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Immunomodulatory effect of *Parkia speciosa* Hassk. pods extract on rat induced by *Salmonella typhimurium*

[Efecto inmunomodulador del extracto de vainas de *Parkia speciosa* Hassk. en ratas inducidas por *Salmonella typhimurium*]

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Abstract

Context: *Parkia speciosa*, which has phenolic compounds and pharmacology activities, is a plant having potential immunomodulatory agents from the extract of its pod.

Aims: To evaluate the potential of *P. speciosa* as an immunomodulatory agent tested on albino rats induced by the bacteria *Salmonella typhimurium* leading to typhoid fever.

Methods: The animals were divided into six groups: normal non-infected group, negative control group, positive control, and three test groups with doses of 200, 400 and 800 mg/kg BW. The test groups were treated by the extract for 12 days. The control and test groups were induced with the *S. typhimurium* on the 8th day. On day 12th, CD4$^+$, total leukocytes, differential cell count, and histology of the spleen organ were evaluated. Statistical analysis was performed by using one-way ANOVA followed *post hoc* LSD test (α 0.05).

Results: The result showed that the ethanol extract of *P. speciosa* pod could increase the number of CD4$^+$. The extract to 200 and 400 mg/kg BW doses increased in the parameters of leukocyte, neutrophil, lymphocyte and monocyte with insignificant differences compared to that of the positive control group ($p>0.05$). However, there were significant differences between the two first parameters between the test group with 800 mg/kg BW doses compared to the positive control group. Moreover, more necroses in the spleen were observed from histological analysis on 800 mg/kg BW doses than 400 mg/kg BW.

Conclusions: In general, *P. speciosa* ethanolic extract can increase the immune system based on the CD4$^+$ count, leukocyte, monocyte, and neutrophil, and causes normal necroses in low doses.

Keywords: *Parkia speciosa;* immunomodulator; *Salmonella typhimurium;* typhoid fever.

Resumen

Contexto: *Parkia speciosa*, que tiene compuestos fenólicos y actividades farmacológicas, es una planta que tiene agentes inmunomoduladores potenciales del extracto de su vaina.

Objetivos: Evaluar el potencial de *P. speciosa* como agente inmunomodulador en ratas albinas inducidas por la bacteria *Salmonella typhimurium* que conduce a la fiebre tifoidea.

Métodos: Los animales se dividieron en seis grupos: grupo normal no infectado, grupo de control negativo, control positivo y tres grupos de prueba con dosis de 200, 400 y 800 mg/kg de peso corporal. Los grupos de prueba fueron tratados con el extracto durante 12 días. El control y los grupos de prueba fueron inducidos con *S. typhimurium* en el octavo día. El día 12, se evaluaron los CD4$^+$, los leucocitos totales, el recuento diferencial de células y la histología del órgano del bazo. El análisis estadístico se realizó mediante el uso de ANOVA unidireccional seguido de la prueba *post hoc* LSD (α 0,05).

Resultados: El extracto etanólico de la vaina de *P. speciosa* podría aumentar el número de CD4$^+$. El extracto a dosis de 200 y 400 mg/kg de peso corporal aumentó en los parámetros de leucocitos, neutrófilos, linfocitos y monocitos con diferencias insignificantes en comparación con el grupo de control positivo ($p>0,05$). Sin embargo, hubo diferencias significativas entre los dos primeros parámetros entre el grupo de prueba con dosis de 800 mg kg de peso corporal en comparación con el grupo de control positivo. Además, se observaron más necrosis en el bazo a partir del análisis histológico con dosis de 800 mg/kg de peso corporal que con 400 mg/kg de peso corporal.

Conclusiones: En general, el extracto etanólico de *P. speciosa* puede aumentar el sistema inmunitario en función del recuento de CD4$^+$, leucocitos, monocitos y neutrófilos, y causa necrosis normales en dosis bajas.

Palabras Clave: *Parkia speciosa;* inmunomodulador; *Salmonella typhimurium;* fiebre tifoidea.

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INTRODUCTION

Infectious diseases are the second leading cause of death in worldwide after cardiovascular disease (WHO, 2004). Infection can be caused by viruses, bacteria, protozoa, worms, and parasitic fungi entering the body or growing on the body surface. An infectious disease with a high occurrence in several Asian countries is typhoid fever (Purba et al., 2016). This acute fever is caused by bacterium *Salmonella enterica* infection, especially its derivative, *Salmonella typhimurium* (Alba et al., 2016). Based on WHO data, the number of typhoid fever patients in Indonesia is relatively high, reaching 81,000 of 100,000 inhabitants (Rahmasari and Lestari, 2018). A person with a weak immune system is at a high risk of developing typhoid fever (Kalia et al., 2016).

The innate immune system, which consists of phagocyte cells, is the most important to defense against microorganisms and malignant cells (Harun et al., 2015; Venkatalakshmi et al., 2016). Activation of the immune system is required to help a body destroying antigenic substances, and it can be stimulated using immunomodulators.

Synthetic immunomodulatory drugs have many benefits, but adverse side effects limit their use. Further studies are thus an important way to find safer and more effective immunomodulatory agents (Venkatalakshmi et al., 2016). Plant extracts are commonly considered as potential agents from their immunomodulatory properties, which have smaller side effects (Alamgir and Uddin, 2010). Their properties are generated by activating a function and efficiency of macrophages, granulocytes, complement, and natural killer cells and by producing effector molecules (Jayathirtha and Mishra, 2004). Plant metabolites such as sterols, polysaccharides, alkaloids, flavonoids, lectins, and glycoproteins are used as immunomodulatory agents (Harun et al., 2015). The pod of *Parkia speciosa* Hassk. (*Leguminosae*) contains phytochemical compounds such as flavonoids, phenolics, alkaloids, and saponins (Kamisah et al., 2013). Pharmacology activities of *P. speciosa* have been reported as an antioxidant, anti-carcinogenic and anti-inflammatory (Kamisah et al., 2013; Mustafa et al., 2018), antianemia (Nursucihta et al., 2014), antihypertensive and heart problems (Siow and Gan, 2013; Kamisah et al., 2017) and antibacterial (Uyub et al., 2010). However, the immunomodulatory activity of *P. speciosa* has not been found in previous studies. Thus, this study aims to reveal the potential of *P. speciosa* as an immunomodulatory agent by observing $CD4^+$ parameters, total leukocytes, differential blood cell counts, and spleen's evaluation of albino rats induced by *Salmonella typhimurium* bacteria.

MATERIAL AND METHODS

Vegetal material and chemicals

P. speciosa was collected from Musi Rawas districts, South Sumatera, Indonesia (2.90°S, 103.28°E). This species has been identified by the Indonesian Institute of Science with Register No. 218/IPH.06/HM/I/2018. *Salmonella typhimurium* ATCC 14028 (10^5 CFU) was obtained from the Central Laboratory of Health, Palembang, South Sumatera, Indonesia. Chemicals as methanol, NaCl (Merck), phosphate buffer pH 6,8 and Giemsa stain (Sigma-Aldrich) were in an analytical grade.

Preparation of *P. speciosa* pod extract

Ethanol extract of *P. speciosa* pod was prepared by a maceration method. Its powder (500 mg) was soaked with 2 L of ethanol 95% for 2 days. A re-maceration process of the residue was carried out twice. The maceration result then was evaporated with a rotary evaporator (Yamato®) at a temperature of 65°C to get a concentrated extract (Harun et al., 2015).

Design of test animal experiments

Wistar male white albino rats (150 - 200 g weight) were used in this study. An ethics certificate for the use of test animals was obtained from the Faculty of Medicine, Sriwijaya University, with No. 064/kepkrsmhfkunsri/2019. The test animals

were acclimatized to the laboratory environment at a temperature of 23 ± 2°C and a 12-hour light-dark cycle for one week. During the acclimation process, these rats were given standard *ad libitium* drink and feed (Johnson et al., 2017). They were divided into 6 groups: the normal group and negative control group were given Tween-80 0.5 mL/kg BW; the positive control group was given 0.54 mL (dose 2.5 mg/kg BW) of Stimuno® syrup (containing extracts of *Centella asiatica*, 5 mg/mL); the test groups I-III received 200, 400 and 800 mg/kg BW of the extract, respectively. All groups (except the normal group) were also given 0.5 mL *Salmonella typhimurium* 10^5 CFU (intraperitoneal) on the 8th day of treatment as the agent inducer of the typhoid fever.

Antigen preparation

S. typhimurium bacterial culture was bred in slant *Salmonella-Shigella* Agar (SSA) media, and it was then incubated at a temperature of 37°C for 48 h. The bacterial culture was suspended with 10 mL of 0.9% NaCl solution in a tube until the concentration reached 10^5 CFU/mL, which was measured using a densitometer.

Immunomodulatory effects test

After the acclimatization period, the test animals were orally treated with the extract once a day. The animals were induced by giving antigen suspension of *S. typhimurium* dose of 10^5 CFU as much as 0.5 mL on the 8th day. Before and 24 h after the induction, the body temperature of the albino rats was measured. Furthermore, blood was taken, and spleen was observed on the 12th day (Susanti et al., 2012).

Evaluation of leukocytes and differential cells

Test animals were intraperitoneally anesthetized with phenobarbital 40 mg/kg BW. By using cardiac puncture, the blood sample was collected as much as 1 mL to transfer it into the EDTA vacutainer tube. Then, the total determination of leukocytes was counted by using a hematology analyzer (Sysmex® KX-21). Meanwhile, $CD4^+$ counts were analyzed using a flow cytometer (Alere® Pima *analyzer)* (Yapo et al., 2011). The differential blood cells were manually analyzed by peripheral blood smear observation under a microscope (Olympus CX21®).

Evaluation of spleen organs

Peritoneal and thoracic cavities were opened after euthanizing by cervical dislocation. Spleen organs were isolated and washed with *distilled* water and 0.9% NaCl, consecutively. A macroscopic examination was observed based on size, weight, color, and consistency of spleen organs. Then the spleen was fixed with 10% formalin for histological analysis.

Statistical analysis

The $CD4^+$ and the differential counts were analyzed using SPSS for Windows. Macroscopic findings of the spleen of each test group were compared to the control group. The normality test was performed by using a Shapiro-Wilk analysis followed by one way ANOVA. A *post hoc* LSD test was performed to determine the significance between groups with α 0.05.

RESULTS

Evaluation of $CD4^+$ counts

The result of the $CD4^+$ count measurement showed in Table 1. ANOVA test showed that the ethanol extract of *P. speciosa* pod could increase the number of $CD4^+$ compared to those of the normal group ($p<0.05$). Administration of the ethanol extract of *P. speciosa* pod in the doses of 400 and 800 mg/kg body weight were sufficient doses to increase $CD4^+$ significantly.

Differential blood cell evaluation

The statistical analysis results of leucocyte and differential blood cell measurements of three test groups (I, II, and III), and the control groups (normal, positive, and negative) based on the significant values are presented in Table 2. Based on these results, even though there are statistically significant differences in leukocyte and lymphocyte counts between all test groups and two control (normal and negative) groups, differences be-

tween the two first test groups and the positive group are insignificant. In addition, the significant value of the difference between the test group III and the positive one was less than 0.05. These values of the significant differences imply that the leukocyte counts can be stimulated in all test groups; however, the group III can increase the leukocyte counts higher than the groups I and II.

Furthermore, the statistically significant values of differences between the test groups (I and II) and the positive control group were higher than 0.05 for two parameters of the differential blood cell, i.e., lymphocyte and monocyte. Therefore, they did not show statistically significant differences in these parameters. Contrary, the comparison between test group III and the positive group showed statistically significant differences ($p<0.05$) for the last parameter (neutrophils).

Spleen evaluation

The shape of a normal spleen is likely an elongated bean with the sharp in one of the edges and red to dark red. Damaged spleen will be blackish, and the edges will tend to be rounded or blunt (Cesta, 2006). The macroscopic spleen of rats can be presented in Fig. 1. It can be clearly seen that the spleen of the negative group has a black-bigger shape and harder consistency than that of other groups. The statistical analysis showed that group III had significantly heavier spleens compared to the positive control group ($p<0.05$) (Table 3).

Based on the histological observations, levels of necrosis occurred in the spleens of the positive control group and the test group were lower than those of the negative control group. The lowest level of necrosis was found in the group I and the positive group. The results of this histological and macroscopic observation of the spleens are in good agreement, which showed group I exhibited a weight, color, and consistency relatively similar to the normal group.

Fig. 2A shows that in the spleen histology of the normal group, many follicles were found, but no necrosis was observed. Otherwise, the most severe necrosis occurred in the negative control group compared to other groups (Fig. 2C). Like the positive control group (Fig. 2B), the test rats treated with the extract of *P. speciosa* pod at the lowest dose, 200 mg/kg BW (Fig. 2D), had the least necrosis. Therefore, it indicates that the extract can reduce necrosis as observed in the test group as well as in the positive control group. The lower doses the extract was given, the less necrosis occurred, and vice versa.

Moreover, at 400 and 800 mg/kg BW doses of the extract, a considerable amount of the necrosis was observed (Fig. 2E-F). It may suggest that the higher the dose was given, the more accumulation of the lymphocyte and macrophage observed in the spleen, and as a consequence, the effect of hyperplasia is higher.

Table 1. $CD4^+$ count in rats with typhoid fever and treated with *P. speciosa* pod ethanolic extract.

Group	Treatment	Dose	$CD4^+$ (cells/mm^3)
Normal	Tween-80	0.5 mL/kg	6.0 ± 0.00
Negative control	Tween-80	0.5 mL/kg	4.0 ± 0.00
Positive control	*Centella asiatica*	2.5 mg/kg	6.5 ± 0.70
Group I	*P. speciosa*	200 mg/kg	7.5 ± 4.95^{a}
Group II	*P. speciosa*	400 mg/kg	8.5 ± 3.54^{ab}
Group III	*P. speciosa*	800 mg/kg	11.0 ± 7.07^{ab}

Values are presented as mean ± SD, n=3; [a]$p<0.05$ statistically significant differences compared with the positive control group: group I ($p=0.016$), group II ($p=0.001$), and group III ($p=0.00$); [b]$p<0.05$ statistically significant differences compared with the normal group with p values for group II ($p=0.023$), and group III ($p=0.00$). *Salmonella typhimurium* 0.5 mL (10^5 CFU, intraperitoneal) was administered on the 8th day of treatment in all groups except normal group.

Table 2. Differential blood cell counts in animals with typhoid fever and treated with *P. speciosa* pod ethanolic extract.

Group	Leukocyte ($10^3/\mu L$)	Lymphocyte (%)	Monocyte (%)	Neutrophils (%)
Normal	7.83 ± 0.52	78.00 ± 3.60	11.67 ± 2.51	10.33 ± 2.88
Negative control	11.17 ± 2.14	76.00 ± 2.00	9.67 ± 1.52	10.00 ± 1.00
Positive control	14.83 ± 5.51	65.00 ± 3.00	13.00 ± 1.73	16.67 ± 1.53
Group I	15.53 ± 7.30^{ab}	69.00 ± 1.00^{ab}	11.67 ± 0.57^{b}	15.33 ± 0.58^{b}
Group II	16.57 ± 0.20^{ab}	67.33 ± 1.15^{ab}	15.00 ± 1.00^{b}	20.00 ± 1.00^{ab}
Group III	19.30 ± 5.54^{ac}	66.00 ± 3.60^{ab}	18.33 ± 0.57^{ab}	24.00 ± 1.00^{ac}

The values are presented as mean ± SD, n=3; $^{a}p<0.05$ compared to the normal group; $^{b}p>0.05$ compared to the positive control group; $^{c}p<0.05$ compared to the positive control group. There were statistically significant differences between leucocyte (all group: p=0.00), lymphocyte (group I, p=0.006; group II, p=0.002; group III, p=0.001), monocyte (group III, p=0.024) and neutrophils (group II, p=0.03; group III, p=0.00) compare to the normal group. There were no statistically significant differences between in leukocyte (group I, p=0.67; group II, p=0.121); lymphocyte (group I, p=0.177; group II, p=0.419; group III, p=0.726); monocyte (group I, p=0.646; group II, p=0.493; group III, p=0.084); neutrophils (group I, p=0.626; group II, p=0.235) compare to positive control group but there were statistically significant differences between leukocyte (group III, p=0.00) and neutrophils (p=0.018) compare to the positive control group. Normal and negative control groups: Tween-80 0.5 mL/kg BW; positive control group: *Centella asiatica* (2.5 mg/kg BW); groups I-III: *P. speciosa* pod ethanolic extract at doses of 200, 400 and 800 mg/kg BW. *Salmonella typhimurium* 0.5 mL (10^5 CFU, intraperitoneal) was administered on the 8th day of treatment in all groups except normal group.

Table 3. Spleen morphology of the animals with typhoid fever and treated with *P. speciosa* pod ethanolic extract.

Morphology	Treatment					
	Normal	Negative control	Positive control	Group I	Group II	Group III
Color	Red	Red Black	Blackish red	Red with little black	Blackish red	Blackish red
Shape	Normal	Normal	Normal	Normal	Normal	Normal
Weight (g)	0.54 ± 0.05	1.03 ± 0.05	0.66 ± 0.01	0.61 ± 0.02	0.75 ± 0.05	0.88 ± 0.03^{a}
Consistency	Springy	Springy hard	Springy little hard	Springy	Springy	Springy

Values are presented as means ± SD, n=3. $^{a}p<0.05$ compared to the positive control group. There were statistically significant differences between group I (p=0.00), group II (p=0.00), group III (p=0.017) compare to the normal group. There were no statistically significant differences between group I (p=0.089), group II (p=0.849) compare to the positive control group, but there were statistically significant differences with the group III (p=0.017). Normal and Negative control groups: Tween-80 0.5 mL/kg BW; Positive control group: *Centella asiatica* (2.5 mg/kg BW); groups I-III: *P. speciosa* pod ethanolic extract at doses of 200, 400 and 800 mg/kg BW. *Salmonella typhimurium* 0.5 mL (10^5 CFU, intraperitoneal) was administered on the 8th day of treatment in all groups except normal group.

Figure 1. Macroscopic of spleens in normal group **(A)**; test groups I-III **(B-D)**; negative control **(E)**; and positive control **(F)**.

Normal and negative control groups: Tween-80 0.5 mL/kg BW; positive control group: *Centella asiatica* (2.5 mg/kg BW); groups I-III: *P. speciosa* pod ethanolic extract at doses of 200, 400 and 800 mg/kg BW. *Salmonella typhimurium* 0.5 mL (10^5 CFU, intraperitoneal) was administered on the 8th day of treatment in all groups except normal group.

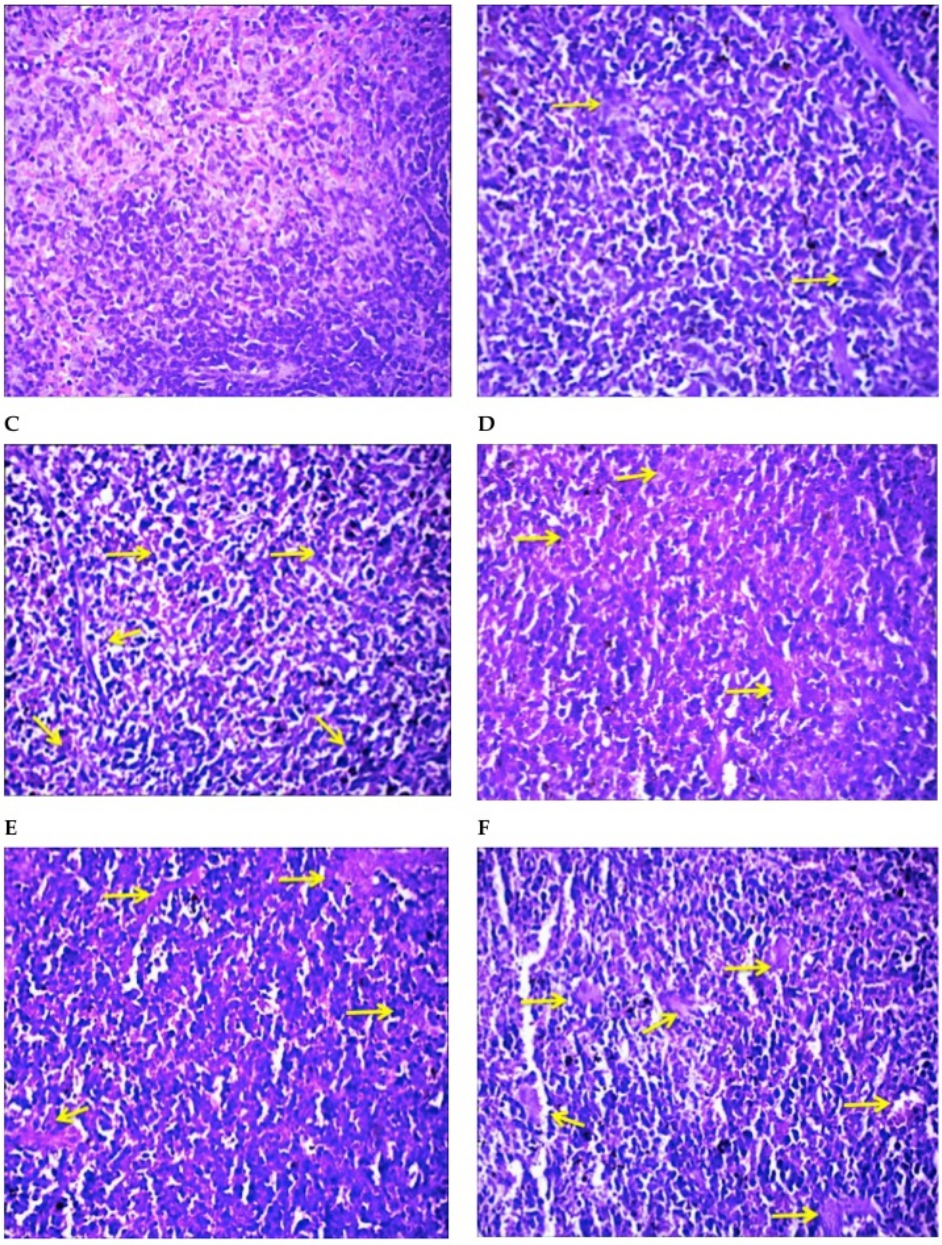

Figure 2. Histopatology of spleen in normal group **(A)**, positive control **(B)**, negative control **(C)**, doses of *P. speciose* ethanolic extract 200, 400, and 800 mg/kg BW **(D-F)**.

Normal and negative control groups: Tween-80 0.5 mL/kg BW; positive control group: *Centella asiatica* (2.5 mg/kg BW). *Salmonella typhimurium* 0.5 mL (10^5 CFU, intraperitoneal) was administered on the 8th day of treatment in all groups except normal group. H & E staining. Magnification 400×.

DISCUSSION

The $CD4^+$ is a marker on the surface of white blood cells of humans, especially lymphocyte cells. It is well known that infection causes a decrease in the immune response by blocking the activity of $CD4^+$ and $CD8^+$ cells. $CD4^+$ has an important role in the immune system. A decrease in its level implies a weak immune system for blocking infection. Therefore, to provide defense against the infection, the immune system needs to be strengthened by stimulating $CD4^+$ production (Yapo et al., 2011).

In addition, the activation of immune cells such as T cells of $CD4^+$ and $CD8^+$ is a possible mechanism to stimulate immune activity. An increase in the activity of the immune cells is considered a good indicator of a better prognosis and active immune response to tumors and infections (Gautam et al., 2009; Ayeka et al., 2017). In this research, an increase in the number of $CD4^+$ is allegedly triggered by flavonoid and phenolic compounds contained in the ethanol extract of *Parkia speciosa*. Flavonoid compounds activate T cells to differentiate into $CD8^+$ or $CD4^+$ cells. The $CD4^+$ cells have no direct killing activity in infected cells but direct other immune cells to act against cells infected with pathogens, mainly by secreting some cytokines (Grigore, 2017).

The levels of the $CD4^+$ cells, leukocyte, monocyte, and neutrophil in the blood circulation can increase due to the activation of immune-promoting compounds. The extract-contained flavonoid stimulates the proliferation of the number of differential T and B lymphocyte (Ketema et al., 2015). This flavonoid increases the secretion of cytokine interleukin-2 (IL-2), acting as proliferation and differentiation factors (Middleton et al., 2000). Meanwhile, phenolic compounds can stimulate the immune system from its hydroxyl groups. These compounds influence enzymes or electron transfer systems to produce immunomodulatory properties, especially a phagocytic activity (Manosroi et al., 2003).

The decrease in the lymphocyte count in this study was affected by two factors. Firstly, the ability of flavonoids to stimulate lymphocyte proliferation resulted in the acceleration of a lymphocyte formation to increase the lymphocyte count. However, as the lymphocyte was formed too quickly, it would die quickly. Therefore, the total number of lymphocytes in the spleen finally decreased. Another factor was the infection, which retained lymphocyte in lymphoid organs such as the spleen. As a consequence, lymphocyte and macrophage would accumulate in the spleen, and the spleen then swelled. (Fahrimal et al., 2014).

The spleen is an organ where immune cells, especially phagocyte cells, carry antigens, interact with, and then produce T lymphocyte. Thus, an increase in the spleen weight is an indicator of the stimulation in the immune system (Ketema et al., 2015). The higher the extract dose was given, the higher ability of the extract stimulated lymphocyte and macrophage cells. As this stimulation affected the accumulation of antigens, hyperplasia due to infection took place in the spleen. This spleen produced more lymphocyte and macrophage to deal with antigen attacks (Cesta, 2006; Vásquez et al., 2015).

CONCLUSIONS

This study proved that the ethanol extract of *Parkia speciosa* pod increases the $CD4^+$ count and the differential cells so that it is potential as an immunomodulatory agent. However, an increase in its doses caused a decrease in lymphocyte count. In addition, necrosis and hyperplasia occurred in the spleen. The extract with 200 mg/kg BW dose can stimulate the immune system even though it creates low necrosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Fitrya F	Amriani A	Novita RP	Elfita	Setiorini D
Concepts or ideas	x			x	
Design	x				
Definition of intellectual content	x	x	x	x	
Literature search	x	x	x	x	x
Experimental studies	x	x	x	x	x
Data acquisition	x	x	x	x	x
Data analysis	x	x	x	x	x
Statistical analysis	x				x
Manuscript preparation	x	x	x	x	x
Manuscript editing	x	x	x	x	x
Manuscript review	x	x	x	x	x

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