ORIGINAL ARTICLE

ATTENUATION OF OXIDATIVE STRESS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS BY *EUCALYPTUS GLOBULUS*

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ABSTRACT

In traditional medicine, Eucalyptus globulus (eucalyptus) was used for the treatment of diabetes mellitus. Hyperglycemia in diabetes has been associated with increased formation of reactive oxygen species (ROS) and oxidative damage to tissue compounds. The aim of this study was to evaluate the effects of eucalyptus in the diet (20 g/Kg) and drinking water (2.5 g/L) on lipid peroxidation, protein oxidation and antioxidant power in plasma and liver homogenate, as well as glycated-Hb (HbA_{1c}) of blood in streptozotocin-induced diabetic rats for a period of 4 weeks. Diabetes induced in rats by a single intraperitoneal injection of streptozotocin (STZ, 65 mg/Kg). At the end of the treatment period, the level of plasma glucose, plasma and liver malondialdehyde (MDA, the main product of lipid peroxidation), protein carbonyl (PC, one of the protein oxidation products) and HbA_{1c} increased and ferric reducing antioxidant power (FRAP) decreased in diabetic rats compared to normal rats. Eucalyptus administration for 4 weeks caused a significant decrease in the plasma glucose levels, plasma and liver MDA, PC and HbA_{1c}, also a concomitant increase in the levels of FRAP in diabetic treated rats. In conclusion, the present study showed that eucalyptus possesses antioxidant activities. Eucalyptus probably restores antioxidant power, due to the improved hyperglycemia in streptozotocin-induced diabetic rats.

KEY WORDS

Diabetes mellitus, Eucalyptus globulus, Ferric reducing antioxidant power, Malondialdehyde, Protein Carbonyl, Glycated-Hb.

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders of glucose characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (1). The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030. The total number of people

with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (2). In spite of the introduction of hypoglycemic drugs, diabetes and related complications continue to be a major medical problem (3). The chronic hyperglycemia was found to increase the production of free radicals that is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (4, 5). Several hypotheses have been reported to explain the genesis of free radicals in diabetes. These include oxidation of glucose, the non-enzymatic and progressive glycation of proteins with consequently increased formation of glucose-derived advanced glycation end products (AGEs) (6, 7). Evidences indicate that free radicals, membrane lipid peroxidation and protein oxidation are significantly increased in diabetic patients and in experimental diabetic animals (8-10). The increased production and/or ineffective scavenging of reactive oxygen

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species (ROS), resulting in tissue damage that in the most instances is assessed by the measurement of lipid peroxides and protein carbonyl (11, 12).

In recent years, there has been renewed interest in plant medicine for the treatment of diseases such as diabetes (13, 14). Furthermore, evidences have indicated that various plants including eucalyptus exert antidiabetic effects (15, 16). *Eucalyptus globulus* (Myrtaceae) grows in wide range of climatic conditions and is widely distributed throughout the Sistan-Balouchestan province of Iran. Moreover, the leaves of eucalyptus plant were traditionally used to treat diabetes mellitus (17). Furthermore, recent studies in streptozotocin-induced diabetic mice were confirmed the anti-hyperglycemic efficacy of eucalyptus (17, 18). The antioxidant activity of *Eucalyptus globulus* leaves has not been previously investigated. Thus, the purpose of the present study was to evaluate the effects of eucalyptus on oxidative stress in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Chemicals: TPTZ (2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine), TBA (2-thiobarbituric acid: 4, 6- dihydroxy-2-mercaptopyrimidine), n-butanol, sodium phosphate, sodium hydroxide, sodium acetate, phosphoric acid, ferric chloride, ferrous sulfate, glacial acetic acid, TCA (trichloroacetic acid), EDTA (ethylene diamine tetra acetic acid), guanidine-HCl, DNPH (2,4-dinitrophenylhydrazine), KCl, NaCl, digitonin, Coomassie blue brilliant G250 and bovine serum albumin were purchased from Merck. Streptozotocin and malondialdehyde standard (malondialdehyde bis dimethyl acetate) were obtained from Sigma.

Plant material: Fresh eucalyptus leaves were collected from Mellat garden of Zahedan, Iran. The leaves were washed with distilled water and dried in 45°C oven. Dried leaves were crushed and then powdered in an electrical grinder. The powder was stored at room temperatures ($25 \pm 2^\circ\text{C}$) until use. For animal foods, plant material was prepared according to method of Gray *et al* (16). Eucalyptus powder was incorporated into powdered rat diet (20 g/Kg), thoroughly mixed, distilled water added and mixed to a stiff paste. The mixture was then pelleted and dried at 45°C. Control diet was prepared by the same manner to ensure there was no difference in vitamin and mineral content as a result of the drying process. Aqueous extract of eucalyptus was prepared by 15 min decoction of the powdered leaves at 25 g/L. In brief, 2.5 g powdered material was placed in 100 ml distilled water, brought to boiling point, then was released from boiling and allowed to infuse

for 15 min. This suspension was filtered by Whatman paper No. 1 and the volume readjusted with distilled water to 100 ml and 10 ml aliquots of the extract were stored at -20°C until use when they were diluted 10-fold with tap water (2.5 g/L).

Preparation of animals: The study was performed on matured normoglycemic male Wistar rats, weighing 200-220 g, which were separately housed in cages (one rat per cage). Animals were maintained in a room at $23^\circ\text{C} \pm 2$, humidity 45% to 55% with a fixed 12 h artificial light period and allowed to eat and drink adlib. Rats were fed with standard rodent diet until initiation of treatment. All animals received humane care, as outlined in the guide for the care and use of laboratory animals.

Induction of diabetes in animals: Diabetes was induced by a single intraperitoneal administration of streptozotocin (65 mg/Kg of body weight) in 0.15 M NaCl with 100 mM sodium citrate buffer (pH 4.5). Control rats received the vehicle alone. After 5 days of development of diabetes, the rats with plasma glucose more than 200 mg/dl were considered as diabetic rats and used for experiment.

Experimental design and treatment: Thirty rats were divided in to three groups: (1) Non-diabetic control group: Rats of this group (n=10) received normal saline containing 100mM sodium citrate and fed with standard rodent diet and tap water throughout study; (2) Diabetic group: Rats of this group (n=10) received a single intraperitoneal administration of streptozotocin and fed with standard rodent diet and tap water throughout study; (3) Treated-diabetic group: rats of this group (n=10) received a single intraperitoneal administration of streptozotocin and fed standard rodent diet and tap water supplemented with 2.5g /L aqueous extract of eucalyptus 5 day after administration of streptozotocin. The experiment was carried out for 4 weeks after the initiation of treatment. Body weight, food and fluid intake of all groups were measured at the end of experiment period. At the end of the treatment period overnight-fasted rats were anesthetized under light ether and blood samples were collected from tip of the tail vein. Blood sample was collected in EDTA for determination of HbA_{1C} and preparation of plasma.

Isolation of liver tissue: After blood collection, animals were killed by cervical decapitation and livers removed and rinsed of any adhering blood. Then, livers were quickly sliced, and fragments were homogenated in appropriate buffers.

Liver homogenization for evaluation of oxidative stress: For TBARS and FRAP assay, a fraction of liver was

homogenized (1:10, w/v) in cold 1.15% KCl and 0.05 M sodium phosphate buffer pH 7.4, respectively with a Silent Crusher S homogenizer (Heidolph homogenizer, Germany). Homogenates were centrifuged at 6000g for 20 min at 4°C. Tissue homogenates for protein carbonyl assay were prepared by the method of Evans et al (19). Briefly, 200mg of tissue was homogenized in 2ml of phosphate buffer containing 0.1% digitonin. After 15 min, streptomycin sulfate was added to final concentration of 1% to the homogenized tissue and mixture was centrifuged at 2800g for 10 min at room temperature. The resulting supernatants were used for biochemical assays.

Assays: For evaluation of hyperglycemia, glucose levels were measured by glucose oxidase standard method. HbA_{1C} was estimated by a commercial Kit (BioSystem, Spain) according to manufacturer method (20). Concentration of lipid peroxidation product (MDA) or thiobarbituric acid reactive substances (TBARS) was determined spectrophotometrically by the method of Uchiyama and Mihara (21). 3 ml of 1% phosphoric acid and 1ml 0.6% w/v thiobarbituric acid aqueous solution was added to 0.5 ml of supernatant or plasma. The mixture was heated for 45 min in a boiling water bath. After cooling, 4 ml n-butanol was added, shaken and upper layer was separated by centrifugation at 1000 xg for 10 min. The light absorbance of the sample was determined at 535 nm and TBARS concentration was calculated using MDA standard curve. Concentration of thiobarbituric acid reactive substances (TBARS) in plasma and tissue was expressed in nmol/ml and nmol/mg of protein, respectively. Antioxidant power of plasma and liver were measured by ferric reducing/antioxidant power (FRAP). FRAP assay was performed according to the method as described (22, 23). The method is based on the reduction of the Fe³⁺-TPTZ (2, 4, 6-tri-(2-pyridyl)-s-triazin) complex to the ferrous form at low pH. This reduction is monitored by measuring the absorption change at 593 nm. Briefly, 3 mL of working FRAP reagent (25 ml 0.3 M sodium acetate buffer, pH 3.6; 2.5 ml 0.01 M TPTZ in 0.04 M HCl; 2.5 ml 0.02 M FeCl₃·6H₂O; preheated to 37°C) was mixed with 100 µL of supernatant or plasma and the absorbance at 593 nm was recorded after a 5 min incubation at 37°C. The absorption of the blue Fell-complex was measured at 593 nm using a WPA biowave II spectrophotometer. FeSO₄·7H₂O solutions from 0.2 to 1 mM were used for calibration. FRAP value in plasma and tissue was expressed as µmoles/L and µmoles/milligram of protein, respectively. Protein concentration was determined by Bradford's method using bovine serum albumin as standard (24).

Protein carbonyls were measured according to procedure described by Reznick and Packer (25) using dinitro-

phenylhydrazine (DNPH) reagent and spectrophotometric method. Four ml of 10 mM DNPH in 2.5 M HCl was added to 1 ml sample (tissue homogenate or 1/5 diluted plasma). In other tube as blank, 4 ml 2.5 M HCl was added to 1 ml sample. Tubes were incubated in room temperature and darkness for 1 hr and swirled every 15 min. Protein was precipitated with 5 ml of 20 % (w/v) trichloroacetic acid and the pellets were washed once with 4 ml of 10 % (w/v) trichloroacetic acid and three times with 4 ml of an ethanol/ethyl acetate mixture (1:1) to remove free DNPH and lipid contaminants. Washings were achieved by mechanical disruption of the pellets in the washing solution using a small spatula and re-pelleting was done by centrifugation at 6000 g for 5 min. Finally, the precipitates were dissolved in 1 ml 6 M guanidine-HCl solution and the absorbance was measured at 370 nm. The results were expressed as nanomoles of carbonyl groups per milligram of protein using a molar extinction coefficient of 22,000 M⁻¹ cm⁻¹. Protein contents were determined on the HCl blank pellets using a bovine serum albumin standard curve in guanidine-HCl and reading the absorbance at 280 nm.

Statistical data analysis: Results were expressed as mean ± SE for ten rats in each experimental group. Statistical analysis was performed using SPSS 11 software. One-way analysis of variance (ANOVA) followed by turkey's post hoc test was used to compare differences between experimental groups. The criterion for statistical significance considered P<0.05.

RESULTS

Table 1 shows body weight, food and fluid intake, plasma glucose concentration and HbA_{1C} levels in different experimental groups. Diabetic rats showed significant (P<0.05) weight loss, polyphagia, and polydipsia compared with control

Table 1: Effect of eucalyptus supplementation on food and fluid intake, body weight, plasma glucose and %HbA_{1C} of rats in different experimental groups

	Control	Diabetic	Treated-diabetic
Food intake (mg/day)	23.4 ± 3.2	52.4 ± 5.6 *	27.7 ± 6.6 **
Fluid intake (ml/day)	50.4±7.5	361.4±29.6 *	58.8± 8.65 **
Body weight (g)	298 ± 27.4	254± 20.5*	280 ± 25.6**
Plasma glucose (mg/dl)	89.5 ± 2.6	268± 8.5*	117.7 ± 5.25 **
HbA _{1C} (%)	6.4 ± 0.54	12.8 ± 9.4 *	7.1 ± 0.85 **

The values represent the mean ±SE for ten rats per group. Comparisons were made by one-way ANOVA test. * P<0.01 compared to control group; ** P<0.01 compared to diabetic group.

at the end of the treatment period. Administration of eucalyptus significantly ($P<0.01$) reduced the body weight loss, polyphagia and polydipsia in treated-diabetic rats compared with untreated-diabetic rats. Plasma glucose concentration and HbA_{1C} level in diabetic rats receiving eucalyptus were significantly lower ($P<0.01$) than untreated-diabetic rats and were not different from those of control animals.

Figure 1 shows concentration of TBARS in plasma and liver of control and diabetic animals. Diabetes caused a significant

Fig 1: TBARS level in plasma and liver of rats in different experimental groups. The values represent the mean $\pm$ SE for ten rats per group. Statistical analysis was performed by One-way ANOVA test (* $P<0.001$ compared to control group; ** $P<0.001$ compared to diabetic group)

Fig 2: FRAP level in plasma and liver of rats in different experimental groups. The values represent the mean $\pm$ SE for ten rats per group. Statistical analysis was performed by One-way ANOVA test (* $P<0.001$ compared to control group; ** $P<0.001$ compared to diabetic group)

Fig 3: Protein carbonyl (PC) level in plasma and liver of rats in different experimental groups. The values represent the mean $\pm$ SE for ten rats per group. Statistical analysis was performed by One-way ANOVA test (* $P<0.01$ compared to control group; ** $P<0.01$ compared to diabetic group)

increase in TBARS concentration in plasma and liver compared to the corresponding treated-diabetic rats ($P<0.001$). Treatment with eucalyptus ameliorated lipid peroxidation. The changes in antioxidant power (FRAP) were shown in Figure 2. FRAP was found to be lower ($P<0.001$) in plasma and liver of diabetic rats compared to control ones. Antioxidant power was significantly decreased in treated-diabetic rats ($P<0.001$). Treatment with eucalyptus improved antioxidant power.

Protein carbonyl concentration in plasma and liver of all animal groups are indicated in Figure 3. The level of PC was increased in untreated-diabetic rats compared to control ones ($P<0.01$). The results shown in Figure 3 indicate that eucalyptus treatment significantly reduced protein carbonyl concentration in treated-diabetic group ($P<0.01$).

DISCUSSION

Streptozotocin (STZ, 2-deoxy-2-(3-methyl-3-nitrosourea)-D-glucopyranose), is synthesized by *Streptomyces achromogenes* and has long been used to generate animal models of diabetes (26). Streptozotocin-induced diabetes animals exhibit most of the diabetic complication (27). During diabetes, advanced glycation end products form when glucose reacts with various proteins such as hemoglobin, albumin, collagen, LDL, or crystalline proteins to form labile Schiff bases, which then undergo further modification to form Amadori products (28, 29). Additional rearrangements or modifications

may give rise to advanced glycation end products seen in prolonged hyperglycemia. A common modification is generated by oxidative cleavage of Amadori intermediates to form epsilon-(carboxymethyl) lysine structures (30). The rate of glycation is proportional to the blood glucose concentration (31). Glycated hemoglobin (HbA_{1C}) was found to increase in the patients with uncontrolled or poorly controlled diabetes mellitus and amount of increase is directly proportional to the hyperglycemic state (32). Evidence showed that glycation itself may induce the formation of oxygen-derived free radicals in diabetic condition, and the level of HbA_{1C} is considered as one of the markers of degree of oxidative stress in diabetes mellitus (33). Therefore, the measurement of HbA_{1C} is supposed to be a very sensitive index for glycemic control (34). In the present investigation, diabetic animals showed higher levels of HbA_{1C}, compared with control rats. Treatment with eucalyptus reduced significantly ($P<0.01$) levels of HbA_{1C}, in treated-diabetic rats that could be due to an improvement in hyperglycemia. On the other hand, streptozotocin-induced diabetes is characterized by loss in body weight, polyphagia and polydipsia and these were also seen in our study. The decrease in body weight observed in uncontrolled diabetic might be the result of protein wasting due to unavailability of carbohydrate for utilization as an energy source. Body weight enhanced significantly ($P<0.01$) in eucalyptus treated diabetic rats when compared with untreated-diabetic ones. Likewise, eucalyptus decreased significantly ($P<0.01$) polyphagia and polydipsia in treated-diabetic rats when compared with diabetic group.

Chronic hyperglycemia induces carbonyl stress, which in turn can lead to increased lipid peroxidation (35). The increased concentration of lipid peroxidation induces oxidative damage by increasing peroxy radicals and hydroxyl radicals (36). Thus, lipid peroxidation is one of the characteristics features of chronic uncontrolled diabetes. The most commonly used indicator of lipid peroxidation is TBARS (37). The increased lipid peroxidation in the plasma and tissues of diabetic animals may be due to the observed remarkable increase in the concentration of TBARS and MDA as a main product of lipid peroxidation in the plasma and liver (38). In the present study, significant ($P<0.001$) elevations of TBARS levels were observed in the plasma and liver homogenate of diabetic rats compared to the corresponding control rats. Administration of eucalyptus decreased significantly ($P<0.001$) TBARS in treated diabetic when compared with diabetic rats. Several studies have been shown that some constituents isolated from *Eucalyptus globulus* significantly reduces free radicals and inhibited lipid peroxidation (39, 40). Oxidative stress in diabetes coexists with a reduction in the antioxidant power (41). Cakatay

et al also indicated that total antioxidant capacity (FRAP) levels in plasma of chronic diabetic animals were decreased significantly as compared to those of control animals (42). The present work, indicated the same results and showed significant ($P<0.001$) reduction in plasma and liver homogenate FRAP of diabetic rats when compared with control animals. Treatment with eucalyptus improved significantly ($P<0.001$) antioxidant power in treated-diabetic rats when compared with untreated-diabetic group. Elevated protein carbonyl levels have been detected in diabetes (10, 43). High plasma PC levels in diabetic children and adolescents without complications compared with control subjects indicate that oxidative protein damage occurs at the onset of disease and tends to increase in the later stages. Furthermore, decreased antioxidant defenses might increase the susceptibility of diabetic patients to oxidative injury (44). The results of the present study also showed that tissue and plasma PC levels were increased in untreated diabetic rats compared to controls ($P<0.01$) and Eucalyptus causes improvement of protein oxidation ($P<0.01$).

In conclusion, the results of this study suggest that *Eucalyptus globulus* possess antidiabetic and antioxidant activity. Previous studies have shown antihyperglycemic and improving effects of eucalyptus on loss of body weight and polydipsia in streptozotocin-induced diabetic animals (17, 18). Also, data of this study indicates that eucalyptus can either increase antioxidant power or reduce the oxidative stress or both. *Eucalyptus globulus* probably improved oxidative stress, due to reduction in plasma glucose level in diabetic rats, which prevents excessive production of free radicals through glycation of the proteins.

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