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Modulation of Inflammatory Cytokines and Islet Morphology as Therapeutic Mechanisms of *Basella alba* in Streptozotocin-Induced Diabetic Rats

Dennis S. Arokoyo[1], Ibukun P. Oyeyipo[2], Stefan S. Du Plessis[2], Novel N. Chegou[3] and Yapo G. Aboua[1]

[1]*Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, Cape Town, South Africa*
[2]*Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa*
[3]*DST/NRF Centre of Excellence for Biomedical Tuberculosis Research and SAMRC Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa*

Abstract

The mechanism of the previously reported antidiabetic effect of *Basella alba* is unknown. This study investigated the role of *B. alba* aqueous leaf extract in the modulation of inflammatory cytokines and islet morphology in streptozotocin-induced diabetic rats. Forty male Wistar rats, between 8 and 10 weeks old, were randomly divided into four groups (n = 10) and administered the following treatments: Healthy control (H-c) and Diabetic control (D-c) animals received normal saline 0.5 mL/100 g body weight daily, while Healthy Treatment (H-Ba) and Diabetic Treatment (D-Ba) rats received the plant extract 200 mg/kg body weight daily. All treatments were administered by oral gavage. Diabetes was induced in D-c and D-Ba rats by a single intraperitoneal injection of streptozotocin (55 mg/kg body). The body weight and fasting blood sugar (FBS) levels were recorded every week for 4 weeks, after which the rats were euthanized and samples collected for further analysis. After the experiment, FBS level was significantly reduced ($p < 0.0001$) in rats in the D-Ba group, but increased ($p < 0.001$) in rats in the D-c group. The absolute (H-c and H-Ba vs D-c, $p < 0.05$) and relative (D-Ba vs H-c, $p < 0.05$; D-Ba vs H-Ba, $p < 0.005$) weights of the pancreases were significantly higher after the experiment. The rats in the D-c group had significantly higher levels of serum interleukin-1β ($p < 0.001$ vs H-c; $p < 0.05$ vs H-Ba and D-Ba) and monocyte chemotactic protein-1 ($p < 0.0001$), but lower levels of interleukin-10 ($p < 0.05$) in comparison with the other groups. Histopathological examination revealed severe interstitial congestion, reduced islet area ($p < 0.0001$), and increased islet cell density in the D-c group compared with those in the D-Ba group. From these findings, it was concluded that the aqueous extract of *B. alba* stimulates the recovery of beta-islet morphology in streptozotocin-induced diabetic rats by modulating the peripheral production of inflammatory cytokines.

***Key words*:** Inflammation, Cytokines, Islet morphology, *Basella alba*, Diabetes

Correspondence to: Dennis S. Arokoyo, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, Cape Town, South Africa
E-mail: dennisarokoyo@yahoo.com

Abbreviations: Ba, Basella alba; STZ, Streptozotocin; DM, Diabetes mellitus; T1DM, Type I Diabetes mellitus; FBS, Fasting blood sugar; IL, Interleukin; TNF, Tumor necrosis factor; IFN, Interferon; MCP-1, Monocyte chemotactic protein; SEM, Standard error of means; ANOVA, Analysis of variance.

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INTRODUCTION

Cytokines are members of a large group of polypeptides that mediate inflammatory and immune responses in tissues and play a central role in the pathophysiology of most chronic diseases, including diabetes mellitus (DM) (1). They can be classified into pro- and anti-inflammatory cytokines, with the process of inflammation believed to be a result of interplay between these two groups (2). However, more recent views on the role of cytokines in inflammation suggest that such a classification may not be all-encompassing, with certain cytokines capable of both activities (2). Conventionally, cytokines such as interleukin-1 (IL-1), IL-12, IL-18, interferon-gamma (IFN-γ), tumor necrosis factor, and granulocyte-macrophage colony stimulating factor are considered pro-inflammatory, whereas IL-4, IL-10, IL-13, transforming growth factor-β, and IFN-α are anti-inflammatory (2). Chemokines, such as monocyte chemotactic protein-1 (MCP-1), are closely associated with cytokines and important in the inflammatory processes. During the progression of type 1 diabetes mellitus (T1DM), pro-inflammatory cytokines invade the islet cells of the pancreas and exert cytotoxic effects on beta cells (3). Therefore, T1DM is essentially an inflammatory disease of the endocrine pancreas, which destroys insulin-producing beta islet cells via a cytokine-mediated autoimmune reaction (4). It is challenging to understand the specific role played by inflammatory cytokines in the pathophysiology of DM, owing to the complex physiological functions of cytokines and their inhibitors. However, ever-changing dynamics exist between the pro- and anti-inflammatory components of the immune system, but in immune mediated diseases such as T1DM, the anti-inflammatory mediators appear to provide insufficient protection against the pro-inflammatory activity (5).

The destruction of insulin-producing islet beta-cells of the pancreas results in insulin deficiency and consequently hyperglycemia, which is the primary anomaly in insulin-dependent DM. The circulating levels of the most pro-inflammatory cytokines are usually elevated in DM, largely as a result of hyperglycemia, which stimulate blood levels of cytokines to increase via an oxidative mechanism (6,7). Hyperglycemia also results in hyperosmolality of the extracellular fluid, a physiological state that enhances the production of cytokines by mononuclear cells in peripheral circulation (8). Otto and colleagues (8) suggested that hy- perglycemia causes tissue inflammation through the stimulation of cytokine production via osmotic stress.

Streptozotocin and alloxan are two diabetogenic agents commonly used to induce experimental DM in laboratory animals through their ability to generate reactive oxygen species via different routes, and subsequently induce cytotoxic effects on the beta-cells of the pancreas (9). Streptozotocin is a methylating agent that causes extensive beta-cell necrosis in rodents after the injection of a single high dose. Multiple low doses induce partial beta-cell apoptosis, which triggers an autoimmune reaction that eventually eliminates the remaining cells (10). It was originally used as an alkylating agent for the treatment of metastatic tumors of islet cells, but was later discovered to be diabetogenic by Rakieten *et al*. in 1963 (11) and has since become a chemical agent of choice for experimental induction of DM (12).

Diabetes continues to pose a serious threat to human survival and constitutes a major health challenge worldwide despite the treatments currently available. Recent studies reported an 8.3% prevalence rate, with 387 million people affected by the disease worldwide and an estimated 46.3% cases still undiagnosed (13), with the majority of these cases in underdeveloped and developing countries where the resources for disease management are scarce. Therefore, there is a need for the continued investigation of new treatments for this disease.

Basella alba (Ba), a leafy green vegetable, belongs to the family *Basellaceae* and is said to be native to South Asia, but is also widely cultivated in tropical Africa. The common names of this perennial vine, which differ in geographical locations, include Ceylon spinach, Malabar spinach, and Saan choy. The plant has a deep green stem with thick and fleshy oval shaped leaves arranged throughout the length of the creeping stem. It is often mistaken for a counterpart in the Basellaceae family, *Basella rubra*, which has a pinkish or purple stem with pink colored veins running through the leaves (14). Ba has been reported to lower blood sugar levels and improve the health of diabetic rats when administered orally as an aqueous extract (15). However, as the mechanism by which Ba exerts its antidiabetic actions is relatively unknown, this is the focus of our present study. This research aimed to investigate modulation of cytokine levels in the serum and stimulation of beta-islet cell regeneration as the possible antidiabetic mechanisms of Ba.

MATERIALS AND METHODS

Preparation of plant extract. The research plant, Ba, was sourced fresh from Osun state in south western Nigeria, identified, and authenticated by the Department of Botany, University of Ibadan, Nigeria (voucher number: UIH-22391). The leaves were then washed and dried at room temperature to preserve heat-labile components. The dried leaves were subsequently ground to a powder and 100 g was extracted in 1,000 mL distilled water in accordance with the method described by Iloki-Assanga *et al* (16). The liquid extract was filtered through muslin cloth and the filtrate was freeze-dried for 24 hr to obtain a powdery extract that was subsequently dissolved in normal saline. This formed the stock solution that was adminis-

tered to the rats by oral gavage during the course of the study.

Animal care and ethical considerations. Male Wistar rats were obtained from Stellenbosch University animal house and acclimatized in the animal experimental laboratory of the Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa. The animals were given free access to food (standard rat chow) and water, with the exception of a period of overnight fasting prior to the collection of blood for fasting blood sugar (FBS) measurement. The rats were housed under standard atmospheric conditions and the animals were exposed to a 12-hr light/dark cycle. All experiments complied with the National Institutes of Health Guidelines (National Institutes of Health Publication No. 80-23, revised 1978) for the handling of laboratory animals. The Health and Wellness Sciences Research Ethics Committee (HWS-REC) of Cape Peninsula University of Technology, Cape Town, South Africa, granted ethical approval for this study (reference number: CPUT/HWS-REC 2015/A04).

Study design. Forty male Wistar rats, between 8 and 10 weeks old, were randomly divided into four groups (n = 10) and administered the following treatments via oral gavage: animals in the healthy control (H-c) and diabetic control (D-c) groups received normal saline at 0.5 mL/100 g body weight daily; animals in the healthy treatment (H-Ba) and diabetic treatment (D-Ba) groups received the plant extract at 200 mg/kg body weight daily.

Induction of DM. The rats from the D-c and D-Ba groups were administered a single intraperitoneal injection of streptozotocin (55 mg/kg) after an overnight fast. Prior to administration, streptozotocin was freshly dissolved in ice-cold citrate buffer (0.1 M), pH 4.5. The animals were subsequently permitted food and water and FBS was measured after 72 hr to confirm diabetes. The rats with an FBS value greater than 11.1 mmol/L (200 mg/dL) were considered diabetic (17) and included in the study.

Measurement of FBS and weight. The FBS and body weight were recorded weekly in all animals from the four experimental groups. The blood samples for FBS were collected from the tail capillaries by pricking the tip of the tail with a sterile lancet and expressing one or two drops of blood. This was applied in the appropriate manner to a glucometer (ONETOUCH® Ultra2) strip to determine the FBS levels. The rat weight was recorded using a portable electronic balance.

Pancreas and blood sample collection. All animals were euthanized via exsanguination with a high dose (100 mg/kg) of intraperitoneal sodium pentobarbital after a 4-week treatment. The blood was collected via cardiac puncture into serum clotting activator tubes (VACUETTE®), centrifuged at 4,000 g for 10 min at 4°C, and the serum was removed and stored at −80°C for the assessment of serum cytokine levels. The pancreas was carefully removed, washed, and weighed and then preserved in formalin for histological studies. The relative weight of the pancreas was calculated from the division of the pancreas weight by the animal's total body weight.

Assessment of serum cytokines. The evaluation of the various cytokine levels was performed on triplicate serum samples by using multiplex technology. The samples were removed from −80°C and allowed to thaw at room temperature prior to the assay. The analysis of cytokine levels was achieved by using the Bio-Plex Pro™ Reagent kit in 1 × 96-well flat-bottomed plate, Cat. #171-304070M (USA). The assays were performed in accordance with the product protocol manual (Merck Millipore, Billerica, MA, USA) and the Bio-Plex platform (Bio-Rad Laboratories, Hercules, CA, USA) was used to read the plate.

Histopathological analysis of the pancreas. Pancrease tissue samples from each rat were dissected, weighed, and immediately fixed in 10% buffered formalin. The tissues were processed by embedding in paraffin wax and sectioned at 4~6 μm thickness before staining with hematoxylin and eosin (H&E stain). The prepared slides were examined microscopically by using a Digital Microscope, VJ-2005 DN model Bio-microscope® (WanTong Precision Instruments Co., Ltd., China).

Determination of islet cell area and density. The morphometric analysis was conducted using TS View CX Image® Software (version 6.2.4.3) and Motic Image 2000 (Motic China Group Co., Ltd., Xiamen, China). For each slide, the islet parenchyma were observed and several images were captured at ×400 magnification. Discreet random islet areas were determined using (Motic China Group Co., Ltd.). The number of viable cells in each area was analyzed. The summation of all islet areas per slide was calculated to estimate the total area and, likewise, the summation of cell count values yielded the total cell count per slide; the total islet density was determined in each slide from the formula: Total cell count/Total area of count.

Statistical analysis. GraphPad Prism version 5.0 was used to analyze all the data. The values were expressed as the mean ± SEM and the differences between the means of each group were determined by one-way analysis of variance (ANOVA). The Bonferroni post-test was applied and values of $p < 0.05$ were considered to indicate statistical significance.

RESULTS

Effect of treatments on FBS levels. After a single intraperitoneal injection of streptozotocin in D-c and D-Ba rats, the FBS levels in both groups before the commencement of treatments were significantly higher than those in FBS in H-c and H-Ba rats ($p < 0.0001$). After a 4-week treatment period, the FBS levels were not significantly different in the rats in the H-c and H-Ba groups. The FBS level in the D-c rats was significantly increased ($p < 0.001$), but was significantly reduced in D-Ba rats ($p < 0.0001$) compared with readings recorded in the respective groups prior to the start of treatment (Table 1).

Effect of treatments on body weight, pancreas weight, and relative pancreas weight. After the 4-week treatment period, the body weight and weight of the pancreas were significantly ($p < 0.05$) lower in the two diabetic groups (D-c + D-Ba) compared with those in the healthy control rats. There were no significant differences in both body and pancreas weights between the two diabetic groups and the two control groups of rats. Although body weight was significantly ($p < 0.05$) lower in the D-Ba group compared with that in the H-Ba group, the weight of pancreas was not significantly different between the two groups. The relative weight of the pancreas was significantly higher in rats in the D-Ba group when compared with that in rats in both the H-c ($p < 0.05$) and H-Ba ($p < 0.005$) groups. There was no significant difference between the relative pancreatic weights of rats in the D-c group and of the other three groups (Fig. 1).

Effect of treatments on serum cytokine levels. The serum levels of IL-1β were significantly higher in samples from the D-c group compared with those in samples from all other groups ($p < 0.001$ v s H-c and $p < 0.05$ vs H-Ba and D-Ba). Similarly, samples from the D-c group had significantly ($p < 0.0001$) higher levels of MCP-1 compared with those from the other three groups (Fig. 2). The serum levels of IL-4 and IL-13 were not differ significantly among any of the four experimental groups. However, IL-10 level was significantly lower ($p < 0.05$) in the samples from the D-c when compared with those in the other groups. Furthermore, the serum IL-10 levels in H-c samples were significantly higher ($p < 0.05$) than the levels in samples from the D-Ba group (Fig. 3).

Table 1. Fasting blood sugar before commencement and after completion of four weeks of *Basella alba* treatment, Islet cell area and Islet cell density of Control and Treatment rats

Groups	Fasting blood sugar (mmol/L)		Islet cell area ($\times 10^5$ Sqμm)	Islet cell density ($\times 10^{-2}$ Cells/Sqμm)
	Before treatment	After treatment		
Healthy control (H-c)	4.48 ± 0.15	4.46 ± 0.14^{ns}	2.54 ± 0.36^{c}	$0.009 \pm 0.00^{\#}$
Diabetic control (D-c)	$18.69 \pm 1.36^{*}$	24.71 ± 1.14^{a}	$0.03 \pm 0.00^{*}$	$1.468 \pm 0.23^{*}$
Healthy treatment (H-Ba)	4.51 ± 0.15	4.48 ± 0.15^{ns}	$6.20 \pm 0.31^{*\#}$	$0.009 \pm 0.00^{\#}$
Diabetic treatment (D-Ba)	$17.74 \pm 1.34^{*}$	10.71 ± 0.41^{b}	$2.68 \pm 0.45^{\#}$	$0.012 \pm 0.00^{\#}$

Values are expressed as mean ± SEM, FBS readings before commencement of treatment in all groups were compared with that of H-c and readings before and after four weeks of treatment were compared in each group. Islet cell area and density in all experimental groups were compared with control groups.
The *p* values were denoted as; ^{ns}Not significant, $^{a}p < 0.001$, $^{b}p < 0.0001$ vs. values before treatment.
$^{*}p < 0.0001$ vs. H-c, $^{\#}p < 0.0001$ vs. D-c.
mmol/L = Millimoles per litre, Sqμm = Square micrometres.

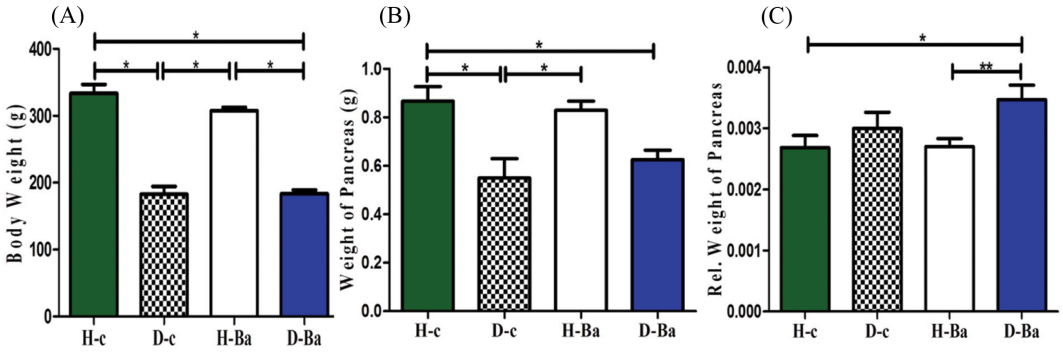

Fig. 1. Body weight (A), weight of pancreas (B) and relative weight of pancreas (C) of rats after 4 weeks of *Basella alba* treatment. $^{*}p < 0.05$, $^{**}p < 0.005$.

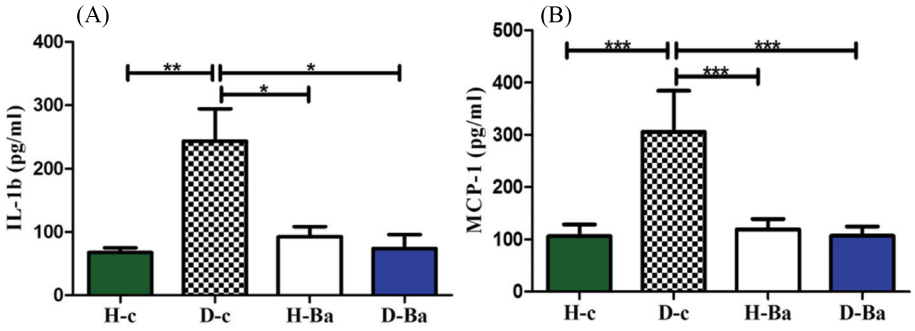

Fig. 2. Levels of a pro-inflammatory cytokine, IL-1β (A) and a chemokine, MCP-1 (B), in the sera of rats after 4 weeks of *Basella alba* treatment. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

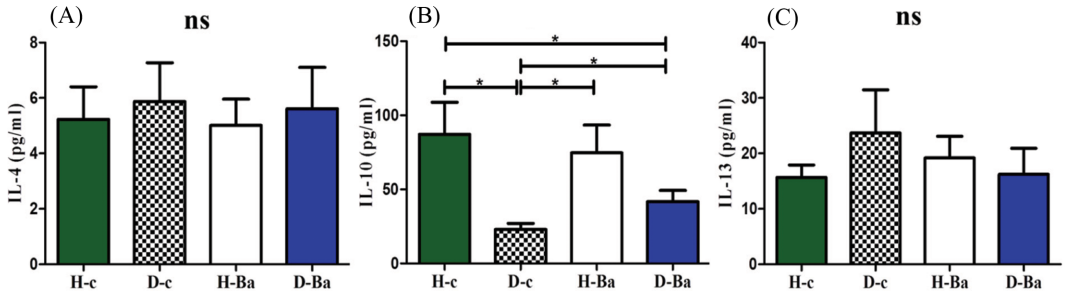

Fig. 3. Levels of the anti-inflammatory cytokines IL-4 (A), IL-10 (B) and IL-13 (C) in the sera of rats after 4 weeks of *Basella alba* treatment [ns = Not significantly different]. *$p < 0.05$.

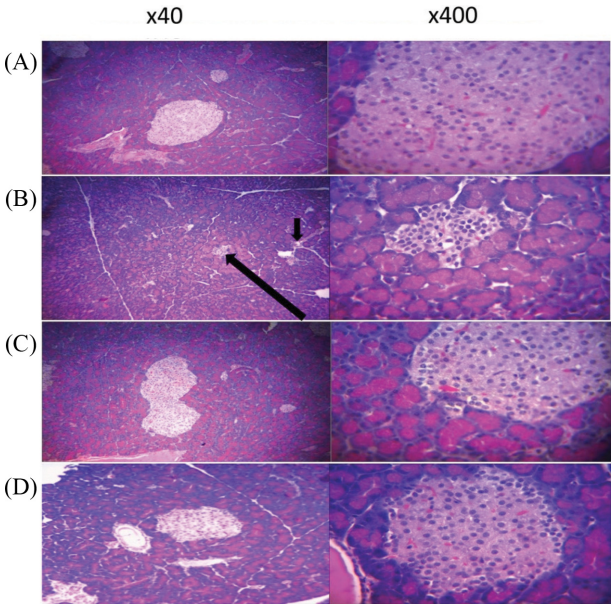

Fig. 4. Histopathological photomicrographs of the pancreas (H & E) showing slides from: (A) Healthy control rat with normal interstitium and islet section; (B) Diabetic control rat with reduced islet section (Long arrow) and severe interstitial congestion (Short arrow); (C) Healthy treatment rat with normal interstitium and islet section; and (D) Diabetic treatment rat with normal interstitium and islet section.

Effect of treatments on pancreatic histology. The histopathological analysis of the pancreas showed normal interstitial architecture and islet sections in slides from the H-c, H-Ba, and D-Ba groups; however, the islet sections in the H-c group appeared to be slightly larger in slides from the H-c group compared with those in the other groups. Severe interstitial congestion and reduced islet sections were observed in samples from the D-c group, in which the rats did not receive the plant extract (Fig. 4).

Effect of treatments on area and density of islet cell. As shown in the table, the islet cell area was significantly reduced in the D-c group when compared with those in the other three groups ($p < 0.0001$), whereas it was increased in the H-Ba group compared with those in the other groups ($p < 0.0001$). Conversely, the islet cell density was significantly lower in the H-c, H-Ba, and D-Ba rats ($p < 0.0001$) compared with that in the D-c group. There was no significant difference in the islet cell density among the rats in the H-c, H-Ba, and D-Ba groups.

DISCUSSION

The reduction in the FBS levels observed in the diabetic rats treated with aqueous Ba extract (D-Ba) con-

firmed the previously reported anti-hyperglycemic effect of the plant (15). Furthermore, levels of potent mediators of inflammation, such as IL-1β (18) and MCP-1, were considerably lower in the serum samples of the treated diabetic rats compared with those in the sera of their untreated counterparts, which alluded to the possibility of a correlation between the antihyperglycemic (15) and anti-inflammatory (19,20) effects of Ba. As mentioned in the introduction part, hyperglycemia stimulates the peripheral production of inflammatory cytokines via oxidative (6,7) and osmotic stress (8) mechanisms. These inflammatory mediators play a major role in the suppression of beta-islet cell function and ultimately in the induction of apoptosis of the cells by initiating and intensifying immune assaults (21). This implies that an unpleasant interplay exists between the high serum levels of inflammatory mediators and hyperglycemia in a vicious cycle that culminates in deterioration of the symptoms of T1DM. The prevention of one or all of the following occurrences (hyperglycemia, production of pro-inflammatory cytokines/chemokines, or the depletion of anti-inflammatory cytokines) is therefore expected to exert a positive impact on beta-islet cell function and survival in ongoing T1DM. In the present study, T1DM was induced by a single intraperitoneal injection of a high dose of streptozotocin (55 mg/kg) to sufficiently ablate the insulin production of beta-islet cells of the pancreas (22). We observed that the reduced levels of blood sugar and proinflammatory cytokines, but the higher IL-10 levels and the enhanced level of IL-10 in the blood serum from diabetic animals treated with the aqueous extract of Ba (i.e., D-Ba group). These clearly highlight the actions underlying the anti-inflammatory role of Ba. Gomphrenin I (15S-betanidin 6-O-β-glucoside), the principal coloring agent in Ba fruit, was found to be a potent antioxidant and inhibitor of inflammation (23). It was observed to inhibit the transcription and expression of genes encoding certain inflammatory cytokines, including IL-1β (23). Although Gomphrenin I was not isolated during the phytochemical analysis of aqueous leaf extract of Ba in a previous study (24), the possibility of its presence in the leaves cannot be excluded in light of the observations from this study. Furthermore, the established chemical constituents of the leaf extract, such as tannins, flavonoids, mucilage, and saponin (19), have strong antioxidant properties (25,26) which may also explain the ability of the extract to suppress the production of inflammatory cytokines. This is because the generation of oxygen free radicals is known to contribute significantly to the initiation and progression of systemic, as well as localized inflammatory responses, via the activation of nuclear factors that stimulate release of cytokines (27).

The histopathological findings in this study are also noteworthy. A recovery of previously lost islet regions in the pancreas of diabetic rats that were treated with the plant extract appears to have occurred, as evidence by the significantly larger islet area in comparison with the pancreas sections from their untreated counterparts. Additionally, even though body weight was significantly lost in both diabetic groups, the relative pancreatic weight loss was lower in the diabetic rats treated with Ba extract, which suggested a protective role for the plant with regard to the degenerative damage of the pancreas. From the present data, we were unable to ascertain whether the larger islet area in the treated rats occurred as a result of regeneration of new beta-islet cells or from the inhibition of progress of streptozotocin-induced apoptosis, but this topic should be explored for further research. It is also not exactly clear why a huge increase in islet area was observed in the healthy treatment group (H-Ba). This may be a partial result of the unchallenged action of Ba in the stimulation of islet cell proliferation. Presently, the curative therapies of T1DM are based on the restoration of endogenous insulin production either by the transplantation of beta-islet cells or the whole pancreas with concurrent immunosuppression (28,29). This has its own disadvantages, especially those that stem from the continuous need to suppress the immune system and the increased risk of vascular and metabolic complications (29). The ability to restart insulin synthesis using a natural medicinal agent that is capable of restoring islet function well enough to achieve better glucose homeostasis in T1DM would therefore be extremely valuable. Interestingly, islet cell density was observed to be highest in the diabetic control (D-c) rats that were not treated with the extract. This can be explained by the increases in the alpha-islet and delta-islet cell mass that usually occur after a selective streptozotocin-induced destruction of the insulin-producing beta-islet cells (30). The inflammatory milieu in the streptozotocin-induced model of T1DM actually allows for the alpha- and delta-cells to expand and proliferate at a faster rate than usual (30) to compensate for the lost beta-cells. There is also a marked increase in beta-islet cell proliferation during the early stages of T1DM in humans (31), which may also be a contributory factor to the extremely high islet cell density observed in the control diabetic rats. Further studies in this respect may include differential staining of the various islet cell types to specifically account for the composition of the islet population and the quantitative analysis of serum insulin and the other hormones produced by each cell type.

It was therefore concluded that the antidiabetic effect of Ba aqueous leaf extract is largely dependent on its ability to modulate the production of inflammatory cytokines and influence the proliferation, and possibly the function, of pancreatic islet. The apparent recovery of islet sections observed in this study offers future hope for an alternative to pancreas/islet transplantation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with regard to the publication of any part of this study.

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