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# Ethyl acetate extract of fungus comb from Malayan termite (*Macrotermes gilvus* Hagen) mound modulates splenic inflammatory responses in mice

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## ABSTRACT

**Background:** The fungus comb is a unique structure inside termites' nests that facilitates the growth of *Termitomyces* sp. as a nutrient source for the termites. It is known to possess immunomodulatory properties that boost the immune system.

**Aim:** The objective of this study was to assess the impact of ethyl acetate extract of fungus comb (EAEFC) on the inflammatory reaction in the spleen of mice induced by intraperitoneal injection of lipopolysaccharide (LPS).

**Methods:** An experimental study was conducted using a post-test-only control group design with male BALB/C mice ($n$ = 24). The mice were divided randomly into four groups, each comprising six mice, and administered substances via gavage. Groups I and III were administered a solution of 5% dimethyl sulfoxide (DMSO) in distilled water, while Groups II and IV were given 500 mg/kg BW EAEFC dissolved in 5% DMSO. On the fifteenth day, Groups I and II received intraperitoneal injections of 5 ml/kg BW saline, while Groups III and IV were injected with 10 mg/kg BW LPS dissolved in saline. After three hours, the mice were euthanized and splenic immunohistology was examined under a light microscope. The results were expressed as mean ± standard deviation, while the group differences were assessed statistically.

**Results:** The expression of interleukin (IL)-1, furin, and activated NK cell was significantly higher in the inflamed model after EAEFC supplementation, while the extract suppressed IL-10.

**Conclusion:** EAEFC was found to alter cytokine expression in the spleen in response to inflammation.

**Keywords:** Fungus comb, Lipopolysaccharide, Inflammation, Interleukin, Cytokine.

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## Introduction

The edible *Termitomyces* is known to produce valuable organic compounds with potential use as fuel and in biological systems (Yang *et al*., 2020; Njouonkou *et al*., 2020; Sharma *et al*., 2022). Some *Termitomyces* mushroom species, such as *T. heimii* and *T. albuminosus*, produce endopolysaccharides and β-glucans that exhibit antibacterial, antioxidant, and cold-alleviating properties. (Venkatachalapathi and Paulsamy, 2016; Ahmad *et al*., 2021). These fungi sprout inside the termite's nest due to their mutual symbiosis with insects, including termites *Macrotermes gilvus* and *M. carbonarius* (Isoptera: Termitidae), which create a suitable environment for the mushrooms to live in (Yang *et al*., 2020; Njouonkou *et al*., 2020; Noknoy *et al.*, 2020). Known as the Indo-Malayan termite, *M. gilvus* is Indigenous to Southeast Asia from Myanmar, Vietnam, Thailand, the Philippines, and Indonesia; moreover, the nest can be found in Yanlappa Experimental Forest, West Java, Indonesia (Singham *et al.,* 2012; Noknoy *et al.*, 2020; Paloi *et al.*, 2023).

Inside the termite nest, there is a unique structure, a fungus comb, that has complex and convoluted structures and is abundant in tiny white spherules of *Termitomyces* fungus (Nandika *et al*., 2021). The fungus comb facilitates the growth of *Termitomyces* as a nutrient source for the termites (Mahamat *et al*., 2018a; Nandika *et al*., 2021; Park *et al*., 2021). In spite of the fungi, they have been studied for their medicinal benefits, such as being antitumor, immunomodulating, antiviral, lipid-lowering, liver-protective agents, antidiabetic, and anti-inflammation (Mahamat *et al.*, 2018a,b; Yang *et al.*, 2020; Park *et al.*, 2021), the fungus combs are known to possess immunomodulatory properties that enhance the immune system (Mahamat *et al.*, 2018a,b).

Inflammation is a natural response of the immune system to various harmful stimuli, originating both from within and outside the host. These stimuli include pathogens, damaged cells, toxic substances, or irradiation. The inflammatory process is crucial in neutralizing these harmful agents and initiating the healing process (Chen *et al*., 2017). The in vivo model for acute systemic inflammation is suitably observed after lipopolysaccharide (LPS) administration. LPS is the characteristic component of the Gram-negative bacteria's cell wall that binds Toll-like receptor 4 (TLR4) to induce an innate immune response (Seemann *et al*. 2017). After LPS and TLR4 linkage, several cascades signaling steps take place, starting with activating *myeloid differentiation primary response* 88 (MyD88) and TRIF, ultimately leading to gene transcription of pro-inflammatory cytokines (IL-1, IL-6, and Interferon) (El Zayat, 2019).

In a previous study conducted by Nandika *et al*. (2021), the chemical composition of fungus comb derived from Indo-Malayan termite *M. gilvus* Hagen was examined. The ethyl acetate extract of the fungus comb showed antioxidant activity and inhibitory activity against pathogenic bacteria and fungi, including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Aspergillus niger* (Witasari *et al*., 2022). However, the bioactivity of the fungus comb extract from Indo-Malayan termite *M. gilvus* mounds as a preventive immunomodulatory agent has not been explored, through the expression of pro-inflammatory (IL-1 and IFN-γ) and anti-inflammatory (IL-10 and furin) markers (Mahamat *et al*., 2018a,b). Therefore, in this experimental study, we aimed to explore the effects of such an ethyl-acetate fungus comb extract (EAEFC) on the expression of cytokines (IL-1, IFN-γ, IL-10, and furin) in the spleen of mice.

## Materials and Methods

### *Material preparation*

#### Specimen collection

Termite nests of *M. gilvus* Hagen were randomly collected from six different nests in five areas of Yanlappa Experimental Forest, located in West Java, Indonesia. The forest is situated at an altitude of 200–300 m above sea level with high humidity levels, ranging from 75% to 90% relative humidity, suitable for optimum development. The temperature varied between 17.5ºC and 26.8ºC, and the region experienced high rainfall, amounting to 3,282 mm per year.

#### Fungus comb extract preparation

Following the collection process, the termite nest samples were thoroughly washed, cut into small pieces, and stored at −18ºC. The samples were then dried using a freeze dryer (Christ Gamma 1–16 Lyo screen control (LSC) type, Germany), and the moisture content was determined using the gravimetric method. The resulting dry samples had a moisture content of less than 10%. The samples are then ground into a powder.

#### Ethyl acetate extraction of fungus comb and identification of EAEFC

The fungus comb powder was first defatted by extraction with *n*-hexane pro-analysis (pa, EMSURE®, Supelco®, Merck, Germany). The remaining residue was extracted at room temperature (21ºC) with pro-analysis grade ethyl acetate (EMSURE®, Supelco®, Merck, Germany) using a 1:10 w/v ratio of fungus comb powder and solvent. The resulting fungus comb extract (EAEFC) was then concentrated using a rotary evaporator at 400 rpm and 40°C until the solvent was fully removed. The concentrated extract was stored in sealed dark glass tubes at −10°C and transported to the animal laboratory.

#### Animals

In this experimental study, we utilized a total of 24 healthy male Swiss albino BALB/c mice (*Mus musculus*) aged 10–12 weeks old, with a body weight (BW) ranging from 20–25 g. The mice were procured from Farma Veterinary Center, Surabaya, Indonesia, and were housed in the animal laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, where the study was conducted. The laboratory maintained a temperature of 30.2°C

with a light: dark cycle of 12 hours:12 hours. During the study, the mice were given access to standard mouse feed (511 HI-PRO-VITE, Charoen Pokphand, Indonesia) and water ad libitum. All treatments in the experiment, particularly the LPS injection, EAEFC gavage, and euthanasia procedures, were conducted using methods that minimize suffering and stress, as explained in the experimental design section.

**LPS**

LPS, derived from *E. coli* strain O111:B4 and purified by phenol extraction, was purchased from Sigma (St. Louis, MO).

***Experimental design***

**Animal grouping**

A post-test-only control group design was employed in this experimental study using mice models. The animals were subjected to an acclimatization period of one week before the experiment to minimize stress. Twenty-four mice were randomly divided into four groups of six mice each (Table 1). Groups I and III were administered 5% dimethyl sulfoxide (DMSO) in distilled water by gavage using a 1 cc syringe. In comparison, groups II and IV received 500 mg/kg BW ethyl acetate of fungus comb extract (EAEFC) in 5% DMSO for 14 days via oral gavage, a process of animal force-feeding using one-milliliter syringe tube. On the fifteenth day, groups I and II were intraperitoneally injected with 5 ml/kg BW saline solution. In contrast, groups III and IV were injected with 10 mg/kg BW LPS in 5 ml/kg BW saline solution. The mice were physically euthanized with cervical dislocation three hours after the injection, and their spleens were collected for analysis.

**Immunohistochemistry evaluation of IL-1, furin, IL-10, and IFN-γ CD56**

In this study, the mice's spleen, as a secondary lymphoid organ was observed to assess the function of innate and adaptive immunity (Lewis *et al*. 2019). In addition to organ-specific markers, systemic inflammatory markers observed through blood examinations have been reported previously (Caesario *et al.*, 2023). After being collected, the spleens were extracted and fixed with 10% formalin buffer for 24 hours, and the tissue was processed using the paraffin method. Subsequently, the paraffin block was sliced using a microtome with a thickness of about 4–6 microns and attached to a glass object that had been coated with poly L-lysine. The incision was then placed on a hot plate for 60 minutes and stained using immunoperoxidase staining, DAB chromogen, and Mayer's hematoxylin counterstain for single staining and additional HRP green chromogen for double staining immunohistochemistry. Next, each marker (i.e., IL-1, furin, IL-10, and IFN-γ/CD56) was visually observed using a light microscope of 400x magnification scale. On average, the white pulp was observed between 6 and 8 random areas, while the counted cells were those that gave a positive reaction (i.e., ones with brownish coloration) to each antibody: anti-IL-1 (bs-6319R-HRP), anti-furin (bsm-54283R), anti-IL-10 (bs-0698R-HRP), anti-IFN-γ (bs-0480R-HRP), and anti-NCAM/CD56 (bsm-52824R) (Bioss, USA).

**Table 1.** The four experimental groups and assigned treatments.

| Group (*n* = 24) | Treatment by gavage (14 days) | Treatment at day 15 |
|---|---|---|
| I (*n* = 6) | DMSO 5% 20 ml/kg BW | Saline 5 ml/kg BW i.p. |
| II (*n* = 6) | EAEFC 500 mg/kg BW in DMSO 5% 20 ml/kg BW | Saline 5 ml/kg BW i.p. |
| III (*n* = 6) | DMSO 5% 20 ml/kg BW | LPS 10 mg/kg BW in saline solution 5 ml/kg BW i.p. |
| IV (*n* = 6) | EAEFC 500 mg/kg BW in DMSO 5% 20 ml/kg BW | LPS 10 mg/kg BW in saline solution 5 ml/kg BW i.p. |

EAEFC = Ethyl-Acetate Fungus Comb Extract; DMSO = Dimethyl Sulfoxide; BW = Body Weight; i.p. = intraperitoneally

**Extract composition identification**

The ethyl acetate extraction chemical properties were determined using the Gas chromatography/mass spectrometry GC-MS®, Agilent Technologies 6890N series with helium as carrier gas. A 6 µl of the extract was placed into the GC-MS inlet. A capillary column with a diameter of 0.25 mm and a length of 60 m was applied for the compound separation and quantitative analyses by GC-MS. In the beginning, the temperature was 70°C and increased by 15°C per minute until reaching 290°C at 20 ms, with 20 minutes last duration. The peak of spectrum data was analyzed with the data in the WILEY 9th library.

***Statistical analysis***

The mean value with standard deviation (SD) was utilized to present the data of the positively stained cells using IBM SPSS Statistics 22 software. The normality and homogeneity of the results were evaluated, and normal and homogenous data were subjected to statistical analysis through one-way ANOVA and Fisher's least significant difference (LSD) test for multiple comparisons. For non-normally distributed data, non-parametric tests such as Kruskal-Wallis followed by the Man-Whitney U test were employed for multiple comparisons. A *p*-value of less than 0.05 was considered statistically significant.

***Ethical approval***

The Institutional Animal Ethical Committee of Airlangga University approved the animal ethical clearance (No 2.KE.029.03.2021) for this study.

## Results

Table 2 displays the results of immunohistochemistry examinations in the four experimental groups. The expression of IL-1 and furin was the highest in Group IV (EAEFC + LPS), while Group III (DMSO + LPS) exhibited abundant expression of IL-10. NK cells expressing IFN-γ showed an increase in the number of cells in the inflammation model or LPS-administered group. In contrast, the number was lower in the absence of inflammation.

### *Effects of fungus comb extract on IL-1 level in the spleen*

The immunohistochemistry of IL-1 (Fig. 1) showed the number of IL-1 positive cells under a light microscope as presented in Table 2. No significant difference was observed in the number of IL-1 positive cells between groups I and II, which did not receive intraperitoneal LPS injection. The mean number of IL-1 positive cells in group I (negative control; DMSO + saline) and group II (EAEFC + saline) were 1.050 ± 0.244 and 1.188 ± 0.282 cells, respectively. The administration of

**Table 2.** The data of IL-1, IL-10, and Furin, NK cells for each group.

| Group | Average of IL-1 positive cells of spleen (cells/156.25 | Average of IL-10 positive cells of spleen (cells/156.25 | Average of Furin positive cells of spleen (cells/156.25 | Average of CD-56+ IFN-γ positive cells of spleen (cells/156.25 |
|---|---|---|---|---|
| I | 1.050 ± 0.244 | 4,992 ± 0.673 | 2.075 ± 0.716 | 6.775 ± 0.531 |
| II | 1.188 ± 0.282 | 5.748 ± 1.412 | 2.083 ± 1.532 | 4.426 ± 0.339 |
| III | 5.938 ±1.310 | 10.167 ± 3.495 | 3.063 ± 2.183 | 4.441 ± 0.691 |
| IV | 15.354 ± 4.249 | 5.542 ± 1.100 | 6.729 ± 3.582 | 6.744 ± 0.171 |

Group I: DMSO 5% (14 days) and saline injection (15th day), group II: DMSO 5% +Extract of Fungus Comb (14 days) and saline injection (15th day), group III: DMSO 5% (14 days) and LPS +saline injection (15th day), group IV: DMSO 5% +Extract of Fungus Comb (14 days) and LPS +saline injection (15th day). Each value indicates the mean  SD of each group.

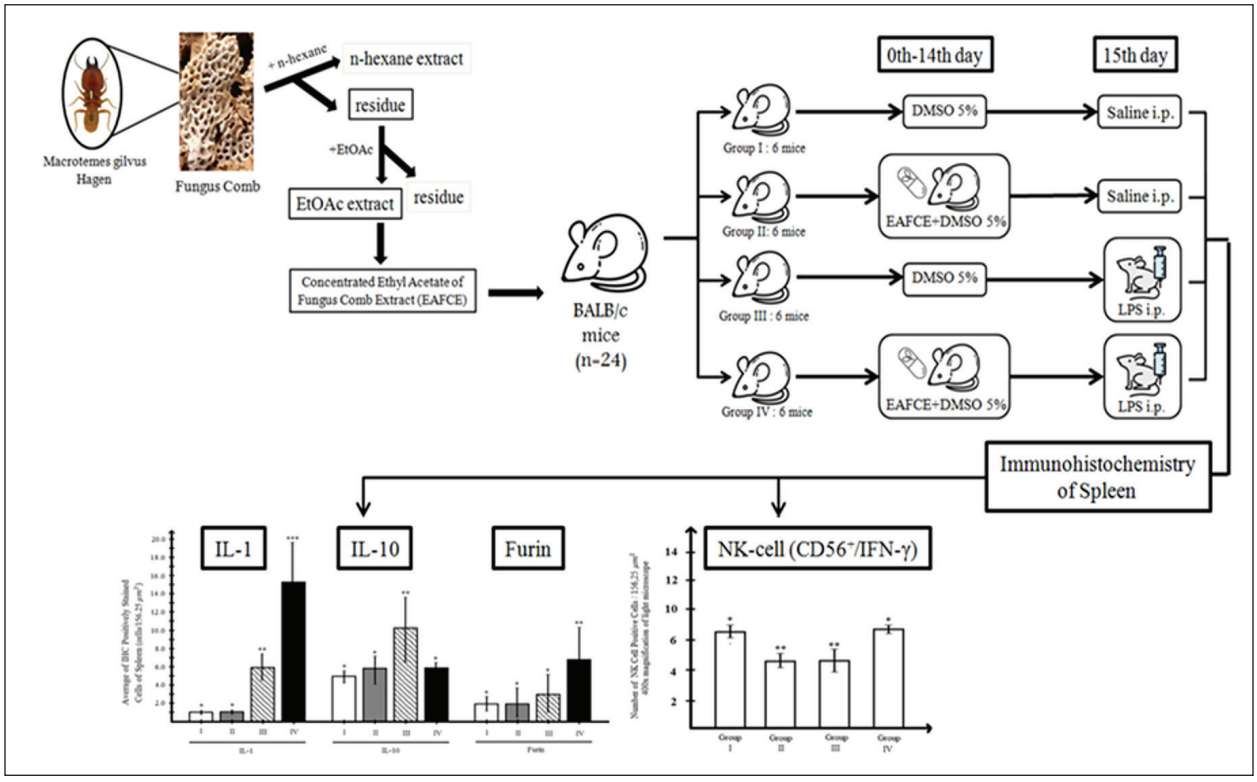


**Fig. 1.** The representative immunohistochemistry images of mice's spleen IL-1of the four experimental groups. (A) Group I (DMSO + saline). (B) Group II (EAEFC + saline). (C) Group III (DMSO + LPS). (D) Group IV (EAEFC + LPS). The yellow arrows indicate IL-1-expressing macrophages and the blue arrows indicate macrophages that do not express IL-1. The immunoperoxidase staining DAB chromogen shows a brownish color and Mayer's hematoxylin counterstain shows blue-purplish color.

LPS in groups III and IV resulted in notable differences in the number of IL-1-expressing spleen cells when compared to the first two groups. Moreover, group IV (EAEFC + LPS) expressed significantly more IL-1 (15.354 ± 4.249 cells) than group III (DMSO + LPS; 5.938 ± 1.310 cells) (Fig. 2). Statistical analyses showed significant differences between the last two groups (Groups III and IV).

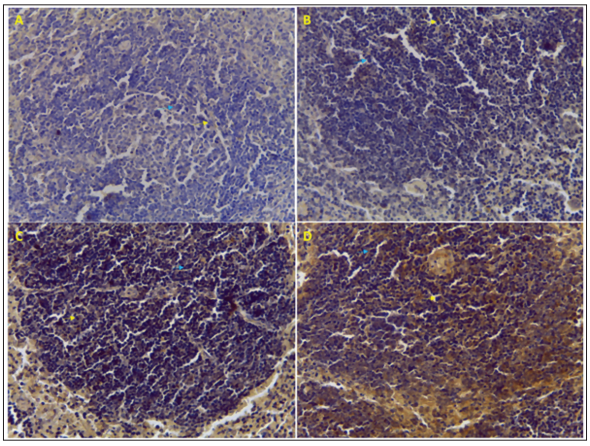

**Fig. 2.** The number of IL-1, IL-10, and Furin positive cells/156.25 μm² for each group under 400x magnification of the light microscope. Group I: DMSO 5% (14 days) and saline injection (15th day), group II: DMSO 5% +Extract of Fungus Comb (14 days) and saline injection (15th day), group III: DMSO 5% (14 days) and LPS +saline injection (15th day), group IV: DMSO 5% +Extract of Fungus Comb (14 days) and LPS +saline injection (15th day). Values with different symbols (*/**/***) in the same column are significantly different at the level of 0.05 ($p < 0.05$).

***Effects of fungus comb extract on IL-10 level in the spleen***

In the spleen cells, the IL-10 quantification result (Fig. 3) showed no significant difference between groups I and II (Table 2). The average IL-10 level of group I (control) was 4.992 ± 0.673 and group II (EAEFC + saline) was 5.748 ± 1.412 cells/156.25 μm². Treatment with EAEFC + LPS in group IV did not exhibit a significant difference compared to the first two groups, with an average IL-10 level of 5.542 ± 1.100 cells/156.25 μm². However, group III (DMSO + LPS) showed an increase in the IL-10 level in spleen cells compared to other groups. The IL-10 level in group III (Fig. 2) was 10.167 ± 3.495 cells/156.25 μm², and the statistical analysis comparing groups III and IV revealed significant results. This finding suggests that administering the fungus comb extract before the intraperitoneal injection of LPS significantly reduced in the IL-10 level.

***Effects of fungus comb extract on furin level in the spleen***

Results of the immunohistochemistry of splenic furin levels as presented in Figure 4 indicated that groups I and II, which received an intraperitoneal saline injection, showed no significant difference in the average number of furin-positive cells (2.075 ± 0.716 and 2.083 ± 1.532, respectively). However, the average

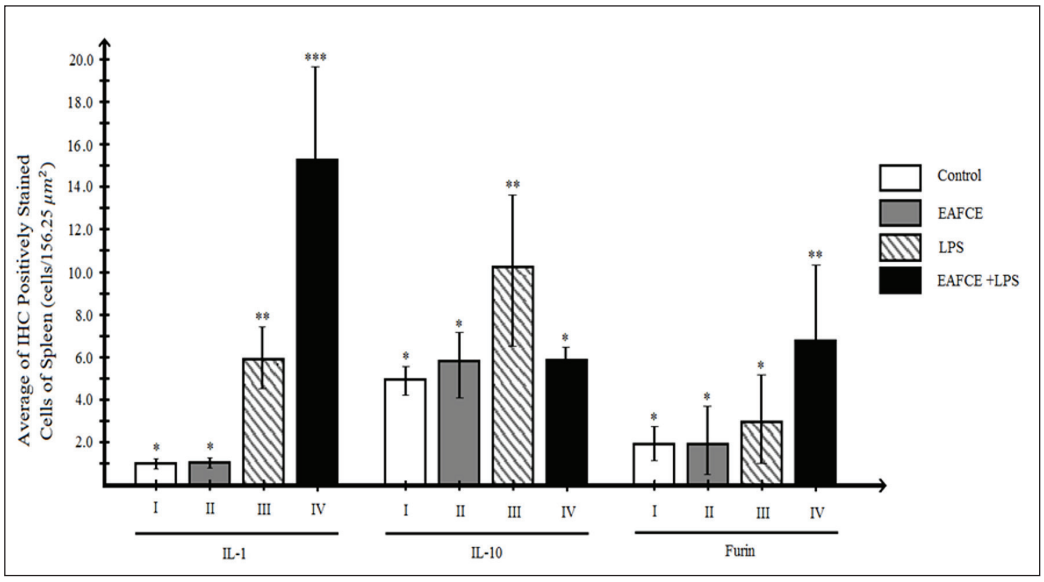


**Fig. 3.** The representative immunohistochemistry images of splenic IL-10 of the four experimental groups. (A) Group I (DMSO + saline). (B) Group II (EAEFC + saline). (C) Group III (DMSO + LPS). (D) Group IV (EAEFC + LPS). The yellow arrows indicate IL-10-expressing macrophages and the blue arrows indicate macrophages that do not express IL-10. The immunoperoxidase staining DAB chromogen shows a brownish color and Mayer's hematoxylin counterstain shows blue–purplish color.

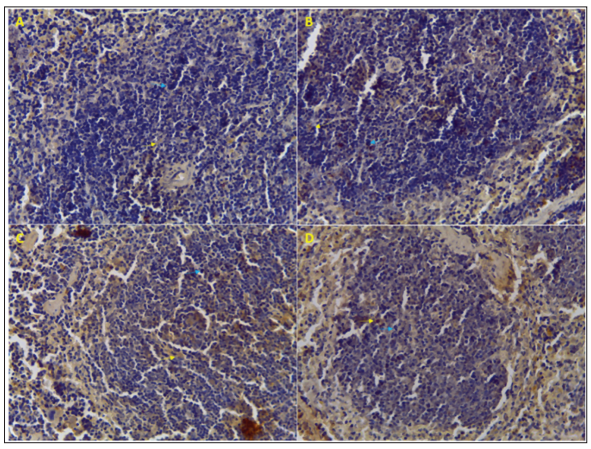

**Fig. 4.** The representative immunohistochemistry images of mice's splenic furin of the four experimental groups. (A) Group I (DMSO + saline). (B) Group II (EAEFC + saline). (C) Group III (DMSO + LPS). (D) Group IV (EAEFC + LPS). The yellow arrows indicate Furin expressing macrophages and the blue arrows indicate macrophages that do not express Furin. The immunoperoxidase staining DAB chromogen shows a brownish color and Mayer's hematoxylin counterstain shows blue purplish color.

number of furin-positive cells in group III, which served as an inflammation model, was not significantly different from the previous groups. However, the administration of the fungus comb extract in group IV resulted in a notable increase in furin levels in spleen cells compared to the other groups (Fig. 2). The average number of furin-positive cells in groups III and IV was 3.063 ± 2.183 and 6.729 ± 3.582 cells, respectively (Table 2).

***Effects of fungus comb extract on NK cells expressing IFN-γ level in the spleen***

The immunohistochemistry double staining results presented in Figure 5 showed an average of 6.775 ± 0.531 and 4.426 ± 0.339 cells expressing CD56 and IFN-γ in groups I and II, respectively. No significant difference was observed between groups I and II, which received intraperitoneal saline injections. However, the administration of the fungus comb extract in group IV resulted in a notable increase in the number of cells expressing CD56 and IFN-γ (4.441 ± 0.691 cells) compared to the inflammation model (group III) with 6.744 ± 0.171 cells (Table 2 and Fig. 6).

***Ethyl acetate extract of fungus comb (EAEFC) composition***

The fungus comb sample underwent initial extraction with n-hexane to isolate the nonpolar substances. Subsequently, the residue of the first extraction was further processed with ethyl acetate to extract semipolar substances, resulting in the ethyl-acetate extract. Analysis via GC-MS revealed the presence of 14 compound types in the ethyl acetate fraction of the fungus comb contained, with four dominant compounds

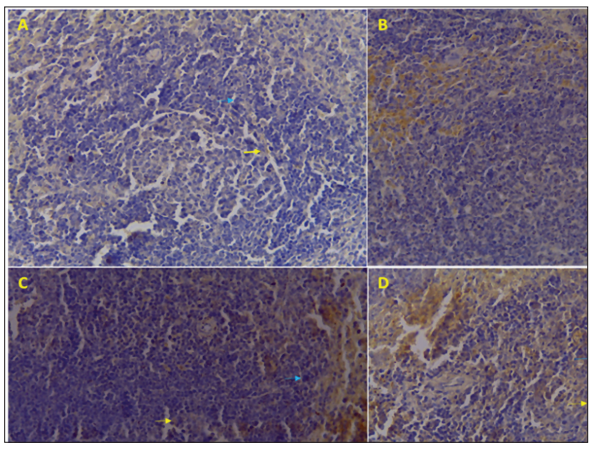

**Fig. 5.** The representative immunohistochemistry images of splenic NK cells expressing IFN-γ of the four experimental groups under 1,000x magnification of the light microscope. (A) Group I (DMSO + saline). (B) Group II (EAEFC + saline). (C) Group III (DMSO + LPS). (D) Group IV (EAEFC + LPS). The yellow arrows indicate NK cells expressing IFN-γ and the blue arrows indicate NK cells that do not express IFN-γ. The immunoperoxidase staining DAB chromogen shows a brownish color, Mayer's hematoxylin counterstain shows blue purplish color, and HRP green chromogen shows a green color.

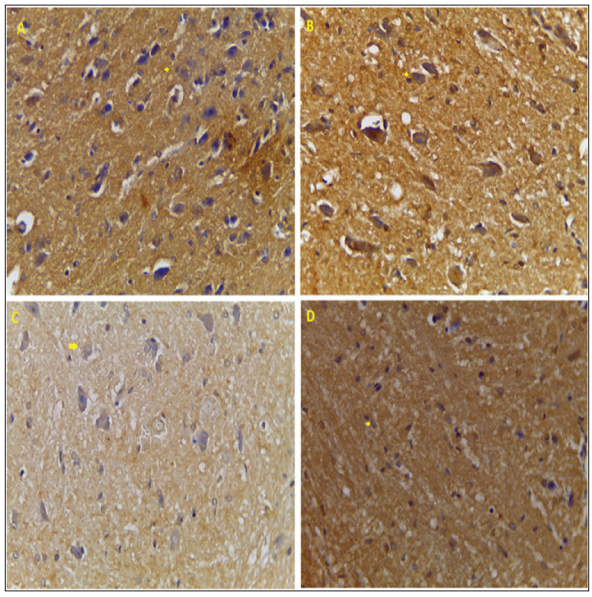

**Fig. 6.** The number of NK cell $CD56^{+}$/IFN-γ positive cells/156.25 $\mu m^2$ for each group under 400x magnification of the light microscope. Group I: DMSO 5% (14 days) and saline injection (15th day), group II: DMSO 5% +Extract of Fungus Comb (14 days) and saline injection (15th day), group III: DMSO 5% (14 days) and LPS +saline injection (15th day), group IV: DMSO 5% +Extract of Fungus Comb (14 days) and LPS +saline injection (15th day). Values with different symbols (*/**) are significantly different at the level of 0.05 ($p < 0.05$).

**Table 3.** Four most dominant compounds identified in EAEFC.

| Compound name | CAS number | Relative content (%) |
|---|---|---|
| 1,2,3-Propanetriol | 56-81-5 | 28.93 |
| Phenol, 2-methoxy- | 90-05-1 | 8.54 |
| Phenol, 2,6-dimethoxy- | 91-10-1 | 6.55 |
| Bis(2-ethylhexyl) phthalate | 117-81-7 | 4.82 |

The four most dominant sompounds identified in the ethyl-acetate extract of fungus comb. EAEFC = Ethyl-Acetate Fungus Comb Extract; CAS= Chemical Abstracts Service.

(Table 3). These compounds, mainly phenol, were identified. Glycerol, also known as 1,2,3-propanetriol, exhibited the highest estimated relative concentration among the identified compounds (Nandika et al. 2021).

## Discussion

The fungus combs inside the termite nest are known to contain various components, including high levels of crude ash, fiber, starch, protein, and bioactive substances (Nandika et al., 2021). These bioactive substances may be derived from the nest itself or from components of the fungus *Termitomyces*, such as phenol hydroquinone, steroids, and terpenoids (Venkatachalapathi and Paulsamy, 2016; Nandika et al., 2021). Polysaccharides, lectins, proteins, and terpenoids, which are commonly extracted from different types of mushrooms, have been found to exhibit immunomodulation effects. Immunomodulators have been traditionally divided into three groups: immunosuppressive agents, immunostimulators, and tolerogens. Fungal metabolites such as Cyclosporine and Mycophenolic acid have been broadly used as immunosuppressive agents to prevent transplanted tissues and organs from being attacked by the host immune system) (Hyde *et al*., 2019).

One of the well-known mushroom species, *Termitomyces sp.*, contains a full range of irreplaceable amino acids and high-concentration minerals and has also been utilized as traditional medicine in rural areas, especially in Africa and Southeast Asia, for blood pressure-lowering agents, anti-cholesterol herbs, and infection, such as typhoid fever and fungal infection (Rahmad *et al*., 2014; Venkatachalapathi and Paulsamy, 2016).

*Termitomyces heimii* and *T. albuminosus*, cultivated in liquid fermentation, are able to produce endopolysaccharide and exopolysaccharide (β-glucan) that exert pharmacological function as an antioxidant, hepatoprotective, and antimicrobial agents (Ahmad *et al*., 2021). In addition, *T.clypeatus* contains AkP, an effective biomolecule with anti-cancer properties (Majumder *et al*., 2016). The chemical composition of EAEFC from Indomalayan termite mounds has been found to consist of various components, predominantly the 1,2,3-propanetriol (28.93%), which has anti-microbial and anti-inflammatory effects (Rawal and Sonawani, 2016; El-Zayat *et al*., 2019), as well as phenol,2-methoxy (8.54%) and phenol, 2,6-dimethoxy (6.55%), and Bis (2-Ethylhexyl) phthalate (4.82%), which have antifungal properties. (6,23,24). Moreover, Mahamat *et al*. (2018a) found that the aqueous extract of fungus comb from certain regions of Cameroon has been demonstrated to possess significant immunostimulatory effects on both the cell-mediated and humoral immune systems in immunosuppressed mice induced by levamisole. Despite these findings, the potential immune response modulation effects of various *Termitomyces* species have yet to be fully characterized.

This study investigated the inflammatory responses of mice's spleen following LPS injection. LPS has been widely used to stimulate an innate immune response, and intraperitoneal injection of LPS can induce systemic inflammation in mice (Daubeuf *et al*., 2007; Seemann *et al*., 2017). Two hours after the LPS injection, the inflammation signal in the spleen should have been established (Olesen *et al*., 2015; Raduolovic *et al*., 2018). In this study, the levels of IL-1 as a pro-inflammatory and IL-10 as an anti-inflammatory cytokine significantly increased in group III after LPS injection. The peak elevation of both IL-1 and IL-10 occurs within 3 hours after the endogenous trigger, indicating the establishment of systemic inflammation (Peranteau *et al*., 2008; Yudhawati *et al*., 2022). Moreover, this study showed a significant modulation of IL-1 positive stained cells after 14 days of EAEFC treatment compared to the group with LPS only (Fig. 1). The dose selection of EAEFC at 500 mg/kg BW was based on prior studies to achieve comparable results (Mahamat *et al*., 2018a; Caesario *et al.*, 2023; Xiao *et al.*, 2024). The most dominant chemical component of EAEFC, 1,2,3 propanetriol can also be observed in Asiatic pennywort *Centella asiatica* methanol extract (Legiawati *et al*., 2018; Radiastuti *et al*., 2021). In contrast to this study, the pennywort extract showed inhibition of IL-1β and IL-6 expression as well as its active compound, 1,2,3 propanetriol (Majumder *et al*., 2016; Legiawati *et al*., 2018; Nandika *et al*., 2021). Previous observation by Nandika has shown that the active component of the Indo-Malayan termite mound also downregulated inflammatory response, highlighting the potential for discovering more active compounds (Nandika *et al*., 2021).

NK cells $CD3^{-}CD56^{+}$ represent an important part of innate immune responses that are also involved in tissue homeostasis. Human NK cells are categorized into two subsets depending on the expression of CD56 and CD16. $CD16^{+}CD56^{dim}$ NK cells represent the majority (~90%) of NK cells in the blood and spleen with high levels of perforin and granzymes demonstrating higher

cytotoxic activity (Kawai and Akira, 2010). The other type of NK cells, $CD16^{-}CD56^{bright}$ are predominantly located in secondary lymphoid organs such as spleen, lymph nodes, and tonsils. $CD16^{-}CD56^{bright}$ NK cells demonstrate reduced cytolytic activity but display increased responsiveness to pro-inflammatory cytokines stimulation. When activated, these cells release cytokines (IFN-γ, TNF-α, and IL-10) as well as chemokines (Hudspeth *et al*. 2016). NK cells produce granzyme B and perforin to induce cellular apoptosis and secrete cytokines such as IFN-γ and TNF-α to further the sequential immune response (Ahlenstiel, 2013). In this study, the microscopic result showed that the number of cells positively stained IFN-γ and $CD56^{+}$ decreased after EAEFC was given orally for 14 days; however, in the inflammation model, the number of cells increased to the same level as the negative control. Secreted IFN-γ might limit the excessive inflammation, in this case, LPS and EAEFC, and also induce tissue regeneration. The EAEFC also significantly decreased and increased the number of activated splenic NK cells in the normal and inflamed mice, respectively. Several fungal components, for instance, polysaccharides, terpenes, and terpenoids from wild mushrooms *T. heimii, T. microcarpus, Auricularia auricula,* and *Pleurotus ostreatus* can similarly have NK cells immunomodulating effect (Rahmad *et al*., 2014; Surayot *et al*., 2021).

IL-10 is recognized as an anti-inflammatory cytokine that downregulates inflammatory immune responses at multiple levels (Peranteau *et al*., 2008). The cytokine is synthesized by a wide range of cell types, including B cells, monocytes, DCs, NK cells, and T cells (Kubo *et al*., 2017). Our study compared the levels of IL-10 in different inflammation models (groups III and IV) and found that the extract had the ability to decrease inhibitory cytokines and enhance inflammatory response. No significant difference was found in the IL-10 level in Group II compared to that in Group I. The suppressing effect of IL-10 can also be observed after supplementing high concentrations of resveratrol, a polyphenol in red wine, grapes, and berries (Mueller *et al*., 2010).

Furin is likely to represent the ubiquitous endoprotease activity and is capable of cleavage effectively at the R-X-K/R-R consensus motif (Devi *et al*., 2022; Dahms *et al*., 2022). In addition, furin also serves as a pro-TGFβ-1 (Transforming growth factor beta) converting enzyme (Devi *et al*., 2022). Furin is an important mediator of regulatory T-cell (Treg) activity via TGFβ-1 co-expression and has been linked to the secretion of interferon-γ (IFN-γ) by T helper type 1 (Th1) cells (Oksanen *et al*., 2014). Furthermore, T cell-expressed furin is essential for the body's physiological and regulatory function. The results of this study demonstrated that the splenic furin level only increased in the inflammation group that received EAEFC supplementation.

In several studies, it has been suggested that the aqueous extract of fungus comb exhibits antibacterial properties against a variety of bacteria, including *Salmonella enterica* and *Bacillus cereus*, as well as highly resistant strains such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* (Hyde *et al*., 2019). In our previous study, we also found that the extract was effective in inhibiting the growth of pathogenic bacteria and fungi such as *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *Aspergillus flavus*, and *A. niger* using Kirby-Bauer disc diffusion and microdilution methods (Witasari *et al*., 2022).

In the present study, the lack of significant differences in IL-1, IL-10, and furin levels between the negative control group and the extract group (Groups I and II, respectively) may suggest that the fungus comb did not elicit an antigenic effect. Several components of fungi are known to cause an inflammatory response in healthy subjects, for example, chitin, glucans, oligosaccharides, proteins, melanin, and phospholipids, which are the main antigenic properties of the principal pathogenic fungi, such as *Histoplasma capsulatum, Aspergillus fumigatus, Candida albicans*, *Cryptococcus neoformans,* and *Sporothrix schenckii* (García-Carnero *et al*., 2020). The lack of a significant difference between groups I and II suggests that the extract had negligible antigenic effects.

Eventually, this study has some limitations that must be acknowledged. In this study, we used healthy male BALB/C mice as an experimental model, not those with pathological conditions (e.g., immunodeficient conditions or those rendered for having immunodeficiency). As a result, it was not possible to investigate the effects of EAEFC on pathological conditions such as immunodeficiency and nude mice Swiss albino (Mahamat *et al*., 2018a,b). In the future, mouse models with various pathologies, including immunodeficiency, can be developed better to understand the effects of EAEFC in the disease states. Our current study also did not evaluate the inflammatory and immune responses in lymph nodes and in the circulation, either humoral or cellular. Future studies measuring such responses in lymph nodes and circulation can be performed to provide a more comprehensive understanding of the effects of EAEFC.

## Conclusion

In conclusion, the present study demonstrated that the administration of EAEFC from *Termitomyces gilvus* Hagen mound modulated the immune system in the mice with an inflammation model. Specifically, the extract led to the upregulation of IL-1, furin, and NK cells in the inflammatory mouse model, while the level of IL-10 decreased, highlighting its role as an immunostimulator.

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***Conflict of interest***

The authors declare there is no conflict of interest.

***Authors' contributions***

Design and conception of the study: Hermawan Susanto, Jefferson Caesario, Ketut Sudiana, Djoko Santoso. Material preparation, conduct the experiment: Hermawan Susanto, Jefferson Caesario, Irmanida Batubara, Ahmad Dzulfikri Nurhan, Mahardian Rahmadi, Dodi Nandika, Arinana Arinana, Lina Karlinasari. Analysis and interpretation of the data: Hermawan Susanto, Jefferson Caesario, Decsa Medika Hertanto, Ketut Sudiana, Djoko Santoso. Statistical analysis: Hermawan Susanto, Jefferson Caesario, Ketut Sudiana, Djoko Santoso. The first drafting the manuscript: Hermawan Susanto, Jefferson Caesario, Kukuh Dwiputra Hernugrahanto, Irmanida Batubara, Djoko Santoso. The critical revision of the manuscript: Irmanida Batubara, Dwikora Novembri Utomo, Nicolaas Cyrillus Budhiparama, Dodi Nandika, Arinana Arinana, Lucia Dhiantika Witasari, Lina Karlinasari, Dikhi Firmansyah, Yanti Rachmayanti, Sairah Abdul Karim, Indang Ariati Ariffin.

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***Data availability***

All data are provided in the manuscript.

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