

# The Influence of Naringin on the Oxidative State of Rats with Streptozotocin-Induced Acute Hyperglycaemia

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The effect of various doses (0, 10, 20, 40, or 80 mg/kg body weight) of naringin (a citrus flavonone) was studied on streptozotocin (STZ)-induced hyperglycaemic rats to evaluate the possible hypoglycaemic and antioxidant activity of naringin in diabetes. In comparison to the normoglycaemic group the treatment of rats with a single dose of STZ (65 mg/kg body weight) only revealed a significant increase ( $P < 0.05$ ) in plasma hydrogen peroxide ( $H_2O_2$ ) by 230%, increased the thiobarbituric acid reactive substances (TBARS) as index of the lipid peroxidation level by 69%, while total antioxidant activity was decreased by 36%, with a consistent significant decrease ( $P < 0.05$ ) in the activity of erythrocytes antioxidative enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and paraoxonase (PON). Exogenous administration of individual gradual doses of naringin to hyperglycaemic rats causes a dose-dependent decrease of the glucose level, an increase of the insulin concentration, a decrease of the  $H_2O_2$  and TBARS levels, as well as the increase of the total antioxidant status with an increase of antioxidant enzyme activities (CAT, SOD, GPx, and PON). From this study, it may be concluded that all doses of naringin provided a significant amelioration of hypoglycaemic and antioxidant activity in STZ-induced diabetic rats, however, the greatest effect of naringin was observed at 80 mg/kg body weight.

**Key words:** Naringin, Hyperglycaemia, Antioxidative Enzymes

## Introduction

Diabetes mellitus is the most important disease involving the endocrine pancreas. Its major manifestations include disordered metabolism and inappropriate hyperglycaemia. Currently there are over 150 million diabetics worldwide and this number is likely to increase to 300 million or more by the year 2025 due to increase in sedentary lifestyle, consumption of energy rich diet, and obesity (Yajnik, 2001). While management of diabetes mellitus includes diet, exercise, oral hypoglycaemic agents, and insulin, these treatments do not effectively prevent the complications of diabetes like nephropathy, neuropathy, cataract, and hypertension (Palumbo, 2001).

It is well known that in diabetes oxidative stress has been found to be mainly due to an increased production of oxygen free radicals and a sharp reduction of antioxidant defences catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Oberley, 1988). In addition, there is a relationship between diabetes and impairment of lipid metabolism; high density lipoprotein (HDL) protects low density lipoprotein (LDL) oxidation, and this protection is impaired in diabetic cases (Sharpe *et al.*, 1998). The antioxidant activity of HDL depends on its associated antioxidant enzyme paraoxonase (PON) (Boemi *et al.*, 2001). PON has been shown to protect LDL from oxidative modifications and lipid peroxidation by hydrolyzing biologically active oxidized phospholipids (Watson *et al.*, 1995). Moreover, Boemi *et al.* (2001) reported that less efficient protection mechanisms of PON may be one feature of the greater susceptibility to oxidation in diabetes with a consistent decrease in its diabetes mellitus activity.

In recent years, flavonoids have attracted the interest of researchers because they show promise of being powerful antioxidants that can protect the human body from free radicals and against oxidative stress (Bors *et al.*, 1996). Flavonoids cannot be produced by the human body and have taken in through the daily diet. The evidence reported that flavonoids play a vital biological role, including the function of scavenging reactive oxygen species (Pietta and Simonetti, 1998). Chemically, there are

three features that confer on flavonoids their remarkable antioxidant properties (Rice-Evans *et al.*, 1997): the hydrogen donating substituents (hydroxyl groups) attached to the aromatic ring structures of flavonoids, which enable the flavonoids to undergo a redox reaction that helps them to scavenge free radicals more easily; a stable delocalization system, consisting of aromatic and heterocyclic rings as well as multiple unsaturated bonds, which helps to delocalize the resulting free radicals; and the presence of certain structural groups which are capable of forming transition metal-chelating complexes that can regulate the production of reactive oxygen species such as hydroxyl radicals and oxygen radicals.

Naringin (glycoside) is the predominant flavonone found in grapefruit and other related *Citrus* species (Swiader and Zarawska, 1996). Like most flavonoids, naringin has metal-chelating, antioxidant and free radical scavenging properties (Chen *et al.*, 1990) and has been reported to offer some protection against lipid peroxidation (Maridonneau-Parini *et al.*, 1986). In addition, naringin has a wide range of biological actions. It is the potent cholesterol-lowering agent (Jeon *et al.*, 2001), anti-atherogenic agent (Lee *et al.*, 2001), and antimutagenic agent (Calomme *et al.*, 1996). Although, the previous beneficial effects of naringin have been established, little attention has been given to the role of naringin in hyperglycaemic conditions.

As it is necessary to look for an economical as well as therapeutically effective treatment for usage in the developing and under-developed countries, diabetologists have been engaged in finding natural product extracts for the treatment of hyperglycaemia. The present study was undertaken to determine whether naringin administration might alter the levels of lipid peroxidation and antioxidative enzymes in rats with streptozotocin-induced acute hyperglycaemia in an attempt to assess the possible therapeutic action of naringin in lowering blood glucose and increasing insulin levels.

## Materials and Methods

### Animals

The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland. Healthy, adult male Sprague-Dawley rats with an average body weight of  $190 \pm 20$  g obtained from the animal house of National Research Centre, Egypt were

used for the study. The animals were housed at room temperature ( $25 \pm 3$  °C) with a 12 h reverse light cycle and had free access to water and food (standard laboratory diet).

### Experimental design

The rats were divided into two experimental groups. (A) The normoglycaemic group received one dose (1.0 ml) *via* intraperitoneal (i.p.) injection of 0.1 M citrate buffer, pH 4.5. (B) The hyperglycaemic group induced to develop diabetes mellitus by a single i.p. injection of the diabetogenic agent streptozotocin (STZ; from Sigma Chemical Co., St. Louis, MO, USA) in a dose of 65 mg/kg body weight. STZ was dissolved in 1.0 ml of 0.1 M citrate buffer, pH 4.5 (Bell and Hye, 1983). After 72 h tail blood samples were obtained from each rat, and the blood glucose concentration was determined spectrophotometrically using Roche Diagnostics kits (Germany). STZ treated rats having blood glucose below 300 mg/dl were excluded from the study (Mayhan *et al.*, 1991).

Each group (A and B) was divided into two subgroups. The control subgroup received 1.0 ml physiological saline i.p. The naringin group was injected with naringin (Sigma Chemical Co., St. Louis, MO, USA), each a single i.p. dose of 10, 20, 40, or 80 mg naringin/kg body weight dissolved in physiological saline.

After 24 h the rats were anaesthetized by i.p. injection with sodium-5-ethyl-5-(1-methyl-butyl)-2-thiobarbiturate (Thiopental) (VUAB, Roztoky, Czech Republic) at a dose of 35 mg/kg body weight. Blood samples were collected from the anaesthetized rats by puncture of the right ventricle in heparinised tubes and centrifuged for 15 min at  $1000 \times g$ . Plasma was carefully removed. The separated cells were then 3 times washed, resuspended in a 0.9% NaCl solution and repeatedly centrifuged. The washed cells were lysed in an equal volume of water and mixed thoroughly. The hemoglobin concentration was estimated in an aliquot of the hemolysate, using a commercial assay kit (Sigma Chemical Co.). The hemolysate was used for estimation of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and paraoxonase (PON) activities. Plasma was used in the assay of the level of glucose, insulin, thiobarbituric acid reactive substance (TBARS), hydrogen peroxide ( $H_2O_2$ ), total antioxidant status as following.

#### *Determination of insulin concentration*

The concentration of insulin in plasma was estimated using an ELIZA kit (Mercodia Ultrasensitive Mouse Insulin, Sweden). It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. The reaction is read spectrophotometrically at 450 nm.

#### *Determination of hydrogen peroxide ( $H_2O_2$ ) level*

The level of plasma  $H_2O_2$  was determined by the method of Wolf (1994), based on that the ferrous oxidation with xylenol orange (Fox-1) reagent and the color development were virtually read spectrophotometrically at 560 nm.

#### *Assessment of lipid peroxidation*

Thiobarbituric acid reactive substances (TBARS) as markers of the lipid peroxidation were measured using the method of Conrad *et al.* (2000), based on the reaction of thiobarbituric acid with malondialdehyde, the second product of lipid, carbohydrate, protein, and DNA peroxidation.

#### *Determination of total antioxidant status*

The antioxidant status was assayed by a Randox kit purchased from Randox Laboratories Ltd (Antrim, UK). The assay is based on the reaction of metmyoglobin with hydrogen peroxide to form ferrmyoglobin, a free radical species. A chromogen 2,2-amino-di-(3-ethylbenzthiazole sulfate) is incubated with ferrmyoglobin to produce a radical cation which has a relatively stable blue color. Antioxidants in the added plasma can suppress this color production to a certain degree proportionally to their concentrations; the intensity of the blue color was measured at 600 nm.

#### *Determination of catalase (CAT) activity*

CAT activity was adapted from the method of Aebi (1984), based on the principle that the disappearance of hydrogen peroxide was monitored spectrophotometrically at 240 nm.

#### *Determination of superoxide dismutase (SOD) activity*

SOD activity was spectrophotometrically measured using a method developed by Marklund and Marklund (1974). Briefly, SOD is detected on the

basis of its ability to inhibit superoxide-mediated reduction. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%.

#### *Determination of glutathione peroxidase (GPx) activity*

GPx activity was measured using the Paglia and Valentine (1967) method, where the oxidation of NADPH by hydrogen peroxide was followed at 340 nm at 25 °C.

#### *Determination of paraoxonase (PON) activity*

PON activity was measured using the method developed by Lee *et al.* (2002), based on the principle that the hydrolysis rate of paraxon (diethyl 4-nitrophenyl phosphate) was assessed by measuring the liberation of *p*-nitrophenol at 405 nm at 25 °C.

#### *Statistical analysis*

Data were expressed as mean  $\pm$  S. E. and analyzed statistically using Student's *t*-test. A probability-value of  $P < 0.05$  was determined to be statistically significant.

### **Results**

#### *Effect of naringin on plasma glucose and insulin levels*

The plasma glucose level (Table I) in the hyperglycaemic group was increased by 325% (from  $78.80 \pm 11.0$  to  $335.00 \pm 18.3$  mg/dl,  $P < 0.05$ ) in comparison to the normoglycaemic group. The administration of gradual doses naringin to rats with hyperglycaemia resulted in the decrease of the glucose concentration by 0, 58, 71, 73%, respectively.

The plasma insulin level (Table I) in the hyperglycaemic group was decreased by 67.5% (from  $4.62 \pm 0.6$  to  $1.50 \pm 0.4$  ng/ml,  $P < 0.05$ ) in comparison to the normoglycaemic group. The administration of gradual doses naringin to rats with hyperglycaemia resulted in the increase of the insulin concentration by 0, 141, 183, 193%, respectively.

#### *Effect of naringin on plasma $H_2O_2$ , TBARS and total antioxidant levels*

The level of  $H_2O_2$  (Table II) in plasma of rats with hyperglycaemia was increased by 230% (from

Table I. The effect of various doses of naringin on the level of plasma glucose and insulin of the normoglycaemic and hyperglycaemic rats.

	Control	Naringin [mg/kg body wt]			
		10	20	40	80
<i>Glucose</i> [mg/dl]					
Normoglycaemic	78.80 ± 11.0	75.21 ± 6.7	79.12 ± 8.3	73.26 ± 6.3	69.70 ± 8.6
Hyperglycaemic	335.0 ± 18.3 <sup>a</sup>	335.00 ± 4.6	140.00 ± 5.7 <sup>a,b</sup>	97.00 ± 7.5 <sup>a,b</sup>	87.60 ± 5.4 <sup>a,b</sup>
<i>Insulin</i> [ng/ml]					
Normoglycaemic	4.62 ± 0.6	4.56 ± 0.7	4.96 ± 0.6	5.11 ± 0.6	5.50 ± 0.7
Hyperglycaemic	1.50 ± 0.4 <sup>a</sup>	1.52 ± 0.3	3.62 ± 0.4 <sup>a,b</sup>	4.25 ± 0.5 <sup>a,b</sup>	4.40 ± 0.5 <sup>a,b</sup>

<sup>a</sup> Significant difference from normoglycaemic group.  
<sup>b</sup> Significant difference from hyperglycaemic group.

Table II. The level of plasma hydrogen peroxide, thiobarbituric acid reactive substance (TBARS), and total antioxidant in the normoglycaemic and hyperglycaemic rats after administration of naringin.

	Control	Naringin [mg/kg body wt]			
		10	20	40	80
<i>H<sub>2</sub>O<sub>2</sub></i> [μM]					
Normoglycaemic	2.30 ± 0.1	2.30 ± 0.2	1.90 ± 0.1	1.66 ± 0.2	1.30 ± 0.2
Hyperglycaemic	7.60 ± 0.4 <sup>a</sup>	7.10 ± 0.5	4.30 ± 0.2 <sup>a,b</sup>	3.92 ± 0.5 <sup>a,b</sup>	2.46 ± 0.3 <sup>a,b</sup>
<i>TBARS</i> [nM]					
Normoglycaemic	3.50 ± 0.3	3.54 ± 0.4	2.96 ± 0.3	2.11 ± 0.2	1.95 ± 0.2
Hyperglycaemic	5.90 ± 0.4 <sup>a</sup>	5.52 ± 0.4	3.92 ± 0.4 <sup>a,b</sup>	3.15 ± 0.4 <sup>a,b</sup>	3.10 ± 0.4 <sup>a,b</sup>
<i>Total antioxidant</i> [nM]					
Normoglycaemic	1.73 ± 0.2	1.80 ± 0.7	2.33 ± 0.3	2.82 ± 0.3	4.10 ± 0.4
Hyperglycaemic	1.10 ± 0.0 <sup>a</sup>	1.40 ± 0.1	1.70 ± 0.2 <sup>a,b</sup>	2.30 ± 0.3 <sup>a,b</sup>	2.86 ± 0.3 <sup>a,b</sup>

<sup>a</sup> Significant difference from normoglycaemic group.  
<sup>b</sup> Significant difference from hyperglycaemic group.

2.30 ± 0.1 to 7.60 ± 0.4 μM, P < 0.05) in comparison to the normoglycaemic group. The TBARS level in rats treated with STZ was increased by 69% (from 3.50 ± 0.3 to 5.90 ± 0.4 nM, P < 0.05). While the total antioxidant level was decreased by 36% (from 1.73 ± 0.2 to 1.10 ± 0.0 nM, P < 0.05) in comparison to the normoglycaemic group.

Comparing to the hyperglycaemia untreated group, administration of gradual doses of naringin to rats with hyperglycaemia resulted in a decrease of the H<sub>2</sub>O<sub>2</sub> level by 7, 43, 48, 68%, respectively, and the level of TBARS by 6, 34, 47, 48%, respectively, while the level of total antioxidant activity was increased by 27, 54, 109, 160%, respectively.

*Effect of naringin on the activity of erythrocytes CAT, SOD, GPx, and PON enzymes*

The activity of CAT, SOD, GPx, and PON (Fig. 1) in erythrocytes of rats receiving STZ decreased by 40% (from 17.32 ± 1.3 to 10.45 ± 1.6 μmol min<sup>-1</sup> gHb<sup>-1</sup>, P < 0.05), 45% (from 355.43 ± 18.4 to 195.62 ± 14.7 unit/g Hb, P < 0.05), 33% (from 5.90 ± 0.5 to 3.94 ± 0.3 μmol min<sup>-1</sup> gHb<sup>-1</sup>, P < 0.05), and 33% (from 7.80 ± 1.1 to 5.22 ± 0.4 nmol min<sup>-1</sup> gHb<sup>-1</sup>, P < 0.05), respectively, in comparison to the results obtained from the normoglycaemic group.

Comparing to the hyperglycaemia untreated group, administration of gradual doses of naringin to rats with hyperglycaemia resulted in the increase of CAT activity by 12, 59, 98, 117%, respec-

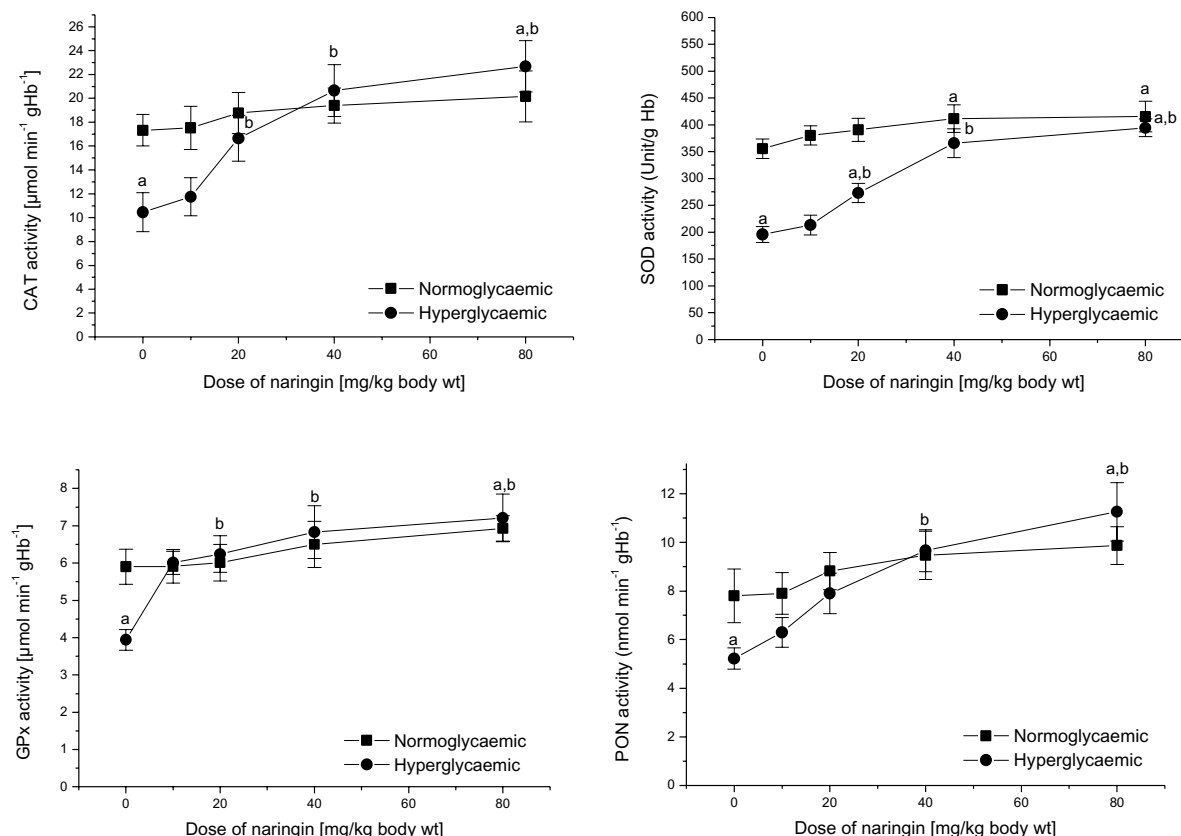


Fig. 1. The activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and paraoxonase (PON) in the normoglycaemic and hyperglycaemic rats after administration of different doses of naringin (10, 20, 40, or 80 mg/kg body wt); a: significant difference from normoglycaemic group at  $P < 0.05$ ; b: significant difference from hyperglycaemic group at  $P < 0.05$ .

tively, increased the activity of SOD by 9, 39, 66, 102%, respectively, increased the activity of GPx by 52, 58, 73, 83%, respectively, and increased the activity of PON by 21, 51, 85, 116%, respectively.

## Discussion

Oxidant free radicals play a relevant role in the etiology and pathogenesis of a variety of diseases such as diabetes mellitus, cancer, hypertension, and cardiovascular diseases and are considered to be the principle causative agents of aging (Jeon *et al.*, 2002). Diabetes mellitus and its sequels, neuropathy and angiopathy, are conditions in which free radicals are involved both in human and in experimental models (Soto *et al.*, 1988).

Several mechanisms may lead to increased oxidative stress in diabetes. Firstly, hyperglycaemia may increase the generation of free radicals

through the ability of glucose to enolize and yield oxidizing intermediates such as superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxide (Hunt *et al.*, 1990; Haluzik and Nedvickova, 2000). Secondly, antioxidant defenses are reduced in diabetes (Tsai *et al.*, 1994). Hence, compounds with both hypoglycaemic and anti-oxidative properties would be useful anti-diabetic agents (Baynes, 1995).

There are several reports describing elevations in specific oxidant stress markers in both experimental STZ and human diabetes mellitus, together with reduced total antioxidant defense and depletion in individual antioxidants (Laight *et al.*, 2000). Such a prooxidant environment was demonstrated in our study by the increased lipid peroxidation products and hydrogen peroxide, accompanied by inhibition of the activity of antioxidative enzymes

CAT, SOD, and GPx as well as total antioxidant status. Our results were in agreement with that reported by Piconi *et al.* (2003) who mentioned that in diabetes overproduction of ROS can lead to a decrease in cell/organism antioxidant defenses, demonstrated as a fall in the concentration of a single antioxidant molecule or by a fall in the total antioxidant status. An increase in TBARS was presumably associated with an increase in ROS, confirming the fact that the singlet oxygen and peroxy radicals inhibited the activity of SOD (Escobar *et al.*, 1996). Also Blum and Fridovich (1985) mentioned that overproduction of superoxide radicals during oxidative stress could inhibit the activity of GPx, while the activity of CAT was inhibited by production of singlet oxygen, superoxide and peroxy radicals (Kono and Fridovich, 1982; Escobar *et al.*, 1996).

Also, in our study, erythrocytes PON activity in the hyperglycaemic rats was significantly lower than in the normoglycaemic group. Our result confirms the previous data reported by Inoue *et al.* (2000), and this can be explained by that there are some inhibitors against the enzyme activity of PON in circulating blood of diabetic patients such as glycosylated proteins or disturbance of the interaction of PON with HDL affecting its activity (Abbott *et al.*, 1995; Inoue *et al.*, 2000).

The present study suggests that exogenous administration of different doses of naringin to hyperglycaemic rats causes a dose-dependent decrease of hydrogen peroxide and lipid peroxidation as well as an increase in the activity of antioxidant enzyme. These results indicate the important role of naringin in the reduction of oxidative stress. However, the mechanism by which naringin could affect free radical and peroxide production, as well as total antioxidant, CAT, SOD, GPx, and PON activities *in vitro*, is not clearly defined. Naringin may exhibit antioxidant activity capacity based on increasing the upregulation of gene expressions in the antioxidant enzymes (CAT, SOD, and GPx) (Jeon *et al.*, 2002) with consequently enhancement the scavenging of reactive oxygen species accompanying with significantly lowering of lipid peroxidation suggests that administration of naringin increase the antioxidative potential. In addition like all the flavonoid compounds, presence of hydrogen donating substituents attached to aromatic rings may enable naringin to scavenge free radicals. Consistent with our result naringin exhibited a dose-dependent

increase in the antioxidant PON activity and this result is in accordance with that reported by Fuhrman and Aviram (2002) who mentioned that consumption of wine flavonoids was shown to preserve PON activity by reducing the oxidative stress in apolipoprotein E-deficient mice, thereby contributed to PON in oxidized lipoproteins.

Kashiwagi *et al.* (1994) reported that the elevation of glucose concentration reduces the activity of GPx, leading to an accumulation of  $H_2O_2$ .  $H_2O_2$  catabolism leads to the formation of the superoxide radical anion, while the decrease in plasma glucose concentration causes the activation of the pentose phosphate pathway, inactivation of the sorbitol pathway and consequently, an increase in the NADPH level (Sinclair, 1993). NADPH is a cofactor required for the resynthesis of reduced glutathione which regulates the GPx activity and indirect activity of other antioxidative enzymes (Togashi *et al.*, 1990). Therefore, the increase of the NADPH level may lead to the activation of all examined antioxidant enzymes. While, decreased the level of hydrogen peroxide and lipid peroxidation observed after naringin administration may point to a reduction in free radical production.

The mechanism underlying the blood-glucose-lowering actions of naringin remains unclear; the protective effect of naringin in hyperglycaemic conditions may be associated with an intensification of glucose uptake by peripheral tissues, a reduction in plasma glucose concentration with stimulation of insulin secretion that is inconsistent with our observation. Our results were in accordance with that mentioned by El-Missiry and El-Gindy (2000) who reported that the active principles from plant sources might act by several mechanisms such as stimulation of insulin secretion, increasing repair/proliferation of pancreatic  $\beta$ -cells, enhancing the effect of insulin and adrenaline as well as increasing the antioxidative capability.

Thus, it is postulated from this study that oxidative damage appears to play a major role in STZ-induced hyperglycaemia as evidenced by significant inhibition of the antioxidant defense mechanism accompanied by an elevation of hydrogen peroxide and lipid peroxidation end products. Intraperitoneal administration of naringin may reveal their action on glucose metabolism through reduction of oxidative stress intensity in hyperglycaemia. The protective effect of naringin may be connected with the normalization of hypergly-

caemia, the inhibition of glucose autooxidation and, as a result, exhibited a potent significant amelioration of oxidative stress in the diabetic animals.

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