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Original Article

Metformin and Bee Venom: a Comparative Detection of Histological Alteration of the Pancreas and Systemic Inflammatory Markers in Diabetic Mice

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Abstract

Metformin is the approved medication for managing global health issues concerning type 2 diabetes mellitus (T2DM). Using natural bioactive compounds as an alternative therapy is crucial to managing several metabolic diseases. Therefore, due to recent limited studies that detected the role of bee venom (BV) in improving diabetic conditions in Iraq, the current study was designed to identify the potential therapeutic role of BV and metformin in diabetic mice. Twenty male mice (Balb/c) aged about 60 days with an average weight of 26.55±2.70 g were randomly divided into four groups (n=5). The animals were placed in plastic cages for acclimatization for one week of access to food and water ad libitum. Overnight fasting was applied to 15 mice which were then injected with 95 mg/kg body weight of prepared alloxan. The mice were supplemented with glucose fluid for 3 days. On day 4, the blood was collected from the tail to measure the circulating glucose level. When blood glucose levels exceed 200 mg/dl, the animals are considered diabetic. After induction of diabetes, the animals were divided as follows: Control group: included five mice that were not subjected to diabetes induction; the animals in this group did not receive any medications. Diabetic group: including five mice confirmed with diabetes without receiving any treatments. Metformin group: including five diabetic mice exposed to a single oral dose of 150 mg/kg of metformin for 30 days. Bee Venom group: including five diabetic mice exposed to a single intraperitoneal dose of 1 mg/kg Bee Venom for 30 days. After 30 days of treatment, blood was drained, and serum was obtained to detect the levels of glucose, insulin, TNFα, IL6, and IL10 by using precise enzyme-linked immunosorbent assay (ELIZA) kits. Also, the pancreas was collected from all mice for histopathological investigation. The result displayed significantly elevated glucose concentration in diabetic mice, while metformin and BV significantly reversed these increases. A significant decline in insulin concentration was seen in diabetic mice, whereas metformin and BV significantly enhanced this reduction in insulin concentration. Furthermore, mice treated with alloxan exhibited remarkable increases in TNFα and IL6 compared to control mice, while supplemented metformin and BV significantly reduced these high concentrations. Moreover, the level of IL10 markedly declined in diabetic mice, which was reversed significantly in response to metformin and BV. Histological detection of the pancreas in diabetic mice showed significant changes in the shape and size of islets associated with the arrangement and number of beta cells with a reduction of islets covering connective tissue. Metformin slightly restored these alterations; however, significant and remarkable restoring of histological changing was promoted by BV. Thus, BV could be a potential agent for managing metabolic disorders including diabetes.

Keywords: Diabetes Mellitus, TNFα, IL-6, IL-10, Pancreas, Iraq

1. Introduction

Diabetes mellitus (DM) is the most prevalent metabolic disease worldwide (1). Globally, approximately 537 million adults worldwide aged 18–99 were diagnosed with diabetes mellitus. This number is expected to increase to 643 million by 2030 and 783 million by 2045 (2). More than 90% of these recorded and expected cases are attributed to type 2 diabetes mellitus (T2DM) (3). The latter is a chronic metabolic disorder that occurs due to the inability of pancreatic beta cells to produce a sufficient amount of insulin and/or the inability of insulin to promote its proper effect on sensitive tissue (4). Accordingly, circulating glucose and insulin levels are impaired, consequently impairing glucose and lipid metabolism (5).

Metformin is a member of the biguanide family; since its discovery, it has been a frequently prescribed medication for people with T2DM. Metformin increases insulin sensitivity, particularly in the liver and peripheral tissues associated with inhibiting liver glucose production (6). This effect is achieved through stimulating AMP-activated protein kinase (AMPK) and protein kinase A (PKA), which inhibits the mitochondrial respiratory chain (complex I) and glycerophosphate dehydrogenase (7). Nowadays, it has broad therapeutic uses with respect to anti-diabetic effects, including antitumor, antiaging, polycystic ovarian disease, and weight loss (8).

Bee Venom (BV) is a water mixture with several peptides, none peptides, and enzyme components (9). These components include melittin, mast cell degranulating, apamin, catecholamines, large phospholipase A2, hyaluronidase, acid phosphomonoesterase, and more, which all possess various actions at the cellular level (10). Thus, it has a potential therapeutic health benefit due to the presence of these constituents in its composition. These benefits include antimicrobial, anti-inflammatory, anti-cancer, and wound healing (9, 11, 12). In Iraq, limited research investigated the role of bee venom as a potential anti-diabetic agent and its effect on the histological structure of the pancreas (13). However, the precise action of bee venom as an anti-diabetic agent is still not well elucidated. Therefore, this study was designed to investigate the comparative effect of metformin and bee venom on modulating diabetic conditions and possible changes in the histological structure of the pancreas.

2. Materials and Methods

2.1. Animal Housing and Diabetes Induction

Twenty male mice (Balb/c) aged about 60 days with an average weight of 26.55±2.70 g. The animals were placed in plastic cages for acclimatization for one week with open access to food and water, under standard light and dark cycles of 12-hour, temperature and humidity. Overnight fasting was applied to 15 mice, then injected with 95 mg/kg body weight of prepared alloxan (Sigma-Aldrich, UK). The mice were supplemented with glucose fluid for 3 days. On day 4, the blood was collected from the tail to measure the circulating glucose level. When blood glucose levels exceed 200 mg/dl, the animals are considered diabetic.

2.2. Preparation of Metformin and Bee Venom

Metformin and BV were obtained from the local market in Iraq. The metformin powder and lyophilized BV were dissolved in doubled distil water, and the prepared metformin was preserved in the refrigerator until required. Whereas prepared BV was transferred to sterile Eppendorf tubes, which were preserved at -20 C° until required.

2.3. Study Design

After induction of diabetes, the animals were divided as follows:

2.3.1. Control Group

including five mice not subjected to diabetes induction, the animals in this group did not receive any medications.

2.3.2. Diabetic Group

Including five mice confirmed with diabetes without receiving any treatments.

2.3.3. Metformin Group

Including five diabetic mice exposed to a single oral dose of 150 mg/kg of metformin for 30 days.

2.3.4. Bee Venom Group

Including five diabetic mice exposed to a single intraperitoneal dose of 1 mg/kg Bee Venom for 30 days.

At the end of the experiment, the mice were anaesthetized with 0.3 mg/kg ketamine and 0.1 mg/kg lidocaine. The blood was drained directly from the heart and centrifuged at 3000 cycles/minute for 15 minutes for serum separation. The obtained serum was kept at -20°C until needed. Furthermore, the pancreas of all mice was collected and placed in 10% formalin for a short time.

2.4. Serological Evaluation

Specific sandwich ELISA kits were applied to estimate the level of glucose (Spinreact, Spain), insulin (Monobind, USA), tumour necrosis factor α (TNFα) (Boster, United Kingdom)**,** interleukin 6 (IL6) (Boster, United Kingdom)**,** and interleukin 10 (IL10) (Boster United Kingdom) in serum samples based on manufacturer procedures.

2.5. Histological examination

Post experiment, the collected pancreas was transferred to 70% alcohol after 48-72 hours of immersing in 10% formalin. All mice's pancreas goes through an ordinary histological process. The histological sections were exposed to hematoxylin, eosin routine stain, and Masson trichrome special connective tissue stain. The resulting histological slides were evaluated under the light microscope (Novel, China), and the images were captured using (camera type and resolution) (Optica, Italy). Due to the mouse pancreas's small, fragile, and inflated consistency, the histological sections might show improper artefacts with respect to the strict and efficient histological process. Thus, the current study acknowledged the efficient histological process and the issue related to the processing of the mouse pancreas.

2.6. Statistical Analysis

Microsoft Office Excel version 2019 (Microsoft, USA) was used to process all the data obtained. GraphPad Prism version 6 (GraphPad Software Inc., USA) was used to analyze data through One-Way ANOVA with multiple comparisons to Turkey's post-hock. The values of *P* were set as <0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****) between groups. The data shown were represented as the mean, standard error of the mean.

3. Results

3.1. Metformin and BV Improved Glucose and Insulin Levels

Alloxan promoted significant increases in circulatory glucose by 197%±3.3% compared to control. This high glucose concentration level was ameliorated significantly by 54.4%±1.3% and 50.2%±1% in response to metformin and BV, respectively, compared to the diabetic group (Figure 1A). In diabetic mice, insulin levels were reduced by 52.3% and 5% compared to control mice. This reduction was significantly reversed by 70.6%±2.8% and 55.3%±3.6% in metformin and BV groups, respectively, compared to diabetic mice only (Figure 1B).

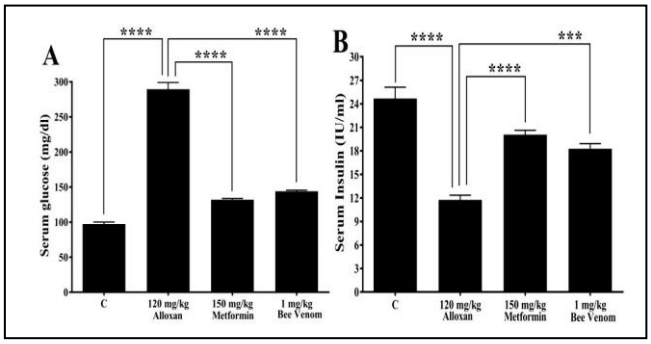

Figure 1. Metformin and bee venom improved glucose and insulin levels in diabetic mice (**A** and **B**).

3.2. Metformin and BV Enhanced the Levels of Inflammatory Markers

The main inflammatory and anti-inflammatory markers levels were investigated in this study's groups. The level of TNFα was significantly increased by 33%±2.3% in diabetic mice compared to control. Metformin and BV promoted a significant decrease of this elevated level of TNFα by 17.4%±3.5% and 25%±2.5%, respectively, compared to diabetic mice

(Figure 2A). Similarly, diabetic mice exhibited significant elevation of the IL6 level by 125%±1.2% compared to control mice. This elevation was reduced significantly by 42.9%±1.6% and 47.4%±2.5% in mice treated with metformin and BV respectively compared to diabetic mice (Figure 2B). While the level of IL10 declined significantly in diabetic mice (17%±2.5%) compared to the control group. This dropdown of IL10 concentration was significantly enhanced by 17.8%±2.2% and 23.6%±2.3% in response to supplemented metformin and BV, respectively, compared to diabetic mice (Figure 2C).

3.3. Metformin and BV Improved the Histological Structure of the Pancreas

Investigation of the histological structure of the pancreas showed normal exocrine tissue with a clear arrangement of acinar cells that exhibited clear basal nuclei and amphophilic cytoplasm stains. The endocrine tissue (islets of Langerhans) showed a normal structure embedded in acinar tissue and bordered by distinct, delicate connective tissue and blood vessels that give the islets a spherical shape. The islets have a regular shape and size with normal cellular pattern arrangement and cell number. The many beta cells looked round, with clear nuclei and eosinophilic cytoplasm (Figures 3, 4, and 5A). Induced diabetes using alloxan promoted variable histological changes in the pancreas. The islets' shape becomes irregular and compressed, associated with a reduction in the number of beta cells.

Furthermore, irregular bleeding, islet cell vocalization, and reduced the connective tissue sheet surrounding the islets (Figure 3, 4, 5 B).

The pancreas of metformin-treated mice showed slightly enhanced islet shape, size, and the number of beta cells associated with remarkable enhancement of surrounding connective tissue. However, minor bleeding and vocalization of the cellular islets are seen (Figure 3, 4, 5 C).

The histological structure of the pancreas of mice treated with BV displayed significant enhancement. The islet's shape and size were restored almost usually. The normal arrangement and number of beta cells concomitant with eosinophilic cytoplasm were retained. Furthermore, the vocalization of the islets disappeared, associated with marked decreases in bleeding and remarkably restored islets covering connective tissue (Figure 3, 4, 5 D).

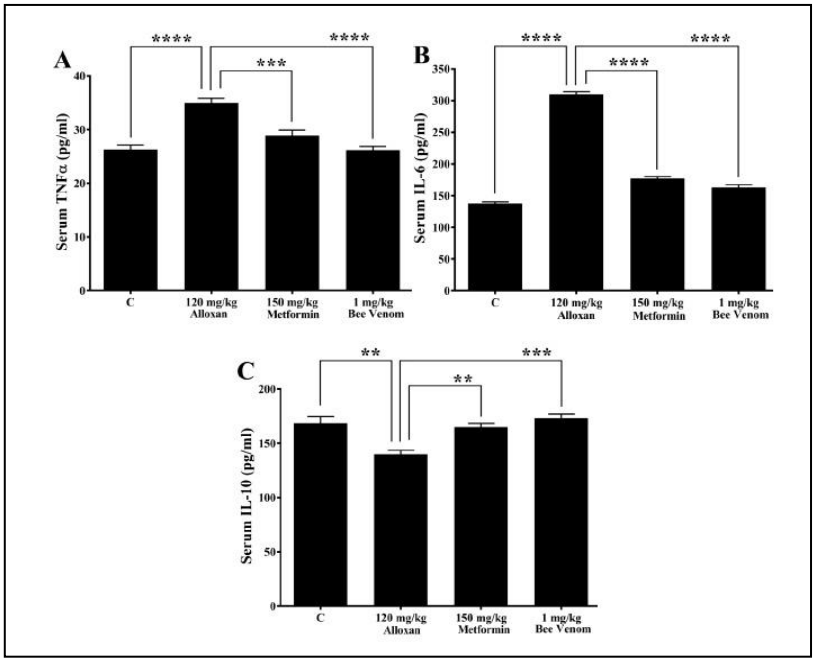

Figure 2. The metformin and bee venom enhanced (A) TNFα, (B) IL6, and (C) IL10 levels in the diabetic mice

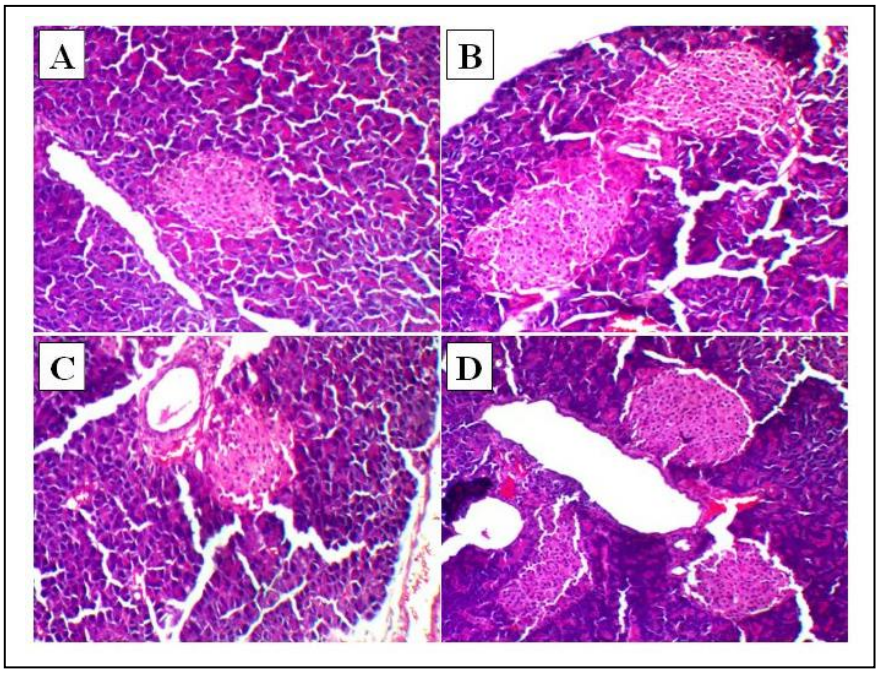

Figure 3. Metformin and BV improve pancreatic structure in diabetic mice. The histological structure of the pancreas was detected in (**A**) control mice, (**B**) diabetic mice, (**C**) metformin-treated diabetic mice (**D**) BV-treated diabetic mice. The tissue was stained with H & E and captured at 10X. The displayed images represent all the studied animals.

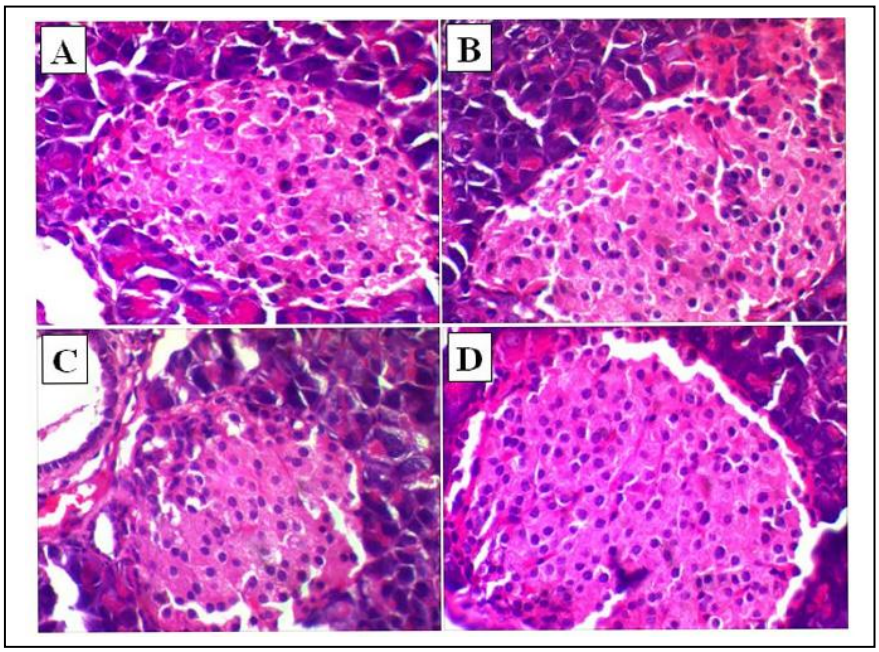

Figure 4. Metformin and BV improve pancreatic structure in diabetic mice. The histological structure of the pancreas was detected in (**A**) control mice, (**B**) diabetic mice, (**C**) metformin-treated diabetic mice (**D**) BV-treated diabetic mice. The tissue was stained with H & E and captured at 40X. The displayed images are represented all study animals.

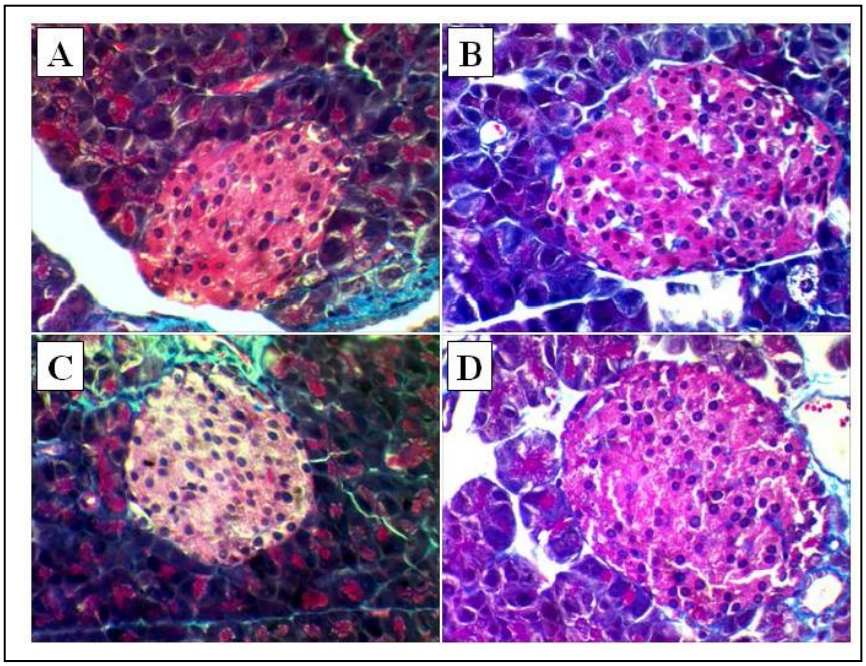

Figure 5. Metformin and BV improve pancreatic structure in diabetic mice. The histological structure of the pancreas was detected in (**A**) control mice, (**B**) diabetic mice, (**C**) metformin-treated diabetic mice (**D**) BV-treated diabetic mice. The tissue was stained with Masson trichrome and captured at 40X. The displayed images are represented all study animals.

4. Discussion

In the last few years, natural animal venom has drawn sparkling attention in the medical field and the development of therapeutic medication. This is based on accumulative evidence of the use of various types of venom as a traditional ancient alternative therapy to manage and cure several diseases (14). The curative effects are mainly attributed to the availability of several bioactive constituents in animal venom (15). Melittin, apamin, phospholipase A 2, adolapine, and more are the major bioactive compounds of the BV, considered crucial natural animal venom that shows several exciting health benefits, including anti-diabetic (16). Thus, detecting any natural plants, animal venom, and remnants possessing anti-diabetic properties is interesting as the disease is widely spreading, causing health issues worldwide. Although BV's health benefits, including anti-diabetic, are well known, limited information is available, particularly in Iraq. Thus, specific in vivo and in vitro research are needed to cope with the missing information and elucidate the precise potential effect of BV.

Impairment of glucose and lipid metabolism with insulin secretion is the major hallmark of T2DM (17). The impairment includes elevated glucose levels in the circulation concomitant with a reduction in the level of insulin secretion with the development of insulin resistance (18). As expected, administration of alloxan disrupted glucose and insulin levels, resulting in a high glucose concentration associated with a decreased insulin level.Metformin, the first and most widely prescribed medication, is involved in improving this glucose and insulin level impairment.The enhancement includes a significant glucose level reduction associated with increased insulin secretion. Several studies have found that metformin plays an important role in diabetic management by lowering blood glucose levels and increasing insulin circulation (19).Similarly, several studies findings demonstrated BV's ability to control diabetic conditions such as metformin by lowering glucose levels while increasing insulin (20).The previous result is parallel with the current study result that suggests the BV is participating strongly in reducing the glucose level by increasing

insulin secretion in diabetic mice. These effects could be increased insulin secretion, thus stimulating insulin-sensitive tissues to increase glucose uptake, causing a lower circulatory glucose concentration (21). While the increased level of insulin could be due to depolarizing the cell membranes of the pancreatic beta cells, which leads to the opening of the Ca_{2+} channels, allowing a large quantity of calcium to enter, which then triggers insulin vesicles and releases insulin (20, 22).

In addition to glucose and insulin deterioration, several other important markers can be identified in diabetic conditions. Prolonged hyperglycemia associated with insulin resistance can cause cellular stress and promote oxidative stress, a condition in which the production of reactive oxygen species (ROS) is increased, affecting the cells and causing varying degrees of systemic inflammation (23).The alteration of circulatory inflammatory markers, including increased levels of TNF and IL-6 and reduced levels of anti-inflammatory marker IL-10, can be distinguished in diabetic patients, which are all strongly related to T2DM (24, 25). Accordingly, in the current study, induction of diabetes using alloxan triggered the body's inflammatory state and caused a modification in the inflammatory and anti-inflammatory markers. These alterations include elevated TNF and IL-6, concomitant with a decreased level of IL-10, which was the expected result. All these alterations in inflammatory markers were modulated firstly by supplemented metformin, which showed a significant effect, and secondly by the administration of BV, which promoted a positive impact on the inflammatory state. Several studies found similar results; however, the results could be mediated by metformin's and BV's ability to modulate the level of ROS via anti-oxidant properties, thus significantly reducing oxidative stress, which affected the level of inflammatory and anti-inflammatory cytokines (6, 26, 27).Based on these collected results, the study suggests that the BV is a potent agent which could be used as an additive in diabetic patients to manage systemic inflammation.

The histological architecture of the pancreas in all diabetic and treated mice was determined using classic histological procedures with routine H & E and a special Masson trichrome stain. Several, but limited, studies have been conducted to identify the histological changes in the pancreas in diabetics and diabetics treated with metformin and BV (22, 28).These results align with the current study that showed clear alteration and restoration in the pancreatic structure, particularly in the endocrine part, in diabetic mice exposed to metformin and BV. Alloxan altered the pancreatic endocrine system, causing changes in islet shape and size, beta cell number and size, blood vessel status, the amount of connective tissue surrounding the islets, and tissue and cell vocalization.Most of these changes were altered nearly to normal after supplementation with metformin and BV. The regenerative effect of both metformin and BV could be due to the ability of both supplemented agents to scavenge free radicals and therefore decrease oxidative stress and inflammatory state, which then improves the histological structure (22). This could be the nearest possible reason for restoring the histological changes in the pancreatic structure. Similarly, multiple studies have associated the regeneration impact of BV on pancreatic endocrine with specific BV components with high anti-oxidant capabilities, which is the only possible reason to reduce inflammation and recover from oxidative stress by scavenging huge amounts of free radicals (20, 29).

According to all these studies, the BV could be a potential anti-diabetic agent that could be used as an alternative to diabetes therapy.

The remarkable hallmark of the prevalence of diabetes, which can be naturally or synthetically induced, is the alteration of glucose and insulin levels in the circulation. In addition to glucose and insulin, inflammatory and anti-inflammatory cytokines disturbances can be exhibited in diabetic conditions. Furthermore, alteration of the histological architecture of the endocrine pancreas can be seen clearly. Fundamentally, the precise effect of metformin as an

anti-diabetic agent widely used to manage diabetic conditions is limited to studies focused on the potential anti-diabetic role of bee venom. As a result, the current study identified this potential role of BV, and the results are promising, demonstrating that BV has a positive effect on glucose and insulin concentration in diabetic mice.

Furthermore, BV can manage inflammatory and anti-inflammatory markers, including TNF, IL-6, and IL-10, in diabetic conditions. Moreover, the BV improved the histological structure impairment of the pancreas, particularly the endocrine tissue. Based on this evidence, the BV could be an interesting anti-diabetic agent that could be applied as an alternative additive to control the diabetic state and prevent diabetes consequences. However, the molecular mechanism of BV's anti-diabetic effect still needs more studies to elucidate the precise effect and pathways.

Authors' Contribution

Study concept and design: S. J. J. A.
Acquisition of data: R. A. H. A.
Analysis and interpretation of data: R. A. H. A.
Drafting of the manuscript: R. A. H. A.
Critical revision of the manuscript for important intellectual content: S. J. J. A.
Statistical analysis: S. J. J. A.
Administrative, technical, and material support: S. J. J. A.

Ethics

This study is approved and performed according to the regulation of Ethical Committees/ College of Pure Sciences at Wasit University (Wasit, Iraq).

Conflict of Interest

The authors declare that they have no conflict of interest.

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