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Effects of *Ononis natrix* on glucose and lipid metabolism: An *in vivo* study

Mohammad A. AL-MTERIN [1], Nour ABOALHAIJA [2], Malek A. ZIHLIF [1], Fatma U. AFIFI [3,4*]

[1] Department of Pharmacology, School of Medicine, The University of Jordan, Amman, Jordan.
[2] Department of Pharmaceutical Sciences, Faculty of Pharmacy, Al-Zaytoonah University of Jordan, Amman, Jordan.
[3] Department of Pharmaceutical Sciences, School of Pharmacy, The University of Jordan, Amman, Jordan.
[4] Department of Pharmaceutical Chemistry and Pharmacognosy, Applied Science Private University, Amman, Jordan.
*Corresponding Author. Email: fatueafi@ju.edu.jo; Tel. +962795737352

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ABSTRACT: Several medicinal plants have been used historically and are claimed to be effective in either preventing or treating diabetes. This study aimed to evaluate the effect and the mechanism of *O. natrix* extract (ONE) as an antihyperglycemic and antihyperlipidemic agents in *in vivo* experiments. Lipid profile was analyzed using fully automated chemistry analyzer. Blood serum samples were used to measure high-density lipoprotein (HDL), triglycerides (TG), total cholesterol (TC) and Low-density lipoprotein (LDL). The expression levels of AMPK alpha-2 and Glut-4 receptors in diabetic rats were investigated using Western blotting. Oral starch tolerance test (OSTT) and oral glucose tolerance test (OGTT) were determined for the plant extracts at three concentrations on Wistar rats. Acarbose or metformin and glipizide were used as positive controls. Blood glucose levels were measured at −30, 0, 45, 90 and 135 min.ONE (250mg/kg and 125 mg/kg), administered before or after induction of diabetes using streptozotocin (STZ), significantly (p<0.05) reduced the blood glucose level by applying preventive and treatment protocols. The expression levels of Glut-4 receptors were significantly increased in rats given ONE (250 mg and 125 mg/kg) compared to the diabetic rats after 8 days of treatment. ONE (250 mg/kg) enhanced significantly (p<0.05) starch tolerance area under the curve (AUC) and glucose tolerance AUC. *O. natrix* extracts can activate the Glut-4 receptor, and enhance the glucose and starch tolerance in experimental rats. Hence, this widely distributed species in Jordan can be considered as a potential candidate for management of diabetes.

KEYWORDS: *O. natrix*; diabetes mellitus; OSTT; OGTT; Glut-4.

1. INTRODUCTION

Diabetes mellitus (DM) is a chronic disorder characterized by impaired production and/or unresponsiveness to insulin, leading to both, short- and long-term serious complications [1]. It is one of the most common endocrine chronic diseases worldwide [2]. However, both, genetic and environmental factors contribute to the disease progression and consequent hyperglycemia [3]. World Health Organization (WHO) stated that more than 400 million people worldwide have diabetes, and 1.5 million deaths directly attributed to diabetes each year [4].

The majority of diabetes sufferers live in low-and middle-income countries [4]. The prevalence of DM in the Middle East and in the North African countries has the second highest rate of increase globally. The number of people with DM in these regions of world expected to increase by 96.2% in 2035 [5]. Historically and still, the use of medicinal plants is very well accepted in preventing or treating diabetes. Numerous studies reported that many herbs with their secondary metabolites having anti-oxidative activities have impact in managing diabetic complications, and oxidative stress related diseases [6-8]. Also in Jordan, the use of medicinal plants in the treatment of many diseases, including DM is widespread, especially by the local inhabitants of the rural areas [9,10].

The species *Ononis natrix* L. belongs to the family Fabaceae. This family has about 20.000 species of trees, shrubs, vines, and herbs distributed worldwide [11]. In Jordan, it is represented by 45 genera, whereas the genus *Ononis* is represented by 14 species [12]. *O. natrix* is one of the most widely distributed species of this genus in Jordan. It is found on roadsides and waste places throughout in Jordan [11,12]. *O. natrix* is reported to have antibacterial, antihypertensive and antirheumatic properties [13-15]. In different folk medicines, *Ononis* species have been also used for healing of wounds, eczema, and rheumatic complaints, and are used also as an antiseptic and antimicrobial agent [16]. Hudaib et al. (2008) have reported that *O.*

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natrix is still in use in traditional practice of the inhabitants in Mujib area, especially to treat constipation and fever and Al-Mubideen et al. (2021) the hypoglycemic effect of *O. natrix* [17,18].

Recently, we reported the identification of several flavonoids in the aqueous and ethanol extracts of *O. natrix* leaves by LC-MS[11]. In continuation of the phytochemical evaluation of *O. natrix*, the present study was designed to evaluate the antidiabetic activity of *O. natrix* water extract before and after induction of diabetes according to preventive and treatment protocols using streptozotocin (STZ) to induce diabetes. The observed activity was compared to the accepted antidiabetic agent Metformin. Additionally, emphasis was given to the effect of the aqueous extract of *O. natrix* L. on glucose metabolism and on lipid profile by *in vivo* experiments. The challenging question was if *O. natrix* extract will reduce glucose and lipid levels and increase insulin sensitivity through glucose metabolism cell signaling pathway.

2. RESULTS

2.1. Effect of *O. natrix* extracts on glucose level before induction of diabetes (preventive treatment)

Significant reductions in fasting glucose levels in the metformin, ONE250, and ONE125 treated groups compared to their initial glucose levels after 7 (p<0.005, p<0.005, p<0.005, respectively) and 14 days (p< 0.05, p< 0.005, p< 0.005, respectively) were detected (Figures 1B, C and D).

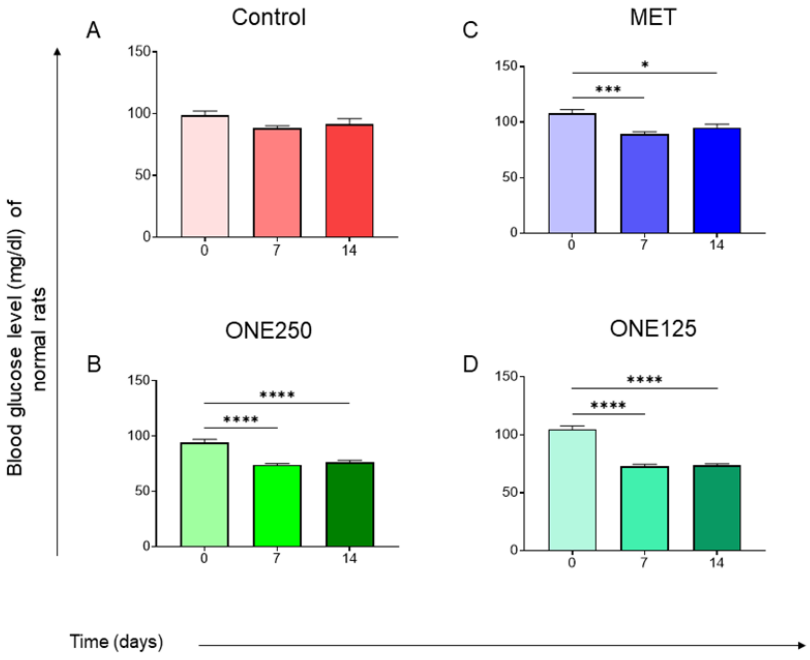

Figure 1. Effects of ONE and Metformin on the plasma glucose levels of normal rats at 0, 7 and 14 days of the experiment. Bar plots show the blood glucose level of normal rats negative control (**A**), 250 mg/kg (**B**), MET 100 mg/kg (**C**), and 125 mg/kg (**D**) Data are presented as mean ± SED. p<0.05 was considered significant *p<0.05, **p<0.05, *** p<0.005 compared to control group.

2.2. Effect of *O. natrix* extracts on glucose level of diabetic rats

None of the treated groups (MET, ONE125 and ONE250) exhibit any difference compared to their initial glucose levels at day 4 of the experiment. After 8 days, there was a significant reduction in glucose level in MET, ONE250, and ONE125 groups compared to their initial glucose levels (p<0.05, p<0.05, and p<0.05, respectively) (Figures 2B, C, and D).

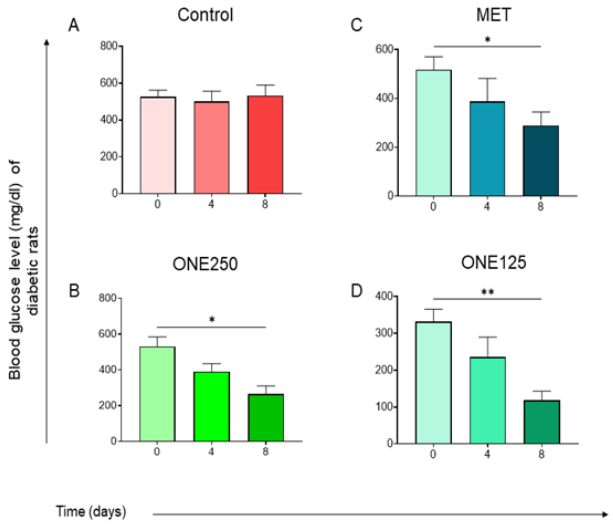

Figure 2. Effects of ONE and Metformin on the plasma glucose levels of diabetic rats at 0, 4 and 8 days of the experiment. Bar plots show the blood glucose level of diabetic rats Negative control (**A**), ONE250 mg/kg (**B**), MET 100 mg/kg (**C**), and ONE125 mg/kg (**D**). Data are presented as mean ± SED, $p<0.05$ was considered significant compared to their initial glucose levels. * $p<0.05$, ** $p<0.05$, *** $p<0.005$ compared to control group.

2.3. Effect of *O. natrix* extracts on protein expression

The expression levels of AMPK alpha-2 and Glut-4 receptors in diabetic rats were investigated using Western blotting. There were no significant differences in the AMPK alpha-2 levels among all treatment groups compared to the untreated group (DM group). The expression levels of Glut-4 were increased significantly in ONE250 and ONE125 groups compared to DM group after 8 days of the experiment ($p<0.05$, and $p< 0.005$, respectively) (Figures 3A, B, and C).

2.4. Oral starch tolerance test (OSTT)

In this experiment the possible effect of ONE on gastrointestinal carbohydrate hydrolyzing enzymes of normal rats was tested. The administration of acarbose (3 mg/kg B.Wt) enhanced significantly ($p< 0.005$) the starch tolerance AUC compared to control rats. Both ONEgroups decreased the starch induced hyperglycemia at 45, 90 and 135min. Rats treated with ONE125 exhibited a decrease in starch induced hyperglycemia only after 135 min post treatment. *O. natrix* in concentration of 250 mg/kg B.Wt enhanced markedly ($p< 0.05$) starch tolerance AUC as effective as acarbose (Figure 4).

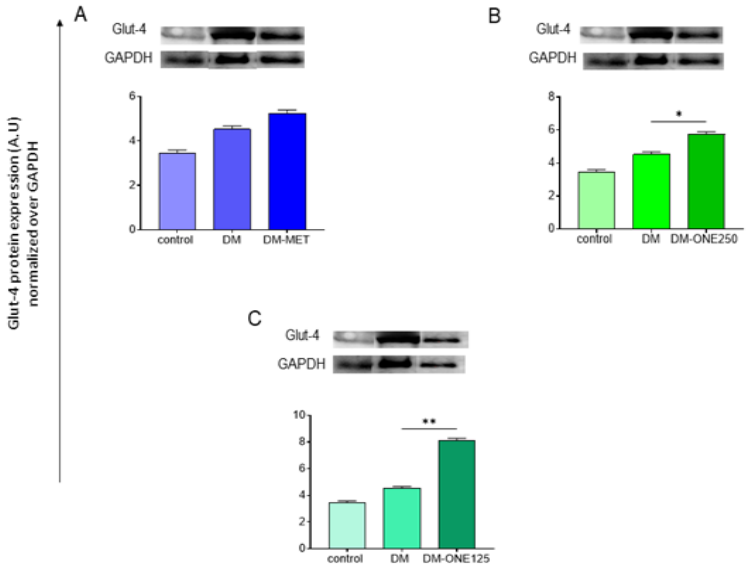

Figure 3. Effects of ONE and Metformin on the expression levels of Glut-4 protein of diabetic rats at 8 days of the experiment. Livers tissues prepared from control and treated rats were separated by SDS-gel electrophoresis and blotted for Glut-4. Bar plots show the expression level of Glu-4 in diabetic rats treated with MET 100 mg/kg (**A**), 250 mg/kg (**B**), and ONE125 mg/kg (**C**). Data in the bar graphs represent the mean ± SEM. * p<0.05, **p<0.05, *** p<0.005 compared to control group.

2.5. Oral glucose tolerance test (OGTT)

Treatment with glipizide (0.6 mg/kg B.Wt) reduced significantly (p<0.005) the overall glycemic diversion compared to the control group. At 45 min post glucose administration, ONE250 significantly (p<0.05) decreased the glucose-induced hyperglycemia compared to the control group (Figure 4). At 90 min all the treatment groups significantly decreased the glucose induced hyperglycemia with exemption of the ONE125 treated rats, while 135 min post ingestion, all the treatment groups significantly decreased the glucose-induced hyperglycemia. Among tested three different concentrations, ONE250 enhanced markedly glucose tolerance AUC similar to MET (Figure 5).

2.6. Effect of *O. natrix* extracts on the lipid profile in normal and diabetic rats

The effect of *O. natrix* extract on the levels of HDL, TG, TC, and LDL-C in normal and diabetic rats was investigated. There were no significant changes in the lipid profiles of the normal rat after receiving ONE and metformin for 14 days. Also lipid profile of the diabetic rats did not show any significant changes after treatment for 8 days with ONE. Earlier study demonstrated that reductions in TC and LDL-C appeared after 14 days, and reduction in TG, after 28 days of treatment with metformin [19,20]. The short duration of the treatment in the present study might be the reason why the lipid profile was not affected.

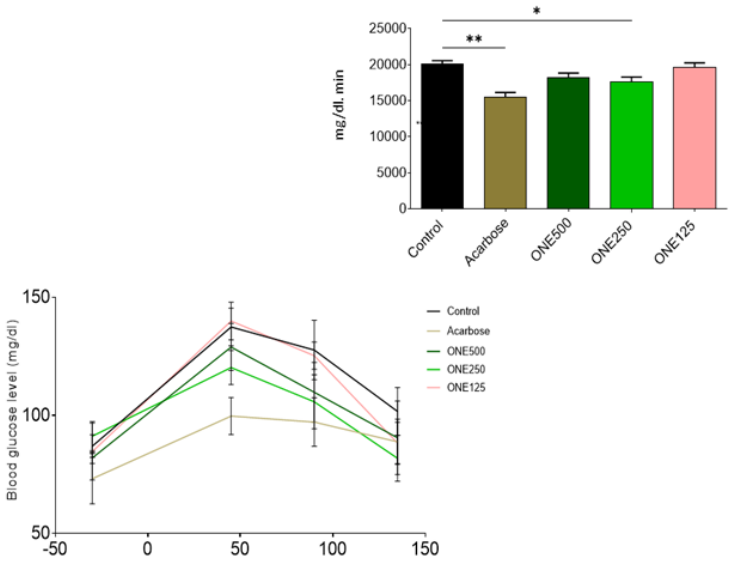

Figure 4. OSTT and AUC of normal rats treated with *O. natrix* aqueous extracts (500 mg, 250mg, and 125 mg/kg B.Wt), and acarbose (3 mg/kg B.Wt). Data are shown as mean ± SEM * p<0.05, **p<0.05, *** p<0.005 compared to control group.

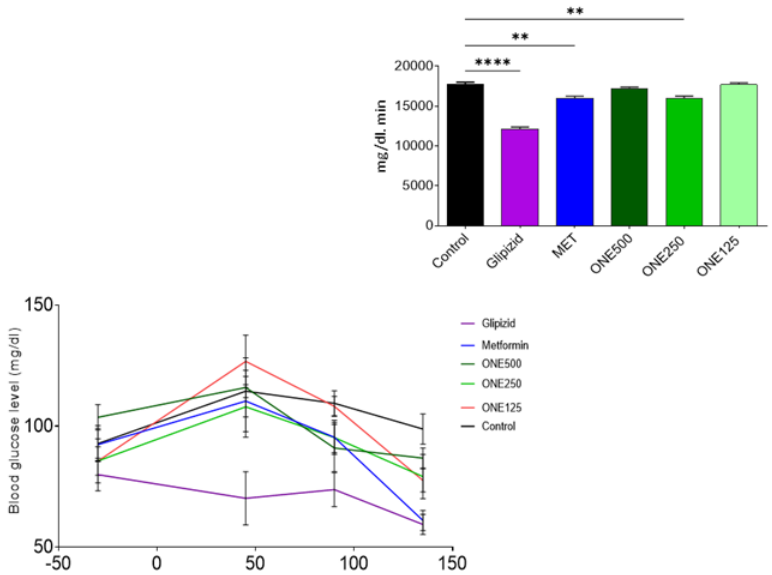

Figure 5. OGTT and AUC of normal rats treated with *O. natrix* aqueous extracts (500 mg, 250mg, and 125 mg/kg B.Wt), metformin (300 mg/kg B.Wt), and glipizide (0.6 mg/kg B.Wt) . Data are shown as mean ± SEM. * p<0.05, **p<0.05, *** p<0.005 compared to control group.

3. DISCUSSION

Throughout the world, for the prevention and treatment of diabetes, the use of medicinal plants in the traditional medicine of several countries is well accepted and is widely reported [21-23]. Under diabetic conditions oxidative stress is increased that can induce tissue damage in patients with diabetes [24].*Ononis* species are reported to have a high content of flavonoids, known for their antioxidant potential [11,21,25]. In our previous study, high concentrations of phenolic substances and flavonoids were detected in the water extract of *O. natrix* with quercetin, luteolin and apigeninas major flavonoids in addition to isoorientin, rutin, hesperidin, hyperoside and kaempferol. The presence of phenolic acids, such as chlorogenic-, ferulic- and ellagic acids was also revealed [11].This high concentration of polyphenolic compounds, including flavonoids in ONE can be linked to the hypoglycemic potential of this extract.

Earlier researchers have reported anti-hyperglycemic effects for these flavonoids [26-28]. Zang et al. (2016) demonstrated the anti-diabetic effects of luteolin and luteolin-7-O-glucoside on KK-A(y) mice [29]. As a potent antioxidant, apigenin can stimulate the metabolism of glucose and transportation of it in the peripheral tissues; as well as enhances secretion of insulin from pancreas [30]. The two well studied flavonoids detected in *O. natrix*, namely rutin and quercetin work as antihyperglycemic agents by decreasing carbohydrates absorption from the small intestine, and stimulation of insulin secretion as well as protecting pancreatic β-cells against degeneration [26,28]. Vessal et al., (2003) reported that quercetin significantly increases the pancreatic islets cells in normal and diabetic rats after receiving intraperitoneal (i.p). injection of 10 and 15 mg/kg once daily for 10 days [31]. Also, it has antioxidant activity which in turn is responsible for protection of pancreatic islets against free radicals [31]. Rutin has antioxidant and anti-diabetic activities. Kamalakkanna et al., (2006) reported that administration of rutin (100 mg/kg) for 45 days, decreased fasting plasma glucose level, increased insulin levels and increased the enzymic and nonenzymic antioxidants of diabetic rats [32]. In the present study, there was a significant decrease in glucose level in diabetic rats that received ONE compared to their blood glucose level on day 0.

In the preventive study, both extracts of *O. natrix* (ONE125 and ONE250) showed their antidiabetic activities only 7 days after administration while studies with other plants with reported antidiabetic activities (*Murraya koenigii* (L), *Olea europaea* L. and *Chamaemelum nobile* L.) exerted hypoglycemic effect after 15-day treatment [33,34]. Earlier studies demonstrated that metformin may need longer duration of administration to exert hypoglycemic activity [35,36].

Our result have shown that the Glut-4 expressions were increased in ONE groups that received 250mg and 125mg/kg B.Wt compared to the control group. The presence of flavonoids may increase the activity and translocation of Glut-4 [37,38]. In addition, quercetin leads to an increase in glucose uptake in skeletal muscle cells by promoting Glut-4 translocation [39]. Moreover, rutin has been confirmed to regulate Glut-4 translocation in adipocytes and skeletal muscle cells by stimulating Akt synthesis and phosphorylation [40]. Ding et al., (2010) reported that the levels of Glut-4 gene expression were significantly increased in adipose cells that were treated with luteolin (20 μmol/L) for 24h [41].

The digestion of carbohydrates in the intestinal lumen is a complex process that starts by converting the complex polysaccharides into simpler polysaccharides and disaccharides. This is catalyzed by the α-amylase isozyme, which is synthesized and secreted by pancreatic exocrine cells into the intestinal lumen [42]. Therefore, inhibitors of α-amylase and α-glucosidases can be used to treat postprandial hyperglycemia [42,43]. Acarbose, an inhibitor of both, α-amylase and α-glucosidases, can delay the degradation of complex carbohydrates to absorbable monosaccharides [44]. However, acarbose is associated with gastrointestinal adverse effects such as flatulence, abdominal distension, and diarrhea, as a result of fermentation of unabsorbed carbohydrates and augments the hypoglycemic effects of sulfonylureas or insulin [45]. Interestingly, *O.natrix* has been shown to have an inhibitory effect on α-amylase and α-glucosidase enzymes in *in vitro* [46]. As seen by the results of OGTT, *O.natrix* can be considered as a hypoglycemic agent. As effectively as glipizide and metformin, a dose of 250 mg/kg B.Wt. of *O.natrix* has markedly enhanced glucose tolerance AUC. In the case of OSTT, *O.natrix* 500 and 250 mg/kg B.Wt significantly decreased the starch induced hyperglycemia. Interestingly, phenolic compounds have the capacity to bind and change digestive enzymes such as α-amylase and α-glucosidases [47]. Also, apigenin can be responsible for the α-amylase and α-glucosidases inhibitory activities [46]. ONE250 exhibited similar efficacy as acarbose.

4. CONCLUSION

To the best of our knowledge, this is the first study to determine the mechanisms of hypoglycemic activity of ONE grown in Jordan. The aqueous extracts of *O. natrix*, in concentrations of 125 mg/kg B.Wt and 250 mg/kg B.Wt have the ability to reduce glucose levels in STZ-induced diabetic rats. Alteration in

Glut-4 and the inhibitory activity of the extracts on two enzymes (α-amylase and α -glucosidases) could be the possible mechanism of *O. natrix* as an antidiabetic agent. Still, further studies are needed with the crude extracts and with the isolated compounds of this species to justify the use of these plants' extracts as adjunct therapy by diabetic individuals in the future.

5. MATERIALS AND METHODS

5.1. Collection of the plant material and Extraction

Fresh plant samples were collected from the northern parts of Madaba governorate, Jordan during May, 2021 and authenticated in comparison with the herbarium specimen of the Department of Biology, School of Science, The University of Jordan as well as using descriptive references [48,49]. A voucher sample was kept in the Department of Pharmaceutical Sciences, School of Pharmacy, The University of Jordan (FMJ-FAB-32-6). Leaves were dried for one week at room temperature (RT) without exposure to direct sunshine until constant weight was obtained. Then, dried leaves were coarsely crushed. Extraction was performed by gentle heating of 10 g dried leaves in 100 mL of distilled water until boiling and kept soaked overnight. After filtration the volume was adjusted with distilled water to obtain 10 % (w/v) concentration for the experiment.

5.2. *In vivo* experiments

5.2.1. Selection of the animals

Sixty-four (male) Wister rats were used in this experiment. The experiments were performed under the approval of the Institutional Review Board (IRB) of the University of Jordan (Approval Number: 16-2019). The experiments were done in the Animal House at the University of Jordan.Animals were housed in colony cages (four rats per cage) at a temperature of 24 ± 2 ^{o}C with 12-h-light/12-h-dark cycle with a converter air system and were given standard rat chow dietand water *ad libitum*. Cage bedding was changed daily following the induction of diabetes. They were housed under standard environmental conditions until treatment or sacrifice. All selected rats were normoglycemic having fasting blood glucose levels (85±5mg/dL). Their initial body weight averaged (200g±30g). Rats were kept for one week to acclimatize to the animal room before starting the experiments.

5.3. Induction of diabetes

Diabetes was induced by a single intraperitoneal injection of streptozotocin 65 mg/ kg (STZ, Sigma-Aldrich, St Louis, MO, USA) freshly dissolved in citrate buffer (trisodium citrate 0.1M and citric acid monohydrate 0.1M) at a pH of 4.5 [50]. Rats were slightly anaesthetized with diethyl ether before the injection. After injection, 10% sucrose solution was added to their drinking water to prevent the hypoglycemic shock that might occur overnight. Glucometer (Accu-Check® Performa; Roche Diagnostics GmbH, Mannhein, Germany) was used to measure the blood glucose level. Animals showing a fasting blood glucose level 250 mg/dL and more were considered diabetic and selected for the experiments [51].

5.4. Experimental design

The animals were divided randomly into eight groups (four groups to test the preventive potential and four groups to test the antidiabetic activity) as follows:
- Group I: untreated control group; neither streptozotocin (STZ) nor ONE/metformintreatment was used.,
- Group II: Negative control group, STZ is used to induce diabetes but no additional treatments are used.
- Group III: preventive group 1 low dose ONE125 (125 mg/kg) was given 2 weeks before and 1 week after STZ injection
- Group IV: preventive group 2 high dose ONE250 (250 mg/kg) was given 2 weeks before and 1 week after STZ injection.
- Group V: preventive MET group; the same as preventive groups 1 and 2 but 100 mg/kg metformin (Glucophage®) was given 2 weeks before and 1 week after STZ injection (positive control group for preventive study).
- Group VI: treatment group 1 low dose ONE125 was given only 1 week after STZ injection.
- Group VII: treatment group 2 high dose ONE250 was given only 1 week after STZ injection.
- Group VIII: Treatment MET group, the same as treatment groups 1 and 2 but metformin (Glucophage ®) (100 mg/kg) was given 1 week after STZ injection (positive control group for the treatment study).

Accordingly, groups III and IV evaluate the protective activity of ONE against STZ-induced diabetes, compared to metformin (group V) while groups VI and VII evaluate the hypoglycemic activity of the *O. natrix* extracts, compared to metformin (group VIII).

Glucose levels were measured daily using one drop of tail blood and checked by a glucose meter according to the manufacturer's instructions. At the end of the experiments, all rats were sacrificed, and 3 mL whole blood was collected from retro-orbital capillary vessels, centrifuged at 3000 rpm for 10 min to obtain serum, which was stored at -80 ºC for lipid profile analysis. Moreover, liver biopsies were obtained from each rat and stored at -80 ºC for protein analysis.

5.5. Protein sample preparation

Ripa lysis buffer (25mM Tris HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) was used in concentration 20:1 of the livers' tissue. The mixtures of tissue and lysate were homogenized thoroughly with an electric homogenizer and then they were kept on constant shaking for 2h at 4 ºC. After that, the samples were centrifuged for 20 min at 12000 rpm at 4 ºC in a microcentrifuge. Thenthe supernatant was aspirated, placed in a new tube and kept on ice. The DC Protein Assay (Bio-Rad Laboratories) was used for the determination of protein concentration. The samples absorbance was read at 750 nm on a microplate reader. Then, all samples were kept at -80 ºC until analysis.

Cell lysates were solubilized by using 4x denaturation buffer (10%SDS, 0.5M Tris/HCL pH 6.8, Glycerol and β-Mercaptoethanol). The proteins were separated by using SDS-PAGE with 12.5% polyacrylamide gels calculated according to the molecular weight of proteins of interest. The voltage used to separate the proteins was (100 V) for 2 h. Then, the transfer took place for 18 hr at 4 ºC. The membranes were blocked for 1 hr by using 5% skim milk which diluted by Tris-buffered Saline (TBS), pH= 8. Five percentage skim milk/Tris-buffered SalineTween-20 (TBST), pH=7.5 was used for primary antibodies dilution. Then the membranes were kept overnight at 4 ºC with continues shaking. The membranes were washed three times by TBST (10 min for each wash) and incubated with Horseradish Peroxidase (HRP) for 1 hr at RT. Finally, the membranes were washed three times by TBST and Enhanced Chemiluminescence (ECL) western blot substrate was used for detection. The detection was done by using Chemi doc XRS+Imaging System (Bio-Rad Laboratories).

5.6. Lipid profile test

Lipid profile was analyzed using fully automated chemistry analyzer Hitachi Cobas c311, under following conditions: Temperature: (18-22 ºC), Humidity: (53%). Blood serum samples were used to measure high-density lipoprotein (HDL), triglycerides (TG), and total cholesterol (TC). Low-density lipoprotein concentration (LDL-C) was calculated using The Martin/Hopkins Equation [52]. This formula estimates LDL-C using an adjustable factor for the TG: VLDL-C ratio.

LDL-C (mg/dL) = TC – (HDL)– (Triglycerides/adjustable factor).

5.7. Oral starch tolerance test (OSTT)

This experiment was carried out as described by Kasabri et al. (2011) [43]. Thirty male Wistar rats were used in this experiment (6 rats per group). OSTT was determined with ONE at doses 125, 250 and 500 mg/kg body weight (B.Wt). Acarbose was used at dose of 3 mg/kg B.Wt. Then, blood glucose levels were determined with 45 min intervals at 45, 90 and 135 min calculated from starch administration time.

5.8. Oral glucose tolerance test (OGTT)

The OGTT experiment was designed and performed as the experiments for OSTT except for the replacement of starch by Dglucose monohydrate (3 g/kg B.Wt.) and the drug acarbose by two classical drugs used in the treatment of diabetes, namely metformin (300 mg/kg B.Wt) and glipizide (600µg/kg B.Wt). Thirty male rats were divided into 6 groups (n = 5 rats per group). Blood glucose levels were determined with 45 min intervals at 45, 90 and 135 min calculated from glucose administration time [43].

5.9. Statistical analysis

All the values are presented as mean ± SED. and were considered significantly different if $p< 0.05$. GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses. In grouped analyses, statistical significance was determined using a one-way ANOVA test as well as the nonparametric counterpart of ANOVA (Kruskal-Wallis test). On samples that passed the Shapiro-Wilk normality test, unpaired t-tests were used to compare groups of normally distributed data, while Mann-Whitney tests were used for samples that did not show normal distribution.

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AUTHORSHIP STATEMENT

Author contributions: Concept – M.Z.; F.A.; Design – M.A., N.A., M.Z., F.A.; Supervision – M.Z., F.A.; Materials – M.A., M.Z.; Data Collection &/or Processing - M.A., N.A.; Analysis &/or Interpretation - M.A., N.A.; Literature Search – M.A., N.A., F.A.; Writing – M.A.; Critical Reviews – M.A., N.A., F.A.

DISCOSURE STATEMENT

The authors declared no conflict of interest.

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