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Antidiabetic and Antioxidant Activities of *Zizyphus lotus* L Aqueous Extracts in Wistar Rats

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Abstract

Objectives: *Zizyphus lotus* L. is a medicinal plant, used in traditional medicine for its multiple therapeutic properties. The aim of the present study was to determine the antidiabetic and antioxidant effects of aqueous extracts of different parts of *Zizyphus lotus* in diabetic Wistar rats.

Research methods and procedures: The male Wistar rats were rendered diabetic by intraperitoneal (i.p.) injection of streptozotocin (40 mg/kg body weight). Aqueous extracts from different parts, i.e, root, leaf, and seed of *Zizyphus lotus* L. were prepared and administrated orally to the animals at the dose of 300 mg/kg for 21 days. Blood glucose level was measured, and antioxidant status was assessed by determining oxygen radical absorbance capacity (ORAC) and the activities of enzymes like catalase, glutathione reductase and peroxidase in pancreas, liver, and erythrocytes. Vitamin C levels were determined by precipitating with 10% trichloroacetic acid. Vitamins A and E concentrations were measured by HPLC.

Results: The leaf and root, but not seed, extracts exerted the glucose lowering effect on 21st day of post-administration. The leaf and root extracts corrected antioxidant status of diabetic animals in pancreas, liver and erythrocytes. The concentrations of different vitamins (vitamin A, C and E) in diabetic rats were also modulated by leaf and root, but not seed, extracts.

Conclusion(s): Our study shows that oral administration of *Zizyphus lotus* L. extracts from roots and leaves exerted antidiabetic and antioxidant effects in diabetic rats. *Z. lotus* L. seems to be a good candidate to lower, in addition to conventional antidiabetic drugs, the hyperglycaemia in diabetic subjects.

Keywords: *Zizyphus lotus* L; Streptozotocin; Diabetes; Antioxidants

Introduction

Zizyphus lotus L., also known as *Jujube*, is a medicinal plant largely found in the Mediterranean region including Algeria [1]. The fruit of this plant is consumed by local population for the treatment of several pathologies such as digestive disorders, obesity, urinary troubles and skin infections [2,3]. In traditional medicine, jujube is used for treatment of liver diseases [4], insomnia and anxiety [5]. Z. jujuba extracts showed protection against hydroquinone-induced cytogenesis [6]. In Algeria, *Zizyphus lotus* L. is used for its antidiabetic, sedative and hypoglycemic properties [7]. The medicinal properties of this plant depend on the part of the plant and the extract used. For instance, fruit has been used for its emollient effects and leaves are known for the beneficial effects in the boils. Interestingly, the root barks are known for their antidiabetic property [8]. The butanol extracts of the leaves of *Zizyphus spina-christi,* another plant of the same family, improved the oral glucose tolerance and potentiated the glucose-induced insulin release in type II diabetic rats [9]. Anti-inflammatory, analgesic and anti-ulcerogenic activities of this plant have been demonstrated in rodents [10,11]. We have recently shown that fruit, leaves and seeds are rich in different vitamins and exhibit *in vitro* antioxidant properties [12]. We have also shown that these extracts exert immunomodulatory properties on human T-cell activation and IL-2 mRNA expression [13].

Diabetes is a serious metabolic disorder affecting the metabolism of carbohydrate, protein and fat. A number of studies have shown that diabetes mellitus is associated with oxidative stress, leading to an increased production of reactive oxygen species. Recently, the protein hydrolysates isolated from *Zizyphus* have been found to possess antioxidant properties [14]. Moreover, the seeds of *Zizyphus* were found to be effective in the improvement of blood glucose and lipid levels in serum of dietary hyperlipidemic rats [15]. Since *Zizyphus lotus* L. has been shown to modulate different physiological parameters [16,17], including glucose concentrations, the role of aqueous extracts of different parts of this plant was investigated to determine their antidiabetic and antioxidant properties in normal and streptozotocin-induced diabetic rats.

Materials and Methods

Chemicals

Anti-radical resistance kit (Kit Radicaux Libres (KRL®), tocol and the HPLC column (HP ODS Hypersil C18) were purchased from Lara Spiral, France. All of the solvents and other chemicals were obtained from Sigma, USA.

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Preparation and extraction of the aqueous phase of different parts of *Zizyphus lotus* L.

Zizyphus lotus L. was collected from south-western part of Algeria (Ain Ouessara and Maessad (willaya de Djelfa) between September and October 2008. The climate is very arid (annual rain fall: around 324 mm according to Office National de Météorologie) with a drought period from half May to half October (5 months). A voucher specimen (ZLI 1320) has been deposited in the Herbarium Center of the Faculty of Pharmacy of the University of Abou Bekr Belkaid [12].

An amount of 300 g of either of the following parts, *i.e,* root, leaf and seed, was suspended in 500 ml distilled water and boiled for 30 min. The decoction obtained was filtered, and the filtrate was frozen at -70°C and, later on, lyophilised and stored at ambient temperature until further use. Lyophilised extracts were stable for several months at room temperature and before use they were suspended in physiological saline (NaCl, 0.9% v/w) at 1 mg/ml.

Animals and diets

Male Wistar rats, 2–3 months old and weighing 200–250 g, were obtained from IFA-CREDO (Abresle, France). After 1 week of acclimatization, animals were divided into five groups. In each group, a total of 6 rats were rendered diabetic by intraperitoneal (i.p.) injection of streptozotocin (40 mg/kg body weight) in 0.1 M citrate buffer, pH 4.5. In control group, 6 rats were injected with citrate buffer alone. Glycaemia was measured in rats by One Touch II Glucometer (LifeScan, Johnson and Johnson, USA). The rats were daily weighed just before per os gavage with the aqueous phase of different parts of *Zizyphus lotus* at 300 mg/kg as this concentration, in a pilot study, was found to exert the most beneficial effects. Besides, Palejkar et al. (2012) have reported that the extracts of this plant exert hypoglycemic effects between 200 and 400 mg/kg. The extracts were administered in the morning for 21 days. The general guidelines for the care and use of laboratory animals, recommended by the council of European Economic Communities, were followed and the protocol was approved by the Regional Ethical Committee.

Oral Glucose Tolerance Test (OGTT)

Following three weeks of gavage with plant extracts, the rats were fasted overnight during 12 h before OGTT. The glycaemia test was performed before gavaging the rats with 3 g/kg of glucose. The glycaemia was measured at different times till 120 min (0, 30, 60, 90 and 120 min).

Animal sacrifice and collection of organs

The weighing of rats and sample collection were done in the morning before the administration of the extracts. After overnight fasting, rats in each group were anesthetized with pentobarbital (30 mg/kg body weight). The abdominal cavity was opened, and whole blood was drawn from the abdominal aorta. The blood samples were also collected, at different time intervals, by bleeding the tail end. Serum was obtained by low-speed centrifugation (1000 g×20 min). Different organs were removed, washed with cold saline solution (0.9%) and immediately frozen in liquid nitrogen and stored at -80°C.

Antioxidant capacity by KRL test

The effects of the plant extracts on the sensitivity to free radical aggression was tested by the capacity of red blood cell (RBC) to withstand free radical-induced haemolysis as described elsewhere [12]. Briefly, washed RBCs from control or diabetic mice were diluted (1:40, vol/vol) with anti-radical resistance [Kit Radicaux Libres (KRL®; Kirial International SA, Couternon, France)] buffer (300 mOsmol/kg), and 50 µl of RBCs suspension was assayed in a 96-well microplate coated with a free radical generator (GRL, Kirial International SA).

Determination of antioxidant enzyme activities

Glutathione peroxidase (GSH-Px EC1.11.1.9) was assessed as described elsewhere [18], using cumene hydroperoxide as the substrate. One unit of GSH-Px activity is defined as the amount of enzyme which gives a 90% decrease in glutathione concentration per minute at 1 mM glutathione. Glutathione reductase (GSSG-Red EC 1.6.4.2) activity was determined by measuring the rate of NADPH oxidation in the presence of oxidized glutathione [19]. The unit of enzyme activity was defined as the amount of enzyme which oxidized 1 mmol of NADPH per minute. The catalase activity was determined by measuring the rate of decomposition of H_2O_2 at a wavelength equal to 420 nm [20]. For total glutathione determinations, 100 µl of red blood cells or homogenized tissues in PBS, pH 7.4, were diluted in 15 volumes of perchloric acid (2 g/l). After centrifugation (1000g for 10 min), 200 µl of the supernatant were taken for determination of total glutathione, using the technique described elsewhere [21]. The protein concentrations were determined as per laboratory routine method.

Evaluation of antioxidant status by ORAC

The total antioxidant status was determined by measuring plasma oxygen radical absorbance capacity (ORAC) as described elsewhere [22]. The fluorescent protein allophycocyanin (APC) was used in this assay. ORAC refers to the capacity of plasma to scavenge free radicals, and then to delay the loss of APC fluorescence. The reaction mixture (2 ml) in the assay contained a final concentration of 76.8 nM APC in 75 mM phosphate buffer pH 7.0, at 37°C, 9 mM of $CuSO_4$ and 0.3% H_2O_2, in the absence (blank) or presence, respectively, of 20 ml of Trolox 1 mM (standard) or plasma (sample). Samples containing APC alone (blank) were prepared to monitor the spontaneous decay of fluorescence under experimental conditions. All readings were automatically corrected for the time-dependent decay. The APC fluorescence, at excitation 598 nm and emission 651 nm, was measured every 5 min, at 37°C, using a spectrofluorometer (SFM25 Kontron Instrument, France) until the disappearance of fluorescence. The ORAC value of each plasma sample was calculated by measuring the net protection area under the quenching curve of APC. One ORAC unit has been assigned as the net protection area provided by 1 mM of Trolox. The ORAC value of the samples was calculated as follows: ORAC= $(A_{samples}-A_{blank})/(A_{trolox}-A_{blank})$, A refers to the area under the quenching curve of APC.

Determination of vitamin C levels

The Vitamin C concentrations were determined in lyophilised extracts by precipitating with 10% trichloroacetic acid and followed by centrifugation [23]. The supernatant (500 µl) was mixed with 100 µl of DTC reagent (2,4-dinitrophenylhydrazine 3%, thiourea 0.4%, and copper sulfate 0.05%) prepared in 9N sulfuric acid, and then incubated at 37°C for 3 h. After the addition of 750 µl of 65% (vol/vol) sulfuric acid, the absorbance was recorded at 520 nm.

Determination of vitamin A and E concentrations

Plasma α-tocopherol (vitamin E) and retinol (vitamin A) were determined by reverse phase HPLC [24]. The stationary phase was constituted of greffed silica (C18 column, HP ODS Hypersil C18; 200 mm x 4.6 mm; Lara spiral, maintenance temperature of analytical column, 35°C). The mobile phase was a mixture of methanol/water

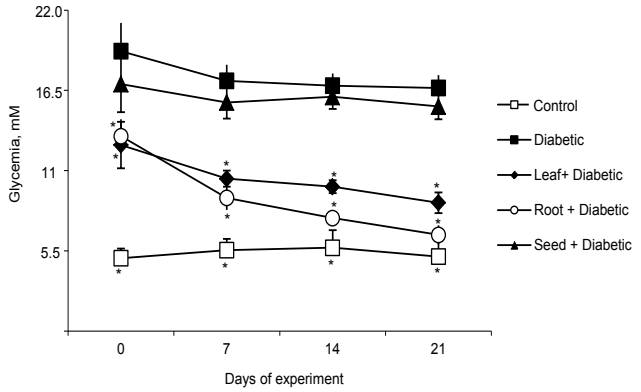

Figure 1: Blood glucose concentrations in control and diabetic rats, treated or not with extracts from leaf, root and seed of *Zyziphus lotus* L. for 3 weeks. The rats were rendered diabetic and given or not different extracts (leaf, root or seed) of *Z. lotus* L. as described in the Materials and Methods section. Each value represents the mean ± SEM (n=6). Asterisks show the significant differences (p<0.001) as compared to diabetic animals on the respective days.

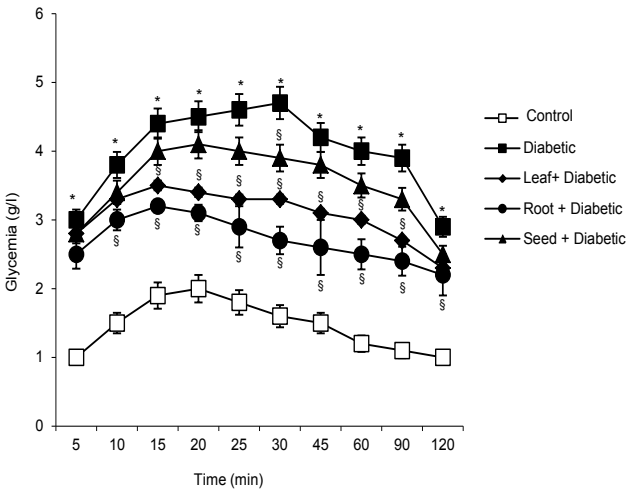

Figure 2: Oral glucose-tolerance test (OGTT) on day 21 in diabetic and control rats, treated or not with extracts from leaf, root and seed of *Zyziphus lotus* L. for 3 weeks. Glycaemia, during OGTT (3g/kg-body weight), was measured after a 15-h fasting, every 5–10 minutes, for 120 minutes following glucose administration as described in the Materials and Methods section. Each value represents the mean ± SEM (n=6). Asterisk (*) shows the significant differences (P<0.001) between control and diabetic animals. The § shows the significant differences (P<0.001) between extract-treated and diabetic animals.

(98/2, v/v) at a flow rate of 1 ml/min. Vitamins were extracted by hexane, dried under nitrogen and resuspended in methanol. The extracted vitamins were injected into the HPLC system. The HPLC peaks were detected by a UV detector at 292 nm for vitamin E and at 325 nm for vitamin A. Representative chromatograms were obtained by injecting standard solutions.

Statistical analysis

Values are mean ± SEM. Statistical analysis of data was carried out using STATISTICA (version 4.1; Statsoft, Paris, France). Data were evaluated by analysis of variance. Duncan's multiple-range test was employed for the comparison between different groups.

Results

Z. Lotus L. extracts decrease hyperglycaemia

Figure 1 shows that streptozotocin induced hyperglycaemia in rats. After gavaging the rats with different extracts of *Z. Lotus* L., we notice that seed extracts do not decrease hyperglycaemia significantly in diabetic animals (Figure 1). However, the leaf and root extracts significantly decreased the glucose levels during 3 weeks, *i.e.,* from 7th day to 21st day of treatment (Figure 1). It is interesting to mention that the extracts administered orally daily at 300 mg / kg to rats were not toxic (results not shown). The extracts of this plant has been shown to exert no toxic effect with daily doses upto 20 g/kg [25].

Z. Lotus L. extracts accelerate recovery of glucose levels in OGTT

The rats were also subjected to oral glucose tolerance test (OGTT) by gavaging 3 g/kg of glucose. The OGTT-induced hyperglycaemia was higher in diabetic rats than control animals (Figure 2). The OGTT-evoked hyperglycaemia is normalized after 2 h in control animals; however, the same phenomenon is decreased, but not normalized, in diabetic animals (Figure 2). It is interesting to note that the extracts from leaf and root of *Z. lotus* L. decreased rapidly hyperglycaemia in diabetic animals, suggesting that these extracts have hypoglycaemic effect, though the animals remained hyperglycemic as compared to control rats.

KRL and ORAC activities are corrected by *Z. Lotus* L. extracts

We used the KRL test to elucidate the antioxidant status by using the serum from control and diabetic animals (Figure 3). We observed that the rate of hemolysis was lower in diabetic animals as compared to control rats. The administration of root and leaf, but not seed, extracts increases hemolysis rate though it was always lower than control rats (Figure 3).

The measurement of ORAC in plasma shows that the antioxidant

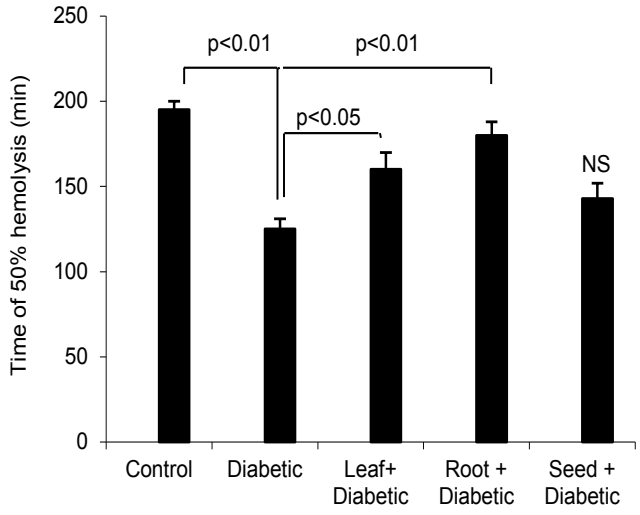

Figure 3: Red blood cell hemolysis test on day 21 in diabetic and control rats, treated or not with extracts from leaf, root and seed of *Zyziphus lotus* L. for 3 weeks as described in the Materials and Methods section. Each value represents the mean ± SEM (n=6). NS=insignificant difference as compared to diabetic rats..

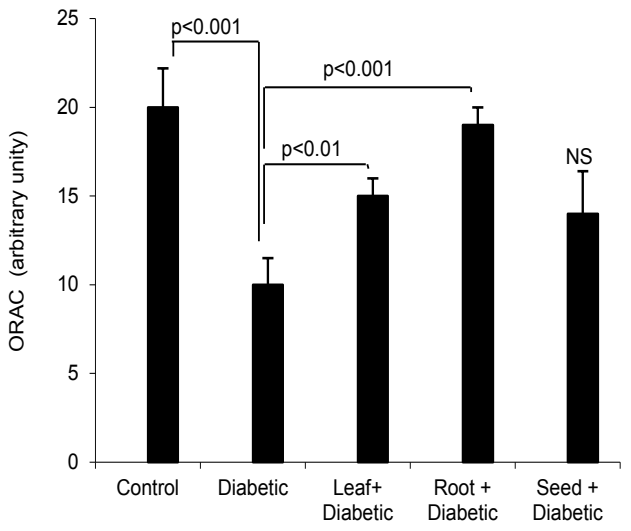

Figure 4: Plasma ORAC values on day 21 in diabetic and control rats, treated or not with extracts from leaf, root and seed of *Zyziphus lotus* L. for 3 weeks. ORAC activity was determined by employing a fluorescent protein, i.e., allophycocyanin (APC), as described in the Materials and Methods. The absorbance decay of APC was measured at 598 nm (excitation) and 651 nm (emission) every 5 min, at 37°C, using a spectrofluorometer until the disappearance of fluorescence. The ORAC value of each plasma sample was calculated by measuring the net protection area under the quenching curve of APC with trolox, sample or blanc. Each value represents the mean±SEM (n=6). NS=insignificant difference as compared to diabetic rats.

Organs/	Catalase	Glutathione reductase	Glutathione peroxidase	Total Glutathione
Groups				
Pancreas				
Control	39 ± 4	0.32 ± 0.02	12 ± 1.06	5.45 ± 1.56
STZ	65 ± 9[§]	0.13 ± 0.02[§]	18 ± 1.36[§]	2.46 ± 0.26[§]
STZ + Leaf	40 ± 5[$]	0.32 ± 0.03[$]	12 ± 1.0[$]	5.09 ± 1.19[$]
STZ + Seed	61 ± 10	0.12 ± 0.07	18 ± 1.99	2.30 ± 1.23
STZ + Root	37 ± 5[$]	0.31 ± 0.01[$]	11 ± 1.52[$]	5.09 ± 1.31[$]
Erythrocytes				
Control	7 ± 1.1	10 ± 2.7	13 ± 1.66	18 ± 1.62
STZ	13 ± 1.[6§]	5 ± 2.0[§]	19 ± 1.29[§]	12 ± 1.60[§]
STZ + Leaf	8 ± 1.7[$]	10 ± 1.9[$]	13 ± 1.43[$]	17 ± 2.09[$]
STZ + Seed	13 ± 2.1	5 ± 1.88	19 ± 2.31	12 ± 3.45
STZ + Root	7 ± 1.1[$]	9 ± 2.09[$]	13 ± 1.52[$]	17 ± 1.05[$]
Liver				
Control	25 ± 2	18 ± 2.05	13 ± 1.56	8.8 ± 1.66
STZ	35 ± 2[§]	8 ± 2.07	18 ± 1.40[§]	4.3 ± 1.16[§]
STZ + Leaf	27 ± 1.3[$]	17 ± 2.07[$]	12 ± 1.25[$]	8.9 ± 1.19[$]
STZ + Seed	34 ± 1.7	7 ± 2.05	18 ± 1.65	4.27 ± 1.23
STZ + Root	20 ± 2.1[$]	18 ± 1.16[$]	13 ± 1.02[$]	8.23 ± 1.34[$]

[§]shows the significant values (p<0.01) compared to the Control group. [$]shows significant differences (p<0.01) compared to STZ group.

Table 1: Activities of antioxidant enzymes in pancreas, erythrocytes and liver

status of diabetic animals is decreased (Figure 4). The extracts from root and leaf increased ORAC values in diabetic rats. These observations suggest that these extracts have beneficial effects on the alteration of antioxidant status induced by diabetes. The seed extracts did not significantly exert any effect on ORAC values in diabetic rats (Figure 4).

Z. Lotus L. extracts modulate the activities of antioxidant enzymes

The catalase activity was determined in three compartments of animals (erythrocytes, liver and pancreas). Diabetes increased catalase activity only in liver, pancreas and erythrocytes. The extracts from leaf and root, but not seed, significantly decreased the catalase activity in diabetic animals (Table 1). The leaf and root extract corrected glutathione reductase activity that was decreased in diabetic animals, and seed extracts did not exert a beneficial effects on this parameter (Table 1). Similarly, the extracts of leaf and root decreased the activity of glutathione peroxidase which was increased in diabetic animals. The seed extract failed to exert a down regulatory effect on this enzyme in pancreas, liver and erythrocytes.

The reserve of total glutathione which was decreased in diabetic animals was increased by the extracts of leaf and root, whereas the seed extract could not normalize the decreased levels of glutathione (Table 1).

Z. Lotus L. extracts upregulate concentrations of A, C and E vitamins

Figure 5 shows that the concentrations of three vitamins (A, C and E) are lower in the blood of diabetic animals as compared to controls. Surprisingly, the seed extract upregulated the concentrations of the two vitamins (C and E) in diabetic animals, without influencing vitamin A levels in diabetic rats (Figure 5). The leaf extract upregulated the concentrations of the three vitamins in both the groups. The root extracts increased vitamin A, C and E in diabetic animals (Figure 5).

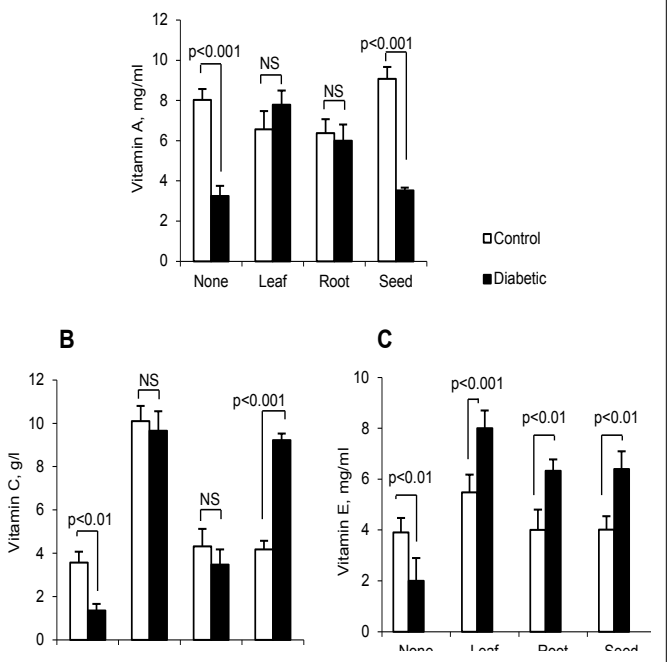

Figure 5: Plasmatic concentrations of three vitamins (A, C and E) day 21 in diabetic and control rats, treated or not with extracts from leaf, root and seed of *Zyziphus lotus* L. for 3 weeks as described in the Materials and Methods section. The determination of vitamin A (in A), vitamin C (in B) and vitamin E (in C) were performed as described in the Materials and Methods section. Each value represents the mean±SEM (n=6). NS=insignificant difference as compared to diabetic rats.

Discussion

Our study highlights the effects of aqueous extracts of *Z. lotus* L. on the progression of diabetes in rats. Hence, we assessed the effects of the extracts from three parts of *Zizyphus*, *i.e.,* leaf, root and seed, on the antioxidant status in blood, pancreas and liver, as well as the ORAC and KRL capacity in control and diabetic rats.

As observed elsewhere [9], streptozotocin induced hyperglycaemia in Wistar rats. We observed that the aqueous extracts of leaf and roots of *Z. lotus* L. presented remarkable hypoglycaemic effects in diabetic rats after three weeks of gavage. Similarly, in the OGTT, the diabetic rats which received leaf and root, but not seed, extracts exhibited lesser glucose tolerance than diabetic animals. Diabetic rats also had low levels of vitamins as observed in diabetic subjects [26]. In the present study, we noticed that leaf and root, but not seed, extracts upregulated the vitamin A concentrations in diabetic animals. The hypoglycemic effect might be due to high vitamin A concentrations in leaves and roots as shown previously [12]. The seed extracts were found to be devoid of vitamin A contents [12]. Vitamin A has been shown to improve insulin sensitivity by increasing insulin receptor phosphorylation through protein tyrosine phosphatase 1B regulation [27]. Vitamin A-enriched diet-fed obese rats had reduced body weight gain, visceral adiposity and improved insulin sensitivity as evidenced by decreased fasting plasma insulin [27]. Other vitamins like C and E may also participate in glucose lowering actions of leaf and root extracts, but they are not sole agents implicated in hypoglycemic effects as seed extracts corrected the concentrations of vitamin C and E, but not glycaemia, in diabetic rats. These vitamins (A, C and E) may protect the animals against oxidant damage, as reported by Catherwood et al. [28].

The total antioxidant status in the blood reflects the overall condition of the body as it corresponds to the balance between oxidant and antioxidant capacities. The oxidative stress plays an important role in diabetes mellitus [29]. Several experimental studies support the role of hyperglycaemia-induced oxidative stress in the development and progression of diabetes-induced complications [30]. An increase in oxidative stress may occur due to an increase in free oxygen radicals. These reactive oxygen species are capable of chemically altering all major classes of bio-molecules like lipids, proteins and nucleic acids, by changing their structure and functions [31], thus leading to cell damage in diabetes [32]. In our study, we observed that leaf and root extracts of *Z. lotus* L. improved the antioxidant status, measured by KRL and ORAC parameters, in diabetic animals.

Catalase, GSSG-Red and GSH-px are important indices of oxidative stress. Our results on the increases in antioxidant status in diabetic rats corroborate the findings of Kakkar et al. [33] who have shown that streptozotocin-induced diabetes is associated with a significant increase in the activity of catalase in liver and heart. We observed that the GSSG-Red activity was decreased in diabetic animals. Our findings are in close agreement with the results of several investigators [34,35] who have also noticed decreased activity of this enzyme in type II diabetes. Interestingly, the leaf and root, but not seed, extracts of *Z. lotus* L. upregulated the activity of this enzyme in diabetic rats. The leaf and root extracts also downregulated the activity of catalase and GSH-px that was very high in diabetes rats [33].

The diabetic animals, in our study, also showed a significant decrease in glutathione concentrations. Glutathione is involved in many pathways, including DNA and protein synthesis and transport of amino acids [36]. Glutathione is also critical for cellular protection, such as the detoxification of reactive oxygen species, the conjugation and excretion of toxic molecules and the control of the inflammatory cytokine cascade [36]. It is interesting to mention that the leaf and seed extracts of *Z. lotus* L. improved the glutathione levels in diabetic animals. It is possible that polyphenols, present in these extracts may be responsible for these beneficial effects. It has been shown that the leaf extracts from P. nitida leaves, rich in polyphenols, exhibited free radical-scavenging activity, significant blood sugar reduction capacity, and reduced the levels of oxidative stress markers like catalase in animal model [37]. Indeed, we have previously shown that *Z. lotus* L. contains polyphenols, responsible for the immunosuppressive effects on Jurkat T-cells [13].

Conclusions

To sum up, we can state that hyperglycaemia of diabetic animals is associated with a decrease of antioxidant status, and leaf and root aqueous extracts of *Z. lotus* L. bear therapeutic potential as they possess antioxidant and hypoglycemic properties. Though the treatment of diabetes requires urgent medication for the patient, some medicinal plants can be useful to reduce the doses of exogenous insulin to treat diabetes mellitus. Hence, *Z. lotus* L. seems to be a good candidate to lower, in addition to conventional antidiabetic drugs, the hyperglycaemia in diabetic subjects.

Authors' Contributions

Study design and supervision (Naim A. Khan), practical work (Chahid Benammar, Selvakumar Subramaniam, Aziz Hichami), critical comments and reading (Choukri Baghdad, Meriem Belarbi, Aziz Hichami).

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