Indonesian Journal of Medical Laboratory Science and Technology
Open Access
p-ISSN 2684-6748
e-ISSN 2656-9825

RESEARCH ARTICLE

Immunomodulatory activity of kersen leaf extract (*Muntingia calabura*) on diabetic rats: analysis of immune response

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Article history:
Received: 2024-03-10
Revised: 2024-06-05
Accepted: 2024-07-10
Available online: 2024-10-27

Keywords:
Immunomodulator
Muntingia calabura
Diabetes mellitus

https://doi.org/10.33086/ijmlst.v6i2.5746

Abstract

The immune response to high blood glucose levels leads to an inflammatory response and also produces inflammation mediators. Immunomodulatory functions of Kersen (*Muntingia calabura*) need further enhancement to ensure that its benefits are more widely recognized by the public. This study aims to determine the immunomodulatory activity of Kersen leaf in inducing and modulating the immune response in diabetic rats. This study was an experimental laboratory with a pre-and post-test with a control group design. The subjects were 30 white rats (*Rattus Novergicus Wistar Strain*), were treated with extract *M. calabura* dose 1 (100 mg/kg bw/day), dose 2 (200 mg/kg bw/day), dose 3 (300 mg/kg bw/day). For clinical evaluation, three control groups were formed, including a Normal Control Group, a Diabetes Mellitus (DM) Positive Group, and a DM Positive Group treated with Anti-Diabetic Drugs. The highest amount of IFN-γ concentrations were found in the DM positive control group + antidiabetic drugs (710.3 ± 27.2 ng/mL). The highest number of Nitrit Oxide (NO) concentration was found in the DM positive control group (103.7 ± 10.2 µmol/L). The highest average amount of pancreatic β cell regeneration was found in the normal control group. The DM positive control group and the treatment group had a significant difference ($p < 0.05$) It means that there is a significant difference in the data of all treatment groups, or these three groups have anti-diabetic activity by repairing or preventing damage to the pancreas organ in DM rats. This study revealed that *M. calabura* possesses immunomodulatory activity, capable of inducing and modulating immune responses in diabetic rats.

1. INTRODUCTION

Chronic Kidney Disease (CKD) is a condition of a state of decline in kidney function that is quite slowly (chronic) caused by various kidney diseases. This disease is progressive and generally irreversible (1). CKD according to the definition of kidney disease: improving global outcome (KDIGO) is a disorder in kidney structure or function for more than 3 months, with implications for health that are classified by cause, category of glomerular filtration rate (GFR) and albuminuria (2). CKD can be caused by various causes such as pre-renal, renal, and post-renal. The pre-renal process means everything, anything that happens before entering the anatomy of the kidney that affects the state of the kidney such as hemodynamics, etc. Renal means all the elements that are in the kidney

Citation: Hasan FE, Yunus R, Magdalena. Immunomodulatory activity of kersen leaf extract (*Muntingia calabura*) on diabetic rats: analysis of immune response. Indones J Med Lab Sci Technol. 2024;6(2):107–120. https://doi.org/10.33086/ijmlst.v6i2.5746

itself. Post-renal refers to the components in the kidney and the genitourinary system that lead to the external urethral meatus (3).

Chronic metabolic disease known as diabetes mellitus is typified by high blood glucose levels brought on by either inadequate insulin synthesis or inefficient insulin utilization by the body (1,2,3). With its many manifestations, including gestational diabetes mellitus, it is a significant global public health concern (4). Diabetic retinopathy, a microvascular disease that can result in visual loss, is one of the many consequences linked to diabetes mellitus (2). Define the relevance of hyperglycemia in various clinical contexts. It has also been noted that patients with hyperglycemia but no diabetes mellitus are at a higher risk of unfavorable outcomes compared to those with diabetes mellitus (5).

Hyperglycemia in diabetes is known to induce dysfunction in the immune response, leading to an impaired ability to control the spread of invading pathogens in individuals with diabetes (6,7,8). This dysfunction can make diabetic subjects more susceptible to infections due to the immune compromise resulting from chronic hyperglycemia (8,9). Chronic hyperglycemia during diabetes has been associated with chronic damage and dysfunction in tissues such as the eyes, kidneys, heart, blood vessels, muscles, and nerves (10). This chronic damage is attributed to the immune system disorder characterized by pancreatic β cell death induced by hyperglycemia (11).

Studies conducted on human pancreatic samples obtained from organ donors have provided valuable insights into the mechanisms underlying the loss of pancreatic β-cells in individuals exposed to chronic high glucose levels, particularly in the islets of Langerhans. These studies have highlighted that increased apoptosis serves as a key mechanism contributing to the reduction in pancreatic β-cell mass in the context of type 2 diabetes mellitus. The exposure of pancreatic β-cells to chronic high glucose levels has been associated with a cascade of events, including the upregulation of inflammatory cytokines, heightened oxidative stress, and the activation of various signaling pathways that promote cell death (12).

One of the important cytokines against infection is Interferon-γ (IFN-γ). Research conducted by Marissa *et al.* (13), found that there were differences in IFN-γ concentrations in DM sufferers, increased fasting blood glucose and dyslipidemia compared to normal people. IFN-γ plays a key role in cellular immunity, coordinating immune responses necessary for combating infections and malignancies. Its functions include activating immune cells like T lymphocytes, natural killer cells, and macrophages, which are vital for effective immune responses against pathogens and tumor cells (14,15,16). IFN-γ also modulates the concentration of immune-related genes, influences immune cell differentiation and function, and regulates the production of other cytokines crucial for immune responses (17,18,19).

One of the key immune system processes dependents on IFN-γ is the generation of Major Histocompatibility Complex (MHC) class II molecules, essential for antigen presentation and activation of T cells. MHC class II molecules play a crucial role in the adaptive immune response by presenting antigens to CD4+ T helper cells, initiating immune activation, and coordinating immune responses (20). Additionally, IFN-γ is involved in the production of nitric oxide (NO), a potent antimicrobial molecule crucial for the immune response against pathogens.

The role of immunomodulators in the mechanisms of the immune system is to restore the imbalance of the impaired immune systems, improve the functioning of the immunological system, and suppress immune responses (21). Clinically, an immunomodulator is used in patients with immune system disorders. Despite this, the use of immunomodulators in therapy is sometimes obstructed. Among the frequently emerging barriers are less affordable prices, drugs available on the market in the form of patents, and the majority imported from abroad. In such circumstances, it is imperative to consider obtaining immunomodulators from natural materials (22,23,24,25).

One of the herbal plants that has potential as an immunomodulator is Kersen plant (*Muntingia calabura*) (26). *M. calabura's* leaves, stem, and roots have all yielded the chemical components. Flavonoids are the primary chemical component of the secondary metabolite. Reports indicate that the flavonoid group is a valuable source for pharmacological aspects. According to most study, *M. calabura* possesses good bioactivity as an antioxidant, antidiabetic, antibacterial, anticancer, anti-inflammatory, and other compounds (27).

Previous research has demonstrated that flavonoids contents in plants have antioxidant and anti-inflammatory properties, as well as the ability to enhance the activity of immune cells in the body (28). Several classes of flavonoids found in Kersen leaves include Quercetin and Kaempferol. These active compounds possess strong anti-inflammatory and antioxidant properties, which can aid in modulating the immune system by enhancing immune response and reducing inflammation (29,30,31).

However, the immunomodulatory functions of Kersen still need to be further explored to increase public awareness of its benefits. In addition, previous research has not investigated the immunomodulatory effects of *M. calabura* L. extract in animals models (26,29). Therefore, further study to evaluate the immunomodulatory effects of *Muntingia calabura* L. extract in animal models of certain disease conditions that cause immune system disorders are required.

2. MATERIALS AND METHODS

The study employed a post-test only control group design -experimental design at March to August 2023 at the Pharmacy Laboratory, Biology Laboratory and Medical Laboratory of Haluoleo University Kendari.

2.1. Preparation Kersen Leaf Extract (*Muntingia calabura*)

Fresh leaves of *M. calabura*, which grow wild, were collected from various location in the Kendari City area, Southeast Sulawesi Province, Indonesia, in March 2023. Kersen leaves used as samples are young leaves in the position of 2-5 leaves from the top of the branch and exposed to sunlight. Identification of plant was asses by Gayuh Agastia, S.Si from Laboratory of the Department of Biology Education, Faculty of Teacher Training and Education, Haluoleo University, Kendari.

M. calabura leaf extract was made through the process of sorting, washing, draining in a shady place at room temperature. Then the leaves were oven dried at 60°C for 24 hours. The dried *M. calabura* leaves were then ground into powder using a grinding machine. Powder samples were stored at 4^{0}C in a closed bottle.

Samples were weighed and then put into a macerator, poured with ethanol 96 % until the surface of the sample was completely submerged, left for 3 x 24 hours in a place protected from light while occasionally stirring. After 3 days the extract was filtered using filter paper, repeat the maceration process 3 times to obtain liquid extract of *M. calabura* leaves. Then the liquid extract was vacuum evaporated to evaporate the solvent, then with the help of a rotary evaporator at a certain temperature a thick extract was obtained. One kg (1,000 grams) of wet *M. calabura* leaves yielded 261.2 grams of dry leaves and 41.93 grams of leaf extract (32,33).

2.2. Experimental Animals

In this study involved male *Rattus novergicus Wistar* rats aged 8 - 12 weeks, with an initial weight ranging from of 100 to 300 grams, provided by the Experimental Animal Laboratory at the Faculty Medicine, Haluoleo University, Kendari, Indonesia. The rats were in a good condition, as indicated by their active movement. Prior to the treatment, the rats were acclimated for one week.

The rats were housed in a polypropylene cage within a controlled rearing room maintained at a temperature of 24°C ± 1°C and relative humidity of 60-70% with standard environmental conditions (12-hour light and 12-hour dark cycle). The rats were fed standard pellet feed and drinking water ad libitum.

All the experimental protocols used in this research were carried out after the ethical approval was granted by the University of Surabaya Ethics Committee with a letter No.220/KE/VIII/2023. The research design included grouping 30 rats into 6 groups with 4 rats in each.

The groups consisted of the normal Control group, a Diabetes Positive Control group (DM), and a DM group treated with antidiabetic drug (glibenclamide 0.65 mg/kg bw). The glibenclamide drug used was First Medifarma brand with catalog number GKL0007113404A1. In addition, there were three treatment groups: Treatment Group I consisted of diabetic rats administered 2 mL of *M. calabura* leaf extract at a dose of 100 mg/kg bw/day through e feeding tube for 14 days; Treatment Group II included diabetic rats administered 2 mL of *M. calabura* leaf extract at a dose of 200 mg/kg bw/day through a feeding tube for 14 days; Treatment Group III comprised diabetic rats receiving 2 mL of *M. calabura* leaf extract at a dose 0f 400 mg/kg bw/day through a feeding tube for 14 days.

2.3. Preparation of Diabetic Rats

To develop diabetes in rats (experimental animals), an agent inducing damage to pancreatic β-cells can be used, such as the chemical *Streptozotocin*/STZ (bioWORLD, Atlanta, USA). *Streptozotocin* was injected to the group of rats at a single dose of 60 mg/kg bw intraperitoneally using a 25-g 1-inch syringe (7). After 5,10, and 15 days of injection, the blood glucose levels were measured using the glucose test/easy touch (Glucometer, Bioptik Technology Inc., Taipei, Taiwan). Rats are categorized as having Diabetes mellitus if there is an increase in the blood sugar levels to ≥ 200 mg/dL. The rat's termination procedure is: preserving several important organs that

have not been used such as; liver, heart, lungs and mandible for further research development. Body parts of experimental animals and other organs that are not used are burned in a special burning container.

The blood extraction procedure involved collecting rat blood samples, with approximately 5 mL taken from the ventricle and then collected in an EDTA tube. In addition, the rats from which blood was taken were sacrificed using anesthesia with ether compounds.

After the rat is unconscious, dissection is carried out with surgical tools (dissecting set) starting from the epigastrium area until the rat's heart is visible. The surgical procedures are performed by trained laboratory technicians.

2.4. Parameters Measurements

The parameters measured from the blood plasma were the concentrations of interferon-γ (IFN-γ) (ng/mL) and Nitric Oxide (NO) (µmol/L). The concentration of IFN-γ (ng/mL) was examined through the *Enzyme-linked Immunosorbent Assay* (ELISA) with the IFN-gamma ELISA kit (rat) Bioassay Technology (Shanghai Korain Biotech Co.,Ltd. Shanghai. China). Meanwhile, the concentration of Nitric Oxide (NO) (µmol/L) was examined by using the Griess reagent followed by reading in UV-VIS Spectrophotometry with a spectrophotometer manufactured by BMG Labtech (Ortenberg. Germany). Meanwhile, the histopathology of the pancreas was examined under a Leica DM 500 microscope (Light Microscope Leica Microsystems GmbH, Wentzler, Germany), in 5 microscope fields of view with 100-fold magnification followed by 400-fold magnification. The histopathological changes were observed for the regeneration and necrosis of pancreatic β-cells in the islets of Langerhans.

2.5. Statistical Analysis

Statistical analysis was conducted to determine the comparison of the mean concentrations of IFN-γ (ng/mL) and the average production of NO (µmol/L) in each group. The initial stage involved the Shapiro-Wilk test, aimed at determining whether the data in all treatment groups were normally distributed. The test results showed a p-value > 0.05, indicating that the samples in all treatment groups were normally distributed. Subsequently, a Homogeneity Test was performed on all treatment groups, yielding a p-value > 0.05, thus meeting the requirements for conducting One Way ANOVA analysis.

One Way *Analysis of Variance* (ANOVA) was employed to test the mean/effect of *M. calabura* leaf extract on the average concentration of IFN-ɣ (ng/mL) and the concentration of NO (µmL/L). The Sig value served as the significance threshold.

By observing the Sig value in the concentration data of the test results across all treatment groups, a p-value < 0.05 was found, indicating the acceptance of H1. Therefore, it can be asserted that there is a significant difference in the test results data for all treatment groups, which justifies the continuation of the LSD Post-Hoc test. The LSD-Post Hoc test indicated a comparison of the mean concentrations of IFN-γ (ng/mL) and the mean concentrations of NO (µmol/L) in each group.

In the positive DM control group compared to the treatment group, there was a significant difference with p < 0.05, indicating a significant difference in the data of all treatment groups or the three groups. Meanwhile, the comparison between the positive DM control group with Anti-Diabetic Drug and the treatment group obtained a sig p-value > 0.05, indicating no significant difference in the data of all treatment groups. Therefore, it can be concluded that dose group 1 (100 mg/kg bw/day), dose 2 (200 mg/kg bw/day), and dose 3 (400 mg/kg bw/day) have anti-diabetic activity by repairing or preventing damage to the pancreas organ in DM rats.

To determine the degree or level of correlation between the average concentration of IFN-γ (ng/mL) and NO concentration (µmol/L) and the average amount of necrosis as well as the average regeneration of β-cell pancreas in Langerhans islets, a Pearson Correlation test was conducted.

3. RESULTS AND DISCUSSION

3.1. Characteristics of Experimental Animals

The experimental animals studied were evaluated based on their average Body Weight (BW) and Glucose Levels. The body weight of the animals across the treatment groups ranged from 100 – 300 grams, with the lowest mean BW recorded at 105.2 ± 4.3 grams in the normal group, and the highest mean BW of 300.8 ± 25.6 grams in the DM with antidiabetics drug positive control.

The profile of the blood glucose levels of the experimental animals based on the treatment groups was in the range 68.8 – 425.5 with the lowest mean glucose level of 68.75 + 11.3 (mg/dL) in the normal group and the

highest average glucose level of 425.5 ± 76.8 (mg/dL) found in The DM with antidiabetics Drug positive control group (Table 1).

Table 1. Blood glucose levels of experimental animals

Variable group	First glucose level (mg/dL)	Glucose level (mg/dL) after STZ induction	Glucose level (mg/dL) after treatment		
			1	2	3
Normal control	89.8 ± 7.9	76 ± 5.4	75.5 ± 6.4	70.3 ± 11.1	68.8 ± 11.3
Diabetes mellitus positive control	103.3 ± 8.5	283.3 ± 26.2	408.3 ± 75.4	419.5 ± 77.8	425.5 ± 76.8
Diabetes mellitus positive control + Drug diabetic	121.3 ± 6.9	364.3 ± 26.7	111.5 ± 7.9	97.3 ± 6.7	87 ± 4.2
Treatment dose 1 (100 mg/kg bw/day)	109.5 ± 3.7	288.8 ± 19.6	242.5 ± 22.6	237.5 ± 21.6	232 ± 24.5
Treatment dose 2 (200 mg/kg bw/day)	113.5 ± 5.8	352.3 ± 41.0	128.5 ± 6.4	118.3 ± 4.6	114 ± 4.2
Treatment dose 3 (400 mg/kg bw/day)	118.8 ± 2.9	426.5 ± 52.2	121.3 ± 5.9	110.3 ± 5.3	101.25 ± 4.3

3.2. Average Concertation Level IFN-ɣ

The result of the average amount of IFN-γ concentration (ng/mL) in the blood plasma of experimental animals based on treatment groups are shown in Figure 1. It shows that the lowest IFN-γ concentration (ng/mL) was found in the positive control group for diabetes mellitus, the average amount was 259.8 ± 67.2 ng /mL and the highest IFN-γ (ng/mL) concentration was found in the positive control group for diabetes mellitus with anti-diabetes drug, with a mean amount of 710.3 ± 27.2 ng/mL.

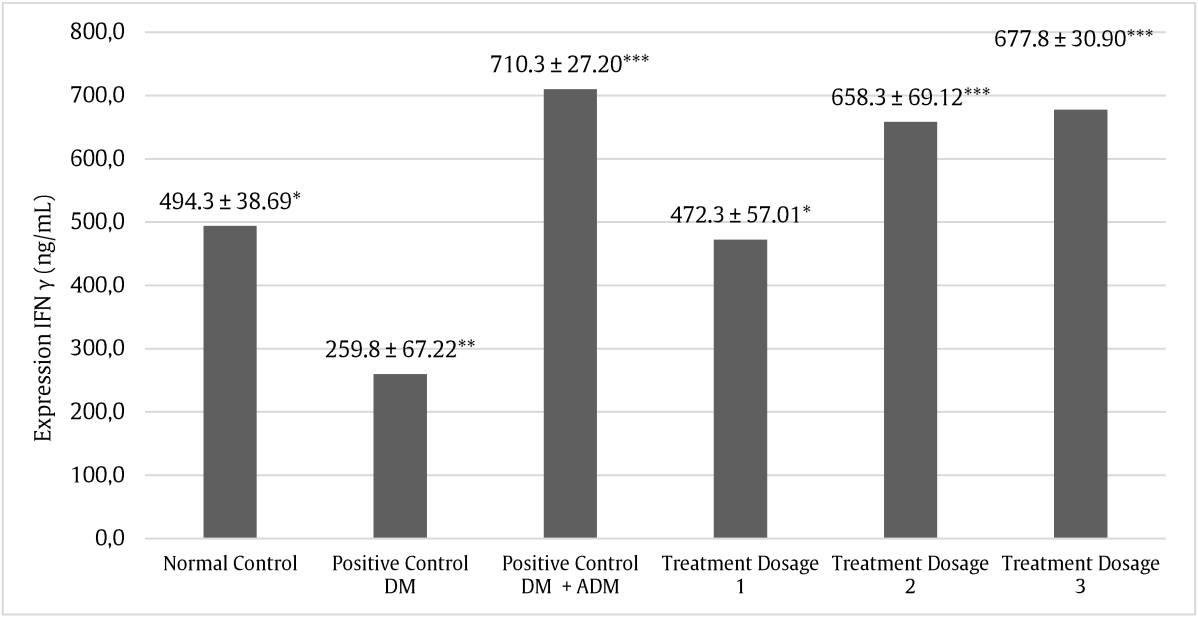


Figure 1. Description of the average concentration levels of IFN-γ (ng/mL) in the blood plasma of experimental animals based on treatment groups. (* significant with normal control; **significant with DM; ***significant with DM with antidiabetic drug)

Interferon (IFN) is a type of natural protein found in the human immune system. Its function is very important, which is to combat disease-causing pathogens (such as bacteria and viruses), or cancer cells in the body. Cells infected by viruses or bacteria release IFN-α and IFN-β as warning signals to the immune system. This triggers white blood cells (part of the immune system) to release IFN-γ to fight the virus or bacteria (34).

IFN-γ works indirectly to prevent viral replication and stimulates certain proteins that lead to the destruction of infected cells. IFN-γ is involved in regulating almost all phases of immune and inflammatory responses, both in the innate and adaptive immune systems, including the activation and differentiation of T cells, B cells, NK cells, macrophages, and others. Therefore, IFN-γ is often referred to as a classic immunoregulatory cytokine (35).

The results of the observations on the average concentration of IFN-γ (ng/mL) in the blood plasma of experimental animals based on treatment groups indicated that the lowest concentration of IFN-γ (ng/mL) was found in the positive control group with diabetes mellitus. In contract, the highest concentration of IFN-γ (ng/mL) was observed in the positive control group with diabetes mellitus treated with antidiabetic drugs.

The condition of increased blood glucose levels in the experimental animals treated for diabetes mellitus implicates changes in the immune mechanism. DM accompanied by chronic inflammation affects the immune system, characterized by a decrease in the concentration of various types of cytokines. This leads to changes in the body's immunity, making it more susceptible to infections.

Meanwhile, the treatment group receiving extracts of *M. calabu*ra leaves, the highest concentration of IFN-γ (ng/mL) was found in the sample group with dose 3. Conversely, the treatment group receiving the lowest dose 1 the least concentration of IFN-γ (ng/mL).

To demonstrate that *M. calabu*ra leaf extract has potential as an immunomodulator, a Post-Hoc LSD (Least Significant Difference) test was conducted. The test results showed a comparison of IFN-γ (ng/mL) concentrations in each group. The control and treatment groups showed significant differences, $p < 0.05$. In other words, the three treatment groups exhibited immunomodulatory activities capable of inhibiting the inflammatory process and modulating immune responses, evidenced by the increase in IFN-γ (ng/mL) concentrations in each group of experimental animals.

The findings of this study are consistent with the study by Marissa *et al.*, (13), which stated that there are differences in IFN-γ concentrations among patients with DM, increased fasting blood glucose, and dyslipidemia compared to normal individuals. Additionally, the test results for the positive DM control group with antidiabetic drugs, compared to the treatment groups with dose 2 and dose 3, showed that the Sig. p values were greater than 0.05, indicating no significant differences between these two treatment groups. Therefore, it can be concluded that the treatment group with dose 2 (Sig. $p > 0.168$) and dose 3 (Sig. $p > 0.381$), after administration of *M. calabu*ra leaf extract, demonstrated immunomodulatory effectiveness and immune response comparable to the positive DM control group with antidiabetic drugs. The immunomodulatory role of IFN-γ in this study is supported by previous studies on molecular and immunological observationssupported by the research conducted by Tan *et al.* (16) on molecular and immunological observations. The immunomodulatory role of IFN-γ in this study is supported by previous studies on molecular and immunological observations (36).

3.3. Concertation of Nitric Oxide (NO)

A description of the average amount of Nitric Oxide (NO) µmol/L concentration in the blood plasma of experimental animals based on treatment groups, shows that the highest concentration of Nitric Oxide (NO) µmol/L was found in the positive control group for diabetes mellitus with a of 103.7 ± 10.2 µmol/L. The lowest L and Nitric Oxide (NO) concentration µmol/L were found in the normal group, with a mean of 21.5 ± 0.7 µmol/L (Figure 2).

Diabetes mellitus is often associated with the early development of cardiovascular complications. The condition of diabetes mellitus due to hyperglycemia can induce the formation of free radicals and a reduction in the antioxidant defense system. Chronic hyperglycemia leads to an increase in the production of free radicals, which consequently results in oxidative stress (37,38).

One of the pathological conditions induced by oxidative stress due to diabetes mellitus is the increased contraction and activation of thromboxane receptors in the coronary arteries, which can trigger tension in the blood vessel walls, coagulation, and inflammation. NO is a key regulatory molecule with metabolic, vascular, and cellular effects.

The results of this study indicated that the average concentration of NO in the blood plasma of experimental animals, based on treatment groups, showed changes in NO concentration. The average concentration of NO in µmol/L in the blood plasma of experimental animals, based on treatment groups, showed that the highest NO concentration was found in the positive control group with diabetes mellitus, while the lowest NO concentration (µmol/L) was found in the normal group. Among the treatment groups, the lowest concentration was found in the sample group with dose 3, and the highest in the sample group with dose 1.

Under physiological conditions, the endothelium plays a protective role against the development of atherosclerosis. Endothelial cells produce NO, a substance capable of maintaining the balance of vascular tone, coagulation, and inflammation. However, in pathological conditions such as diabetes mellitus, the activity of NO can be disrupted (39).

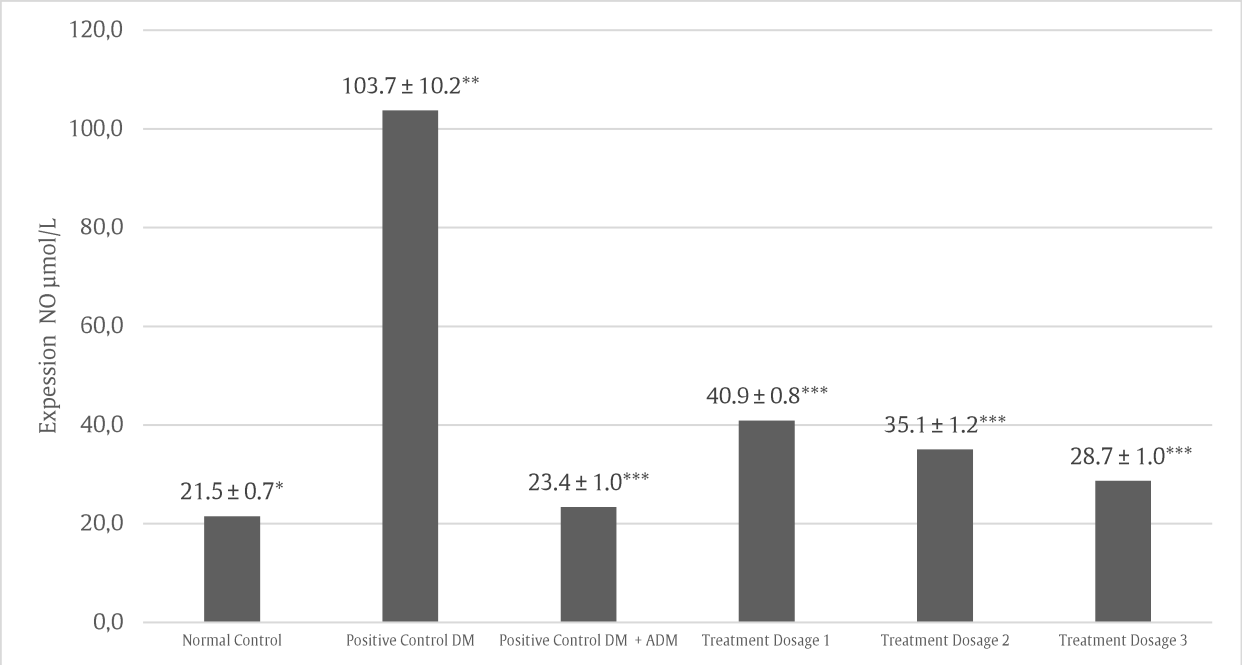


Figure 2. Description of the average concentration levels of nitric oxide (NO) µmol/L in the blood plasma of experimental animals based on treatment groups. (* significant with normal control; **significant with DM; ***significant with DM + antidiabetic drug)

The test results showed a significant difference in the concentration levels of NO µmol/L between the positive control DM group and the treatment groups, with $p < 0.05$. This indicates that all three treatment groups (doses 3, 2, and 1) exhibited antidiabetic activity by reducing NO concentration in diabetic rats.

The high concentration of NO in the positive control group of DM indicates adaptation of NO to vascular tension reactivity caused by increased blood glucose levels in experimentally induced diabetes mellitus animals. If NO concentration decreases, disturbances in endothelial relaxation of blood vessels may occur, potentially triggering tension in vessel walls leading to intravascular pressure (40). Decreased NO availability is not only relevant to the development of atherosclerotic complications in diabetes but can also interfere with postprandial glucose disposal mediated by insulin and may contribute to the development of insulin resistance (35,41).

Previous research on experimentally induced type II diabetes mellitus in white rats of the *Rattus novergicus* species, administered with oral extracts of *M. calabura* leaves, has demonstrated the anti-diabetic activity *of M. calabura* leaves (42).

Rattana and Sungthong (43) stated that methanol extracts from the roots and leaves of the *M. calabura* showed good antioxidant activity. The highest total flavonoid content was found in the methanol extract of leaves in previous studies stated that methanol extracts from the roots and leaves of the *M. calabura* showed good antioxidant activity. The highest total flavonoid content was found in the methanol extract of the leaves.

The results of qualitative and quantitative analysis of the bioactive components of the *M. calabura* plant in several solvents (water, methanol, ethanol, chloroform, ether and citric acid), found antioxidant bioactive components, namely, saponins, flavonoids and tannins in all solvents (38,39).

Based on observations of changes in Nitric Oxide (NO) concentration and the statistical tests conducted, it can be concluded that the treatment groups (doses 3, 2, and 1) exhibit similar activity and effectiveness to the positive control group of DM with OAD in reducing NO levels in diabetic rats.

3.4. Differences in the Number of Cell Necrosis and Cell Degeneration Based on Groups

In Figure 3, it is demonstrated that the lowest number of necrotic cells is found in the normal group, with 0.0 cells, while the highest number is found in the positive DM control group, with 32.3 cells. Among the groups treated with *M. calabura* leaf extract, the highest number of necrotic cells is observed in the Dose 1 group.

Conversely, the lowest number of regenerating cells is found in the normal group, with 31.8 cells, while the highest number is observed in the positive DM control group treated with antidiabetic medication. In the groups treated with *M. calabura* leaf extract, the highest number of regenerating cells is found in the Dose 3 group.

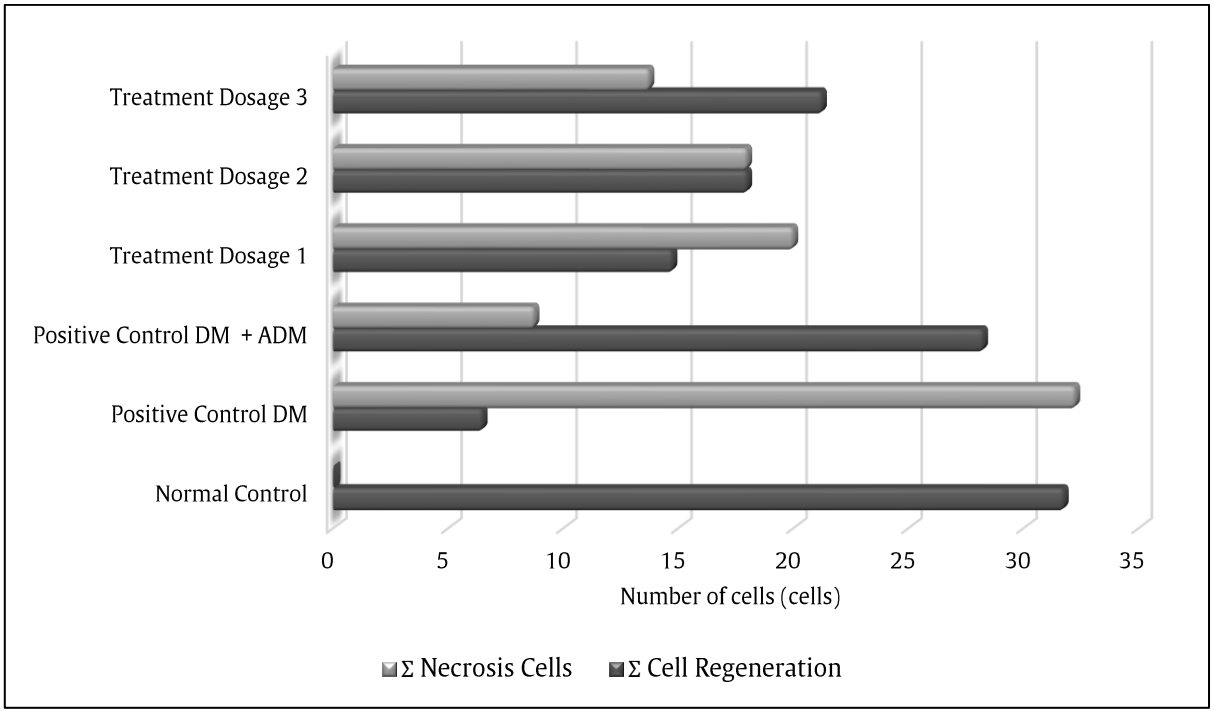


Figure 3. Differences in cell necrosis and regeneration cell counts by group

Several studies indicate that DM, particularly type 2, can lead to various conditions that cause cellular necrosis and inhibit cellular regeneration, including oxidative stress, inflammation, and cell death. There is a significant correlation between necrosis and cell regeneration with diabetes mellitus (44). This study revealed that varying patterns in the number of necrotic cells and regenerating cells in both control and treatment groups before and after administering *M. calabura* leaf extract.

One of the critical aspects of the pathophysiology of type 2 diabetes is the reduction in the number of cells that produce functional insulin. Factors that can cause cell necrosis include glucose toxicity, dyslipidemia, cytokines, leptin, autoimmunity, and certain medications. These factors also encompass oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction. These factors not only affect cell mass but also their secretory function, leading to a gradual decline in insulin production (45,46).

The study revelaed that the normal group had the lowest number of necrotic cells, reporing 0.0 cells, whereas positive DM control group exhibited the highest number at 32.3 cells. In the treatment groups with kersen leaf extract, the highest number of necrotic cells was observed in the Dose 1 group. The highest number of necrotic cells was found in the positive DM control group, with 32.3 cells. In the treatment groups with kersen leaf extract, the highest number of necrotic cells was observed in the Dose 1 group (47,48).

Studies on the relationship between diabetes mellitus and cell necrosis, particularly those focusing on the processes leading to cell death in diabetic patients, demonstrate that high glucose levels can cause cell death through various pathways, such as the IL-1–Fas pathway, which links the inflammatory response to cell death. Dyslipidemia and chronic inflammation exacerbate cell dysfunction and death, indicating a complex interplay between metabolic and inflammatory factors in diabetes (49).

The inflammatory environment exacerbates oxidative stress and impairs cellular function, leading to necrosis and apoptosis. Chronic inflammatory conditions in diabetes are one of the critical factors that promote necrosis in various cell types. High levels of pro-inflammatory cytokines, such as TNF-α and IL-1β have been shown to promote cell death in pancreatic islets of Langerhans, contributing to both type 1 and type 2 diabetes (44,45).

Studies on *M. calabura* have shown that ethanol extract of this leaf has significant pharmacological effects on cell necrosis and regeneration. In research conducted on alloxan-induced hyperglycemic rats, administration of cherry leaf extract reduced pancreatic cell damage and enhanced cell regeneration in the islets of Langerhans. The optimal dose for repairing cell damage is 500 mg/kg body weight per day (50).

This study demonstrated that ethanol extract of *M. calabura* leaves increased the number of pancreatic beta cells in STZ-induced Wistar rats. The group given Dose 3 experienced the highest pancreatic beta cell regeneration, while the positive DM control group treated with antidiabetic drugs showed the highest pancreatic beta cell regeneration.

This is supported by previous studies indicating a relationship between DM and cell regeneration, particularly those focusing on the regeneration process of pancreatic beta cells in diabetic patients. Antioxidants are needed to enhance the regeneration of pancreatic beta cells. The antioxidant content in kersen leaves can repair beta cell damage caused by high blood sugar levels by reducing ROS (Reactive Oxigen Species) and NO (Nitric Oxide) thereby decreasing oxidative stress and increasing the number of pancreatic beta cells (51). Research on cell regeneration through bioinformatics approaches and cellular therapy to restore pancreatic β-cell function and reduce diabetes complications is significant due to its ability to promote pancreatic cell regeneration, which is crucial for diabetes management (52).

3.5. Histopathological of Pancreatic β-cells

The histopathology of the pancreas was examined under a microscope in 5 microscopic fields of view, with a magnification of 100 X then continued with a magnification of 400 X. The histopathological description of the pancreatic β-cells in the experimental animals in each group at the initial state, before the treatment, and after the treatment presented in the Table 2.

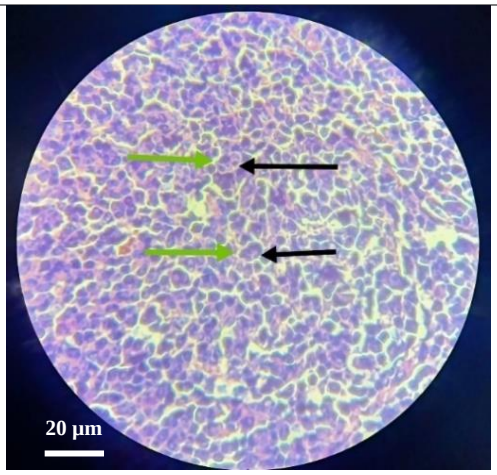
The histopathological changes in the β cells of the islets of Langerhans in the pancreatic tissue of the experimental animals in the control groups and samples treated with diabetes mellitus were observed after exposure to *M. calabura* leaf extract based on the number of cells experiencing necrosis and the number of cells undergoing regenerations.

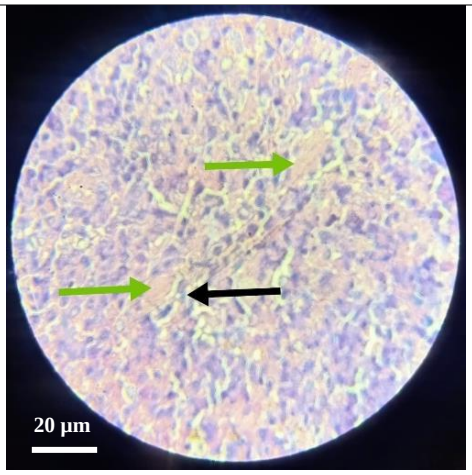
The histopathological observation shows that there are differences in the shape of the β cells of the islets of Langerhans in each group, including the normal group, DM positive group, DM with Antidiabetic drug positive group, and treatment groups (dose 1, dose 2, and dose 3). Changes in shape were identified based on differences in the amount of necrosis and the number of regenerations of β cells of the islets of Langerhans in the pancreatic tissue of the experimental animals in each group.

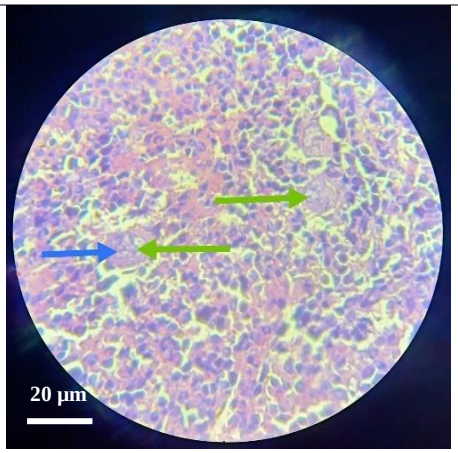
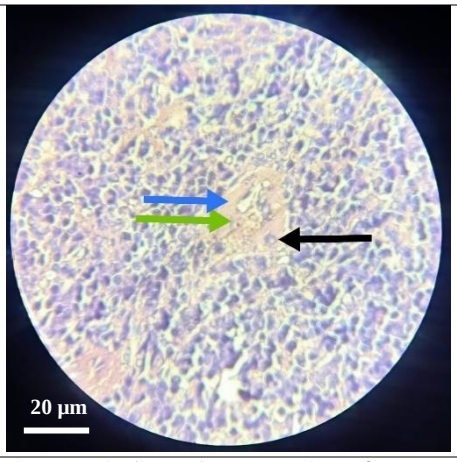
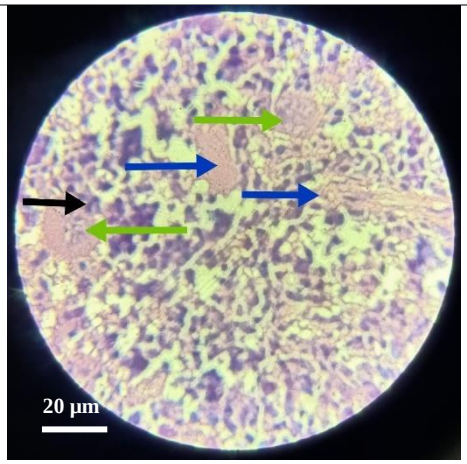
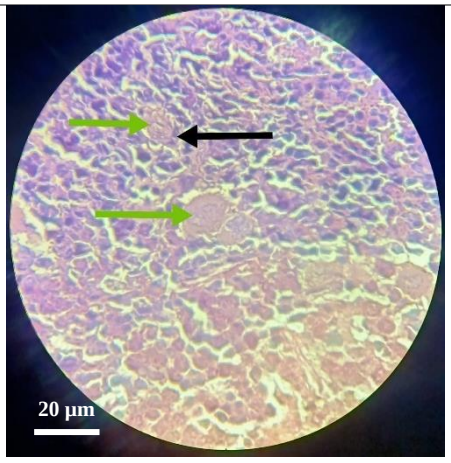
The LSD – Post Hoc test results showed a comparison of the number of necrotic cells among the pancreatic β-cells in each treatment group. The DM positive control group and the treatment group had a significant difference with $p < 0.05$, indicating that the data of all the treatment groups was significantly different. In other words, all the three treatment groups showed anti-diabetic activity by inhibiting damage to the pancreas of diabetic rats.

The LSD – Post Hoc test results in the DM + antidiabetics drug positive control group and in the treatment groups with dose 1, dose 2 and dose 3 demonstrated a sig p value > 0.05, indicating no significant difference among them. Therefore, *M. calabura* leaf extract has the potential as an immunomodulator that can induce and modulate the immune response, with the same effectiveness as the DM with antidiabetic drug positive control group, as an immunomodulator by inhibiting necrosis in the pancreas of rats with DM.

Table 2. Histopathological picture of pancreatic β-cells





The histopathological image magnification 100 X of the pancreatic tissue of the experimental animals in the normal control group shows that in the islet of Langerhans area (Green Arrow) there are islet cells surrounded by the acinar cells (Black Arrow). No necrosis is found in these cells.	The histopathological image of the pancreatic tissue of the experimental animals in the diabetes mellitus positive control group shows the condition of the islet of Langerhans (Green Arrow) with the cavity (intralobular cavity) since some of the islet cells have experienced necrosis. The same condition occurred in acinar cells (Black Arrow). The image shows the presence of intralobular cavity in these cells.
	
The histopathological image of the pancreatic tissue of the experimental animals in the Diabetes Mellitus + antidiabetic drug Positive Control Group shows the islet of Langerhans (Green Arrow) with the islet cells surrounded by the acinar cells which are completed with small vacuoles (Blue Arrow). There are cavities in the interlobular of the acinar cells.	The histopathological image the of pancreatic tissue of the experimental animals in the Dose 1 Treatment Group shows that the pancreatic β-cells in the islet of Langerhans (Green Arrow) are found to contain only a few islet cells (Blue Arrow), indicating that there are numerous cell deaths but a few regenerations (Black Arrow).
	
The histopathological image of the pancreatic tissue of the experimental animals in the Dose 1 Treatment Group shows that, in the islet of Langerhans (Green Arrow), the islet cells are surrounded by acinar cells. There are cavities in the interlobular acinar cells which indicates cell necrosis (Blue Arrow) and cell regenerations (Black Arrow).	The histopathological image of the pancreatic tissue of the experimental animals in the Dose 3 Treatment Group shows that in the islet of Langerhans (Green Arrow) there are islet cells surrounded by acinar cells which are densely arranged but there are still gaps in the interlobular acinar cells, indicating cell death and cell regenerations (Black Arrow).

The description of the average number of pancreatic β-cells of the experimental animals that underwent regenerations (cell replacement/repair) by treatment groups showed that the highest average cell regeneration

occurred in the normal control group with 31.8 cells. This number was then followed by the DM with antidiabetic drug positive control group with an average number of cell regenerations of 28.25 cells. Meanwhile, the lowest average number of regenerations occurred in the positive control group with 6.5 cells.

Meanwhile, in the treatment groups, the highest number of cells that experienced regeneration was found in the sample group given dose 3 of *M. calabura* leaf extract with an average number of regenerations of 21.3 cells. This was followed by the sample group given dose 2 of *M. calabura* leaf extract with the average number of regenerations of 18 cells. Meanwhile, the treatment group given dose 1 of *M. calabura* leaf extract was at the lowest position with an average number of cell regenerations of 14.8 cells.

M. calabura has garnered interest due to its possible therapeutic qualities; the plant's active components can be found in the fruit, leaves, and roots, among other sections. Studies on the antioxidant and therapeutic qualities of *M. calabura* have been conducted by (42,43), with a focus on the high concentration of vitamins, minerals, flavonoids, and secondary metabolites that support the plant's ability to scavenge free radicals and act as an antioxidant. These investigations demonstrate Kersen's therapeutic potential as a homeopathic treatment for a range of illnesses, including diabetes mellitus.

Moreover, research by Pertiwi *et al.* (30) has explored the immunostimulatory effects, antibacterial activities, and radical scavenging properties of *M. calabura* leaves, underscoring its role as an immunomodulator and antimicrobial agent.

3.6. Correlation Between Total IFN-ɣ Concentration, Total Nitric Oxide (NO) Production Concentration and Histopathological Changes of Pancreatic Langerhans Island β-Cells in Control and Diabetes Mellitus Treated Rats After Exposure to *M. calabura* Leaf Extract

The results of the correlation test between IFN-ɣ concentration levels (ng/mL) and the number of necrosis cells showed a significance value of $p < 0.05$, indicating a relationship between IFN-ɣ concentration levels (ng/mL) and the number of Pancreatic Langerhans Island β-cells that undergo necrosis. Likewise, the level or degree of relationship between IFN-ɣ concentration levels (ng/mL) and the number of pancreatic Langerhans islet β cell necrosis is a perfect correlation with a negative relationship direction.

The correlation between the concentration of IFN-ɣ (ng/mL) and the amount of β cell regeneration in the pancreatic Langerhans islets demonstrates a significance value of $p < 0.05$, suggesting that the research data is correlated or shows a relationship between the concentration level of IFN-ɣ (ng/mL) and the amount of β cell regeneration in the pancreatic Langerhans islets. Similarly, the level of correlation between the concentration of IFN-ɣ (ng/mL) and the amount of β cell regeneration in the pancreatic Langerhans islets is perfect with a positive direction of relationship.

Furthermore, the correlation test between the production level of NO µmol/L and the number of β cell necrosis in the pancreatic Langerhans islets shows a significance value of $p < 0.05$, indicating a relationship between the concentration level of NO µmol/L and the number of β cell necrosis in the pancreatic Langerhans islets. Similarly, the degree or level of correlation between the level of NO µmol/L and the number of β cell necrosis in the pancreatic Langerhans islets is perfectly correlated with a positive direction.

The correlation test results between the concentration of NO µmol/L and the number of necrotic cells show a significance value of $p < 0.05$, indicating a relationship between the concentration of NOµmol/L and the number of necrotic β cells in the pancreatic Langerhans islets. Similarly, the degree or level of correlation between the concentration of NO µmol/L and the amount of β cell necrosis in the pancreatic Langerhans islets is perfectly correlated with a positive direction.

Based on the above analysis, it is indicating that there is a relationship between the level of IFN-ɣ concentration, NO production, and histopathological changes in Pancreatic Langerhans Island β-cells (necrosis and regeneration) in the control group and the experimental group treated with diabetes mellitus after exposure to *M. calabura* leaf extract. Therefore, this study proves that *M. calabura* leaf extract has immunomodulatory activity.

Although previous studies have shown that various parts of the *M. calabura* plant, including its leaves and fruits, contain active compounds that can fight free radicals and provide various health benefits. However, the difference of this study compared to previous studies lies in the observation of immunomodulatory activity of *M. calabura* leaf extract, with the aim of inducing and modulating immune responses in diabetic rats.

The limitation of this study is that it mainly assessed the immune response in diabetic rats and did not explore the potential side effects or long-term implications of using *M. calabura* leaves for immunomodulation.

Studies have shown that *M. calabura* leaf extract has great potential for use in the treatment of problems related to the immune system. By increasing immunostimulatory activity and reducing inflammation, the bioactive compounds in *M. calabura* leaves have the ability to alter immune responses. Preliminary results are very promising, but further research is needed to find out if *M. calabura* leaf extract is safe and effective for use in humans as an immunomodulator.

4. CONCLUSIONS

M. calabura leaf extract has immunomodulatory activity that can inhibit the mechanism of β-cell loss in pancreatic Langerhans islets (inhibit necrosis) in inducing and modulating immune response and reduce the risk of cardiovascular complications due to impaired Nitric Oxide (NO) production in rats treated with Diabetes mellitus.

The recommended effective dose of *M. calabura* leaf extract that can increase immunomodulatory activity in inhibiting the mechanism of β-cell loss in pancreatic islets of Langerhans and the risk of cardiovascular complications due to impaired NO production in rats treated with diabetes mellitus is a dose of 400 mg/kg bw/day.

Based on the limitations of the research, potential future studies could focus on investigating the long-term effects and potential side effects of using *M. calabura* leaves for immunomodulation in diabetic animal models, and research on how safe and effective the immunomodulatory role of *M. calabura* leaf extract is in humans.

Author contributions: FEH: supervision, technique, and conception. RY: data curation, inquiry, writing, preparing original drafts, visualization, editing. M: investigation, data collection, writing, writing-reviewing, and editing; original draft preparation.

Funding: This study was funded from the research budget of the Health Polytechnic of the Ministry of Health Kendari in 2023.

Acknowledgements: To the Institution of the Ministry of Health and the Director of the Health Polytechnic of the Ministry of Health Kendari.

Ethics statement: This research has been conducted in adherence to the highest ethical standards in the use of experimental animals to ensure the welfare, respect, and humane treatment of the animals used in the study.

Conflict of interest: There are no conflicts of interest.

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