Anti-Diabetic Effect of Balanites Aegyptiacea Leaves Extract (Heglig) by Regulation of Erythrocyte Glucose Uptake in Diabetic Patients Type 2 in Vitro

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ABSTRACT

Background: in Diabetes, the increase in the oxidative stress and decrease in the antioxidant defense may elevate he susceptibility of diabetic patients to many pathological complications, oxidative induced cell damage has been proposed to play an important role in the etiology of numerous pathological conditions. So, the aim of the present study was to investigate the antioxidant potential of Alcoholic Leaves extract of Balanites aegyptiacea (Heglig) due to the presence of phenolic and flavonoids compounds on uptake of glucose in vitro by erythrocytes of diabetic patients.

Results: in hyperglycemic patients, erythrocytes malondialdehyde level was highly significantly increased ($P<0.0001$) than that of control. However, the erythrocytes glutathione content was highly significantly decreased ($P< 0.0001$) when compared to that of corresponding control values. The glucose uptake by erythrocytes of diabetic patients was highly significantly decreased ($P < 0.0001$) with increasing hyperglycemia (Fasting Blood glucose), while it was highly significantly elevated ($P< 0.0001$) after addition of Balanites aegyptiacae leaves extract to the incubation medium. On the other hand, the malondialdehyde concentration was highly significantly reduced ($P < 0.0001$) on adding the extract. So, it could be concluded that , an appreciate support for enhancing Antioxidant supply from natural sources such Balanites aegyptiace leaves extract may help control blood glucose levels and prevent pathological complications of diabetes

Keywords: Diabetes Mellitus, Balanites Leaves Extract, phenolic and flavonoids cpd., Oxidative Stress, Human Erythrocytes.

INTRODUCTION

Diabetes Mellitus (DM) is a group of metabolic disease Characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both[1].Several distinct types of DM caused by a complex interaction of genetics, environmental factors and life style choices. Pazdro and Burgess [2] reported that; arise in blood glucose concentration causes increased oxidative stress which contributes to the development and progression of diabetes –associated complications. Diabetic Ketoacidosis (DKA) and hyperosmolar state (HHS) are acute complications of diabetes. Although chronic hyperglycemia is an important etiologic factor leading to complications of DM, the mechanism(s) by which it leads to such diverse cellular and organ dysfunction is unknown[3].

Free radicals are constant products produced from aerobic cell metabolism[4].excessively high levels of free radicals cause damage to cellular proteins, lipids and nucleic acids, and eventually cell death[5]. A system of nutritional and endogenous enzymatic antioxidant defense generally holds the production of free radicals in check[6].

Oxidative stress is defined as either an overproduction of free radicals or a diminution in antioxidant defenses, the result of either leads to excessive levels of free radicals[7].Oxidative stress in cell and tissue plays an important role in the pathogenesis of DM. oxidative stress manifests by increased levels of free radicals; it suppresses glycolysis, protein and nucleic acids production, and enzyme activities and promotes oxidation – phosphorylation uncoupling[8].In living animal's cells, peroxidized membranes lose their permeability, becoming rigid, reactive and nonfunctional. Malondialdehyde (MDA) is an end product of cell membrane lipid peroxidation and its concentrations have been widely used as a measure of cell membrane lipid Peroxidation[9].

Hyperglycemia promotes hyper production of active oxygen and nitrogen forms, including superoxide radicals, nitrogen monoxide, and peroxinitrite which is to be one of the main factors in the development of DM complications, and modifies structural and mechanical characteristics of erythrocyte membrane and cytoskeleton and erythrocyte shape[10].

Halliwell and Gutteridge[11]have defined antioxidants as substances that are able, at

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relatively low concentrations, to compete with other oxidazable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates. This definition includes the enzymes: Superoxide dismutase (SOD), Glutathione peroxidase (GPx), and catalase, as well as nonenzymic compounds such as α-tocopherol (vitamin E), β-carotene, ascrobate (vitamin C) and glutathione [12].

Membrane lipids are vital for the maintenance of cellular integrity and survival. Peroxidation of membrane lipid can result in the activation of enzymes and cross-linking of membrane lipids and proteins and in cell death [13]. Since hyperglycemia induces oxidative stress, it seems to be the main cause of diabetic complications [14].The ideal anti-diabetic drugs should combine both hypoglycemic and antioxidant properties.

In the present study, the antioxidant defense and the lipid peroxidation have been studied by investigating erythrocytes reduced glutathione content and Malondialdehyde levels, as well as the activities of superoxide dismutase in diabetic patients and healthy subjects. Also the effect of Balanites aegyptiacae leaves extract with high antioxidant potentials on the uptake of glucose by erythrocytes was studied.

PATIENTS AND METHODS

Patients and groups

This study was conducted on 90 diabetic patients who were admitted to National Institute of Diabetes and Endocrinology. Patients were chosen according to the criteria presented in 2010 by the American Diabetes Association[15] where diabetes is diagnosed if patient has fasting plasma glucose equal to or higher than 126 mg/dl or who has plasma glucose 2 hours after 75 g glucose load equal to or higher than 200 mg/dl. Their ages ranged 24-75 years. Results were compared with those obtained from thirty healthy non diabetic volunteers with normal glucose levels who were recruited from outpatient clinics. Informed consent was obtained from all participants. The study subjects were arranged in four groups as follows:

- **Group I:** included thirty diabetic patients whose fasting blood glucose equal to or lower than 150 mg/dl.
- **Group II:** included thirty patients whose blood glucose levels ranged from 150-300 mg/ orally treated with drugs.
- **Group III**: included thirty patients with fasting blood glucose levels higher than 300 mg/dl and treated with insulin.
- **Group IV**: included 30 apparently healthy non diabetic individuals. They were matched with the group 1 patients for age and gender.

Methodology

The present study was divided into two parts The First part included the study of the effect of an extract of Balanites aegyptiacae Leaves extract with high antioxidant activity on erythrocytes uptake of glucose.

The Second part included the evaluation of some antioxidant as reduced glutathione (GSH), superoxide dismutase (SOD) in addition to lipid peroxidation represented by Malondialdehyde (MDA) in erythrocytes. The assay of LDH, GST activities in erythrocytes also included. The antioxidant biomarkers estimated with and without addition of the extract to the incubation medium for 2 h at 37C.

Preparation of plant extract

Preparation of Balanites aegyptiace leaves extract according to Kahsay method [22]. The identification of extract active constituents was performed by HPLC analysis to phenolic and flavonoids compounds chemical analysis of total flavonoids according to method of Violeta Ivanova.[15].

Blood Samples

Samples of diabetic patients were collected by the doctors at National Institute of diabetes and endocrinology, all blood samples divided into two parts the first part withdrew on fluoride for plasma glucose estimation and the second part withdrew on EDTA to prepare packed red blood.

Whole blood was collected from diabetic patients. Human erythrocytes were separated by centrifugation at 3000 r.p.m the plasma and buffy coat were removed then the erythrocytes washed three times with phosphate buffered saline (pH.4), then washing buffer eluted by micropipette.

Biochemical tests

Fasting blood glucose (FBG) levels was calorimetrically determined according to enzymatic method of Barham and Trinder[16], the activity of erythrocyte glutathione (GSH) was determined by method of Beutler et al[17]. Red blood cells superoxide dismutase (SOD) was determined by the method of Nishikimi[18]. Malondialdehyde (MDA) levels was determined by the method Stocks and Donnady[19].

Erythrocyte Lactate dehydrogenase (LDH) activity was determined by the method of Heiden et al .[20]. Glutathione–S-transferase (GST) activity was determined by the method of Habig et al.[21].

Preparation of Balanites aegyptiacae Leaves Extract

Fresh leaves of Balanites aegyptiaca delile were collected from Green House of National Gene Bank in Agriculture ResearchCenter. After the washed leaves of BalanitesAegyptiaca were shade dried at room temperature for 5days, dried powdered leaves (300 g) of Balanites aegyptiaca were extracted using one liter methanol at room temperature for three days by maceration and then the obtained extract was filtered using Whatman filter paper. The second successive extraction was performed using recycled methanol on the residue left, following formerly mentioned technique to get the methanolic extract. The obtained methanolic extracts were concentrated in rotary evaporator and dried in a vacuum oven so as to get thick, viscous mass Kahsay et al. [22].

The identification of extract active constituents was performed by Agilent HPLC model No.1100analysis to phenolic and falvonoids compounds, chemical analysis of total flavonoids determined according to VoiletaIvanova method.

Incubation of erythrocytes in hyperglycemic condition

After washing erythrocytes from controls and diabetic patients with phosphate buffered saline (PBS), the erythrocytes were incubated with (PBS) containing (100 mg glucose /100 PBS) for 2 hours at 37C. The capability of erythrocytes to uptake glucose, with and without addition of Balanites aegyptiace Leaves extract, was investigated. Erythrocytes glucose uptake, erythrocytes GSH content, erythrocytes SOD activity , erythrocytes MDA levels , erythrocytes LDH activity and erythrocytes G-S-T activity were determined with and without addition of extract to the incubation media after 2 h. the erythrocytes glucose uptake determined according to the method of Jain[23].The washed erythrocytes can consume glucose from PBS solution that contains glucose with a given concentration, when incubated for 2 hours in 37C. The remaining consumed glucose can determined and the subtracted value can be considered as the glucose uptake value.

Statistics

Data were statistically described in terms of Mean ± standard deviation (M±SD). Comparisons between control groups and diabetic groups were assessed by T-test analysis using the excel software (Microsoft). A probability value (P value) less than 0.05 was considered statistically significant and less than 0.001 was considered statically highly significant. Correlation coefficient (r) was done by statistical program package, Medcalc program version 11.3.3.0.

RESULTS

Table 1: Shows results of FBG, GSH, MDA, SOD, GST and LDH in control and diabetic patient without addition of Balanites leaves extract to the incubation medium.

Parameter	Control group (n= 30) (Mean±SD)	Diabetic group 1 (n=30) (Mean±SD)	diabetic group 2 (n=30) (Mean±SD)	diabetic group3 (n=30) (Mean±SD)
FBG mg/dl	96.5±14.6	123±19.12	203±42.48	372.4±65.99
GSH mmol/L cell	4.44± 0.49	3.54 ± 0.80b	3.38 ± 0.69b	3.52 ± 0.40 b
MDA mmol/L cell	0.1± 0.01	0.1 ± 0.01a	0.13 ± 0.01b	0.16 ± 0.02b
SOD unit/L cell	0.21± 0.02	0.15 ± 0.05b	0.13 ± 0.02b	0.13 ± 0.02b
LDH unit/L cell	23.49± 1.8	17.1 ± 3.81b	15.9 ± 3.12b	15.77 ± 2.7b
G-S-Tunit/ L cell	5.8± 1.13	4.5 ± 1.3a	3.05 ± 0.82b	3.5 ± 0.7b

N: number of cases , a: P value is significant b: P value is highly significant, compared to values of control.

A Highly significant decrease (P< 0.001) were found in erythrocytes GSH content and erythrocytes GST activities of the patients of the three diabetic groups compared to controls. A highly significant (P< 0. 01) and (P<0.0001) increase in MDA levels is observed in diabetic patients, SOD activities in red blood cells showed a highly significant decrease (P< 0.0001), the mean activities of LDH in red blood cells is highly significant decrease (P<0.0001) when compared to control.(Table 1).

Table 2: Chemical analysis of total flavonoids determined according to VoiletaIvanova method measured byJenwayVis spectrophotometer 6705UV.

Sample	*Test Method	Test Results(%)
Heglig Leaves	Violeta IVANOVA et al(2010) J. Serb. chem. Soc. 75 (1) 45-59 (2010) JSCS-3940	Total Flavonoids (mg/ml)
		2.38

Balanites Leaves extract showed total flavonoid concentration of 2.38 mg/ml extract and HPLC analysis showed several phenolic compounds includes (Catechein ,catechol, e-vanillic, pyrogallol and benzoic ect.) and flavonoid compounds includes (Acacetin, Quercetrin, Kampferol and Quercetinect.) which have high antioxidant potentials (**Table 2**).

Table 3: Glucose uptake (mg/dl) by red blood cells of controls and diabetic patients, with and without the addition of Balanites leaves extract.

Group	Glucose uptake (%) Without extract	with extract
Control		
Range	(38-67)	(45-88)
Mean ±SD	56.2±12.25	66±12.55
n	30	30
Diabetic grp1		
Range	(10-66)	(38-88)
Mean ±SD	36.16±15.44	56.44±14.88
n	30	30
Diabetic grp2		
Range	(6-57)	(23-75)
Mean ±SD	27.71±13.9	48.7±11.9
n	31	31
Diabetic grp3		
Range	(2-36)	(22-66)
Mean ±SD	19.45±9.6	49.45±12.5
n	31	31

After incubation of erythrocytes of diabetic patients in hyperglycemic buffered saline at 37C for 2 h without addition of 5mg Balanites aegyptiace leaves extract the mean glucose uptake levels in all diabetic patients are highly significantly decreased ($P < 0.0001$) compared to that of control. However, the erythrocytes glucose uptake is elevated after addition of the extract (**Table 3**).

Table 4: Correlation between erythrocytes glucose uptake and GSH, MDA and FBG levels

Correlation between		r value	P value
Erythrocyte glucose uptake	GSH	0.335	<0.01
	MDA	-0.365	<0.01
	F.B.G	-0.4073	<0.001

Erythrocytes glucose shows negative correlations with both plasma FBG and MDA levels and shows the positive correlation with erythrocytes GSH content (**Table 4**).

Table 5: Malondialdehyde (MDA) (mmol/L red blood cells) levels with and without addition of Balanites Leaves Extract.

Group	MDA (mmol/L packed cell) Without extract	with extract
Control		
Range	(0.09-0.1)	(0.04-0.09)
Mean ±SD	0.1± 0.01	0.06±0.01
n	30	30
Diabetic grp1		
Range	(0.11-015)	(0.04-0.09)
Mean ±SD	0.1±0.01	0.06 ±0.01
n	30	30
Diabetic grp2		
Range	(0.12-0.15)	(0.06-0.1)
Mean ±SD	0.13±0.01	0.07±0.01
n	31	31
Diabetic grp3		
Range	(0.13-0.18)	(0.05-0.09)
Mean ±SD	0.16±0.02	0.07±0.01
n	31	31

Erythrocytes MDA of patients of the three diabetic groups after incubation in high glucose concentration without Balanites aegyptiacea leaves extract are highly elevated compared to that of controls. After addition of extract to the incubated erythrocyte the MDA levels of the same diabetic groups are highly reduced compared to the levels of the same diabetic groups before addition of that extract (**Table 5**).

Table 6 : Lactate dehydrogenase (LDH) (mmol/L red blood cells) activity with and without addition of Balanites Leaves Extract.

Group	LDH (mmol/L packed cell) Without extract	with extract
Control		
Range	(21-26.7)	(23.8-27.1)
Mean ±SD	23.49± 1.8	25.29±1.06
n	30	30
Diabetic grp1		
Range	(10.9-21.4)	(18.7-28)
Mean ±SD	17.1 ± 3.81	23.53. ±3.4
n	30	30
Diabetic grp2		
Range	(11.46-19.7)	(14.2-31.5)
Mean ±SD	15.9±3.12	22.9±6.12
n	31	31
Diabetic grp3		
Range	(11.7-20.45)	(15.3-26.5)
Mean ±SD	15.77±2.7	20.42±3.4
n	31	31

The mean erythrocytes activities of LDH of patients of the three diabetic groups showed highly significant increase after incubation with the extract compared with their values incubated without the extract
(**Table 6**).

DISCUSSION

Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanism can lead to damage of cellular organelle and enzymes, increasing of lipid peroxidation, and development of insulin resistance. These consequences oxidative stress can promote the development of complications of diabetes mellitus.

Many studies showed that, the biomarkers of oxidative stress increase in diabetes and its complications[24].The result of the present study showed highly increased in erythrocytes MDA levels in diabetic patients(**Table 1**) confirming the pervious reporting significant increase in lipid peroxides in both type 1 and type 2 diabetic patients[25].

The tripeptide glutathione (GSH) is the most prevalent antioxidant in cells and participate in many cellular functions, including detoxification processes. Thus the decrease in GSH content in the diabetic patients may disturb antioxidant defenses, which together with increased oxygen free radicals activity will result in acceleration of the oxidative damage already present in early stages of diabetes[28]The present results showed highly depleted erythrocytes GSH contents in diabetic patients (**Table1**), a finding which is in agreement with the finding of other investigators[29,30], who reported that , GSH metabolism was altered in both type1 and type 2 diabetes.Beard et al.[29]suggested that α-oxoaldehydes produced through glycolytic and pentose phosphate pathways of carbohydrates may deplete cellular GSH. As a result of GSH depletion, subsequent glycoxidative stress affects erythrocyte function and contributed to diabetic complications.

The activities of SOD in erythrocytes are enough to protect against free radicals injury. Some studies [30], reported reduced SOD activities while others, reported increased activity of this enzyme in diabetes. In the present study, there was highly significance decrease in RBCs SOD activity in diabetic patients **(table 1**) which in agreement with [31].

The highly significant decrease in LDH activity in diabetic patients (**table 1**) is agreeable with the results of Abd El-Baky et al.[32]and may reflect the free radical inhibition effect on the active site of the enzyme.

GSTs are group of enzymes that play a very important role in the detoxification of dangerous compounds to less toxic compounds[33]. Age associated changes in GST has been reported. [34] GSTs also play a significant role in drug resistant development in tumor cells, Alzheimer's and Parkinson's disease, atherosclerosis. GST are involved in many biological functions in mammals which includes detoxification of toxicants, catalysis of several biological processes, several functions associated with metabolism, resistance towards drugs and inhibiting age associated disorders[35].In our study,The highly significant increased in GST activities with addition of Balanites aegyptiacae Leaves extract is agreeable with the results of Qusti *et al.*[36].

D-glucose is transported in the red blood cell by facilitated diffusion, this metabolism is unaffected by insulin and independent of ATP requirements. In red blood cell, about 90% of glucose is consumed by the glycolytic pathway while the rest (10%) flow through the pentose shunt. In diabetes, many alternations have been found in the erythrocytes, such as decreased life span, reduced deformability, increased red cell aggregation, and reduced membrane cholesterol and sialic acid contents [37]. All these changes may affect greatly the erythrocytes glucose uptake which is actually the case in the present data where the erythrocytes glucose uptake in DM patients was highly significantly decreased (**table3**). Increased lipid peroxidation and altered RBCs membrane composition observed in DM patients may be due to oxidative stress [38]. Which may also affect membrane fluidity and deformability, and consequently erythrocytes glucose uptake. Elevated glucose levels can cause peroxidation of membrane lipids and increased membrane osmotic fragility in vitro. Since RBCs do not need insulin for glucose uptake [39]. It seems likely that increased glucose oxidation leads to accumulation of glucose metabolites such as NADPH. Apparently in hyperglycemia, greater NADPH formation stimulates the NADPH-dependant cytochrome P450 system like activity of hemoglobin, which may form oxygen radicals and results in the membrane lipid peroxidation of RBCs.

Lipid peroxidation and MDA accumulatation can disturb organization of phospholipids in the erythrocytes membrane bilayer. This impairment in RBCs membrane in diabetic patients may explain the significant inverse correlation between MDA level and erythrocytes glucose uptake (**Table 4**). The impairment of erythrocytes membrane, therefore, leads to decreased glucose

uptake by RBSc and consequently increases plasma glucose level. In our study the Balanites aegyptiacea leaves extract with high antioxidant potentials was tested for its capability to relieve the oxidative stress and repair the RBCs membrane. The obtained data demonstrated increased erythrocytes glucose uptake (50%), (table 2). The results were confirmed by determination of MDA, and LDH with and without addition of the Blanites aegyptiacea leaves extract to the incubation media. The results revealed a highly significant decrease in MDA level in erythrocytes of diabetic patients after addition of the extract compared with controls and erythrocytes without addition of that extract.
Abdel Motaal et al. And Qusti1 et al. found that, Balanites aegyptiacae has the ability to reduce the squeal of hyperglycemia-induced ROS overproduction. Since Balanites aegyptiaca leaves extract also has beneficial effects on other target tissues as kidney, and shows beneficial effects of mediators of large vessel damage, this concept appears attractive for the prevention or delay of diabetic nephropathy.In other words, the addition of Balanites leaves extract increase the erythrocytes glucose uptake,LDH activity, and decrease the oxidative stress as represented by MDA **.**

CONCLUSION

From the above mentioned observations, we can conclude that, Balanites aegyptiacae Leaves Extract has hypoglycemic and antioxidant effects which may render its use in treatment and control of DM and its complications.

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