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Ameliorative effect of diosmin, a citrus flavonoid against streptozotocin-nicotinamide generated oxidative stress induced diabetic rats

Subramani Srinivasan, Leelavinothan Pari *

Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar 608002, Tamilnadu, India

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ABSTRACT

Oxidative stress has been suggested as a contributory factor in development and complication of diabetes. The aim of the study was to evaluate the effect of diosmin (DS) in oxidative stress in streptozotocin-nicotinamide (STZ-NA)-induced diabetic rats by measuring the lipid peroxidation (LPO) as well as the ameliorative properties. Experimental diabetes was induced by a single intraperitoneal (i.p) injection of STZ (45 mg/kg body weight (b.w.)) dissolved in 0.1 mol/L citrate buffer (pH 4.5), 15 min after the i.p administration of NA (110 mg/kg b.w.). Diabetic rats exhibited increased plasma glucose with significant decrease in plasma insulin levels. The activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST) and the levels of low-molecular weight antioxidants vitamin C, vitamin E and reduced glutathione (GSH) were decreased while increases in the levels of LPO markers were observed in liver and kidney tissues of diabetic control rats as compared to normal control rats. Oral treatment with DS (100 mg/kg/day) for a period of 45 days showed significant ameliorative effects on all the biochemical parameters studied. Biochemical findings were supported by histological studies. These results indicated that DS has potential ameliorative effects in addition to its antidiabetic effect in type 2 diabetic rats.

1. Introduction

Diabetes mellitus (DM) is the most significant chronic disease and cause of death in modern society. Diabetes comprises a group of chronic disorders characterized by hyperglycemia or diminished insulin secretion, or both. DM involves high levels of blood glucose, which contributes to an increase in free radical production [1]. Defects in glucose metabolizing machinery and consistent efforts of the physiological system to correct the imbalance in glucose metabolism place an over exertion on the endocrine system. Continuing deterioration of endocrine control exacerbates the metabolic disturbances and leads primarily to hyperglycemia [2]. The elevated levels of blood glucose in diabetes are associated with increased lipid peroxidation (LPO), which may contribute to long term tissue damage [3]. Various studies have shown that DM is associated with oxidative stress, leading to an increased production of reactive oxygen species (ROS), including superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) or reduction of antioxidant defense system [4,5]. ROS are involved in the pathogenesis of many diseases including hypoxia, hypercholesterolemia, atherosclerosis, hypertension, ischemia reperfusion injury and heart failure [6].

Streptozotocin (STZ), an antibiotic produced by *Streptomyces achromogenes*, has been widely used for inducing diabetes in the experimental animals through its toxic effects on pancreatic β-cells [7]. The cytotoxic action of STZ is associated with the generation of ROS causing oxidative damage [8]. LPO is a key marker of oxidative stress. The increased oxidative stress, as measured by indices of elevated LPO, depletion of endogenous antioxidant, and antioxidant enzymes activities in plasma and tissues, are commonly found in rats with STZ-NA-induced diabetes, and these alterations may cause tissues to be more susceptible to oxidative damage [9]. The significant extent of LPO byproducts that was measured as thiobarbituric acid reactive substances (TBARS) has been reported in diabetes [10].

Antioxidant refers to a compound that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions and which can thus prevent or repair damage done to the cells by oxygen. They act by one or more of the following mechanisms: reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. The formation of ROS is prevented by an antioxidant system that included non-enzymatic antioxidants (vitamin C, vitamin D and glutathione), enzymes regenerating the reduced forms of antioxidants, and ROS-scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST)

* Corresponding author. Tel.: +91 04144 239141; fax: +91 04144 238145.
E-mail address: jayampari@gmail.com (L. Pari).

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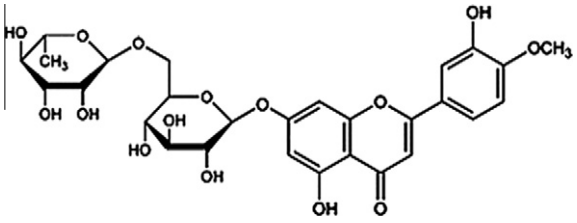

Fig. 1. Chemical structure of diosmin.

[11,12]. Epidemiological studies have shown that many phytonutrients of fruits and vegetables might protect the human body against damage by ROS. The consumption of natural antioxidant phytochemicals were reported to have potential health benefits [13]. In recent years, considerable focus has been given to an intensive search for novel type of antioxidants from numerous plant materials [14]. Phytochemicals isolated from plant sources are used for the prevention and treatment of various diseases [15]. It has been reported that chemicals with antioxidant effect may help to regenerate β-cells and protect pancreatic islets against cytotoxic effects of STZ [16].

Diosmin (DS) (diosmetin 7-*O*-rutinoside; Fig. 1), a natural flavone glycoside is readily obtained by dehydrogenation of the corresponding flavanone glycoside, hesperidin that is abundant in the pericarp of various citrus fruits [17]. DS possesses blood lipid lowering [18] and anticarcinogenic activities [19]. It enhances venous tone and microcirculation and protects capillaries [20], mainly by reducing systemic oxidative stress [21]. Pharmacokinetic investigations have shown that DS is rapidly transformed by intestinal flora to its aglycone form, diosmetin. Diosmetin is absorbed and rapidly distributed throughout the body with a plasma half-life of 26–43 h. Diosmetin is degraded to phenolic acids or their glycine-conjugated derivatives and eliminated through the urine. The presence of degradation products such as alkyl-phenolic acids confirmed a metabolic pattern similar to other flavonoids. In our previous studies, we reported the effects of DS on rate-limiting enzymes of carbohydrate metabolism and reversed the abnormalities in the levels of glycoprotein components in type 2 diabetic rats [22,23].

The present study was undertaken to determine whether the liver and kidney were subjected to oxidative damage during experimental diabetes as well as to examine the accompanying changes in antioxidant status. Histopathological studies were also carried out to assess the effect of DS on liver and kidney cells against STZ-nicotinamide (NA) damage in rats.

2. Experimental procedure

2.1. Animals

Male albino Wistar rats (200–220 g) were bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, India, used in this study. The rats had free access to water and a commercial standard pelleted diet (Lipton India Ltd., Mumbai, India). The rat diet consisted of 21% protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 55% nitrogen free extract (carbohydrate), and 15,067 kJ metabolizable energy. The animals were housed in standard polypropylene cages and maintained under controlled room temperature (22 ± 2 °C) and humidity (55 ± 5%) with 12:12 h light and dark cycle. The rats used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India. The study protocol was approved (Reg. No. 160/1999/CPCSEA, Vide No. 566, 2008) by the Committee for the Purpose of Control and Supervision on Experimental Animals at Annamalai University, Annamalainagar, India.

2.2. Chemicals

DS and STZ were purchased from Sigma Chemical Company, St. Louis, MO, USA and NA was obtained from Ranbaxy Chemicals Ltd., Mumbai, India. All the other chemicals and reagents were used of analytical grade.

2.3. Induction of type 2 diabetes in experimental rats

STZ was freshly dissolved in (0.1 M, pH 4.5) citrate buffer and NA was dissolved in normal physiological saline and maintained on ice prior to use. Non-insulin-dependent diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal (i.p) injection of STZ (45 mg/kg body weight (b.w.)), 15 min after the i.p administration of NA (110 mg/kg b.w.) [24]. The levels of plasma glucose were determined and those rats with fasting glucose >250 mg/dL served as diabetic rats and used in this study. Treatment with DS was started on the third day after STZ induction.

2.4. Experimental procedure

The animals were separated into four groups ($n = 6$), a total of 24 rats (12 diabetic surviving rats, 12 normal control rats) were used. DS was dissolved in vehicle solution of 0.6% dimethylsulfoxide (DMSO) and DS (100 mg/kg b.w.) was administered orally using an intragastric tube for a period of 45 days [22,23].

- Group I: Normal control (vehicle treated; DMSO: 1 mL/kg b.w.)
- Group II: Normal + DS (100 mg/kg b.w.)
- Group III: Diabetic control
- Group IV: Diabetic + DS (100 mg/kg b.w.)

At the end of the experimental period, the animals were fasted overnight, anesthetized using ketamine hydrochloride (24 mg/kg b.w., intramuscular injection), and sacrificed by cervical decapitation. Blood samples were collected in tubes containing potassium oxalate and sodium fluoride (3:1) mixture for the estimation of plasma glucose and insulin. Liver and kidney were immediately dissected, washed in ice-cold saline to remove the blood. The liver and kidney were selected because it is one of the tissues showing a high rate of free radical generation.

2.5. Preparation of tissue homogenate

The tissues were weighed and 10% tissue homogenate was prepared with 0.025 M Tris–HCl buffer, pH 7.5. After centrifugation at 10,000×*g* for 10 min, the clear supernatant was used to measure TBARS and hydroperoxides. For the determinations of vitamin E level the liver and kidney tissues were weighed and lipids were extracted from tissues by the method of Folch et al. [25] using chloroform–methanol mixture ($CHCl_3$: MeOH) (2:1; v/v). The extract used for the estimation of vitamin E. For the estimation of non-enzymatic and enzymatic antioxidants, tissues were minced and homogenized (10% w/v) in 0.1 M phosphate buffer (pH 7.0) and centrifuged for 10 min and the resulting supernatant was used for enzyme assays.

2.6. Analytical procedure

2.6.1. Measurement of plasma glucose and plasma insulin

Plasma glucose levels were estimated using a commercial kit (Sigma Diagnostics Pvt. Ltd., Baroda, India) by the method of Trinder [26]. Plasma insulin was assayed by an enzyme linked

immunosorbent assay (ELISA) method using a commercial kit (Catalog No. SP-401) from United Biotech Inc., Mountain View, CA, USA.

2.6.2. Estimation of LPO in plasma and tissue

LPO in liver and kidney were estimated UV/VIS spectrophotometer (SL 177, Elico Ltd., Hyderabad, India) by measuring TBARS and hydroperoxides using the methods of Fraga et al. [27] and Jiang et al. [28], respectively. In brief, 0.1 mL of tissue homogenate was treated with 2 mL of thiobarbituric acid (TBA)–trichloroacetic acid (TCA)–HCl reagent (0.37%TBA, 0.25 M HCl and 15%TCA, 1:1:1 ratio), placed for 15 min in a water bath and then cooled and centrifuged at 3500×*g* for 10 min at room temperature, the absorbance of clear supernatant was measured at 535 nm against a reference blank. Values were expressed as mM/100 g-tissue. Hydroperoxides were expressed as mM/100 g-tissue. Tissue homogenate (0.1 mL) was treated with 0.9 mL of Fox reagent (88 mg of butylated hydroxy toluene (BHT), 7.6 mg of xylenol orange and 0.8 mg of ammonium iron sulfate were added to 90 mL of methanol and 10 mL of 250 mM sulfuric acid) and incubated at 37 °C for 30 min. Then the absorbance was read at 560 nm.

2.6.3. Activity of CAT

The activity of CAT was estimated by the method of Sinha [29]. The reaction mixture (1.5 mL, vol) contained 1.0 mL of 0.01 M phosphate buffer (pH 7.0), 0.1 mL of tissue homogenate and 0.4 mL of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 mL of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). Then the absorbance was read at 620 nm; CAT activity was expressed as μM of H_2O_2 consumed/min/mg protein.

2.6.4. Activity of SOD

The activity of SOD was assayed by the method of Kakkar et al. [30]. 0.5 mL of tissue homogenate was diluted with 1 mL of water. In this mixture, 2.5 mL of ethanol and 1.5 mL of chloroform (all reagents chilled) were added and shaken for 1 min at 4 °C then centrifuged. The enzyme activity in the supernatant was determined. The assay mixture contained 1.2 mL of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 mL of 186 μM potassium metabisulfite (PMS), 0.3 mL of 30 μM nitroblue tetrazolium (NBT), 0.2 mL of 780 μM NADH, appropriately diluted enzyme preparation and water in a total volume of 3 mL. Reaction was started by the addition of NADH. After incubation at 30 °C for 90 min the reaction was stopped by the addition of 1 mL glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 mL of *n*-butanol. The intensity of the chromogen in the butanol layer was measured at 560 nm against butanol blank. A system devoid of enzyme served as control. One unit of the enzyme activity is defined as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute under the assay conditions.

2.6.5. Activity of GPx

The activity of GPX was measured by the method described by Rotruck et al. [31]. Briefly, the reaction mixture contained 0.2 mL 0.4 M phosphate buffer (pH 7.0), 0.1 mL 10 mM sodium azide, 0.2 mL tissue homogenized in 0.4 M, phosphate buffer, pH 7.0, 0.2 mL glutathione and 0.1 mL 0.2 mM H_2O_2. The contents were incubated for 10 min at 37 °C, 0.4 mL 10% TCA was added to stop the reaction and centrifuged at 3200×*g* for 20 min. The supernatant was assayed for glutathione content using Ellman's reagent (19.8 mg 5,5′-dithiobisnitrobenzoic acid (DTNB) in 100 mL 0.1% sodium nitrate). The activity was expressed as μg of GSH consumed/min/mg protein.

2.6.6. Activity of GST

GST activity was determined spectrophotometrically by the method of Habig et al. [32]. The reaction mixture contained 1.0 mL 100 mM phosphate buffer (pH 6.5), 0.1 mL 30 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 0.7 mL double distilled water. After pre-incubating the reaction mixture for 5 min at 37 °C, the reaction was started by the addition of 0.1 mL tissue homogenate and 0.1 mL of 30 mM glutathione as substrate. After 5 min the absorbance was read at 340 nm. Reaction mixture without the enzyme was used as a blank. The activity of GST is expressed as mM of GSH–CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6/mM/cm.

2.6.7. Activity of GR

Glutathione reductase (GR) was assayed by the method of Horn and Burns [33]. The reaction mixture containing 1 mL of phosphate buffer, 0.5 mL of EDTA, 0.5 mL of GSSG and 0.2 mL of NADPH was made up to 3 mL with water. After the addition of 0.1 mL of tissue homogenate, the change in optical density at 340 nm was monitored for 2 min at 30 s intervals.

2.6.8. Estimation of reduced glutathione

Reduced glutathione was measured according to the method of Beutler and Kelly [34]. The technique involved in protein precipitation by metaphosphoric acid and spectrophotometric assay at 412 nm of the yellow derivative obtained by the reaction of supernatant with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB).

2.6.9. Estimation of oxidized glutathione

Oxidized glutathione was measured according to the method described by Aseni et al. [35] based on the principle of glutathione reductase enzyme reducing GSSG to GSH with the concomitant oxidation of NADPH to $NADP^+$. To 0.9 mL of 1.75 mol/l K_3PO_4 buffer (pH 7.0) containing 20 mmol/l NEM was added 0.05 mL of sample extract and 0.025 mL of 10 mg/mL of NADPH–Na solution. Absorbance at 340 nm was measured for 30 s immediately after addition of 0.025 mL of (10 mg/mL) glutathione reductase (GR) to the assay mixture.

2.6.10. Estimation of vitamin C

Vitamin C was estimated by the method of Omaye et al. [36]. 0.5 mL of tissue homogenate was mixed thoroughly with 1.5 mL of 6% TCA and centrifuged for 10 min at 3500×*g*. After centrifugation, 0.5 mL of the supernatant was mixed with 0.5 mL of dithiobis-2-nitrobenzoic acid (DNPH) reagent and allowed to stand at room temperature for an additional 3 h then added 2.5 mL of 85% sulfuric acid and allowed to stand for 30 min. Then the absorbance was read at 530 nm. A set of standards containing 10–50 μg of ascorbic acid were taken and processed similarly along with a blank. Ascorbic acid values are expressed as μM/mg tissue.

2.6.11. Estimation of vitamin E

Vitamin E was determined by the method of Baker et al. [37]. 0.1 mL of lipid extract, 1.5 mL of ethanol and 2 mL of petroleum ether were added, mixed and centrifuged for 3000×*g* for 10 min. The supernatant was evaporated to dryness at 80 °C then 0.2 mL of 2,2-1-dipyridyl solution and 0.2 mL of ferric chloride solution was added and mixed well. This was kept in dark for 5 min and added 2 mL of butanol. Then the absorbance was read at 520 nm. Standards of α-tocopherol in the range of 10–100 μg were taken and treated similarly along with blank containing only the reagent. The values are expressed as μM/mg-tissue.

2.6.12. Determination of protein

Protein content in the tissue homogenate was determined by the method of Lowry et al. [38]. 0.5 mL of the tissue homogenate

was precipitated with 0.5 mL of 10% TCA and centrifuged for 10 min and the precipitate was dissolved in 1.0 mL of 0.1 N sodium hydroxide (NaOH). From this 0.1 mL of aliquot was taken and made up to 1.0 mL with distilled water. Then, 4.5 mL of alkaline copper reagent was added and allowed to stand at room temperature for 10 min. After incubation, 0.5 mL of Folin's-Ciocalteau reagent was added and the blue color developed was read at 620 nm after 20 min. A standard curve was obtained using bovine serum albumin.

2.7. Histopathological study

The liver and kidney samples fixed for 48 h in 10% formal saline were dehydrated by passing successfully in different mixture of ethyl alcohol–water, cleaned in xylene and embedded in paraffin. Sections of liver and kidney (4–5 μm thick) were prepared and then stained with hematoxylin and eosin (H&E) dye, which mounted in neutral deparaffinated xylene medium for microscopic observations.

2.8. Statistical analysis

The statistical significance of the data has been determined using one-way analysis of variance (ANOVA) and significant difference among treatment groups were evaluated by Duncan's multiple range test (DMRT). The results were considered statistically significant at $p < 0.05$ [39]. All statistical analyses were made using SPSS 16.0, SPSS Inc., and Cary, NC.

3. Results

Figs. 2 and 3 show fasting plasma insulin levels were significantly ($p < 0.05$) decreased and the levels of plasma glucose significantly ($p < 0.05$) increased in diabetic control rats compared to normal control rats. Plasma glucose levels were significantly ($p < 0.05$) decreased and insulin levels were increased in diabetic rats treated with DS compared to diabetic control rats.

Table 1 and Fig. 4 depict the concentration of TBARS and hydroperoxides in plasma and tissues of normal control and diabetic rats. In diabetic rats, TBARS and hydroperoxides were increased significantly ($p < 0.05$). Treatment of diabetic rats with DS significantly ($p < 0.05$) reversed the concentration of TBARS and hydroperoxides in the liver and kidney.

Table 2 illustrates the activities of enzymatic antioxidants namely SOD, CAT, GPx, GST and GR in the liver and kidney of control and experimental rats. A significant ($p < 0.05$) depletion in the activities of enzymatic antioxidants in STZ treated rats was observed. Treatment of DS increased the levels of enzymatic antioxidants in the liver and kidney.

The levels of GSH, vitamin C and vitamin E in tissues (Table 3) and plasma (Fig. 5) of diabetic rats were significantly ($p < 0.05$) decreased. Administration of DS to diabetic rats exhibited a significant ($p < 0.05$) increase in the levels of these non-enzymatic antioxidants and the levels of GSH was normalized in the plasma and tissues.

Fig. 6 summarizes the concentration of GSSG and the level of GSH/GSSG ratio in control and experimental rats. There was a significant ($p < 0.05$) increased level of GSSG and concomitant decreased in the level of GSH/GSSG in diabetic rats when compared to normal control rats. Oral treatment of DS restored GSSG and GSH/GSSG ratio towards near normal levels.

Fig. 7A–D represents the microphotographs of H&E staining of hepatic and renal tissues of normal control and experimental rats. Pathological changes of STZ-NA-induced diabetic rat liver include

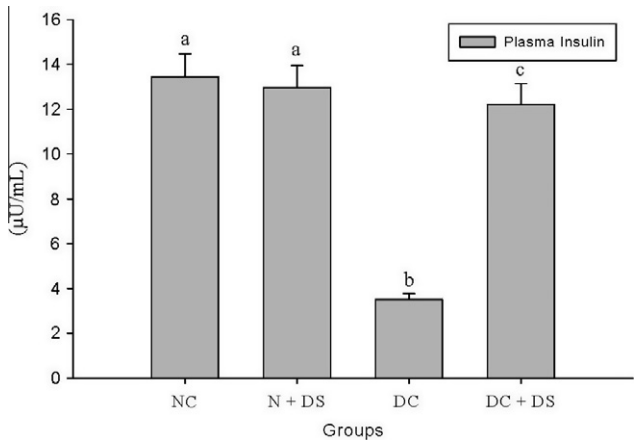

Fig. 2. Changes in the levels of plasma insulin in normal control and experimental rats. Each value is mean ± S.D. for 6 rats in each group. [a–c]In each bar, means with different superscript letter differ significantly at $p < 0.05$ (DMRT). DC: diabetic control, NC: normal control, DS: diosmin.

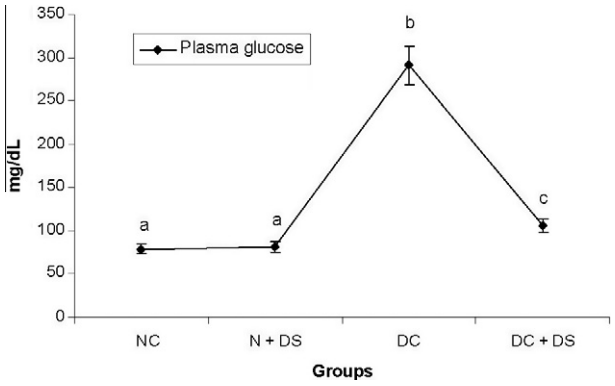

Fig. 3. Changes in the levels of plasma glucose in normal control and experimental rats. Each value is mean ± S.D. for 6 rats in each group. [a–c]In each point, means with different superscript letter differ significantly at $p < 0.05$ (DMRT). DC: diabetic control, NC: normal control, DS: diosmin.

Table 1
Effects of DS on the levels of TBARS and HP in the tissues of normal control and experimental rats.

Groups	Normal control	Normal + diosmin (100 mg/kg)	Diabetic control	Diabetic + diosmin (100 mg/kg)
TBARS				
Liver (mM/100 g tissue)	10.23 ± 0.78[a]	0.71 ± 0.05[a]	1.95 ± 0.13[b]	1.12 ± 0.08[c]
Kidney (mM/100 g tissue)	1.72 ± 0.13[a]	1.68 ± 0.13[a]	2.53 ± 0.19[b]	1.93 ± 0.15[c]
HP				
Liver (mM/100 g tissue)	65.32 ± 5.67[a]	68.45 ± 5.71[a]	102.33 ± 8.81[b]	85.47 ± 6.66[c]
Kidney (mM/100 g tissue)	48.65 ± 5.34[a]	44.78 ± 4.78[a]	75.93 ± 6.79[b]	56.42 ± 5.45[c]

Each value is mean ± S.D. for 6 rats in each group. [a–c]In each row, means with different superscript letter differ significantly at $p < 0.05$ (DMRT).

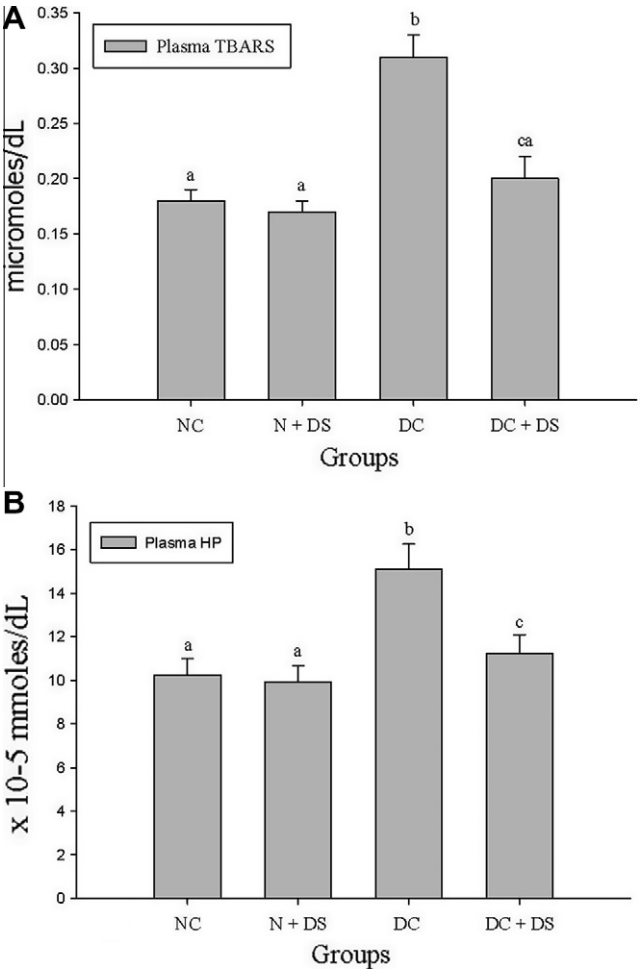

Fig. 4. Effect of diosmin on plasma TBARS (A) and HP (B) in normal control and diabetic rats. [a–c]In each bar, means with different superscript letter differ significantly at $p < 0.05$ (DMRT). DC: diabetic control, NC: normal control, DS: diosmin.

congestion of sinusoidal dilatation, inflammation of the central vein and focal necrosis in the hepatocytes in diabetic control rats. The above pathological changes were reduced in rats treated with DS. Fig. 8A–D represents the diabetic control rat kidney showed multiple foci of hemorrhage, necrosis and swelling of tubules. These changes were reduced in DS treated rats. No histological alterations were observed in liver and kidney of normal rats.

4. Discussion

STZ is a 2-deoxy-D-glucose with an *N*-nitrosomethylurea moiety at the second carbon atom and the existence of 2-deoxy-D-glucose facilitates preferential uptake of STZ into the pancreatic β-cells through GLUT2 [40] and the *N*-nitrosomethylurea moiety triggers DNA fragmentation in pancreatic β-cells through the formation of alkylating free radicals leading to hasty necrosis of the β-cells thereby the rate of insulin synthesis is diminished [43]. It has been reported that administration of NA, a poly-ADP-ribose synthetase inhibitor, protected the islets function by preventing the decrease in the levels of NAD and proinsulin thereby partially reversing the inhibition of insulin secretion to prevent the aggravation of experimental diabetes following the administration of β-cell toxins, such as, STZ and alloxan [41]. This condition contributes a number of features similar to type 2 diabetes mellitus, and is exemplified by stable hyperglycemia, glucose intolerance, and significantly altered glucose-stimulated insulin secretion both *in vivo* and *in vitro* [24]. DS is a common constituent in many citrus species [42]. DS has been reported to stimulate the pancreatic β-cells, which play a crucial role in the production and secretion of insulin [22]. In our study, we examined the oxidative stress markers of STZ-NA-induced diabetic rats. Damaged β-cells often display extensive degranulation and are clinically associated with the development of diabetes [43]. Several studies have shown that antioxidant treatments improve markers of oxidative stress and LPO in diabetic patients and animals. The administration of DS to diabetic rats decreases the blood glucose concentration near to normal, which is an essential trigger for the liver and kidney to revert back to its normal homeostasis during experimental diabetes. Our study revealed that the mechanism of antihyperglycemic action of DS may be through its scavenging ability to protect the pancreatic islets from free radical-induced damage by STZ in experimental rats.

Hyperglycemia results in the generation of free radicals which can exhaust antioxidant defenses thus leading to the disruption of cellular functions, oxidative damage to membranes and enhanced susceptibility to LPO [44]. Lipid peroxides and hydroperoxides are the secondary products of oxidative stress and are unleashed as a result of the toxic effect of ROS produced during LPO in diabetes [45]. In the present study, STZ treatment significantly increased LPO products and decreased enzymatic and

Table 2
Effect of DS on the activities of SOD, CAT, GPx and GST in normal control and experimental rats.

Parameters	Normal control	Normal + DS (100 mg/kg)	Diabetic	Diabetic + DS (100 mg/kg)
SOD (*units/mg of protein*)				
Liver	5.32 ± 0.41[a]	5.65 ± 0.43[a]	3.63 ± 0.28[b]	4.76 ± 0.36[c]
Kidney	5.87 ± 0.45[a]	6.18 ± 0.47[a]	3.85 ± 0.29[b]	4.92 ± 0.38[c]
CAT (*μmol/(min mg protein)*)				
Liver	72.60 ± 5.56[a]	74.93 ± 5.74[a]	51.97 ± 3.96[b]	67.39 ± 5.16[c]
Kidney	36.22 ± 2.76[a]	37.40 ± 2.86[a]	20.03 ± 1.53[b]	29.46 ± 2.25[c]
GPx (*μg/(min mg protein)*)				
Liver	8.79 ± 0.67[a]	9.02 ± 0.69[a]	5.38 ± 0.41[b]	7.45 ± 0.57[c]
Kidney	8.40 ± 0.64[a]	8.99 ± 0.69[a]	5.23 ± 0.40[b]	7.53 ± 0.58[c]
GST (*μmol/(min mg protein)*)				
Liver	4.98 ± 0.38[a]	5.27 ± 0.40[a]	3.29 ± 0.25[b]	4.17 ± 0.32[c]
Kidney	4.97 ± 0.38[a]	5.21 ± 0.40[a]	3.22 ± 0.25[b]	4.02 ± 0.31[c]
GR (*nmol/(min mg protein)*)				
Liver	0.54 ± 0.04[a]	0.58 ± 0.04[a]	0.32 ± 0.02[b]	0.44 ± 0.03[c]
Kidney	0.36 ± 0.03[a]	0.38 ± 0.03[a]	0.22 ± 0.02[b]	0.28 ± 0.02[c]

Each value is mean ± S.D. for 6 rats in each group. [a–c]In each row, means with different superscript letter differ significantly at $p < 0.05$ (DMRT).

Table 3
Effect of DS on the levels of vitamin C, vitamin E and GSH in normal control and experimental rats.

Parameters	Normal control	Normal + DS (100 mg/kg)	Diabetic	Diabetic + DS (100 mg/kg)
Vitamin C (*mg/dl*)				
Liver	1.31 ± 0.10[a]	1.39 ± 0.11[a]	0.86 ± 0.07[b]	1.21 ± 0.09[c]
Kidney	1.01 ± 0.08[a]	1.09 ± 0.08[a]	0.63 ± 0.05[b]	0.88 ± 0.07[c]
Vitamin E (*mg/dl*)				
Liver	0.73 ± 0.06[a]	0.76 ± 0.06[a]	0.46 ± 0.04[b]	0.63 ± 0.05[c]
Kidney	0.52 ± 0.04[a]	0.55 ± 0.04[a]	0.31 ± 0.02[b]	0.41 ± 0.03[c]
GSH (*mg/dl*)				
Liver	3.82 ± 0.29[a]	4.12 ± 0.32[a]	2.43 ± 0.19[b]	3.12 ± 0.24[c]
Kidney	2.85 ± 0.22[a]	3.06 ± 0.23[a]	1.67 ± 0.13[b]	2.29 ± 0.18[c]

Each value is mean ± S.D. for 6 rats in each group. [a–c]In each row, means with different superscript letter differ significantly at $p < 0.05$ (DMRT).

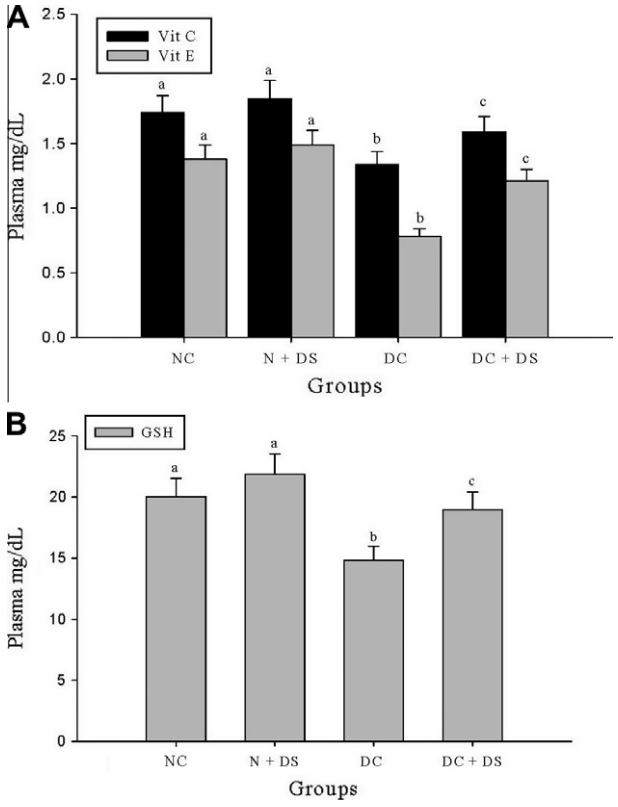

Fig. 5. Effect of DS on plasma vitamin C, vitamin E (A) and GSH (B) in normal control and experimental rats. Each value is mean ± S.D. for 6 rats in each group. [a–c]In each bar, means with different superscript letter differ significantly at $p < 0.05$ (DMRT). DC: diabetic control, NC: normal control, DS: diosmin.

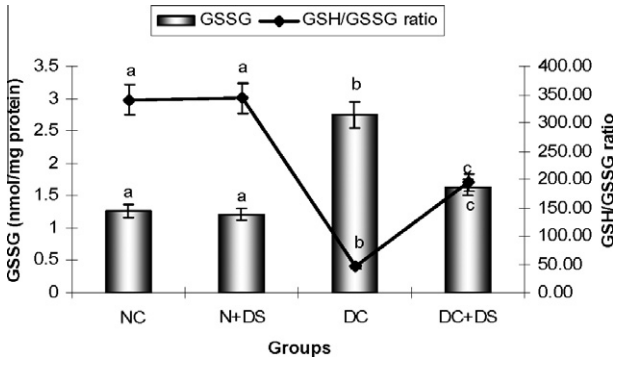

Fig. 6. Effect of DS in the levels of GSSG and GSH/GSSG ratio in normal control and experimental rats. [a–c]In each bar, means with different superscript letter differ significantly at $p < 0.05$ (DMRT). DC: diabetic control, NC: normal control, DS: diosmin.

nonenzymetic antioxidant levels in the plasma and tissues of rats. Elevated LPO in STZ-induced diabetes is the reduction in the levels of reduced glutathione, a potent endogenous antioxidant. LPO mediated damage has been observed in the development of type 1 and type 2 diabetes mellitus. Insulin secretion is also closely associated with lipoxygenase-derived peroxides [46]. Low levels of lipoxygenase peroxides stimulate the secretion of insulin, but when the concentration of endogenous peroxides increases, it may initiate uncontrolled LPO leading to cellular infiltration and islet cell damage. The observed increase in the level of TBARS in diabetic rats is generally thought to be a consequence of increased production and liberation into the circulation of tissue lipid peroxides due to pathological changes. The increased LPO during diabetes, as found in the present study may be due to the inefficient antioxidant system prevalent in diabetes [47]. Our present exploration showed a significant increase of plasma TBARS and hydroperoxides levels in diabetic rats. Administration of DS to diabetic rats significantly decreased the levels of TBARS and hydroperoxides. DS act as antioxidant by scavenging free radicals which result in decreased LPO in diabetic rats.

Antioxidants constitute the foremost defense system that limit the toxicity associated with free radicals. Oxidative stress in diabetes is coupled with decrease in the antioxidant status, which can increase the deleterious effects of free radicals [48]. Antioxidant enzymes form the first line of defense against ROS in the organism includes the enzymes SOD, CAT, GPx and GST, which play an important role in scavenging the toxic intermediate of incomplete oxidation. SOD and CAT are the two major scavenging enzymes that remove radicals *in vivo*. A decrease in the activities of these antioxidant enzymes can lead to an excess availability of $O_2^{\cdot-}$ and H_2O_2, which in turn generate $OH^{\cdot}$, resulting in initiation and propagation of LPO. SOD protects tissues against oxygen free radicals by scavenging $O_2^{\cdot-}$, which damages the membrane and biological structures [49]. SOD can catalyze dismutation of $O_2^{\cdot-}$ into H_2O_2, which is then deactivated to H_2O by CAT or GPx [50]. Thus, SOD can act as a primary defense against $O_2^{\cdot-}$ and prevents further generation of free radicals. The activity of SOD was found to be lower in diabetic subjects. The observed decrease in SOD activity could result from inactivation by H_2O_2 or by glycation of the enzyme, which have been reported to occur in diabetes [51]. CAT is a hemeprotein, which is present virtually in all mammalian cells and is responsible for the reduction of H_2O_2 and protects tissues from highly reactive $OH^{\cdot}$ radicals [51]. The decrease in CAT activity could also result from inactivation by glycation of the enzyme. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of oxidative damage. Therefore removing $O_2^{\cdot-}$ and $OH^{\cdot}$ is probably one of the most effective defense against diseases [52]. Treatment with DS increased the activities of SOD and CAT in diabetic rats when compared with diabetic control rats. In fact, the reactivation in SOD activity promoted by DS may accelerate the dismutation of $O_2^{\cdot-}$ to

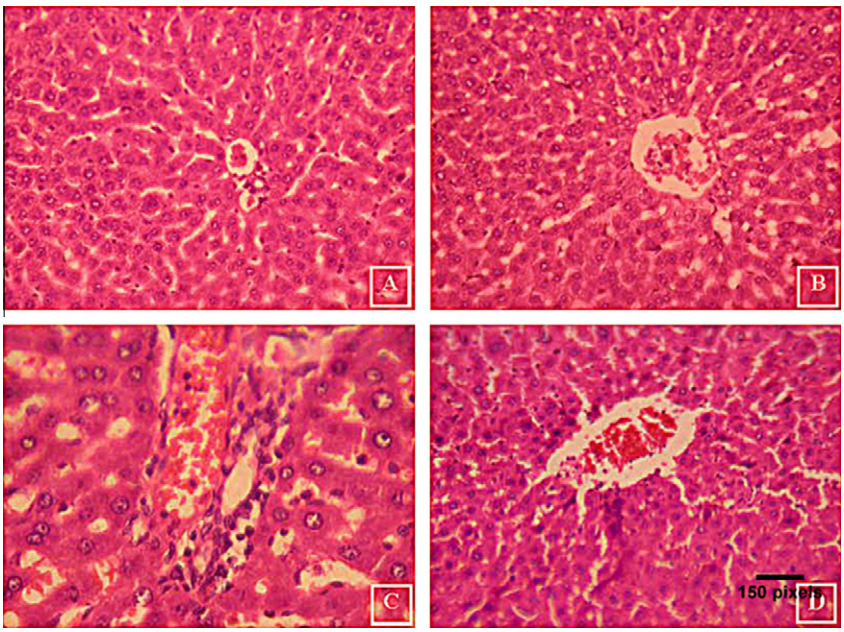

Fig. 7. Histology of liver on treatment with DS, STZ-NA; H&E staining 100×. Scale bar 150 pixels (Image J analysis software, NIH, 6th Version). (A) Shows normal hepatocytes around the central vein with sinusoidal cards around the central vein and portal tracts. (B) Shows normal architecture of the hepatocytes. (C) STZ-NA-induced diabetic liver shows the congestion of sinusoidal dilatation, inflammation of the central vein and focal necrosis in the hepatocytes. (D) Diabetic rats treated with DS shows near normal hepatocytes, mild sinusoidal dilatation with mild inflammation around central vein when compared to diabetic liver.

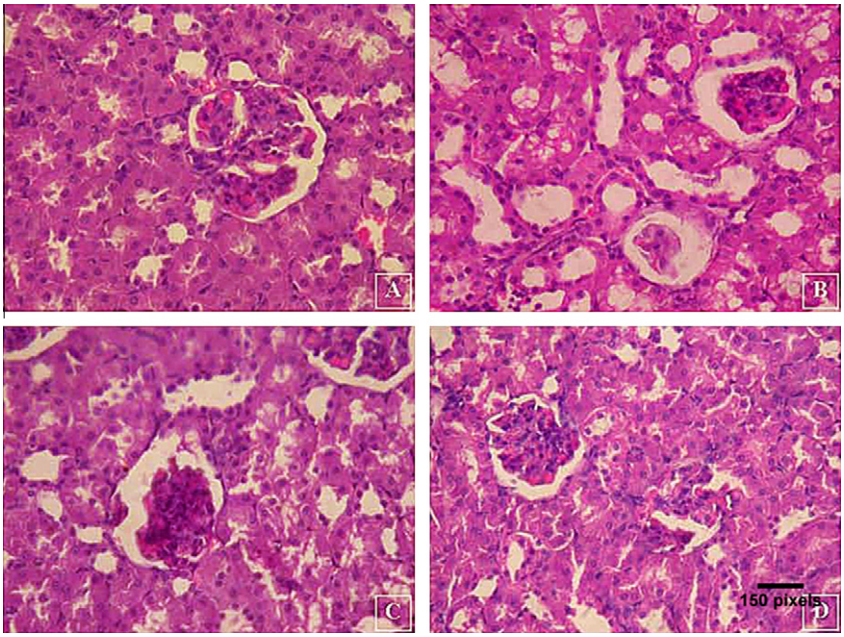

Fig. 8. Histology of kidney on treatment with DS, STZ-NA; H&E staining 100×. Scale bar 150 pixels (Image J analysis software, NIH, 6th Version). (A) Shows normal appearance of kidney, normal glomeruli and tubules. (B) Shows normal appearance of glomeruli. (C) STZ-NA-induced diabetic kidney shows multiple foci of hemorrhage, necrosis and swelling of tubules. (D) Diabetic rats treated with DS shows almost normal appearance of kidney of glomeruli and tubules when compared to diabetic kidney.

H_2O_2, which is quickly removed by CAT protecting the hepatic and renal tissues of diabetic rats against highly reactive and toxic $OH^{\cdot}$ and consequently preventing the LPO. The increased activities of antioxidant enzymes may act as an added compensation mechanism to maintain the cell integrity and protection against free radical damage. This showed that free radical scavenging ability of DS could exert a beneficial action against pathogenic alterations caused $O_2^{\cdot-}$ and $OH^{\cdot}$.

GPx and GST are important antioxidant enzymes, whose activities were significantly decreased in diabetic liver and kidney tissues, indicating impaired scavenging of H_2O_2 and lipid hydroperoxides [53,54]. GPx catalyzes the reaction of hydroperoxides with GSH to form glutathione disulfide. GPx uses GSH as a proton donor, converts H_2O_2 to H_2O and molecular oxygen [55]. Reduced activity of GPx may result from radical-induced inactivation and glycation of the enzyme [56]. Decreased GPx and GST

activities could be due to excess ROS [57], which are increased in diabetic rats. The decrease may also be due to the decreased availability of its substrate, GSH, which has been shown to be depleted during diabetes [58]. However, administration of DS could reverse the progress of disease, and increased the activities of GPx and GST in diabetic rats [59].

GR belongs to a family of flavin containing pyridine nucleotide-disulphide oxido reductases. GR serves to regenerate reduced GSH from oxidized GSSG by the activation of GPx [60]. In the current study, GR activity was decreased in diabetic rats. The decrease in GR activity may reflect the decline of the production and availability of GSH to overcome H_2O_2 [61]. GR requires NADPH for its activity, which is maintained at high level in the cell by the action of glucose-6-phosphate dehydrogenase through HMP shunt [62]. Decrease in the activity of glucose-6-phosphate dehydrogenase in diabetes results in reduced availability of NADPH and hence decreased level of GSH. Administration of DS has been reported to increase the activity of glucose-6-phosphate dehydrogenase in diabetic rats [22], which in turn enhances NADPH levels and the activity of GR. Thus GSH is replenished by the administration of DS, which may in turn maintain the antioxidant status in the tissues.

GSH is one of the essential compounds for maintaining cell integrity against ROS, as it can scavenge free radicals and reduce H_2O_2 [63]. The liver plays a major role in glutathione homeostasis and is the main export organ for glutathione [64]. The depletion of GSH below its basal level promotes the generation of ROS and oxidative stress with a cascade of effects on the functional and structural integrity of cells and organelle membranes [65]. GSH is required for the recycling of vitamin C and acts as a co substrate for GPx and GST which are involved in preventing the deleterious effect of oxygen radicals. Studies have shown that the tissue GSH concentrations of STZ-induced diabetic rats are significantly lower when compared with the control rats [55]. Decreased levels of GSH in the liver of diabetic rats may increase susceptibility to oxidative damage. In the present study, the elevation of GSH levels in plasma and tissues were observed in the DS treated diabetic rats [66]. This indicates that the DS can increase the biosynthesis of GSH and reduce the oxidative stress.

GSH and GSSG levels are commonly used markers for oxidative stress. Increased GSSG and, consequently, decreased GSH and GSH/GSSG ratio were implicated in the disruption of the intracellular antioxidative system [67]. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the oxidised form (GSSG), When cells are exposed to increased levels of oxidative stress, GSSG will accumulate and the ratio of GSH to GSSG will decrease. Therefore, the determination of the GSH/GSSG ratio and the quantitation of GSSG are useful indicators of oxidative stress in cells and tissues. The ratio of GSH/GSSG plays a critical role in glucose homeostasis of diabetes because thiol groups are important in intracellular and membrane redox state [68]. It has been reported that, under hyperglycemic conditions, as much as 30% of glucose is shunted to the polyol pathway [69], causing a marked depletion of NADPH, and consequently, a significant decrease in the GSH level. In our study, DS administration to diabetic rats significantly reduced GSSG and increased the GSH level and GSH/GSSG ratio in tissues of diabetic rats.

Earlier research have shown that diabetes rats have low levels of vitamin C and vitamin E. Supplementation of vitamin E helps to prevent the development of glucose intolerance and diabetes [70]. Vitamin E is a well-known physiological antioxidant and membrane stabilizer. It interrupts the chain reactions of LPO by reacting with lipid peroxy radicals, thus protecting the cell structures against damage. It is suggested to be a beneficial antioxidant for treatment of diabetic complication in humans as well as in STZ-treated rats [71]. Vitamin C is a major antioxidant that is essential for the scavenging of toxic free radicals in plasma and tissues. The disturbances in vitamin C metabolism in diabetes are might be important in the pathogenesis of diabetic complications [72]. Hyperglycemia has also been shown to inhibit the uptake of dehydroascorbic acid, the oxidized species of vitamin C [73]. Vitamin C scavenges ferociously destructive hydroxyl radicals. In our study, both the vitamin C and vitamin E significantly decreased in the plasma and tissues of diabetic rats. This could be due to the increased oxidative stress. Administration of DS increases the levels of vitamin C and vitamin E by directly scavenging the free radicals which in turn reduced oxidative stress.

In the present study, histopathological observation in diabetic control rats displayed the congestion of portal triad with mild inflammation; sinusoidal congestion in the liver and tubular epithelial damage; mesangial capillary proliferation in the kidney. The reaction is provoked by the increased production of highly reactive intermediates of STZ, which are normally detoxified by endogenous GSH but when present in excess, can deplete GSH stores, allowing the reactive intermediate to react with and destroy hepatic, renal cells. The above pathological changes were reduced in diabetic rats treated with DS. Thus, in addition to the blood glucose-lowering effect, histopathological observations also support the notion that DS produced significant increase of antioxidant enzymes and protected the hepatic and renal tissues in diabetic rats.

From the above findings, we conclude that DS has the ability to ameliorate oxidative stress in plasma and tissues STZ-NA induced diabetic rats as evidenced by improved glycemic and antioxidant status along with decreased lipid peroxidation. In addition, it protects histological changes from peroxidative injury through its antioxidant properties.

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