centrifuged at 3000 rpm for 10 min to separate the plasma from the erythrocytes. Plasma was stored at -80 °C to determine. Plasma lipid peroxides as malondialdehyde (MDA) were measured spectrophotometrically after the reaction with thiobarbituric acid [24]. Plasma reduced glutathione (GSH) was assayed by Spectrophotometric technique; the method is based on reductive cleavage of 5.5. dithiobis 2-nitrobezoic acid (DTNB) by sulphydryl group to yield yellow colour with maximum absorbance at 412 nm [25]. Plasma glucose level determined according to [26]. Plasma insulin level was assayed according to the method of [27]. Plasma triglyceride concentration was determined according to the method of [28]. Plasma total cholesterol concentration was estimated according to the method of [29]. Plasma LDLc concentration was determined according to [30]. Plasma HDLc concentration was measured according to the method of [31].

To obtain packed erythrocytes, the remaining erythrocytes washed repeatedly with an isotonic solution of NaCl (0.9%) until a colorless supernatant was observed. To obtain erythrocyte hemolysate, 500 µl packed erythrocyte were destroyed by addition of four volumes of cold redistilled water. The resulting suspension was centrifuged twice to eliminate all of the cell membranes: first for 10 min in the tube centrifuge at 3500 rpm at 4°C, then in an Eppendorf centrifuge at 7800 rpm for 5 min at 4°C [32]. Clear supernatant was obtained as hemolysate to determine. The activity of glutathione peroxidase (GPx) was determined chemically using cumene hydroperoxide as substrat[33]. Catalase activity (CAT) was determined according to the method of [34]. Superoxide dismutase activity (SOD) was determined according to the method of [35].

E. Statistical analysis

The results are expressed as Mean ± SE. Analysis of data was performed by one-way analysis of variance (ANOVA). P value less than 0.05 was considered statistically significant.

III. Results

A. Changes in plasma MDA, GSH concentration and erythrocyte antioxidant enzymes activities of normal and STZ-diabetic rats

All diabetic rats had a significant increase in oxidative stress after induction of diabetes as judged by the significant increase of oxidative stress marker MDA, significant decrease of GSH and antioxidant enzymes. Table 1 demonstrates the differences in plasma MDA, GSH and erythrocyte GPx, CAT and SOD activities. A significant decrease of plasma MDA and significant increase in plasma GSH and erythrocyte GPx, CAT and SOD were observed in OLE-treated group compared with diabetic control group.

B. Changes in blood glucose, insulin and lipid profiles of normal and STZ-diabetic rats

Table II illustrates the variation in blood glucose, insulin and lipid profiles of normal control, diabetic control and OLE-treated rats during 30 day period of study.

The levels of blood glucose, cholesterol, triacylglycerol and LDLc were significantly decreased while plasma insulin concentration and HDLc were significantly increased in OLE-treated rats as compared with diabetic control rats who continued to exhibit elevated glucose levels and lipid profiles except HDLc throughout the study period.

TABLE I
EFFECT OF STZ (45MG/KG I.P.) AND OLE (0.5G/KG), ON PLASMA MDA, GSH LEVEL AND ERYTHROCYTE GPX, CAT AND SOD ACTIVITIES OF RATS

Groups	Plasma MDA (nmol /g protein)	Plasma GSH (µmol/g protein)	Erythrocyte GPx(IU/gm Hb)	Erythrocyte CAT (K/Sec/g Hb)	Erythrocyte SOD (U/ g Hb)
Control	33.88±1.91b	34.73±0.35b	0.329 ± 0.017a	0.166 ± 0.014b	1.538 ± 0.127a
STZ	50.64±2.47a	13.45±0.43d	0.221 ± 0.013c	0.118 ± 0.012d	1.113 ± 0.113c
OLE	18.05±1.91c	47.87±0.26a	0.332 ± 0.017a	0.189 ± 0.016a	1.539 ± 0.132a
STZ+OLE	37.87±2.47b	24.65±0.44c	0.298 ± 0.033b	0.141 ± 0.031c	1.417 ± 0.151b

Means within the same column carrying different letters are significantly different (P<0.05).

TABLE II
EFFECT OF STZ (45MG/KG I.P.) AND OLE (0.5G/KG), ON PLASMA GLUCOSE, INSULIN, CHOLESTEROL, TRIGLYCERIDE LDLC AND HDLC CONCENTRATION OF RATS

Groups	Glucose (mg/dl)	Insulin (µIU/ml)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	LDLc (mg/dl)	HDLc (mg/dl)
Control	119.00 ± 1.31c	18.84± 0.56a	156.12±2.66c	129.12±2.43c	113.03±2.43c	28.43±1.72b
STZ	377.12 ± 3.04a	9.80± 0.32bc	266.12±2.55a	189.62±3.23a	194.54±3.34a	16.06±0.64c
OLE	96.50 ± 1.53d	19.03±1.13a	132.12±2.56d	127.87±2.43c	82.43±2.43d	35.12±1.53a
STZ+OLE	182.62 ±3.24b	11.07±0.35b	187.62±3.76b	171.62±3.24b	142.06±4.54b	33.24±1.36ab

Means within the same column carrying different letters are significantly different (P<0.05).

IV. Discussion

Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. The various antioxidants exert their effect by scavenging superoxide, or by activation of a battery of detoxifying/defensive proteins. Recently, much attention has been focused on antioxidants in food that are potential compounds for preventing diseases caused by oxidative stress including diabetes because of their distinctive biological activity and low toxicity. In fact, previous studies reported that scavengers of oxygen radicals are effective in preventing diabetes in experimental animal models [36]. Furthermore, diabetes can be produced in animals by intraperitoneal injection of alloxan and streptozotocin, which are toxic to β-cells and are widely used for such purposes. This induction produces active oxygen species responsible for diabetes complications [37].

The result postulated in Table (1) revealed that, the injection of STZ significantly decreased erythrocyte enzymatic antioxidant activities such as GPx, SOD and CAT as well as plasma GSH respectively and increased TBARS level in the plasma of diabetic rats. Our results are in agreement with other findings showing that hyperglycemia is accompanied with an increase in marked oxidative impact as evidenced by the significant increase in hepatic lipid peroxidation resulting in the formation of TBARS and a significant decrease in hepatic antioxidants enzymes [38].

[36]-[39] concluded that, rats treated with STZ showed a significant increase in lipid peroxidation and significant decrease in the activities of CAT, SOD and GPx in liver and kidneys compared with controls. The same authors revealed that, the increased lipid peroxidation observed in DM returned to increased oxidative stress due either to (hyperglycemia or Streptozotocin which gives rise to oxygen free radicals). The antioxidant enzymes are known to be inhibited in diabetes mellitus as a result of increased ROS in DM and non-enzymatic glycation of these enzymes due to persistent hyperglycemia. [39]-[40].

On the other hand, the present results disagree with those obtained by [41] who found that, no difference in serum conjugated diene levels between otherwise healthy diabetic patients and healthy control subjects. Also, TBARS levels in both poorly and well controlled type II DM patients did not differ from control subjects, whereas hydroxyl radical formation was elevated in DM patients [42]. Moreover, plasma TBARS levels were similar in type 1 DM and type 2 DM patients as in control subjects [43]. Furthermore, baseline lipid hydroperoxide levels were similar in 75 subjects with normal glucose tolerance, impaired glucose tolerance, and type 2 DM [44]. These controversial data might be due to difference of study subjects, duration of diabetes or differences in methodology and study design. The present results disagree also with [45] who revealed that, increased blood GSH levels in the DM men could represent an adaptive response to increased oxidative stress mediated possibly in part through increased red cell GRD activity.

All of these found perturbations in the antioxidant system were restored by the administration of OLE, table 2. The olive leaf extract known to be efficient antioxidants in vivo [9] as well as invitro [46]. The administration of oleuropein and hydroxytyrosol-rich extracts improved the antioxidant status in liver [47]. OLE administered to rats prior to stress induction attenuated the inhibition of SOD and CAT activity and, thus, additionally implicated its role in the modulation of the oxidative balance in liver [48]. OLE supplementation to aged males rabbits, significantly increased blood plasma of glutathione s-transferase (GST) activity and Superoxide dismutase (SOD) activity and decreased blood plasma of Thiobarbituric acid reactive substances [49]. In fact, several studies show [50]that polyphenolic substances increased the expression of SOD and CAT enzymes at the transcriptional level. The same authors revealed that, these antioxidants could inactivate the circulating free radicals that quench NO before it reaches pancreatic β-cells, where they induced their damage and/or death. Also, phenolic compounds of OLE has been shown to be scavengers of superoxide anions and inhibitors of the respiratory burst of neutrophils and hypochlorous acid derived radicals [13]. Moreover, similar results were achieved by [51], who suggested that olive leaf extract is effective in scavenging radicals and protecting lipid oxidations. Furthermore,[52] showed that, the combination of olive leaf extract phenolics possessed antioxidant and antimicrobial activities. The positive impact of treatment with OLE on these enzymes observed in the present study could be explained with two possible mechanisms. First, the antioxidative effect of OLE may prevent further glycosylation and peroxidation of proteins by interacting with free radicals and hence minimizing their noxious effects. Second, OLE may induce protein synthesis of these enzymes that explains the observed elevated activity after treatment. In support with this view is the observation of [53] who found that oleuropein increased the expression of glutathione-related enzymes at the transcriptional level. Olive leaf extract was also shown to have a modulatory effect on the expression of the enzyme SOD in response to oxidative stress in vitro [54]. Also, in diabetes mellitus, various hypoglycemic agents reduce oxidative stress indirectly by lowering blood glucose level and preventing hyper insulinemia and directly by acting as free radical scavengers. This study demonstrated another potential and beneficial effect of OLE in attenuating oxidative stress and enhancing of body's own antioxidant defenses in diabetic rats with established oxidative stress and may add another explanation of the hypoglycemic effect of OLE through its action as an antioxidant. Our results could be useful to elucidate one of the polyphenolic mechanisms in glucose metabolism regulation. OLE acts as an antioxidant at both prevention and intervention levels. Prevention of free radicals formation by OLE may occur through its ability to chelating metal ions, such as Cu and Fe, which catalyze free radical generation reactions [55], and through its inhibitory effect on several inflammatory enzymes like lipoxygenases [56]. Intervention of OLE with already present free radicals may come about through providing hydroxyl group to directly neutralize and quench free radicals [13]….

The data summarized in Table (2) revealed that, STZ injection caused significant increase in serum glucose, Ch, TG, LDL-c and decreased HDL-c and insulin level. The present

findings come in accordance with those obtained by [57] who reported that, significant increase in blood sugar and decrease in insulin concentrations were recorded in STZ induced diabetic rabbits compared to control rabbits. The same authors reported that, the increased in blood glucose and decreased in insulin concentrations reflect abnormalities in beta cell function induced by STZ. Moreover, [58] reported that, diabetic patients were characterized by significant increase in lipid profile except HDL cholesterol which is decreased has been found as compared to controls. The same authors revealed that, the high levels of total cholesterol appear due to increased cholesterol synthesis, the triglyceride levels may be increased due to overproduction of vLDL-TG, also insulin increases the number of LDL receptor, so chronic insulin deficiency might be associated with a diminished level of LDL receptor, this causes the increase in LDL particles and result in the increase in LDL-cholesterol value in diabetic patients.

Our results showed that OLE had significant hypoglycemic, hypolipidemic effects in STZ-induced diabetic rats, table 2. In agreement with the present results, [47]-[59] reveled that, the administration of oleuropein- and hydroxytyrosol-rich extracts significantly decreased the serum glucose and cholesterols. The eventual mechanism responsible of the hypoglycemic activity of OLE may result from a potentiation of glucose-induced insulin release or increased peripheral uptake of glucose [60]. The inhibitory action of an ethanol extract of olive leaves (OEE) on the activities of amylases might be the other mechanism of its hypoglycemic effects in which, two anti-α-amylase components were purified from a 50% ethanol soluble fraction of OEE using Sephadex LH-20 column chromatography. One was identified as luteolin-7-*O*-β glucoside and the other as luteolin-4′-*O*-β glucoside [61].

Also, our study indicated that OLE can decrease the total cholesterol levels in diabetic rats, which is important in preventing or treating the complications of diabetes. Moreover, the previous studies confirmed the hypocholesterolemic effects of olive tree byproducts such as phenolics [62]. This has clinical implications in as much as olive leaves extract, if used as hypoglycemic agents, may also reverse hypercholesterolemia associated with diabetes and prevent the cardiovascular complications which are very prevalent in diabetics. Furthermore, the levels of plasma lipids are usually raised in diabetes, and such an elevation represents a risk factor for cardiovascular disease [63]. Consistently, in agreement with our results, other studies have reported that Oleaeuropaea has hypolipidemic effects in diabetic rats [64]. The current results are in agreement also with findings obtained by [21], who noted that the olive leaves significantly decreased triglyceride and cholesterol. The major constituent of the olive leaves is oleuropein [11]-[51].

[65] found that the addition of oleuropein to the standard diet reduces plasma levels of total cholesterol and increases the ability of low density lipoprotein (LDL) to resist oxidation in the rabbits. On the same line, [66] noted that treatment with oleuropein reduced total cholesterol and triglyceride concentrations, and reduced circulating lipids. Also, [67] reported that the oleuropein exert potent antioxidant activities, such as inhibition of low density lipoproteins oxidation and free radical scavenging. Administration of olive leaf extract caused a significant decrease in blood levels of glucose, cholesterol and triglycerides, whereas an increase in insulin and HDL-C levels was seen, with no significant changes in LDL-c values in diabeti.[68] OLE supplementation to aged males rabbits, significantly decreased plasma cholesterol and triglyceride [49].

The mechanism of this hypocholesterolaemic action may be due to inhibition of the absorption of dietary cholesterol in the intestine or its production by the liver [69] or stimulation of the biliary secretion of cholesterol and cholesterol excretion in the feces [70]. Also OLE enriched with oleic acid (mono unsaturated fatty acids that accelerate cholesterol metabolism in which cholesterol esters of unsaturated fatty acids is easily and rapidly metabolized than esters of saturated fatty acid. [71] found that, the role of polyphenols in reducing the risk of coronary heart disease based on the antioxidant activity of these compounds.

V. Conclusion

We demonstrate that olive leaf extracts, exhibited a pronounced hypoglycemic and hypolipidemic effects, reduced the lipid peroxidation process, and enhanced the antioxidant defense system in an experimental diabetic rat model. These effects highlighted once again the olive tree byproduct as a source of antioxidants able to reduce the frequency of oxidative stress-related metabolic diseases such as diabetes.

According to the biochemical parameters, the first step has been made in that it was shown that OLE synchronized antioxidant enzymes and inhibited lipid peroxidation in RBCs hemolysate. Thus, this effect is worthy of further investigation of its potential in the regulation of cellular signaling, gene expression and protein synthesis; in one word, investigation at the molecular level.

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