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The ability of earthworm *Lumbricus rubellus* extract in slowing down the activation of NFkB and TNF-α in lipopolysaccharide-induced *Rattus norvegicus*

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ABSTRACT

Background: Earthworm (*L. rubellus*) contains many compounds such as Lumbricin I, G-90 glycoprotein, and polyphenols as antimicrobial, antioxidant, and hepatoprotective toward bacterial infections. Tumor necrosis factor (TNF-α) has been identified as a mediator for necrosis tumor inside animal serum treated by lipopolysaccharide (LPS). High concentrated LPS can cause tissue damage and death. The increasing number of Nuclear Factor Kappa Beta (NFkB) activation has been responded by macrophage to produce cytokines pro-inflammation TNF-α as an indicator of inflammation.

Methods: The research was conducted in the Animal Laboratory Pharmacology Unit, Biomolecular Laboratorium, Faculty of Medicine Udayana University and the Pathology Clinic Sanglah General Hospital with a post-test only control group design. Thirty-two experimental rats were made as samples and divided into four groups, which are negative control, positive control, treatment 1, and treatment 2. Treatment was performed for 14 days. The containment of NFkB and TNF-α was measured on the 16th day using quantitative technique sandwich enzyme immunoassay (ELISA).

Results: The results of the Shapiro-Wilk test and Levene's test showed that the data were normally distributed and homogeneous, with $p > 0.05$. As the data were normally distributed, the one way ANOVA was used to analyze the data, followed by the Least Significant Difference (LSD) test. There was a significant difference between the average amount of TNFα found in each treatment group $p = 0.001$ (< 0.05). There were also significant differences between the average amount of NFkB found in each treatment group $p = 0.001$ (< 0.05). Advanced test using LSD shows more significant differences by $p < 0.05$.

Conclusion: It can be concluded that earthworm extract (*L.rubellus*) raises the amount of NFkB and TNF-α in male experimental rats injected with LPS.

Keywords: Earthworm extract, *Lumbricus rubellus,* lipopolysaccharide, NFkB, TNF-α

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INTRODUCTION

Infectious disease is a major problem in different tropical countries around the world, including Indonesia. A clinical syndrome that occurs due to the body's excessive systemic inflammatory response to infection is called sepsis,[1] which is also referred to as Systemic Inflammatory Response Syndrome (SIRS), particularly in the intestines, lungs, kidneys, and other organs.[2] In the digestive tract, sepsis prompts the intestinal hypoperfusion as inflammatory disorders.

Sepsis is caused by gram-negative bacteria (60 – 70% of cases) and gram-positive bacteria including *Staphylococci* and *Pneumococci* (20 – 40% of cases). On the other hand, fungi, virus, or protozoa were also reported to cause sepsis.[3] Sepsis is the leading cause of mortality in the world.[4] The occurrence of sepsis continues to rise in the extent to the human population.[5]

Over the last 30 years, numerous investigations on sepsis have been carried out at no little cost. Endotoxin utilizing Lipopolysaccharide (LPS) is usually used in sepsis models. Endotoxin originates from one component of another cell wall, which also contributes to the systemic inflammatory response.[6] LPS is a membrane component of gram-negative bacteria, which is a pathogenic cause of sepsis.[7]

Several types of antibiotics such as chloramphenicol, ampicillin, and cotrimoxazole have been utilized for quite a long time for the treatment of typhoid fever until the resistance issue emerged, known as the multidrug-resistant (MDR) *S. thypi*. The causes of resistance include irrational administration, consumption of antibiotics that are not in accordance with guidelines, and intrinsic changes in the microbes themselves. The issue of antibiotic resistance is what underlies the researchers to attempt to find additional medicinal ingredients, specifically an ingredient from the Indonesian nature. One of the natural ingredients obtained from animals that can be used as an additional medicine is earthworm (*Lumbricus rubellus*). Extracts of earthworm (Lumbricus sp) contain antibacterial properties that can inhibit the growth of pathogenic bacteria from both gram-positive and gram-negative bacteria.

Cytokines are mediators (in the form of proteins or glycoproteins) produced by cells in inflammatory

or immunologic responses that serve as signals between cells to form communication networks in the immune response. Included in cytokines are interleukin (IL-1, IL-2), interferon (IFN α, β, and γ), Tumor Necrotic Factor (TNF), growth factors and chemokines (chemotactic cytokines). Almost all inflammatory processes result in the activation of tissue macrophages and the infiltration of blood monocytes.

Cytokines serve to maintain the inflammatory processes to be always interconnected. For example, in sepsis syndrome, endotoxins produced by bacteria and other toxic products will stimulate the production of TNF-α and IL-1. The above conditions occur in not only infection but also other pathobiology processes. The cytokine stimulation depends on the characteristics of the cell and the type of tissue.

Nuclear Factor Kappa Beta (NFkB) can be activated by various stimuli, cytokines, and LPS, which results in the release of inhibitory kB binding so that the NFkB is within the cell nucleus and regulates various inflammatory mediators such as TNF-α. TNF-α acts as a mediator of endogenous inflammation and directs numerous cellular responses, including the activation of genes involved in immune inflammation and regulation, cell proliferation, antiviral response, growth inhibition, and cell death.

Complications that occur are usually due to an imbalance of pro- and anti-inflammatory mediator production. Prolonged production of anti-inflammatory mediators could result in harm to some tissues and organs. The cytokine is an inflammatory mediator. Inflammatory reactions that occur in the digestive tract can instigate endothelial cell death (necrosis).

Regarding the above prelude, this study aimed to investigate the ability of earthworm extracts in inhibiting the activation of NFkB and TNFα in lipopolysaccharide-induced *Rattus norvegicus*. This study used animal models of white rats (*Rattus norvegicus*) because it is a standard model that is often utilized to examine the mechanism of infection caused by LPS exposure.[8,9]

MATERIALS AND METHODS

Animal models

The study subject was male rats (Ratus norvegicus; Wistar strain), which were 3-4 months old with a body weight of 200-250 grams. The rats were injected with lipopolysaccharide and fulfilled the inclusion and exclusion criteria. The mice were excluded from the test (drop out) if they died during the study period.

The sample size in this study was estimated based on standard procedures in determining the number of samples using mouse animal models. Based on the calculation, the minimum number of replications (r) required was six mice per group. The study comprised of 4 groups: (1) no treatment group, (2) the positive control group, (3) treatment group, and (4) treatment group; subsequently the total number of rats required was 24. To anticipate dropouts, the sample size was increased to 32 rats to allow adjustment for an attrition rate of 10%.

Study variable

The measured variables in this study were independent variables and dependent variables. The independent variable was earthworm extract of Lumbricus rubellus. The dependent variables were NFkB and TNF-α levels in the blood of LPS-induced rats (Rattus norvegicus). The controlled variables were gender, age, rat weight, temperature, humidity, nutrition, and cage.

Research procedure

This was an experimental study with a posttest-only control group design (Federer, 2008). The mice were kept in a well-ventilated room with an adequate light source and were singly-confined. Wistar strain male mice were adapted for one week in a room with a temperature of around 20°C and a cage measuring 23 cm x 17 cm x 9.5 cm. Food and drinks were given ad libitum in the form of pellets and mice food. The lighting was regulated with a 12-hour bright-dark cycle (the bright period started at 6:00 a.m. to 6:00 p.m.).

The animal models were assigned to four groups. The negative control group (P0) consisted of six mice that were given standard food and drink, while the positive control group (P1) consisted of six mice that were injected with LPS on the first day and given standard food and drink until day 14. In treatment group 1 (P2), subjects were injected with LPS on the first day and given earthworm extract until day 14, whereas in treatment group 2 (P3), subjects were given earthworm extracts on days 1-6, and on day-7, they were injected with LPS followed with earthworm extract until day 14.

Blood sampling was done using anesthesia with 0.3 ml of ketamil per mouse. Blood was taken using 3 – 4 cc syringe and collected in a vacuum tube with a gel separator. Blood sampling was carried out at the medical canthus of the orbital sinus because there were large blood vessels, so it was easier to retrieve and has faster recovery time. Blood sampling was performed by trained personnel so that the rats did not experience severe trauma due to spuid puncture in the medical canthus of the

orbital sinus. The separation of serum and blood cells was carried out by centrifuging the vacuum tube using a centrifugator with a speed of 4000 rpm for 5 minutes. The serum was then transferred to a 1.5 ml aliquot tube using a micropipette.

Procedure for making the Earthworm extract

The collected earthworms were washed with flowing water to remove the mucus on the surface. Earthworms were then soaked for 6 – 8 hours in distilled water to remove soil from the body and then rinsed again using distilled water. After being cleaned, they were dried constantly in an oven with a temperature of 40°C for 24 hours.

After being dried, the worms were then mashed by cutting them into small pieces and then smoothed and moved into a glass tube. Following this, 80% ethanol solvent was added, which then evaporated, leaving the crude extract. Soaking earthworms using 80% ethanol was carried out for two days.

NFkB and TNF-α examination

The intestinal tissue that had been washed with PBS was weighed with a total of 100 mg, then frozen with a temperature of -80°C. The freezing was done to break the cell membrane. The frozen tissue was crushed with mortar, then it was homogeneous in 200 µl PBS and stored overnight at -20°C. It was centrifuged for 15 minutes at a speed of 1500. The supernatant was taken, and if not examined, it was immediately stored at -20°C, and centrifuged once more before testing.

The NFkB and TNF-α levels were examined using the quantitative sandwich enzyme immune assay (ELISA) method according to the manufacturer's instructions. In general, the material used was serum (for examination of NFkB) and tissue fluid as much as 100 mg (for TNF-α examination). The results of the examination were then analyzed to check the concentration between the calculated by the dilution formula obtained from the absorbance readings of spectrophotometer with a wavelength of 450 nm.

Data analysis

In this study, statistical analysis was performed using the SPSS 20.00 software for Windows. The obtained data were analyzed for normality using the Shapiro-Wilk test, homogeneity using the Levene's test, and comparison using the ANOVA followed with LSD post-hoc test.

RESULTS

Data normality and homogeneity

Data of NFkB and TNF-α levels in each group (negative control (P0), positive control (P1), treatment group 1 (P2) and treatment group 2 (P3)) were tested for normality using the Shapiro-Wilk test (Table 1) and homogeneity using the Levene's test (Table 2). The results show that the data were normally distributed ($p > 0.05$) and homogeneous ($p > 0.05$) so that they fulfilled the prerequisite for the ANOVA test.

The effect of Earthworm (*L. rubellus*) extract on NFkB and TNF-α levels

The results of the measurement of NFkB showed that the highest NFkB level in the negative control group (P0) was 1,809 ng/ml and the lowest was 2,780 ng/ml, with an average level of 2,316 ng/ml. In the positive control group (P1), the highest level was 3,196 ng/ml and the lowest level was 2,500 ng/ml, with a mean level of 2,894 ng/ml. In the 1st treatment group (P2), the highest level was 3,527 ng/ml and the lowest level was 2,263 ng/ml, with a mean level of 2,649 ng/ml. In treatment group 2 (P3) the highest level was 2,773 ng/ml and the lowest level was 2,109 ng/ml, with a mean level of 2,470 ng/ml (Figure 1).

The highest Tumor Necrosis Factor (TNF α) level in the negative control group (P0) was 6.73 pg/ml

Table 1 The result of data normality test

Subject group	r	*P*	Note
NFkB Level Neg. Control (P0)	6	0.084	Normal
NFkB Level Pos. Control (P1)	6	0.933	Normal
NFkB Level Treatment 1 (P2)	6	0.853	Normal
NFkB Level Treatment 2 (P3)	6	0.563	Normal
TNF-α Level Neg. Control (P0)	6	0.241	Normal
TNF-α Level Pos. Control (P1)	6	0.718	Normal
TNF-α Level Treatment 1 (P2)	6	0.415	Normal
TNF-α Level Treatment 2 (P3)	6	0.405	Normal

Note : r = the amount of replication, *p* = signifinancy

Table 2 The result of data homogeneity test

Variable	F	*p*	Note
TNFα level	0.534	0.668	Homogeneous
NFkB level	0.823	0.580	Homogeneous

Note : r = the amount of replication, *p* = signifinancy

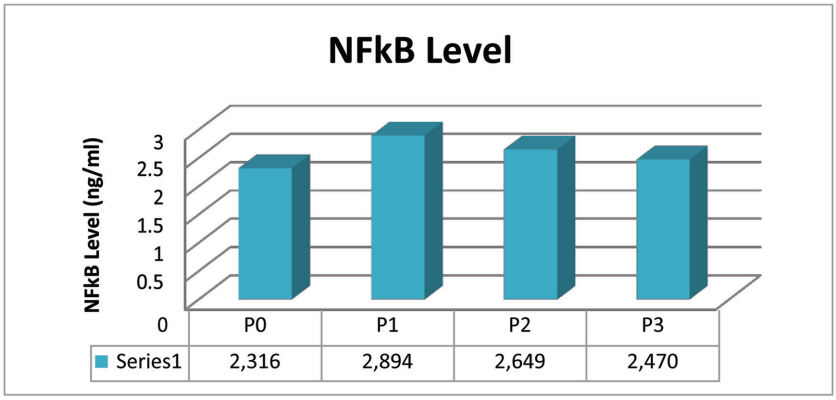

Figure 1 The comparison of the mean of NFKB levels

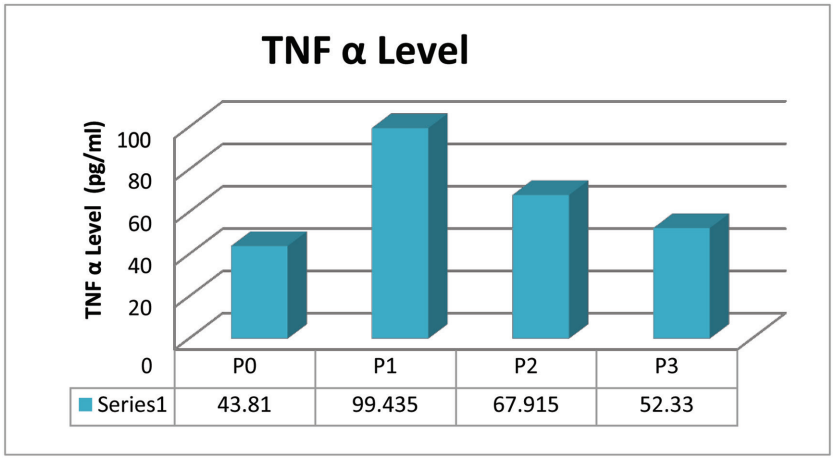

Figure 2 The comparison of the mean of TNF-α levels

and the lowest was 40.59 pg/ml, with an average level of 43.66 pg/ml. In the positive control group (P1) the highest level was 75.83 pg/ml and the lowest level was 62.04 pg/ml, with a mean level of 67.405 pg/ml. In the first treatment group (P2) the highest level was 52.85 pg/ml and the lowest level was 43.66 pg/ml, with a mean level of 49.023 pg/ml. In the second treatment group (P3) the highest level was 98.84 pg/ml and the lowest level was 90.56 pg/ml, with a mean level of 93.518 pg/ml (Figure 2).

DISCUSSION

Tumor Necrosis Factors (TNF-α) are found to be elevated in acute and chronic inflammatory conditions, such as trauma, sepsis, infection, rheumatoid arthritis, and dyslipidemia. TNF-α plays a vital role in this process and is likely to be a potential target for therapy.[10]

TNF-α are identified as mediators for tumor necrosis in the serum of animals injected with LPS (lipopolysaccharide). High concentration of LPS causes tissue damage and death.[11] Several different types of LPS can be found in microbial molecules. Toll Like Receptors (TLRs) are the specific components of different microbes. In this case, the TLRs are essential for macrophages in response to lipopolysaccharide. TLRs4 activates a transcription factor called NFkB, where this factor stimulates the production of cytokines, enzymes, and proteins involved as antimicrobials.[11]

Lipopolysaccharide is an outer membrane component of a gram-negative bacteria that could induce sepsis. The pathophysiology of sepsis is well known, although its therapy is still limited, and its mortality remains high. The LPS exposure causes the release of several cytokines (TNF-α, NFkB, IL-1, IL-8) as a response against foreign objects that could have both positive and negative effects. In sepsis, there is a release of proinflammatory cytokines such as TNF-α, NFkB, IL-1, IL-8 that are associated with endothelial and tissue damage.

The earthworm used in this study was *Lumbricus rubellus (L. rubellus)*, obtained from an earthworm farm in Denpasar. The earthworms were obtained in the form of flour powder. To get the extract, the earthworms were soaked with 80% ethanol for two days, which then evaporated, leaving the *L. rubellus* crude extract.

The phenolic acid is the component of polyphenol compounds of *L. rubellus*. The working mechanism of phenolic acid is assumed to be similar to gallic acid, which inhibits the activation of NFkB, Mitogen-activated Protein Kinase (MAPK) and Protein-1 Activator (AP-1). The target work of TNF-α is similar to gallic acid in suppressing the production of pro-inflammatory mediators through the inhibition of NFκB. The inhibition of NFκB will result in a suppressed production of pro-inflammatory mediators.

The dosage of *L. rubellus* earthworm extract used in this study was 100 mg/kg body weight and was converted for the mice body weight of 200-250 grams to 25 mg/kg. The dose used was in accordance with previous studies conducted by Omar (2012) by inducing white rats using CCl4 and administering the earthworm extracts with a dose of 100 mg/kg body weight, which was proven to provide an antioxidant effect and liver cells protection from CCl4 induction.

The results of measurements of NFkB and TNF-α levels in the first treatment group (P2) and second treatment group (P3) showed lower results than the positive control (P1). In the positive control (P1), the rats were given LPS only, but the results showed a higher level, which may be due to this study not

using rats that met the Specific Pathogen Free (SPF) requirements; hence the rats were not completely free of disease, both infectious diseases and degenerative diseases.

Previous studies about the antibacterial effects of earthworm *L. rubellus* have been carried out in vitro. A study by Chauhan et al.[12] showed that earthworm extracts could inhibit the growth of *P. aeruginosa* bacteria with a comparable level to the standard antibiotic Streptomycin. The results of a study conducted by Purwaningroom[13] using *L. rubellus* and *Pheretima aspergillum* found that *L. rubellus* earthworm extract was better and significant in inhibiting the growth of *S. typhi* bacteria in vitro with 50'C processing temperature.

The components of earthworm extracts could destruct the microorganisms by recognizing the LPS and/or peptidoglycan on the cell wall of bacteria. The earthworm molecules may recognize the foreign molecules of these bacteria, which is called pattern-recognition proteins (PRPs).[12] This antibacterial compound may prevent the synthesis of peptidoglycan in bacterial cells. Therefore, it is more sensitive to gram-positive bacteria, although it could also affect the gram-negative ones.[14]

The study by Chauhan[12] found that the earthworm extract from the genus *Eudrilus eugeniae* has an anti-microbial effect and could be a new candidate for antibiotics. *S. typhimurium* bacteria belong to the gram-negative bacteria, where it is known that the walls of gram-negative bacteria have a more complex structure compared to gram-positive ones. This structure is a molecular complex of LPS that can protect the cells from toxic compounds and antibiotics, and make the structure of cell walls of gram-negative bacteria more stable.[9]

Lilis et al. confirmed that the earthworms have an antibacterial effect on gram-positive and gram-negative pathogens, which act as a bacteriostatic and bactericidal. The presence of an antibacterial property on earthworms was caused by the active compounds such as alkaloid compounds.

A study by Salzet et al.[15] demonstrates that *L. rubellus* earthworms are a group of Annelids that have peptide compounds as the first-line defense against microorganisms with their anti-microbial property. These compounds minimized the infection caused by pathogenic bacteria. According to Tasiemski[16], *L. rubellus* also has antibacterial compounds called Lumbricin I, which is a broad-spectrum antimicrobial against gram-negative and gram-positive bacteria. Lumbricin I is an antibacterial compound found in earthworms that are thought to be present in the digestive tract of earthworms (chloragocytes). Willey et al.[9] stated that antimicrobial peptides could damage the plasma membrane of pathogenic bacteria by electrostatic interaction with the bacterial cell wall to form an ionic hole (pore) which changes the permeability of cell membranes so that bacteria will be more prone to lysis.

Chang et al.[17] stated that the earthworm extract has three main components that could be beneficial. Earthworm extracts consist of Polyphenolic, which has anti-oxidation effects and also serves as an anti-inflammatory, G-90 glycoprotein, as well as the fibrinolytic enzyme. G-90 glycoprotein is a molecule that plays a role in the anti-microbial effects of earthworms. According to Popovic et al.,[18] the G-90 glycoprotein molecule could be found almost in all types of earthworms. G-90 glycoprotein is found in *E. feotida* earthworms; it is known that with a concentration of 10 mg/mL, it could inhibit the growth of pathogenic bacteria. G-90 glycoprotein has an even higher sensitivity of 17±0.43% compared to antibiotics Gentamicin with a dose of 10 µg and Enrofloxacin 20 µg against the *Staphylococcus sp*.

A study by Parwanto et al.[19] found that in 5 gr powder of *L. rubellus* precipitated crude extract, the dialysate fractions was found to contain 2487 ug/L of protein, and there were 4 dominant types of proteins, which are protein molecules with a molecular weight of 12.2, 13.3, 14.6, and 29.2 kDa.

From a series of chemical studies, it is known that the active compound that provides the antipyretic property of earthworm extract is a class of alkaloid compounds. The group of alkaloid compounds has the characteristic of nitrogen atoms (compared to the structure of paracetamol, which also has a nitrogen atom) and alkaline (pH more than 7). The examples of the most famous alkaloids are nicotine obtained from tobacco. Like most active compounds, if consumed in an excessive amount, could be toxic. The alkaloid group is already widely found in plant and animal extracts, and most of them have pharmacological effects.[13,20]

The components of *L. rubellus* earthworm extract could kill microorganisms by recognizing its LPS. Peptide compounds are the first defense against microbes with their anti-microbial property. TNF-α can be stored in proactive cells that reduce TNF-α levels in acute inflammatory conditions, sepsis, infection, and dyslipidemia. TNF-α was identified as a mediator of tissue necrosis in the serum of LPS-induce animals. High content of LPS may damage the tissue and lead to necrosis. TLRs are essential components in response to LPS. TLRs4 will activate NFKB to stimulate the production of cytokines involved in anti-inflammation. With the results of the measurements of NFkB and TNF-α levels, which showed a significant decrease between the treatment groups, this study showed that *L. rubellus* earthworm extract has an anti-inflammatory property. In accordance to several prior reports, it is

expected that *L. rubellus* earthworm extract may be used as an alternative to antibiotics.

CONCLUSION

Based on the results of this current study, it can be concluded that the extract of *L. rubellus* earthworms serves as an anti-inflammatory due to its ability to reduce the activation of NFkB and TNF-α of lipopolysaccharide-induced *Rattus norvegicus*. Further clinical trials on humans are necessary to determine the anti-inflammatory effect of *L. rubellus* earthworm extract.

CONFLICT OF INTEREST

The authors declare that there is no competing interest regarding manuscript.

ETHICAL CLEARANCE

The ethical approval of study has been obtained by Ethics Committee of Udayana University, Bali, Indonesia.

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AUTHOR CONTRIBUTION

Neiny Prisy Foekh is responsible for data analysis. I Dewa Made Sukrama and Anak Agung Wiradewi Lestari are responsible for preparing manuscript, data collection, and data synthesis.

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