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Clinacanthus nutans leaf extract reduces pancreatic β-cell apoptosis by inhibiting JNK activation and modulating oxidative stress and inflammation in streptozotocin-induced diabetic rats

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Abstract

Background: Controlling apoptosis induced by oxidative stress in pancreatic β-cells provides promising strategies for preventing and treating diabetes. *Clinacanthus nutans* leaves possess bioactive constituents with potential antioxidant and anti-diabetic properties.

Aim: This study aimed to investigate the molecular mechanisms by which *C. nutans* extract protects pancreatic β-cells from apoptotic damage in streptozotocin (STZ)-induced diabetic rats.

Methods: Diabetes was induced in male Wistar rats by intraperitoneal injection of 45 mg/kg STZ, followed by 28 days of treatment with *C. nutans* leaf extract and Glibenclamide as the standard drug. At the end of the study, blood samples were collected to measure glucose levels, oxidative stress markers, and inflammation. Pancreatic tissue was stained immunohistochemically to detect c-Jun N-terminal kinase (JNK) and Caspase-3 expression.

Results: The administration of *C. nutans* leaf extract to diabetic rats significantly reduced fasting blood glucose, malondialdehyde, and tumor necrosis factor-α levels, while concurrently enhancing the activity of superoxide dismutase. The immunohistochemical studies revealed a decrease in the expression of JNK and caspase-3 in the pancreatic islets of diabetic rats.

Conclusion: *Clinacanthus nutans* exhibits the potential to protect pancreatic β-cells from apoptosis by suppressing oxidative stress and inflammation.

Keywords: Apoptosis, *Clinacanthus nutans*, Diabetes, Inflammation, Oxidative stress.

Introduction

The prevalence of diabetes mellitus (DM) has reached 10.5% of the worldwide population by 2021, which makes it a significant global health problem (Sun *et al*., 2022). DM is distinguished by elevated levels of glucose in the bloodstream, which arise from chronic progressive metabolic abnormalities related to insufficient production and/or utilization of insulin by the body (Banday *et al*., 2020; ADA, 2021) The persistence of chronically elevated blood glucose levels has detrimental effects on several tissues, including the pancreatic β-cells. This condition is responsible for the advancement of DM and the development of multiple complications affecting both macrovascular and microvascular systems (Harding *et al*., 2019; Goyal and Jialal, 2020).

Alternative pathways for glucose metabolism are activated when oxidative phosphorylation becomes saturated due to hyperglycemia. As a consequence, the production of reactive oxygen species (ROS) is upregulated (Costes *et al*., 2021). Conversely, it has been observed that hyperglycemia may suppress the synthesis of endogenous antioxidants, such as superoxide dismutase (SOD) (Gerber and Rutter, 2017). ROS production that exceeds the body's antioxidant defense capacity can result in a state of oxidative stress (Wang and Wang, 2017). ROS can oxidize lipid components inside cellular structures, generating malondialdehyde (MDA). Therefore, quantifying MDA levels becomes a marker for assessing cellular damage caused by oxidative stress (Ohiagu *et al*., 2021). On the other hand, the production of proinflammatory cytokines, mainly tumor necrosis

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factor-alpha (TNF-α), is enhanced in the presence of high quantities of ROS, exacerbating oxidative stress. Both conditions, oxidative stress, and inflammation, have been identified as the primary mechanisms underlying hyperglycemia-induced dysfunction and death of pancreatic β-cell (Ortega-Camarillo, 2019).

One of the molecular pathways contributing to pancreatic β-cell damage by oxidative stress and inflammation is the involvement of c-Jun N-terminal kinase (JNK) (Kaneto *et al*., 2005). It has been reported that multiple stress signals, such as cytokines and glucolipotoxicity, can activate the JNK pathway. Previous research has demonstrated that the levels of JNK protein expression are elevated in cultures of pancreatic β-cell and pancreas of diabetic rats exposed to STZ (Tang *et al*., 2018; Liu *et al*., 2019). The JNK pathway modulates apoptotic signaling in pancreatic β-cell by modulating the caspase cascade (Dhanasekaran and Reddy, 2017). Caspase-3 is recognized as one of the key apoptotic executors, and its elevated activity indicates the presence of cell apoptosis (Palai and Mishra, 2015).

Regarding the potential detrimental effect of higher ROS generation on pancreatic β-cell, modulating the capacity for antioxidants could provide advantageous outcomes. Therefore, controlling cellular stress-induced apoptosis in pancreatic β-cell suggests a potential therapeutic strategy for diabetes (Salazar-García and Corona, 2021). Several studies have demonstrated that natural compounds with antioxidant capability can reduce oxidative stress and proinflammatory cytokines, protecting β-cell from damage caused by ROS (Tabatabaie and Yazdanparast, 2017). Hanchang *et al*., 2019). *Clinacanthus nutans* is a widely utilized herbal plant that has been investigated for its properties in treating diabetes (Chia *et al*., 2022) *C. nutans* has demonstrated anti-diabetic properties by inhibiting α-glycosidase in vitro (Alam *et al*., 2017). This plant also showed the ability to decrease blood glucose levels and oxidative stress markers while enhancing markers of antioxidant status in diabetic rats (Sarega *et al*., 2016a; Umar-Imam *et al*., 2019). However, the exact mechanisms of how *C. nutans* exhibits its beneficial effect on pancreatic β-cells in diabetes remain unknown.

This study examines the molecular mechanisms by which *C. nutans* extract preserves pancreatic β-cells from apoptotic damage in diabetic rats. Specifically, the study intends to determine how the extract modulates the JNK pathway through the antioxidant and anti-inflammatory defense systems.

Material and Methods

Chemicals

Streptozotocin (STZ) was acquired from Santa Cruz Biotechnology USA. The ELISA kit used to detect TNF-α was acquired from Bioassay Technology Laboratory China (No. E0764Ra). The Total SOD Activity (No. E-BC-K019-S) and MDA Colorimetric (No. E-BC-K025-S) Assay Kit were obtained from Elabscience Biotechnology China. The polyclonal antibodies against JNK (No. bs-2900R) and Caspase3 (No. bs-0081R) were acquired from Bioss USA.

Plant extraction

Extraction of *C. nutans* leaves was according to the method described in our previous study (Susanti *et al*., 2023). The leaves of *C. nutans* were acquired from the UPT Herbal Laboratory of Materia Medica Batu, Indonesia, with determination number 074/042/102.7-A/2022. The leaves undergo the process of drying in an oven set at a temperature of 50°C, followed by subsequent grinding and sieving procedures. One hundred grams of *C. nutans* powder was dissolved in 1,000 ml of 70% ethanol as solvent (ratio 1:10). The extraction process was carried out using the sonication method with the SONICA 2400EP S3 instrument. Solvents were evaporated under reduced pressure before drying in an oven. The resulting dry extract was subsequently stored in a glass container at a temperature of 4°C for subsequent experimental procedures.

Animals

This study used male Wistar rats weighing 190.63 ± 19.00 g and aged approximately three months. Rats were obtained from animal farms in Malang, Indonesia, and were certified healthy by a veterinarian from the Department of Agriculture of Malang City No. 524.3/088/35.73.309/2022. All rats were kept in cages at a regulated temperature with alternate light cycles for 12 hours and ad libitum access to standard feed and water. The animals were housed according to the Guidelines for the Care and Use of Laboratory Animals of the US National Institutes of Health and the EU recommendations (Directive 2010/63/EU) for animal experiments.

Diabetes induction

Following a period of acclimatization lasting seven days, rats that had undergone overnight fasting were induced to develop diabetes with the intraperitoneal injection of a single dose of Streptozotocin (STZ). Before injection, STZ was freshly prepared in a 0.1 M citrate buffer pH 4.5. The dosage administered was 45 mg/kg of body weight. The rats were given a 10% sucrose solution for 48 hours following injection to prevent the risk of sudden death caused by hypoglycemia. Seven days after the injection of STZ, fasting blood glucose levels were measured by a glucometer (Accu-Check Active, Roche Diagnostics). Rats with fasting blood glucose levels of more than 200 mg/dl were identified as diabetic and used in this experiment. The increase in blood sugar belongs to the intermediate level category (151–250 mg/dl) (Fajarwati *et al*., 2023).

Experimental design

The rats were divided into six groups using a random allocation method. Each group included six rats. Group 1 consisted of rats not injected with STZ (normal control). Group 2 consisted of STZ-injected rats (diabetes control). Group 3 consisted of rats

injected with STZ and administered 0.45 mg/kg BW Glibenclamide. Rats in Groups 4, 5, and 6 were injected with STZ and administered *C. nutans* leaf extract at 100, 200, and 400 mg/kg BW, respectively. The administration of *C. nutans* leaf extract and Glibenclamide were given using an intragastric tube for 28 days. The dosage was adjusted based on weekly variations in body weight to ensure a consistent dosage per kilogram of body weight in rats.

Measurement of biomarkers for oxidative stress and inflammation

On the 29th day of the experiment, after an overnight fasting period, the rats were sacrificed following anesthetization with Ketamine (100 mg/kg) and Xylazine (10 mg/kg) injection. Blood samples were obtained by cardiac puncture and deposited in a vacutainer without ethylenediaminetetraacetic acid (EDTA). The blood samples were left at room temperature for approximately two h and then centrifuged at 4000 g, 4°C for 15 minutes to obtain serum. The assessment of oxidative stress markers involved the quantification of MDA and measuring SOD, a key antioxidant enzyme. In addition, inflammatory activity was determined by measuring TNF-α levels.

Serum levels of TNF-α were measured with a rat TNF-α ELISA kit, while serum SOD activity was assessed using the SOD Assay kit following the instructions provided by the manufacturer. In addition, MDA concentrations were measured by the thiobarbituric acid (TBA) reactive substances (TBARSs) method (Đorđević *et al*., 2019). The reaction mixture of 0.1 ml serum, 0.2 ml sodium dodecyl sulfate 8.1%, 1.5 ml acetic acid 20% (pH 3.5), and 1.5 ml TBA 0.8% was heated for 60 minutes at 95°C. Following the cooling step, the mixture was centrifuged at 4,000 rpm for 10 minutes at a temperature of 4°C. Subsequently, the supernatant was quantified its absorbance at 532 nm. The preparation of calibration curves was conducted using MDA standards. The serum MDA levels were calculated using the equation based on the calibration curve.

Immunohistochemical staining of pancreas

The histological examination was conducted on pancreatic tissue samples obtained from all experimental groups. The samples were washed using a normal saline solution to remove any remains of blood stains. After that, the samples were weighed and fixed in a solution of 10% formalin. Relative organ weight is determined by the percentage ratio of organ to body weight. The tissues were dehydrated using increasing concentrations of ethanol solutions, followed by embedding in paraffin blocks. Tissues embedded in paraffin were sliced into sections of 5 µm thickness. For immunohistochemical staining, tissue sections were incubated overnight with anti-JNK and Caspase 3 primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. The peroxidase reaction used Diaminobenzidine as the substrate, whereas hematoxylin was used for counterstaining. Images were visualized by a microscope with a digital camera at 400× magnification and ten fields of view, displaying different Langerhans islets. The analysis was conducted with the ImageJ software program. The percentage of immunopositive cells was determined by dividing the number of brown-stained cells by the total number of cells in the islets of Langerhans.

Statistical analysis

All data were presented as the mean ± standard deviation. The statistical analysis was conducted utilizing SPSS 26 software. One-way analysis of variance (ANOVA) followed by a Tukey post hoc test was used to analyze the differences between groups. A *p*-value less than 0.05 indicated a statistically significant difference.

Ethical approval

Animals were maintained following institutional guidelines for animal care. All efforts were taken to minimize any discomfort or pain. Experiment procedures were approved by the Health Research Ethics Committee of the State Islamic University of Maulana Malik Ibrahim, Indonesia (083/EC/KEPK-FKIK/2022).

Results

Effect of C. nutans extract on body weight, blood glucose, and pancreas and relative pancreas weight in diabetic rats

After treatment, body weight, blood glucose level, pancreas weight, and relative pancreas weight were analyzed, and the data are displayed in Figure 1. There was a reduction ($P<0.05$) in the body weight of diabetic rats that were not treated in comparison to the body weight of normal rats. Treatment with *C. nutans* extract at 400, 200, and 100 mg/kg BW doses have increased body weight by 13%, 10%, and 8%, respectively. Diabetic rats also displayed increased blood glucose levels in comparison with normal rats, whereas rats that received *C. nutans* displayed a hypoglycemic effect ($p < 0.05$). There was no significant difference between pancreas and relative pancreatic weight in all groups ($p > 0.05$).

Effect of C. nutans extract on oxidative stress and inflammation markers in diabetic rats

As illustrated in Figure 2, induction of diabetes led to an increase in MDA and TNF- α levels, together with a decrease in SOD levels ($p < 0.05$). This effect was reversed after all doses of *C. nutans* extract were given for four weeks. There was a decrease in MDA and TNF-α levels and an increase in SOD levels ($p < 0.05$). The study's findings indicated that *C. nutans* extracts decreased markers associated with oxidative stress and inflammation in rats with diabetes.

Effect of C. nutans extract on the expression of JNK and Caspase-3 in the pancreas of diabetic rats

The expression of JNK dan Caspase-3 was determined by immunohistochemical staining of the pancreas, which gives a brown appearance on immunopositive

islet cells. Only a small brown-stained islet of Langerhans was found in the pancreas of the normal rats. In contrast, the number of brown-stained islets of Langerhans increased markedly in the diabetes rats. Administration of *C. nutans* extracts decreased the distribution area of the brown-stained islets (Figs. 3A and 4A). JNK and Caspase-3 immunopositive cells are shown in Figures 3B and 4B. The percentage of JNK immunopositive cells in the diabetes rats was higher than in the normal rats ($p < 0.05$). Treatment with *C. nutans* extract decreased the percentage of JNK and Caspase-3 immunopositive cells compared to untreated diabetic rats ($p < 0.05$). The data suggest that the administration of *C. nutans* extract has the potential to attenuate apoptotic signaling pathways and markers in rats with diabetes.

Discussion

Preserving functional β-cell mass offers a promising therapeutic approach to managing diabetes (Zhong and Jiang, 2019; Marrano *et al*., 2020). Oxidative stress has been considered a promising therapeutic target for decelerating β-cell loss due to its detrimental effects (Puddu *et al*., 2013). Some evidence indicates that antioxidants in plant extract formulations may efficiently reduce oxidative stress (Adwas *et al*., 2019; Hrelia and Angeloni, 2020). *Clinacanthus nutans* is widely consumed as a traditional medicinal plant in Southeast Asia because of its anti-diabetic properties (Yeo *et al*., 2018). Recent studies have shown that *C. nutans* can potentially reduce blood glucose levels and enhance insulin production in rats with diabetes (Sarega *et al*., 2016b; Azemi *et al*., 2020). Nevertheless, the effect of this plant on alleviating the damage in rat pancreatic β-cells by STZ remains uncertain.

Elevated blood glucose levels and decreased body weight are common signs of diabetes (Erukainure *et al*., 2020). This condition was also observed in rats with diabetes in our research study. The cellular catabolic processes ultimately result in the breakdown of fat and muscle tissue, which causes weight loss. The formation of ROS caused by STZ induces β-cells death, thus reducing insulin release and elevating blood glucose levels (Šoltésová and Herichová, 2011; Eleazu *et al*., 2013). Nevertheless, the administration of *C. nutans* extracts relieved these symptoms, indicating its potential as an anti-diabetic agent.

Hyperglycemia induces an overproduction of ROS and triggers oxidative stress (Yaribeygi *et al*., 2020). Based on phytochemical analyses, the active compounds of *C. nutans* extract contain flavonoids such as vitexin, isovitexin, orientin, isoorientin, apigenin,

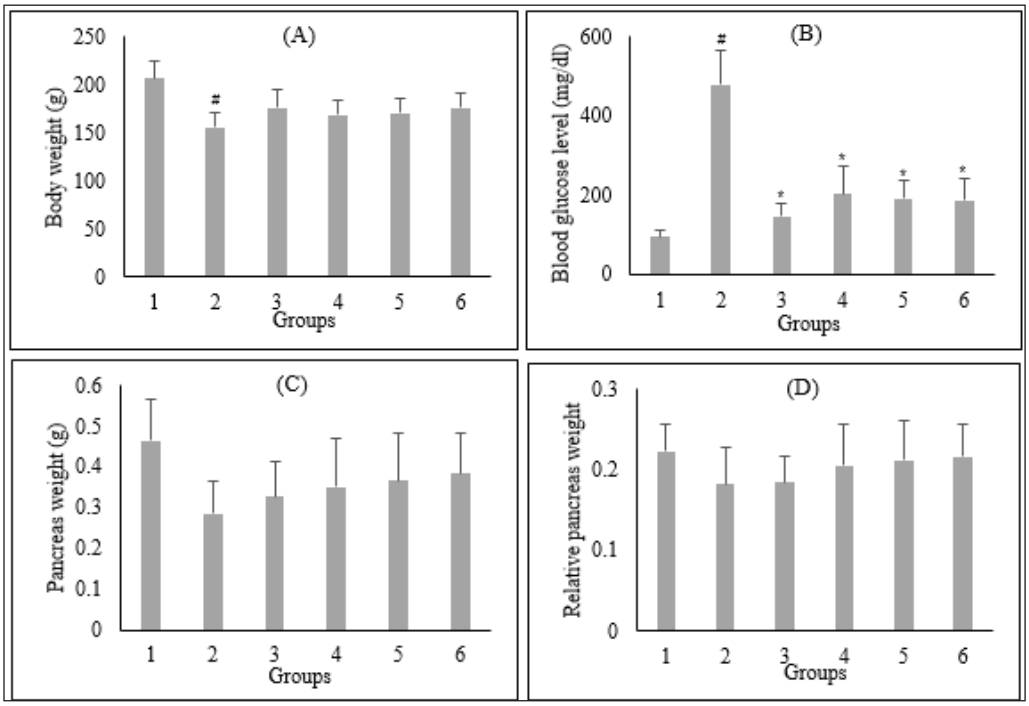

Fig. 1. Effect of *C. nutans* extract on body weight (A), blood glucose levels (B), pancreas weight (C), and relative pancreas weight (D). Data represented mean ± SD. *$p < 0.05$ significantly different from group 2; #$p < 0.05$ significantly different from group 1. Group 1 normal control; Group 2 diabetes control; Group 3 diabetic rats were administered Glibenclamide 0.45 mg/kg BW; Group 4 diabetic rats were administered *C. nutans* leaf extract at 100 mg/kg BW; Group 5 diabetic rats were administered *C. nutans* leaf extract at 200 mg/kg BW; Group 6 diabetic rats were administered *C. nutans* leaf extract at 400 mg/kg BW.

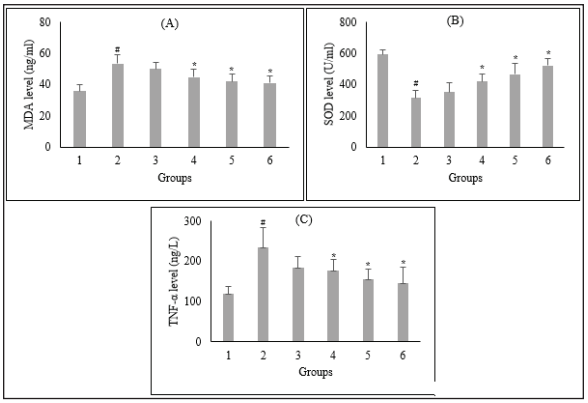

Fig. 2. Effect of *C. nutans* extract on serum levels of MDA (A), SOD (B), and TNF-α (C). Data represented mean ± SD. **p* < 0.05 significantly different from group 2; #*p* < 0.05 significantly different from group 1. Group 1 normal control; Group 2 diabetes control; Group 3 diabetic rats were administered Glibenclamide 0.45 mg/kg BW; Group 4 diabetic rats were administered *C. nutans* leaf extract at 100 mg/kg BW; Group 5 diabetic rats were administered *C. nutans* leaf extract at 200 mg/kg BW; Group 6 diabetic rats were administered *C. nutans* leaf extract at 400 mg/kg BW.

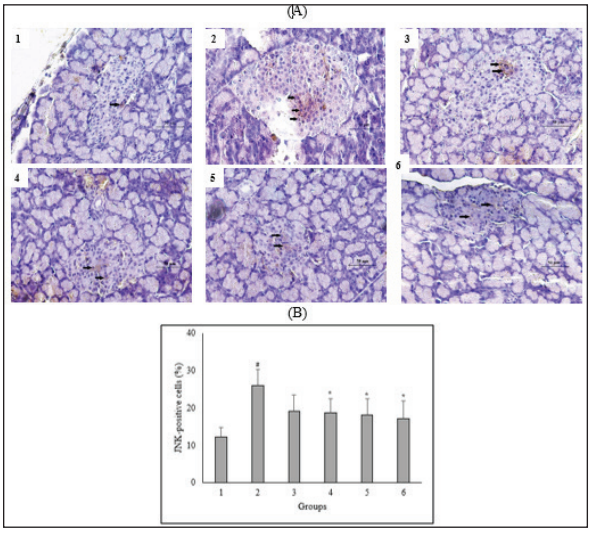

Fig. 3. Effect of *C. nutans* on Immunohistochemical features of JNK expression from the pancreas of diabetic rats (A) and the percentage of JNK-positive cells of Langerhans islet (B). Magnification 400×, scale 50 µm, black arrows indicate the representative immunopositive cells. Data represented mean ± SD. **p* < 0.05 significantly different from group 2; #*p* < 0.05 significantly different from group 1. Group 1 normal control; Group 2 diabetes control; Group 3 diabetic rats were administered Glibenclamide 0.45 mg/kg BW; Group 4 diabetic rats were administered *C. nutans* leaf extract at 100 mg/kg BW; Group 5 diabetic rats were administered *C. nutans* leaf extract at 200 mg/kg BW; Group 6 diabetic rats were administered *C. nutans* leaf extract at 400 mg/kg BW.

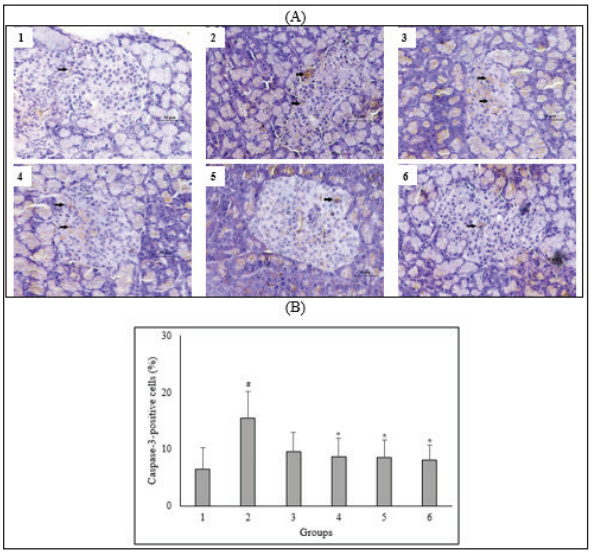

Fig. 4. Effect of *C. nutans* on Immunohistochemical features of Caspase-3 expression from the pancreas of diabetic rats (A) and the percentage of Caspase-3-positive cells of Langerhans islet (B). Magnification 400×, scale 50 µm, black arrows indicate the representative immunopositive cells. Data represented mean ± SD. **p* < 0.05 significantly different from group 2; #*p* < 0.05 significantly different from group 1. Group 1 normal control; Group 2 diabetes control; Group 3 diabetic rats were administered Glibenclamide 0.45 mg/kg BW; Group 4 diabetic rats were administered *C. nutans* leaf extract at 100 mg/kg BW; Group 5 diabetic rats were administered *C. nutans* leaf extract at 200 mg/kg BW; Group 6 diabetic rats were administered *C. nutans* leaf extract at 400 mg/kg BW.

and shaftoside, which contribute potent antioxidant and anti-inflammatory activities (Khoo *et al*., 2018; Susanti *et al*., 2023). Previous studies demonstrated that flavonoids can prevent oxidative stress in rats with diabetes by scavenging free radicals and improving the endogenous antioxidant capacity of the body (Ghorbani *et al*., 2019; Wickramasinghe *et al*., 2021). In these results, MDA levels were decreased, and SOD activity was significantly increased after four weeks of treatment with *C. nutans* extract. This study corresponds with previous studies demonstrating that *C. nutans* extracts in rats induced by a high-fat and high-cholesterol diet can decrease MDA levels and increase SOD activity in the liver (Sarega *et al*., 2016b).

Hyperglycemia was also reported to increase proinflammatory cytokines released via NF-κB activation mediated by oxidative stress (Boarescu *et al*., 2022). These cytokines enhance the generation of ROS by activating NADPH oxidase, thereby exacerbating oxidative stress and ultimately damaging pancreatic β-cell. Furthermore, proinflammatory cytokines play a significant role not only in the process of inflammation but also in the initiation of pancreatic β-cell death. Prior studies showed

increased proinflammatory cytokine marker TNF-α concentrations in animals with diabetes (Yapislar *et al*., 2022). The study findings suggest that *C. nutans* extract can decrease TNF-α levels in diabetic rats, thus confirming its anti-inflammatory properties.

Oxidative stress induced by prolonged hyperglycemia can adversely affect pancreatic β-cells. Much evidence points to the involvement of the JNK pathway in oxidative stress-induced pancreatic β-cell damage (Dhanasekaran and Reddy, 2017; Baumel-Alterzon and Scott, 2022). ß-cell dysfunction was observed in rats infused with glucose, but not in JNK-1-null rats treated with JNK inhibition using SP600125 (Tang *et al*., 2018). On the other hand, STZ-induced rats lead to JNK activation, negatively impacting pancreatic β-cells survival (Liu *et al*., 2019; Sadek *et al*., 2017). This condition supports the evidence that the JNK pathway plays a role in the impairment of β-cell function mediated by glucose. In this study, we found that *C. nutans* extract could decrease JNK expression in the pancreas of diabetic rats. The findings presented in this study correspond with prior research, which has demonstrated that plants possessing antioxidant properties can protect against diabetes-induced pancreatic β-cell damage by attenuating oxidative stress and inhibiting the JNK pathway (Tabatabaie and Yazdanparast, 2017).

JNK-mediated signaling pathways regulate apoptosis through extrinsic and intrinsic pathways (Šrámek *et al*., 2016). The extrinsic pathway is activated following the ligation of the cell surface death receptor, triggering downstream effector mechanisms and activating caspase-3. The intrinsic pathway is triggered by the translocation of the pro-apoptotic protein Bcl to mitochondria. It causes the Cytochrome-C release to form the apoptosome complex and thus activates caspase-3 (Tomita, 2016). Caspase-3 facilitates the release of caspase-activated endonucleases from their inhibitory states, thereby leading to DNA fragmentation (Kitazumi and Tsukahara, 2011). These findings indicate that *C. nutans* extract can reduce caspase-3 expression in the pancreas of diabetic rats, strengthening the protective effect of this plant against diabetic-induced pancreatic β-cell apoptosis. This anti-apoptotic effect has also been proven in previous studies where *C. nutans* extract exerts a neuroprotective effect by reducing neuronal apoptosis and repairing ischemic brain injury (Wu *et al*., 2018).

Conclusion

This study provides scientific evidence that *C. nutans* may possess a protective effect against pancreatic β-cell apoptosis in diabetic rats. The observed effect is associated with its ability to regulate oxidative stress and inflammatory response, stimulate antioxidant enzymes, suppress the JNK pathway, and inhibit caspase-3 activation. Further research is necessary to validate the molecular pathways underlying the anti-diabetic properties of *C. nutans* before clinical application.

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Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contributions

NS: conceptualized the study, conducted the experiment and laboratory examination, analyzed the data, and wrote and revised the manuscript; AM: supervised the study, wrote and reviewed the manuscripts; JK: supervised the study, wrote and reviewed the manuscripts.

Data availability

All data are provided in the manuscript.

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