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Original Article

Effect of *Gynura procumbens* (Lour.) Merr. on the histopathology of the diabetic pancreas via caspase family expression

[Efecto de *Gynura procumbens* (Lour.) Merr. sobre la histopatología del páncreas diabético a través de la expresión de la familia de las caspasas]

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Abstract

Context: Diabetes is a prominent cause of mortality globally, particularly in Indonesia. Elevated glucose levels stimulate insulin secretion from pancreatic β cells. A variety of drugs, including oral treatments and synthetic insulin, have been developed to address the needs of individuals with diabetes mellitus. Nevertheless, these medications are often too expensive. *Gynura procumbens*, a plant prevalent in Indonesia, demonstrates notable antidiabetic potential.

Aims: To analyze apoptosis via caspase family in pancreas tissue after administration of *G. procumbens*.

Methods: The ethanol extract *of G. procumbens* leaves was chitosan nanoencapsulated by amalgamating chitosan with a glacial acetic acid solution, followed by filtration with Na-TPP solution. This study utilized Wistar rats divided into six groups, comprising two control groups, one comparison group receiving metformin, and three treatment groups administered *G. procumbens* at doses of 100, 200, and 300 mg/kg BW orally for two weeks. The rats underwent dissection for immunohistochemistry examination of caspase family proteins.

Results: Administering *G. procumbens* leaves to rats in the diabetic cohort markedly reduced blood glucose levels, particularly in the P3. The glucose levels in this cohort were nearly equivalent to those observed in the group receiving the metformin. The injection of the maximum dosage of *G. procumbens* at 300 mg/kg BW can inhibit apoptosis via caspase-3, -8, and -9. Furthermore, it can regenerate pancreatic tissue by mitigating necrosis and vacuolization.

Conclusions: *G. procumbens* inhibits apoptosis by decreasing the expression of the caspase family in the pancreatic tissue of diabetic rats.

Keywords: apoptosis; caspase-3; caspase-8; caspase-9; diabetic; pancreas.

Resumen

Contexto: La diabetes es una causa importante de mortalidad a nivel mundial, particularmente en Indonesia. Los niveles elevados de glucosa estimulan la secreción de insulina de las células β pancreáticas. Se han desarrollado diversos medicamentos, incluidos tratamientos orales e insulina sintética, para abordar las necesidades de las personas con diabetes mellitus, in embargo, estos medicamentos suelen ser demasiado caros. *Gynura procumbens*, una planta predominante en Indonesia, demuestra un notable potencial antidiabético.

Objetivos: Analizar la apoptosis a través de la familia de las caspasas en el tejido pancreático después de la administración de *G. procumbens*.

Métodos: El extracto de etanol de las hojas de *G. procumbens* fue nanoencapsulado con quitosano mediante la amalgama de quitosano con una solución de ácido acético glacial, seguido de filtración con una solución de Na-TPP. En este estudio se utilizaron ratas Wistar divididas en seis grupos: dos grupos de control, un grupo de comparación que recibió metformina y tres grupos de tratamiento a los que se les administró *G. procumbens* en dosis de 100, 200 y 300 mg/kg de peso corporal por vía oral durante dos semanas. Las ratas se sometieron a disección para el examen inmunohistoquímico de las proteínas de la familia de las caspasas.

Resultados: La administración de hojas de G. procumbens a ratas de la cohorte diabética redujo notablemente los niveles de glucosa en sangre, en particular en el P3. Los niveles de glucosa en esta cohorte fueron casi equivalentes a los observados en el grupo que recibió la metformina. La inyección de la dosis máxima de *G. procumbens* a 300 mg/kg de peso corporal puede inhibir la apoptosis a través de las caspasas-3, -8 y -9. Además, puede regenerar el tejido pancreático al mitigar la necrosis y la vacuolización.

Conclusiones: *G. procumbens* inhibe la apoptosis al disminuir la expresión de la familia de las caspasas en el tejido pancreático de ratas diabéticas.

Palabras Clave: apoptosis; caspasa-3; caspasa-8; caspasa-9; diabético; páncreas.

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INTRODUCTION

Diabetes mellitus is a prominent cause of global mortality, including in Indonesia (Soeatmadji et al., 2023). Indonesia is fourth worldwide in diabetes prevalence, trailing the United States, China, and India (Pulungan et al., 2021). A 2017 study by the International Diabetes Federation (IDF) predicts that the annual incidence of diabetes in children and adolescents would surpass 96,000 cases (Pulungan et al., 2021). Diabetes mellitus, sometimes described as a chronic condition of increased blood glucose levels, is characterized by metabolic dysfunctions arising from disturbances in hormone secretion and response. When blood glucose levels are increased, pancreatic β cells release insulin; conversely, when blood glucose levels decrease, these cells suppress insulin production (Soeatmadji et al., 2023).

β-cell apoptosis is a critical event that facilitates the onset of type 1 diabetes mellitus (Liadis et al., 2005). Apoptosis is the principal mechanism of β-cell destruction, and it also contributes to the formation of type 1 diabetes mellitus via antigen cross-presentation, which activates β-cell-specific T lymphocytes (Tomita, 2017). Caspase-3 functions as the principal caspase that mediates the apoptotic process. Evidence indicates the importance of β-cell apoptosis in the pathogenesis of type 1 diabetes, whereas the specific roles of caspase-3, along with caspases-8 and 9, are currently being examined (McIlwain et al., 2013). Caspase-8, referred to as apical caspase, is involved in various forms of programmed cell death within mammalian immunology, including necroptosis and pyroptosis (Orning and Lien, 2021). Recent findings suggest that several forms of cell death communicate with one another, and caspase-8 performs several non-apoptotic roles. These roles encompass the regulation of necroptosis, the modulation of inflammatory cytokine release, the activation of inflammasomes, and the promotion of cell division (Han et al., 2021). Numerous *in vitro* investigations indicate that the caspase-dependent apoptotic pathway is essential in β-cell apoptosis (Orning and Lien, 2021). This route is initiated by increased glucose concentrations, resulting in the overexpression of Fas, a receptor that activates the extrinsic apoptotic pathway (Han et al., 2021). Fas suppresses Fas-induced apoptosis and enhances β-cell proliferation by obstructing caspase-8-mediated apoptosis (Han et al., 2021). The detection of caspase-3 antibody signifies that apoptotic β cells are effectively removed soon after the onset of diabetes (Aggarwal and Gupta, 1999).

A range of treatments, encompassing oral agents and synthetic insulin, has been formulated for persons with diabetes mellitus (Sugandh et al., 2023). Nonetheless, the use of insulin pharmaceuticals is quite expensive, leading many persons to seek particular plants for their potential in diabetes treatment (Sugandh et al., 2023). *Gynura procumbens*, commonly referred to as life-giving, is a herbal plant that may function as an alternative therapy for diabetes. *Gynura procumbens*, also referred to as lifeline leaves, is a medicinal plant within the *Asteraceae* family (Tahsin et al., 2022). Studies demonstrate that the life-sustaining plant has multiple benefits, including a decreased risk of acquiring diabetes mellitus (Tan et al., 2016). Leaves possessing life-saving attributes demonstrate an anti-diabetic effect on rats rendered diabetic (Tahsin et al., 2022). The splicing life plant comprises flavonoids that can diminish glucose absorption and regulate the synthesis of enzymes associated with carbohydrate metabolism (Zhang and Tan, 2000). The ethanol extract of *G. procumbens* leaves has exhibited anti-hyperglycaemic properties, significantly lowering blood glucose levels in diabetic rats (Unuofin and Lebelo, 2020).

The production of caspases in the pancreas may be linked to the mechanism of apoptosis, or programmed cell death, which is crucial for maintaining cellular equilibrium and preventing the buildup of damaged cells (Liadis et al., 2005). Plants rich in antioxidants, such as *G. procumbens,* may mitigate oxidative stress, which frequently initiates caspase activation in the death pathway of pancreatic cells. The antioxidants present in this plant can counteract free radicals that harm pancreatic cells, therefore diminishing the body's requirement to initiate the apoptosis process involving caspases. Consequently, caspase expression in the pancreas subjected to natural plant antioxidants may diminish, suggesting a protective effect against cellular injury (Gao et al., 2021; Situmorang and Ilyas, 2018). The correlation between caspase expression and antioxidants warrants additional investigation, as it may elucidate the potential of natural plant-derived compounds in the prevention or treatment of pancreatic diseases, including diabetes and pancreatitis. This study sought to investigate the histological changes generated by the treatment of *G. procumbens* leaves and the modifications in expression related to apoptosis via caspase activity in pancreatic tissue from alloxan-treated rats.

MATERIAL AND METHODS

Chemical and reagents

The immunohistochemistry reagents included Invitrogen Caspase-3 Monoclonal Antibody (CPP32 4-1-18) Catalog #MA1-16843 (200 µL), Invitrogen Caspase-8 (cleaved Asp384) Polyclonal Antibody Catalog #PA5-99435 (100 µL), and Invitrogen Caspase-9 (cleaved Asp353) Polyclonal Antibody Catalog #PA5-105271 (100 µL). A phosphate-buffered saline (PBS) solution containing 50% glycerol and 1% bovine serum albumin (BSA) (Catalog Number BS-0812R).

Vegetal material

The *G. procumbens* leaves were derived from indigenous flora found in the karst region of the Southern Mountains, specifically in Bungur Village and Tulakan Village in Pacitan Regency, East Java, Indonesia. The coordinates of Bungur Village are -8° 10' 22.8" latitude and 111° 16' 37.2" longitude. The area encompasses 596,616 hectares and features an elevation varying from 150 m to 450 m above sea level. The samples were transported to the Herbarium Laboratory for type analysis and assigned voucher number 1321/MEDA/2023.

Preparation of *Gynura procumbens* leaves

The leaves of *G. procumbens* were pulverized using a blender until a fine powder was achieved. The powder was immersed in a 70% ethanol solvent until fully submerged and allowed to sit for 24 h, followed by stirring. The macerate was gathered, and the subsequent maceration process was conducted 24 h later. The resultant macerate was subsequently filtered and concentrated utilizing a rotary evaporator at low pressure at a temperature of 60°C. This procedure yields nanochitosan extract by combining chitosan with a glacial acetic acid solution in a 1:1 ratio. The mixture was agitated for 24 hours and subsequently filtered using a Na-TPP solution prepared by swirling NaTPP in distilled water for two hours. The *G. procumbens* extract will be solubilized in the NaTPP solution. During the nanoencapsulation procedure, the *G. procumbens* extract was incorporated into the Na-TPP solution and blended with the chitosan solution utilizing a magnetic stirrer. Additionally, a spill occurred from the hypodermic syringe carrying the catechin solution. Subsequent to the addition of the *G. procumbens* solution, the chitosan solution was agitated for 15 minutes at 28°C. The chitosan nanoencapsulation of *G. procumbens* extract exhibited a spherical morphology with dimensions ranging from 140 to 185 nm. Particle size analysis indicated an average size of approximately 150 nm, accompanied by a zeta potential of -30 mV, which conferred substantial stability.

Animal handling

The study included male Wistar rats (*Rattus norvegicus* L.) as subjects, aged 10-12 weeks and weighing between 150-200 g. The rats employed in the study were healthy, displaying traits typical of healthy rats, like smooth fur, pure white coloration, bright eyes, normal behavior, and no weight loss during the acclimatization period. The rats were acclimatized for two weeks in plastic tub-shaped cages. Throughout the acclimatization phase, rats were supplied with consistent meals in pellet form and granted unlimited access to water. The cage was lit for 12 hours, followed by 12 hours of darkness. The male rats were allowed unlimited access to water, grain, and pellets. The rats were placed in a plastic container measuring 40 cm by 30 cm. The research was approved by the Health Research Ethics Committee of USU FMIPA Medan (No.0009/KEPH-FMIPA/2024).

Animal model of diabetes

The rats were categorized into six research groups. The rats underwent overnight fasting followed by alloxan induction, excluding the standard treatment group. An intraperitoneal dose of 160 mg/kg body weight of alloxan was delivered on day 0. After a 3-day induction phase, blood glucose levels were evaluated. Animals subjected to testing are categorized as diabetic if their fasting blood glucose levels are 200 mg/dL or above. The treatment is delivered orally over a period of 14 days. Blood glucose levels were evaluated in diabetic rats on days 3, 7, and 14.

Study design

This research utilized an experimental methodology with a fully randomized design. A total of 30 male Wistar rats were employed in this experiment. They were subsequently assigned at random to six treatment groups, each comprising five replications. Group P0 functioned as the untreated control group. Group P- comprised rats that were solely administered alloxan. Group P+ comprised diabetic rats administered metformin at a dosage of 45 mg/kgBW. Group P1 comprised diabetic rats administered *G. procumbens* leaves at a dosage of 100 mg/kgBW. Group P2 comprised diabetic rats administered *G. procumbens* leaves at a dosage of 200 mg/kgBW. Group P3 comprised diabetic rats administered *G. procumbens* leaves at a dosage of 300 mg/kgBW. The oral administration of *G. procumbens* was performed over a period of two weeks. Thereafter, the rats were anesthetized with chloroform. The neck dislocation procedure was subsequently executed while the rats were anesthetized. Subsequently, abdominal surgery was performed to excise the pancreas from each rat.

Performing tissue preparations using hematoxylin-eosin staining

The pancreatic organ was submerged in a 10% formalin solution with a pH of 7. The fixation fluid was initially eliminated, followed by multiple washes of the organ with 70% alcohol until it was clean. The tissue underwent dehydration with a sequential application of graded alcohol concentrations, commencing at 30% and then escalating to 50%, 70%, 80%, and 90% alcohol. The tissue underwent treatment with 100% alcohol for 60 minutes at each concentration. Following the dehydration procedure, dealcoholization was performed utilizing a blend of pure xylol and alcohol in varying ratios (1:3, 1:1, 3:1). The tissue was submerged in pure xylol for a duration of 4 to 10 hours. The infiltration procedure was conducted in an oven maintained at 60°C. The specimen was included in the mixture. This procedure entails positioning the pancreatic tissue within a mold containing liquid paraffin and permitting the paraffin to solidify. Additionally, the solidified paraffin block was affixed to a wooden substrate to guarantee stable adhesion. The sample was thereafter stored in the refrigerator for several nights to achieve complete hardening. The paraffin block was subsequently sectioned into many segments using a microtome knife, achieving a thickness of 3-5 µm. The paraffin removal procedure was conducted using xylene 1 and 2 in succession for 4 minutes each. The sample was subsequently immersed in 100% pure alcohol for 2 minutes, then in 95% alcohol for 2 minutes, followed by 90% alcohol for 2 minutes, 80% alcohol for 2 minutes, and finally 70% alcohol for 2 minutes. The thing was ultimately cleaned by rinsing it with flowing water. The hematoxylin-eosin staining procedure involved immersing the glass slide in a hematoxylin solution for 3-7 seconds, followed by a 1-minute rinse with water, and subsequently washing with distilled water. The slide was immersed successively in 30%, 50%, and 70% alcohol solutions. The specimen was thereafter immersed in a 0.5% eosin solution for 1 to 3 minutes. The specimen was subsequently immersed in 70%, 80%, 96%, and 100% alcohol solutions (absolute alcohol), followed by a 10-minute exposure to xylene. The slide was coated with Canadian balsam by applying it from above, followed by the placement of the cover slide (Situmorang et al., 2023).

Immunohistochemistry of caspase proteins

Pancreatic tissue was immersed in xylol I and II for 10 minutes. Absolute alcohol I, II, and III were introduced at concentrations of 95%, 80%, and 70% for a duration of five minutes, respectively. The slide was rinsed twice for two minutes using deionized water. A citric acid buffer solution with a concentration of 0.01M and a pH of 6.0 was created to prevent tissue desiccation. The tissue was subjected to microwave radiation for a duration of 10 minutes. Subsequent to cooling the tissue, it was washed three times for 3 minutes each in PBS (pH 7.4). A 3% hydrogen peroxide (H_2O_2) solution was incrementally administered to the slide to diminish cell peroxidase activity (Situmorang et al., 2024a). Following a 15-minute immersion of the slide in a 30% H_2O_2 solution at ambient temperature, the slide was subsequently washed with PBS for three minutes. A solution comprising 5% antibody and secondary antibody was introduced. The block was thereafter operated for thirty minutes at 37°C. Prior to the dilution of primary antibodies, surplus water from the tissue was eliminated using absorbent paper. Following the dilution and incubation of the primary antibodies (caspase-3, caspase-8, and caspase-9), the slides were preserved in a humidified chamber at 4°C overnight. Following three 2-minute rinses with PBS, absorbent paper was employed to eliminate moisture from the slides. The samples were subsequently re-incubated with HRP-conjugated secondary antibodies at 37°C for 30 minutes. The tissue sections were analyzed microscopically following a three-minute washing technique conducted four times, and subsequently dried with absorbent paper (Situmorang et al., 2024b; Simanullang et al., 2022a). The DAB substrate was subsequently applied. Efficient oversight is crucial to avert excessive color enhancement. During the hematoxylin staining procedure, the specimens were initially submerged in Harris hematoxylin solution for 30 seconds and thereafter rinsed with water. The test objects were thereafter submerged in a hydrochloric acid and 1% alcohol mixture for 1 min (Simanullang et al., 2022b). The slides were saturated with a sequence of solutions comprising absolute alcohol I and II at concentrations of 70%, 80%, 90%, and 95%, as well as xylol I and II. Following a two-minute immersion in each solution, the slides were meticulously washed with water. The slides were ultimately dried in a fume hood. The object glass was fitted with a microscope and readied for analysis (Simanullang et al., 2022c).

Statistical analysis

The data is presented with the mean and standard deviation, followed by analysis using ANOVA and Duncan's post hoc test. The Kruskal-Wallis test was employed for non-parametric data, followed by the Mann-Whitney test. The testing procedure encompassed hypothesis development, data presentation, test statistic calculation, and conclusion derivation.

RESULTS

Gynura procumbens reduces blood sugar levels

The ANOVA statistical analysis indicated that the administration of *G. procumbens* leaves significantly reduced blood sugar levels in male rats induced by alloxan. The average blood glucose levels of rats in both the P- group and the treatment group exhibited an inclination to increase after alloxan induction, relative to the non-induced P0 group. The increase in blood glucose levels in rats is due to the pancreatic toxicity of alloxan, resulting in the destruction of β cells in the islets of Langerhans (Yin et al., 2018). This degradation transpires via the generation of reactive oxygen species, particularly superoxide radicals, due to the redox cycle. Superoxide radicals subsequently produce highly reactive hydroxyl compounds that damage pancreatic β cells, leading to elevated blood sugar levels in rats (Yin et al., 2018). In the treatment group, the average blood sugar levels of rats on day 0 (before alloxan induction) showed negligible change and did not reveal any statistically significant variations. After the injection of alloxan to the rats, the average increase in blood glucose levels among all groups surpassed 200 mg/dL. The reduction in blood glucose levels in the treatment group began on the fifth day after the administration of *G. procumbens* leaves. The most notable decrease in blood glucose levels occurred on day 14 in the treatment group at the highest dosage, especially P3, as well as in the control group (P+). The data in Fig. 1 indicate that providing *G. procumbens* leaves to rats in the diabetic group significantly lowered blood sugar levels, especially in the P3 group (highest dose). The blood glucose levels in this cohort were almost identical to those recorded in the group administered the reference medication, metformin.

Histological analysis of the pancreas with hematoxylin-eosin staining

The research findings in Table 1 indicate a substantial variation in the average diameter of the Langerhans islands between the treatment groups ($p<0.05$). P0 demonstrates an average diameter of 142.48 µm for the islets of Langerhans, aligning with the standard range of 100-200 µm for normal pancreatic histology. Consequently, P0 will act as a benchmark for delineating the mean diameter of the islets of Langerhans within each group. Alloxan induction causes alterations in the pancreas that result in a reduction in the size of the islets of Langerhans. Consequently, P- functions as a standard for unusual histological anomalies. The administration of *G. procumbens* leaves significantly affected ($p<0.05$, Fig. 2) the histological damage to the pancreas, leading to necrosis and vacuolization. The P0 rats demonstrated a minimal average of histological damage, signifying a normal state. Rats only generated by alloxan demonstrated markedly increased histological damage relative to the normal group (P0). Alloxan induces cytotoxicity in pancreatic β cells, resulting in degeneration marked by necrosis and vacuolization.

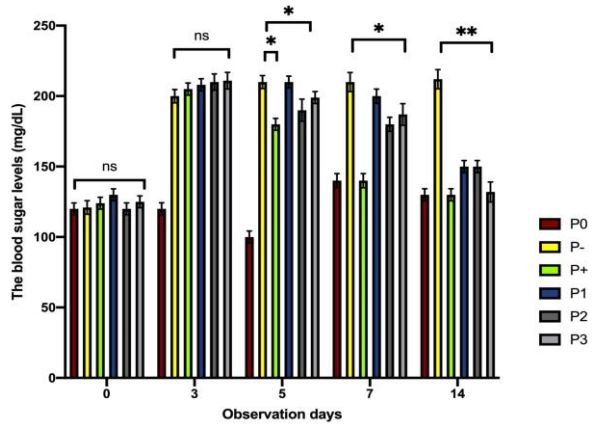

Figure 1. Results of measuring blood sugar levels in rats after administration of *Gynura procumbens* (GB).

Values are expressed as mean ± SD (n = 30). $^{ns}p>0.05$, $*p<0.05$; $**p<0.01$ compared day 3 with day 14 in the same group.

P0: Untreated rats, P-: Only induced by alloxan, P+: induced by alloxan + metformin. P1: Induced by alloxan GB leaves at a dose of 100 mg/kg BW, P2: induced by alloxan GB leaves at a dose of 200 mg/kg BW, P3: induced by alloxan GB leaves at a dose of 300 mg/kg BW.

Expression of caspase-8 in pancreatic histology after administration of *Gynura procumbens*

The findings demonstrated a reduction in caspase-8 expression across all dosage levels, with the untreated group (P0) exhibiting the lowest expression, succeeded by the group administered metformin at a dosage of 300 mg/kgBW (Fig. 3). Histological analysis revealed significant variations in caspase-8 expression among groups, as determined by the Kruskal-Wallis test. No statistically significant differences in protein expression were observed between the P1 and P2 groups when compared to the P- group, which underwent only alloxan induction. Histological analysis revealed significant changes following the administration of a 300 mg/kgBW dose of *G. procumbens*. It was found that the expression of caspase-8 in pancreatic tissue declines with increasing doses of *G. procumbens* (Fig. 4).

Expression of caspase-9 in pancreatic histology after administration of *Gynura procumbens*

The administration of *G. procumbens* had an impact on the expression of caspase-9 within pancreatic histology. There are significant differences among all types. At a dosage of 100 mg/kgBW, there was a notable reduction in caspase-9 expression, which coin-

cided with observable improvements in the histology of the tissue (Fig. 5). At the maximum dosage, *G. procumbens* mitigated the reduction in nuclear cells, consequently leading to a decrease in the expression of caspase-9 (Fig. 6).

Expression of caspase-3 in pancreatic histology after administration of *Gynura procumbens*

G. procumbens exhibited a significant decrease in caspase-3 expression at doses of 200 and 300 mg/kgBW ($p<0.001$). The administration of the highest dosage of this plant effectively inhibits the formation of caspase-3. The results of the Kruskal-Wallis test applied to this non-parametric data reveal a significant difference in caspase-3 expression across the groups (Fig. 7). The Mann-Whitney test reveals a notable statistical difference between the P0 and P- groups. The doses of *G. procumbens,* P2 (200 mg/kg BW), and P3 (300 mg/kg BW) failed to achieve the statistical significance required to identify any differences. The data shown in the table align with the histological changes noted in pancreatic tissue due to caspase-3 expression. The P- group exhibited the highest caspase expression levels, while the P0, P+, and *G. procumbens* groups demonstrated the lowest levels at a dosage of 300 mg/kgBW (Fig. 8).

Table 1. Histological analysis of the pancreas after administration of *Gynura procumbens* (GB).

Groups	Langerhans islands diamater ± SD (µm)	Vacuolated cells	Necrotic cells
P0	142.48 ± 5.591	2.86±0.51	6.3±1.48
P-	65.28 ± 9.754$^{\#\#}$	12.3±6.99$^{\#}$	28.45± 6.58$^{\#\#}$
P+	90.48 ± 9.980	11.05±3.77	31.18± 9.51
P1	92.25 ± 8.281*	10.08±1.67	19.38± 3.28
P2	126.73 ± 7.473*	6.95±3.49*	16.65± 2.55*
P3	129.75 ± 7.344*	5.13±1.60*	10.98±1.19*

Values are expressed as mean ± SD (n = 30). $^{\#}p<0.05$ vs. P0, $^{\#\#}p<0.01$ vs. P0, *$p<0.05$ compared P-, **$p<0.01$ vs. P-

P0: Untreated rats, P-: Only induced by alloxan, P+: induced by alloxan + metformin. P1: Induced by alloxan GB leaves at 100 mg/kg BW, P2: induced by alloxan GB leaves at 200 mg/kg BW, P3: induced by alloxan GB leaves at 300 mg/kg.

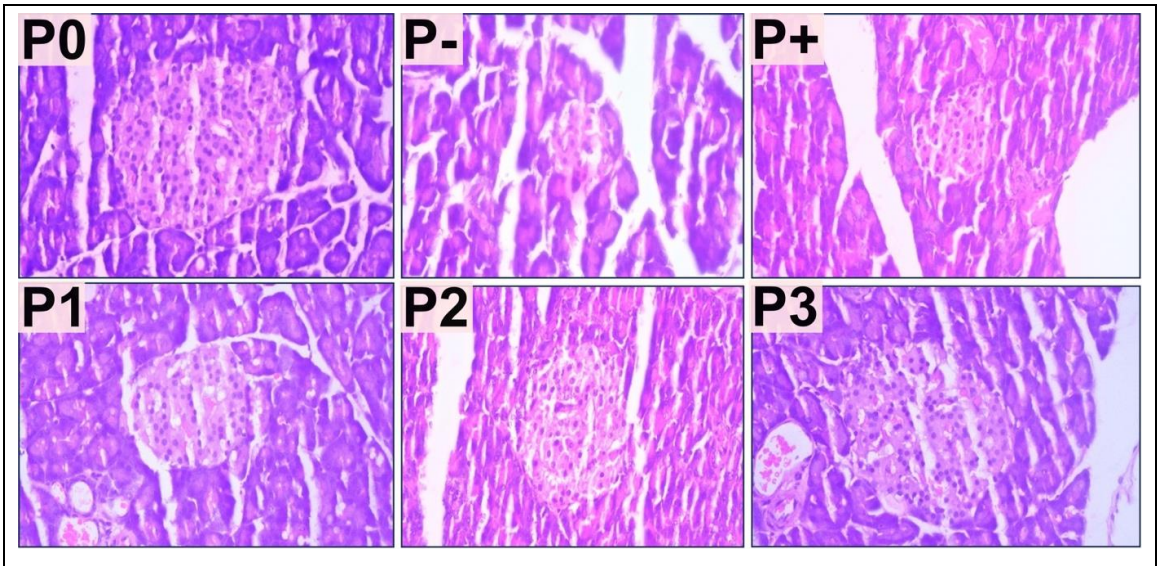

Figure 2. Histological analysis of the pancreas after administration of *Gynura procumbens* (GB).

P0: Untreated rats, P-: Only induced by alloxan, P+: induced by alloxan + metformin. P1: Induced by alloxan GB leaves at a dose of 100 mg/kg BW, P2: induced by alloxan GB leaves at a dose of 200 mg/kg BW, P3: induced by alloxan GB leaves at a dose of 300 mg/kg BW. Hematoxylin-eosin staining Magnification 400x

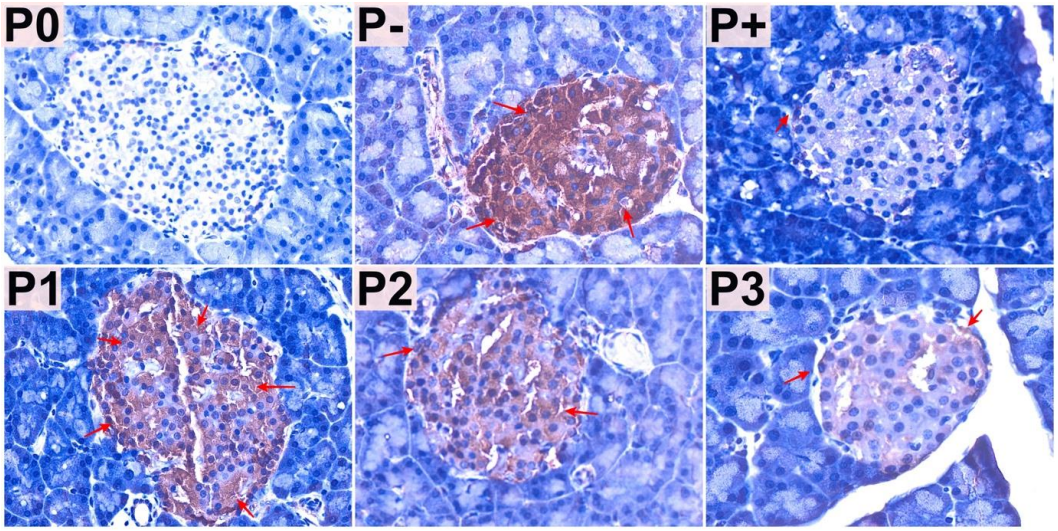

Figure 3. Expression of caspase-8 in pancreatic histology after administration of *Gynura procumbens* (GB).

P0: Untreated rats, P-: Only induced by alloxan, P+: induced by alloxan + metformin. P1: Induced by alloxan GB leaves at 100 mg/kg BW, P2: induced by alloxan GB leaves at 200 mg/kg BW, P3: induced by alloxan GB leaves at 300 mg/kg BW. Magnification 400×.

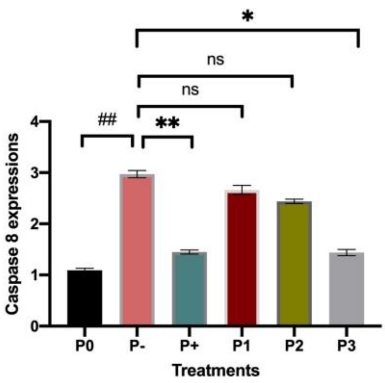

Figure 4. Caspase-8 expression score after administration of *Gynura procumbens* (GB).

Values are expressed as mean ± SD (n = 30). #p<0.05 vs. P0, ##p<0.01 vs. P0, *p<0.05 compared P-, **p<0.01 vs. P-

P0: Untreated rats, P-: Only induced by alloxan, P+: induced by alloxan + metformin. P1: Induced by alloxan GB leaves at 100 mg/kg BW, P2: induced by alloxan GB leaves at 200 mg/kg BW, P3: induced by alloxan GB leaves at 300 mg/kg BW.

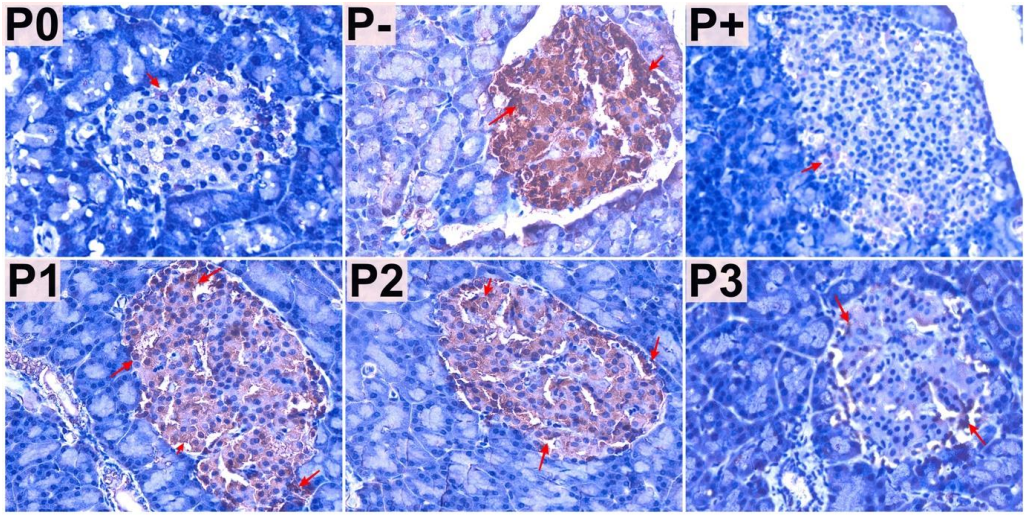

Figure 5. Expression of caspase-9 in pancreatic histology after administration of *Gynura procumbens* (GB).

P0: Untreated rats, P-: Only induced by alloxan, P+: induced by alloxan + metformin. P1: induced by alloxan GB leaves at a dose of 100 mg/kg BW, P2: induced by alloxan GB leaves at 200 mg/kg BW, P3: induced by alloxan GB leaves at 300 mg/kg BW. Magnification 400×.

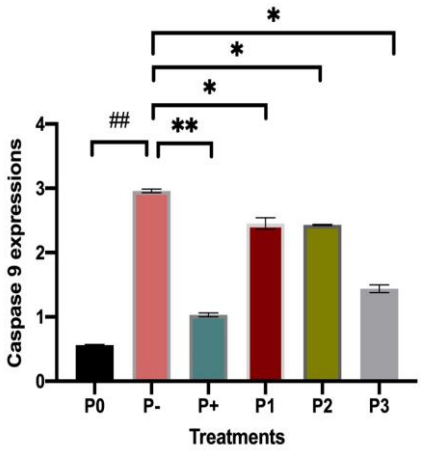

Figure 6. Caspase-9 expression score after administration of *Gynura procumbens* (GB).

Values are expressed as mean ± SD (n = 30). #p<0.05 vs. P0, ##p<0.01 vs. P0, *p<0.05 compared P-, **p<0.01 vs. P-

P0: Untreated rats, P-: Only induced by alloxan, P+: induced by alloxan + metformin. P1: Induced by alloxan GB leaves at 100 mg/kg BW, P2: induced by alloxan GB leaves at 200 mg/kg BW, P3: induced by alloxan GB leaves at 300 mg/kg BW.

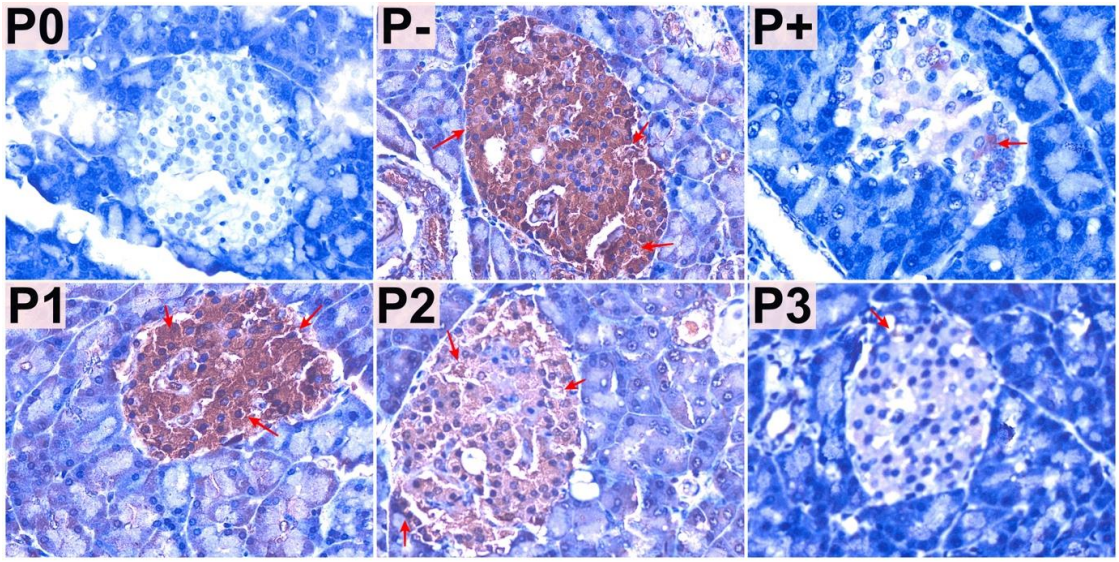

Figure 7. Expression of caspase-3 in pancreatic histology after administration of *Gynura procumbens* (GB).

P0: Untreated rats, P-: Only induced by alloxan, P+: induced by alloxan + metformin. P1: induced by alloxan GB leaves at 100 mg/kg BW, P2: induced by alloxan GB leaves at 200 mg/kg BW, P3: induced by alloxan GB leaves at 300 mg/kg BW. Magnification 400×.

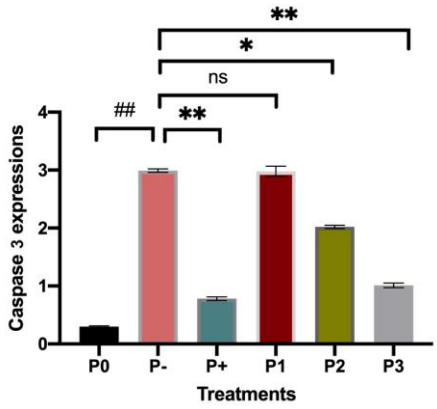

Figure 8. Caspase-9 expression score after administration of *Gynura procumbens* (GB).

Values are expressed as mean ± SD (n = 30). #p<0.05 vs. P0, ##p<0.01 vs. P0, *p<0.05 compared P-, **p<0.01 vs. P-

P0: Untreated rats, P-: Only induced by alloxan, P+: induced by alloxan + metformin. P1: Induced by alloxan GB leaves at 100 mg/kg BW, P2: induced by alloxan GB leaves at 200 mg/kg BW, P3: induced by alloxan GB leaves at 300 mg/kg BW.

DISCUSSION

The administration of *G. procumbens* leaves to diabetic rats resulted in a significant reduction in blood glucose levels, especially observed in the P3 group, which received the highest dosage. The blood glucose levels observed in this group closely matched those documented in the group receiving metformin treatment. *G. procumbens* is a herb recognized for its notable antihyperglycaemic effects. The PI3K/Akt signaling pathway plays a significant role in mediating the effects of *G. procumbens,* as demonstrated by Cao et al. (2022). The antidiabetic effect of *G. procumbens* was investigated in streptozotocin-induced diabetic rats (Hassan et al., 2010). Following a 14-day period, there was a reduction in blood sugar levels, which appeared to have minimal effects on the β cells within the islets of Langerhans in the pancreas (Hassan et al., 2010). Furthermore, *G. procumbens* enhanced the uptake of glucose by muscle tissues. The application of *G. procumbens* therapy showed no adverse effects or mortality during the treatment phase or for at least 6 months following it (Hassan et al., 2010). All rats demonstrated typical development, appearance, and behavior, reflecting their overall well-being (Cao et al., 2022). The experimental group demonstrated that the administration of alloxan alone (P-) led to histological damage in pancreatic beta cells. Alloxan specifically infiltrated the pancreatic beta cells prior to being absorbed by them (Szkudelski, 2001). The ability of pancreatic beta cells to absorb substances from alloxan plays a significant role in assessing the level of toxicity and the characteristics associated with diabetes (Lenzen, 2008). The absorption of chemicals leads to simultaneous damage in pancreatic beta cells through various mechanisms, such as the oxidation of sulfhydryl groups and the production of free radicals. The oxidation reaction of alloxan specifically affects the DNA within the cells of the islets of Langerhans located in the pancreas (Lenzen, 2008). The necrosis seen in pancreatic beta cells is thought to result from the depolarization of the cell membrane initiated by alloxan induction (Fu et al., 2013). However, the *G. procumbens* treatment group demonstrated blood sugar levels comparable to those observed in the metformin group. It improves insulin sensitivity and glucose utilization, thereby demonstrating notable hypoglycemic effects (Fu et al., 2013).

With an increase in the dosage of *G.procumbens*, there was a corresponding decrease in the expression level of caspase-8 within pancreatic tissue. Caspase-8 is present in the cytoplasm of glandular cells, individual islets of Langerhans, and ductal epithelial cells within healthy pancreatic tissue (Orning and Lien., 2021). Apoptosis, commonly known as programmed cell death, is a biological process characterized by the activation of caspases, which function as the main signaling pathway that culminates in cell death (Jakubowska et al., 2016; Situmorang and Ilyas, 2018). Caspases are classified into two distinct groups: effectors, which include caspase-3, -6, and -7, and initiators, comprising caspase-8 and -9. The inactive form of caspase-8 undergoes activation upon the binding of its death receptor to a protein that possesses a death domain (Jakubowska et al., 2016). Upon activation, the protein facilitates the action of effector caspases, such as caspase-3 (Parrish et al., 2013). Caspase-8 is identified in the cytoplasm of glandular cells, individual islets of Langerhans, and ductal epithelial cells within normal pancreatic tissue (Jakubowska et al., 2016). Caspase-8 is present in all cell types constituting the pancreatic structure, with elevated levels of expression noted in inflammatory regions surrounding the islets of Langerhans (Parrish et al., 2013). The administration of this herb resulted in the stimulation of insulin-secreting cell lines and led to notable changes in plasma insulin levels in diabetic rats (Liadis et al., 2007). This consequently hindered the activation of caspase-8 and obstructed the process of apoptosis. Additionally, following a 14-day investigation, it was demonstrated that a 25% *G. procumbens* extract, containing the same components as metformin (chlorogenic acid, rutin, astragalin, and kaempferol-3-O-rutinoside), exhibited a mode of action that closely resembled that of metformin. Multiple investigations have identified various active chemical constituents, including saponins, flavonoids, terpenoids, tannins, and sterol group glycosides, in different extracts of *G. procumbens* leaves (Algarigi et al., 2013). This substance has the potential to induce a hypoglycaemic response in diabetic animals. Research indicates a notable reduction in fasting blood glucose levels and an inhibition of glucose elevation during a glucose tolerance test in diabetic rats, while no such effects were observed in normal rats (Algarigi et al., 2013).

The administration of *G. procumbens* at the highest dosage led to a reduction in cell nuclei, which subsequently resulted in decreased expression of caspase-9. Although there was a clear decrease in the expression of caspase-9 across all dosage levels, none demonstrated a statistically significant difference. Excessive expression of caspase-9 has the potential to trigger apoptosis (Avrutsky and Troy, 2021). A possible explanation is that heightened expression levels could result in the closer proximity of caspase-9 molecules, thereby aiding the necessary dimerization process for its activation (Druskovic et al., 2006). Selective inhibition of caspase-9 can be achieved

through the application of dominant negative caspase-9 mutants and pharmacological inhibitors derived from the XIAP protein. The Bir3 domain of the XIAP protein demonstrates significant efficacy in suppressing the inherent function of caspase-9 (Avrutsky and Troy, 2021). However, the exact implications of caspase-9 expression and activation remain to be determined across various diseases (Druskovic et al., 2006). The molecular docking study demonstrates strong molecular interactions between digestive enzymes (α-glucosidase and α-amylase) and particular compounds present in *G. procumbens*, which exhibit significant antidiabetic effects (Khan et al., 2023). The objective of these interactions is to obstruct the caspase-9 mechanism that plays a role in the apoptotic process (Khan et al., 2023). Additionally, the compounds isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C, extracted from *G. procumbens*, demonstrate a notable concentration and possess strong anti-inflammatory and antioxidant properties (Elwekeel et al., 2022). The cohort administered *G. procumbens* exhibited a significant reduction in caspase-9 expression, likely linked to elevated antioxidant levels (Khan et al., 2023).

The process of T-cell priming, an essential initial event in type 1 diabetes, necessitates β-cell death that is mediated by caspase-3. Type 1 diabetes mellitus represents an autoimmune disorder characterized by the targeted destruction of pancreatic β-cells by T lymphocytes, which specifically recognize certain antigens involved in insulin production (Li et al., 2014). Caspase-3 plays a role in the advancement of diabetic kidney disease by enhancing secondary necrosis via gasdermin E during the process of apoptosis (Wen et al., 2020). Apoptosis, also known as programmed cell death, appears to play a crucial role in the molecular depletion of pancreatic β cells (Sing et al., 2019). The reduction observed is intricately associated with the emergence of insulin deficiency and the onset of disease advancement. Green tea contains a notable concentration of antioxidants that may play a role in inhibiting programmed cell death in pancreatic tissue by reducing the production of caspase-3 (Kim et al., 2014). The antioxidant route and the apoptotic pathway play a vital role in reducing damage by blocking the activity of oxidative stress ligands (Chaudhary et al., 2023). The histological examination of pancreatic tissue indicated that the P-group exhibited the highest levels of caspase-3 expression, whereas the P0, P+, and *G. procumbens* groups showed the least expression at a dosage of 300 mg/kgBW. The treatment of pancreatic islets with caspase-3 antibodies appears to facilitate the acceleration of programmed cell death within these islets. This process is associated with an increase in amyloid deposits, which ultimately contributes to the demise of the cells. Apoptosis suppressors that act before caspase activation can be influenced by three key factors: cytokines, inhibitors, and antioxidants (Elwekeel et al., 2022). Additionally, a compound derived from *G. procumbens* may contain antioxidants that inhibit apoptosis through the downregulation of caspase-3 expression.

CONCLUSION

Gynura procumbens demonstrates significant anti-diabetic potential by reducing blood glucose levels in diabetic rats, comparable to the effects of metformin. The *G. procumbens* inhibits apoptosis in pancreatic tissue by decreasing the expression of caspase-3, -8, and -9. This effect contributes to the regeneration of pancreatic tissue, reducing necrosis and vacuolization. These findings suggest that *G. procumbens* could be a cost-effective alternative for diabetes management, particularly in regions like Indonesia, where access to expensive medications may be limited.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Situmorang PC	Dewatisari WF	Setyawati A	Manurung RD	Setiawan E
Concepts or ideas	x	x			
Design	x	x			
Definition of intellectual content		x	x	x	
Literature search			x		
Experimental studies			x	x	x
Data acquisition	x	x			
Data analysis	x	x			
Statistical analysis	x				
Manuscript preparation	x		x	x	x
Manuscript editing	x				
Manuscript review	x	x	x	x	x

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