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Hypoglycemic and antioxidant effect of oleuropein in alloxan-diabetic rabbits

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Abstract

Patients with diabetes mellitus are likely to develop certain complication such as retinopathy, nephropathy and neuropathy as a result of oxidative stress and overwhelming free radicals. Treatment of diabetic patients with antioxidant may be of advantage in attenuating these complications. Oleuropein, the active constituent of olive leaf (*Olea europaea*), 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 oleuropein in reducing oxidative stress and hyperglycemia in alloxan-induced diabetic rabbits. After induction of diabetes, a significant rise in plasma and erythrocyte malondialdehyde (MDA) and blood glucose as well as alteration in enzymatic and non-enzymatic antioxidants was observed in all diabetic animals. During 16 weeks of treatment of diabetic rabbits with 20 mg/kg body weight of oleuropein the levels of MDA along with blood glucose and most of the enzymatic and non-enzymatic antioxidants were significantly restored to establish values that were not different from normal control rabbits. Untreated diabetic rabbits on the other hand demonstrated persistent alterations in the oxidative stress marker MDA, blood glucose and the antioxidant parameters. These results demonstrate that oleuropein may be of advantage in inhibiting hyperglycemia and oxidative stress induced by diabetes and suggest that administration of oleuropein may be helpful in the prevention of diabetic complications associated with oxidative stress.

Keywords: Oleuropein; Olive leaf; Diabetes; Alloxan; Rabbits; Antioxidant; Hypoglycemic

Introduction

Diabetic patients, both type 1 and 2, exhibit abnormal antioxidant status, auto-oxidation of glucose, and excess glycosylated proteins (Jones et al., 1985; Ceriello et al., 1991; Young et al., 1992; Mak et al., 1996). Oxidative stress in diabetes leads to tissue damage, with lipid peroxidation, inactivation of proteins, and protein glycation as intermediate mechanisms (Wolffe et al., 1991) for complications including retinopathy, nephropathy, and coronary heart disease (Oberley, 1988; Jennings et al., 1991; Lyons, 1991; Valezquez et al., 1991). It has been postulated that supplementation with dietary antioxidant compounds, such as ascorbic acid and vitamin E may offer some protection against these complications through their roles as inhibitors of glycation and as free radical scavengers (Davie et al., 1992; Sinclair et al., 1992). Recent interests were also in the use of non-vitamin antioxidants such as flavonoids and polyphenols in reducing the negative effect of oxidative stress and free radicals in diabetic patients (Lean et al., 1999; Asgary et al., 2002).

Olive tree, botanically known as *Olea europaea*, and its products have been recognized as important components of a healthy diet because of their phenolic content (Visioli et al., 2002). Large body of epidemiological studies has shown that the incidence of coronary heart disease (CHD) and certain cancers, e.g., breast and colon cancers, is lowest in the Mediterranean basin where the diet is rich in olives and olive products (Keys, 1995). The principal active component in olive leaf extract is oleuropein, a natural product of the secoiridoid group (Fig. 1). Upon hydrolysis, oleuropein can produce other bioactive substances, namely elenolic acid and 3, 4-dihydroxy-phenylethanol (hydroxytyrosol). Studies have

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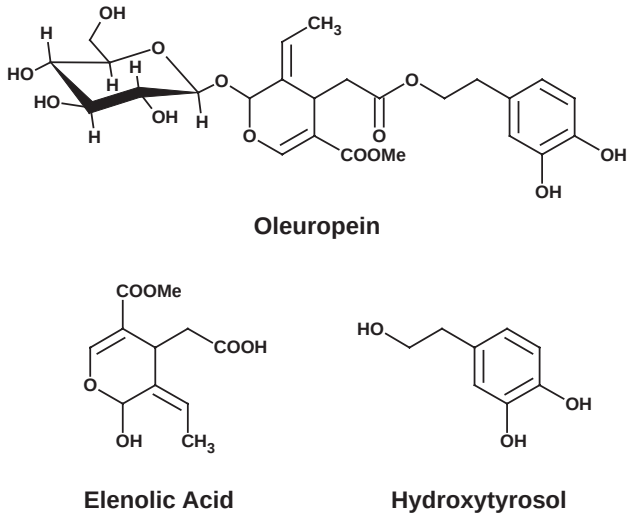

Fig. 1. Structure of oleuropein and its metabolites elenolic acid and hydroxytyrosol.

shown that oleuropein possesses a wide range of pharmacologic and health promoting properties including antiarrhythmic, spasmolytic, immune-stimulant, cardioprotective, hypotensive, and anti-inflammatory effects (Petkov and Manolov, 1978; Visioli et al., 1995, 1998; Diaz et al., 2000; Somova et al., 2004). Many of these properties have been suggested as a result from the antioxidant character of oleuropein (Visioli et al., 2002). Previously, oleuropein reported to have an antihyperglycaemic effect in diabetic rats (Gonzalez et al., 1992). However, regarding the antioxidant feature of oleuropein, it is still unknown if oleuropein may exert other beneficial effects in diabetes as in attenuating oxidative stress.

This study aimed to evaluate the effect of oleuropein obtained from olive leaves on oxidative stress and enzymatic and non-enzymatic antioxidants in alloxan-induced diabetic rabbits. Furthermore, most of the reported antioxidant characteristics of oleuropein are drawn from in vitro investigations (Amro et al., 2002; Stupans et al., 2002; Ferroni et al., 2004), and even those who involved animals or human subjects the antioxidant activity of oleuropein was demonstrated in a condition at which there is no established oxidative challenge (Visioli et al., 2000). The results obtained from this study may provide further information on the antioxidative effect of oleuropein in an animal model of oxidative stress.

Materials and methods

Material

Experiments were carried out on *O. europaea* leaves of Labeeb variety commonly cultivated in different areas of Iraq. Leaves were obtained by handpick and collected at the end of March. Panasonic 1330 microwave oven was used for the drying of leaves three times for 2 min at maximum power (2680 W). Dried leaves were powdered and stored in a dry and dark place.

Extraction of oleuropein

Oleuropein was extracted from dried leaves of *O. europaea* according to Savournin et al. (2001). In brief, 500 g of powdered leaves were extracted with 75% ethanol. After evaporation of ethanol the aqueous phase was extracted with chloroform followed by ethyl acetate. The resulting solution was evaporated to dryness to obtain 23.5 g dried extract. 6.0 g of the extract were separated by column chromatography using 200 g silica gel 60 (Merck, England) and eluted with 1 l of chloroform–methanol (9:1), then with 400 ml of chloroform–methanol (4:1). Combined fractions gave 5.0 g oleuropein. Identification was established using TLC, UV, IR and NMR, and found to be comparable to that of authentic standards reported in literature (Savournin et al., 2001). Oleuropein content in the extract was 82%.

Animals and diets

All animals used in this work were male New Zealand rabbits purchased from Al-Razzi Research Center for Diagnostic Kits in Baghdad. Male rabbit (850–1000 g weight) were used at 3 months of age. Groups of rabbits were housed at room temperature with a lighting schedule of 12 h light and 12 h dark. Animals had free access to a standard pellet diet and tap water ad libitum. Rabbits were categorized into three groups, 8 animals each: healthy animals as controls (C), control diabetic rabbits that received no treatment (CD), and diabetic rabbits supplemented with oleuropein (OLE). Rabbits were rendered diabetic by treatment with alloxan intravenously in a dose of 150 mg/kg, and those who exhibited significant elevation in blood glucose above 200 mg/dl were used in the subsequent procedure. Diabetic animals of both CD and OLE groups were then given placebo and OLE, respectively. A daily oral dose of 20 mg/kg of OLE was used in the treatment group for the next 16 weeks. Blood glucose, body weight and fluid intake of all animals were recorded at weekly interval. The study was performed in accordance of the International Guidelines regarding animal experiment.

Blood sampling

Blood samples were drawn in the fasting state and processed within 1 h of collection. Samples were centrifuged for 5 min at 1500 *g*, then plasma and buffy-coat were removed by aspiration. Erythrocytes were washed thrice with phosphate buffered saline (PBS) of pH 7.4 (0.02 M phosphate; 0.123 M NaCl). The packed erythrocyte volume (PCV) after the final wash was used for the assay of glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GRx), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA). Plasma was stored at −20 °C and used later for the other biochemical parameters.

Analytical procedures

A standard enzymatic method using glucose oxidase from commercially available kits was performed for the determination of glucose. MDA was assayed according to the method of Ohkawa et al. (1979) with minor modification, being butylated hydroxytoluene used in the assay system to prevent the alteration in lipid peroxidation during heating. In brief, 0.1 ml of plasma or erythrocyte suspension was transferred to screwed-cap Pyrex tube. 0.1 ml of 8.1% SDS, 0.75 ml of 20% acetic acid solution and 0.75 ml of 0.8% aqueous solution of TBA were then added to the sample. All samples included butylated hydroxytoluene (BHT) (10 μl of 1% [w/v] BHT in absolute alcohol). The mixture was made up to 2.0 ml with D.W., homogenized, and heated in a water bath at 95 °C for 60 min. After cooling, 2.5 ml of *n*-butanol/pyridine (15:1) and 0.5 ml D.W. were added, vortex vigorously and centrifuged at 3000 rpm for 15 min. The TBA-RS was measured by following the increase in absorbance at 532 nm in the *n*-butanol/pyridine phase. Erythrocyte GSH was assayed with the method of Beutler (1975). Plasma α-tocopherol and β-carotene were determined by RP-HPLC with ultraviolet detection at 280 nm and with visible detection at 435 nm, respectively (Bieri et al., 1979, 1985). Serum vitamin C was measured colorimetrically according to the method of Liu et al. (1982) using a commercially available kit (Randox, England). The activities of erythrocyte GPx, GRx, SOD, and CAT were assayed as described elsewhere (Paglia and Valentine, 1967; Misra and Fridovich, 1972; Lee et al., 1975; Aebi, 1987). Hemoglobin concentration was obtained for erythrocyte suspension and whenever needed with Drabkin's method. Assays of GSH, MDA and enzymes were performed within 2 h of blood sampling.

Statistical analysis

The Student's *t*-test (unpaired) was used to analyze the significance of differences between control and the experiment groups. All results are presented as mean ± SD unless otherwise stated. Changes in blood glucose, enzymatic antioxidant activity and non-enzymatic antioxidant activity levels calculated as the difference between pre- and post-treatment values were carried out with paired Student *t*-test. A p value of 0.05 was chosen as the level of significance.

Results

Changes in blood glucose, body weight and fluid intake of normal and alloxan-diabetic rabbits

Fig. 2 illustrates the variation in blood glucose, body weight and fluid intake of normal control, diabetic control and oleuropein-treated rabbits during 16 weeks period of study. The levels of blood glucose were significantly decreased in oleuropein-treated rabbits after initiation of treatment, being strictly apparent at week 8 as compared with diabetic control rabbits who continued to exhibit elevated glucose levels throughout the study period (Fig. 2A).

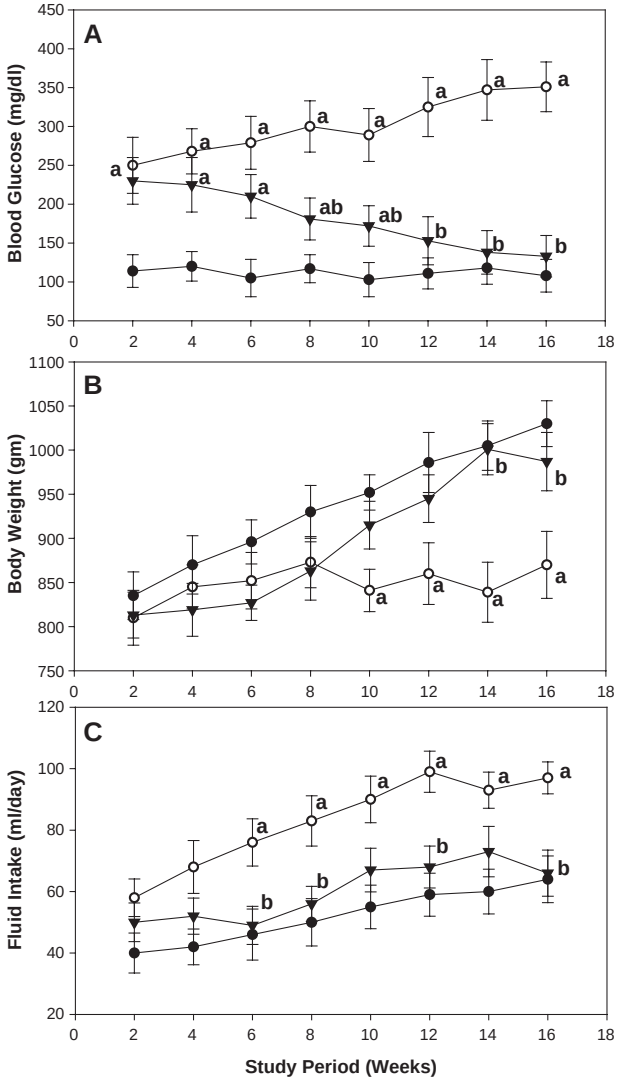

Fig. 2. Changes of plasma glucose (A), body weight (B) and fluid intake (C) in normal control (⋆), diabetic control (>) and oleuropein-treated (▲) rabbits. Data represent mean ± SD of eight rabbits in each group. Significance is indicated from the normal (a) and diabetic control (b) groups at $p < 0.05$.

Both normal rabbits and oleuropein-treated diabetic group showed a constant augmentation in body weight during the 16 weeks period, while no change was observed in that of untreated diabetic rabbits (Fig. 2B).

As shown in Fig. 2C, all animal groups demonstrated a continuous rise in fluid intake during the 16 weeks of the study with the highest levels seen in diabetic control rabbits.

Changes in plasma and erythrocyte MDA of normal and alloxan-diabetic rabbits

All diabetic rabbits had a significant increase in oxidative stress after 20 days of induction of diabetes as judged by the oxidative stress marker MDA. Fig. 3 demonstrates the differences in plasma and erythrocyte MDA levels before and

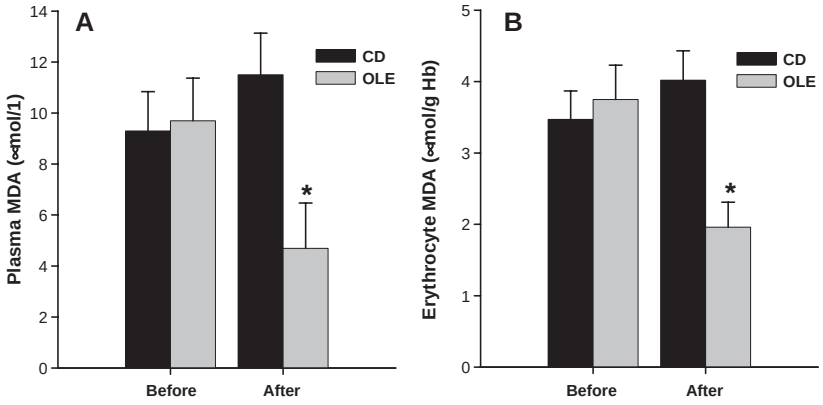

Fig. 3. Differences in plasma (A) and erythrocyte (B) MDA of diabetic rabbits after 16 weeks of treatment. Data represent the mean ± SD of eight rabbits in each group. *$p < 0.05$ as compared with basal value.

after 16 weeks of treatment of diabetic rabbits. A significant decrease of both plasma (Fig. 3A) and erythrocyte (Fig. 3B) MDA was observed in oleuropein-treated group compared with no change in diabetic control group. The variation in plasma and erythrocyte MDA levels during the sixteen weeks of study is presented in Fig. 4. Oleuropein-treated rabbits had a gradual decline in plasma (Fig. 4A) and erythrocyte (Fig. 4B) MDA to reach a significant difference at week 10 as compared with untreated diabetic rabbits. On the other hand, untreated diabetic rabbits had their plasma and erythrocyte MDA constantly and significantly elevated as compared with normal control group (Fig. 4).

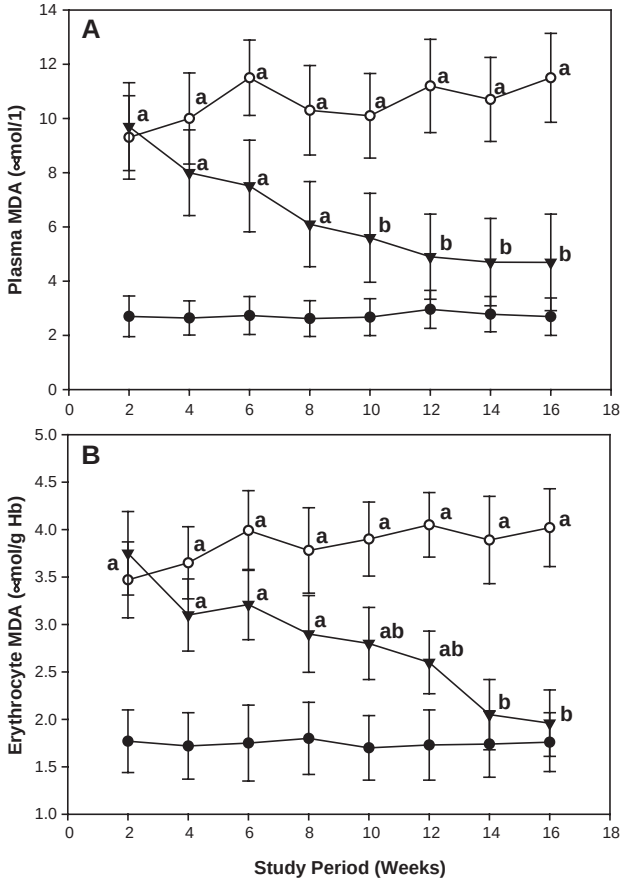

Fig. 4. Changes of plasma (A) and erythrocyte (B) MDA in normal control (⋆), diabetic control (>) and oleuropein-treated (▲) rabbits. Data represent mean ± SD of eight rabbits in each group. Significance is indicated from the normal (a) and diabetic control (b) groups at $p < 0.05$.

Changes in enzymatic and non-enzymatic antioxidants of normal and alloxan-diabetic rabbits

The activities and levels of enzymatic and non-enzymatic antioxidants of all animal groups are presented in Table 1, respectively. After induction of diabetes and before initiation of treatment all diabetic rabbits had a significant decrease in the activities and levels of all the studied parameter, with the exception of SOD being significantly elevated, as compared with normal rabbits. Treatment with oleuropein for sixteen weeks resulted in a significant restoration in most of the evaluated parameters to values that were not different from that of normal control animals. However, although significantly increased after oleuropein treatment, plasma levels of α-tocopherol and β-carotene were still lower than that of normal controls. On the other hand, the mean (±SD) values of all of the enzymatic and non-enzymatic antioxidants remained significantly aggravated among untreated diabetic rabbits.

Discussion

An accumulating body of evidence has been showing that diabetic patients manifest oxidative stress resulting from hyperglycemia, hyperinsulinemia and insulin resistance. Overwhelming free radicals generated due to oxidative stress may develop several adverse effects commonly seen in diabetes mellitus such as neuropathy, nephropathy, retinopathy and vascular disorders (Oberley, 1988; Jennings et al., 1991; Lyons, 1991; Valezquez et al., 1991). As a strategy to counteract the negative effect of oxidative stress, antioxidant-based therapy is promising to minimize the complications associated with oxidative stress in diabetes mellitus (Davie et al., 1992; Sinclair et al., 1992; Lean et al., 1999; Asgary et al., 2002). Recent observations have shown that many of these complications are diminished upon supplementation with certain dietary

Table 1
Mean (±SD) values of enzymatic and non-enzymatic antioxidants of control rabbits, and diabetic control (DC) and oleuropein-treated (OLE) rabbits before and after 16 weeks of supplementation with placebo and 20 mg/kg oleuropein, respectively

Parameter[a]	Control ($n=8$)	DC ($n=8$)		OLE ($n=8$)	
		Week 0	Week 16	Week 0	Week 16
GPx (U/g Hb)	12.5±2.50	8.34±1.63[b]	7.55±2.08[a]	7.55±1.92[a]	10.5±1.74[c,d]
GRx (U/g Hb)	4.40±0.27	2.58±0.29[a]	2.66±0.34[a]	2.71±0.25[a]	3.56±0.32[b,c]
SOD (U/g Hb)	0.22±0.05	0.46±0.04[a]	0.44±0.07[a]	0.44±0.08[a]	0.25±0.04[b,c]
CAT (U/g Hb)	1.12±0.30	0.69±0.16[a]	0.66±0.15[a]	0.66±0.12[a]	0.85±0.23[b,c]
GSH (μmol/g Hb)	4.55±0.50	2.31±0.34[a]	2.09±0.37[a]	2.25±0.30[a]	3.95±0.45[b,c]
α-Tocopherol (μM)	9.0±2.80	4.2±2.63[a]	3.6±2.45[a]	4.7±1.61[a]	6.7±1.37[a,b,c]
Ascorbic acid (μM)	23.8±6.81	11.3±6.24[a]	12.5±7.38[a]	13.6±7.57[a]	19.8±7.49[b,c]
β-Carotene (μM)	0.76±0.07	0.35±0.06[a]	0.34±0.09[a]	0.36±0.08[a]	0.55±0.09[a,b,c]

[a] GPx: glutathione peroxidase, GRx: glutathione reductase, SOD: uperoxide dismutase, CAT: catalase, GSH: glutathione.
[b] $p<0.05$ as compared with control.
[c] $p<0.05$ as compared with Week 16 (between groups).
[d] $p<0.01$ as compared with Week 0 (within group).

antioxidants such as vitamin E, C, and α-lipoic acid (Davie et al., 1992; Sinclair et al., 1992). The use of other non-nutrient antioxidants such as flavonids and polyphenols has been reported with the same advantage (Lean et al., 1999; Asgary et al., 2002).

Oleuropein, the active principle of olives, is a phenolic compound which has been shown to possess diverse healing properties for its vasodilatory (Petkov and Manolov, 1978), hypotensive (Ribeiro Rde et al., 1986; Khayyal et al., 2002), anti-inflammatory (Diaz et al., 2000), anti-rheumatic (Visioli et al., 1998), diuretic (Ribeiro Rde et al., 1986), anti-atherogenic (Visioli and Galli, 2001) and antipyretic (Visioli et al., 1995) effects. Many of these pharmacologic features of oleuropein are due to its potent antioxidant action (Visioli et al., 2002). Oleuropein acts as an antioxidant at both prevention and intervention levels. Prevention of free radicals formation by oleuropein may occur through its ability to chelating metal ions, such as Cu and Fe, which catalyze free radical generation reactions (Andrikopoulos et al., 2002), and through its inhibitory effect on several inflammatory enzymes like lipoxygenases (de la Puerta et al., 1999). Intervention of oleuropein with already present free radicals may come about through providing hydroxyl group to directly neutralize and quench free radicals (Visioli et al., 2002). Oleuropein and its metabolite hydroxytyrosol both possess the structural requirement (a catechol group) needed for optimum antioxidant and/or scavenging activity. Both oleuropein and hydroxytyrosol have been shown to be scavengers of superoxide anions, and inhibitors of the respiratory burst of neutrophils and hypochlorous acid-derived radicals (Visioli et al., 2002). Both compounds also scavenged hydroxyl radicals with oleuropein showing greater activity (Chimi et al., 1991). Hydroxytyrosol and oleuropein are also reported to be effective scavengers of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Gordon et al., 2001).

In diabetes mellitus, various hypoglycemic agents reduce oxidative stress indirectly by lowering blood glucose level and preventing hyperinsulinemia, and directly by acting as free radical scavengers. For example, gliclazide, a sulfonylurea normally used to augment insulin release, is an effective scavenger of superoxide and hydroxyl radicals. Indeed, oleuropein has been reported with an effective hypoglycemic action in diabetic animals (Gonzalez et al., 1992). There have been two mechanisms suggested explaining the hypoglycaemic effect of oleuropein in diabetes: 1) potentiation of glucose induced insulin release and 2) increased peripheral uptake of glucose (Gonzalez et al., 1992). This study demonstrated another potential and beneficial effect of oleuropein in attenuating oxidative stress and enhancing of body's own antioxidant defenses in diabetic rabbits with established oxidative stress and may add another explanation of the hypoglycemic effect of oleuropein through its action as an antioxidant.

As presented in the Result section, by the end of study period most of the evaluated parameters exhibited a significant restoration which was comparable that of normal control. On the other hand, the enzymatic and non-enzymatic antioxidants, as well as the oxidative stress marker MDA remained significantly altered in the diabetic untreated control rabbits.

The antioxidant pool is drastically aggravated in hyperglycemia due to the persistent challenge by reactive oxidants and free radicals (Jennings et al., 1991 Wolffe et al., 1991; Mak et al., 1996). The antioxidant enzymes GPx, GRx, CAT and SOD are known to be inhibited in diabetes mellitus as a result of non-enzymatic glycosylation and oxidation (Lyons, 1991). The positive impact of treatment with crude oleuropein on these enzymes observed in the present study could be explained with two possible mechanisms. First, the antioxidative effect of oleuropein may prevent further glycosylation and peroxidation of proteins by interacting with free radicals and hence minimizing their noxious effects. Second, oleuropein may induce protein synthesis of these enzymes that explains the observed elevated activity after treatment. In support with this view is the observation of Masella et al. (2004) 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 (Madar et al., 2004). However, since mature erythrocytes lack the ability

of protein synthesis, the last explanation may be restricted to young-aged red blood cells and increased erythropoiesis.

As the case with enzymatic antioxidants, non-enzymatic antioxidants such as glutathione, β-carotene and vitamin E and C were also known to be decreased in diabetes (Jennings et al., 1991; Wolffe et al., 1991; Mak et al., 1996). Treatment with crude oleuropein to diabetic rabbits in this study showed a significant restoration in the levels of glutathione, β-carotene and vitamin E and C. There is evidence that elevation in glucose concentration may depress natural antioxidant defense agents like vitamin C and glutathione (Inouye et al., 1999). The observed enhancement in the levels of these antioxidants could be attributed to the sparing effect of oleuropein in competing with free radicals that burden the antioxidative function of these antioxidants. Another factor is that oleuropein has been suggested to provide protection from degradation to vitamin E and C while in intestine (Edgecombe et al., 2000). Such protection could maximize the level of vitamins available for absorption, and hence increase their serum level. It has been shown that oleuropein is rapidly absorbed from the intestine with t_{max} of 2 h reaching a peak of 200 ng/ml of plasma after administration of 20 mg/kg oleuropein in rats (Del Boccio et al., 2003). In addition, oleuropein detected in plasma only in its glycoside form suggests that it is absorbed intact from the intestine. The high availability of oleuropein in its active form in vivo may explain the positive impact on the enzymatic and non-enzymatic antioxidants observed in our study, and may demonstrate similar events to those reported in vitro.

In conclusion, this study demonstrated the beneficial effect of using oleuropein as an effective hypoglycemic and antioxidant agent in alleviating oxidative stress and free radicals as well as in enhancing both enzymatic and non-enzymatic defenses diabetes. The use of oleuropein may be of prophylactic value in reducing the complications usually resulting from oxidative stress in diabetes mellitus.

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