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Antioxidant Activity and Immunomodulatory Effect of *Stachytarpheta* sp. Leaf Extract on Phagocytic Activity of Mice Peritoneal Macrophages in Alloxan Induction

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Abstract: *Hyperglycemia is a condition characterized by elevated blood glucose levels, which is a hallmark of diabetes mellitus. Prolonged hyperglycemia not only disrupts glucose homeostasis but also impairs the immune response. The condition of hyperglycemia in the body can increase free radicals and oxidative stress, thus can impair various macrophage functions, including their ability to engulf and eliminate pathogens. The bioactive constituents in Stachytarpheta sp. may contribute to reducing oxidative damage and enhancing immune system function. This study aims to evaluate both the antioxidant capacity and the immunomodulatory effects of Stachytarpheta sp. leaf extract on the phagocytic activity of peritoneal macrophages in alloxan-induced diabetic mice. Alloxan is widely used in research to induce hyperglycemia (diabetic conditions) in experimental animals. The antioxidant activity of the extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, while phagocytic activity of peritoneal macrophages was performed in vivo using male mice. Stachytarpheta sp. leaf extract from Beteng Sari Village, East Lampung has very strong antioxidant activity against DPPH with IC_{50} value 14,85 ± 0,30 µg/mL. This extract at doses of 200, 300, and 400 mg/kgBW can be an immunomodulator that maintains the phagocytic activity of macrophage cells in alloxan-induced mice.*

Keywords: *Antioxidant; Hyperglycemia; Immunomodulatory; Macrophage; Stachytarpheta sp.*

1. INTRODUCTION

Hyperglycemia is a condition characterized by elevated blood glucose levels, which is a hallmark of diabetes mellitus. This condition often associated with various metabolic disturbances. One of the major contributing factors to cellular damage in hyperglycemic conditions is oxidative stress, which arises due to the excessive generation of reactive oxygen species (ROS) (Tang et al., 2014). These free radicals can damage lipids, proteins, and DNA, leading to impaired cellular function and tissue injury, and involved in several diseased conditions such as diabetes mellitus (Brownlee, 2001; Phaniendra et al., 2015).

Prolonged hyperglycemia not only disrupts glucose homeostasis but also impairs the immune response. The innate immune system, which serves as the first line of defense, is significantly affected. Several innate defense mechanisms work less than optimally due to diabetes mellitus condition (Berbudi et al., 2020). The innate immunity fighting primary infections through macrophages. Macrophages are effector cells of

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the innate immune system that phagocytose bacteria and secrete both pro-inflammatory and antimicrobial mediators. The condition of hyperglycemia in the body can increase free radicals and oxidative stress. Oxidative stress can impair various macrophage functions, including their ability to engulf and eliminate pathogens or apoptotic cells. Furthermore, diabetes condition leads to a reduced expression of Fcγ receptors on macrophages and monocytes (Kirkham, 2007; Restrepo et al., 2014; Hirayama et al., 2017). Thus, managing oxidative stress is crucial not only for glycemic control but also for maintaining immune competence in diabetic individuals.

In recent years, there has been growing scientific interest in natural antioxidants and immunomodulatory agents derived from medicinal plants. One such plant with notable potential is white snakeweed (*Stachytarpheta* sp.), a wild species known for its diverse pharmacological properties. Snakeweeds also known as porter weeds (*Stachytarpheta* spp.) are a member of the family of Verbenaceae which consists of 2600 species and 100 genera. This plant mostly grows in the tropical regions of America, as well as in the subtropical forests of Africa, Asia, and Oceania (Idu et al., 2007; Liew & Yong, 2016). Snakeweed leaves contained flavonoids, alkaloids, steroids, glycosides, terpenoids, resins, tannins, saponins, carbohydrates, reducing sugars, and proteins (Estella et al., 2020; Harini et al., 2022; Patil et al., 2023). Several studies reported that snakeweed leaf has antioxidant, antibacterial, antidiarrheal, antifungal, antiinflammatory, antinociceptive, antihypertensive, antidyslipidaemia, anticancer, hepatoprotective, antitrypanosomal, and wound healing properties (Liew & Yong, 2016; Nazir et al., 2020; Udo et al., 2020). *Stachytarpheta jamaicensis* leaves posses hypoglycemic (antidiabetic property) and antioxidant effects and may therefore serve as a potential source of hypoglycaemic agent as well as antioxidant agents for the prevention and management of free radical induced metabolic diseases, and is also not toxic to body organs on long term administration (Egharevba et al., 2019; Estella et al., 2020). Therefore, the white snakeweed plant (*Stachytarpheta* sp.) contains phytochemical constituents with promising potential for development as phytopharmaceutical agents.

The bioactive constituents in *Stachytarpheta* sp. may contribute to reducing oxidative damage and enhancing immune system function. However, research on *Stachytarpheta* is still limited, especially on its immunomodulatory potential in hyperglycemic conditions. Investigating the effects of *Stachytarpheta* sp. leaf extract on peritoneal macrophage activity could provide valuable insights into its ability to support immune function during oxidative stress under hyperglycemic conditions. This study aims to evaluate both the antioxidant capacity and the immunomodulatory effects of *Stachytarpheta* sp. leaf extract on the phagocytic activity of peritoneal macrophages in alloxan-induced diabetic mice. Alloxan is widely used in research to induce hyperglycemia (diabetic conditions) in experimental animals (Wulandari et al., 2024).

2. RESEARCH METHOD

This research is part of a series of studies we previously published, titled "*Effect of Stachytarpheta sp. Leaf Extract In Alloxan-Induced Mice*" (Khairani, Lusiana, et al., 2024). That publication focused on qualitative phytochemical tests of white snakeweed extract and its protective effect against alloxan induction in mice as seen from the parameters of blood sugar levels, hemoglobin levels, total erythrocytes, and total leukocytes. Meanwhile, the data of the white snakeweed's antioxidant activity and its immunomodulatory potential will be explained in this study.

Stachytarpheta sp. Leaf Extraction

The white snakeweed was collected from Beteng Sari Village, Jabung District, East Lampung Regency, Lampung Province, Indonesia. The leaves were air-dried, then blended into powder. The simplicia powder was processed using maceration method with ethanol 96% solvent (1: 5 w/v ratio) and soaked for 72 hours. The macerate was filtered and evaporated using a rotary evaporator at 50°C to obtain the crude extract (Khairani, Lusiana, et al., 2024).

The ethanol-free test

The ethanol-free test was conducted on previously prepared white snakeweed extracts, to ensure the absence of ethanol content. 1 ml of extract was added 2 drops of H_2SO_4 and 2 drops

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of CH_3COOH then heated in a test tube. If it does not emit the typical ester aroma of ethanol, then the extract is free from ethanol content (Tivani et al., 2021). Furthermore, a validation test was carried out, 1 ml of extract was added to 2 drops of H_2SO_4 and 2 drops of $K_2Cr_2O_7$, then heated. The extract is positive for ethanol-free if it does not change colour to blue (Sukadiasa et al., 2023). Extracts to be used in this study must be free from ethanol.

Antioxidant activity test

The antioxidant activity of the extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The extract was initially dissolved in methanol, and five different concentrations were prepared (9.375, 18.75, 37.5, 75, and 150 ppm). For each concentration, 100 µL of the extract and a positive control (ascorbic acid) were transferred into individual wells of a 96-well microplate, with each treatment performed in triplicate. Subsequently, 100 µL of DPPH solution (final concentration 125 µM in methanol) was added to each well. The mixture was incubated in the dark at 30°C for 30 minutes. Following incubation, absorbance readings were taken at 514 nm using a microplate reader (Thermo Scientific Varioskan Flash).

Antioxidant activity was calculated using the equation :

$$\%\ inhibition = \frac{Abs.Control - Abs.Extract}{Abs.Control} \times 100\%$$

The absorbance of the sample is the absorbance of the test sample or positive control, the absorbance of the control is the absorbance of the methanol + DPPH (Prastya et al., 2019).

The percentage inhibition values were applied to a linear regression model (y = ax + b), and the resulting equation was utilized to calculate the IC_{50} value, representing the antioxidant capacity of *Stachytarpheta* sp. leaf extract to inhibit 50% of DPPH radicals (Khairani et al., 2025).

Immunomodulatory test using animal experimental

This animal experimental study has obtained ethical approval from Faculty of Medicine University of Lampung with a number 736/UN26.18/PP.05.02.00/2024. The design of the experiment was carried out using 15 mice which were divided into 5 treatment groups (shown in **Table 1**).

Table 1. The design of the experiment

Groups	Treatment
K1	Control (aquadest)
K2	Aquadest and alloxan
P1	200 mg/kgBW extract and alloxan
P2	300 mg/kgBW extract and alloxan
P3	400 mg/kgBW extract and alloxan

Mice were acclimatized for 7 days. Next, mice were given the aquadest (K1 and K2 groups), and the extracts at doses of 200 mg/kgBW (P1), 300 mg/kgBW (P2), and 400 mg/kgBW (P3) orally for 15 days. On the 15th day, mice were induced with alloxan at a dose of 170 mg/kgBB subcutaneously (K2, P1, P2, P3). About the 5th day after alloxan induction, blood glucose levels were measured to observe the effects of hyperglycemia. Then, immunomodulatory test was conducted the next day.

This study used *Staphylococcus aureus* bacteria for initiating an immune response in mice. Mice received an intraperitoneal injection of 0.5 mL *S. aureus* suspension for each, equivalent to the 0.5 McFarland standard (approximately 1.5 × 10^8 CFU/mL). After one hour of exposure, mice were euthanized and abdominal dissection was performed. The peritoneal fluid was collected using a 1 mL syringe. Samples were smeared onto clean glass slides, fixed with methanol for 5 minutes, and stained with 10% Giemsa solution. After a 20-minute incubation, the slides were rinsed with running water and air-dried. The peritoneal macrophage were observed and analyzed microscopically to determine phagocytic activity. Macrophages were classified as active and passive based on morphological characteristics. Active macrophages shown an irregular (amoeboid) shape with enlarged nuclei, while passive macrophages were rounded with smaller nuclei. The immunostimulatory potential was assessed by calculating the phagocytic activity (Khairani et al., 2023; Digyo et al., 2024) :

$$\%\ phagocytic\ activity = \frac{total\ of\ active\ macrophages}{total\ of\ macrophages\ observed} \times 100\%$$

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3. RESULTS AND DISCUSSION

The picture below was the white snakeweed (*Stachytarpheta* sp.) a member of the family of Verbenaceae used in this study (see **Figure 1**).

Figure 1. The white snakeweed (*Stachytarpheta* sp.) from Beteng Sari Village, East Lampung.

Stachytarpheta Vahl is a monophyletic genus with 130 species distributed in the Americas, Africa, Asia, and Oceania, with abundant species in Brazil (81 spp.). *Stachytarpheta* sp. is an annual sometimes perennial weedy herbaceous plant. It grows 60-120 cm tall and is reproduced from seeds. The stem is smooth and somewhat woody especially at the base. It is dark green, often covered with powder which gives it a bluish shine. The leaves are opposite, rounded to broadly acute at the apex, smooth on both surfaces and with short petioles. The inflorescence is made up of flowers in slender spikes on a long and swollen rhachis about 30-40 cm long. The flowers are white with tubular corolla about 10 mm long and lobes about 3 mm long. They are more or less sparsely grouped along and immersed in the axis of the inflorescence. The whole plant and leaves of most species of *Stachytarpheta* has been widely used by the people throughout the world for the various medicinal purposes (Idu et al., 2007; Yadav et al., 2021).

Antioxidant activities of Stachytarpheta sp. extract

The antioxidant activity assay was conducted to evaluate the capability of a compound to neutralize free radicals and prevent oxidative damage to cells and tissues. In this study, the antioxidant potential of *Stachytarpheta* sp. (white snakeweed) leaf extract was quantitatively assessed using the DPPH (*2,2-diphenyl-1-picrylhydrazyl*) radical scavenging method. DPPH is a stable free radical commonly used to screen the radical scavenging ability of bioactive compounds (Gulcin & Alwasel, 2023). Ascorbic acid, a water-soluble antioxidant known for its protective effects on cells and organs through free radical neutralization, was employed as the positive control (Hieu et al., 2022). The antioxidant capacity was determined based on the IC_{50} value, which represents the concentration of extract required to inhibit 50% of DPPH radicals. The results demonstrated that the linear regression model of the extract was y = 3.037x+3.6815 (R^2 = 0.9973), while the linear regression model of the positive control was y = 14.902x + 2.7669 (R^2 = 0.9939), shown in **Figure 2 and 3**.

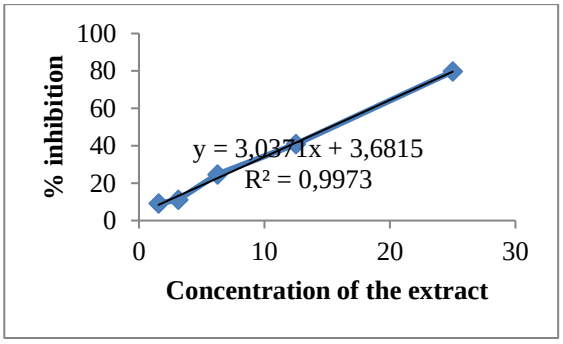

Figure 2. The linear regression model of the extract

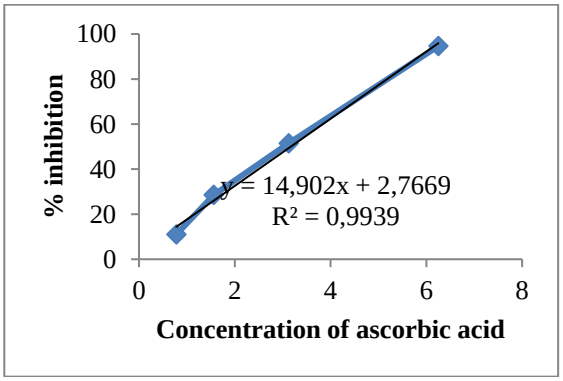

Figure 3. The linear regression model of the positive control

The IC_{50} value of the *Stachytarpheta* sp. leaf extract was 14.68 ± 0.30 µg/mL, while the ascorbic acid 3,10 ± 0,06 µg/mL (**Table 2**). According to Molyneux, (2004), compounds with IC_{50} values below 50 ppm (µg/mL) are classified as very strong antioxidants; those within the range of 50–100 ppm are considered strong, 100–150 ppm as moderate, and 151–200 ppm as weak. Lower IC_{50} values indicate higher antioxidant

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activity. So, the antioxidant activity of this extract is classified as very strong antioxidant.

Table 2. Antioxidant activity (IC_{50} value)

Sample	IC_{50}(µg/mL) ± STDEV
Stachytarpheta sp. extract	14,85 ± 0,30
Ascorbic acid	3,10 ± 0,06

This study in line with Amin et al., (2024), *S. jamaicensis* leaf ethanolic extract from North Kolaka, Indonesia has very strong antioxidant activity against DPPH with IC_{50} value 12.17 µg/mL. Furthermore, antioxidant activity has also been observed in *Stachytarpheta* extracts prepared using another solvent, Egharevba et al., (2019) reported that *S. jamaicensis* leaves extract from Nigeria has antioxidant effects that are categorized as very strong activity, with an IC_{50} value 16.95 µg/mL (methanolic extract) and 33.12 µg/mL (ethyl acetate extract). Another study showed that *S. jamaicensis* leaves methanolic extract from Ambon, Indonesia has antioxidant activity against DPPH and ABTS radicals with IC_{50} values of 19.36 ± 0.34 µg/mL and 27.37 ± 0.11 µg/mL respectively (Fatmawati et al., 2023). These findings suggest that the extract possesses antioxidant potential for the prevention and management of metabolic disorders associated with oxidative stress.

Free radicals may arise from exogenous sources such as air and water pollution, cigarette smoke, alcohol consumption, heavy metals, pharmaceuticals, industrial solvents, and radiation exposure. Endogenously, they can be generated through physiological processes including inflammation, psychological stress, intense physical activity, carcinogenesis, and aging, often via mechanisms like auto-oxidation or molecular inactivation. An overproduction of free radicals can lead to oxidative stress, a pathological condition implicated in the development of various diseases, including diabetes mellitus. Therefore the body relies on antioxidants as a compounds which capable of inhibiting or neutralizing reactive species generated through free radical-mediated chemical reactions (Phaniendra et al., 2015).

The ability of the extract to reduce free radicals may be due to its phenol and flavonoid phytochemicals. Phenolic compounds exhibit potent antioxidant activity, primarily due to their ability to donate hydrogen atoms from hydroxyl groups, thereby stabilizing free radicals and preventing the oxidation of organic substrates. Consequently, the total phenolic content in plant extracts is often correlated with their overall antioxidant capacity, serving as an important indicator of bioactivity (Gülçin, 2012; Gulcin & Alwasel, 2023). Flavonoids are the most common polyphenolic compounds found mainly in fruits and vegetables. Flavonoids contain aromatic rings bearing free hydroxyl, peroxyl and superoxide radicals, capable of donating hydrogen atoms, thereby neutralizing free radicals. The antioxidant efficacy of these compounds is generally proportional to the number of hydroxyl groups present on the aromatic ring (Banjarnahor & Artanti, 2015; Embuscado, 2015). Our previous research demonstrated that the total phenolic and flavonoid contents were positively correlated with antioxidant activity. Extracts exhibiting the highest levels of total phenols and flavonoids also showed the strongest antioxidant effects (Khairani, Anisa, et al., 2024).

Immunomodulatory effect of Stachytarpheta sp. extract on phagocytic activity of mice peritoneal macrophages in alloxan induction

The measurement of blood glucose levels has been previously published. Detailed data can be found in our published article (Khairani, Lusiana, et al., 2024). The results indicated that following alloxan induction, the K2 group exhibited a significant increase in blood glucose levels, with an average of 402 mg/dL (indicating hyperglycemia). In contrast, blood glucose levels in the K1, P1, P2, and P3 groups remained within the normal range of 71–124 mg/dL.

The day after the final blood sugar level measurement, immunomodulatory assays were performed. The phagocytic activity of mice peritoneal macrophages was assessed through microscopic examination of Giemsa-stained peritoneal fluid smears. Phagocytic activity was quantified as the percentage of macrophages actively to the total number of cells observed. The percentage values of phagocytic activity are presented in **Table 3**. Statistical analysis using *one-way ANOVA* revealed no significant differences among the treatment groups ($p > 0.05$), suggesting that administration of the extract did not produce a measurable effect on the

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phagocytic activity of peritoneal macrophages in alloxan-induced mice. Nonetheless, based on the mean percentage of phagocytic activity, the K2 group (characterized by hyperglycemia) exhibited the lowest phagocytic response compared to the other experimental groups, while K1 (the normal control without alloxan induction) showed the highest phagocytic activity (see Table 3).

Table 3. Phagocytic activity of mice peritoneal macrophages

Groups	Phagocytic activity (%) ± STDEV
K1	77,66 ± 03,08
K2	58,94 ± 05,65
P1	65,55 ± 13,03
P2	66,48 ± 09,57
P3	68,92 ± 15,66

Macrophages are phagocytic cells that can be derived from monocytes or from proliferating cells that form resident macrophage colonies. Monocytes will differentiate into macrophages after leaving the circulation and entering the tissue, when inflammation occurs (Ginhoux & Jung, 2014; Guan et al., 2025). The peritoneal provides an easily accessible site for harvesting moderate numbers of resident macrophages, which are essential in mediating immune responses to infection and inflammation. Peritoneal macrophages are the major cell type of peritoneal cells that contribute significantly to both innate and adaptive immune functions within the peritoneal cavity (Zhang et al., 2008; Liu et al., 2018). Collection of peritoneal fluid samples was performed one hour after *S. aureus* induction. Within an hour of induction, macrophages exhibit significant activation, primarily involving phagocytosis and the release of pro-inflammatory cytokines. This initial response aims to control the infection by neutralizing the bacteria (Pidwill et al., 2021).

Microscopic examination at 1000× magnification showed differences between active macrophages and passive macrophages (**Figure 4**). Macrophages initiate phagocytosis by extending their plasma membrane to form pseudopodia and engulfing the antigen by surrounding it with the membrane. Antigens are enclosed within phagosomes, which subsequently fuse with lysosomes to form phagolysosomes. Lysosomes contain hydrolytic enzymes and acidic components that facilitate the degradation and elimination of engulfed particles or pathogens. The activated macrophages are characterized by the shape and size of macrophages that increase in size with varying pseudopodia, and are characterized by *S. aureus* phagocytosed in their cells. Whereas passive macrophages have a smaller shape and size than active macrophages (Rengganis and Baratawidjaja, 2018; Wahyuni et al., 2019; Jusuf et al., 2023).

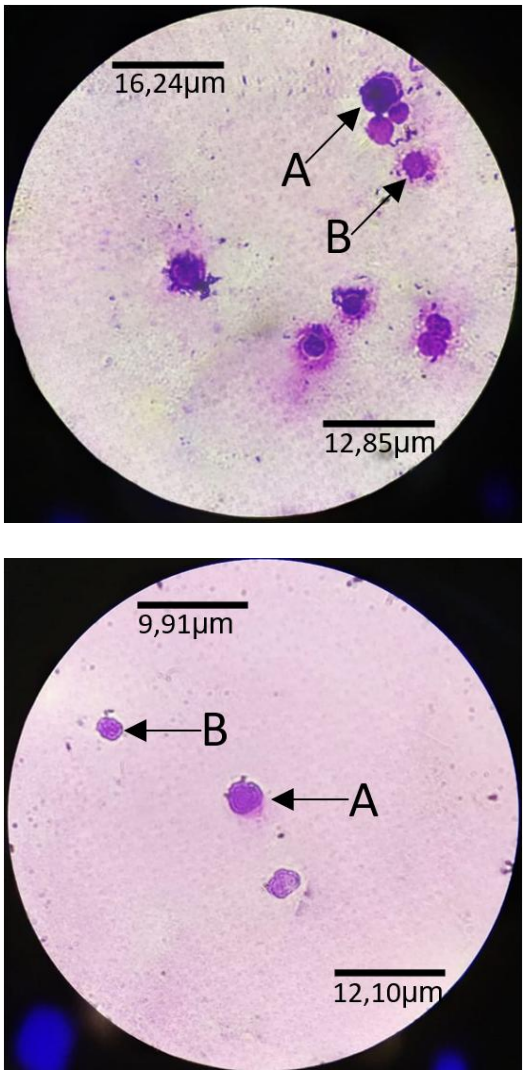

Figure 4 (upper and lower). Activate and passive macrophages, (A) active macrophages, (B) passive macrophages.

The phagocytic activity observed in the K1 group (normal control) reflects a functional innate immune response, which acts immediately to

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protect the body from the pathogens (Coico & Sunshine, 2015). In the phagocytosis activity of mice in all alloxan-induced groups (K2, P1, P2, P3) showed lower activity than group K1. However, group K2 which was not given the extract showed the lowest percentage of activity. Alloxan is a diabetogenic compound that selectively damages pancreatic beta cells, the cells that produce insulin, leading to insulin-dependent diabetes, characterized by hyperglycemia (Ighodaro et al., 2017). This selective toxicity is achieved through mechanisms involving sulfhydryl group oxidation and the generation of reactive oxygen species (free radicals). Alloxan promotes the formation of reactive oxygen species (free radicals), which are highly reactive molecules that can damage cellular components, including DNA, proteins, and lipids (Furman, 2007; Puspitasari & Choerunisa, 2021). Hyperglycemia affects the immune system and can lead to defects in host immunity, such as impaired cell migration, phagocytosis, and intracellular killing (Abu-Ashour et al., 2018; Jafar et al., 2016).

P1, P2, and P3 which receiving white snakeweed (*Stachytarpheta* sp.) leaf extract at doses of 200, 300, and 400 mg/kgBW exhibited phagocytic activity levels lower than the K1 group but higher than the K2 group. This suggests that white snakeweed leaf extract can be an immunomodulator that maintains the phagocytic activity of macrophage cells in alloxan-induced mice. The immunomodulatory effects may be attributed to the presence of bioactive compounds. Most of the plant-derived phenolics have been reported to modulate the innate immune response, primarily by stimulating the phagocytic function and proliferative activity of macrophages and neutrophils (Grigore, 2017). Flavonoids exert immunomodulatory effects by influencing lymphokine activity derived from T lymphocytes, thereby promoting the phagocytic function of immune cells. These compounds enhance the activation of key effector cells, including lymphocytes and macrophages, which subsequently secrete pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF-α. At elevated concentrations, flavonoids have been shown to augment the phagocytic efficiency of macrophages, facilitating more effective bacterial engulfment and degradation (Aldi et al., 2016; Zalizar, 2013).

Flavonoids and alkaloids are also known to modulate immune responses by activating natural killer (NK) cells, which in turn stimulate the production of interferon-gamma (IFN-γ)—a key macrophage-activating cytokine that enhances phagocytic activity (Sulistiani & Rahayuningsih, 2015). According to Asti (2015), the flavonoid content in white snakeweed leaf extract exerts immunomodulatory effects that contribute to the restoration of immune function and enhancement of phagocytic cell activity. Secondary metabolites such as alkaloids are thought to have similar roles to the cytokine IFN-γ in maintaining the immune response and enhancing the nonspecific immune response in the form of increased leucocyte responsiveness or specific immune response to activate macrophages to perform their function in phagocytosis of infectious agents entering the body (Ilyas Y. et al., 2023; Priyani, 2020). Tannins also can act as immunostimulators by optimizing immune system function, increasing the phagocytic activity of macrophages in destroying microbes (Hikmah & Triastuti, 2022).

4. CONCLUSION

The *Stachytarpheta* sp. leaf extract from Beteng Sari Village, East Lampung has very strong antioxidant activity against DPPH with IC_{50} value 14,85 ± 0,30 µg/mL. This extract at doses of 200, 300, and 400 mg/kgBW can be an immunomodulator that maintains the phagocytic activity of macrophage cells in alloxan-induced mice. The further research is recommended to identify the specific active compounds responsible for these effects, and also testing the extract in diabetic animal models over a longer period would help evaluate its safety and effectiveness for potential use in managing immune and oxidative stress conditions caused by hyperglycemia.

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