ORIGINAL ARTICLE

Hematological Profile of Mice After Ethyl Acetate Extract of Fungus Comb of Indo-Malayan Termite (*Macrotermes gilvus* Hagen) Mound Supplementation in Regulating Lipopolysaccharide-induced Inflammatory Response

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ABSTRACT

Introduction: *Termitomyces sp.* is an edible fungal species commonly found in tropical forests in Africa and Southeast Asia. It is consumed as an immunomodulatory agent to enhance the immune system. This study explored the hematological profile of ethyl acetate extract of fungus comb(EAEFC) on mice's inflammatory response induced by intraperitoneally-injected lipopolysaccharide. **Methods:** An experimental study with a post-test-only control group design using BALB/C mice(n = 24, bodyweight/BW:25-30 grams) was conducted. The animals were randomly allocated into 4 groups, each consisting of 6 mice, and received substance(s) via gavage. Groups I and III received 5% Dimethyl Sulfoxide(DMSO) in water, groups II and IV received 500mg/kg BW EAEFC in 5% DMSO. On day 15, Group I and II were injected intraperitoneally with 5 ml/kg BW saline, whereas Group III and IV with 10 mg/kg BW lipopolysaccharide(LPS) in 5 ml/kg BW saline. After three hours, the mice were sacrificed; their blood was collected, and a hematological profile was observed. The results were displayed as mean ± standard deviation (SD), and the differences between groups were statistically analyzed using one-way ANOVA. The non-normally distributed data were analyzed using Kruskal-Wallis followed by Man-Whitney U test. **Results:** Peripheral blood study revealed that the levels of leukocyte count from the EAEFC group(group II) increased significantly when compared to the negative control(group I). Group IV had higher granulocyte and lower lymphocyte percentages than group III, suggesting the potential inflammation-regulating effect of EAEFC, while there is no marked difference in group I and II without LPS-induced inflammatory response. **Conclusion:** Results indicated that EAEFC altered leukocyte responses when the inflammation occurred.

Keywords: *Termitomyces*, ethyl acetate extract of fungus comb, lipopolysaccharide, inflammation, hematological profile.

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INTRODUCTION

Termitomyces sp. is an edible fungal species commonly found in tropical forests in Africa and Southeast Asia (1,2). The mushroom's edible part is its fruiting body which can only be observed in a short period at the end of a rainy season under a specific temperature and humidity (3). In Africa, people consume the mushroom because of its delightful taste and for various medical purposes, for instances *Termitomyces* heimii, being called Khumb', 'Tanna', 'Sootree' or 'Naadu' by

indigenous agrarian people, is traditionally consumed by Indians to control high blood pressure, regulate blood lipids, enhance immune response, and encounter infection (1,4). *Termitomyces clypeatus* is generally used in Africa and South Asia for curing pox by applying it to the infected area (5).

In addition to their highly nutritious composition, *Termitomyces* have been investigated to possess several medicinal benefits as antitumor, immunomodulating, antiviral, lipid-lowering, and liver-protective agents (1,6,7). Many bioactive substances such as polysaccharides, lectins, proteins, terpenoids, and other organic compounds have also been extracted from mushrooms (8). In our previous study, Nandika et al. (2021) explored the chemical components of fungus comb from Indo-Malayan termite *Macrotermes gilvus* Hagen (Isoptera: Termitidae) (9). The study also showed that ethyl acetate extract of the Indo-Malayan termite inhibited the growth of some pathogenic bacteria and fungi such as E*scherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Aspergillus flavus,* and *Aspergillus niger* (10).

Lipopolysaccharide (LPS) are characteristic components of the cell wall of Gram-negative bacteria that stimulate the innate immune system via Toll-like receptor 4 (TLR4). The LPS model of systemic inflammation revealed to be most suitable for further expolration of acute inflammation. The LPS has high reproducibility in order to increase the levels of pro-inflammatory cytokines in circulating serum, besides it is feasible. Therefore, LPS can be used to study the pathophysiological processes of endotoxemia or SIRS and the anti-inflammation agents observed, but not of sepsis in general (11,12).

To date, the bioactivity of the ethyl acetate extract of fungus comb as an immunomodulatory (e.g. inflammation-modulating) agent has not been fully explored. The immune response can easily be observed in the circulating number of blood cells such as WBCs (13). Therefore, in this experimental study, we sought to explore the hematological profile of an ethyl acetate extract of fungus comb on mice's inflammatory response induced by intraperitoneally-injected lipopolysaccharide in the peripheral blood.

MATERIALS AND METHODS

Material Preparation

Specimen collection

The termite nests of *Macrotermes gilvus* Hagen in Yanlapa Experimental Forest, West Java, Indonesia, were collected in five different areas. The Forest is 200-300 meters above sea level, with 75-90% humidity, 17.5-26.8°C temperature, and 3282 mm/year of rainfall.

Extract preparation

After being collected, the samples were washed, sliced into small pieces, and stored in a freezer (temperature -18 C). The samples were dried using a freeze dryer Brand CHRIST Gamma 1-16 LSC type. The moisture content of the sample was determined by the gravimetric method. The dry sample had less than 10% moisture.

Ethyl Acetate Fungus Comb Extraction

The fungus comb powder first defatted by extraction with n-hexane pro-analysis (pa). Then, the residue was extracted with ethyl acetate pro-analysis grade at room temperature (21 C) with a ratio of fungus comb powder and the solvent 1:10 w/v. The fungus comb extract (EFC) was concentrated with a rotary evaporator (400 rpm for 15-25 minutes). Then, the concentrated extract was stored and delivered to Surabaya in sealed dark glass tubes at -10°C.

Animals

Twenty-four healthy adult male Swiss albino BALB/c mice (Mus musculus) aged 10-12 weeks old and of 20-25 grams body weight (BW) were used in this study. They were raised at the animal laboratory of the experimental model of the Veterinary Faculty of Airlangga University, Surabaya, Indonesia, where the study was conducted. The colony was obtained from Pusat Veteriner Farma, The Laboratory of Animal Model Cultivation of the Ministry of Agriculture of Indonesia. The experiments were conducted under the temperature of 30.2 oC and light: dark of 12h:12h. The animals were fed with a standard mouse feed (511 HI-PRO-VITE, Charoen Pokphand) procured from the animal laboratory and water ad libitum. Every effort was made to minimize animal suffering and stress, especially during LPS challenge and euthanasia. This study was approved by the Institutional Animal Ethical Committee of Airlangga University with approval letter No 2.KE.029.03.2021.

Lipopolysaccharide

Lipopolysaccharide (LPS) from Escherichia coli O111:B4, purified by phenol extraction, was purchased from Sigma (St. Louis, MO, USA).

Experimental Design

Animal Grouping

This was an experimental study with a post-test-only control group design using mouse models which were acclimatized one week before the experiment to eliminate stress. Twenty-four mice were randomized and divided into four groups, six mice in each group (Table I), i.e., Groups I and III received 5% Dimethyl Sulfoxide (DMSO) in aqua bidest, groups II and IV received 500 mg/kg BW ethyl acetate extract of fungus comb extract (EAEFC) in 5% DMSO for 14 days. The treatments were administered orally by gavage, a process of animal force-feeding using a plastic tube. On day 15, Groups I and II were injected intraperitoneally with 5 ml/kg BW

saline solution, whereas groups III and IV were injected intraperitoneally with 10 mg/kg BW lipopolysaccharide (LPS) in 5 ml/kg BW saline solution. After three hours, the blood was extracted and the mice were sacrificed (12).

Table I: The four experimental groups and assigned treatments

Group (n = 24)	Treatment by gavage (14 days)	Treatment at Day 15
I (n = 6)	5% DMSO in water	Saline 5 ml/kg BW i.p.
II (n = 6)	EAFCE 500 mg/kg BW in 5%DMSO	Saline 5 ml/kg BW i.p.
III (n = 6)	5% DMSO in water	10 mg/kg BW LPS in 5 ml/kg BW saline i.p.
IV (n = 6)	EAFCE 500 mg/kg BW in 5%DMSO	10 mg/kg BW LPS in 5 ml/kg BW saline i.p.

EAFCE = EthylAcetate Fungus Comb Extract; DMSO = Dimethyl Sulfoxide; BW = Body Weight; i.p. = intraperitoneally.

Blood Extraction and Hematological Analysis

The mice's blood was collected intracardially after the mice were physically euthanized with cervical dislocation and using isoflurane as general anesthesia. Calculation of red blood cells (RBC), hemoglobin (Hb), hematocrit, white blood cells (WBC), lymphocytes, monocytes, and granulocytes levels used a fully automated hematology analyzer ABX Micros 60 (Horiba ABX SAS, Montpellier, France) and printed.

Statistical Analysis

Data of the hematological profile were presented as mean ± standard deviation (SD). Obtained results were tested for normality and homogeneity. Normal homogenous data were statistically analyzed using one-way ANOVA followed by Fisher's least significant difference (LSD) multiple comparison tests. Non-normally distributed data were analyzed using Kruskal-Wallis followed by Man-Whitney U test for multiple comparisons. P<0.05 was considered statistically significant.

RESULTS

Effects of Fungus Comb Extract on RBCs, Hemoglobin, Hematocrit, and Platelets in the Blood

As shown in Table II, hematological profiling of the four experimental groups revealed that the number of RBCs and Hb showed no significant difference after receiving EAEFC (group I vs group II and group III vs group IV). The RBCs data for group I, II, III, and IV were 7.420 ± 0.763, 7.733 ± 0.690, 8.500 ± 0.901, and 9.083 ± 0.915, respectively. Whereas the Hb data from each group was 13.96 ± 1.605, 14.18 ± 0.731, 14.98 ± 1.714, and 16.38 ± 2.110, respectively. The RBCs, and Hb data of group IV, treated with EAEFC and LPS, were significantly higher (p<0.05) than groups I and II. The number of platelets and hematocrit percentage throughout the four groups had no difference.

Effects of Fungus Comb Extract on WBCs in the Blood

The WBCs result of group II (Fig. 1), after EAEFC supplementation, showed an elevating number of cells in the blood than the control group, while the increase of WBCs in group III induced by LPS injection was suppressed in group IV after EAEFC was administered for 14 days. The number of leukocytes in group I was 11.700 ± 0.167, while group II was 13.117 ± 0.478. The data exhibited a significant difference (p<0.05) after 14 days of EAEFC supplementation. The average of WBCs of groups III and IV were 11.900 ± 0.111 and 11.733 0.160 respectively as shown in Table II. The leukocytes number in 1 mcl blood was suppressed in group IV, compared to group III, to reach the same level as the control group (group I).

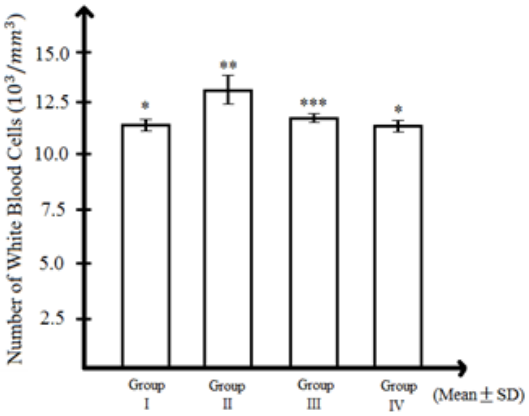

Fig. 1: The number of white blood cells from each group of treatment. Group I: DMSO 5% (14 days) and saline injection (15th day), group II: DMSO 5%+EAEFC (14 days) and saline injection (15th day), group III: DMSO 5% (14 days) and LPS+saline injection (15th day), group IV: DMSO 5% +EAEFC (14 days) and LPS+saline injection (15th day). Each value indicates the mean SD of each group (*p<0.05, **p<0.01, and ***p<0.05). DMSO: Dimethyl Sulfoxide, EAEFC: Ethyl Acetate Extract of Fungus Comb, mm: millimeter.

Table II. The data of Erythrocytes, Hemoglobin, Hematocrit, Leukocytes, and Thrombocytes for each group.

Group	Erythrocytes (10^6/ mm^3)	Hemoglobin (g/dL)	Hematocrit (%)	Leukocytes ($10^3/mm^3$)	Thrombocytes ($10^3/mm^3$)
I	7.420 ± 0.763*	13.96 ± 1.605*	40.88 ± 4.979*	11.700 ± 0.167*	421.2 ± 286.6*
II	7.733 ± 0.690*	14.18 ± 0.731*	44.23 ± 2.769*	13.117 ± 0.478**	565.0 ± 254.5*
III	8.500 ± 0.901*.**	14.98 ± 1.714 *.**	46.27 ± 4.900*	11.900 ± 0.111***	601.5 ± 166.4*
IV	9.083 ± 0.915**	16.38 ± 2.110 **	49.37 ± 5.285*	11.733 ± 0.160*	539.3 ± 249.7*

Group I: DMSO 5% (14 days) and saline injection (15th day), group II: DMSO 5%+EAEFC (14 days) and saline injection (15th day), group III: DMSO 5% (14 days) and LPS+saline injection (15th day), group IV: DMSO 5% +EAEFC (14 days) and LPS+saline injection (15th day). Each value in Erithrocytes, leukocytes, and thrombocytes column represents the number of cells in 1 mcl blood. Each value indicates the mean SD of each group. Values with different symbol in the same column are significantly different at the level of 0.05 (*p<0.05, **p<0.05, and ***p<0.05). DMSO: Dimethyl Sulfoxide, EAEFC: Ethyl Acetate Extract of Fungus Comb, mm: milimeter, g/dL: gram/deciliter.

The administration of LPS in groups III showed an increase in mice's leukocytes. Moreover, the EAEFC did not synergistically elevate the WBCs in the blood after LPS injection. The extract, on the contrary, suppressed the number of leukocytes circulating in the blood.

Effects of Fungus Comb Extract on The Types of WBCs in The Blood

The hematological profiling also quantitatively presented the percentage of lymphocytes, monocytes, and granulocytes in the blood. Granulocytes collectively contained eosinophils, basophils, and neutrophils,

Table III. The data of Lymphocytes, Monocytes, and Granulocytes percentage for each group.

Group	Lymphocytes Percentage (%)	Monocytes Percentage (%)	Granulocytes Percentage (%)
I	35.8 ± 1.720 *	5.6 ± 1.020*	58.6 ± 1.855*
II	35.8 ± 1.572*	5.7 ± 1.491*	58.5 ± 2.217*
III	36.7 ± 1.374*	6.2 ± 1.067 *	57.2 ± 1.951*
IV	31.5 ± 0.763**	5.0 ± 0.816*	63.5 ± 1..258**

Group I: DMSO 5% (14 days) and saline injection (15th day), group II: DMSO 5%+EAEFC (14 days) and saline injection (15th day), group III: DMSO 5% (14 days) and LPS+saline injection (15th day), group IV: DMSO 5% +EAEFC (14 days) and LPS+saline injection (15th day). Each value indicates the mean ± SD of each group. Values with different symbol in the same column are significantly different at the level of 0.05 (*p<0.05 and **p<0.05). DMSO: Dimethyl Sulfoxide, EAEFC: Ethyl Acetate Extract of Fungus Comb.

where the latter were the most dominant.

The percentage of lymphocytes, monocytes, and granulocytes of the control group and group receiving EAEFC/ group II (Table III) showed insignificant differences (p ≥0.05) while group IV exhibited remarkable elevating granulocytes percentage and a decrease in lymphocytes percentage (p<0.05).

Table IV. The data of types of WBCs; Lymphocytes, Monocytes, and Granulocytes for each group.

Group	Lymphocytes (10^3/mm^3)	Monocytes (10^3/mm^3)	Granulocytes (10^3/mm^3)
I	4.191 ± 0.256 *	0.655 ± 0.117 *	6.854 ± 0.172*
II	4.701± 0.281**	0.735. ± 0.178 *	7.677 ± 0.471**
III	4.376 ± 0.180 *	0.736 ± 0.126 *	6.822 ± 0.236*
IV	3.696 ± 0.114 ***	0.587 ± 0.095 *	7.451 ± 0.171 **

Group I: DMSO 5% (14 days) and saline injection (15th day), group II: DMSO 5%+EAEFC (14 days) and saline injection (15th day), group III: DMSO 5% (14 days) and LPS+saline injection (15th day), group IV: DMSO 5% +EAEFC (14 days) and LPS+saline injection (15th day). Each value in lymphocytes, monocytes, and granulocytes column represents the number of cells in 1 mcl blood. Each value indicates the mean SD of each group (*p<0.01, **p<0.01, and ***p<0.01). DMSO: Dimethyl Sulfoxide, EAEFC: Ethyl Acetate Extract of Fungus Comb, mm: milimeter.

The absolute number of the lymphocytes, monocytes, and granulocytes were measured and the result showed a marked difference of lymphocytes and granulocytes from group II and IV when being compared to the other groups (p<0.05). The average of lymphocytes and granulocytes of group II indicated an increased amount when compared to group I. Group I and group III both received DMSO 5% exhibited no dissimilarity even

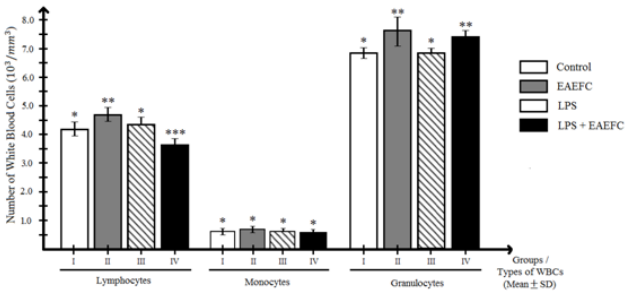

Fig. 2: The number of white blood cells from each group of treatment. Group I: DMSO 5% (14 days) and saline injection (15th day), group II: DMSO 5%+EAEFC (14 days) and saline injection (15th day), group III: DMSO 5% (14 days) and LPS+saline injection (15th day), group IV: DMSO 5% +EAEFC (14 days) and LPS+saline injection (15th day). Each value indicates the mean SD of each group (*p<0.01, **p<0.01, and ***p<0.01). DMSO: Dimethyl Sulfoxide, EAEFC: Ethyl Acetate Extract of Fungus Comb, mm: millimeter.

though group III was given LPS intraperitoneally.

The lymphocytes of group IV declined concerning group III, while granulocytes increased as shown in Fig. 2. There was no remarkable difference in the monocyte number of the cells between the four groups.

DISCUSSION

For decades, mushroom metabolites such as Cyclosporine and Mycophenolic acid have been used as a preventive agent for graft rejection (i.e., to prevent transplanted tissues and organs from being attacked by the host immune system) (14). Cyclosporine A, synthesized from the fermentation of T*olypocladium inflatum*, selectively binds to cyclophilin A, an inhibitor of calcineurin, which results in the depletion of the transcription process of interleukin-2 (IL-2) (15,16). Mycophenolic acid, which is originally from *Penicillium stolonifera, Penicillium brevicompactum,* and *Penicillium echinulatum*, inhibits inosine monophosphate dehydrogenase (IMPDH) in the T- and B-lymphocytes selectively (17).

The use of *Termitomyces* as traditional medicine is popular in rural areas, especially in Africa and Southeast Asia, for blood pressure-lowering agents, anti-cholesterol herbs, and infection, such as typhoid fever (18). Some studies indicated that aqueous extract of fungus comb had antibiotic activities to some bacteria such as *Salmonella enterica* and *Bacillus cereus* including some high resistant bacteria such as *Methicillin-resistant Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (18). In addition to their ability to inhibit bacterial growth, these fungi are widely consumed because they are believed to be able to regulate the immune system inside the human body, so-called the immunomodulatory effect.

Modulation of immune responses is classified into immunostimulants, immunosuppressants, and tolerant.

Mahamat et al found an aqueous extract of fungus comb was capable to create a significant immunostimulatory effect on both the cell-mediated and humoral immune systems in the mice (6,7). In our study, the increase of WBCs in peripheral blood after EAEFC supplementation, without LPS, can explain the immune enhancement effect in relatively normal conditions. Both lymphocytes and granulocytes took a role in the significant WBCs elevation after 14 days of oral EAEFC.

LPSs have previously been used to stimulate innate immune response and the intraperitoneal injection of LPS has been shown to induce systemic inflammation in mice (20,21). Two hours after LPS injection, the inflammation signal in the spleen should have been established (22,23). Comparing the WBCs level, after being injected by LPS, between EAEFC and normal diet, the result showed a decrease in total of WBCs number due to lymphocyte deprivation. The results were contrary to the previous findings that the water extract of fungus comb could induce cellular immunity mediated by T cell (7). There was a remarkable decrease in lymphocytes number in the blood for the group receiving EAEFC while the granulocytes still increased. The reason for the decrease in peripheral blood lymphocytes might be due to the redistribution and migration of lymphocytes to the sites of inflammation, and lymphocyte transport mediated by cytokines also took a role in this process (24).

As previously investigated, the analysis of EAEFC from Indomalayan termite mound had chemical composition dominantly 1,2,3-propanetriol (28.93%) which functioned as anti microbes and anti-inflammation (9,24). Besides there were several components such as Phenol, 2-methoxy- (8.54%), Phenol, 2,6-dimethoxy- (6.55%) and Bis(2-Ethylhexyl) phthalate (4.82%) that all had antifungal purpose (9,26,27). The extract of fungus comb also inhibited the growth of some pathogenic bacteria and fungi such as *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Aspergillus flavus*, and *Aspergillus niger* in Kirby-Bauer disc diffusion and microdilution (10).

In our study, the ethyl acetate extract of fungus comb showed an increase in erythrocyte and hemoglobin concentration for the group with EAEFC and LPS when being compared to the control group and the second group receiving EAEFC without LPS. The result indicated that RBCs formation was independent to inflammation process. Meanwhile, the levels of platelets and monocytes had no effect. Although platelets are related to sepsis, and monocytes recruitment will be active in inflammation conditions, their level insignificantly differed (28).

Finally, this study has several limitations that need to be acknowledged. In this study, we used healthy male BALB/C mice with no pathological conditions (e.g., immunodeficient conditions or those rendered for having immunodeficiency), therefore precluding the investigation of the effects of EAEFC on those particular situations. Our current study also did not evaluate the inflammatory responses in lymph nodes, either humoral or cellular. In the near future, a study that measures such responses in lymph nodes as well as in the spleen can be performed.

CONCLUSION

The present study showed that the ethyl acetate extract of fungus comb from *Termitomyces* gilvus Hagen mound altered the immune system in the mice with inflammation model. The ethyl acetate extract of fungus comb upregulated both monocytes and granulocytes in normal conditions while in inflamed circumstances, the leukocyte decreased to normal value.

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