

Evaluation of Hypoglycemic Effect of *Achillea biebersteinii* Afan., Growing in Syria, in Induced Diabetic Rats

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ABSTRACT

Supported by many studies and botanical surveys, the potential role of medicinal plants as antidiabetic agents has dramatically raised. The present study was designed to investigate the hypoglycemic effect of *A. biebersteinii* Afan. ethanolic extract in both types of diabetes, using blood glucose and insulin levels as markers for its efficacy, morphological changes of pancreatic β -cell islets as a potential marker for a protective role, and high fat diet-streptozocin (HFD-STZ) induced diabetic rats and STZ-induced diabetic rats as diabetic models. Aerial part ethanolic extract was administered by oral gavage for 16 days at a dose of 400 mg/kg. Glibenclamide at a dose of 3 mg/kg, and metformin at a dose of 300 mg/kg were used as reference standards. *A. biebersteinii* Afan. reduced significantly fasting blood glucose (FBG) levels, improved oral glucose tolerance, tended to raise serum insulin levels, enhanced regeneration of β cells, and seemed to be more effective than the reference standards. It is assumed that *A. biebersteinii* Afan. increases utilization of glucose by tissues and/or enhances insulin release from remanent and/or regenerated β -cells. In conclusion, *A. biebersteinii* Afan. possesses potent antihyperglycemic activity and it may prove to be effective for the treatment of both types of diabetes.

Keywords: *Achillea biebersteinii* Afan., high fat diet-streptozocin, oral glucose tolerance test (OGTT), fasting blood glucose (FBG).

INTRODUCTION

Diabetes mellitus is a metabolic disease, characterized by hyperglycemia resulting from defect in insulin secretion, and reduced sensitivity to its actions¹. These abnormalities of insulin secretion and its actions also lead to impaired lipid and protein metabolism and increased risk of macrovascular and microvascular diseases^{2,3}. In 2012 there were 1.5 million deaths worldwide caused by diabetes. It was the eighth leading cause of death among both sexes and the fifth leading cause of death in women in 2012⁴. Current approved prescribed treatments are concerned with a day to day control of blood glucose levels, and haven't shown to modify the course of diabetic complications. Furthermore, they produce undesirable side effects which may increase cardiovascular risks^{5,6}. Therefore, it is of great urgency to find a novel therapy and prevention for diabetes.

The plant kingdom is a wide and promising field to look for new hypoglycaemic agents which can be successfully employed to treat diabetes. The potential role of medicinal plants as antidiabetic agents has dramatically raised, supported by many studies and botanical surveys^{7,8}. Hypoglycemic effect of some *Achillea* species had been established by animal experimentation (*A. santolina*)⁹ or by α amylase inhibition *in vitro* experimentation (*A. ligustica*)¹⁰. *Achillea biebersteinii* Afan., which grows wild in different areas of the world has been used in Syrian

folk medicine to treat abdominal pain, wounds and stomachache. It has antioxidant activities¹¹ and recently, it has been reported as an antihyperlipidemic agent¹², and a potent α amylase inhibitor¹³.

A. biebersteinii Afan. belongs to Asteracea family. It is a perennial herb with villous erect stems. Leaves are oblong-lanceolate in outline and Pinnatilobate. Heads are radiate and corymbose. Flowering occurs during April and May. It grows on clay soils¹⁴.

This study was aimed to investigate the hypoglycemic effect of *A. biebersteinii* Afan. in both types of diabetes, using its ethanolic aerial part extract, blood glucose and insulin levels as markers for its efficacy, and morphological changes of pancreatic β -cell islets as a potential marker for its protective role in high fat diet-streptozocin (HFD-STZ) induced diabetic rats and in STZ-induced diabetic rats.

MATERIALS AND METHODS

Chemicals and Reagents

Glibenclamide and metformin, of analytical grade, were kindly supplied by local pharmaceutical companies (Ibn Al Haytham Co., and Unipharma Co.). Streptozotocin (STZ) was purchased from Sigma-Aldrich Co. (UK). Ultra-sensitive mouse insulin ELISA kit was purchased from Crystal Chem Inc. (Downers Grove, USA). Cholesterol, 95% was purchased from Alfa Aesar (Karlsruhe,

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Germany). Casein was purchased from Acros Organics (Geel, Belgium). Glucose was purchased from Panreac (Barcelona, Spain). A glucometer (Glucolab™) was purchased from infopia21 Co. (Korea) with a maximum measuring capacity of 600 mg/dL. All other reagents and chemicals used in the study were of analytical grade. Deionized water was employed for preparing the reagents.

Animals

Eighteen male and nine female Wistar rats (200-300±22 g; 10 months old) were obtained from the Animal House Centre of faculty of pharmacy, Aleppo University. The animals were housed in standard polypropylene cages (three rats/cage) and maintained under controlled room temperature and humidity with a 12/12-hour light-dark cycle. The rats had free access to water and rodent chow, either standard rat chow or high fat diet (HFD).

Plant material and Preparation of ethanolic extract

The flowering aerial parts of *A. biebersteinii* Afan. were collected in May 2016, from Saraqip, Idlib, Syria, and identified by Pharmacognosy Department, Aleppo University, Syria. The plant sample (100 g of *A. biebersteinii* Afan.) was air-dried under shade, coarsely ground, then extracted with 1 liter ethanol (80%). The mixture was sonicated for 60 minutes at 30 °C in an ultrasound water bath (POWERSONIC 405, Hwashin Technology Co., Korea), then macerated overnight and filtered. The obtained residue was remacerated for two other days. The combined filtrates were dried, using a rotary evaporator (Laborota4000, Heidolph, Germany), under reduced pressure at 40°C to yield waxy residue (12.3 % w/w). Plant extract was reconstituted with deionized water.

Experiment protocol

Type I Diabetes

A freshly prepared solution of STZ (30 mg/kg), dissolved in 0.1 mM sodium citrate buffer (pH 4.4), was injected intraperitoneally to the overnight fasted male rats. The injected dose was repeated after 48 hours. The animals were allowed to drink 5% glucose solution overnight to overcome induced hypoglycaemia¹⁵. After one week of STZ administration, FBG levels were measured by using the glucometer strips. Animals with a severe hyperglycemia (FBG level > 300 mg/dL or above) were considered to be type I diabetic and were used in the study¹⁶.

The study was designed as follows:

Male rats were divided randomly into three different groups of six animals: control group (orally administered deionized water for 14 days), *Achillea* group (orally administered a daily single dose of 400 mg/kg for 14 days) and glibenclamide group (orally administered a daily single dose of 3 mg/kg for 14 days).

After overnight fasting (water allowed *ad libitum*), animals of each group were administered orally either deionized water, or extract, or glibenclamide via oral metallic cannula. Blood glucose levels were measured nearly every other day at 0, 30, 60, 120, and 180 minutes after administration by using the glucometer strips. Oral glucose tolerance test (OGTT) and serum insulin level measurements were done on days 15 and 16 of the

experiment, respectively. At the end of experiment, rats were sacrificed for histopathological study.

Type II diabetes

The female rats had been allocated into HFD dietary regimen (ingredients: 29% pure vegetable ghee (Lulu®, Arij Co., Oman) 12% protein (casein), 58% carbohydrate, and 1% cholesterol) for three months, then they were injected with freshly prepared solution of STZ at a dose of 35 mg/kg, dissolved in 0.1 mM sodium citrate buffer (pH 4.4). FBG levels were measured in the blood obtained from tail veins one week after STZ injection, using glucometer strips. Animals with hyperglycemia (FBG level > 200 mg/dL) were considered to be Type II diabetic^{17,18}.

The study was designed as follows:

The female rats were divided randomly into three different groups of three animals: control group (orally administered deionized water for continuous 14 days), *Achillea* group (orally administered a daily single dose of 400 mg/kg/day for 14 days) and metformin group (orally administered a daily single dose of 300 mg/kg for 14 days).

After overnight fasting (water allowed *ad libitum*), animals of each group were administered orally either deionized water, or extract, or metformin via oral metallic cannula. Blood glucose levels were measured nearly every other day at 0, 30, 60, 120, 180 minutes after administration by means of glucometer strips. OGTT and serum insulin level measurements were done on days 15 and 16 of the experiment, respectively. At the end of experiment, rats were sacrificed for histopathological study.

Oral glucose tolerance test (OGTT)

On day 15, after overnight fasting, all animals of both types of diabetes were administered either deionized water, or extract, or glibenclamide, or metformin by oral gavage at doses mentioned above. After 30 minutes, blood glucose levels (minute 0) were measured, followed by oral administration of a liquid glucose meal (2 g/kg). Blood glucose levels were measured again at minute 15, 30, 60, 90, 120 and 180 after liquid meal administration¹⁹.

Measurement of Serum Insulin level

On day 16, after overnight fasting, all animals of both types of diabetes were administered either deionized water, or extract, or glibenclamide, or metformin by oral gavage at doses mentioned previously. After 2 hours, blood samples were collected using capillary tubes from retro-orbital plexus under light diethyl ether anesthesia. Blood samples allowed to clot, and centrifuged at 4°C for 20 min at 2000 × g. The supernatant serum, ultra-sensitive mouse insulin ELISA kit, and microplate reader (ELX™, BioTek Instrument, Inc., VT, USA) were used for the measurement.

Histological procedures

Rats were anaesthetised with diethyl ether and euthanized by decapitation. Pancreatic tissues were harvested and tissue fragments were fixed in Bouin's solution, dehydrated with alcohol, embedded in paraffin, cut into 7 µm thick sections, and stained with hematoxylin-eosin dye for photomicroscopic observation²⁰.

Statistical analysis

All data were expressed as mean ± S.E.M (Standard Error of the

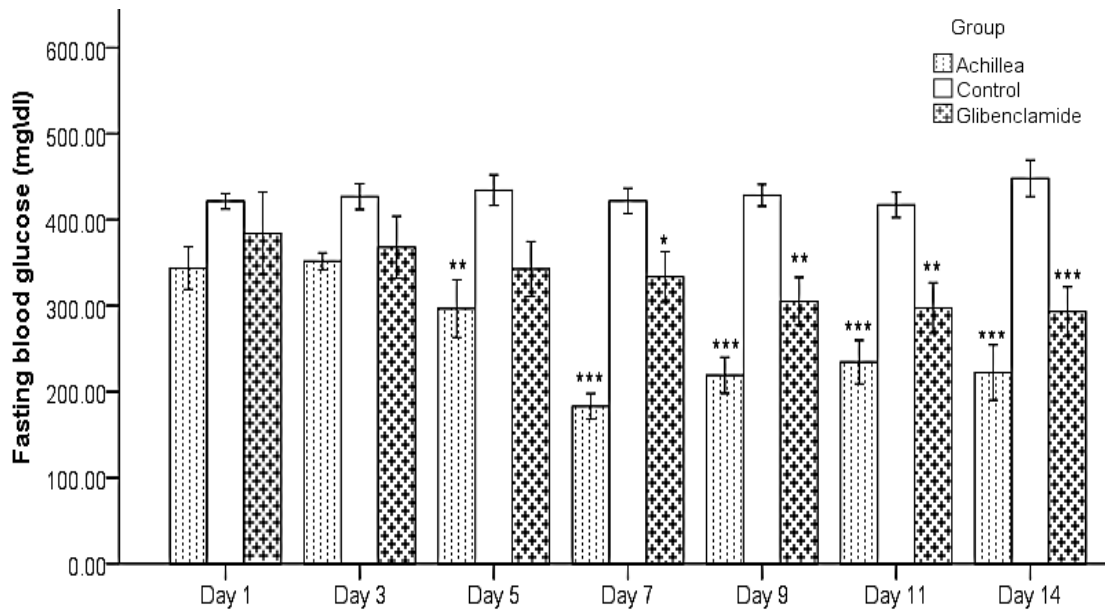


Figure 1: Effect of *A. biebersteinii* Afan. and glibenclamide on FBG levels in type I diabetic rats over a 14 day period. Values are means \pm SEM, *P<0.05; **P<0.01; ***P<0.001 vs. control group (n=6).

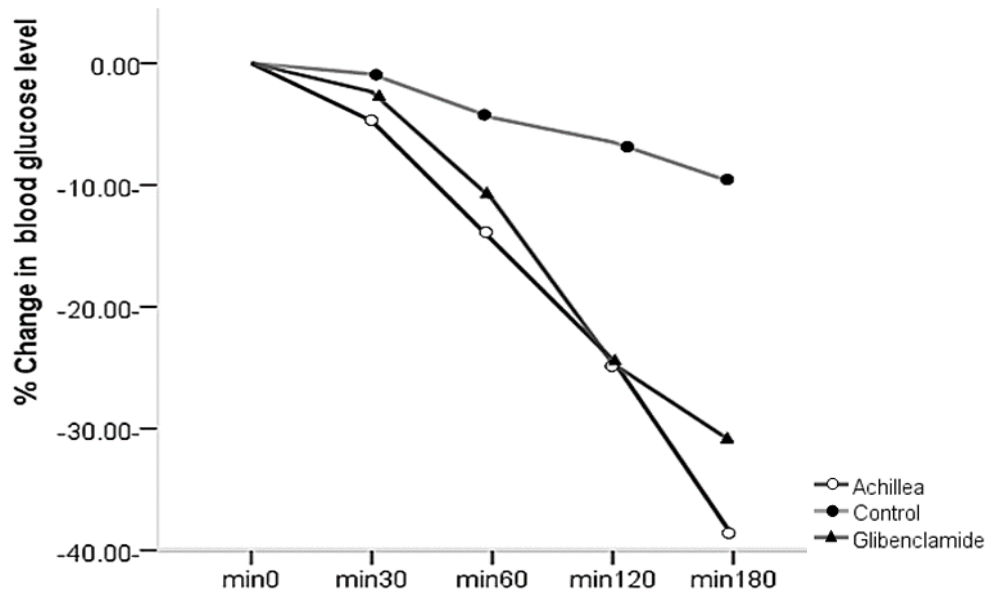


Figure 2: Percentage change in BG levels in type I diabetic rats after administration of *A. biebersteinii* Afan and glibenclamide at different time points.

Table 1: Blood glucose Levels (mg/dl) of OGTT in type I diabetic rats treated with *A. biebersteinii* Afan., and glibenclamide at different time points. Values are means \pm SEM, ***P<0.001 vs. control group (n=6).

	min0	min15	min30	min60	min90	min120	min180	Peak Change
Control	331.8 \pm 27.67	398.5 \pm 34.28	434.1 \pm 32.88	528.3 \pm 30.90	496.5 \pm 27.28	404.1 \pm 25.88	298.5 \pm 25.63	196.5 \pm 8.78
Glibenclamide	340.6 \pm 34.96	405.3 \pm 21.87	414.6 \pm 19.99	408.1 \pm 18.99	379.8 \pm 28.43	342.1 \pm 39.72	332.1 \pm 35.79	74 \pm 7.2***
Achillea	215.5 \pm 18.01	254.1 \pm 19.01	278.5 \pm 40.58	250.3 \pm 18.65	211.1 \pm 21.31	200.50 \pm 20.11	169.1 \pm 9.00	63 \pm 26.8***

Mean). The data were analysed by a statistical software package (SPSS version 18). One-way ANOVA (analysis of variance) and Tukey's tests were used to evaluate the

significance between means. The values were considered significantly different at $p < 0.05$.

RESULTS

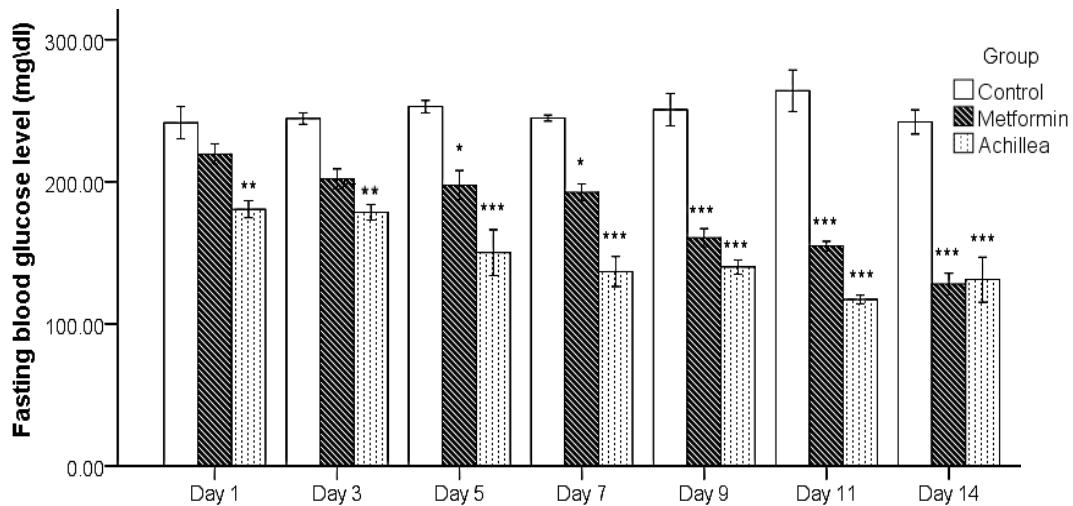


Figure 3: Effect of *A. biebersteinii* Afan. and metformin on FBG levels in type II diabetic rats over a 14day period. Values are means \pm SEM, *P<0.05; **P<0.01; ***P<0.001 vs. control (n=3).

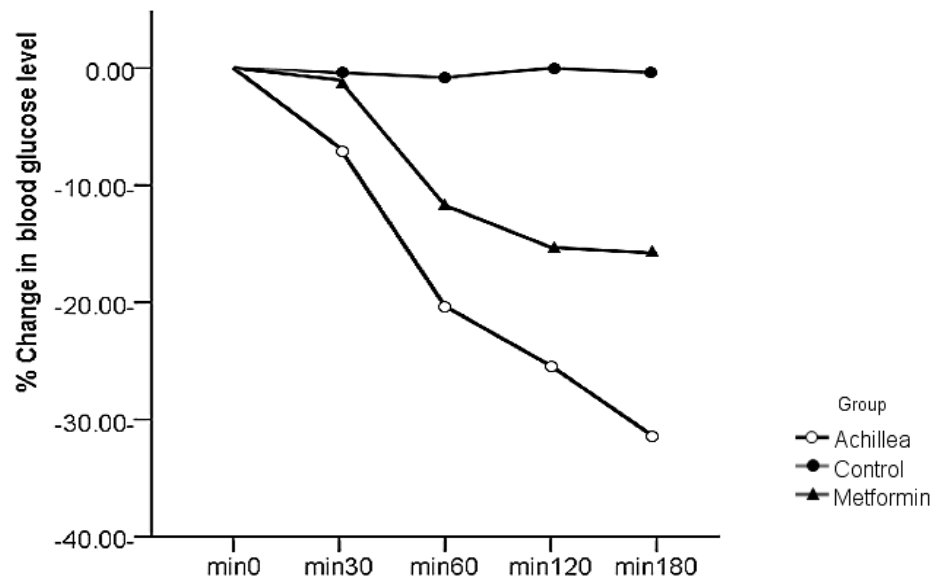


Figure 4: Percentage change in BG levels in type II diabetic rats after administration of *A. biebersteinii* Afan. and metformin at different time points.

type I diabetic rats

A. biebersteinii Afan. and glibenclamide decreased FBG levels significantly on day 5 and 7, respectively, and when compared to control group this effect persisted along the remaining experiment duration (figure 1). It was noticed on day 9 that FBG levels in *Achillea* and glibenclamide groups reached to a steady value. *A. biebersteinii* Afan. was found to produce a significant blood glucose lowering effect after 2 hours of administration ($p < 0.001$). The percentage change in BG levels over 180 minutes after administration is shown in figure 2.

Effect of A. biebersteinii Afan. on OGTT in type I diabetic rats

After loading a single dose of glucose (2 g/kg), maximum blood glucose levels were shown at min 30 in *Achillea* and glibenclamide groups, but in control group, it was shown at min 60. *A. biebersteinii* Afan. and glibenclamide limited significantly ($p < 0.001$) the peak in blood glucose levels

Table 2: Serum insulin levels (ng/ml) in type I diabetic rats treated with *A.biebersteinii* Afan., and glibenclamide. Values are means \pm SEM (n=6).

Control	Glibenclamide	<i>Achillea</i>
0.201 \pm 0.02	0.2890 \pm 0.03	0.378 \pm 0.039

when compared to the control group. The results are shown in table 1.

Effect of A. biebersteinii Afan. on Serum Insulin levels in type I diabetic rats

Serum insulin levels were measured on day 16, after 2 hours of administration of *A. biebersteinii* Afan. and glibenclamide. They were found to be higher in both groups but they weren't significantly different from the control group (table 2).

Effect of A. biebersteinii Afan. on FBG and BG levels in type II diabetic rats

Compared to the control group, *A. biebersteinii* Afan. and metformin began to reduce FBG levels significantly on the

Table 3: Blood glucose Levels (mg/dl) of OGTT in type II diabetic rats treated with *A. biebersteinii* Afan., and metformin at different time points. Values are means \pm SEM, **P<0.01; ***P<0.001 vs. control (n=3).

	min0	min15	min30	min60	min90	min120	min180	Peak Change
Control	210 \pm 1.15	326 \pm 5.19	381.3 \pm 14.5	334.6 \pm 22.36	301.3 \pm 13.77	263.6 \pm 8.08	232.3 \pm 6.88	171.3 \pm 14.37
Metformin	221.3 \pm 9.56	250.6 \pm 8.81	283.6 \pm 18.8	205.6 \pm 40.18	211.3 \pm 43.6	143.6 \pm 3.48	129.0 \pm 14.73	62.3 \pm 9.24**
Achillea	156.6 \pm 26.76	196.3 \pm 30.36	214.6 \pm 25.20	198.0 \pm 41.96	162.3 \pm 34.20	161.6 \pm 32.40	151.3 \pm 28.59	39.6 \pm 4.25***

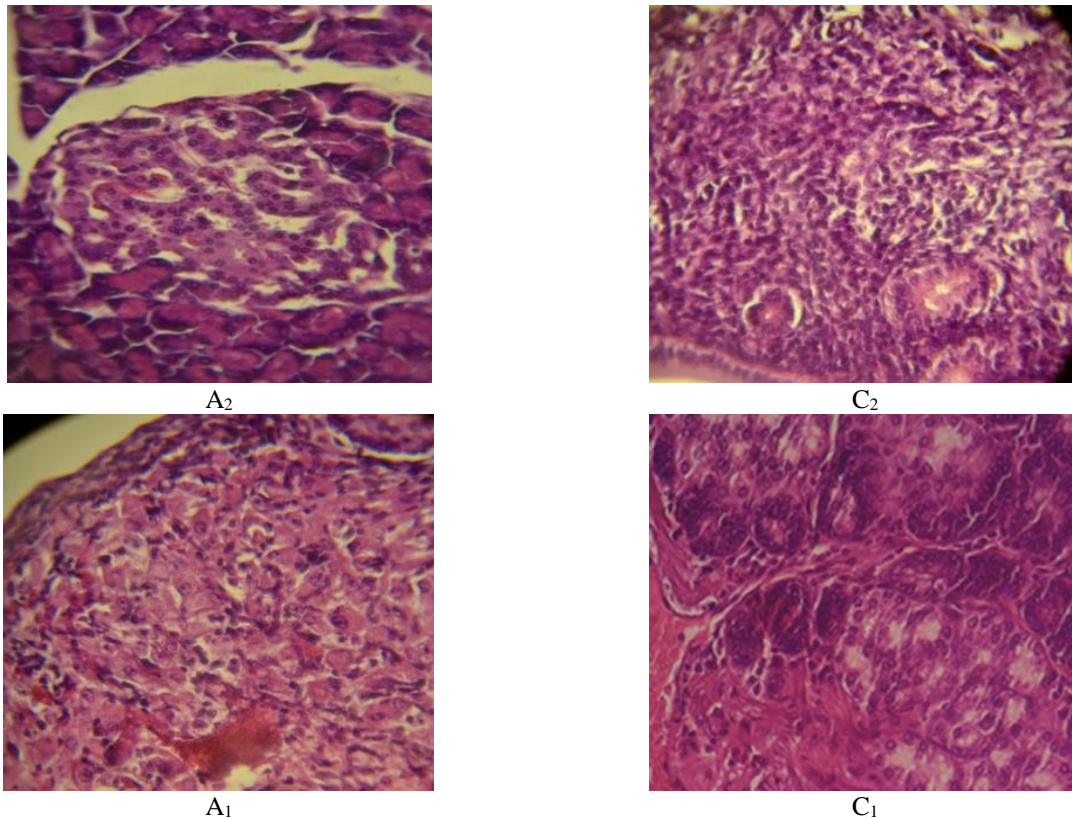


Figure 5: Photomicrographs of pancreatic tissues stained with H& E, (X400), (A₂) pancreatic tissue of the *A. biebersteinii* Afan-treated type II diabetic rats, (C₂) pancreatic tissue of the untreated type II diabetic rats, (A₁) pancreatic tissue of the *A. biebersteinii* Afan-treated type I diabetic rats, (C₁) pancreatic tissue of the untreated type I diabetic rats.

first and fifth day of the experiment, respectively (figure 3). This effect seemed to persist till the end of the experiment. *A. biebersteinii* Afan. was found to exert significant blood glucose lowering effect after 1 hour of administration ($p < 0.05$). Metformin was found to produce the significant blood glucose lowering effect after 2 hours of administration ($p < 0.05$). The percentage change in FBG levels over 180 minutes after administration is shown in figure 4.

Effect of A. biebersteinii Afan. on OGTT in type II diabetic rats

The result of OGTT in type II diabetic rats has been presented in table 3. After loading a single dose of glucose (2 g/kg), all groups showed maximum blood glucose levels at min 30. *A. biebersteinii* Afan. and metformin limited significantly the peak in blood glucose levels ($p < 0.001$

and $p < 0.01$, respectively) when compared to the control group.

Effect of A. biebersteinii Afan. on Serum Insulin levels in type II diabetic rats

Two hours after administration of *A. biebersteinii* Afan. and metformin, serum insulin levels were measured on day 16 of the experiment. They were found to be higher in *Achillea* group and lower in metformin group but they weren't significantly different from the control group (table 4).

Effect of A. biebersteinii Afan. on pancreatic β -cell islets in type I and type II diabetic rats

light microscopic examination of pancreatic sections showed them divided into lobules by connective tissue septa. Lobules were composed largely of grape-like clusters of exocrine cells called acini. Islets of Langerhans were embedded forming the endocrine component of the

Table 4: Serum insulin levels (ng/ml) in type II diabetic rats treated with *A.biebersteinii* Afan., and metformin. Values are means \pm SEM (n=3).

Control	Metformin	Achillea
0.34 \pm 0.04	0.286 \pm 0.049	0.439 \pm 0.009

pancreas. They appeared as pale stained rounded or oval areas inside the pancreatic lobules. The histologic sections of the pancreases of untreated group showed that STZ resulted in severe necrotic changes in pancreatic islets, and severe reduction in β cell mass of type I diabetic rats (figure 5C₁). However, in pancreatic islets of type II diabetic rats, STZ causes moderate necrotic changes together with a moderate reduction in β cells (figure 5C₂). The histologic sections of the pancreases of *A. biebersteinii* Afan-treated group showed increases in size and number of pancreatic islets. Moreover, pancreatic islets revealed to have regenerated β cells in comparison with untreated control rats (figure 5A1 and 5A2).

DISCUSSION

The plant kingdom is a wide and promising field to look for new hypoglycaemic agents which can be successfully employed to treat diabetes. The present study was designed to investigate the hypoglycemic effect of *A. biebersteinii* Afan., growing in Syria, in both types of diabetes.

plant constituents that contribute to the hypoglycemic activity are various, including phenolic compounds, flavonoids, terpenoids, coumarins, glycopeptide, alkaloids, steroids, and other constituents²¹. Because of plant hypoglycemic constituent variety, it was difficult to predict which constituent of *A. biebersteinii* Afan. should be isolated, so the extract was prepared by general extraction method using ethanol 80%.

HFD-STZ induced diabetic rats and STZ- induced diabetic rats were used as diabetic models. Multiple low doses of STZ have replaced the use of STZ at a single high dose because of demonstrating minimal toxic effects in animals and producing clinical features and pathological changes resembling to those of human type I diabetes in which β -cell destruction is mediated by activation of immune mechanisms^{22,23}. The destruction of β -cell islets was obviously demonstrated in figure 5C₁.

Type 2 diabetes has been shown to be induced by various approaches such as STZ injection following nicotinamide administration, HFD feeding followed by low-dose STZ injection, and STZ injection during the neonatal period^{17,18,23,24}. To induce type II diabetes, rat models were allocated into a HFD regimen followed by a single low STZ dose (35 mg/kg). This was found to be an efficient and useful way to generate a rat model that mimic the natural history and metabolic characteristics of human type II diabetes^{17,18}.

Flavonoid content of ethanolic extracts of *A. biebersteinii* Afan. have been reported to contain quercetin, rutin, and myricetin^{25,26}. Myricetin was found to ameliorate insulin resistance. Moreover, myricetin can execute the functions including anti-inflammation, anti-oxidative stress, anti-aldoase reductase, anti-non-enzymatic glycation and anti-

hyperlipidemia. All of these functions may provide the contribution to the prevention of diabetes and diabetic complications²⁷. Quercetin was also found to have antidiabetic activity. Several studies have reported quercetin mechanism of action in diabetes, such as decreases in lipid peroxidation, increases in antioxidant enzymes activities, reduction in intestinal glucose absorption by inhibiting glucose transporter-2 (GLUT-2), and stimulating GLUT4 translocation and expression in skeletal muscle²⁸⁻³⁰. Rutin has been reported to reduce significantly plasma glucose and HbA1c, and restore the depleted liver antioxidant status and serum lipid profile in HFD-STZ induced diabetic rats³¹. Rutin was also found to increase insulin levels, and decrease the levels of fasting blood glucose^{32,33}. During 16 days of treatment, *A. biebersteinii* Afan. extract results indicated clearly that it is efficient in both types of diabetes; it reduced significantly FBG levels, reduced significantly BG levels during 3 hours after administration, improved oral glucose tolerance, tended to raise serum insulin levels, and enhanced regeneration of β cells.

Although *A. biebersteinii* Afan. extract was given once daily, it produced highly significant antihyperglycemic effect on FBG levels in type I diabetic rats (figure 1). This result indicates that *A. biebersteinii* Afan. extract may have a long duration of action when it is compared to glibenclamide which its duration of action is estimated by 12-24 hours. The results in figure 1 also indicate that extract effect seemed to persist cumulatively till the day 9 when FBG levels in *Achillea* group reached to a steady values estimated by 219-222 mg/dl. The explanation for this is not clear, but it may attributed to reaching to a steady state concentration that exerts effect matching to the given dose, or more likely the extract reached the maximum effect matching remanent and/or regenerated β -cell capacity.

OGTT data (table 1 and table 3) demonstrate that *A. biebersteinii* Afan. extract improved significantly oral glucose tolerance in type I diabetic rats and in type II diabetic rats and seemed to be more effective than the reference standards. These results could be interpreted in different ways. The study of Lui Z *et al.*, 2012, inferred that improving glucose intolerance was caused by increasing the insulin action on the target tissues responsible for glucose uptake³⁴, whereas the study of Brockman DA *et al.*, 2012, and the study of Deng YX *et al.*, 2012 inferred that it could be caused by increasing the release of insulin or by providing resistance to intestinal glucose absorption^{35,36}.

Although serum insulin levels, measured on day 16 of the experiment, were not significantly different from control group (table 4 and table 2), *A. biebersteinii* Afan. tended to raise serum insulin levels. This raise was accompanied to significant antihyperglycemic effect and regeneration of β cells (figure 5A1 and 5A2).

It is well known that oxidative stress plays a role in the causation of both types of diabetes, mediated by pancreatic injury and inhibiting insulin signalling cascade^{37,38}. *A. biebersteinii* Afan. may have antioxidant activity due to its phenols and flavonoids content¹¹. The protective activity

of *A. biebersteinii* Afan. ethanol extract against DNA damage *in vitro* showed significant inhibition of DNA damage induced by reactive oxygen species (ROS)³⁹. This antioxidant activity of *A. biebersteinii* Afan. might protect and enhance β -cell regeneration and/or increase tissue sensitivity to insulin.

According to Srinivasan *et al.*, 2005 study, the combination of HFD and low dose of STZ associated with significant increase in triglycerides and total cholesterol levels¹⁸. In the study of Babelly *et al.*, 2016, *A. biebersteinii* Afan. extract reduced significantly the levels of serum cholesterol, triglycerides and LDL-C¹². This potential regulating role in lipid metabolism together with antioxidant effect of *A. biebersteinii* Afan., may partly explain the hypoglycemic effect of *A. biebersteinii* Afan. in type II diabetic rats (figure 4 and table 3).

According to *A. biebersteinii* Afan results and the results of studies mentioned above, it could be inferred that *A. biebersteinii* Afan. exerted its antihyperglycemic effect by enhancing insulin release from remanent and/or regenerated β -cells, and by extra pancreatic effects contributed to increasing utilization of glucose by tissues, and/or decreasing hepatic glucose output, and/or providing resistance to intestinal glucose absorption. These proposed mechanisms of *A. biebersteinii* Afan. have to be clarified in further molecular studies.

Although *A. biebersteinii* Afan. extract at dose of 400 mg/kg did not reveal any physical signs of toxicity or mortality even after 2 weeks of treatment, it is necessary to analyse other vital biochemical parameters with various doses in order to fully assess the toxic dose.

This study was carried out in regard to the whole aerial parts of the plant with one extraction method and for a relatively short term period, so there is a need for further studies to detect the optimum extraction method and to detect the part of plant responsible for the hypoglycemic effect.

CONCLUSION

It is concluded from the data that *A. biebersteinii* Afan. possesses potent antihyperglycemic activity and it may prove to be effective for the treatment of both types of diabetes.

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