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The Immunomodulatory Activity of *Cinnamomum burmanni* Bark Extract on Leucocyte Differentiation of Mice (*Mus musculus*) In Diabetes Mellitus Model

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Abstract: Diabetes mellitus frequently coexists with infection, leading to an immunological response characterised by an elevation in leukocyte levels. This research aimed to assess the immunomodulatory activity of an extract derived from the bark of *Cinnamomum burmanni* on the differentiation of leukocytes in a diabetes mellitus model using mice (*Mus musculus*). Prior to the administration of therapy, the glucose levels of all mice were assessed. Subsequently, groups K (+), P1, P2, and P3 were administered STZ at 0.2 ml per individual. The blood glucose levels (post-STZ blood glucose) were checked once more on the seventh day. In the event of a rise in blood glucose levels over (>200 mg/dL), subjects belonging to groups P1, P2, and P3 were administered *Cinnamomum burmanii* extract under the prescribed dosage. The intervention was administered for 14 days, and blood glucose levels were assessed on the final day of experimentation. On the final day of the experiment, blood samples were collected from the mice's tails in order to prepare blood preparations. The findings indicate that the methanol extract derived from cinnamon bark possesses immunomodulatory properties, as evidenced by its ability to decrease the population of eosinophils, lymphocytes, and monocytes while concurrently increasing the amount of neutrophils.

Keywords: Cinnamon; Diabetes mellitus; Immunomodulatory; Leucocyte differentiation

Introduction

Diabetes mellitus is a medical condition characterised by the dysfunction of pancreatic endocrine hormones, specifically insulin and glucagon (Galicia-Garcia et al., 2020; Lassie et al., 2023; Wardani, 2023). Diabetes mellitus arises from insufficient insulin activity, manifesting as diminished insulin production or reduced peripheral tissue responsiveness to insulin (insulin resistance) (Tandi et al., 2023; Ukratalo et al., 2023). Additionally, pancreatic β-cells may exhibit less capacity to release insulin in response to glucose stimulation. The primary signs encompass dysfunctions in lipid, carbohydrate, and protein metabolism, leading to the stimulation of hyperglycemia (Ahmed et al., 2021). Hyperglycemia, suppose left untreated, can progress into Diabetes mellitus and give rise to a range of problems (ADA, 2013).

According to Berbudi et al. (2020), diabetes mellitus can also lead to immunological insufficiency through many pathways. Elevated blood glucose levels can impede the chemotaxis of phagocytes and the movement of inflammatory cells, hindering their accumulation at the site of inflammation. Diabetes mellitus frequently coexists with infection, leading to an immunological response characterised by elevated leukocyte levels (Dowey et al., 2021; McElwain et al., 2021).

An increased leucocyte count may indicate inflammation and infection. This often occurs in patients with chronic diseases such as DM (Chmielewski et al., 2018). Inflammation in DM is also associated with

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oxidative stress, which results in tissue damage. Tissue damage resulting from oxidative stress or oxygen deprivation will increase inflammation and result in cell death. Inflammation induces the release of leukocytes into the blood circulation and other tissues, resulting in an increase in the number of leukocytes and activating other leukocytes in the area of inflammation as a form of body defense. The systemic inflammatory response will stimulate the hemopoietic system, especially the spinal cord, to release leucocytes into the blood circulation, resulting in an increase in the number of leucocytes (Ardina, 2018). An excessive increase in leucocytes causes oxidative stress conditions with an uncontrolled amount of reactive oxygen species (ROS). This causes cell and tissue damage that promotes chronic inflammation (Ayutama et al., 2020; Chelombitko, 2018).

Cinnamomum burmannii is a frequently seen botanical species in Indonesia, known for its medicinal properties. Cinnamon is recognised as a dietary source of antioxidants. Cinnamon possesses a diverse array of constituents that have been found to confer health benefits. These include potential efficacy in managing conditions such as uric acid levels, hypertension, gastric ulcers, vertigo, common colds, diarrhoea, flatulence, vomiting, hernias, constipation, asthma, canker sores, urinary pain, antirheumatic effects, perspiration regulation, flatulence reduction, and hunger stimulation. Moreover, scientific research has demonstrated that cinnamon possesses anti-inflammatory, anti-fungal, antioxidant, antidiabetic, insecticidal, and nematicidal properties (Qosimah et al., 2023).

Cinnamon comprises various constituents, including essential oil, safrole, sinamadehid, eugenol, tannins, resin, calcium oxalate, tanning agents, flavonoids, saponins, and nutritional components such as sugar, protein, crude fat, and pectin. These constituents have been suggested to contribute to the modulation of immune response (Awaluddin et al., 2023; Farias et al., 2020; Winda et al., 2023; Wulandari et al., 2023). Hosseinzade et al. (2019) reported that flavonoids had immunomodulatory properties. Flavonoids, being polyphenolic chemicals, have antioxidant properties. Specifically, within blood cells, they serve as reservoirs for hydroxyl and superoxide radicals, thereby safeguarding membrane lipids. Flavonoids exert their effects on lymphokines, specifically Interferon γ, synthesized by T cells. This interaction leads to the activation of phagocytic cells, hence promoting phagocytic responses. Additionally, flavonoids can enhance lymphocyte proliferation, augment the population of T cells, and promote the secretion of IL-12. Flavonoids can enhance the synthesis of IL-2, a crucial cytokine involved in lymphocyte proliferation.

Method

Type of Research

This research is a laboratory experimental research.

Research Design

This study utilised a Completely Randomised Design (CRD) with five treatments and three replicates. The division and treatment of each group are as follows:

K+ : Positive control group (mice given streptozotocin)
K- : Negative control group (without treatment)
P1 : Injected with streptozotocin and given a dose of cinnamon bark extract 200 mg / g BB
P2 : Injected with streptozotocin and given a dose of cinnamon bark extract of 400 mg / g BB
P3 : Injected with streptozotocin and given a dose of cinnamon bark extract 800 mg / g BB

Tools and Materials

The equipment utilised in this investigation comprised enclosures for mice, digital weighing scales, erlenmeyer flasks, sonde instruments, surgical equipments for experimental animals (*scalpel*), tweezers, scissors, needles, a wax table, Petri dishes, a microscope, and a digital camera. Simultaneously, the utilised elements encompass cinnamon bark, distilled water, streptozotocin (STZ), mice, aluminium foil, 70% alcohol, filter paper, tissue, mice feed, PAM water, distilled water, Giemsa, absolute methanol, and immersion oil.

Conducting Research

Animal Preparation

Prior to commencing the research, the necessary provisions for housing experimental animals were made, including the arrangement of cages (specifically plastic tubs), bedding material, as well as designated areas for mice to consume food, water, and feed. Subsequently, a period of one-week acclimatisation was conducted within the laboratory setting. The mice were categorised into five distinct groups: the positive control group, the negative control group (consisting of non-diabetic mice), and three experimental groups with dosages of 200mg/g BW, 400mg/g BW, and 800mg/g BW, respectively.

Preparation of Test Materials

Cinnamon bark is obtained by removing the outer layer of trees. The outer skin of cinnamon bark is thoroughly cleaned to remove impurities, then split into segments of 3-4 cm in length. Subsequently, the split bark is dried in ambient room conditions. Following the

drying process, the cinnamon bark underwent pulverisation using a blender, resulting in the production of cinnamon bark powder (simplisia). Subsequently, the simplisia was subjected to extraction using the Maceration method.

Extraction

Cinnamomum burmanii bark specimens were collected and subsequently fragmented into smaller segments, which were subsequently subjected to a drying process within a controlled laboratory environment. After drying, the *Cinnamomum burmanii* bark was pulverised using a blender to obtain a powdered form. Subsequently, the powder was subjected to extraction utilising metabolic solvents by the maceration method. The resulting extract was then concentrated using a rotary evaporator.

Testing Procedure

Prior to the initiation of treatment, the glucose levels of all mice were assessed. Subsequently, groups K (+), P1, P2, and P3 were administered STZ at 0.2 ml per individual. The blood glucose levels (post-STZ blood glucose) were checked once more on the seventh day. In the event of a rise in blood glucose levels over 200 mg/dL, subjects in groups P1, P2, and P3 were administered *Cinnamomum burmanii* extract under the prescribed dosage. The intervention was administered for 14 days, and blood glucose levels were assessed on the final day of experimentation. Blood smear preparations were made by extracting blood from the tail of the mice on the final day.

Observation of blood smear preparations

The blood smear preparations were examined under a microscope at a magnification 1000x, utilising immersion oil. Daily observations were conducted following the onset of the parasite infection. The purpose of this experiment was to determine the percentage of parasitemia and leucocyte differentiation in mice.

Leucocyte differentiation calculation

The leukocytes were enumerated in groups of 100 and subsequently classified into distinct types, including neutrophils, eosinophils, basophils, lymphocytes, and monocytes. The process of leucocyte amount involves examining multiple fields of vision along the smear, which are systematically shifted towards the centre. Parallel to the edge of the smear and subsequently shifted back to the edge. This shifting pattern is repeated until 100 leukocytes have been counted. The expression of the relative value of each type of leukocyte is denoted in percentage units (Sosilawati, 2011).

Data Analysis

The data about the relative value of each leukocyte type was subjected to statistical analysis using the *Analysis of Variance* (ANOVA) test. Subsequently, the analysis was further extended by employing the Least Significant Difference (LSD) test to ascertain the disparities in the administered treatment.

The workflow procedure in this research can be seen in the following diagram.

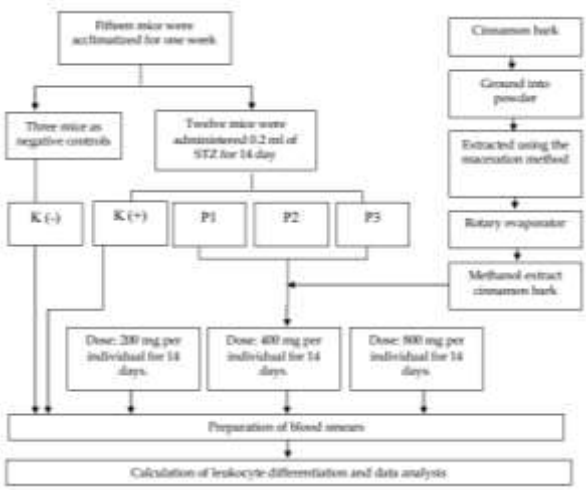

Figure 1. Research flow diagram

Result and Discussion

Table 1 displays the computed average percentages of neutrophils, eosinophils, lymphocytes, and monocytes in various groups of mice, including the negative control group, cheerful group, STZ-induced group, and the group treated with methanol extract of cinnamon bark at doses of 200 mg/g BW, 400 mg/g BW, and 800 mg/g BW.

Table 1. Average Percentage of Neutrophil, Eosinophil, Lymphocyte and Monocyte

Treatment	Percentage of Leukocyte types (%)			
	Neutrophil	Eosinophil	Lymphocyte	Monocyte
Control -	66.00 ± 4.36^{a}	10.67 ± 3.51^{a}	22.00 ± 2.65^{a}	4.67 ± 1.16^{a}
Control +	36.00 ± 3.00^{b}	19.67 ± 2.08^{b}	29.00 ± 2.00^{b}	14.00 ± 2.00^{b}
Dose 200 mg/g BB	48.67 ± 1.16^{c}	14.67 ± 1.53^{c}	26.67 ± 1.53^{b}	9.00 ± 2.00^{c}
Dose 400 mg/g BB	56.00 ± 3.00^{d}	13.00 ± 1.00^{d}	26.33 ± 2.52^{b}	6.00 ± 1.00^{a}
Dose 800 mg/g BB	59.67 ± 3.06^{d}	9.33 ± 1.53^{e}	23.33 ± 1.53^{b}	3.67 ± 2.08^{d}

Notes: Superscripts with the same letter are not significantly different

According to the data presented in Table 1, the average percentage of neutrophil varied across different experimental groups. The negative control group exhibited an average percentage of 66%; on the other hand, the positive control group had an average percentage of 36%. In the group of mice induced by STZ and treated with cinnamon bark methanol extract at a dose of 200 mg/g BW, the average percentage was 48.67%. For the dose of 400 mg/g BW, the average percentage was 56%, and for the dose of 800 mg/g BW, the average percentage was 59.67%. The negative control group exhibited an average eosinophil of 10.67%. In the positive control group, the average eosinophil was 19.67%. Among the mice induced by STZ and treated with cinnamon bark methanol extract, a dose of 200 mg/g BB has resulted in an average eosinophil of 14.67%. At a dose of 400 mg/g BB, the average eosinophil was 13%; on the other hand, at a dose of 800 mg/g BB, it was 9.33%.

The negative control group exhibited an average lymphocyte percentage of 22%; on the other hand, the positive control group demonstrated a percentage of 29%. In the group of mice induced by STZ and treated with a 200 mg/g BB dose of methanol extract of cinnamon bark, the lymphocyte percentage was 26.67%. Similarly, at a dose of 400 mg/g BB, the lymphocyte percentage was 26.33%, and at a dose of 800 mg/g BB, it was 23.33%. The negative control group exhibited an average monocyte percentage of 4.67%; on the other hand, the positive control group demonstrated a percentage of 14%. In the group of mice induced by STZ and treated with cinnamon bark methanol extract, the monocyte percentages were 9% for a dose of 200 mg/g BB, 6% for a dose of 400 mg/g BB, and 3.67% for a dose of 800 mg/g BB. The figure depicted in Figure 2 provides a more precise representation of the average percentages of neutrophil, eosinophil, lymphocyte, and monocyte in various groups, including the negative control mice group, cheerful group, STZ-induced mice group, and those administered cinnamon bark methanol extract doses of 200 mg/g BB, 400 mg/g BB, and 800 mg/g BB.

Figure 2 illustrates that the average neutrophil in mice treated with varying dosages (200 mg/g BW, 400 mg/g BW, and 800 mg/g BW) of cinnamon bark methanol extract following STZ induction has a tendency towards augmentation in contrast to the observed decline in the positive control group. The average proportions of eosinophils, lymphocytes, and monocytes in the cohort of mice treated with STZ and cinnamon bark methanol extract exhibited a decline as the administered dosage increased, in contrast to the positive group, where these proportions displayed an upward trend.

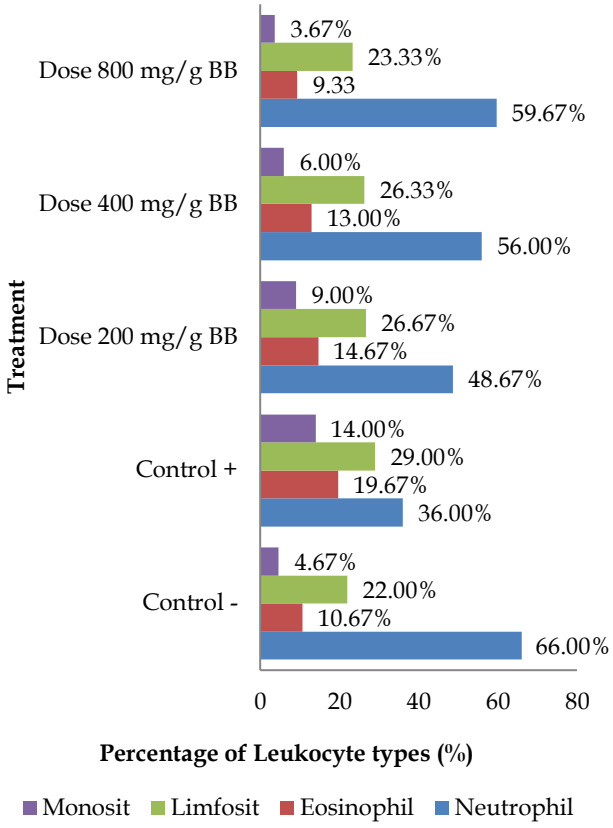

Figure 2. Histogram of the average percentage of neutrophil, eosinophil, lymphocyte and monocyte

According to the findings of the *Analysis of Variance* (ANOVA), the oneway test indicates that the $F_{value} > F_{table}$ value. This suggests that the methanol extract derived from cinnamon bark has a significant impact on the average quantities of neutrophils, eosinophils, lymphocytes, and monocytes in mice. The findings from the BNT test indicate that there is a statistically significant difference in the average percentage of neutrophils among groups K-, K+, P1, and P2. However, there is no statistically significant difference observed between groups P2 and P3 in terms of neutrophil percentages in mice. There was a significant difference observed in the average number of eosinophils across all treatment groups. Conversely, the average number of lymphocytes in groups K+, P1, P2, and P3 did not significantly differ. There is a significant difference in the average number of monocytes across groups K +, P1, and P3; however, no significant difference was seen between groups K- and P2.

The induction of Diabetes mellitus was achieved with the administration of a streptozotocin injection, as reported by Furman in 2015. This diabetes disease is associated with an elevation in eosinophils, lymphocytes, and monocytes. The administration of STZ without extracts to the K+ mice group resulted in a

reduction in the number of neutrophils, as depicted in Figures 1 and 1. Eosinophils are a subset of leukocytes characterized by their relatively low abundance. Eosinophils are actively involved in the regulation of acute allergies and inflammatory processes, as well as in the regulation of parasite infestation. Additionally, they are responsible for the phagocytosis of bacteria, antigen-antibody complexes, mycoplasma, and yeast (Wen et al., 2017). The observed elevation in eosinophil levels in this investigation is hypothesized to result from mice being subjected to acute allergic reactions or infestation caused by toxins derived from streptozotocin.

Lymphocytes are considered to be the predominant leukocytes in ruminant species. Lymphocytes, which are a type of leukocytes, exhibit a response to antigens by generating antibodies that circulate within the bloodstream or by engaging in the process of cellular immunity (Galvin, 2008). The observed elevation in lymphocyte within the scope of this investigation can be attributed to introduction of a toxic agent, specifically streptozotocin, into the organism, necessitating the production of antibodies by the body. Furthermore, the elevation in lymphocyte can be attributed to immunosuppression or lymphoid tissue impairment caused by specific causes or animals experiencing stress.

Monocytes, a subset of leukocytes, play a crucial role in combating infections of a less severe kind (Espinoza et al., 2022). The lifespan of leukocytes in the bloodstream is limited to a few days. Monocytes, being juvenile blood cells, may exhibit more efficient participation in phagocytosis while in circulation, as compared to their function as macrophages within tissues. Monocytes are observed to emerge in instances where antigens are resistant to phagocytosis by neutrophils, leading to their transformation into more giant macrophages. Activated macrophages are capable of producing interleukin-1 alpha (IL-1α). Furthermore, IL-1α is also synthesized by activated endothelial cells. Interleukin-1 alpha (IL-1α) plays a crucial role as the primary mediator of the acute phase response. This response prompts hepatocytes to enhance protein synthesis during the acute phase, resulting in the production of several proteins such as anti-proteases, haptoglobin, components of C3, C4, and factor B, as well as fibrinogen and ferritin. The impacts of IL-1α on endothelial cells encompass several outcomes, such as heightened synthesis of prostaglandin I2 and prostaglandin E2, augmented production of plasminogen activator inhibitor, and elevated expression of intercellular adhesions molecule-1 (ICAM-1) (Prame Kumar et al., 2018).

The observed phenomenon of endothelial adherens being added to circulating mononuclear cells due to IL-1α can perhaps be elucidated by this explanation. Furthermore, activated macrophages can secrete various enzymes, such as neutral proteases like collagenase and elastase, potentially harming connective tissue. Additionally, they can release procoagulant molecules like tissue factor or factor VII, leading to localised coagulation through the extrinsic coagulation pathway. Moreover, these macrophages can also produce plasminogen activators. The conversion of plasminogen into plasmin by the elastase enzyme leads to the degradation of fibrin, gradually reversing its synthesis and ultimately culminating in clot dissolution. Simultaneously, the occurrence of oxidative stress triggers the activation of monocytes, leading to the production of pro-inflammatory cytokines such as IL-1, TNF-α, IL-6, IL-8, and IL-12. Furthermore, the activated monocytes generate growth factors that induce the proliferation and migration of smooth muscle cells from the tunica media to the tunica intima, resulting in the thickening of the tunica intima. Both of the processes mentioned above can lead to the development of sclerosis and the formation of obstructions inside blood vessels (Yulianti et al., 2023).

Neutrophils are the predominant subset of leukocytes. According to Rosales (2018), neutrophils serve as the principal line of defence against the infiltration of foreign entities into bodily tissues. The observed reduction in neutrophil in the present study can be attributed to the manifestation of Diabetes mellitus in the mice. In the context of theileriosis, a blood parasite infection, it is seen that a reduction in the number of neutrophils may occur due to the loss of lymphoid organs responsible for the production of leukocytes.

In this investigation, the administration of methanol extract derived from cinnamon bark resulted in a reduction in the number of eosinophils, lymphocytes, and monocytes. The observed effect was an elevation in neutrophil within the P1, P2, and P3 cohorts. This phenomenon can be attributed to secondary metabolite components in cinnamon bark, which possess antioxidant properties. These compounds include flavonoids, saponins, tannins, and others. The findings of this study provide evidence that cinnamon bark possesses secondary metabolite chemicals with antioxidant properties. The findings of this study demonstrate that the methanol extract of cinnamon bark contains secondary metabolite chemicals that possess immunomodulatory properties.

According to the research by Novianti et al. (2023), flavonoids are known to modulate protein kinase by inhibiting transcription factors that play a role in cytokine production. Cytokines are crucial in the mechanism of the inflammatory process and the increase in the number of leukocytes. Controlled cytokine

production will maintain the leukocyte count within normal limits. Flavonoids have been found to induce the secretion of interleukin-12 (IL-12) by T cells. IL-12 stimulates the generation of IFN-γ by NK cells, and it has been observed that IFN-γ is involved in the activation of macrophages (Ma et al., 2015).

Saponin compounds can induce the production of cytokines, including interleukins and interferons, which are known to contribute to immunostimulatory effects. Interleukins and interferons exhibit immunoreactivity towards antigens, which are exogenous entities that infiltrate the organism. According to Harun et al. (2020), saponins exhibit immunostimulatory effects when present in moderate quantities but can operate as immunosuppressants when their levels surpass the usual range. The augmentation of T lymphocyte cells and B lymphocyte cells is not solely attributed to the presence of a substance that can induce the proliferation of lymphocyte cells. It is also influenced by the exposure to antigens that prompt lymphocyte cells to replicate at an accelerated rate, facilitated by the leptin and IL-6 pathways generated by adipose tissue in individuals with Diabetes mellitus. Consequently, this leads to an elevation in lymphocyte production and differentiation. The initiation of the acute-phase response is considered a crucial consequence of IL-6 activity. During the acute-phase reaction, the liver synthesizes and secretes *acute-phase proteins*.

C-reactive protein (CRP) is classified as one of the *acute-phase proteins* (Sproston et al., 2018). C-reactive protein (CRP) can initiate the complement cascade by interacting with C1q, the initial component involved in complement activation within the classical pathway. Furthermore, interleukin-6 (IL-6) plays a crucial role in innate and adaptive immune responses. This particular cytokine has diverse functionalities. Within the context of innate immunity, it can induce the production of acute-phase proteins by hepatocytes, hence contributing to the manifestation of systemic inflammatory responses. Interleukin-6 (IL-6) can induce the generation of neutrophils from precursor cells located in the bone marrow, typically in conjunction with the Colony Stimulating Factor (CSF). Within adaptive immunity, interleukin-6 (IL-6) is a stimulant for the proliferation of specialised B cells, which subsequently undertake the crucial task of generating antibodies. The T-helper (Th) cells play a crucial role in the regulation and development of immunological responses, as stated by de De Bruin et al. (2014).

Conclusion

The administration of streptozocin in mice induces the development of diabetes mellitus, resulting in an elevation in the population of eosinophils, lymphocytes, and monocytes while concurrently leading to a reduction in the number of neutrophils. The immunomodulatory properties of the methanol extract derived from cinnamon bark are attributed to its ability to decrease the population of eosinophils, lymphocytes, and monocytes while concurrently increasing the count of neutrophils.

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Conflicts of Interest

The authors declare no conflict of interest.

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