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Original Research Article

MOLECULAR INVESTIGATION OF ANTI-DIABETIC EFFECT OF *BALANITES AEGYPTIACA* FRUITS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Abstract: *Balanites aegyptiaca* (*B. aegyptiaca*) is an anti-diabetic medicinal plant traditionally used in Egyptian folk medicine as alternative therapy for the treatment of type 2 diabetes. No available studies revealed the mechanism(s) of the associated anti-diabetic effect especially at the molecular level. This study aimed to explore the possible molecular mechanism(s) that underline *B. aegyptiaca* fruits aqueous extract administration in diabetic and non-diabetic rats. Four equal groups (n=10) of albino rats were used. The prepared aqueous extract of *B. aegyptiaca* fruits was given orally (80 mg/kg body weight for 4 weeks) to normal control and streptozotocin (65 mg/kg BW, i. p.) -induced-diabetic rats. Administration of *B. aegyptiaca* fruits aqueous extract in diabetic rats significantly elevated the serum insulin (91%) and reduced serum glucose (54%), cholesterol (26%), triglycerides (16%) and LDL cholesterol (25%) compared to the diabetic control. Produced hypoglycemia in treated diabetic rats simultaneously accompanied at least by significant improving (p<0.05) of pancreatic insulin and α-amylase; hepatic insulin receptor A, glucose transporters (GLUT-2 and GLUT-4), and adipocyte leptin gene expressions. In conclusion: The anti-diabetic effect of *B. aegyptiaca* fruits aqueous extract was achieved by increasing insulin level as well as stimulating endogenous insulin secretion and enhancing its action at the target tissues. So it covered at least most of the main therapeutic strategies of diabetes. More studies are needed for preparation of a standardized dose and dosage regimen of active constituents of this promising fruit that can play a significant role in the management of type 2 diabetes and related complications.

Key words: *Balanites aegyptiaca*; streptozotocin; α-amylase; insulin receptor A; GLUT-2; GLUT-4; leptin

Introduction

Diabetes mellitus (DM) is one of the most common chronic diseases that cause serious damage to many different body systems, especially nerves and blood vessels**.** The second type of diabetes (type 2) is the most public form of diabetes due to defects in the secretion of insulin or insulin resistance**.** More than 80% of people living with diabetes are present in low- and middle-income countries as reported by WHO and the mortality is expected to double between 2005 and 2030 (1).

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Side effects and limited efficacy associated with the oral anti-diabetic drugs leads to increased demand of research on natural anti-diabetic products. Herbal remedies have been used as alternative medicines for the treatment of many diseases, including DM due to their remarkable effectiveness, relatively low costs, and the low side effects of their use (2). One of the most widely used in this field is *Balanites aegyptiaca* (*B. aegyptiaca)*.

Balanites aegyptiaca (L.) Delile, belongs to the family Zygophyllaceae probably called the 'desert date' (Heglig in Arabia). It is an evergreen tree present in wild areas and savannah areas in Africa and South Asia. Mesocarp of fruit contains 1.2 to 1.5% protein, 35 to 37% sugars, 15% organic acids and other constituents. Phytochemical studies on *B. aegeptiaca* isolated several classes of metabolites many of which possess biological activities such as cumarins, flavonoids and steroidal saponins. Balanitoside (furostanol glycoside) and 6-methyldiosgenin, balanitin-3 (spirostanol glycoside) and Balanitin-6 and -7 have been reported from fruits (mesocarp) of *B. aegyptiaca*. The different parts of the plant (leaves, bark, fruit or root) characterized by multipurpose medicinal application as antidiabetic, hypocholesterolemic, hepatoprotective, cardioprotective**,** antioxidant, antiviral, antibacterial, anti-inflammatory, anti-nociceptive, analgesic and anthelmintic (3).

In Egyptian folk medicine, its fruits mesocarp extract is traditionally used as a hypoglycemic agent **(**4-6) and as an anti-diabetic (7). The exact mode of action of this extract is still unclear. Some previous *in vitro* studies suggested that *B. aegeptiaca* may produce its hypoglycemic action through an intestinal reduction of glucose absorption by inhibiting α-amylase activity that considered as the first line therapy in diabetes treatment (5). Another hypothetical possible mechanism suggested that the hypoglycemic action might be produced through potentiation of insulin secretion from β-cells or due to glucose transport enhancement to peripheral tissue **(**6).

To our knowledge, there are no available studies revealed the possible anti-diabetic molecular mechanism(s) of *B. aegyptiaca* fruits extract on normal or diabetes-induced experimental animals. For this reason, the current study was planned to explore, *in vivo*, the possible mechanism (s) by which *B. aegyptiaca* fruits aqueous extract may succeed to produce hypoglycemic or anti-diabetic effect in streptozotocin (STZ) - induced diabetic (hyperglycemic) and normal (normoglycemic) rats.

Material and methods

Preparation of B. aegyptiaca fruits aqueous extract

The *B. aegyptiaca* fruits were purchased from a commercial source; local markets in Sharkia Governorate. According to the commercial produced company (Al Tahhan company to fill the dates), the fruits were obtained from Heglig trees that grow in the southern desert of Egypt; New Valley. The dried fruits (1 Kg) were soaked in distilled water for 24 h and filtrated after the seeds were discarded. This freshly prepared filtrate was freeze dried (using labcono, freeze dryer, model 18) to give thick dark brown extract. The dosed extract was prepared (100 mg extract dissolved in 10 ml distilled water) immediately before administration. The prepared aqueous extract of *B. aegyptiaca* fruit was given orally in a dose of 80 mg/kg body weight (BW) (4) by an orogastric tube daily for 4 weeks.

Animal housing and management

Forty male albino rats were used. Their age and weight at the beginning of the experiment were approximately 6 months and 120±20 gm. Rats were acclimatized for two weeks under standard laboratory conditions including good aerated room with suitable temperature, provided *ad libitum* with food and drinking water. The experimental procedures were conducted according to the guidelines for experimental animal care of the Faculty of Vet. Med. Zagazig University, Egypt and approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary.

Animal grouping

Rats were divided into four groups (n=10): the first group served as normal control,

received saline, the second group received *B. aegyptiaca* aqueous extract in a dose of 80 mg/kg BW. The 1st and 2nd groups assigned as normoglycemic groups. Diabetes type 2 was induced in the 3rd & 4th groups by a single intraperitoneal injection of STZ (Sigma-Aldrich Co., USA) freshly prepared, dissolved in 0.01 M cold sodium citrate buffer (pH 4.5) immediately before use, in a dose of 65 mg/kg BW (8). The third group used as diabetic control while the fourth group treated with *B. aegyptiaca* aqueous extract in a dose of 80 mg/kg BW. To avoid the circadian rhythm, each of the drug or saline was administered between 7:00 and 8.00 am. The dosing was for 4 weeks using orogastric tube. STZ-injected rats were fed with glucose solution (5%) next 24h to avoid drug induced hypoglycemia. After three days, rats with fasting blood glucose levels >200 mg/dl were considered as diabetic rats.

Sampling

At the end of experimental period, over-night-fasted rats were scarified. Blood samples were individually collected and the sera were separated and stored at -20°C until used for biochemical investigation. Liver, pancreas and adipose tissues were rapidly taken on liquid nitrogen for molecular analysis.

Biochemical analysis

Calorimetrically serum glucose, total lipids (TL), triacylglycerol (TAG), total cholesterol (TC) and HDL-c levels were determined by SPINREACT kit according to manufacture instructions (kit obtained from Girona, Spain). Serum insulin levels were determined by specific ELISA kit for rat insulin according to manufacture instructions (Cat. No. ezermi-13 kelisa, Billerica, MA, USA). LDL-c was estimated by Friedewald formula (9).

Relative quantitative polymerase chain reaction (RQ-PCR)

Hepatic, pancreatic and adipose tissue total RNA were extracted from liquid nitrogen saved samples, grinding occurred in sterilizing mortars and 30 mg were used for extraction using RNeasy Mini Kit (Qiagen, Germany, cat. no. 74104). One µl of extracted total RNA was checked for purity and quantity at OD260/OD280 by NanoDrop® ND-1000 Spectrophotometer, (NanoDrop Technologies, Wilmington, Delaware, USA). Only samples with purity more than 1.8 were used for frequent steps. First strand cDNA was synthesized using Revert Aid™ First Strand cDNA Synthesis kit (Fermetas, life science, Pittsburgh, PA, USA). Relative quantitative real-time PCR using cDNA, QuantiTect® SYBR® Green PCR kit (Qiagen, Germany) and designed primers for insulin, IRA, GLUT2, GLUT4, α-amylase and leptin genes using a Rotor-Gene Q cycler (Qiagen, Germany). The conditions of PCR were 95℃ for 5min, 30 cycle (95℃ for 45sec, 60℃ for 30sec and 72° C for 45sec) for insulin 1 and Glut-4; 95℃ for 4min, 35 cycle (95℃ for 30sec, 58℃ for 30sec and 72° C for 30 sec) for IRA, Glut-2 and β-actin; 94℃ for 4min, 35 cycle (95℃ for 30sec, 56℃ for 30sec and 72° C for 30 sec) for amylase and leptin genes. β-actin gene was used as a constitutive control for normalization. Primers were designed according to references listed in Table (1). $2^{-\Delta\Delta Ct}$ were estimated and the relative fold changes were calculated according to the method of Litvak and Schmittgen (10).

Statistical analysis

All data are expressed as mean ± SD. Data was compared among groups using One-way analysis of variance (ANOVA) with statistical package for social science (SPSS, 21 software, 2015). For testing the inter-grouping homogeneity, the Duncan's multiple rang test was applied. *P* values of less than 0.05 represent statistically significant difference.

Results

Induction of diabetes type 2 by STZ caused a significant reduction in the relative mRNA expression of pancreatic α-amylase and insulin; hepatic insulin receptor A (IRA) and GLUT-2; adipocyte GLUT-4; with significant increase of leptin hormone in comparison with the normal control rats. Molecular investigations in normoglycemic rats treated with *B. aegyptiaca*

Table 1: The primer sequences used in this study

Target Gene	Primer Sequence (5'→3')	Product size(bp)	Gene bank ID	References
Insulin1	F: ATGGCCCTGTGGATGCGCTT R:TAGTTGCAGTAGTTCTCCAGCT	331	3630	(11)
IRA	F: TTCATTCAGGAAGACCTTCGA R:AGGCCAGAGATGACAAGTGAC	222	24954	(12)
GLUT-2	F: TTAGCAACTGGGTCTGCAAT R: TCTCTGAAGACGCCAGGAAT	243	25351	(11)
GLUT-4	F: GAGCCTGAATGCTAATGGAG R: GAGAGAGAGCGTCCAATGTC	187	442992	(13)
α-amylase	F: TTGCGTTCAGGAGACCAAC R: CATAGGTTTGTGAGGCGGT	158	24203	(14)
Leptin	F:ATCAAGACCATTGTCACCAGGATC R:CTGGTCCATCTTGGACAAACTCA	129	25608	(15)
ß-actin	F- TCACTATCGGCAATGTGCGG R- GCTCAGGAGGAGCAATGATG	260	81822	(12)

IRA = insulin receptor A; GLUT2 = Glucose transporter 2; GLUT4 = Glucose transporter 4.

watery extract showed no significant differences with the normal control. In the opposite, STZ diabetic rats treated with *B. aegyptiaca* (group 4) produced a significant improvement of all relative gene expressions of α-amylase, insulin, IRA, GLUT-2 and GLUT-4 in comparison with diabetic non treated rats but they still significantly less than that of the control. Relative leptin mRNA expression significantly decreased under the effect of *B. aegyptiaca* treatment in comparison with diabetic non treated rats but it still higher than control level (Figure 1).

Treatment with *B. aegyptiaca* significantly decreased serum levels of TL, TC in non-diabetic rats than control group. While STZ-diabetic rats showed significant elevation of serum fasting blood glucose, TL, TAG, TC and LDL-c, whereas serum insulin and HDL-c levels were significantly decreased. Treatment with *B. aegyptiaca* fruits successfully ameliorated these bad effects through decreasing the serum fasting blood glucose (54%), TL (40%), TAG (16%), TC (26%) and LDL-c (25%) and increasing the levels of serum insulin (91%) and HDL-c (43%) compared to diabetic rats but they did not reach to the levels of control group (Table 2).

Table 2: Blood glucose, insulin levels and lipid profile in STZ-diabetic and non- diabetic rats treated with *B. egyptiaca* aqueous extract (80 mg/kg BW)

Parameters	Control	Non diabetic+*B.egyptiaca*	Diabetic control	Diabetic+ *B.egyptiaca*
Blood glucose (mg/dl)	103.32±5.76^{c}	89.21.8c ± 1.7^{c}	418.4±12.43^{a}	193 ± 3.5^{b}
Insulin (µIU/ml)	0.963±0.004^{a}	0.97 ± 0.006^{a}	0.4± 0.002^{c}	0.763 ±0.08^{b}
Total lipids (mg/dl)	308.4 ± 4.28^{c}	299.4 ± 1.9^{d}	824.4± 10.5^{a}	493.7± 5.5^{b}
Triacylglycerol (mg/dl)	105.42±3.21^{c}	96.07 ± 3.4^{c}	138.2± 4.32^{a}	116.6 ± 1.53^{b}
Total cholesterol (mg/dl)	164.5± 3.42^{c}	156.4 ± 1.7^{d}	226.8 ± 4.3^{a}	197.9 ± 1.6^{b}
HDL-c (mg/dl)	65.4±3.25^{a}	63.13 ± 1.7^{a}	36.8±0.87^{c}	52.56±0.98^{b}
LDL-c (mg/dl)	74.43±2.48^{c}	71.17 ± 1.7^{c}	109.3±3.2^{a}	81.5 ± 0.43^{b}

All data are expressed as mean ± SD.

Means which have different superscript in the same row are significantly different from each other at $p<0.05$ and vice versa.

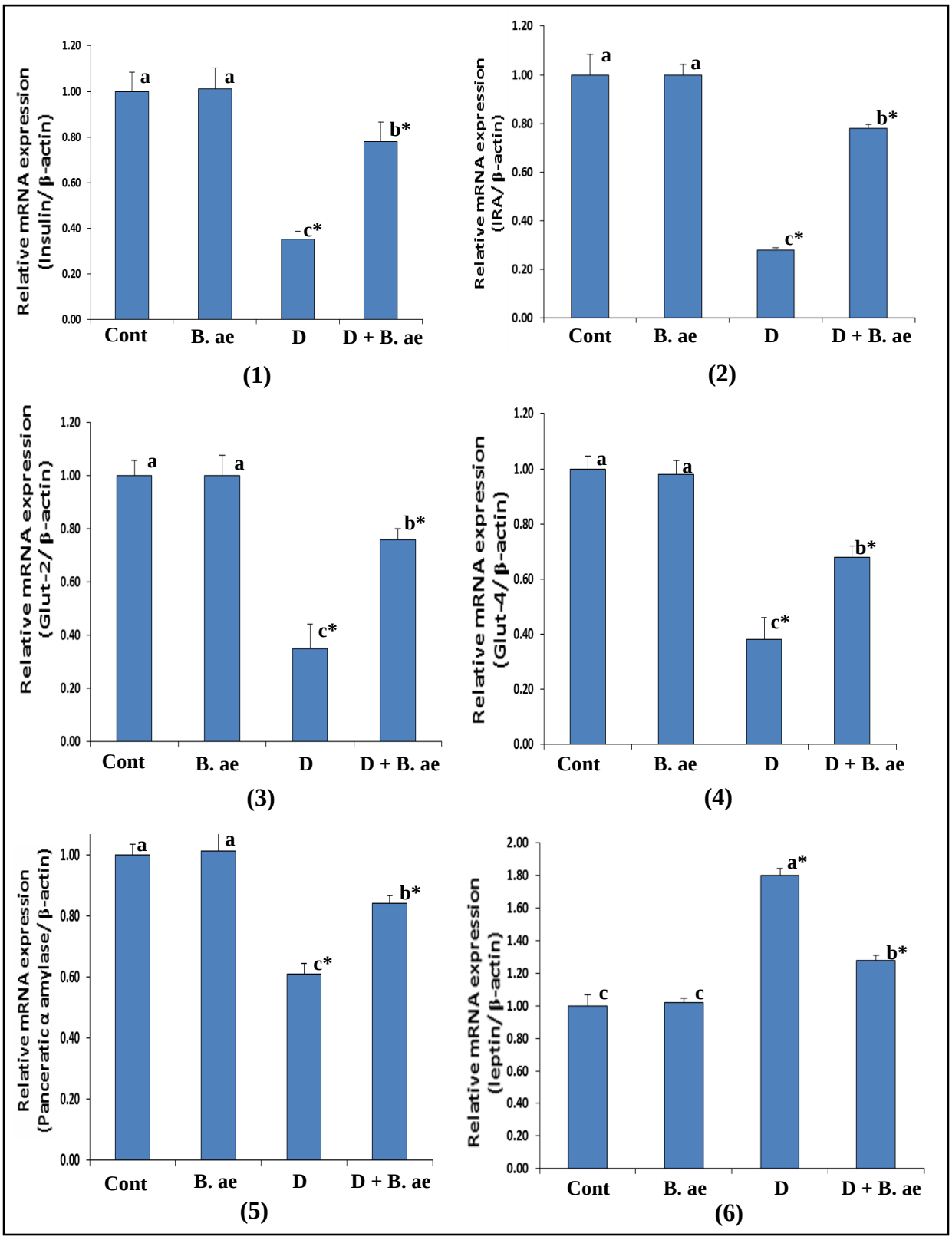

Figure 1: Effect of *B.aegptiaca* fruits extract (B.ae) (80 mg/kg BW) on the relative mRNA expression of insulin 1 (1), insulin receptor A (IRA) (2), glucose transporter 2 (GLUT2) (3), glucose transporter 4 (GLUT4) (4), α-amylase (5) and leptin (6) in STZ diabetic and non- diabetic rats. Cont = control=group 1, B.ae=group 2 treated with *B. aegyptiaca*, D= Diabetic= group 3, D+ B.ae =Diabetic rats treated with B.ae=group 4

Results

Induction of diabetes type 2 by STZ caused a significant reduction in the relative mRNA expression of pancreatic α-amylase and insulin; hepatic insulin receptor A (IRA) and GLUT-2; adipocyte GLUT-4; with significant increase of leptin hormone in comparison with the normal control rats. Molecular investigations in normoglycemic rats treated with *B. aegyptiaca* watery extract showed no significant differences with the normal control. In the opposite, STZ diabetic rats treated with *B. aegyptiaca* (group 4) produced a significant improvement of all relative gene expressions of α-amylase, insulin, IRA, GLUT-2 and GLUT-4 in comparison with diabetic non treated rats but they still significantly less than that of the control. Relative leptin mRNA expression significantly decreased under the effect of *B. aegyptiaca* treatment in comparison with diabetic non treated rats but it still higher than control level (Figure 1).

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Discussion

B. aegptiaca succeeds to induce hypoglycemic effect in the hyperglycemic diabetic rats but not in normo-glycemic ones that is a desirable feature as hyperglycemia was found to cause a cascade of adverse effects (16).

The observed hyperglycemia and hypo-insulinemia in STZ diabetic rats in the present study are consistent with several previous studies (17,18). STZ in a single low dose induced type 2 diabetes characterized by partial destruction of pancreatic β-cells (19) which induced hyperglycemia and leaved many of surviving β- cells can be regenerated (20). Enhancement of such regeneration is flourished by administration *Balanites* extract as it is rich in flavonoids (21). Flavonoid genistein (a soy derived isoflavone) was found to reduce β-cell apoptosis, preserve islet mass and promote islet β-cells survival (22). In support Helal et al. **(**23) reported that aqueous extract of *B. aegyptiaca* (seeds) was able to ameliorate beta-cell dysfunction in alloxan induced diabetic rats. Those stimulate insulin synthesis and release by the β-cells of the islet of Langerhans expressed as elevation of insulin gene expression and raising its level in the blood. Consequently, insulin decreased the elevated blood glucose level by 54%, as it accelerates the rate of glucose uptake from blood vessels into peripheral tissues (24). *Balanites* fruit extract and *Fenugreek* extract were able to reduce blood glucose level by 24% and 58% respectively in STZ-diabetic rats (5). That was through induction of the gene expression of insulin receptor (IRA) and glucose transporter (GLUT2) in hepatic cells. Both of them form a receptor-transporter complex on the rat hepatocyte membrane that forms a mechanism of insulin-mediated hepatic glucose regulation (25). Studies on GLUT2-null mice proved that GLUT2 orderly the function of central glucose sensors (26). The GLUT4 transporter is functionally characterized as an insulin-responsive glucose transporter. It was redistributed from an intracellular location to the plasma membrane following insulin stimulation (24). Glucose transporters; GLUT2 and GLUT4 play key roles in the control of blood glucose concentrations (27). Induction of these transporters with *Balanites* treatment impressive an anti-diabetic effect of *Balanites*.

The increment of insulin under the effect of *B. aegptiaca* fruits aqueous extract in our *in vivo* study ran in parallel with an *in vitro* study (28) which demonstrated that *B. aegyptiaca* stimulates insulin secretion and increases the number and affinity of insulin receptors in β-cells. Recently in 2016, *B. aegyptiaca* fruit aqueous extract was found to increase plasma insulin and liver

pyruvate kinase (L-PK) levels in STZ induced diabetic rats (29). L-PK responsible for increased liver glucose utilization and catalyzes the conversion of phosphoenolpyruvate to pyruvate, the last irreversible steps of glycolysis in the liver (30,31).

Inhibition of carbohydrate–hydrolyzing enzymes, α-glucosidase and α-amylase considers the first line therapies in diabetes treatment by reducing post-prandial glucose levels. Pancreatic α - amylase play of an important role for breakdown of α (1,4) glycosidic linkages of starch and other glucose polymers (32). The inhibition of α-amylase activity determines the anti-diabetic potency. Pancreatic α- amylase gene expression in our *in vivo* study behaves like insulin; it reduced in diabetic rats and induced under the effect of *B. aegyptiaca* fruit aqueous extract treatment. It may be because of 5'-flanking region of the pancreatic amylase gene that contains an insulin-dependent element mediates the loss of expression in diabetic animals (33). It was found that insulin administration *in vivo* has a stimulant effect on amylase activity in the pancreatic acinar cells of STZ diabetic rats, which confirm this relation (34).

In contrast in *in vitro* studies, it was found that *B. aegyptiaca* bark aqueous extract have α-amylase inhibitory activity between 45-75 % at 200 mg/ml concentration (35). And it was able to inhibit intestinal alpha-amylase activity in dose - dependent manner (5). Recently in 2018, Gawade and Farooqui (36) reported that the bioactive phytochemicals present in *B. aegyptiaca* leaves ethanol extract shows *in vitro* alpha amylase inhibition activity.

The observed gap between *in vivo* and *in vitro* studies revealed that the anti-diabetic effect of *Balanites* fruit, *in vivo*, is not dependent on pancreatic α-amylase gene expression. This gap raises an interesting question; whether the increase of pancreatic α-amylase gene expression in the treated animals was a compensatory response to α –amylase inhibition? To answer this question the enzyme activity should be estimated before that must be taken into account in the future. To date there is no experimental indication of pancreatic amylase enzyme and adipose leptin hormone gene expressions in *Balanites* treated diabetic rats. On the other hand, the differences of the used doses, the extracted different parts of the plant and the used methods of extraction may increase this gap.

Leptin is a polypeptide hormone releases from adipocytes. Its production is controlled by the ob/gene. Leptin play a role to control food suppressing and stimulates energy expenditure which lowers body weight (37,38). Hyperin-sulinemia increased serum leptin as they correlated to each other (39).

Hypoglycemic and insulinomimetic effect of the extract together with the elevation of leptin hormone in adipocytes, herein, and probably in serum (4) may be the cause of decreased serum TL and TAG. Consequently they improved lipid profile by decreasing TC, LDL-c and increasing HDL-c. The enhancement of lipase activity in the pancreatic acinar cells by insulin (34) may increase over mobilization of lipid from blood vessel to liver and decrease hepatic lipogenesis mechanism that result in decrease lipid mobilization to blood.

In harmony, anti hyperglycaemic and anti - hyper lipidemic activity of *B. aegyptiaca* were previously reported (4,40). Morsy et al. (6) suggest this fruit as a factor for diabetes and hyperlipidemia control.

Conclusion

This investigation provides new evidence about the possible molecular mechanisms of anti-diabetogenic effect of *B. aegyptiaca* fruit aqueous extract in STZ induced diabetic rats. The extract succeeds to reduce insulin demand as well as to stimulate endogenous insulin secretion and enhances its action at the target tissues. So it covered at least most of the main therapeutic strategies of diabetes treatment. Reduced blood lipid could be an additional benefit in diabetes management. The promising obtained result encourages the using of *B. aegyptiaca* fruit aqueous extract in traditional folk medicine for the management of type 2 diabetes and its related complications. Complementary studies are needed for

preparation of a standardized dose and dosage regimen of active constituents.

Conflict of interest

None of the authors have any conflict of interest to declare.

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