

Vasorelaxant Effects of Aqueous Extract of *Zygophyllum Album* and Antihyperglycemic Activities in Streptozotocin-Induced Diabetic Mice

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Abstract

Zygophyllum album has been mentioned in Tunisian system of traditional medicine to be of value in the treatment of diabetes mellitus. The aqueous extract from *Zygophyllum album* were examined for relaxant effects in isolated mice thoracic aorta, Nitric Oxide (NO) spin-trapping technique combined with Electron Paramagnetic Resonance (EPR) spectroscopy has been employed to measure the *ex vivo* production of NO in liver, heart, pancreas and aorta. The antihyperglycemic effects of aqueous extract from *Zygophyllum album* (100 and 300 mg/kg bw) during 15 days on important enzymes of carbohydrate metabolism in livers of non-diabetic and streptozotocin-induced diabetic mice was evaluated.

Administration of the aqueous extract plant (100 and 300 mg/kg bw) for 15 days resulted in significant reduction in hepatic Glucokinase (GK), glycogen in STZ diabetic mice. In addition to that, significant increase in hepatic Phosphofructokinase (PFK), Glucose-6 Phosphate Dehydrogenase (G6PDH) observed in STZ diabetic mice.

Keywords: *Zygophyllum album*; Nitric Oxide (NO); Streptozotocin; Antihyperglycemic; Vasorelaxant

Introduction

Diabetes is a serious metabolic disorder with micro and macrovascular complications that results in significant morbidity and mortality. The estimated incidence of diabetes mellitus and projection for year 2010, as given by International Diabetes Federation is 239 million [1]. Trends in the last 10 years are influencing the supply and demand for healthcare in diabetes [2].

Traditional medicines derived mainly from plants play major role in the management of diabetes mellitus [3-5]. Herbal remedies are apparently effective, produce minimal or no side effects in clinical experience and are of relatively low costs as compared to oral synthetic hypoglycemic agents [6]. In recent years, the role of alternative therapeutic approaches has become very popular and since a single plant may have many pharmacological activities (anti-diabetic, antioxidant and anti-stress activity) they can be effectively utilized to delay or counter diabetic complications [7]. Hence treatment with herbal drugs has an effect on protecting B-cells and smoothing out fluctuation in glucose levels [8,9]. *Zygophyllum album* (Zygophyllaceae), is one of the most commonly prescribed drug in Tunisian pharmacopoeia. It is used as an antihyperglycemic, antioxydant and antilipedimic [10,11].

Since the discovery of the endothelial dependence of acetylcholine-induced vasorelaxation *in vitro* by Furchgott and Zawadzki [12]. NO has been identified as the prototypic endothelium-derived relaxing factor. NO is synthesized from the amino acid L-arginine by a family of enzymes, the NO synthases (NOSs). NO synthesized by the endothelium of small vessels is involved in the control of vascular tone and plays an important role in the regulation of blood pressure [13-17]. Shear stress acting on the endothelial lining of blood vessels is believed to be the most important physiological stimulus for the release of NO [18]. Studies have demonstrated the presence of endothelial NOS (eNOS) enzyme in and the production of NO by all of the vessels of the coronary circulation [19,20].

Vascular dysfunction is closely related to the high blood pressure. Maintenance of vascular homeostasis is one of the targets for the

control of high blood pressure. Endothelial cells are an intimate modulator for the control of vascular homeostasis. Endothelial cells respond to humoral and physical stimuli by releasing endothelium-dependent vasodilators including endothelium-derived relaxing factor and prostacyclin. Nitric oxide (NO) is endothelium-derived relaxing factor [12,21,22].

Nevertheless, vascular activities claimed for *Zygophyllum album* have never been proved. Thus, we considered it interesting to investigate whether there is a scientific basis for the traditional use of this plant as an anti-hypertensive drug. In the present study, we examined the *in vivo* effect of the aqueous extract of *Zygophyllum album* on *in vitro* effects on the vasomotor tone of aortic rings isolated from mice.

Materials and Methods

Experimental animals

Adult Swiss *albino* mice of either sex (*Mus musculus*) weighing approximately 25 g (housed 4 to 5 per cage) were used in this study. Animals were housed in an air-conditioned animal room at 23 ± 2°C, with 12-h/12-h light/dark photoperiod, relative humidity (50–60%) and maintained with *ad libitum* feeding and water.

Chemicals and reagents

Reagents were obtained from Sigma Aldrich (Milano, Italy) and

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Received April 04, 2014; Accepted August 26, 2014; Published September 07, 2014

Citation: Ghoul JEI, Ben-Attia M (2014) Vasorelaxant Effects of Aqueous Extract of *Zygophyllum Album* and Antihyperglycemic Activities in Streptozotocin-Induced Diabetic Mice. J Diabetes Metab 5: 426 doi:10.4172/2155-6156.1000426

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Merck (Darmstadt, Germany), and were of the highest commercial grade available.

Preparation of the aqueous extract

Fresh whole *Zygophyllum album* (Zygophyllaceae) plants were collected from Southern Tunisia (Douz/Gbéli) between May and July 2007. The plant material was dried at ambient temperature and stored in a dry place prior to use. The plant was washed well with water, dried at room temperature in the dark, and then ground in an electric grinder to give a coarse powder. A 100 g of the powdered aerial parts were suspended in 1000 mL distilled water, heated and boiled under reflux for 30 min. The decoction obtained was filtered, and the filtrate frozen at -20°C and then lyophilised. The average yield of the lyophilised material (Za-extract) was approximately 18.28%. It was stored at ambient temperature until further use. The yield of this extract was 8.06%.

Preparation of isolated mice thoracic aorta rings

A method previously described was used [23,24]. Briefly, animals were sacrificed by cervical dislocation. The thoracic aorta was cleaned of adhering connective tissue and was cut into 5–7 mm length rings. Then, tissue segments with endothelium were mounted in stainless steel hooks, under an optimal tension of 3 g, in 10 mL organ baths containing warmed (37°C) and oxygenated (O₂:CO₂, 19:1) Krebs solution (composition, mM: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0; EDTA, 0.026 and glucose, 11.1, pH 7.4). Isometric tension was measured and recorded by Grass-FT03 force transducers (Astromed, West Warwick, RI, USA), connected to a MP100 analyzer (Biopac Instruments, Santa Barbara, CA, USA). After a 90-min-equilibration period under a resting tension of 1 g, the stabilization period the tissues were stimulated with KCL (8 mM). The aortic rings in bath tubes were allowed to equilibrate for 1 h before experiment and the Krebs solution was changed every 15 min. The viability of the ring preparation was assessed by contracting vessels with 10⁻⁷ M U46619 (9,11-dideoxy-9 α , 11 α -methanoepoxy prostaglandin F_{2a}) before each experiment. The artery endothelium viability and integrity were checked by dilatary response of the ring to acetylcholine (10⁻⁶ M) as described by Furchgott and Zawadzki and Fiscus et al. [12,25].

NO spin trapping and EPR spectroscopy

NO radical determination by Electron Paramagnetic Resonance (EPR) was estimated by the method of Komarov et al. [26]. Detection of NO production was performed using Fe₂⁺ diethyldithiocarbamate (DETC, Sigma Aldrich) as spin trap. Briefly, cells were seeded on 24-well plates and used when 80–90% cell fluency were reached. Endothelial cells were either stimulated with the tested compounds (25 mM) or the reference thapsigargin 10 mM (TG, Sigma–Aldrich) for 10 or 30 min, respectively; the medium then was replaced with 250 μ L of Krebs solution, then treated with 250 μ L of colloid Fe(DETC)₂ and incubated for 45 min at 37.8°C. Cells were then scrapped and frozen in plastic tubes. NO detection was measured in situ by EPR. NO measurement was performed on a table-top x-band spectrometer Miniscope (Magnetech, MS 200, Berlin, Germany). Recordings were made at 77 K, using a Dewar flask. Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation, 100 kHz of modulation frequency, 150 s of sweep time and 5 scans. Signals were quantified by measuring the total amplitude, after correction of baseline as done previously. Values are expressed as amplitude of signal per protein concentration (units/mg / mL of endothelial cell proteins).

Induction of experimental diabetes

The overnight fasted mice were made diabetic with Streptozotocin (STZ) (Sigma, St Louis, MO) at a dose of 45 mg/kg intraperitoneally. The STZ was freshly dissolved in citrate buffer (0.01 M, pH 4.5) and kept on ice prior to use [27]. Control mice received only the buffer. A week after injection of streptozotocin, diabetes was confirmed in STZ-treated mice with fasting blood glucose levels above 250 mg/dl.

Experimental design

The mice were randomly divided into the following five groups with eight mice per group:

Group I: normal control mice or “negative control” received only vehicle solutions.

Group II: diabetic control mice served as positive control group received single dose of STZ (45 mg/kg, i.p.).

Group III: diabetic mice received the aqueous extract of *Z. album* (100 mg/kg, orally (p.o.)).

Group IV: diabetic mice received the aqueous extract of *Z. album* (300 mg/kg, p.o.).

Group V: diabetic mice received gliclazide (25 mg/kg, i.p.).

Animals were treated by oral gavage once a day for a period of 15 days.

Glucokinase

Glucokinase was estimated by the method of Newgard et al. [28]. Weighed amounts of liver tissues (0.3–0.5 g) were homogenized in nine volumes of 50 mM Tris/HCl buffer, pH 7.4. After centrifugation at 12,000 \times g for 20 min at 4°C, the supernatants were used to measure the enzyme activity. The assay mixture contained 100 mM KCl, 10 mM DTT, and 1mM EDTA.

Glucose-6-phosphate dehydrogenase (G6PD)

G6PD was estimated by the method of Bergmeyer [29]. Weighed liver tissues were homogenized in 10 times their weight in volume of 0.1 M Tris/HCl buffer at pH 7.6 containing 1 mM EDTA. The homogenates were centrifuged at 10,000 g for 15 min and the supernatants were used for the enzyme assay. Increase in absorbance of NADPH produced was measured at 340 nm.

Phosphofructokinase

Phosphofructokinase was estimated by the method of Castano et al. [30]. Weighed amounts of liver tissues (0.3–0.5 g) were homogenized with ten times their weight (in volume) of 50 mM Tris/HCl buffer pH 8.2 containing 5 mM MgSO₄ 7H₂O, 1 mM EDTA and 10 mM DTT. The homogenates were centrifuged at 20,000 g for 15 minutes at 4°C. The enzyme activity was measured at 37°C by following the appearance of NADH at 340 nm for 3 minutes at 30 seconds interval.

Hepatic glycogen: Glycogen content was determined by the method described by Ong and Khoo [31]. Weighed amounts of liver tissues (0.3–0.5 g) were homogenized in 10 volumes of ice-cold 30% KOH and boiled at 100°C for 30 min. Glycogen was precipitated with ethanol, pelleted, washed, and resolubilized in distilled water. Glycogen content was determined by treatment with anthrone reagent and measured at 625 nm.

Protein determination

Protein content was determined by the method of Bradford [32] using assay kit (Sigma Diagnostics).

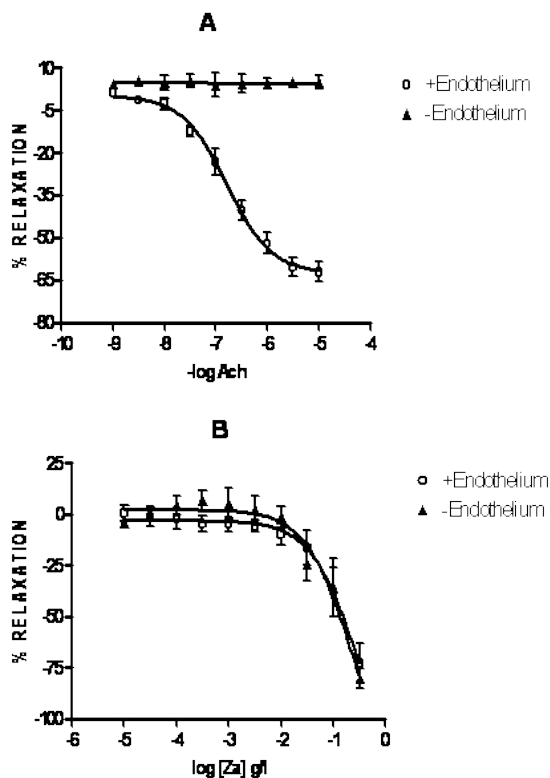


Figure 1: Vasorelaxant effects of aqueous extract of *Zygophyllum album* (Za) (A) and Acetylcholine (B) on the endothelium denuded or endothelium intact mice aortic rings precontracted by 10^{-7} M U46619. The relaxation effect was expressed as a percentage of decrement to the maximum tension caused by 10^{-7} M U46619. n = 6 (six isolated aortic rings from six mice).

Statistical analysis

Results are expressed as the means \pm SD. Student's *t*-test for unpaired samples was performed using GraphPad Prism (GraphPad Software, Version 4.0). A value of $p < 0.05$ was considered significant.

Result

Effect of acetylcholine and aqueous extract of *Zygophyllum album* on U46619-induced precontractions

Figure 1A and B shows that the acetylcholine and the aqueous extract of *Zygophyllum album*, dose dependently and respectively relaxed U46619-induced contractions of aorta with or without endothelium. Acetylcholine (10^{-9} - 10^{-5} M) caused an endothelium-dependent relaxation of 60 mM KCl-induced contractions. The aqueous extract of *Zygophyllum album* caused relaxation of isolated mice aortic rings in a concentration-dependent manner for both endothelium-intact and endothelium-denuded aortic rings. These vasorelaxant effects of aqueous extract at all concentrations tested were reversible to the levels as before the administration of aqueous extract after aqueous was washed out with Krebs solution. Figure 1(B) also shows that aqueous extract of *Zygophyllum album* at low concentrations ($< 10^{-2}$ g/l) caused relaxation of endothelium-intact aortic rings and for endothelium-denuded aortic rings. The EC_{50} of the aqueous extract required to effect these observations were 120 mg/l for U46619-induced contractions.

Effect of aqueous extract of *Zygophyllum album* on *ex vivo* production of NO in aorta, heart, liver and pancreas

Figure 2 shows the variation *ex vivo* production of NO by RPE in the aorta, liver, pancreases and the heart treated by the aqueous extract of *Zygophyllum album* (Za) with 100 mg/l in mice. The graphic analysis shows that the significantly increase production of NO ($p < 0.05$) in the batches of the bodies treated by the aqueous extract of the *Zygophyllum*

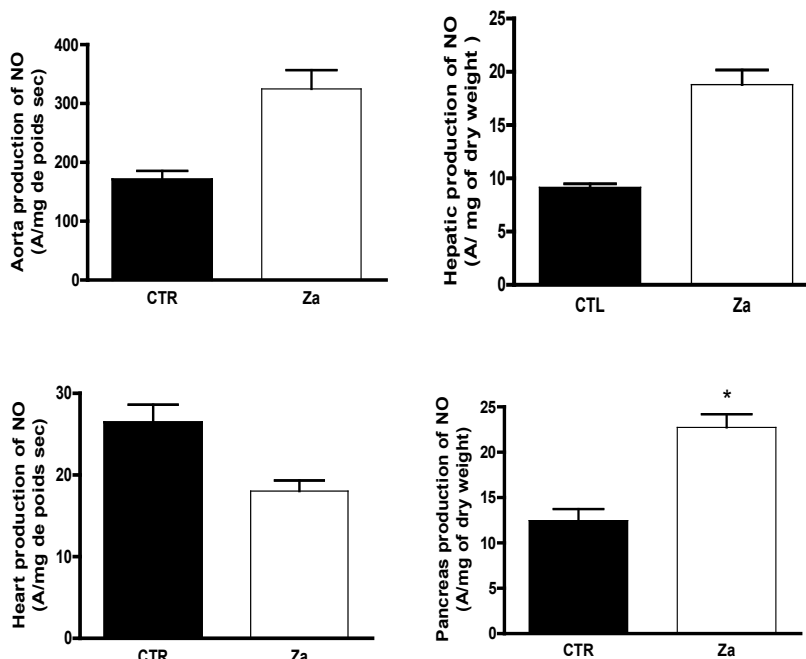


Figure 2: Effects of aqueous extract of *Zygophyllum album* (100 mg/l) on *ex vivo* production of nitric oxide (NO) in the aorta, pancreas, liver and heart. The data are expressed as mean \pm SD (n = 6). Values are given as mean \pm SD for groups of eight animals each. Values are statistically significant at $p < 0.05$ (*) (*test-t of Student*).

album, except on the level of the heart showed a no significant decrease production of NO ($P < 0.05$) (Figure 3).

Effects of aqueous extract of *Zygophyllum album* on hepatic enzymes

The activities of hepatic Hexokinase (HK), Glucokinase (GK) and Phosphofructokinase (PFK) in non-diabetic and diabetic mice treated repeatedly with aqueous extract from *Zygophyllum album* for 14 days is

shown in Figure 4. Significantly increased activities of the glucokinase and decreased activities of the glucose 6 phosphate deshydrogenase and phosphofructosamine were observed in STZ-induced diabetic mice (group II) in the liver when compared with the normal controls (group I). The aqueous extract of *Zygophyllum album* was able to significantly decrease the GK activity and increase the activities of PFK and G6PDH in diabetic mice (group III and IV). The oral administration of glicazide, 25 mg/day to diabetic mice restored the hepatic activities of GK, PKK and G6PDH to near control levels.

Discussion

Although *Zygophyllum album* is widely used in traditional medicine for the treatment of diabetes, the compounds responsible for its vasorelaxant activity have never been identified.

Vascular tone is regulated by a number of receptor- and ion channel-mediated processes, and the contribution of an intact vascular endothelium is considered important. Modulation of vascular tone by endothelium is regulated by the synthesis and release of vasorelaxing factors such as nitric oxide and prostacyclin as well as by vasoconstricting factors such as endothelin and angiotensin II [33]. Removal of the endothelium in the present study led to a reduction of the relaxant activity of aqueous extract of *Zygophyllum album*, suggesting that an endothelium-dependent mechanism is involved.

The aqueous extract of *Zygophyllum album* show inhibition on U46619-induced contraction in isolated mice aorta rings while these extracts provoked an important relaxation of U46619-induced contraction. On the other hand some fractions obtained from methylene chloride-methanol induced relaxation both KCl and NA-induced contraction although in variable proportion. Preliminary

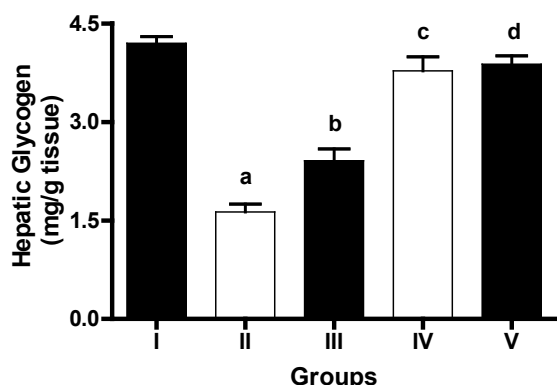


Figure 3: Effect of aqueous extract of *Zygophyllum album* on hepatic glycogen level in STZ-induced diabetic mice. The data are expressed as mean \pm S.D (n = 8). Values are given as mean \pm S.D for groups of eight animals each. Values are statistically significant at $P < 0.05$. Diabetic control vs. control. Diabetic + *Zygophyllum album* extract 100 mg/kg vs. Diabetic control. Diabetic + *Zygophyllum album* extract 300 mg/kg vs. diabetic control. Diabetic + gliclazide vs. diabetic control.

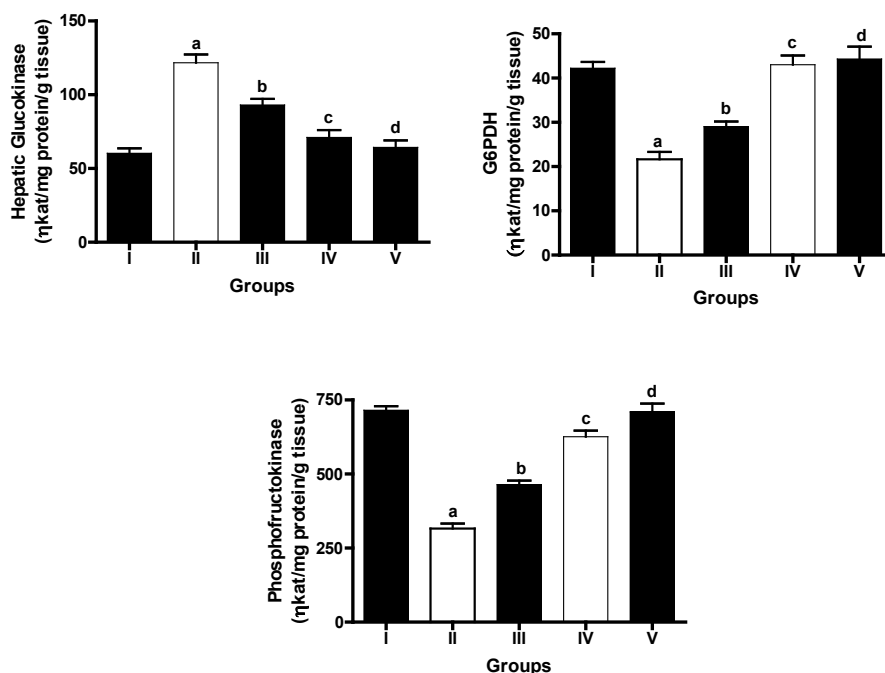


Figure 4: Effects of aqueous extract of *Zygophyllum album* on hepatic glucokinase, G6PDH and phosphofructokinase levels in STZ-induced diabetic mice. The data are expressed as mean \pm S.D (n = 8). Values are given as mean \pm S.D for groups of eight animals each. Values are statistically significant at $P < 0.05$ (test-t de Student). Diabetic control vs. control. Diabetic + *Zygophyllum album* extract 100 mg/kg vs. Diabetic control. cDiabetic + *Zygophyllum album* extract 300 mg/kg vs. diabetic control. dDiabetic + gliclazide vs. diabetic control.

chemical analysis demonstrated that the MeOH and CH₂Cl₂-MeOH (1:1) extracts and fraction of *Vitex cincinnensis* contained a number of terpenoids and some flavonoids. These classes of compounds may be responsible for the activities observed since it has been demonstrated that phenolic compounds and triterpenoids possess vasodilator properties [34-36]. The vasorelaxant properties induced by these compounds clearly show that they are at least partly responsible for the activities observed. These compounds were able to relax, in a concentration-dependent fashion, the contractions induced by noradrenaline in rat aortic rings with functional endothelium. However, these responses were less marked in arteries precontracted by KCl and in endothelium-denuded arteries (results not shown). High KCl concentrations cause contractions in vascular smooth muscle by depolarising cell membranes and by increasing the influx of Ca₂⁺ through long-lasting voltage dependent channels [37]. In this way, the absence of relaxation in KCl (60 mM) evoked contractions might probably remove either the influence of membrane hyperpolarisation is an indication that the blockage of Ca entry through voltage-stimulated Ca₂⁺ channels relaxant responses to extracts, pentacyclic triterpenoids, and ceramide. It has been previously reported in a well-established in vitro model of vasomotion that triterpenoid-related compounds, commonly found in plant species, elicit vasorelaxation through the direct release of NO from vascular endothelium [34,35]. In this way, it was expected that oleanolic acid and β-sitosterol (β-sitosterol relaxed noradrenaline precontracted endothelium intact rings slightly but not significantly) would have vasoactive effects related to NO derived from endothelium. On the other hand ceramide induced vasorelaxation. Another study demonstrated that cellular and molecular mechanisms underlying the relaxant actions of ceramide remain to be established but a reduction in intracellular Ca₂⁺ is a key factor in causing relaxation [38].

The present results indicate that aqueous extract of *Zygophyllum album* induces concentration-dependent relaxation in aortic strips precontracted by U46619. When the aqueous extract was added during the tonic contraction induced by U46619, it also exerted relaxation demonstrating a vasorelaxant activity of the plant extract. The inhibitory effects of *Zygophyllum album* on vascular stimulating agents were reversible, indicating that it does not cause tissue damage or tissue tolerance. The vascular endothelium plays an important role in controlling vascular tone via the secretion of both relaxant and contractile factors. The endothelial cells respond to chemical and physical stimulation by producing relaxant factors such as bradykinin, prostacycline and nitric oxide [39]. The vasorelaxant action of the aqueous extract of *Zygophyllum album* persisted on denuded aortic strips. Because our results did not show a significant difference between the effects on intact and denuded aortic strips, it can be argued that the activity of the plant extract is endothelium-independent. The extract may therefore act directly on the vascular smooth muscle. In addition, the relaxant action of *Zygophyllum album* in intact aortic strips was neither significantly affected by indomethacin (a cyclooxygenase inhibitor) nor by L-NAME (a nitric oxide synthase inhibitor) suggesting that the effect was not mediated via endothelium-derived prostacycline or nitric oxide [39-42]. The concentration of L-NAME used in our experiments (2.5×10⁻⁴ M) is more than sufficient to fully inhibit NO synthetase activity. This was verified in the present studies by testing the response to acetylcholine, which was completely inhibited in the presence of L-NAME (data not given). It is widely known that vascular relaxation, induced by many vasodilators such as acetylcholine, needs the presence of endothelium layer and the increase of Endothelium-Derived Relaxing Factor (EDRF), which has been demonstrated to be nitric oxide (NO) [11].

Diabetes is a chronic metabolic disorder affecting a reduction in hyperglycemia will decrease the risk of developing micro vascular diseases and reduce their complications. Diabetes mellitus is characterized by a reduced capacity of the β-cells of the pancreas to release sufficient insulin to induce the activity of glucose metabolizing enzymes whether the cells are destroyed as in type 1 diabetes (IDDM) or intact as in type 2 diabetes (NIDDM) [43]. In our investigation, the activities of some carbohydrate metabolizing enzymes such as G6PDH, GK, PFK, and glycogen content were measured in the livers of non-diabetic and STZ induced diabetic mice and the effects of repeated 2-week oral treatment with an aqueous extract from *Zygophyllum album* were evaluated. Our results show that decreased glucose in animals may be correlated with inhibition of glycogenolysis as suggested by increased liver glycogen [44,45]. The level of insulin in diabetic mice as well as in normal mice after the treatment with aqueous extract of this plant [10]. In this context, a number of other plants have been reported to have antihyperglycemic activity with a stimulatory effect on insulin release [46,47]. Glycogen level in various tissues especially in liver and skeletal muscle indicates direct reflection of insulin activity since it causes glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase. Glycogen levels in tissues (muscle and liver) decrease as the influx of glucose in the liver is inhibited in the absence of insulin and recovers on insulin treatment [48]. Treatment with aqueous extract of the *Zygophyllum album* for 15 days significantly increased the hepatic glycogen levels in STZ diabetic treated mice, indicating the insulin secretagogue activity. Plants like *Syzygium cordatum*, *Syzygium alternifolium* increase the concentration of hepatic glycogen which is similar to our results [49,50].

In summary, our results clearly indicate that aqueous extract of *Zygophyllum album* has been shown to have, besides antihyperglycemic and vasorelaxant properties which act by improving insulin secretion and the alterations in the carbohydrate. The hypoglycaemic mechanisms of *Zygophyllum album* extract remain unclearly. Further chemical and pharmacological investigation should be carried out to evaluate the mechanism of *Zygophyllum album* extract's hypoglycemic action. More studies are warranted to evaluate whether such therapy can be administered as an auxiliary beneficial therapeutic regimen in diabetic population.

In conclusion, the present study demonstrates that *Zygophyllum album* possesses different vasodilatory properties. Further chemical and pharmacological experiments are required to investigate its potential anti-diabetic activity in diabetic mice and to identify the active principle(s) responsible for the vascular activity attributed to the plant in Tunisian pharmacopoeia.

Acknowledgment

We are grateful to the Tunisian Ministry of Superior Education and Scientific Research for financial support.

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