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Evaluation of Antidiabetic Potentiality of Truffles and Balanites Aegyptiaca among Streptozotocin Induced Diabetic Rats

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ABSTRACT

The potential application of Truffle (Terfeziaceae) and Desert Date tree (Balanites aegyptiaca) for modulating the diabetes mellitus related symptoms has gained much interest. However, less firm evidence has come from data to increase the understanding of the mechanism by which Truffle and Balanites protect pancreatic β-cells. The present study aimed to evaluate the effect of methanolic extract of Truffle and Balanites aegyptiaca on the fasting blood glucose (FBG) level; the changes in pancreatic histology as well as the changes in iNOS and IL-1β genes expression level among STZ induced diabetic rats which might help in better clarification of possible mechanisms beside the beneficial effects of the studied plants on diabetes. The elevation of the FBG level, pathological pancreatic changes and the level of both IL-1β and iNOS gene expressions in diabetic rats were observed in comparison with negative control rats. This increase was declined significantly due to the administration of Truffle and Balanites extracts. All of the studied parameters did not completely reverse to the normal levels as compared with negative control rats. The obtained results concluded that the beneficial effect of Truffle and Balanites aegyptiaca on STZ-induced diabetes was at least partly due to the reduction of IL-1β and iNOS gene over the expression which can have a protective effect on β cell.

Key words: *Diabetes, Rats, Truffles, Balanites Aegyptiaca, IL-1β, iNOS.*

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia caused by the defects in the secretion and/or action of insulin [1]. Worldwide DM has been considered as a major reason of morbidity and mortality as it leads to long term health complications and affect the quality of life [2, 3]. The numbers of diabetic persons have been expected to increase from one hundred and seventy one million in 2000 up to three hundred and sixty six million in 2030 [4]. DM has been the most challenging metabolic disorder as it cannot be cured but needs only to be managed [5]. In the medical sectors, side effects-free managements of diabetes have been still a big challenge.

The management of diabetes basically starts with a life style modification including types of diet and exercise. Nonetheless, most diabetic patients eventually require pharmacotherapeutic drugs, for example the injection of insulin and/or the administration of many other kinds of antidiabetic drugs. Many oral hypoglycemic synthetic drugs for DM treatments have been available. These synthetic drugs have been the mainstay of diabetic treatment and have been effective in controlling hyperglycemia but they have not been free from harmful side effects. The possible side effects may be hypoglycemia, hepatic toxicity, gaining weight, abdomen enlargement,

and gastrointestinal discomfort [6, 7]. Herbal medicines have provided rational means for the treatment of many diseases that are obstinate and incurable in other systems of medicine [8, 9]. Therefore, discovering natural, safe and effective oral antidiabetic drugs obtained from medicinal plants to manage DM without side effects has been of great interest, and has been greatly recommended by WHO [10].

Truffle (Terfeziaceae) is a fruiting part of subterranean fungi, most of them belong to the genus Tuber. Mushrooms are important sources of nutrients, and physiologic beneficial and safe medicines [11]; they have been used in traditional medicine all over the world for a long time. The mushroom has been characterized by having potential anti-inflammatory, hypoglycaemic and hypocholesterolemic nature because it has high percentage of acidic polysaccharides, dietary fibres, and antioxidants such as folate, ergothioneine, polyphenol and vitamins such as C, B12 and D [2]. Epidemiological studies revealed that the higher the levels of dietary fibre intake, the lower the dietary glycemic load, they also showed a potent hypocholesterolemic effects [12].

Desert Date tree (*Balanites aegyptiaca*) (Zygophyllaceae) has traditional values. The tree has been for food and fodder, as agro forestry tree and has had many considerable medicinal uses. The seed kernel contains high percentages of oils and proteins that varies among different sources. It has been known as 'desert date' and it has been widely distributed in the dry areas of South Asia and Africa. It contains many organic compounds such as lipids, proteins, carbohydrates, alkaloids, saponins, flavor-noids, and organic acids. Different extracts of *B. Aegyptiaca* (L.) Del have shown antidiabetic and hypoglycemic effects as reported by many studies done to prove and understand the possible mechanisms involved using different tree parts such as fruit extracts, the mesocarp of fruits, bark extract and the roots [13].

This study was aimed at evaluating the antidiabetic potential of Methanolic extracts of Truffle (Terfeziaceae) and *Balanites aegyptiaca* (Desert Date tree) in non-diabetic and streptozotocin (STZ) induced diabetic rats.

MATERIALS AND METHODS

Preparation of methanolic extract

Truffle and *Balanites aegyptiaca* plant materials were collected and dried from Arar, Northern Border, Kingdom of Saudi Arabia region. The dried parts of the plant were powdered, and ten grams of the powder were extracted with 200 ml of 95 % ethanol. The residue was filtered and concentrated to a dry mass by vacuum distillation, the obtained filtrate thus was used as plant extracts.

Experimental Animals:

Male albino rats with body weight of 130-160 g each were supplied from The Nile For Pharmaceuticals & Chemical Industries Cairo, Egypt. They were grown in the Animal House Lab., Medical Research Centre, Faculty of Medicine, Ain Shams University. The animals were housed in a room with controlled air temperature (25°C ± 2°C) and maintained in a light: dark (12: 12) cycle. The rats were provided with pellet diet *ad libitum* and had a free access to water. Prior to the experimentation, they were adapted for a period of two days.

Healthy males were randomly selected and divided into four groups (five rats each). The first group included non-diabetic control animals. The second group STZ induced diabetic rats as a positive control. The third group included diabetic animals treated with 200mg/kg BW of Truffle extract. The fourth one, included diabetic animals which received 200mg/kg BW of *Balanites* [14, 15].

Induction of Diabetes:

To induce diabetes, one week-acclimatized rats were subjected to overnight fasting, and a single intraperitoneal injection of freshly prepared Streptozotocin (STZ) (40mg/kg BW) in 0.1M citrate buffer (pH 4.5) was administered [1]. Diabetes was confirmed after 72 hrs of STZ injection using blood samples collected from rat tails, and fasting blood glucose level (FBG) was estimated via glucometer (On Call Plus Blood Glucose Monitoring System, ACON Laboratories, Inc. San Diego, USA) and compatible strip [16]. Diabetic rats were characterized by having FBG level exceeding above 160 mg / dl. FBG levels were measured at the diabetes induction phase until the stable hyperglycemia, usually a week after STZ administration.

Fasting blood sugar levels analysis

The FBG level of all rats in each experimental group was measured one time each fifteen days for one and half month study period.

Tissue sampling

Immediately after rat scarifying, pancreas was quickly removed and divided into two sections. The first section was stored in Trizol reagent for real-time gene expression analysis and stored in -80 °C. Second sectioned immerged in 15 % formaldehyde solution for pathological examination.

Pathological examination:

Formalin fixed specimens were routinely dehydrated in ascending series of alcohol, cleared in xylol, and finally embedded in paraffin. 4-5 µm-thick tissues were sectioned and processed for H&E staining [17]. Tissue slides were examined, and were compared to their corresponding controls.

Gene expression analysis via Real-Time PCR assay

All procedures were based on [18] with some modifications as follows, the quantity of pancreatic IL-1β and inducible nitric oxide synthase (iNOS) mRNA was quantitatively examined by real-time PCR. Total RNA was extracted from pancreas using Trizol reagent according to the manufacturer's instructions. After DNase treatment and normalization by spectrophotometric method, 1 µg of RNA was reversely transcribed to first-strand cDNA according to [19]. The obtained cDNA was amplified to examine the expressions of IL-1β and iNOS genes. An internal control GAPDH was used as a standard for the real-time PCR reaction. The sequences of the primers used were as follows: (1) IL-1β forward primer: 5- CAGAATCTATACCTGTCCTG- 3, reverse primer: 5-TGCAGACTCAAACTCCACT- 3 which yields a PCR product size of 129 bp; (2) iNOS: forward primer: 5-GGTGTTCTTTGCTTCTGTGCTAAT- 3, reverse primer: 5-CGTGTTTGCCTTATACTGTTCCA- 3; yielding a 157 bp size product and (3) GAPDH: forward primer: 5-GCAAGTTC GCAAGTTCAACGGCACAGTCAAG- 3, reverse primer: 5- GTACTCAGCACCAGCATCACC- 3 yielding a 153 bp size product. The PCR conditions were an initial denaturation for 5 min at 94 °C followed by 40 cycles with denaturation at 94 °C for 30 sec, annealing at 51 °C (IL-1β), 54 °C (iNOS) and 57 °C (GAPDH) for 40 sec and extension at 72 °C for 45 sec followed by a step of final extension at 72 °C for 5 min. The quantitative RT-PCR was performed by a real time PCR kit (Bioneer, Seoul, South Korea) using SYBR Green in a Mini OpticonTM thermocycler (Bio-Rad laboratories Inc., CA, USA). A negative control of DEPC water was used instead of the cDNA template. The SYBR Green RT-PCR assay was performed according to [20]. The results of IL-1β and iNOS mRNA expression were presented in relation to the expression of GAPDH.

Statistical analysis

The obtained data were analyzed by One Way ANOVA using SPSS Version 20 software and expressed as mean ± SEM. The differences between means were considered significant when $P < 0.05$ (LSD post hoc test).

RESULTS

Blood Glucose Level

STZ-induced diabetic rats revealed significant ($P \leq 0.01$) elevation of FBG level in comparison with negative control rats. In addition, the STZ diabetic rats after treatment with Truffle as well as *Balanites* showed significant decreases ($P \leq 0.01$) of FBG level (Table 1 and Fig. 1). The difference between the effects of two plants on the FBG level was significant ($P \leq 0.05$). However, the effect through the three trials of the same group was insignificant.

Table 1: Effect of *Balanites* and Truffle extract treatments on Fasting blood glucose levels (mg/dl) in diabetic rats.

Treatment	Mean± SE.
Negative control	79.88 ± 1.37
STZ (Diabetic)	216. 80** ± 6.90
Diab+Bala	127. 80** ± 3.01
Diab+Tru	113. 80** ± 2.49

** High significant ($P \leq 0.01$)

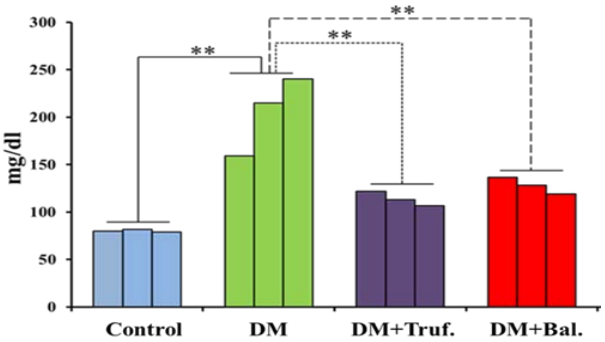

Figure 1: Variations in the FBG levels in non-diabetic control rats, STZ diabetic rats (DM), diabetic-rats received oral Truffles extract (DM+Truf) extract and diabetic-rats received oral *Balanites aegyptiaca* (DM+Bal) for three times with time interval= 15 days. (** = p <0.01).

Pathological examination

Microscopic examination of pancreatic sections of negative control rats showed normal appearance of histological structure of the islets of Langerhans (the endocrine part) and pancreatic acini (exocrine part) (Fig. 2). The examination of the various sections of the pancreas of diabetic rats (streptozocin administrated rats) positive control showed marked swelling and vacuolar degeneration of the islets' cells with many necrotic cells

appeared with pyknotic nuclei (Fig. 3). Most of the islets of Langerhans showed many empty spaces devoid of cells. Necrosis was prominent among the islets' cells, some of these necrotic cells appeared without nuclear structure or with pyknotic nuclei (Fig. 4). The pancreatic acini showed severe wide spread vacuolar degenerative appearance and necrosis of their lining cells, with obvious nuclear pyknosis of the necrotic cells or complete loss of the nucleus in other necrotic cells (Fig. 5). While the examination of various pancreatic sections of the diabetic rats that were treated with Truffle and *Balanites* extracts revealed that, the Truffle extract had more restorative effect on both islets 'cells and acinar cells than that achieved by *Balanites* extract. The pancreas of diabetic rats that were treated with Truffle extract showed good restoration of the pancreatic islets' cells with some scattered granular and vacuolar degeneration and few necrotic cells with either pyknotic nuclei or appeared eosinophilic structureless without any nuclei (Fig. 6). The acinar cells showed vacuolar degeneration and necrosis. The islets in most parts were restored in size and cells with few scattered degenerated and necrotic cells (Fig. 7). The examination of the pancreas of diabetic rats that were treated with *Balanites* extract revealed a moderate degree of the amelioration of the islet's cells, and some necrotic cells were observed, that many of which appeared dark with pyknotic nuclei (Fig. 8). The islets showed empty spaces devoid of cells. The pancreatic acini showed scattered degeneration and necrosis of the acinar cells (Fig 9).

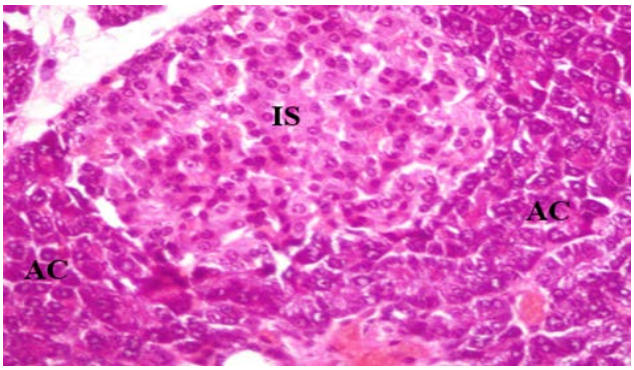

Figure 2: Pancreas of non-treated group (as negative control diabetic rats) showing normal histological appearance of the islets cells (IS) and pancreatic acinar cells (AC). **(H&E, X400).**

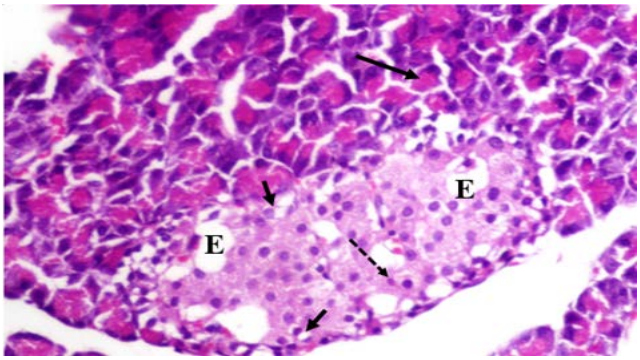

Figure 3: Pancreas of STZ-treated group (as positive control diabetic rat (DM)) showing marked swelling and vacuolar degeneration of the islets' cells, (short arrow), necrotic cells with pyknotic nuclei (dashed arrow) and many empty spaces (E). Notice the degeneration and individualization of the acinar cells (long arrow). **(H&E, X400).**

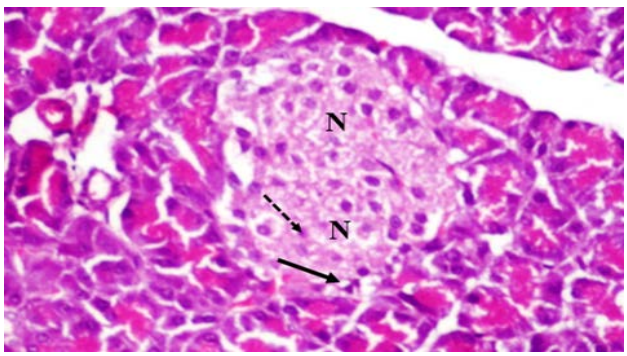

Figure 4: Pancreas of positive control diabetic rat (DM) showing marked necrosis of the islets cells (N), most of them appeared without nuclear structure or with pyknotic nuclei (dashed arrow) and others showed vacuolation of their cytoplasm (arrow). **(H&E, X400)**.

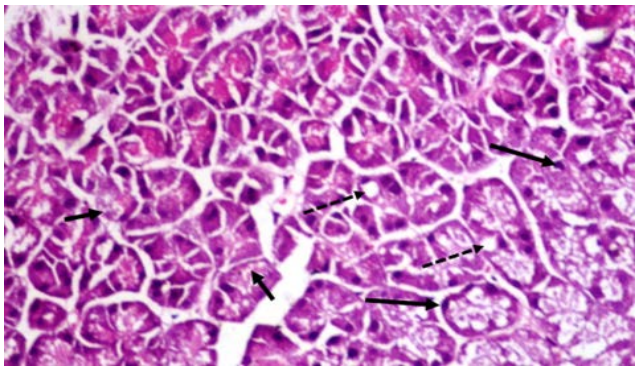

Figure 5: Pancreas of positive control diabetic rat (DM) showing severe wide spread vacuolar degeneration (dashed arrow) and necrosis of the acinar cells. Notice the pyknotic nuclei (long arrow) or absence of nuclei (short arrow) of the necrotic cells. **(H&E, X400).**

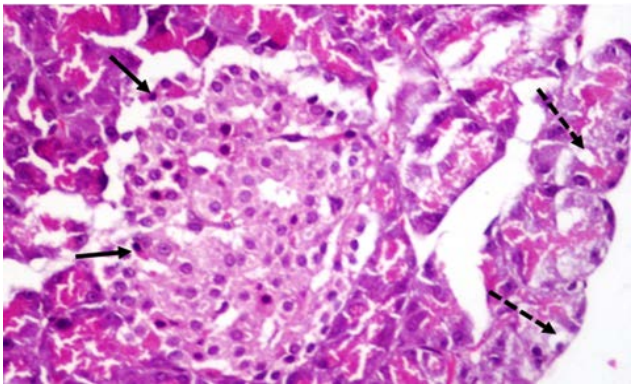

Figure 6: Pancreas of Truffles-treated diabetic rats (DM+Truf) showing good restoration of the pancreatic islets cells with some degenerated cells and few necrotic cells with pyknotic nuclei (arrow). Notice some of the acinar cells still showing vacuolar degeneration (dashed arrow) and necrosis **(H&E, X400)**.

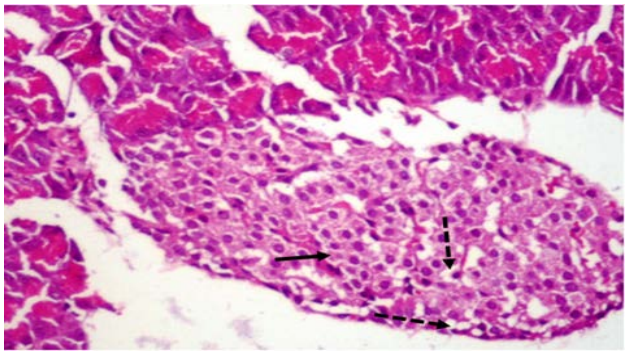

Figure 7 Pancreas of Truffles-treated diabetic rats (DM+Truf) showing restoration of the islets size and cells with only scattered necrotic cells (arrow) and few ones with vacuolar degeneration (dashed arrow) **(H&E, X400)**.

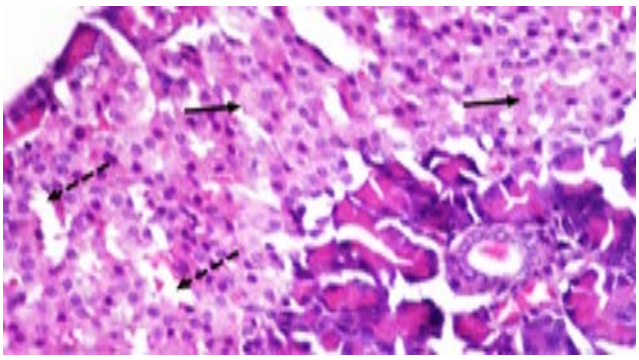

Figure 8: Pancreas of Balanites aegyptiaca-treated diabetic rats (DM+Bal) showing moderate degree of amelioration of the islets cell. Notice necrotic cells (arrow), many of which are dark with pyknotic nuclei and empty spaces (dashed arrow) (H&E, X400).

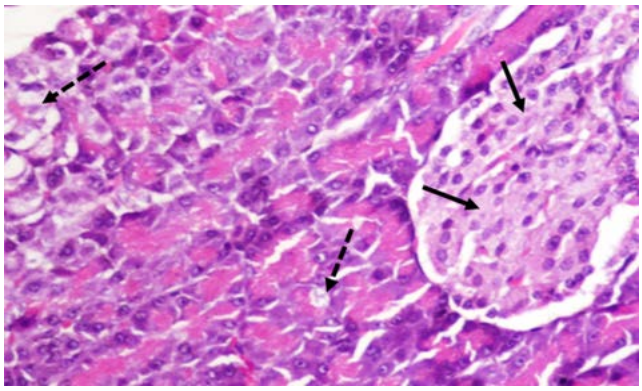

Figure 9: Pancreas of *Balanites aegyptiaca*-treated diabetic rats (DM+Bal) showing moderate degree of restoration of the islets' cells. Notice the scattered necrotic cells (arrow) as well as the degeneration and necrosis of the acinar cells (dashed arrow) **(H&E, X400)**.

Gene expression analysis

The expression level of mRNA of iNOS and IL-1β genes was assessed by real-time PCR in the all studied groups (Fig. 10). The treatment with Truffle caused significant down-regulation ($P \leq 0.01$) in both iNOS and IL-1β genes expression (about 92 and 73 percent, respectively) in comparison with STZ-induced diabetic rats as a positive control group. In the same direction, treatment with *Balanites aegyptiaca* extract reduced the gene expression percentages in both iNOS and IL-1β (about 66 and 56 percent, respectively). However, the decline in the IL-β and iNOS expressions after the treatment with the two extracts did not touch the values of the negative control.

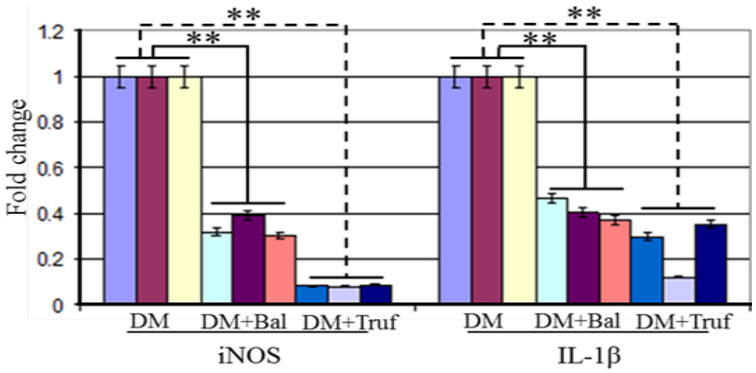

Figure 10: Variations in the (iNOS and IL-1β) genes expression level in STZ diabetic rats, diabetic-rats received oral *Balanites aegyptiaca* and Truffles extracts. (** = P<0.01).

DISCUSSION

Streptozotocin (STZ; N-nitro derivative of glucosamine) is commonly used to induce diabetes in the experimental animals [18, 21]. It destroys islet cells through several mechanisms, including production of reactive oxygen species (ROS) [22], activation of pancreatic NF-kB [23] and induction of pronounced immune and inflammatory responses [24]. It was postulated that the production of excess of free radical NO under the influence of STZ may play crucial role in the destruction of the β-cell during the development of diabetes mellitus [25].

It was found that STZ has a cytotoxic chemical effect on β-cell of the islets of Langerhans which is responsible of producing insulin in mammalian pancreas [26-28]. STZ injection results in the degeneration of β-cells [27, 29], it also causes death of the pancreatic β-cells, thus reduces the population of these cells. The effect of STZ on β-cells leads to the enhancement and development of the insufficient production of insulin and consequently, the increase of blood glucose level [30].

A lot of results from different research disciplines on medicinal plants which were accumulated from evidences confirmed that, there are abundant medicinal plants with antidiabetic and antioxidative characteristics [31]. These studies suggested that, these plants may protect tissues against deleterious undesired effects of free radicals which can lead to cytokine activation. Different groups of Truffles and mushrooms showed antidiabetic and antioxidant activities based on their high polyphenolic and ergosterol biological provitamin D2 contents [32-34]. The modulation of the oxidative stress and enhancement of antioxidant activity as well as antidiabetic potentiality of *Balanites* was reported [35, 36].

In diabetes caused by autoimmune disorders, IL-1β may has a role in β-cell destruction by stimulating the induction of iNOS that triggers the overproduction of NO free radical. The role of IL-1β in both Type1 and Type2 of DM has been established before [37, 38]. The suppression of IL-1β synthesis or inhibition of its interaction with corresponding cellular receptors significantly downregulated IL-1β mediated deleterious effects on β-cells [39, 40] and direct blockage of IL-1β has been observed as a therapeutic strategy for T1D at the preclinical level [41]. In vitro observations suggested that the cytotoxic effects of IL-1β on islet cells included the induction of iNOS and the production of NO [42, 43].

The present study evaluated the effect of methanolic extract of Truffle and *Balanites aegyptiaca* on the FBG level; the changes in pancreatic histology as well as the changes in iNOS and IL-1β gene expression level among STZ-induced diabetic rats which might help in better clarification of possible mechanisms beside the beneficial effect of the studied plants on diabetes. The obtained data showed that, the elevated level of both IL-1β and iNOS gene expression declined appreciably due to the administration of Truffle and *Balanites* extracts; however the treatments did not completely restore their normal values of expressions if compared with the negative control rats. Thus, this result might explain the other results of low FBG levels and restoration of the pancreatic tissue changes after the treatment with the plant extracts.

Truffle and *Balanites aegyptiaca* extract caused a significant decrease in IL-1β and iNOS genes mRNA expression, which can combat the cytotoxic effect of STZ on pancreatic β-cells that was reflected in lower FGB level, and the restoration of pancreatic histopathological parameters of diabetic rats that received the extract compared to the positive control diabetic group. It was suggested that, this advantageous effect might be due to, at least in part, the inhibitory effect of antioxidants presented in polyphenolic and ergosterol and vitamins contents of the studied plants extract on ROS production; immune and inflammatory response as well as NF-Kβ activation [34, 36, 44-48]. Several plant polyphenolic compounds have been shown to inhibit the expression of NF-Kβ- dependent cytokines, iNOS [49-51].

CONCLUSION

The present study concluded that, the methanolic extract of Truffle and *Balanites aegyptiaca* caused a significant decrease in IL-1β and iNOS genes mRNA expression, which can combat the cytotoxic effect of STZ on pancreatic β-cells that was reflected in lower FGB level and restoration of pancreatic histopathological parameters of diabetic rats that received the extract compared to the positive control diabetic group. Further studies are needed to evaluate the potentiality of each fraction of the studied plant's extracts separately in order to determine which chemical component was responsible for the obtained results.

CONFLICT OF INTERESTS

The authors proclaimed that there was no conflict of interests with any other works.

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