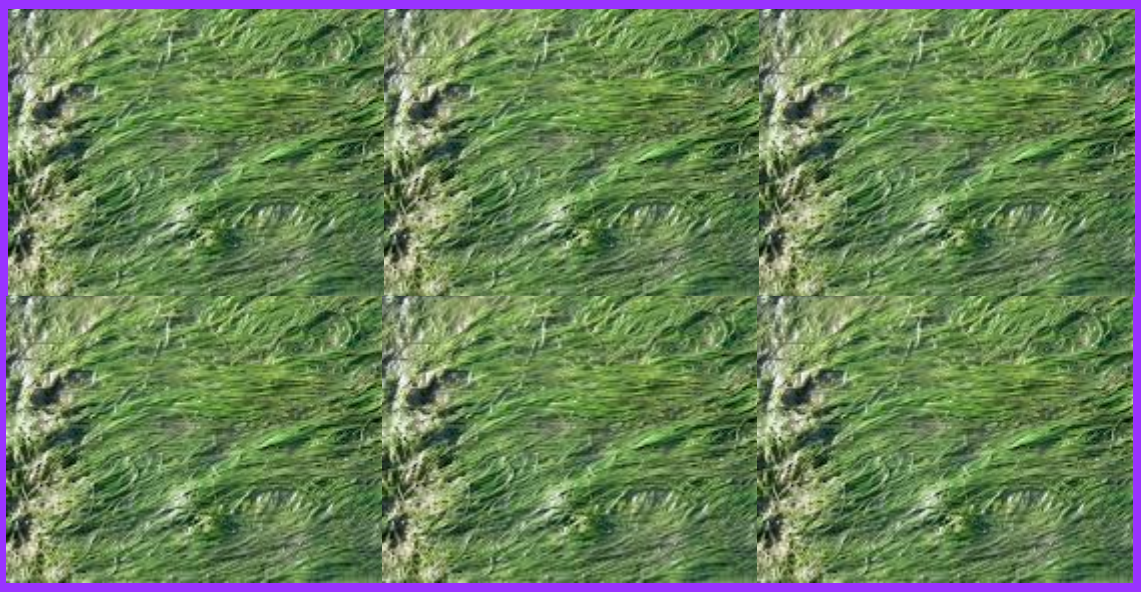

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Zostera noltii extract lowers blood glucose and restores vascular function in diabetic rats

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Abstract

The antidiabetic effect of seagrass *Zostera noltii* extract was investigated through a crosstalk between its anti-oxidant and vasoprotective properties. The extract was orally administered to alloxan-diabetic rats (50, 150, 250 mg/kg body weight). Serum glucose was determined; liver and kidney functions, body weight, total leukocyte counts were measured; liver oxidative markers were assayed. Acetylcholine, phenylephrine and 5-HT responses were tested. eNOS levels and generation of ROS in aortic tissue were quantified. The extract of *Z. noltii* lowered blood glucose in all tested dose levels. Extract at a concentration of 50 mg/kg failed to preserve the levels of anti-oxidants and did not alter lipid peroxidation whereas higher doses improved liver oxidative status. Impaired acetylcholine relaxations, augmented phenylephrine and 5-HT contractions in alloxan-diabetic aortic rings were restored by *Z. noltii* treatment. This recovery was accompanied by increased eNOS synthesis and a reduction in ROS generation. The extract lowers blood glucose and prevents hyperglycemia-induced endothelial dysfunction in alloxan-diabetic rats.

Introduction

Diabetes mellitus is a life-threatening metabolic disorder of multiple etiology. Oxidative stress has been recognized as a critical player in the pathogenesis of diabetes (Murugan and Pari, 2006). Furthermore, chronic hyperglycemia itself, enhances the production of reactive oxygen species (ROS) by glucose auto-oxidation and/or non-enzymatic protein glycosylation (Giugliano et al., 1996; Signorini et al., 2002). Elevated oxidants and markers for oxidative tissue damage have been well documented in patients with diabetes (Rehman et al., 1999; Chowienczyk et al., 2000; Fava et al., 2002; Zitouni et al., 2005). Therefore, anti-oxidants gained considerable attention in prevention of diabetes and its micro- and macro-vascular complications which are regarded as the most common reasons of morbidity and mortality (Guo et al., 2005; Gokce and Haznedaroglu, 2008).

While a damaged endothelium is the starting point to diabetic macroangiopathy, impairments in endothelium - and nitric oxide (NO)-dependent micro-vascular function, may contribute to several other corollaries of diabetes, such as hypertension, dyslipi-demia and insulin resistance (Laight et al., 1999). Anti-oxidant therapy may conceivably confer both cardio-vascular and metabolic benefits in diabetes. This notion is well grounded in the theory surrounding the role of oxidative stress in disease and supported by evidence of reduced anti-oxidant defenses in diabetes and also by experimental findings that anti-oxidants improve endothelium-dependent vasodilation and insulin sensitivity (Laight et al., 1999). Moreover, there is an increasing demand by patients to use natural anti-diabetic products due to undesirable side effects of insulin and

oral hypoglycemic drugs (Rao and Rao, 2001; Gong et al., 2012; Mohan et al., 2014)

Zostera noltii Hornemann (Zosteraceae) is a perennial phanerogam with small leaves growing permanently submerged. It is distributed in shallow sheltered sea bays from the southern coasts of Norway to the Mediterranean Sea, the Black Sea, besides Northwest Africa coasts (Mireia et al., 2011). As with other seagrasses, *Z. noltii* plays important roles in marine ecosystems for biodiversity, ecological, sedimentary and economic reasons (Rende et al., 2012). Seagrasses are thought to be chemically defended against herbivores and pathogenic infections such as wasting disease by phenolic compounds (Grignon-Dubois et al., 2012). *Z. noltii* is rich in phenolic compounds and flavonoids such as, chlorogenic acid, caffeic acid, rosmarinic acid zosteric acid, apigenin, diosmetin, luteolin and luteolin-7-O-glucoside (Lamaison et al., 1990; Zeljan and Misko, 2000; Achamlale et al., 2009; Newberry et al., 2011; Sato et al., 2011). Additionally, crude extracts of seagrasses are shown to exert antibacterial, antifungal, antiviral, anti-oxidant, anti-inflammatory, anti-diabetic and anti-cancer activities (Papenbrock, 2012). Moreover, we have shown that total extract of another seagrass, *Posidonia oceanica* has anti-oxidant, antidiabetic and vasoprotective effects (Gokce and Haznedaroglu, 2008). Present study aims to investigate the glucose lowering activity of *Z. noltii* in alloxan-induced diabetes, through a cross-talk between its anti-oxidant and vasoprotective properties.

Materials and Methods

Plant material

Z. noltii was collected from Urla, Izmir, Aegean Sea in October 2011 at 1 m depth. The plant was identified at the Department of Pharmaceutical Botany, Faculty of Pharmacy, Ege University, Izmir and specimen vouchers are kept at IZEF Herbarium (IZEF5887). The epiphytes on the leaves were removed with paper towel without damaging the organs. Leaves were dried in shadow and at controlled room conditions (25°C).

Extraction

Chopped leaves were infused with aqueous ethanol 50% (v/v) for 3 hours in a water bath at 40°C with a reflux system. Homogenate was filtered and acidified (pH=3) with HCI. Ethanol was evaporated under vacuum at 45°C. Obtained aqueous residue was extracted with ethyl acetate. Water is removed from organic phase with anhydrous sodium sulfate, filtered and evaporated under vacuum. Dry samples were prepared following filtration, evaporation and lyophilization. After 25 repetitions with overall 1.2 kg of plant material 24 g of extract was obtained (Cuny et al., 1995).

Chemicals

All drugs were purchased from Sigma (USA). Solvents used in extraction procedure were purchased from Labscan (Ireland), while hydrochloric acid and anhydrous sodium sulfate were from Riedel Haen (Germany).

Animals

Three-month-old male Wistar rats (200-225 g; n=30; Lemali Ltd, Ankara, Turkey) were used. Animals were maintained under 22 ± 2°C and a 12 hours light/dark cycle day and had unrestricted access to pelleted food and water. At end of the experimental protocol final body weights were recorded and animals were killed by means of an overdose of sodium pentobarbital.

Glucose tolerance

Rats were fasted overnight and rando-mized to three groups (n=6). Control group received 1 mL of distilled water orally (Group I). *Z. noltii* was administered at concentrations of 100, 500 mg/kg (p.o, Groups II and III, respectively). Following *Z. noltii* administration, all groups received glucose (p.o, 2 g/kg). Blood samples were taken from the tail vein just prior to and 30, 60, 120 and 240 min after glucose loading. Glucose was assayed by glucose oxidase method (Trinder, 1976). Data obtained from glucose tolerance test were used as a hypothetical reference to determine the dose level which will be used in evaluation of short- and long-term effects of *Z. noltii* on diabetic rats.

Antidiabetic effects

Diabetes was induced by a single intraperitoneal injection of alloxan monohydrate (120 mg/kg) (Cooperstein and Walkins, 1981). After 72 hours, animals with glucose levels higher than 250 mg/dL were considered diabetic (Perfumi and Tacconi, 1996) and randomized to five groups (n=6). Group I (no alloxan treatment) and Group II (diabetic control) rats were given 1 mL of distilled water. Groups III–V received aqueous suspension of *Z. noltii* on the 3rd day after alloxan administration (respectively, p.o, 50, 100, and 150 mg/kg). Blood samples were taken from the tail vein (fasted rats) just prior to administration of the extract and at 2, 4, 6 and 8 hours intervals. Serum was separated and glucose levels were estimated. These rats were given the same doses of the extract once daily for 15 days in the multidose study. Blood (non-fasted rats) was taken on 6, 9, 12, 15 and 18th day after alloxan administration (Sabu et al., 2002) and serum glucose levels were measured as mentioned above.

Liver and kidney functions

Alkaline phosphatase (ALP) (King et al., 1980), glutamate pyruvate transaminase (GPT) (Bergmeyer et al., 1980), blood urea nitrogen (BUN) (Haslam et al., 1966), and creatinine (Brod et al., 1948) were measured.

Protein content was determined by the method of (Lowry et al., 1951). Total white blood cell count was determined using a hemocytometer.

Anti-oxidant status

In liver homogenates, reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase, and malondialdehyde (MDA levels) were determined spectrophotometrically (Victor III; Perkin Elmer, Finland) using commercially available assay kits and according to the manufacturer's instructions (Bioxytech GSH-412, GPx-340, SOD-525, Catalase-520, and MDA-586, respectively; Oxis International, USA). Total nitrite in aortic tissue was estimated colorimetrically using Griess reagent (Guevara et al., 1998). Nitrite concentration in the sample was calculated using sodium nitrite as standard and normalized to the protein content of aorta.

Effects of extract on vascular responses

Aortic rings (2 mm) were mounted under 2 g resting tension on stainless steel hooks within 25 mL tissue baths and maintained at 37°C in Krebs solution (mM): NaCl, 118; KCl, 4.7; $CaCl_2 \cdot 2H_20$, 2.5; KH_2PO_4, 1.20; $MgSO_4 \cdot 7H_20$, 1.17; Glucose, 11.1; $NaHCO_3$, 25, gassed with 95%O_2 and 5%CO_2. Tension was recorded isometrically by a data acquisition system (Biopac, MP100) equipped with a Grass FT03 transducer. Vascular reactivity studies were performed as previously described (Gokce and Haznedaroglu, 2008).

Reactive oxygen species

Levels of superoxide anion and other reactive species in aortic rings were determined according to the method described by Wang et al. (2001), with slight modifications. 2 mm cut aortic segments were placed in a 96 well plate containing PBS-HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.4). After addition of chemiluminescence enhancers, lucigenin or luminol (final concentration of 5 µmol/L for either), ROS were quantified using a multi-plate reader (Victor III-1420, Perkin Elmer, Finland). Results were corrected for wet tissue weight and expressed as relative light units rlu/mg tissue) (Haklar et al., 2003).

Determination of endothelial nitric oxide synthase (eNOS)

Endothelial nitric oxide synthase levels in aortic tissue was determined using a commercially available ELISA kit (USCNlife, USA) according to the manufacturer's instructions. Optical density was measured at 450 nm using a microplate reader (Victor III-1420, Perkin Elmer, Finland). A standard linear regression curve was generated and the concentration of each sample was calculated by the curve equation.

Statistical analysis

Data are expressed as the mean ± S.E. Student's t-test for unpaired samples (GraphPad Prism, Version 3.02, USA). A value of $p<0.05$ was considered significant. Agonist stimulated vasoactivity was evaluated in means of maximal effect (E_{max}) and pD_2 (negative logarithm of the concentration that produced half of the E_{max}, as a measure of sensitivity) values. Acetylcholine-induced relaxant responses were normalized by the initial phenylephrine contraction. Contractile responses to phenylephrine and 5-HT were normalized with maximal contractions induced by potassium chloride.

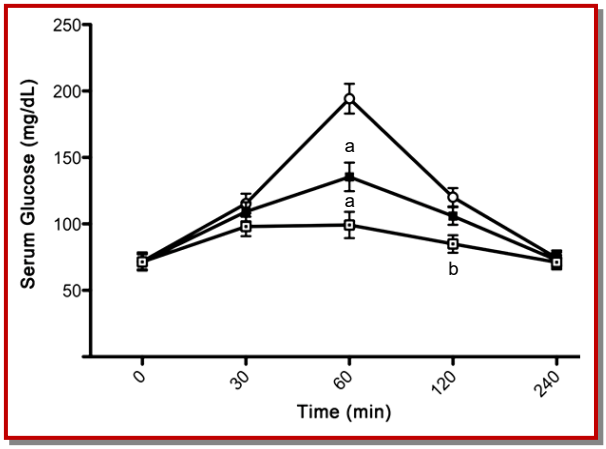

Figure 1: Effects of *Z. noltii* extract on glucose tolerance. Glucose levels obtained from Group I (control) (¡); Group II (*Z. noltii* extract 100 mg/kg) (<); and Group III (*Z. noltii* extract 500 mg/kg) (o) are shown. Data are expressed as mean ± S.E. ($^a p<0.01$, compared to control at 60 min and $^b p<0.05$ compared to control at 120 min; n = 6

Results

Sixty min after glucose loading, serum glucose level of 71.7 ± 6.8 mg/dL increased up to 198.0 ± 8.1 mg/dL and returned to baseline at 240 min. *Z. noltii* extract improved glucose tolerance in a dose-dependent manner (Figure 1, $^a p<0.01$; control vs. *Z. noltii*-100 and *Z. noltii*-100 *vs. Z. noltii*-500). Effect of the extract on glucose tolerance at the dose level of 500 mg/kg remained statistically significant at 120 min (Figure 1, $^b p<0.05$; control *vs. Z. noltii*-500).

In the single dose study, effects of *Z. noltii* extract on blood glucose levels were evaluated on the 3rd day after alloxan administration. *Z. noltii* extract (50 mg/kg) did not reduce serum glucose. At dose levels of 150 and 250 mg/kg, glucose levels were decreased by 24.8 and 29.9% at the 6th hour, respectively. Antidiabetic effect of the extract was slightly decreased at the 8th hour, but remained statistically significant (Table I).

In parallel experiments, *Z. noltii* extract was administered to diabetic rats for 15 days at aforementioned doses (Table II). *Z. noltii* extract (50 mg/kg) started to lower serum glucose on the 9th day and an overall reduc

Table I

Effects of *Z. noltii* extract (single dose) on serum glucose levels in alloxan-induced diabetic rats

Group	Treatment (mg/kg)	Serum glucose (mg/dL)				
		0 hour	2 hour	4 hour	6 hour	8 hour
I	Normal	71.7 ± 6.8	68.4 ± 7.1	65.2 ± 8.1	68.6 ± 8.3	70.3 ± 8.1
II	Control (alloxan)	269.1 ± 11.7[a]	275.3 ± 10.9[a]	278.2 ± 12.1[a]	274.3 ± 9.2[a]	268.6 ± 8.8[a]
III	*Z. noltii* extract (50)	262.6 ± 10.8	265.6 ± 9.8	259.4 ± 9.7	256.4 ± 11.1	253.7 ± 9.9
IV	*Z. noltii* extract (150)	259.3 ± 9.7	254.2 ± 9.3	238.6 ± 8.8	206.1 ± 10.4[b]	215.4 ± 10.2[c]
V	*Z. noltii* extract (250)	255.1 ± 12.1	243.1 ± 11.1	231.7 ± 9.1	192.4 ± 9.6[b]	199.5 ± 9.7[b]

Serum glucose levels were obtained from fasted rats on the 3rd day after alloxan administration. Data are expressed as mean ± S.E; (n=6); [a]p<0.0001 (compared to normal group with corresponding hour). [b]p<0.01 and [c]p<0.05 (compared to control group with corresponding hour)

Table II

Effects of *Z. noltii* extract (daily treatment) on serum glucose levels in alloxan-induced diabetic rats

Group	Treatment (mg/kg)	Serum glucose (mg/dL)					
		3 days	6 days	9 days	12 days	15 days	18 days
I	Normal	73.7 ± 6.1	71.6 ± 7.5	70.1 ± 9.4	72.9 ± 7.6	69.6 ± 5.6	73.8 ± 7.1
II	Control (alloxan)	271.2 ± 11.8[∂]	278.2 ± 10.4[∂]	270.3 ± 10.4[∂]	272.5 ± 9.8[∂]	267.4 ± 9.9[∂]	261.8 ± 8.3[∂]
III	*Z. noltii* extract (50)	268.7 ± 9.7	248.4 ± 9.8	229.3 ± 8.2[*]	217.3 ± 7.9[*]	207.5 ± 8.7[*]	198.5 ± 7.9[*]
IV	*Z. noltii* extract (150)	274.1 ± 8.5	230.2 ± 9.3	209.9 ± 11.1[**]	173.1 ± 8.8[***,a]	149.8 ± 9.5[***,b]	130.2 ± 10.2[+,c]
V	*Z. noltii* extract (250)	270.3 ± 9.4	219.5 ± 8.1[**]	165.2 ± 9.9[***]	140.2 ± 7.6[+,d]	129.6 ± 8.4[+,e]	103.4 ± 11.1[+,f]

Values are of serum glucose levels obtained from alloxan-induced diabetic rats in the absence and in the presence of 15 day Z. noltii extract treatment (from the 3rd to the 18th day) and expressed as mean ± S.E; n=6; [∂]p<0.0001 (compared to normal group with corresponding day); [*]p<0.05, [**]p<0.01, [***]p<0.001 and [+]p<0.0001 (compared to control group with corresponding day). [a]p<0.05, [b]p<0.01 and [c]p<0.001 (compared to Group III with corresponding day). [d,e,f]p<0.05 (compared to Group IV with corresponding day)

Table III

Effects of *Z. noltii* extract on liver and kidney functions

Treatment (mg/kg)	Liver	Liver	Kidney	Kidney
	ALP (KA/dL)	GPT (U/mg protein)	BUN (mg/dL)	Creatinine (mg/dL)
Normal	33.7 ± 2.8	159.4 ± 10.1	9.2 ± 0.6	0.7 ± 0.0
Control (alloxan)	50.3.1 ± 6.7[a]	335.3 ± 21.9[b]	19.3 ± 2.2[c]	2.1 ± 0.1[c]
Z. noltii extract (50)	42.6 ± 4.8	285.6 ± 19.8	16.4 ± 11.1	1.7 ± 0.1
Z. noltii extract (150)	35.3 ± 2.7[e]	244.2 ± 12.3[e]	13.6 ± 0.7[f]	1.5 ± 0.0[e]
Z. noltii extract (250)	31.1 ± 12.1[f]	206.1 ± 9.1[f]	11.5 ± 0.6[g]	1.2 ± 0.0[g]

ALP: alkaline phosphatase; GPT: glutamate pyruvate transaminase; BUN: blood urea nitrogen. Liver and kidney markers were measured on the 18th day after aloxan administration. Data are expressed as mean ± S.E; n=6; [a]p<0.01, [b]p<0.001 and [c]p<0.0001; compared to normal group. [e]p<0.05, [f]p<0.01 and [g]p<0.001 (compared to control group)

Table IV

Effects of Z. noltii extract on body weight, total leucocyte count and liver glycogen

Treatment (mg/kg)	Body weight		Total leukocyte count (mm^3)	Liver glycogen (µg/g tissue)
	Initial	Final		
Normal	241.7 ± 7.8	259.4 ± 9.1	13234.6 ± 456.7	76.9 ± 4.6
Control (alloxan)	245.3 ± 9.6	205.3 ± 21.9[a]	8567.2 ± 389.2[c]	58.1 ± 4.8[d]
Z. noltii extract (50)	249.6 ± 10.1	245.6 ± 19.8[b]	9453.6 ± 511.2	67.7 ± 4.1
Z. noltii extract (150)	243.3 ± 8.1	249.2 ± 12.3	11103.6 ± 658.1[e]	74.5 ± 5.1[e]
Z. noltii extract (250)	247 ± 12.1	263.1 ± 9.11	13002.5 ± 703.1[f]	77.6 ± 5.5[e]

Data are expressed as mean ± S.E; n=6; [a]p<0.001 (compared to initial body weight of the same group), [b]p<0.05 (compared to initial body weight of the same group), [c]p<0.0001 and [d]p<0.05 (compared to normal group), [e]p<0.05 and [f]p<0.001 (compared to control group)

Table V

Effects of *Z. noltii* extract on oxidative status

Parameter	Normal	Control (alloxan)	*Z. noltii* extract (50 mg)	*Z. noltii* extract (150 mg)	*Z. noltii* extract (250 mg)
GSH (nmol/mg protein)	7.4 ± 0.4	4.2 ± 0.2^{c}	4.5 ± 0.3	5.9 ± 0.4*	6.3 ± 0.4***
GSSG (nmol/mg protein)	0.3 ± 0.0	0.9 ± 0.1^{c}	0.7 ± 0.1	0.6 ± 0.1*	0.3 ± 0.1***
GPx (U/mg protein)	0.2 ± 0.0	0.1 ± 0.0^{a}	0.1 ± 0.0	0.1 ± 0.0*	0.2 ± 0.1*
MDA (nmol/mg tissue)	354.2 ± 10.2	442.2 ± 14.9^{b}	422.3 ± 13.1	390.2 ± 9.2*	355.6 ± 11.6**
SOD (U/mg protein)	6.3 ± 0.4	3.3 ± 0.3^{c}	3.6 ± 0.3	4.4 ± 0.6**	5.6 ± 0.5***
Catalase (U/mg protein)	153.6 ± 6.7	98.3 ± 7.0^{b}	119.2 ± 8.3	126.2 ± 7.5*	142.7 ± 10.6**

GSH: reduced glutathione, GSSG: oxidized glutathione; GPx: glutathione peroxidase, MDA: malondialdehyde; SOD: superoxide dismutase. Data are expressed as mean ± S.E; n = 6; ^{a}p<0.01, ^{b}p<0.001, ^{c}p< 0.0001 and **p<0.01 (compared to normal group); *p<0.05 and ***p<0.001 (compared to control group)

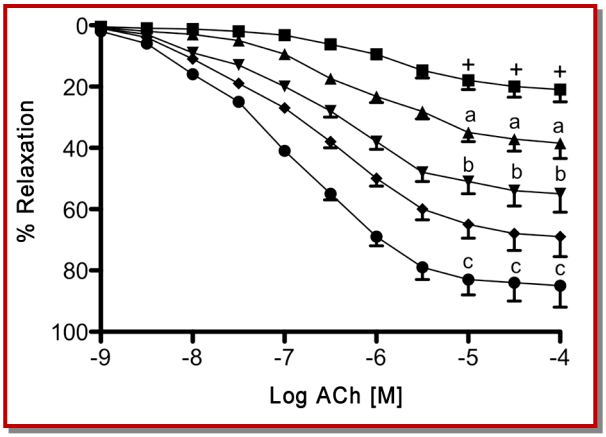

Figure 2: Effects of *Z. noltii* extract on acetylcholine relaxations in alloxan diabetic rats. Concentration–response curves obtained from Group I (normal) (l); Group II (diabetic control) (n); Group III (*Z. noltii* extract 50 mg/kg) (▲); Group IV (*Z. noltii* extract 150 mg/kg) (▼); and Group V (*Z. noltii* extract 250 mg/kg) (¿) are shown. Data are expressed as mean ± S.E; ($^{+}$p<0.0001; compared to normal group, ^{a}p<0.01, ^{b}p<0.001 and ^{c}p<0.0001; compared to diabetic control group; n = 6

-tion of 26% was observed on the 18th day (p<0.01). At dose level of 150 mg/kg, serum glucose was found to decrease by 52.5% on the 18th day (p<0.0001). The effect of the extract was more pronounced at 250 mg/kg, starting on the 6th day with a reduction rate of 18.9% (p<0.01) and and finally reaching to its maximum on the 18th day by 61.9%. Between the 12th and 18th days, antidiabetic effect of *Z. noltii* extract was in a concentration dependent manner (Table II).

Table III summarizes the effects of *Z. noltii* extract on hepatic and renal function in alloxan-diabetic rats. As seen, treatment with 50 mg/kg did affect neither the significantly high levels of ALP, GPT, BUN and creatinine, nor the overall oxidative status. Conversely, rats treated with higher doses of the extract (150 and 250 mg/kg) showed significant improvements in hepatic and renal function (Table III). Additionally, these two dose levels recovered the weight loss and low white blood cell count observed in alloxan-diabetic rats while decreasing liver glycogen (Table IV). *Z. noltii* extract (150 and 250 mg/kg) also showed a protective effect on liver oxidative status (Table V). Anti-oxidants namely GSH, GPx, SOD and catalase were increased by *Z. noltii* extract administration. When compared to alloxan-diabetic rats, MDA formation, as an indirect measure of lipid peroxidation, was found to be significantly low in high dose *Z. noltii* extract-treated rats (Table V).

Table VI

Effects of *Z. noltii* extract on acetylcholine, phenylephrine and 5-HT-induced vascular responses

Group	Treatment (mg/kg)	Acetylcholine					
		E_{max}	pD_2	E_{max}	pD_2	E_{maks}	pD_2
I	Normal	85.1 ± 7.3	6.9 ± 0.08	146.1 ± 12.1	6.2 ± 0.06	126.1 ± 8.1	5.9 ± 0.04
II	Control (alloxan)	21.2 ± 4.2^{a}	5.9 ± 0.05^{a}	370.2 ± 24.2^{a}	6.8 ± 0.06^{a}	280.2 ± 14.2^{a}	6.6 ± 0.05^{a}
III	Z. noltii extract (50)	38.5 ± 5.1*	6.3 ± 0.06*	278.3 ± 15.2*	6.5 ± 0.05^{d}	227.3 ± 10.2*	6.5 ± 0.03
IV	Z. noltii extract (150)	55.2 ± 6.2^{**,b}	6.5 ± 0.05^{**,b}	226.3 ± 11.4^{**,b}	6.4 ± 0.03^{d}	180.4 ± 9.1^{**,b}	6.3 ± 0.04
V	Z. noltii extract (250)	69.3 ± 6.5^{***,c}	6.8 ± 0.04^{***,c}	177.4 ± 8.5^{***,c}	6.3 ± 0.03*	130.2 ± 8.3^{***,c}	6.2 ± 0.05*

E_{max}: maximal effect; pD_2: negative logarithm of the concentration that produced half of the E_{max}. Data are expressed as mean ± S.E: n=6; ^{a}p<0.0001; compared to normal group; ^{d}p<0.05, *p<0.01, **p<0.001 and ***p<0.0001; compared to control group); ^{b}p<0.05; compared to Group III; ^{c}p<0.05 (compared to Group IV)

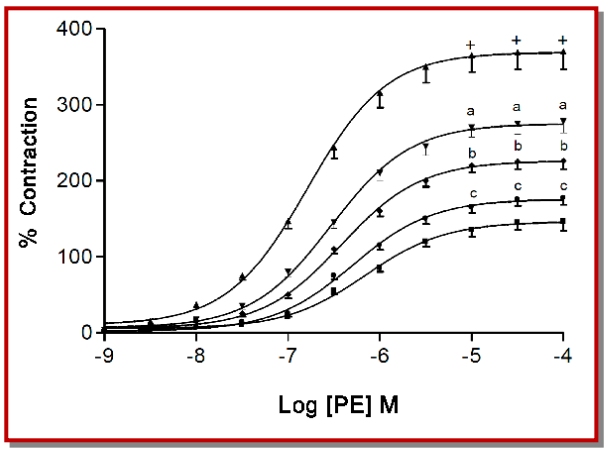

Figure 3: Effects of *Z. noltii* extract on phenylephrine contractions in alloxan diabetic rats. Concentration–response curves obtained from Group I (normal) (n); Group II (diabetic control) (▲); Group III (*Z. noltii* extract 50 mg/kg) (▼); Group IV (*Z. noltii* extract 150 mg/kg) (¿); and Group V (*Z. noltii* extract 250 mg/kg) (=) are shown. Data are expressed as mean ± S.E. ($^{+}p<0.0001$; compared to normal group; $^{a}p<0.01$, $^{b}p<0.001$ and $^{c}p<0.0001$; compared to diabetic control group; $n = 6$

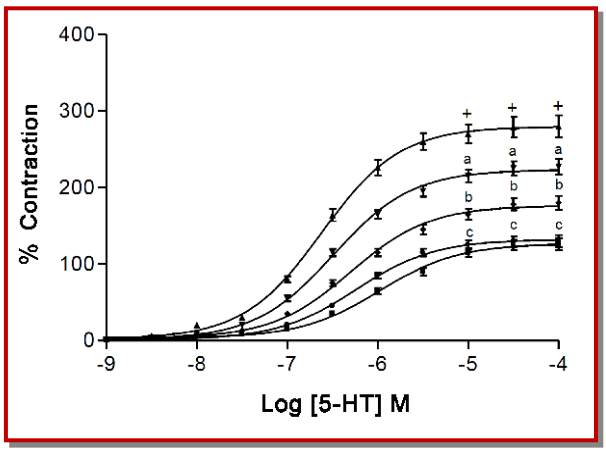

Figure 4: Effects of *Z. noltii* extract on 5-HT contractions in alloxan diabetic rats. Concentration–response curves obtained from Group I (normal) (n); Group II (diabetic control) (▲); Group III (*Z. noltii* extract 50 mg/kg) (▼); Group IV (*Z. noltii* extract 150 mg/kg) (¿); and Group V (*Z. noltii* extract 250 mg/kg) (=) are shown. Data are expressed as mean ± S.E; $^{+}p<0.0001$; compared to normal group, $^{a}p<0.01$, $^{b}p<0.001$ and $^{c}p<0.0001$; compared to diabetic control group; $n = 6$

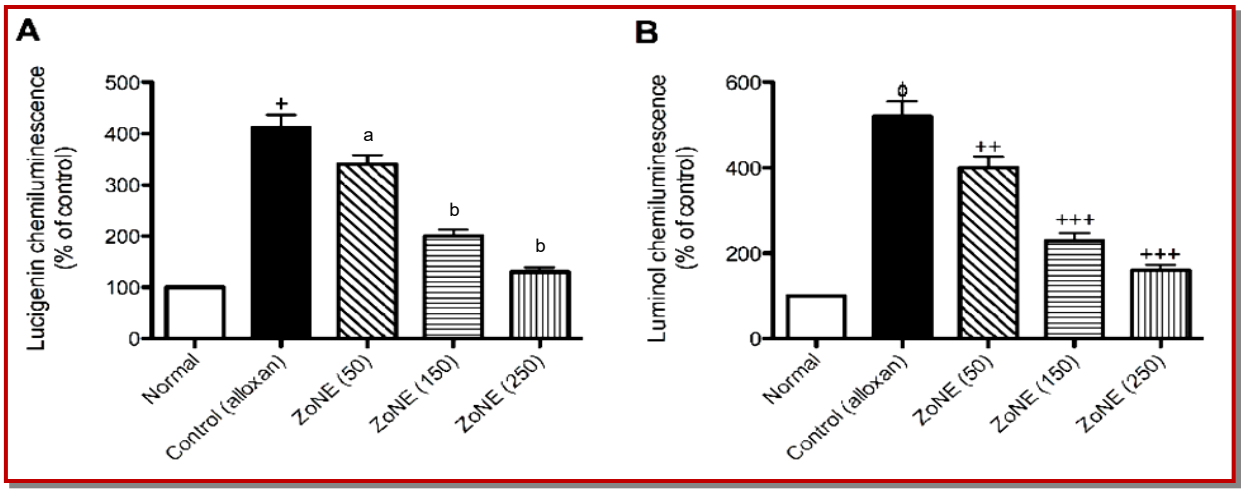

Figure 5: Lucigenin (A) and luminol (B) -enhanced chemiluminescence in rat thoracic aorta. Results are expressed as mean ± standard error of mean of area under curve (AUC) for a counting period of 5 min n = 6, AUC of relative light units (rlu)/mg wet tissue (as % of normal values). $^{+,\phi}p<0.001$ indicates a significant difference in the production of superoxide anion or other ROS from normal group; $^{a}p<0.05$ and $^{b}p<0.001$ indicate a significant reversal of the increased superoxide anion production by *Z. noltii* extract. $^{++}p<0.01$ and $^{+++}p<0.001$ indicate a significant reversal of the increased ROS production by *Z. noltii* extract

In order to evaluate the effects of *Z. noltii* extract on vascular responses, cumulative concentration–response curves for acetylcholine, phenylephrine and 5-HT were obtained in thoracic aorta. *Z. noltii* extract treatment, at all tested dose levels, recovered the impaired acetylcholine relaxations observed in alloxan-diabetic rats (Figure 2) and resulted in a concentration-dependent increase in sensitivity (Table VI). Addition-ally, contractile responses to phenylephrine (Figure 3) and 5-HT (Figure 4) which were significantly augmented in hyperglycemic rats were dose-dependently ate-nuated by *Z. noltii* extract treatment (Table VI). These changes in vascular reactivity by *Z. noltii* extract treatment were accompanied by significant alterations in ROS production in rat aorta. Lucigenin- and luminol-enhanced chemiluminescences in alloxan-diabetic aorta- were approximately 4 and 5 times higher than in those of control tissues, respectively (Figure 5A and B). Starting from the lowest dose, *Z. noltii* extract, significantly inhibited superoxide anion generation and forma-tion of other reactive species (Figure 5A and B). Moreover, concentrations of eNOS in aortae from *Z. noltii* extract treated diabetic rats were significantly higher when compared to diabetic control (Figure 6A). On the other hand, total nitrite levels in aorta were found to be similar among experimental groups (Figure 6B).

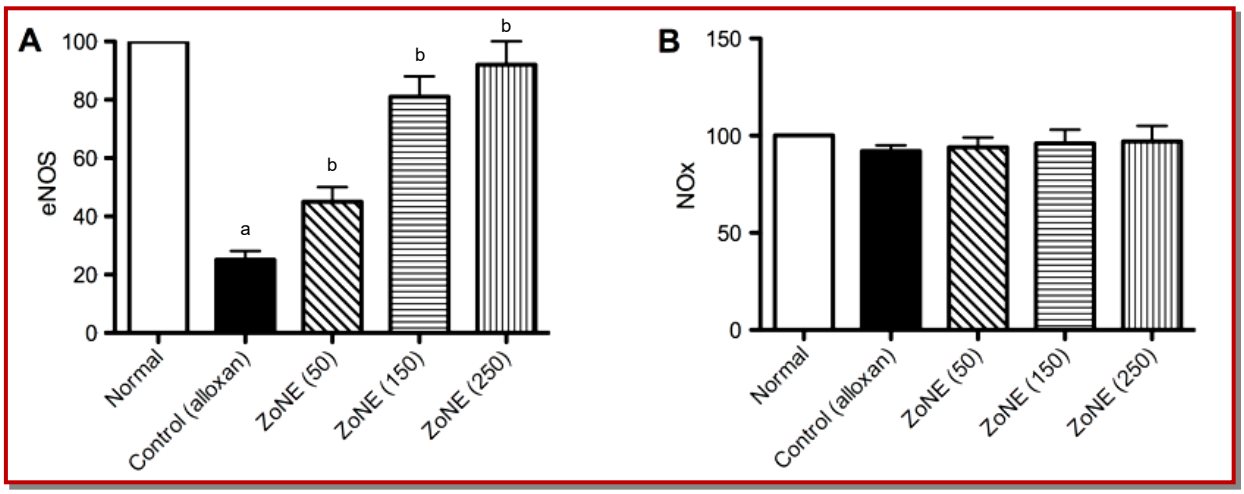

Figure 6: eNOS (A) and NOx (B) levels in rat thoracic aorta. Results are expressed as mean ± S.E; n = 6, as % of normal values; [a]p<0.001 indicates a significant difference from normal group; [b]p<0.001 indicate a significant increase in eNOS levels in comparison with control (alloxan) group

Discussion

In the present study, we for the first time demonstrate that *Z. noltii* extract lowers blood glucose and protects vascular endothelium from the harmful effects of hyperglycemia in alloxan-diabetic rats. As is known, alloxan increases production of reactive oxygen species (ROS) leading to cytotoxicity in pancreatic β-cells, and thereby inhibits insulin activity while affecting major organs and haemopoietic system (Sakurai et al., 2001; Sabu et al., 2002). Additionally, alloxan impairs endothe -lium-dependent vasorelaxation and increases contractile responses to agonists such as phenylephrine and 5-HT (Gokce and Haznedaroglu, 2008). Our results indica -ted that administration of *Z. noltii* extract to alloxan-diabetic rats lowers blood glucose and recovers vascular endothelial function in a dose-dependent manner. Also, at dose levels of 150 and 250 mg/kg, *Z. noltii* extract improves oxidant status and inhibits lipid peroxidation. Since pancreatic tissue damage mediated by lipoxygenase-derived peroxides is closely related with insulin secretion (Metz, 1984; Walsh and Pek, 1984), we firstly hypothesized that glucose lowering activity of *Z. noltii* extract would be related to its anti-oxidant effects. However, *Z. noltii* extract 50 mg/kg, while showing significant antidiabetic activity, failed to preserve the levels of anti-oxidants and did not alter lipid peroxidation. Taking the above debate into consideration, it is conceivable that antidiabetic effects of *Z. noltii* extract may possibly not only be related to its anti-oxidant properties.

Endothelial dysfunction which can be defined as loss of the balance between vasoconstrictors and vasodilators, is a major complication of diabetes and a well-documented phenomenon in various experimental models of hyperglycemia (Oyama et al., 1986; Meraji et al., 1987; Shukla et al., 2004; Gokce and Haznedaroglu, 2008). Increased superoxide anion ($O_2^{\bullet -}$) generation and hydrogen peroxide (H_2O_2) accumulation have been demonstrated to decrease agonist-stimulated activity of nitric oxide (NO) in diabetic aorta (Karasu, 2000). In the present study, *Z. noltii* extract, at all tested dose levels, restored acetylcholine relaxations and increased pD_2 values in a concentration-dependent manner. This recovery by *Z. noltii* extract was completely inhibited by the NO synthase inhibitor L-NAME (data not shown), ruling out the possibility that *Z. noltii* extract causes vasodilation by directly affecting vascular smooth muscle. These findings have given rises to the question whether protective effect of *Z. noltii* extract on endothelium-dependent relaxation may be related to alterations in NO bioavalibility and/or eNOS synthesis. It has been previously shown that polyphenolic compounds in red wine activates eNOS via PI3K pathway (Ndiaye et al., 2005). Also, *Hancornia speciosa* extract which is rich in polyphenolics was shown to produce NO-dependent vasorelaxation in rat aorta (Ferreira et al., 2007). Moreover, *Posidonia ocenica*, another seagrass widely allocated in the Mediterrenean Sea, has been shown to lower blood glucose and prevent hyperglycemia-induced endothelial dysfunction in a similar pattern (Gokce and Haznedaroglu, 2008). Taking into consideration that phosphatidyl-inositol 3-kinase (PI3K) pathway is crucial to many of the effects of insulin (Epstein, 1999), we have therefore measured eNOS and total nitrite (NOx) levels in aortic tissue. While not affecting NOx, *Z. noltii* extract elevated eNOS levels in a dose-dependent manner.

On the other hand, our results indicated that increased contractile responses to phenylephrine and 5-HT were normalized by *Z. noltii* extract treatment with an accompanying reduction in sensitivity. Impaired NO synthesis is known to increase vasocontractility in diabetic animals (Benter et al., 2005). However, in our study, inhibition of NO synthesis by L-NAME did not result in further increments in phenylephrine and 5-HT contractions. Thus, we have questioned the role of ROS produc

-tion in the contractile responses. Indeed, as reflected by lucigenin and luminol chemiluminescence, *Z. noltii* extract, inhibited the production of $O_2^{\bullet -}$ and other reactive species. Since ROS, especially $O_2^{\bullet -}$ are regarded as endothelium-derived contracting factors which play major roles in the regulation of arterial tone (Katusic and Vanhoutte, 1989), free radical scavenging activity of the extract may therefore account for the attenuated contractile responses observed in *Z. noltii* extract-treated alloxan-diabetic animals.

Conclusion

Z. noltii extract is shown to lower blood glucose and prevent hyperglycemia-induced endothelial dysfunction. Anti-oxidant/free radical scavenging properties of the extract are unlikely to be the only mechanisms underlying its anti-diabetic action.

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Ethical Issue

The experiments were carried out in accordance with the guidelines of Local Ethics Committee of Animal Experiments, Ege University, Izmir, Turkey (B.30.2.EGE.0.01.00.01/04-44-215a).

Conflict of Interest

Authors declare no conflict of interest

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