The effect of Zn-Rich Sambiloto (*Andrographis paniculata*) Simplicia Powder Diet to SGOT, SGPT and SOD of *Stapylococcus aureus* induced Wistar

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Abstract. One source of natural antioxidants considered safer is the bitter plant, which contains many phenolics and flavonoids and high levels of chlorophyll, so it has the potential to act as an immunomodulator. However, the drying process of bitter leaves can reduce chlorophyll levels. This study aims to evaluate the effect of metallochlorophyll formation on the chemical properties of bitter powder, antioxidant activity, and the immunomodulatory effects of Zn-rich bitter powder in vivo. The materials of this study were bitter powder (SP) and SP powder with metallochlorophyll treatment (SZP). Twenty five male Wistar rats were divided into five groups: G1: normal, G2: negative control, G3: commercial immune-boosting supplement intake (SC), G4: SP intake, and G5: SZP intake. Group G1-5 was fed a standard feed of 20 g/day, and group G2-5 was injected with Staphylococcus aureus at a dose of 0.1 ml/head (108 CFU) intraperitoneally. The results showed that forming metallochlorophyll complexes in sambiloto simplicia will increase total chlorophyll and Zn contents and its antioxidant activity compared to natural sambiloto simplicia powder. Intake of Zn-rich sambiloto simplicia powder was able to significantly reduce SGPT, SGOT levels, and increase SOD compared to intake of original sambiloto simplicia powder.

1 Introduction

The immune system plays an important role in protecting against foreign organisms and is involved in modulating infections, inflammatory diseases, and autoimmunity. The immune system refers to a collection of cells, chemicals, and processes that protect the skin, respiratory tract, intestinal tract, and other areas from foreign antigens, such as microbes (organisms such as bacteria, fungi, and parasites), viruses, cancer cells, and toxins. Immunity is a state of protection against injury caused by pathogens through rapid immune elimination of pathogenic attackers due to previous antigen contact or a state of specifically acquired response (WHO). The immune system protects the body by neutralizing, inactivating, or eliminating potentially pathogenic attackers such as microorganisms (bacteria and viruses).

Therefore, the normal function of the immune system or mechanism is important for a person's natural self-protection against infectious diseases. Agents that have the ability to normalize or modulate pathophysiological processes, which have stimulating or suppressive effects, are called immunomodulatory agents. Immunomodulators are natural and synthetic substances that have therapeutic benefits by altering the immune system and have the ability to develop, replace, or help produce the desired immune response. Immunosuppression involves a decrease in resistance to infection, environmental factors, and drugs [1].

A decrease in the body's immune system can be caused by stressful lifestyle changes, poor environmental quality, and unpredictable weather changes. All of these can make the body susceptible to disease and ultimately reduce the quality of human life. An increase in the body's immune system can be achieved in various ways, one of which is through immunomodulator or antioxidant supplements. Antioxidants are compounds that can counteract the negative effects of oxidants in the body by donating one of their electrons to oxidant compounds, thereby inhibiting the activity of these oxidants. Oxidant compounds, which are present in the form of free radicals or other reactive compounds, can cause severe damage to the body due to low antioxidant levels in the body, which are unable to counterbalance the oxidative reactivity of radical compounds. A decrease in antioxidants in the body is directly proportional to an increase in disease. Various other studies have also linked antioxidant activity with the immunomodulatory activity of various herbal ingredients.

One type of herbal plant that has potential as an immunomodulator is sambiloto (*Andrographis paniculata*). Sambiloto is a herbal plant that contains androghapolide, phenolic, and flavonoid compounds as well as chlorophyll, which can act as antioxidants [2]. Chlorophyll is a green pigment found in chloroplasts along with carotene and xanthophyll. However, the drying process causes chlorophyll degradation and a decrease in phenolic and flavonoid content. Therefore, a stabilizing agent is needed to maintain the green color of sambiloto. This is because chlorophyll pigments are easily degraded into pheophytin due to the loss of Mg^{2+} ions in the porphyrin ring. One way to increase chlorophyll stability is by forming a metallochlorophyll complex with metals that can produce more stable complexes than Mg, such as Zn, Mn, and Fe In this study, Zn was chosen because it has the ability to boost immunity. Previous studies have shown that the formation of chlorophyll complexes with Zn^{2+} ions can maintain antioxidant activity, and it is known that the formation of Zn-chlorophyll is best when using $ZnCl_2$ reagent at a concentration of 300 ppm [3].

Various studies show that sambiloto can also act as an antioxidant, immunomodulatory anti-inflammatory, and antidiabetic. This plant compound also reduces oxidative stress by neutralizing Reactive Oxygen Species, protecting vital organs such as the liver and spleen through antioxidant defense. However, this study has not examined the effects of administration in the form of powder treated with Zn-chlorophyll complex formation. As a comparison, there is a commercial product containing *Phyllanthus niruri* Linn extract, which is believed to have effective immunomodulatory effects [4]. Meniran herb concentrate extract is an extract made from the herb Phyllanthus niruri L., family Euphorbiaceae, containing total flavonoids of not less than 3.20% calculated as quercetin. Meniran thick extract is black in color, odorless, bitter in taste, and standardized with the following criteria: moisture content $\leq 17\%$, ash $\leq 3.5\%$, acid-insoluble ash $\leq 1.5\%$ [5]. Therefore, this study aims to evaluate the effect of metallochlorophyll complex formation on the immunomodulatory activity of simplicial sambiloto powder compared to commercial immunity-boosting products.

2 Materials and Methods

2.1 Materials

The material used in this study was sambiloto simplisia from the Tawangmangu Medicinal Plant Research Center. The chemical used for the formation of metallochlorophyll complexes was $ZnCl_2$ (Sigma, Aldrich). The materials used for chlorophyll content analysis, phenolic content analysis, and total flavonoids were aquadest, acetone, and methanol, as well as Whatman No. 1 and 42 paper, gallic acid standard, quercetin, Folin-Ciocalteu reagent (Sigma Chemical Co.), 95% ethanol, 90% acetone, methanol, HCl, and acetate buffer. Chemicals for antioxidant activity testing were linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), $FeSO_4.7H_2O$, $AlCl_3.6H_2O$, $NaNO_3$, $FeCl_3.6H_2O$, HCl, NaOH, ethanol, potassium phosphate buffer, ammonium thiocyanate, and deionized water were obtained from Merck with standard specifications for analysis (pro analysis).

2.1.1 Material preparation
This research has been recommended by the research ethics committee with no:KE/AA/III/10111548/EC/2024. The first stage began with sorting to obtain good quality dried sambiloto herb leaves, free from defects, removing impurities, and separating the hard stems of the sambiloto plant. Next, the selected sambiloto leaf simplisia was crushed by blending for 1 minute × 3 times, then sieved with a 60 mesh sieve. The sambiloto powder that did not pass the sieve was then reground and sieved again. 50 g of sambiloto simplisia powder was weighed to be treated for the formation of Zn-chlorophyll metallochlorophyll. The Zn-chlorophyll formation process began by mixing a Zn-chloride ($ZnCl_2$) solution with a reagent concentration of 300 ppm with a ratio of sambiloto powder to reagent of 50:200 (w/v). Mixing was carried out by spraying while stirring to ensure homogeneity. After mixing, it is heated in an autoclave for 10 minutes at a temperature of 110°C. The next process is to dry it again using a cabinet dryer at a temperature of 50°C for 3 hours until dry. The sambiloto simplisia powder that has undergone metallochlorophyll formation (SZP) is packaged in 0.7 mm PP plastic.

2.1.2 Chemical analysis
Chemical analysis includes: total phenolic content analysis total flavonoid content analysis total chlorophyll content analysis and antioxidant activity. Sample preparation for these analyses consisted of 0.5 g of sambiloto herb powder and 10 ml of 80% methanol, which were homogenized and filtered using Whatman No. 42 filter paper, adjusting the volume to 25 ml.

a. The DPPH radical scavenging assay [6] was performed by placing 1 ml of methanol extract sample or BHT (33.3µg/ml) in a test tube, adding 2 ml of 0.2 mM DPPH methanol solution, then left to stand for 30 minutes. Absorbance was measured at 517 nm.

b. The Fe (II) cation chelation ability test was analyzed using the method [7] with slight modifications using the herbal extract sample obtained previously. Three milliliters of herbal extract was added to 0.3 ml of 1 mM $FeCl_2$(new solution), then incubated for 30 minutes, and the reaction was initiated by adding 1 mM ferrozin (0.3 ml). After the mixture reached equilibrium (10 minutes), the absorbance was measured at 562 nm. As a comparison, the sample was replaced with EDTA at the same concentration (5 ppm). The percentage of ability to inhibit the formation of ferrozin-Fe2+ complexes was determined using the equation below with the control absorbance A0 at 562 and the sample absorbance A1.

2.1.3 Immunomodulatory activity

a. Preparation of *Staphylococcus aureus* bacterial antigen according to [8]. One mL aliquot of *Staphylococcus aureus* (ATCC 25923) was diluted with nutrient broth to obtain a cell concentration of 1×10^8 cells/mL. The cell concentration was calculated based on the turbidimetric method using a spectrophotometer with an optical density of 530 nm. The mixture was centrifuged at 25°C for 10 minutes at 10,000 rpm. The cells were separated and then rinsed with 1 mL of phosphate-buffered saline (PBS) (Sigma, USA).

b. The commercial products SC, SP, and SZP suspensions were prepared using the method described by [29]. The SC, SP, and SZP suspensions were prepared in the following steps: 3 tablets of SC or 6 g of SP or SZP powder sample were dissolved in 100 ml of 0.5% CMC-Na and stirred until homogeneous.

c. Twenty-five male Wistar rats aged 2 months were prepared as test animals. The rats were divided into 5 groups and adapted for one week by being fed standard feed. Each group was then given the following treatments: 1) standard diet, 2) standard diet, 3) standard diet + SC suspension, 4) standard diet + SP suspension, and 5) standard diet + SZP suspension. After the adaptation period, the rats were given the treatments and administered the test preparations orally once daily for 16 days. On day 14, groups 2 to 5 were injected with 0.1 ml of *Staphylococcus aureus* at a concentration of 10^8 cells/ml via intraperitoneal injection.

d. The determination of immunity effects was measured using the parameters SOD, SGPT, and SGOT using an ELISA kit. The analysis was performed on day 0 (after a 7-day adaptation period), on day 14 after the *Stapylococcus aureus* injection, and at the end of the study on day 16 using the retro-orbital sinus method.

2.1.3 Statistical analysis

Data obtained from three replicate analyses were statistically analyzed using ANOVA, and if significant differences were found, they were further processed using *the Duncan Multiple Range Test (*DMRT*)*.

3 Results and Discussions

3.1 Chemical Properties

Based on the data in Table 1, it can be seen that the chlorophyll content of SZP sambiloto simplisia is higher than SP. This is because chlorophyll degraded during the drying of sambiloto leaves forms pheophytin bound to Zn from the $ZnCl_2$ reagent. The formation of a Zn complex with pheophytin produces a green-colored Zn-pheophytin complex, resulting in *a regreening effect* on the sambiloto simplisia powder. Zn-pheophytin is also detected as chlorophyll during chlorophyll content analysis using a spectrophotometer. In this study, treatment with $ZnCl_2$reagent,which forms a metallochlorophyll complex, can increase chlorophyll stability and increase the total chlorophyll content (TCC) in sambiloto simplisia. The chlorophyll content in sambiloto simplisia is one of the important parameters that indicate the quality of the material because chlorophyll in the body can reduce oxidative damage by capturing free radicals [9].

Table 1. Chlorophyll content (TCC), total phenolic content (TPC), total flavonoid content (TFC), ash, and Zn s of sambiloto herb powder (SP) and Zn-enriched sambiloto herb powder (SZP)

Sample	TCC (mg/100 g bk)*	TPC (mg/100 g bk)*	TFC (mg/100 g bk)*	Ash content (% bk)*	Zn content (mg/100 g bk)*
SP	284.26 ± 11.86a	642.35 ± 34.32a	84.92 5.90 a	12.42 ±0.27 a	8.48 ±1.11a
SZP	325.55 ± 37.83a	402.05 ±40.13a	23.99 5.55 a	13.64 ±0.11 a	14.46 ±2.51a

Note: *Same letters indicates not significantly difference (P>0.05).

Conversely, the total phenolic and flavonoid content in SZP decreased due to the autoclaving process during the formation of metallochlorophyll complexes. This is because the autoclaving process uses a high temperature of 115°C, causing the phenolic and flavonoid components to degrade significantly. This is supported by previous studies showing that heating at 50-70°C has caused phenolic components to degrade [10]

The process of forming metallochlorophyll complexes in sambiloto herb powder also significantly increases the ash and Zn content. Ash content is an indicator of plant material mineralization and reflects the inorganic mineral content in it. Treatment with $ZnCl_2$ can affect the mineral composition in sambiloto simplicial powder. In addition to increasing chlorophyll stability, the formation of Zn-chlorophyll complexes will increase the Zn content in sambiloto simplicia powder, which is expected to increase its immunomodulatory activity. This is because zinc is one of the important minerals in the body as it can boost immunity [11].

3.2 Antioxidant Activity

Based on the data in Table 2, it can be seen that the DPPH radical scavenging activity (RSA) of Zn-rich sambiloto herb powder is not different from that of the original sambiloto herb powder, but lower than the BHT control. Although the TPC and TFC of SZP powder are lower, the TCC is higher, and the DPPH radical scavenging ability is not significantly different. [34] stated that Zn-phytophytin derivatives have a higher DPPH radical scavenging ability than the original chlorophyll. Unlike RSA, the metal chelating ability of P-Zn powder decreases. This is because the complex formed with Zn metal is stronger than with Fe metal. The formation of Zn-pheophytin complexes is more stable, so Zn^{2+} ions are not easily released and are not easily replaced by Fe, resulting in lower detected metal chelation capacity. The percentage of lipid peroxidation inhibition from SP and P-Zn powders was the same and did not differ significantly from the BHT control. Previous studies have shown that the percentage of lipid peroxidation inhibition correlates with TFC [2] but in this study, despite a decrease in TFC, the percentage of lipid peroxidation inhibition in SZP was the same as in SP. This is because the andrographolide content in SZP is more stable.

When viewed from its reducing power, it can be seen that the reducing ability of SP and SZP is lower than that of vitamin E (Figure 1). Based on the straight line equation obtained, the IC50 value can be calculated as presented in Table 2. It is known that the IC50 value of SZP is greater than that of SP, indicating that the reducing ability of SZP is lower than that of SP. This is because during the autoclaving process in the production of the Zn-pheophytin complex, there was a decrease in the total phenolic and flavonoid content, as detailed in Table

1. The decrease in total phenolic and flavonoid content resulted in a decrease in the reducing ability of SZP. Various studies have shown that total phenolic and flavonoid content is highly correlated with antioxidant activity.

Table 2. Antioxidant activity of sambiloto herb powder (SP) and zinc-rich sambiloto herb powder (SZP)

Sample	RSA	Chelating agent	LPI	IC50
SP	72.97±5.96 a	40.47±11.99 b	59.87±2.61 a	3.75±0.73a
SZP	69.20±0.89 a	24.23± 1.64a	60.67±7.32 a	4.84±0.09 b
Comparator	89.48±0.39 c	90.24 2.39c	60.04±4.41 a	2.52±0.73a

Note: Numbers followed by letters in the same column indicate significant differences ($P<0.05$). The comparator for RSA and LPI analysis is BHT, the comparator for chelating agent analysis is EDTA, and the comparator for IC50 analysis is vitamin E.

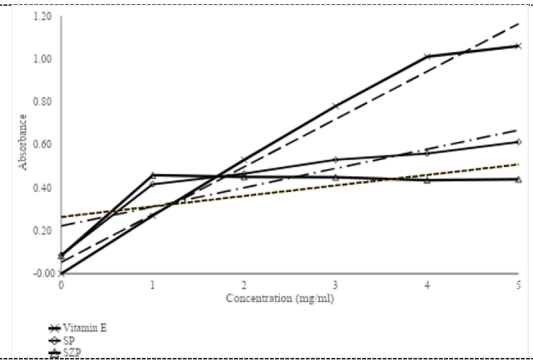


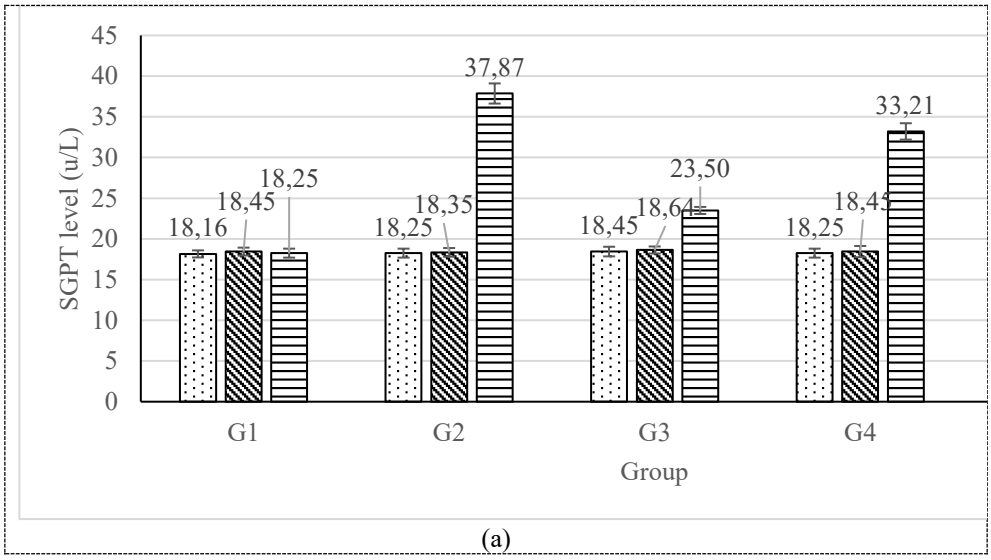
Figure 1. Reducing ability of sambiloto simplicia powder (SP) and Zn-rich sambiloto simplicia powder (SZP) with vitamin E as a comparator

3.3 Serum SGPT and SGOT levels

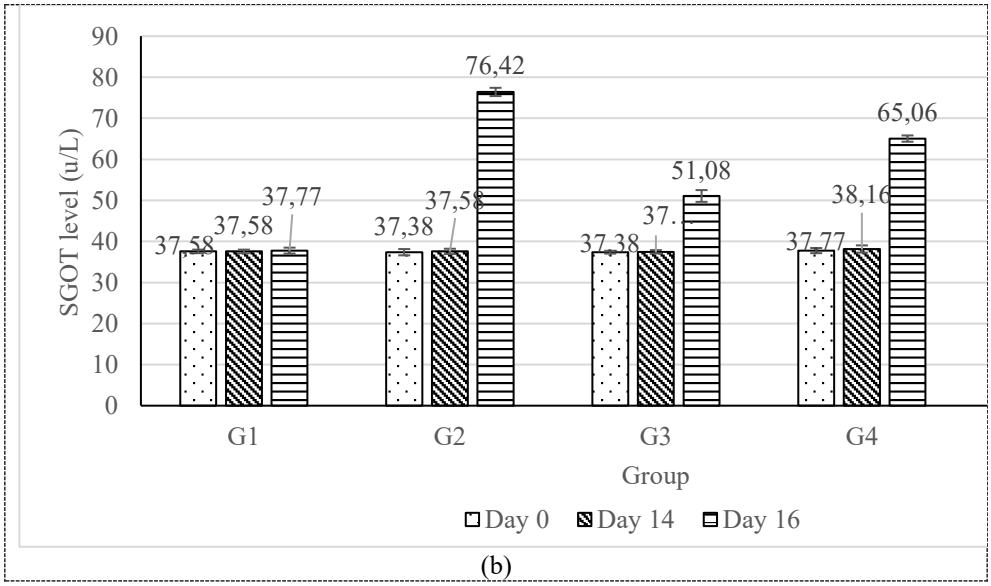
Theoretically, Serum Glutamic Pyruvic Transaminase (SGPT) and Serum Glutamic Oxaloacetic Transaminase (SGOT) levels will increase if there is damage or inflammation in the liver tissue. Increased SGPT and SGOT levels in the blood are parameters of liver activity and function. An increase in SGPT and SGOT levels indicates that liver function is impaired. Based on the data in Figures 2a and 2b, it can be seen that before the S.*aureus* injection treatment on day 14, all groups of mice had SGPT and SGOT levels that were not significantly different from day 0. This indicates that the condition of the mice before and after treatment with powdered sambiloto simplisia (SP), zinc-rich sambiloto simplisia powder (SZP), and commercial supplements (SC) for 14 days did not affect the health of the mice because they had SGPT and SGPT levels similar to normal mice (normal group).

The data in Figures 2.a and b show that two days after *S. aureus* injection (day 16), the group of rats without supplement intake (G2) had the highest SGPT and SGOT levels, while the groups of rats with SP, SZP, and SC supplement intake had lower SGPT and SGOT levels. This indicates that the phenolic and flavonoid components in SP and SZP can act as hepatoprotectors. Phenolic components can regulate immunity by affecting immune cell regulation, proinflammatory cytokine synthesis, and gene expression. Polyphenols inactivate NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and modulate the mitogen-activated protein kinase (MAPk) and arachidonic acid pathways.

Polyphenolic compounds inhibit phosphatidylinositol 3-kinase/-activated protein kinase B (PI3K/AkT), inhibitor kappa kinase/c-Jun amino-terminal kinase (IKK/JNK), mammalian target of rapamycin complex 1 (mTORC1), which is a protein complex that controls protein synthesis, and JAK/STAT. Polyphenols can suppress the expression of toll-like receptor (TLR) and pro-inflammatory genes. Antioxidant activity and the ability to inhibit enzymes involved in eicosanoid production also contribute to their anti-inflammatory properties. This inhibits the production of enzymes involved in the production of reactive oxygen species (ROS) such as xanthine oxidase and NADPH oxidase (NOX) and increases other endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase (Px) [12].



(a)



(b)

Figure 2. Serum SGPT (a) and SGOT (b) levels in rats in group G1: normal group, G2: negative control group, G3: group with commercial supplement (SC) intake, G4: group with SP intake, G5: group with SZP intake Groups G1-5 were fed a standard diet of 20 g/day per animal, and groups G2-5 were injected with *Staphylococcus aureus* at a dose of 0.1 ml/animal (10^8 CFU) intraperitoneally.

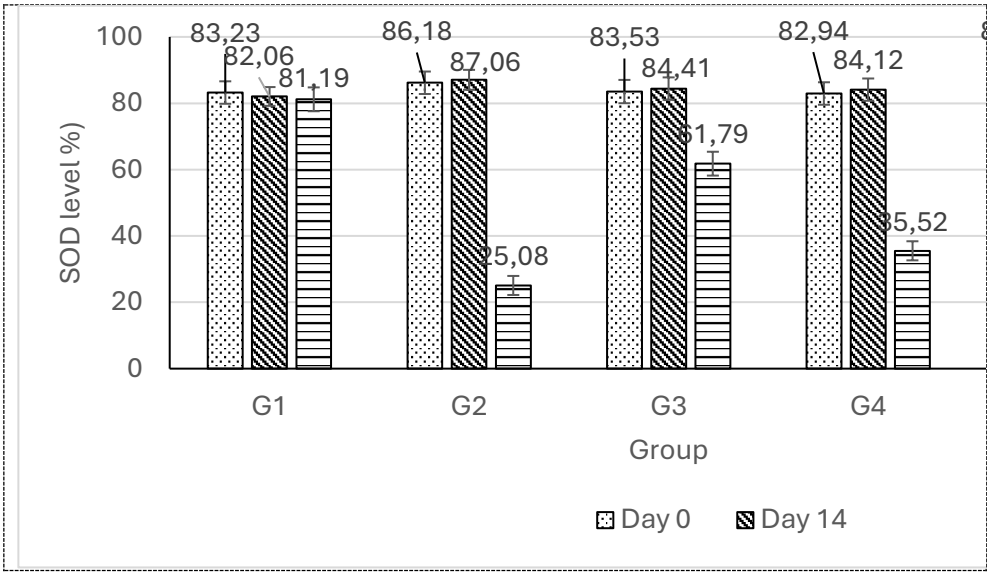


Figure 3. Serum SOD levels in rats in group G1: normal group, G2: negative control group, G3: group with commercial supplement intake (SC), G4: group with SP intake, G5: group with SZP intake Groups G1-5 were fed a standard diet of 20 g/day per animal, and groups G2-5 were injected with *Staphylococcus aureus* at a dose of 0.1 ml/animal (10^8 CFU) via intraperitoneal injection.

3.4 Serum SOD Levels in Rats

Superoxide Dismutase (SOD) is an endogenous antioxidant that protects the body from oxidative stress by neutralizing harmful free radicals, converting them into more stable oxygen (O_2) and hydrogen peroxide (H_2O_2). This enzyme is essential for the survival of organisms and includes several types of SOD, such as Cu/Zn-SOD and Mn-SOD. Based on the data in Figure 3, it can be seen that the SOD levels of mice in all groups on days 0 and 14 did not differ significantly. This indicates that under healthy/normal conditions, the intake of SP, SZP, and SC supplements does not affect antioxidant activity in the body. However, two days after S. *aureus* injection (day 16), it was observed that the SOD levels in group 2, the group without supplement intake, had the lowest serum blood SOD levels, while those with SP, SZP, and SC supplements had higher levels. This is consistent with the trend in SGPT and SGOT levels in Figures 3.a and b. The presence of phenolic compounds can inhibit the production of reactive oxygen species (ROS) and increase other endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase (Px). These results are in line with 26 previous studies showing that flavonoid supplementation can significantly increase antioxidant enzyme levels (superoxide dismutase, catalase, glutathione, glutathione peroxidase, and glutathione-S-transferase), reduce the

production of oxidative agents (malonaldehyde) and proinflammatory mediators (tumor necrosis factor-a, interleukin-6, IL-1b, C-reactive protein, immunoglobulin G, nitric oxide, vascular endothelial growth factor, and myeloperoxidase) [13].

Based on the data in Figure 3, it is known that SZP intake is most effective as an immunomodulatory compared to SP. Based on the results of SGPT, SGOT, and SOD analysis, it can be seen that the intake of sambiloto herb powder (SP), Zn-rich sambiloto herb powder (SZP), and commercial SC supplements can increase the immunity of mice injected with S. aureus. It is known that the most effective is the intake of Zn-rich sambiloto simplisia powder (SZP), followed by commercial SC supplements and natural sambiloto simplisia powder (SP).

Based on the results of the study, it can be concluded that Zn-rich sambiloto simplisia powder is more effective as an immunity booster. Zinc is an essential mineral that plays an important role in the immune system [33]. Zinc is involved in the development and activation of immune cells, such as T lymphocytes and macrophages. Zinc deficiency can lead to a decrease in the immune response, so supplementing with zinc can enhance the body's ability to fight infections. The active compounds in stevia can help regulate the immune response by reducing inflammation and increasing the activity of immune cells. Meanwhile, sambiloto contains active compounds such as phenolics, flavonoids, and andrographolide, which have been proven to have immunomodulatory effects. Phenolics and flavonoids increase the production of endogenous antioxidants, and andrographolide stimulates the production of cytokines, which are proteins that play a role in communication between immune system cells.

4 Conclusion

The formation of metallochlorophyll complexes in sambiloto simplisia increases total chlorophyll and Zn levels. Still, it decreases total phenolic and flavonoid levels and antioxidant activity (RSA, chelating agent, LPI, and IC50) compared to natural sambiloto simplisia. Consumption of Zn-rich sambiloto simplicia powder significantly reduces SGPT and SGOT levels and increases SOD compared to consumption of natural sambiloto simplicia powder, thereby acting as an immunomodulator.

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