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Expresion of Tumor Necrosis Factor Alfa (TNF-α) in The Pancreas of Diabetic Mice (*Mus musculus*) Following Adminstration of *Catharantus roseus* Extract

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Abstract
Diabetes mellitus is a chronic, non-communicable disease that develops gradually and is caused by various interrelated factors. This disease progresses from a metabolic disorder to an inflammatory condition influenced by pro-inflammatory cytokines such as TNF-α. These cytokines are involved in insulin signaling pathways and ultimately exacerbate insulin resistance in pancreatic cells, thereby worsening the diabetic condition. In addition to synthetic drugs, the treatment of diabetes mellitus (DM) can also be approached traditionally using various types of medicinal plants. *Catharanthus roseus* (commonly known as Madagascar periwinkle) contains compounds such as alkaloids, flavonoids, saponins, and carotenoids that act as antioxidants. This study aimed to investigate the effect of *Catharanthus roseus* extract on reducing TNF-α expression in the pancreas of diabetic mice (*Mus musculus*). In this study, mice weighing approximately 30 g were induced with streptozotocin at a dose of 0.1 ml/BB to induce diabetes mellitus. Subsequently, diabetic mice were administered *Catharanthus roseus* extracts prepared using methanol and petroleum benzene solvents at a concentration of 5%, with a dose volume of 1.5 ml/BB, for 14 days. On the final day, the mice were euthanized, and pancreatic tissues were harvested and processed for histological examination using immunohistochemistry. The results demonstrated that administration of *Catharanthus roseus* extracts with methanol and petroleum benzene solvents reduced TNF-α expression. The methanol extract of *Catharanthus roseus* was more effective in decreasing TNF-α expression.

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease resulting from endocrine dysfunction, which leads to impaired glucose regulation and consequently elevated blood glucose levels (hyperglycemia) (Perkeni, 2021; Kaihena *et al*., 2024; Ukratalo *et al*., 2024; Syuaib *et al*., 2025). According to the 2021 report from International Diabetes Federation (IDF) Atlas, the global prevalence of diabetes among individuals aged 20 to 79 is estimated at 10.5% (536.6 million people), and it is projected to rise to 12.2% (783.2 million people) by 2045. The prevalence of diabetes is nearly equal between men and women, with the highest rates occurring in the 75 to 79 age group (Sun *et al*., 2022). In Indonesia, approximately 19.47 million people are affected by diabetes, representing a prevalence of 10.6% of the total population.

Diabetes mellitus is a non-communicable chronic disease that develops gradually and is caused by various interrelated factors (Haamid, 2019). One of the primary contributors to tissue damage in diabetic patients is hyperglycemia, which is triggered by increased oxidative stress (Kurniawan *et al*., 2024). According to Budianto *et al*. (2022), insulin resistance, impaired insulin secretion, and elevated glucagon levels are key factors in developing hyperglycemia in DM. Numerous studies suggest that subclinical inflammation plays a significant role in developing insulin resistance and the pathogenesis of DM (Ukratalo & Sangadji, 2023; Kaihena *et al*., 2024a). This disease progresses from metabolic disturbances to an inflammatory condition, influenced by pro-inflammatory cytokines such as TNF-α, which are involved in insulin signaling pathways and ultimately worsen insulin resistance in pancreatic cells, further exacerbating diabetes (Daniele *et al*., 2014). Chronic inflammation, initiated by adipocyte fat accumulation, triggers an intensified inflammatory response. During this process, immune cells, particularly macrophages, infiltrate adipose tissue. These macrophages play a crucial role in the production and secretion of inflammatory cytokines such as TNF-α (Soták *et al*., 2024). Elevated cytokine levels directly impact the effectiveness of insulin signaling, contributing to insulin resistance (Phosat, 2017).

According to Ovaditya (2022), Tumor Necrosis Factor-alpha (TNF-α) is one of the first cytokines known to be involved in systemic inflammatory responses, and it is closely associated with the development of insulin resistance and diabetes. This cytokine is primarily produced by monocytes, lymphocytes, adipose tissue, and muscle and and it plays a role in the pathogenesis of metabolic syndrome associated with obesity (Narto *et al*., 2024). TNF-α's role in insulin resistance includes enhancing the release of free fatty acids (FFAs) from adipose tissue, inhibiting the synthesis of adiponectin (a molecule that has insulin-sensitizing effects when present at high concentrations in adipose tissue), and interfering with tyrosine phosphorylation of the insulin receptor substrate, which is essential for the development of intracellular insulin signaling. Additionally, TNF-α activates Nuclear Factor-Kappa B (NF-κB), leading to increased expression of adhesion molecules on the surface of endothelial cells and smooth muscle cells in blood vessels, thereby exacerbating inflammation in adipose tissue, endothelial dysfunction, and ultimately atherosclerosis (Sun *et al*., 2022). Furthermore, TNF-α can inhibit insulin signal transduction and affect glucose metabolism, potentially contributing to the onset and progression of diabetes mellitus. TNF-α can reduce insulin production by pancreatic cells and suggested that this cytokine could serve as a potential biomarker mediating the link between insulin resistance and diabetes mellitus.

Patients with DM generally rely on synthetic drugs, which often lead to unwanted side effects. One commonly used medication for DM is metformin, a biguanide (Hardianto, 2020; Ukratalo *et al*., 2023). Metformin works by reducing gluconeogenesis and enhancing glucose uptake and utilization by peripheral tissues (Herawati *et al*., 2021; Kuna *et al*., 2022). It helps lower blood glucose levels without causing weight gain and carries a lower risk of hypoglycemia compared to other antidiabetic agents (Achmad, 2017; Sasmita *et al*., 2024).

In addition to synthetic drugs, diabetes mellitus (DM) can also be treated using traditional approaches that involve various medicinal plants (Rosa & Lestari, 2018; Ukratalo *et al*., 2022). According to Muslikh & Prasetyawan (2024), Indonesia possesses a rich diversity of

medicinal plants, including *Catharanthus roseus*. This plant is highly variable and consists of several varieties. Traditionally, *Catharanthus roseus* has been used to treat various conditions such as malaria, constipation, and hypertension, as well as to act as a diuretic and to manage diabetes mellitus. Scientifically, it has been shown to exhibit antihyperglycemic (Nammi et al., 2006), antibacterial (Kabesh *et al*., 2015), antioxidant (oxidative stress-reducing), and antidiabetic properties (Singh *et al*., 2001). According to Nejat *et al*. (2018), *Catharanthus roseus* contains various active compounds such as flavonoids, saponins, tannins, and anticancer alkaloids like vinblastine (VLB), vincristine (VCR), and leurosin.

Flavonoids, as chemical compounds, play a significant role in regulating the production and activity of TNF-α in the body. These compounds can inhibit the synthesis of TNF-α at the cellular level by disrupting the signaling pathways involved in cytokine regulation. Flavonoids work by inhibiting the activation of NF-kB, a key regulator in TNF-α production. By preventing NF-kB activation, flavonoids reduce the excessive production of TNF-α and alleviate inflammation. Additionally, this mechanism may involve modulation of TNF-α receptors or other molecules involved in its signaling pathways. Flavonoids can block the binding of TNF-α to its receptors or interfere with downstream signal transduction processes, thereby reducing the pro-inflammatory activity and effects of TNF-α.

This study aims to investigate the effects of *Catharanthus roseus* extract on the reduction of TNF-α expression in the pancreas of diabetic mice (Mus musculus).

RESEARCH METHODS

Research Design

This study is an experimental research design

Materials and Instruments

The instruments used in this study include a Soxhlet apparatus (for extract reflux), a rotary evaporator (for solvent removal after extraction), filter paper, pipettes, tweezers, a hot plate, a separating funnel, glassware, mice drinking bottles, scissors, aluminum foil, cloth wipes, a digital weighing scale, a syringe, a gavage needle, cages, a glucotest apparatus, a surgical board, a dissecting set, roll film (for organ fixation), an incubator, a microtome, cover glass, a microscope (Olympus BX51), and a digital camera.

The materials used in this study include *Catharanthus roseus* leaves, streptozotocin (STZ), aquadest (distilled water), methanol, chloroform, petroleum benzene, male mice, ethanol solution at 50%, 70%, 80%, 90%, and 100% concentrations, 4% formalin, xylene I, xylene II, paraffin, albumin-glycerin, PBS (Phosphate Buffer Saline), immersion oil, H2O2, and TNF-α antibodies.

Procedure

Animal Preparation

The test animals used in this study were healthy male mice (characterized by shiny fur, active movement, and a pink-colored tail) aged 2-3 months, and with an average body weight of approximately 30 g.

Preparation of Test Materials

Fresh *Catharanthus roseus* leaves (1 kg) were air-dried. Once dried, the leaves were ground using a blender to obtain a fine powder, which was then used for extraction.

Extraction Procedure

The powdered *Catharanthus roseus* leaves were extracted using methanol and petroleum benzene in Soxhlet extractor for 6 hours with. The extraction process was repeated three times until the presence of extract was visibly evident in the Soxhlet chamber. The collected extract was then concentrated using rotary evaporator to remove any remaining solvent.

Determination of Dose and Administration of Test Solution (Oral)

The dose for the test animals was determined based on the DL50 of *Catharanthus roseus* leaf extract. The oral DL50 for male mice is 27.50 g/kg (Miller-Tainter method). For an average mice weight of 30 g, the DL50 is calculated as:

27.50 g/kg = 27.50 g/1000 g = 0.0275 g/g

For a 30 g mice, DL50 = 30 × 0.0275 = 0.825 g.

Three treatment groups were determined: methanol extract 5%, petroleum benzene extract 5%, and control. The dose administered was 1.5 ml for 24 hours. For the 5% treatment, 5 g of extract was diluted with 100 ml of aquadest. Each treatment resulted in a dose below the DL50 of 0.825 g.

5% treatment = 1.5 ml × 5% = 0.075 g.

The methanol and petroleum benzene extracts were tested for safety in a preliminary oral test over 24 hours.

Streptozotocin Induction in Mice

Streptozotocin (STZ) at a dose of 0.3 ml per mice was used to induce diabetes (Ukratalo *et al*., 2024).

Testing Procedure

The testing procedure involved four groups of mice, each consisting of three male mice, as follows:

a. Negative Control: Healthy mice (standard control)
b. Positive Control: Diabetic mice (positive control)
c. Methanol Extract 5% Treatment: Diabetic mice administered *Catharanthus roseus* methanol extract
d. Petroleum Benzene Extract 5% Treatment: Diabetic mice administered *Catharanthus roseus* petroleum benzene extract

The treatment lasted two weeks, with daily monitoring and blood glucose measurements using a glucotest apparatus. At the end of the study, the animals were euthanized for organ collection.

Surgical Procedure

The test animals were euthanized by inhaling chloroform. Each mice' s paws were pinned to a surgical board using pins. Dissection began with an incision along the lower abdomen, extending to the chest. The pancreas was carefully removed intact and placed into a 4% formalin-filled bottle for fixation.

Immunohistochemical Preparations (IHC) for TNF-α

The immunohistochemistry procedure started with placing the tissue in xylene I and xylene II, followed by sequential immersion in ethanol solutions (100%, 90%, 80%, 70%) and aquadest for 5 minutes at each stage. The tissue was washed thrice with PBS (pH 7.4), lasting 5 minutes. The tissue was incubated in 3% H2O2 for 10 minutes, followed by another PBS wash. The tissue was then incubated with primary antibody (anti-rat TNF-α) for 24 hours at 4°C. After incubation, the tissue was washed thrice with PBS (pH 7.4) for 5 minutes at room temperature. Secondary antibody incubation was performed for one hour at room temperature, followed by three PBS washes. Streptavidin-Horse Radish Peroxidase (SA-HRP) was added for 40 minutes, followed by PBS washes. The tissue was then incubated with DAB chromogen for 2-4 minutes, washed with running water for 5 minutes and stained with hematoxylin for 5 minutes. After rinsing with running water, dehydration was carried out with alcohol and xylene, followed by aquadest and drying. The tissue was then mounted with Entellan and covered with a cover glass.

TNF-α Expression Observation

The prepared pancreas tissue slides were observed under an Olympus BX51® microscope at 400x magnification. The antigen-antibody complex reacting with the DAB chromogen produced a brown deposit (chromogen), allowing the product to be visualized (Kartanegara, 2019).

Data Analysis

The TNF-α expression results were analyzed descriptively.

RESULTS AND DISCUSSION

Figure 1 shows the histological appearance of the pancreas in the normal control group, positive control group, and diabetic mice groups treated with methanol and petroleum benzene extracts of *Catharanthus roseus*.

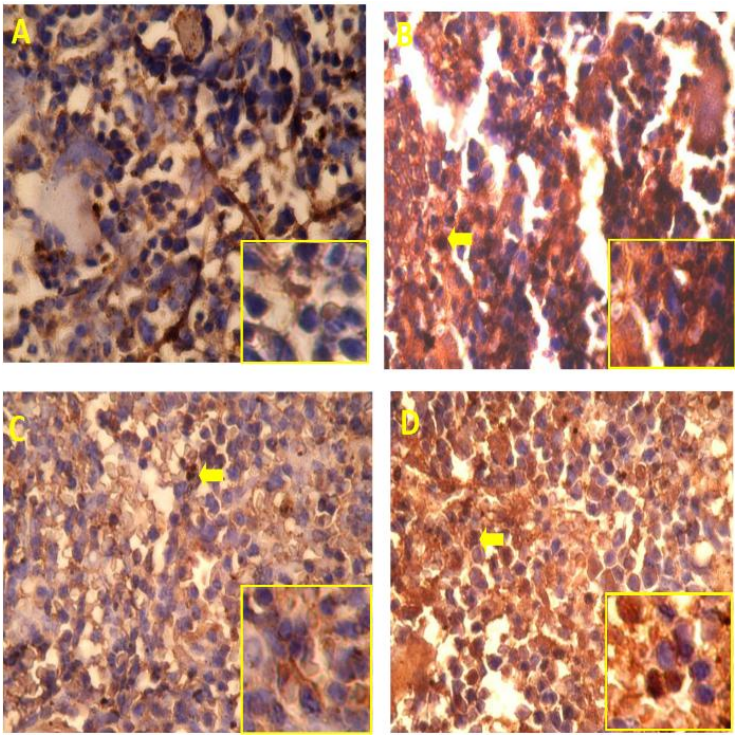

Figure 1. Expression of TNF-α in diabetic mice treated with *Catharanthus roseus* extract. (A) Histology of normal pancreas (without treatment/negative control), (B) Histology of pancreas with STZ treatment (positive control), (C) Histology of the pancreas with methanol extract of *Catharanthus roseus*, and (D) Histology of the pancreas with petroleum benzene extract of *Catharanthus roseus*. **Note:** ⇨ Expression of TNF-α (histological pancreas stained brown). Magnification 400x

In the observation of TNF-α expression in the pancreatic histology of mice not induced by STZ (standard control), TNF-α was not detected. In contrast, STZ-induced mice that did not receive Catharanthus roseus extract (positive control group) showed TNF-α expression in pancreatic cells, indicating cellular damage due to STZ induction. In STZ-induced mice treated with either methanol or petroleum benzene extracts of Catharanthus roseus, TNF-α expression was still present but at lower levels compared to the positive control group. These findings suggest that Catharanthus roseus extract may reduce TNF-α expression, indicating a potential protective effect against pancreatic cell damage under diabetic conditions.

TNF-α is a key marker in the acute inflammatory response and is primarily produced by activated mononuclear phagocytes. Its expression in pancreatic tissue can indicate pathological conditions and can be evaluated using immunohistochemical methods.

The observations indicate that in the positive control mice (DM mice without treatment), TNF-α expression was evident, marked by brown-stained cell nuclei and reddish-brown stained cytoplasm. The TNF-α expression in the pancreatic β-cells observed in this study was triggered by increased intracellular glucose levels, resulting in hyperglycemia following STZ induction. Elevated glucose levels can caused tissue damage, beginning with endothelial dysfunction in blood vessels. This condition leads to increased metabolic activity, where mitochondria produce excessive superoxide (O_2), resulting in oxidative stress. Consequently, DNA damage

occurs along with the activation of the PARP enzyme. PARP activation inhibits Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), an enzyme involved in glycolysis, thereby halting the glycolytic process. This disturbance negatively affects large and small blood vessels (Narto & Restami, 2024).

Tumor necrosis factor-alpha (TNF-α) is a soluble protein involved in various pathological conditions in humans, including autoimmune diseases, obesity-associated insulin resistance, cardiovascular disorders, and cancer (Burke *et al*., 2014). According to Lampropoulou et al. (2020), TNF-α also plays a role in chronic inflammatory responses in the kidneys, a common complication of type 2 diabetes. TNF-α can impact insulin signaling pathways and disrupt normal insulin function. One consequence is the increased activity of serine/threonine protein kinase, which can inhibit insulin receptor efficacy and interfere with insulin signaling (Velikova *et al*., 2021).

Akash *et al*. (2018) noted that TNF-α production plays a critical role in initianting inflammation in specific tissues. This process involves the generation of reactive oxygen species (ROS) and the activation of various transcriptional pathways. Elevated TNF-α levels can disrupt insulin signaling pathways and induce insulin resistance in adipose and peripheral tissues. This mechanism primarily occurs through serine phosphorylation, which is a key contributor to the development of diabetes mellitus (DM) or chronic insulin resistance.

The administration of methanol and petroleum benzene extracts of *Catharanthus roseus* to diabetic mice for 14 days resulted in a reduction of TNF-α expression in the β-cells of the pancreatic islets. The decrease in TNF-α expression following treatment was attributed to secondary metabolites present in the plant. It is known that, *Catharanthus roseus* contains flavonoids with anti-inflammatory and antioxidant properties (Husna *et al*., 2022). Flavonoids act as anti-inflammatory agents by reducing the expression of pro-inflammatory cytokines and inhibiting reactive oxygen species (ROS) production. Specifically, flavonoids can inhibit several pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and suppress the activation of NF-κB induced by elevated ROS levels. The reduction of NF-κB activity due to flavonoids in the ethanol extract of *Catharanthus roseus* leaves leads to decreased TNF-α expression. This reduction in NF-κB activity promotes regeneration of β-cells that had previously undergone apoptosis, resulting in decreased TNF-α expression in β-cells (Utami, 2022).

In addition to flavonoids, *Catharanthus roseus* contains saponins, tannins, and alkaloids, which act as antioxidants by scavenging free radicals. The scavenging activity of these compounds helps reduce damage to pancreatic tissue, thereby decreasing mononuclear cell infiltration into the pancreas for the phagocytosis of damaged β-cells. This leads to a reduction in inflammation, which in turn lowers TNF-α production and facilitates the repair of pancreatic β-cells responsible for insulin production.

CONCLUSION

Methanol and petroleum benzene extracts of *Catharanthus roseus* have been shown to reduce TNF-α expression in a diabetic mice model. Administration of *Catharanthus roseus* extracts during the observation period significantly decreased the pro-inflammatory cytokine TNF-α expression in the pancreases of STZ-induced mice, a commonly used model for type 1 diabetes.

This reduction in TNF-α expression suggests that *Catharanthus roseus* extracts have the potential to alleviate pancreatic inflammation, which may support the restoration of pancreatic function and the reduction of blood glucose levels in diabetic mice.

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