Journal of Research in Pharmacy

Research Article

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Protective effect of *Clinacanthus nutans* on pancreatic β-cell mass and function in diabetic rats

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Received: 28 November 2023 / Revised: 1 February 2024 / Accepted: 2 February 2024

ABSTRACT: Preserving pancreatic β-cell mass and function provides a potentially effective approach for mitigating the development and aggravation of diabetes. This study aimed to prove the effect of *Clinacanthus nutans* on pancreatic β-cell mass and function in rat models of diabetes. The study involved grouping male Wistar rats into six different groups: normal control, diabetes control, diabetes treated with *Glibenclamide*, and diabetes treated with *C. nutans* doses of 100, 200, and 400 mg/kg body weight. Body weight, food intake, and fasting blood glucose levels were documented periodically during the study. Serum insulin levels were determined by the Enzyme-linked immunosorbent assay method. The Homeostatic model assessment of β-cell function (HOMA-β) value was calculated based on the given formula. Histopathological examination of pancreatic tissue was performed by Hematoxylin Eosin and Aldehyde Fuchsin staining. The results showed that administration of *C. nutans* extracts in diabetic rats for twenty-eight days significantly decreased fasting blood glucose levels. Additionally, there was an observed increase in serum insulin levels and HOMA-β values. Similarly, the morphometric analysis findings demonstrated enhancements in the area and number of islets, along with an increase in β-cells count and density. The findings suggest that *C. nutans* exhibits promising antidiabetic properties through its ability to enhance pancreatic β-cells mass and function.

KEYWORDS: *Clinacanthus nutans*; diabetes; fasting blood glucose; insulin; morphometric analysis, pancreatic β-cell.

1. INTRODUCTION

Diabetes mellitus (DM) has emerged as an important health issue due to its high worldwide prevalence, which reaches almost all ages, genders, and urban and rural areas. It affected 537 million people in 2021, predominantly in middle and low-income countries. This number is estimated to exceed 783 million by 2045, which is an extraordinary amount [1]. DM is a complex disease marked by hyperglycemia caused by insulin deficiency and impaired insulin sensitivity in peripheral organs. Absolute insulin deficiency is generated by autoimmune β-cells damage, whereas relative insulin deficiency is triggered by β-cell glucotoxicity [2]. Long-term hyperglycemia is responsible for significant morbidity in many organs due to macro- and microvascular complications such as cardiovascular disease, nephropathy, and amputation. This condition reduces patient productivity, threatens life, and increases mortality worldwide, thus becoming a socio-economic burden and forcing the state to spend numerous healthcare expenditures [3].

Pancreatic β-cells are vital in regulating glucose metabolism through adequate insulin production and secretion, which allows glucose in the blood to enter the cells and undergo metabolism to produce energy [4,5]. Long-term hyperglycemia promotes glucotoxicity and causes a negative impact on pancreatic β-cell mass and function [6]. A decrease in the number of pancreatic β-cell causes reduced insulin synthesis and further promotes disease progression in both types 1 and 2 of DM [7,8]. Pancreatic β-cell mass can change based on variations in insulin requirements both in physiological and pathological conditions [9,10]. An increase in pancreatic β-cell mass can occur by pancreatic β-cell progenitor differentiation, enhanced β-cell proliferation, and minimized β-cell mortality [11]. Therefore, pancreatic β-cell preservation may exert an

How to cite this article: Susanti N, Mustika A, Khotib J. Protective effect of *Clinacanthus nutans* on pancreatic β-cell mass and function in diabetic rats. J Res Pharm. 2024; 28(6): 2104-2113.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.885

effective strategy to prevent the aggravation of diabetes. Herbal plants, either in the form of single or mixed extracts and isolated active constituents, have been extensively studied for this purpose [12].

Clinacanthus nutans is a herbal plant with a wide distribution in the Southeast Asia, including Indonesia, Malaysia and Thailand [13]. This plant has long been utilized by the community to treat a variety of conditions, such as skin diseases, cancer, diabetes, and inflammation [14]. Its leaves contain a wide range of phytochemicals, including phenolics, flavonoids, triterpenoids, and phytosterols, which have been widely explored for their antioxidant, anti-inflammatory, analgesic, antidiabetic, anti-dyslipidemia, anticancer, antiviral, antimicrobial, antivenom, antifungal, immunomodulatory, and neuroprotective properties [15]. Previous studies have discovered that *C. nutans* can modulate fasting blood glucose levels in diabetic experimental rats [16-18]. However, the comprehensive understanding of its effect on dysfunction of pancreatic β-cells remains unclear. Hence, this research aims to prove the beneficial effects of *C. nutans* on insulin levels, HOMA-β value and histomorphometry of the pancreas.

2. RESULTS

2.1 Effect of *C. nutans* on body weight and food intake

Figure 1A-B displays the development of body weight and the amount of food intake over the study. The diabetes control (DC) group demonstrated a gradual decrease in weight loss ($P<0.05$) and an increase in food intake ($P>0.05$) in comparison with the normal control (NC) group. The body weight (BW) of the DC group at the end of the study was 14% lower than the initial value, while the food intake increased by 39% compared to the initial amount. The final weight loss rate compared to the initial value was the lowest in the diabetic rats were given *Glibenclamide* (0.45 mg/kg BW) (GLB) and *C. nutans* extract (400 mg/kg BW) (CN400) groups at 4%, followed by (200 mg/kg BW) (CN200) and (100 mg/kg BW) (CN100) groups at 6% and 7%, respectively. Likewise, the rate of increase in food intake compared to the initial amount ranged from lowest to highest in the GLB, CN400, CN200 and CN100 groups, i.e. 16%, 21%, 21% and 26%, respectively.

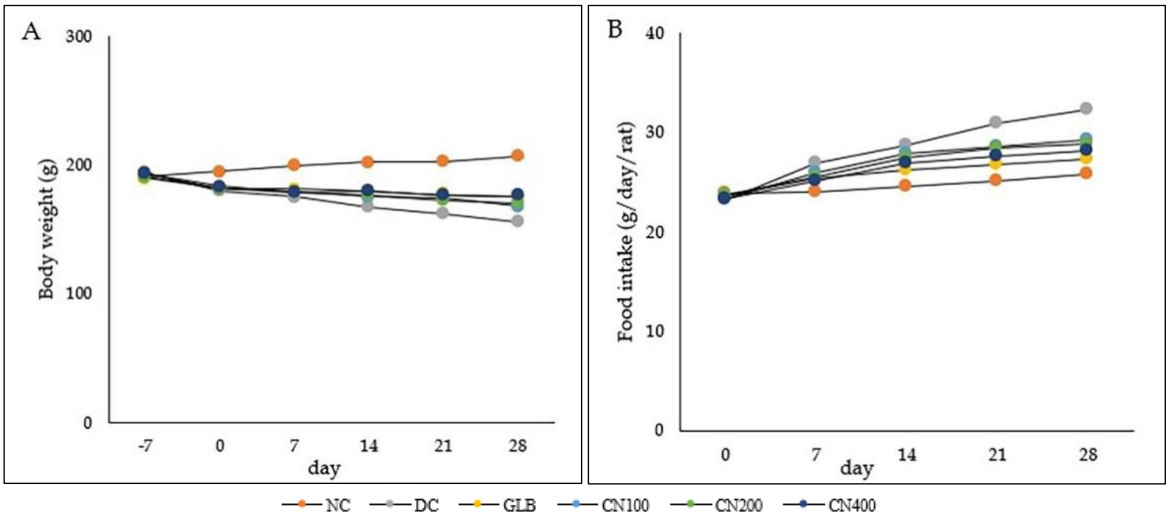

Figure 1. Effect of *C. nutans* on body weight in diabetic rats before (-7 days) and after STZ injection at 0, 7, 14, 21, and 28 days of treatment (A) and food intake in STZ-induced diabetic rats before (0 day) and after (7, 14, 21, and 28 days) of treatment (B).
NC: normal control; DC: diabetic control; GLB: diabetic rats were given *Glibenclamide* (0.45 mg/kg BW); CN100: diabetic rats were given *C. nutans* extract (100 mg/kg BW); CN200: diabetic rats were given *C. nutans* extract (200 mg/kg BW); CN400: diabetic rats were given *C. nutans* extract (400 mg/kg BW).

2.2 Effect of *C. nutans* on fasting blood glucose level

Figure 2 shows variations in fasting blood glucose levels during the study. According to the data, fasting blood glucose levels in all groups were within the normal range, with an average of 91.46 mg/dl before Streptozotocin (STZ) injection. The fasting blood glucose of the normal control remained at normal levels throughout the study period. Meanwhile, fasting blood glucose levels in the DC group increased significantly ($p<0.05$) compared to the NC group and stayed high until the end of the study. Treatment with

C. nutans extract and *Glibenclamide* reduced fasting blood glucose levels significantly ($p<0.05$) compared to the DC group. The hypoglycemic rate was calculated based on the percentage decrease in blood glucose levels on day 28 compared to day 0. The hypoglycemic rate in the groups that received *C. nutans* extract at doses of 100, 200, and 400 mg/kg, was respectively 41%, 45%, and 46%. In the group that received *Glibenclamide*, the hypoglycemic rate was the highest, i.e. 58%.

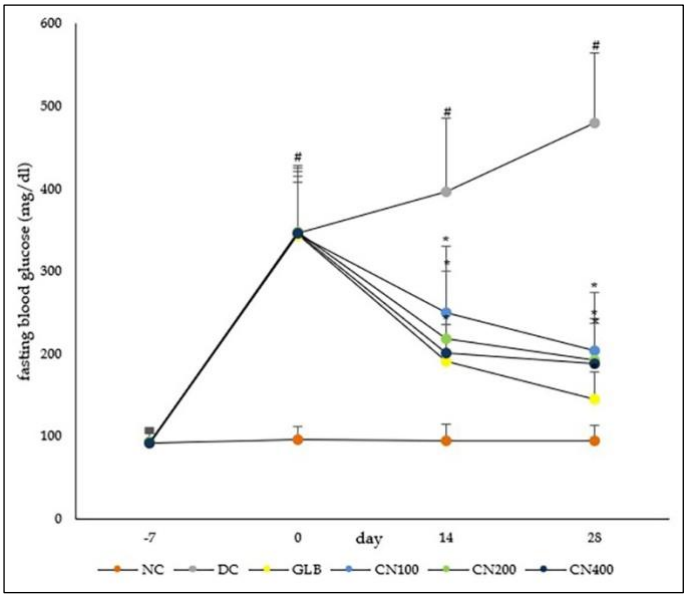

Figure 2. Effect of *C. nutans* on fasting blood glucose level in diabetic rats before (-7 days) and after STZ injection at 0, 14, and 28 days of treatment.
Data represented mean ± SD. * P<0.05 significantly different from DC group; # P<0.05 significantly different from NC group. NC: normal control; DC: diabetic control; GLB: diabetic rats were given *Glibenclamide* (0.45 mg/kg BW); CN100: diabetic rats were given *C. nutans* extract (100 mg/kg BW); CN200: diabetic rats were given *C. nutans* extract (200 mg/kg BW); CN400: diabetic rats were given *C. nutans* extract (400 mg/kg BW).

2.3 Effect of *C. nutans* on fasting serum insulin levels and HOMA-β value

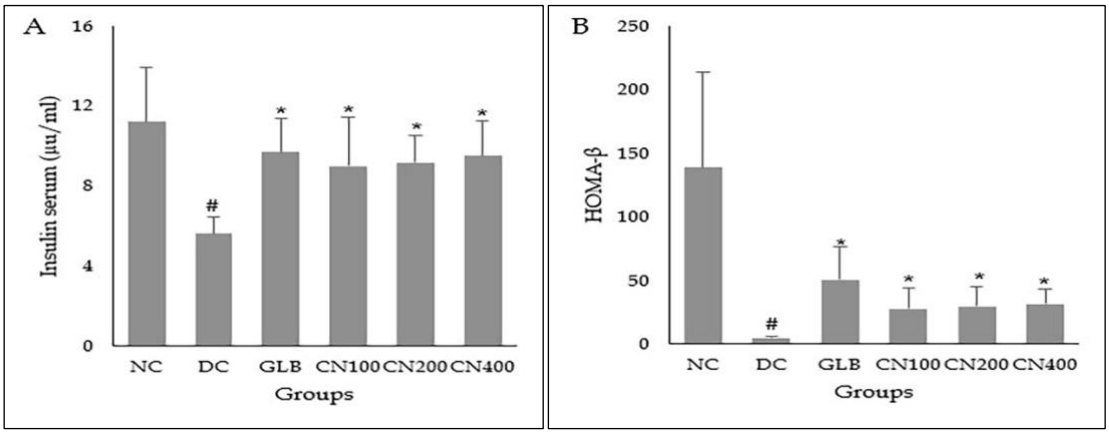

Figure 3. Effect of *C. nutans* on fasting serum insulin levels (A) and HOMA-β values (B) in diabetic rats.
Serum insulin was measured by the ELISA method and HOMA-β value was calculated as (360xserum insulin)/(blood glucose level-63). Data represented mean ± SD. * P<0.05 significantly different from DC group; # P<0.05 significantly different from NC group. NC: normal control; DC: diabetic control; GLB: diabetic rats were given *Glibenclamide* (0.45 mg/kg BW); CN100: diabetic rats were given *C. nutans* extract (100 mg/kg BW); CN200: diabetic rats were given *C. nutans* extract (200 mg/kg BW); CN400: diabetic rats were given *C. nutans* extract (400 mg/kg BW).

As shown in Figure 3A, fasting serum insulin levels in the DC group were significantly lower ($p<0.05$) than in the NC group. Treatment with *Glibenclamide* and *C. nutans* extract given to diabetic rats can significantly reverse this effect, as evidenced by an increase in fasting serum insulin levels in comparison with the DC group ($p<0.05$). Based on the HOMA-β value as demonstrated in Figure 3B, β-cell function decreased sharply in the DC group in comparison with the NC group. Conversely, the HOMA-β value

increased significantly at all doses of *C. nutans* extract and *Glibenclamide* in comparison with the DC group ($p<0.05$).

2.4 Effect of *C. nutans* on pancreatic histomorphometry

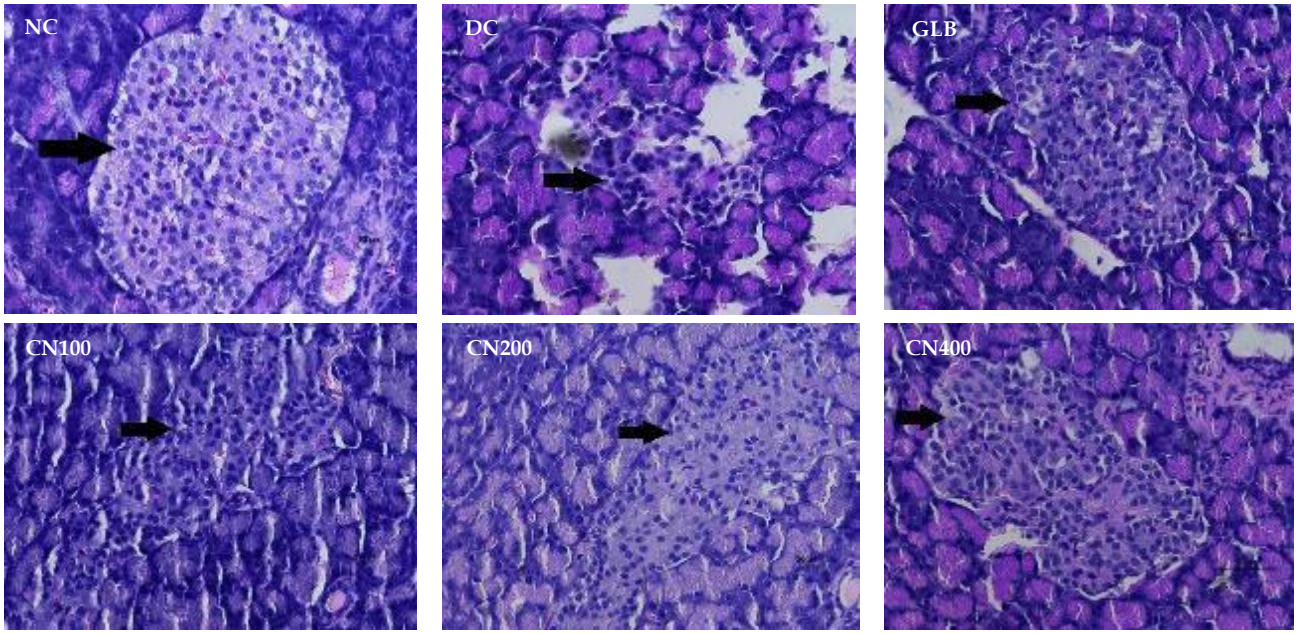

Figure 4. Histopathological feature of pancreatic islets with HE staining (magnification 400x, scale 50 µm, black arrows indicate pancreatic islets)
NC: normal control; DC: diabetic control; GLB: diabetic rats were given *Glibenclamide* (0.45 mg/kg BW); CN100: diabetic rats were given *C. nutans* extract (100 mg/kg BW); CN200: diabetic rats were given *C. nutans* extract (200 mg/kg BW); CN400: diabetic rats were given *C. nutans* extract (400 mg/kg BW).

Histopathological examination of pancreatic tissue with Hematoxylin Eosin (HE) and Aldehyde Fuchsin (AF) staining is demonstrated in Figures 4 and 5. As shown in Figure 4, the pancreatic islets of the NC group exhibited normal morphology. In contrast, the pancreatic islets of the DC group showed severe damage, vacuolization, and smaller islet sizes. A lower degree of damage was observed in the group that received *Glibenclamide* and *C. nutans* extract compared to the DC group. AF staining makes the β-cells stained purple so they can be observed. The NC group showed abundant β-cells scattered inside the pancreatic islets. In contrast, the DC group showed a marked decrease in β-cells counts observed in the pancreatic islets. An increase in β-cells counts was observed in the group that received *Glibenclamide* and *C. nutans* extract compared to the DC group (Figure 5).

The histomorphometric analysis presented in Table 1 includes islets area and number, β cells count and density. Area was calculated on large (>10,000 µm) and small (<10,000 µm) islets. The results showed that the area of large and small islets decreased significantly in the DC group in comparison with the NC group. A significant increase in the area of the large islets was observed in the CN200 and CN400 groups in comparison with the DC group, whereas in the GLB and CN100 groups there was no significant effect. However, no significant difference in small islet areas in all treated groups in comparison with the DC group. The number of islets was determined per field of view. The DC group exhibited a significant decrease in the number of islets compared to the NC group, while the treated groups displayed a significant enhancement in the CN200 and CN400, but not in the GLB and CN100 groups. β-cells counts were determined by quantifying the nuclei stained with AF staining, while β-cells density was calculated by quantifying β-cells counts per observed area. β-cells count and density significantly decreased in the DC group in comparison with the NC group. Treatment with *C. nutans* extract at doses of 200 and 400 mg/kg BW significantly increased the β cells count and density in comparison with DC group. However, the increase observed in the GLB and CN100 groups was not statistically significant.

3. DISCUSSION

Dysfunction of pancreatic β-cell is an important factor in the advancement of diabetes. During the early stages of diabetes, there is an observed decrease in the mass of β-cells before the onset of impaired β-

cell function. The reduction in β-cell mass affects the regulation of glucose levels, leading to persistent hyperglycemia [19,20]. One of the diabetes therapeutic strategies is to preserve pancreatic β-cells mass and function so they can produce and secrete adequate insulin for glucose metabolism in the cells and maintain normal blood sugar levels [21,22]. Herbal plants have been widely studied to have beneficial effects against the dysfunction of pancreatic β-cells [12]. Therefore, this research evaluated the beneficial effects of *C. nutans* on pancreatic β-cell mass and function of diabetic rats.

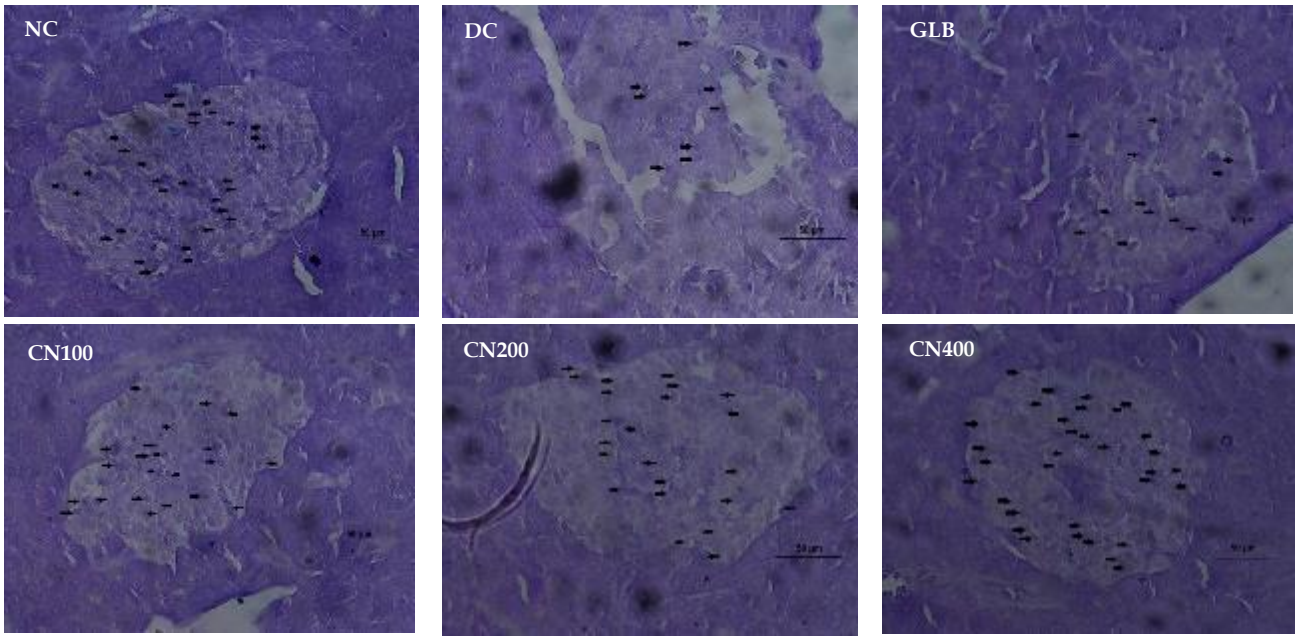

Figure 5. Histopathological feature of pancreatic islets with AF staining (magnification 400x, scale 50 μm, black arrows indicate pancreatic β-cells).
NC: normal control; DC: diabetic control; GLB: diabetic rats were given *Glibenclamide* (0.45 mg/kg BW); CN100: diabetic rats were given *C. nutans* extract (100 mg/kg BW); CN200: diabetic rats were given *C. nutans* extract (200 mg/kg BW); CN400: diabetic rats were given *C. nutans* extract (400 mg/kg BW).

Table 1. Effect of *C. nutans* on pancreatic histomorphometry in diabetic rats.

Groups	Large islets area (μm^2)	Small islets area (μm^2)	Number of islets	β-cells count	β-cells density (cells/ 10^3 μm^2)
NC	28,360 ± 5,616	7,452 ± 2,003	2.60 ± 0.54	118.22 ± 19.71	4.29 ± 0.67
DC	14,576 ± 3,797 #	3,979 ± 1,433 #	1.45 ± 0.44 #	30.25 ± 18.43 #	2.00 ± 1.25 #
GLB	18,193 ± 6,118	5,717 ± 2,201	1.67 ± 0.46	61.17 ± 17.77	3.40 ± 1.00
CN100	19,832 ± 5,824	6,061 ± 2,127	1.82 ± 0.42	71.51 ± 16.95*	3.55 ± 0.92
CN200	23,895 ± 5,009 *	6,140 ± 1,824	2.30 ± 0.54*	73.85 ± 16.67*	3.70 ± 0.92
CN400	24,854 ± 3,125 *	6,301 ± 1,903	2.37 ± 0.39 *	74.70 ± 20.81*	3.78 ± 1.15*

Data represented mean ± SD. * P<0.05 significantly different from DC group; # P<0.05 significantly different from NC group. NC: normal control; DC: diabetic control; GLB: diabetic rats were given *Glibenclamide* (0.45 mg/kg BW); CN100: diabetic rats were given *C. nutans* extract (100 mg/kg BW); CN200: diabetic rats were given *C. nutans* extract (200 mg/kg BW); CN400: diabetic rats were given *C. nutans* extract (400 mg/kg BW).

Diabetic rats display signs of diabetes, induced by streptozotocin, characterized by increased blood glucose levels, excessive food intake, and weight loss [23]. These characteristics correspond to the finding in this study, as indicated in Figure 1 and Figure 2. Streptozotocin is an antibiotic widely used to induce diabetes in animal models due to its capacity to act specifically on pancreatic β-cells and trigger DNA alkylation [24]. Streptozotocin gains entry into pancreatic β-cells via glucose transporter (GLUT)-2 protein, leading to overabundance of reactive oxygen species (ROS) and nitric oxide (NO), which further triggers oxidative stress and inflammation [25]. Consequently, increased inflammatory cytokine release promotes

endoplasmic reticulum (ER) stress, which exacerbates oxidative damage [26,27]. These two conditions then induce pancreatic β-cells destruction and result in hyperglycemia [28]. On the other hand, weight loss and increased food intake are triggered by impaired utilization of carbohydrates as an energy source, thereby increasing the metabolism of other macromolecules and leading to fat and muscle tissue loss [29]. Treatment with *C. nutans* controlled food intake, prevented excessive weight loss, and reduced blood glucose levels.

In this study, pancreatic β-cells function was evaluated from serum insulin levels and HOMA-β values (Figure 3). Decreased serum levels of insulin in diabetic rats indicate impaired insulin secretion by pancreatic β-cells. Once produced, insulin is restructured into its mature form and stored in secretory granules. A metabolic signal stimulates K^+-ATPase channel closure, which causes Ca^{2+} influx and triggers the release of insulin granules from pancreatic β-cells [30]. Administration of *C. nutans* significantly increased fasting serum insulin levels. The ability to stimulate insulin secretion can be assessed by the HOMA-β value, a mathematical model calculated from fasting blood glucose and serum insulin levels [31]. Diabetic rats showed a sharp decrease in HOMA-β value of more than 90% compared to normal rats, while β-cell function significantly increased in all treated groups. Hence, the ability of *C. nutans* to minimize diabetic symptoms and improve pancreatic β-cells function indicates the potential value of this herb in diabetes treatment.

The regulation of β-cell mass is determined by the equilibrium between β-cell proliferation and apoptosis and the presence of islet hyperplasia and new islet production from the exocrine duct [32]. Pancreatic-duodenal homeobox-1 (PDX-1) plays multiple roles in pancreatic β-cell proliferation and survival [33]. PDX-1 promotes insulin gene transcription and other genes, including glucose transporter-2 (GLUT-2) and glucokinase (GCK) [34]. The levels of PDX-1 mRNA and protein are reduced by prolonged hyperglycemia [35]. In addition, oxidative stress in diabetic conditions inhibits PDX-1 localization in the nucleus and reduces PDX-1 binding to promoter of insulin gene, thereby inhibiting insulin synthesis [36]. Previous research has shown that diabetic rats had lower PDX-1, GCK, and GLUT-2 protein expression levels, leading to impaired β-cell proliferation, insulin biosynthesis and secretion [37-39]. Furthermore, glucose toxicity in pancreatic β-cell triggers the caspase cascade activation, leading to DNA damage and subsequent cellular apoptosis [40].

Histopathological findings showed recovery of the area and quantity of pancreatic islets in groups treated with *C. nutans*. This condition was proven in this study, where the area of the large islets and the number of islets were significantly larger compared to untreated diabetic rats. However, significant differences were not found in the area of small islets. The mass of pancreatic β-cells will continue to decrease in line with the development of diabetes, contributing to impaired insulin secretion and further hyperglycemia [41,42]. Besides repairing islets, *C. nutans* increased β-cells counts and density compared to the untreated group. From the data of this study, it is not certain whether the increase in area and number of islets in the group receiving *C. nutans* extract therapy was due to the formation of new islets or protection against damage. Likewise, the number and density of pancreatic β-cells was increased due to cell proliferation or apoptosis inhibition induced by streptozotocin. Both may be the mechanism underlying the effect of *C. nutans* on the repair of pancreatic β-cell mass and function.

The remarkable capacity of *C. nutans* to reduce hyperglycemia and improve the dysfunction of pancreatic β-cells in diabetic rats can be attributed to its flavonoid content. *C. nutans* has been confirmed from previous studies as a source of flavonoids in the flavon class, consisting of isoorientin, orientin, isovitexin, vitexin, shaftoside, and apigenin [43-45]. Prior research has demonstrated that flavonoids can potentially preserve the function and viability of β-cell. The protective effects of flavonoids against β-cell mortality in diabetes can be attributed to their antioxidant and anti-inflammatory activities and their capacity to activate anti-apoptotic and suppress pro-apoptotic proteins [46]. Nevertheless, the precise mechanism through which *C. nutans* elicits this action has not been further elucidated in the present study. Consequently, a more extensive investigation is warranted to establish the molecular pathway responsible for ameliorating pancreatic β-cell dysfunction following the administration of this herb.

4. CONCLUSION

Our findings prove that *C. nutans* exhibits promising effects on the pancreatic β-cells mass and function in rats with diabetes. The observed effect is supported by hypoglycemia, an elevation in serum

insulin levels, an augmentation in the HOMA-beta value, an improvement in the area and quantity of islets, and an increase in β-cells counts and density. Conducting more extensive investigations to validate the antidiabetic efficacy of this herb is important before implementing it in clinical settings.

5. MATERIALS AND METHODS

5.1 Preparation of *C. nutans* extract

Leaves of *C. nutans* were obtained from UPT Herbal Laboratory of Materia medica Batu, Indonesia. The dry powder was extracted with 70 % ethanol (1:10) by the sonication method. The filtrate was evaporated with a Rotary Evaporator (Heidolph Hei-VAP ML Adv/Pre) and then concentrated in an oven at 40 ^{o}C (45). The dry extract obtained was kept at 4 ^{o}C for further experiments.

5.2 Experimental Design

This study used male Wistar rats aged three months with an average weight of approximately 200 g. Rats were obtained from animal farms in Malang, Indonesia. The research was conducted in the Animal and Biomedical Laboratory, Faculty of Medicine and Health Sciences, Maulana Malik Ibrahim State Islamic University, Malang, Indonesia. Histopathological examination was carried out in the Anatomical Pathology Laboratory of Dr. Soetomo Hospital, Surabaya, Indonesia. All rats were placed in collective cages at room temperature with alternate light cycles. All rats had unlimited access to regular food and water before and during the experiment. The research obtained ethical clearance from the Health Research Ethics Commission number 083/EC/KEPK-FKIK/2022.

All rats were induced diabetes by a single intraperitoneal injection of Streptozotocin (STZ, Santa Cruz Biotech, USA) at a dose of 45 mg/kg body weight [47]. Fasting blood glucose (FBG) levels were assessed from the tail vein seven days post-injection utilizing a glucometer (Accu-Check Active, Roche Diagnostics). Rats having FBG levels of more than 200 mg/dL were classified as diabetics and then divided into six groups.

NC: Normal rats as normal control
DC: diabetic rats as diabetes control
GLB: diabetic rats were given *Glibenclamide* (0.45 mg/kg BW)
CN100: diabetic rats were given *C. nutans* extract (100 mg/kg BW)
CN200: diabetic rats were given *C. nutans* extract (200 mg/kg BW)
CN400: diabetic rats were given *C. nutans* extract (400 mg/kg BW)

The extract and *Glibenclamide* were given by intragastric gavage for 28 days. The dose of *C. nutans* used refers to research by Umar Imam (2019) [18]. This dose has been categorized as safe to use in rats based on toxicity tests in previous research [48,49]. The dose was adjusted based on weekly changes in body weight to maintain the same dose per kg BW. During treatment, food intake was calculated daily, body weight was calculated weekly, and FBG was measured every two weeks.

5.3 Measurement of Insulin Levels

After 12 hours of fasting, the rats were sacrificed with a Ketamine-Xylazine injection. Blood samples were obtained by cardiac puncture, left for about 20 minutes, and then centrifuged at 3000 rpm for 20 minutes to get serum. Serum insulin levels were measured using a Rat Insulin Enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory, China) as directed by the manufacture. Homeostatic model assessment of β-cell function (HOMA-β) is determined based on the formula [50]:

$$\text{HOMA-}\beta = \frac{360 \times \text{serum insulin}}{\text{Blood glucose level} - 63}$$

5.4 Histopathological examination

After removal, the pancreatic samples obtained from each rat were rapidly fixed in a 10% buffered formalin solution. Samples were embedded in paraffin and subsequently sliced at a thickness of five micrometres. The slices underwent staining with Hematoxylin Eosin (HE) and Aldehyde Fuchsin (AF)

according to the routine protocol. Images were captured on a light microscope (Nikon E200, Japan) with a magnification of 400x in 10 fields of view containing different Langerhans islets. The evaluation was done with ImageJ 1.53k software (NIH, USA). The histomorphometric analysis was adapted from Majd et al. and Arokoyo et al., including the area and number of islets and β-cells count and density [51,52]. To accurately estimate the area, islets were grouped into two categories: large (>10,000 µm) and small (<10,000 µm). The average area per field of view was calculated for each islet category. The number of islets, both large and small, were counted at 100x magnification in 10 different fields of view. The number of β-cells was counted from the sample section with AF staining where the nuclei were stained with purple and a colourless background. Cell density in different islets was calculated by the formula: number of β-cells/area of islets.

5.5 Statistical Analysis

The data reported in this study is expressed as the mean value ± standard deviation. Statistical calculations were conducted utilizing SPSS 26 statistic software. Differences between groups were analyzed by one-way variance analysis (ANOVA) and a post-hoc test. Statistical significance was determined if the p-value was less than 0.05.

Acknowledgements: We thank the Biomedical Laboratory of the Faculty of Medicine and Health Sciences, Maulana Malik Ibrahim State Islamic University for the research facility and the Anatomical Pathology Laboratory of Dr. Sutomo for the histopathological examination. The first author also expresses gratitude for the scholarship provided by Project Management Unit under scheme The Development of Maulana Malik Ibrahim State Islamic University of Malang Phase II in East Java Project.

Author contributions: Concept – N.S.; Design – N.S., A.M., J.K.; Supervision – A.M. J.K.; Resources – N.S.; Materials – N.S.; Data Collection and/or Processing – N.S.; Analysis and/or Interpretation – N.S.; Literature Search – N.S.; Writing – N.S.; Critical Reviews – N.S., A.M., J.K.

Conflict of interest statement: The authors declared no conflict of interest.

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