# LUNG DAMAGE PREVENTION OF ETHANOLIC EXTRACT OF PROPOLIS AND SYNBIOTICS IN INHALATION ANTHRAX MODEL

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Received: 1/12/2022
Accepted: 26/06/2023
Published:1/07/2023

**ABSTRACT**

**Introduction:** Inflammatory response and oxidative stress can be found in inhalation anthrax, characterized by the increased serum Tumor Necrosis Factor Alpha (TNF-α) and Malondialdehyde (MDA). Ethanolic Extract of Propolis (EEP) and synbiotics were known to have anti-inflammatory and antioxidant properties. This study aimed to determine the effects of EEP and synbiotics on the level of TNF-α and MDA in the inhalation anthrax model.
**Methods:** This was an experimental study with a post-test-only control group design on 40 samples of *Rattus norvegicus* with inhalation anthrax. Samples were randomized into five groups: control (K), antibiotic and EEP (P1), antibiotic and synbiotic (P2), EEP (P3), and synbiotics(P4). TNF-α and MDA were measured seven days after the spore's inhalation and the 14$^{th}$ day after the treatment.
**Results:** EEP and synbiotics significantly reduced TNF-α and MDA levels compared to the control group. P2 showed the greatest changes on TNF-α and MDA, which TNF-α decreasing from 14.2825 ± 0.41623 to 8.4363 ± 0.44938 pg/ml and MDA changed from 14.2825 ± 0.41623 to 2.9638 ± 0.39885 nmol/ml. Followed by P4, P1, and P3 groups.
**Conclusion:** EEP and synbiotics significantly reduce TNF-α and MDA in the model of inhalational anthrax.

**Keywords: anthrax; ethanolic extract of propolis; synbiotics; TNF-α; MDA**

## INTRODUCTION

Anthrax is an acute infectious disease caused by *Bacillus anthracis* that produce spores. This disease is also known as malignant pustule, malignant oedema, charbon, ragpicker disease, or Woolsorter disease [1]. Once spores are inhaled, inhalation anthrax may be developed. Anthrax toxin complex includes three proteins that work synergistically: Protective Antigen (PA), Lethal Factor (LF), and Edema Factor (EF). The combination of LF with PA (lethal toxin) and EF with PA (oedema toxin) are thought to be responsible for the sign and symptoms of anthrax [2], related to the inflammatory process and oxidative stress. Tumour Necrosis Factor Alpha (TNF-α) has been known as a marker of inflammation. At the same time, Malondialdehyde (MDA) can be used as a marker of oxidative stress in the cell [3,4].

Propolis refers to resin substances collected by bees from various plants [5]. Propolis has anti-inflammatory and antioxidant properties. It contains flavonoid that inhibits the production of some inflammatory markers, including Nitric Oxide (NO), Interleukin-1 (IL-1), IL-6, C-Reactive Protein (CRP), and TNF-α [6]. Propolis also reduces the level of MDA by the action of Caffeic Acid Phenethyl Ester (CAPE) [7].

Synbiotics are a mixture of probiotics and prebiotics that significantly contribute to health [8]. Probiotics as part of synbiotics have been shown to reduce the inflammatory process by decreasing the pro-inflammatory cytokine, such as IL-4, IL-6, TNF-α, Interferon-γ (INF-γ) and high sensitivity CRP [9]. Co-administration with prebiotics will boost the growth and activity of probiotics. Prebiotics also have a role in decreasing the level of TNF-α and oxidative stress [10].

Based on the literature study, the study about inhalational anthrax is limited, especially about the effect of ethanolic extract of Propolis (EEP) and synbiotics. This study investigates whether EEP and synbiotics positively reduce the inflammatory process and oxidative stress on inhalational anthrax.

## METHODS

### Study Design

This study was an experimental study with a post-test-only control group design on 40 samples of *Rattus norvegicus* with inhalation anthrax. The health research ethics committee of Dr Moewardi General Hospital approved this trial.

### Population and Sample

The population of this study was white male rats (*Rattus norvegicus*) with inhalation anthrax obtained from the Center of Inter-University Biotechnology Laboratory, Gajah Mada University, Yogyakarta, Indonesia. Samples were chosen by purposive sampling, and the intervention was decided by randomization. The sample size was counted, and eight samples per group were used in this study. The inclusion criteria were aged 3 - 4 months, weighing 175–200 grams, and healthy (with shining eyes, no dull hairs, active, and good appetite). The exclusion criteria were samples that showed some sign of sickness.

### Data and Sources of Data

A total of 40 samples were randomized and placed into five groups: control (K), antibiotic and EEP (P1), antibiotic and synbiotic (P2), EEP (P3), and synbiotic (P4). The values of TNF-α and MDA were measured seven days after the spore inhalation and the 14$^{th}$ day since the given treatment based on the groups.

### Statistical Analysis

Data were analyzed using SPSS 22.0 for Windows with a p-value of $< 0.05$, considered statistically significant. To know and compare the effects of EEP and synbiotics, we used the two-way ANOVA continued by the LSD posthoc tests (if the data is normally distributed) or the Kruskal Wallis continued by the Mann Whitney tests (if the data is not normally distributed).

## RESULTS

### Preliminary Study

This study was divided into two: preliminary and primary study. A preliminary study was held to decide the spore dose that would be used in the preliminary study. From the preliminary study, the onset of alveolar damage could be assessed.

The preliminary study was conducted for seven days on 15 white male rats (*Rattus norvegicus*): 5 rats received $2x10^{10}$ Colony Forming Units (CFU) of spores, five rats received $4x10^{10}$ CFU of spores, and five rats received $6x10^{10}$ CFU of spores (Table 1).

Table 1. The result of the preliminary study

| Group | Number of Samples | Dose (CFU) | End Result |
|---|---|---|---|
| 1 | 5 | $2x10^{10}$ | No death |
| 2 | 5 | $4x10^{10}$ | No death |
| 3 | 5 | $6x10^{10}$ | No death |

After seven days, the lung tissues were evaluated by histopathology examination using Hematoxylin and Eosin (HE) staining. Spore's dose at $2x10^{10}$ CFU did not show any damage to the lung. Lung damage could be seen at doses $4x10^{10}$ CFU and 6x1010 CFU, so the primary study used the $4x10^{10}$ CFU spore's dose. The comparison of histopathological images from the preliminary study is given in Figure 1.

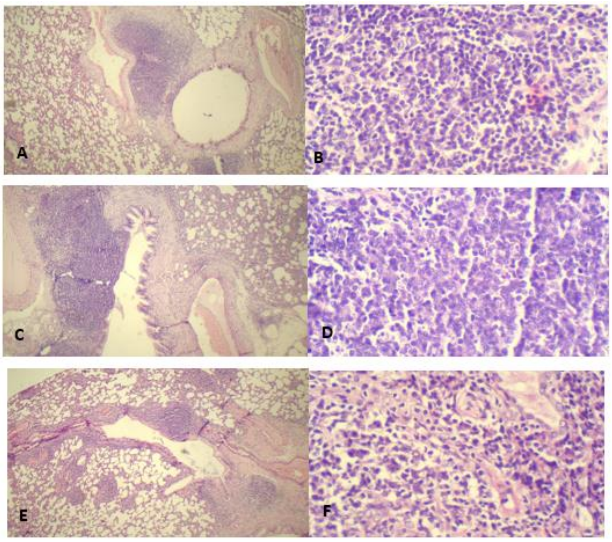


Figure 1. Histopathology of lung tissue in the preliminary study

Notes: A. $2x10^{10}$ CFU, magnification 10x; B. $2x10^{10}$ CFU, magnification 400x; C. $4x10^{10}$ CFU, magnification 10x; D. $4x10^{10}$ CFU, magnification 400x; E. $6x10^{10}$ CFU, magnification 10x; F. $6x10^{10}$ CFU, magnification 400x.

The primary study began with the spore's inhalation of 40 samples. Seven days later, the blood was drawn to be cultured. The culture showed positive results (Figure 2), and the samples were confirmed to be infected with anthrax after seven days of spore inhalation. The 40 samples were placed into five treatment groups using randomization: K, P1, P2, P3, and P4.

Figure 2. Positive blood culture of samples after seven days of spore inhalation

**Effects of EEP and Synbiotics on TNF-α**

This study has five groups (K, P1, P2, P3, P4) and two times measurements of TNF-α (before and after treatment). From the data collected (see Table 2), normality was tested using the Shapiro-Wilk test with p = 0.583 or normally distributed. At the end of the study, the highest level of TNF-α was found in the K group, 14.3600 ± 0.41037 pg/ml and the lowest level in the P2, 8.4363 ± 0.44938 pg/ml.

Table 2. The result of the TNF-α measurement

| Group | Date | N | Mean ± SD |
|---|---|---|---|
| K | June 28th 2021 | 8 | 14.1925 ± 0.40471 |
| | July 12th 2021 | 8 | 14.3600 ± 0.41037 |
| P1 | June 28th 2021 | 8 | 14.3988 ± 0.30272 |
| | July 12th 2021 | 8 | 9.2950 ± 0.37921 |
| P2 | June 28th 2021 | 8 | 14.2825 ± 0.41623 |
| | July 12th 2021 | 8 | 8.4363 ± 0.44938 |
| P3 | June 28th 2021 | 8 | 14.2825 ± 0.28293 |
| | July 12th 2021 | 8 | 10.5350 ± 0.27061 |
| P4 | June 28th 2021 | 8 | 14.4363 ± 0.44100 |
| | July 12th 2021 | 8 | 8.9988 ± 0.43594 |

After the homogeneity was tested using the *Levene Test of Equality Variances*, the significance value was 0.917. Thus, the variance between groups was homogenous, which could be continuously tested by the *two-way ANOVA*. The result showed that treatment groups, measurement date, and the interactions between those two significantly affected the TNF-α level (p = 0.000). The LSD posthoc test was then conducted to compare each group, and it showed that all four treatment groups significantly reduced the TNF-α compared to the control group with p = 0.000 (Table 3).

Table 3. Comparison of the serum TNF-α

| Group | p | Notes |
|---|---|---|
| K – P1 | 0.000 | Significant |
| K – P2 | 0.000 | Significant |
| K – P3 | 0.000 | Significant |
| K – P4 | 0.000 | Significant |
| P1 – P2 | 0.001 | Significant |
| P1 – P3 | 0.000 | Significant |
| P1 – P4 | 0.345 | Not Significant |
| P2 – P3 | 0.000 | Significant |
| P2 – P4 | 0.010 | Significant |
| P3 – P4 | 0.000 | Significant |

Every two groups compared also showed a significant difference (p < 0.05), except the P1-P4 (p = 0.345). Based on the mean difference before and after treatment, the most significant changes in TNF-α consecutively were P2, P4, P1, and P3.

**Effects of EEP and Synbiotics on MDA**

This study has five different groups (K, P1, P2, P3, P4), and measurements of MDA were done on two different dates (before and after treatment). From the data collected (see Table 4), the distribution was tested using the Shapiro-Wilk normality test with p = 0.688 or normally distributed. At the end of the study, the highest level of MDA was found in the K group, 9.6413 ± 0.25870 nmol/ml, and the lowest level was found in the P2, 2.9638 ± 0.39885 nmol/ml.

Table 4. The result of the MDA measurement

| Group | Date | N | Mean ± SD |
|---|---|---|---|
| K | June 28th 2021 | 8 | 14.1925 ± 0.40471 |
| | July 12th 2021 | 8 | 14.3600 ± 0.41037 |
| P1 | June 28th 2021 | 8 | 14.3988 ± 0.30272 |
| | July 12th 2021 | 8 | 9.2950 ± 0.37921 |
| P2 | June 28th 2021 | 8 | 14.2825 ± 0.41623 |
| | July 12th 2021 | 8 | 8.4363 ± 0.44938 |
| P3 | June 28th 2021 | 8 | 14.2825 ± 0.28293 |
| | July 12th 2021 | 8 | 10.5350 ± 0.27061 |
| P4 | June 28th 2021 | 8 | 14.4363 ± 0.44100 |
| | July 12th 2021 | 8 | 8.9988 ± 0.43594 |

After the data was tested using the *Levene Test of Equality Variances*, the significance value was 0.430. Thus, the variance was homogenous, and the two-way ANOVA could continuously test it. The result showed that treatment groups, date of measurement, and the interactions between those two significantly affected the level of MDA (p = 0.000). The *LSD posthoc* test was then conducted to compare each group.

From the *LSD posthoc* test, all four treatment groups significantly reduced the MDA compared to the control group with p = 0.000 (see Table 5). Every two groups compared also showed significant differences (p < 0.05), except the P1-P2 (p = 0.081), P1-P4 (p = 0.345), and P2-P4 (p = 0.333). Based on the mean difference before and after treatment, the most significant changes in MDA consecutively were P2, P4, P1, and P3.

Table 5. Comparison of the serum MDA

| Group | p | Notes |
|---|---|---|
| K – P1 | 0.000 | Significant |
| K – P2 | 0.000 | Significant |
| K – P3 | 0.000 | Significant |
| K – P4 | 0.000 | Significant |
| P1 – P2 | 0.081 | Not Significant |
| P1 – P3 | 0.012 | Significant |
| P1 – P4 | 0.428 | Not Significant |
| P2 – P3 | 0.000 | Significant |
| P2 – P4 | 0.333 | Not Significant |
| P3 – P4 | 0.001 | Significant |

**Effects of EEP and Synbiotics on IHC TNF-α**

The effects of EEP and synbiotics on alveolar damage in this study were assessed microscopically using IHC staining. The higher the TNF-α expression, the greater the alveolar inflammatory process. This study has only drawn some samples in each group to be examined due to the limited time study and resources. Thus, the result could not be statistically tested. All the treatment groups expressed lower TNF-

α percentages than the control group. The lowest expression can be seen in P3 (45%), followed by P1 (47.5%), P2 (52.5%), and P4 (65%).

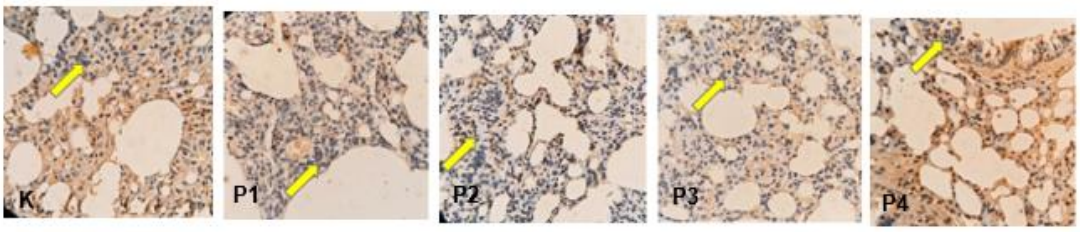


Figure 3. Expression of TNF-α in IHC staining (yellow arrows show the expression of TNF-α).

## DISCUSSION

This experimental study aimed to know whether administering EEP and synbiotics for 14 days affects the TNF-α and MDA levels in the rat model of inhalation anthrax. Inhaled spores induce an inflammatory response marked by TNF-α. This response might develop into oxidative stress, where Reactive Oxygen Species (ROS) are produced. This oxidative stress could be assessed by measuring the serum MDA level. If there is excessive ROS production, endothelial dysfunction or necrosis might happen [11-12]. EEP and synbiotics have been known to reduce the inflammatory response and oxidative stress [13-15]. Thus, EEP and synbiotics might have the potency to be used as additional anthrax therapy.

This study showed that all treatment groups have significant differences in the level of TNF-α and MDA compared to the control group ($p = 0.000$). P2 showed the highest change of TNF-α from $14.2825 \pm 0.41623$ to $8.4363 \pm 0.44938$ pg/ml, followed by P4, P1, and P3. P2 also showed the greatest change of MDA from $14.2825 \pm 0.41623$ to $2.9638 \pm 0.39885$ nmol/ml, followed by P4, P1, and P3.

The effect of EEP in this study is relevant to a previous study in rat models of cutaneous anthrax, which showed no cutaneous manifestation after EEP was given [16]. In another study with rat models of inhalation anthrax, EEP reduced the inflammatory response in the lung tissue [17]. The result on MDA is also relevant to the study that stated EEP has an antioxidant effect and reduces ROS production [17]. However, the effect of synbiotics on inhalational anthrax has never been studied before.

No study has compared EEP and synbiotics efficacy in reducing the inflammatory response and oxidative stress. EEP is a herbal substance with antioxidant, anti-inflammatory, and prebiotic properties [18], so it might be compared indirectly to synbiotics which contain probiotics and prebiotics. A systematic review and meta-analysis showed that synbiotics have a better effect on reducing the inflammatory marker than administering probiotics or prebiotics only [19]. Synbiotics promote the growth of probiotics, thus reducing the pathogenic organisms and inflammatory response [20]. Synbiotics also showed a better effect on the biomarker of the inflammatory process and oxidative stress [21].

The interaction between the EEP and synbiotics with the ciprofloxacin used in this study also might affect the results. Synbiotics used in this study contain *Lactobacillus acidophilus* and *Bifidobacterium longum* as probiotics, which are insensitive to ciprofloxacin [22]. While EEP and ciprofloxacin have antagonist effects when being co-administered [23]. This could explain why combining synbiotics and ciprofloxacin has a better effect than combining EEP and ciprofloxacin.

As additional information from this study, IHC staining showed lower expression of TNF-α in some samples taken from each treatment group compared to the control group (72.5%). The group with the lowest expression of TNF-α in the alveoli was P3 (45%), followed by P1 (47.5%), P2 (52.5%), and P4 (65%). However, the results are not statistically tested due to the limited samples. This result is relevant to a study that showed that EEP is potentially enough to treat lung inflammation by reducing the inflammation process and oxidative stress oxidative [24]. In contrast, the efficacy and safety of synbiotics in treating some lung diseases are still questionable [25].

## CONCLUSION

Based on the results of this study, EEP and synbiotics supplementation for 14 days significantly reduced TNF-α and MDA as biomarkers of the inflammatory process and oxidative stress.

## ACKNOWLEDGEMENTS

There is no acknowledgement in this work.

## CONFLICT OF INTEREST

The author declares there is no conflict of interest.

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