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The Effect of Ethanolic Extract of Propolis on Skin Manifestation and Skin Tissue Necrosis in Cutaneous Anthrax Animal Model

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ABSTRACT

Cutaneous anthrax is responsible for 95% of anthrax cases around the world. Transmission of *Bacillus anthracis* facilitated by direct contact with animal or animal product with abraded skin. The toxins produced after infection could trigger the production of inflammatory cytokines and leads to the cutaneous manifestation of anthrax and its complications. Antibiotics are the current and common therapy for anthrax. The adverse effects and bacterial resistance increase the need for alternative regimens. Propolis has been widely used as traditional medicine containing anti-inflammatory, antiproliferative, antioxidant, antiviral, and antibacterial effects. This experimental post-test only control group study was conducted in cutaneous anthrax animal models receiving ethanolic extract of propolis (EEP). Skin manifestation was assessed clinically by score, while skin tissue necrosis was evaluated and scored histologically through a skin biopsy. In this study, all anthrax animal models had skin manifestations include papules, nodules, and eschar, while most animal models given EEP did not have skin manifestations. Histologically, skin tissue necrosis was found in all control group animals and mostly not found in other groups receiving EEP before or after induction. This proves that administering EEP either before or shortly after exposure can prevent skin manifestations and at the same time reduce inflammatory reactions when clinical manifestations appear.

Keywords: Cutaneous anthrax, Propolis, Skin necrosis

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INTRODUCTION

Anthrax is a zoonotic infection caused by *Bacillus anthracis*.[1] Cutaneous anthrax (CA) is one of the common clinical manifestations during the anthrax outbreak. Nearly 95% of anthrax cases worldwide are CA.[2] The cases of anthrax in Indonesia first occurred in Purwakarta, Subang, Bekasi, and Karawang, in 1996 and there were other outbreaks in 1997, 1999, and 2000. The anthrax outbreak is back in 2011 on Boyolali and Sragen.[3] The latest outbreak occurred in 2019 in Kulonprogo District, Yogyakarta, Indonesia and most of these cases were cutaneous anthrax. Indonesian Ministry of Health reported that from 2010-2016 there were 172 cases in which 97% of them were CA.[4]

Bacillus anthracis can be transmitted to humans by direct contact with animals or animal products through abraded skin area or insect bite.[5] The first lesion occurs within three to five days after spore inoculation and it is usually seen on exposed body areas, such as extremities (hand and finger), head, and neck. Cutaneous anthrax appears as painless, erythematous papules that look like an insect bite and it develops into an ulcer with central eschar.[6] Cutaneous anthrax lesion is self-limited and scarless. About 10% of CA develops into systemic anthrax with a high mortality rate.[7] The inoculation of *B. anthracis* causes cellular immune reaction triggering a humoral response, IgG and Ig M which finally result in skin clinical manifestation. This early response precipitates pro-inflammatory cytokines expression including TNF-α, IL-6, IL-8, and IL-16.[8] If this happens simultaneously, it will lead to endothelial dysfunction, which is characterized by an increase in E-selectin production and finally result in skin necrosis.[9]

Prevention to the occurrence of target organ dysfunction in individuals exposed to anthrax is currently using antibiotics. However, antibiotic therapy has various adverse effects such as nausea, vomiting, and antibiotic resistance.[10] Thus, it is necessary to find an alternative therapy with fewer side effects, non-toxic, and natural. Propolis is also known as "bee glue" is the generic name of the resinous product which is collected by bees from various plant sources. Methods of propolis extraction used in biological assays may influence its activity. The common method is solid-liquid extraction, which uses ethanol in different concentrations. The extract contains an amino acid, flavonoids, terpenes, and cinnamic acid derivates.[11] The biological activity of propolis is mainly due to the presence of flavonoids. Based on studies, there are several therapeutic properties in flavonoids, including antioxidant, anti-inflammatory, antiviral, anticancer, antibacterial, and antiallergic properties.[12] The administration of flavonoids is expected to inhibit the progression of dendritic cells to stimulate the secretion of IL-6 and TNF-α so that the necrosis process does not occur. We conducted this study to know the role of EEP to

prevent clinical skin manifestation and skin tissue necrosis of animal models induced with anthrax spores.

METHODS

Research Design and Data Collection Techniques

A true post-experimental post-test only control group study was performed in male *Rattus norvegicus* which has ANTXR2. This study was conducted in Veterinary Research Centre (BALITVET Bogor), University Research Centre of Universitas Gadjah Mada, Yogyakarta, and Anatomic Pathology Laboratory of Universitas Sebelas Maret, Surakarta, Indonesia. The study subjects were 40 healthy male *Rattus norvegicus* aged between 3 and 4 months old and weighing around 180-200 g. We excluded sick rats with dimmed eyes, dull fur, and inactive as well as decreased weight at the end of the study.

These animal anthrax models were randomly assigned into 5 groups : cutaneous anthrax models receiving EEP 200 mg/ kg body weight 7 days prior to induction (P1) (fig.1), cutaneous anthrax models receiving EEP 200 mg/ kg body weight for 14 days (P2) (fig.2), cutaneous anthrax models receiving EEP 200 mg/ kg body weight for 7 day (P3) (fig.3), cutaneous anthrax models receiving EEP 200 mg/ kg body weight and amoxicillin 9mg/ 200 mg (P4) (fig.4) and cutaneous anthrax models without any treatment (K) (fig.5). Each group consisted of 8 male animal models. All animal models were inoculated subcutaneously with *B. anthracis* spore diluted in 10 ml of normal saline on their back in the dose of 0.2 ml.

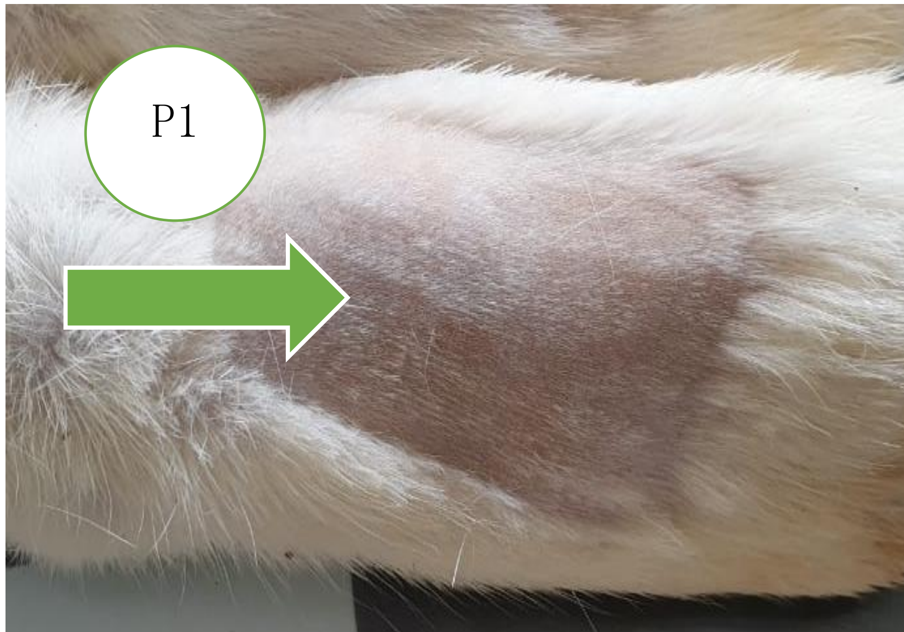

Figure 1. Model group anthrax + EEP 200 mg / Kg BW 7 days before induction

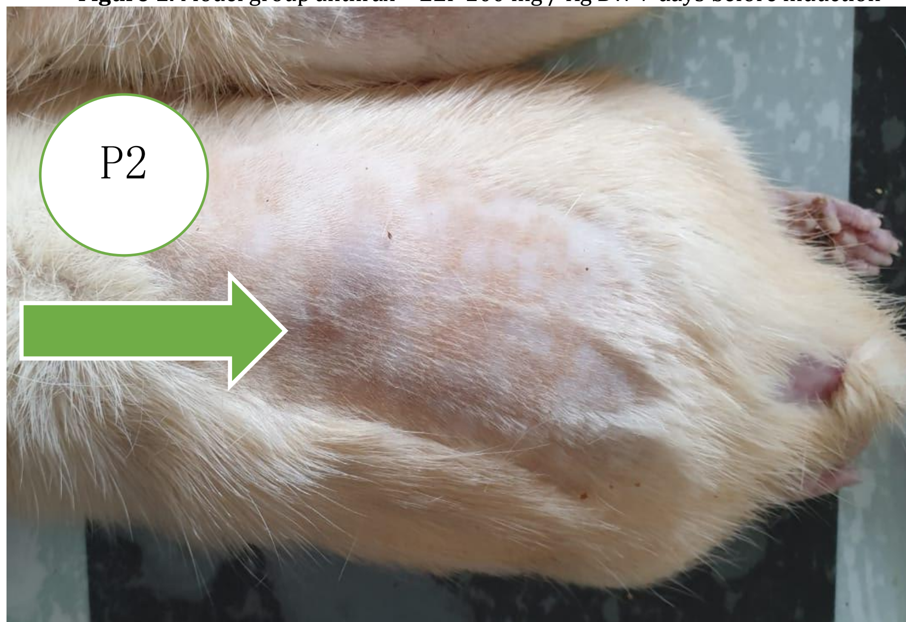

Figure 2. The model group anthrax + EEP 200 mg / Kg BW for 14 days

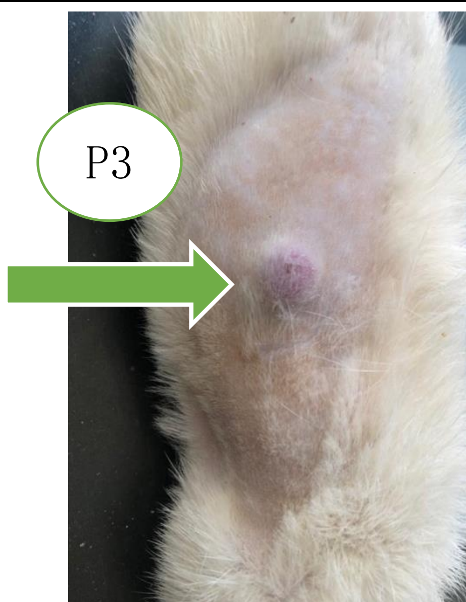

Figure 3. Model group anthrax + EEP 200 mg/Kg BW for 7 days

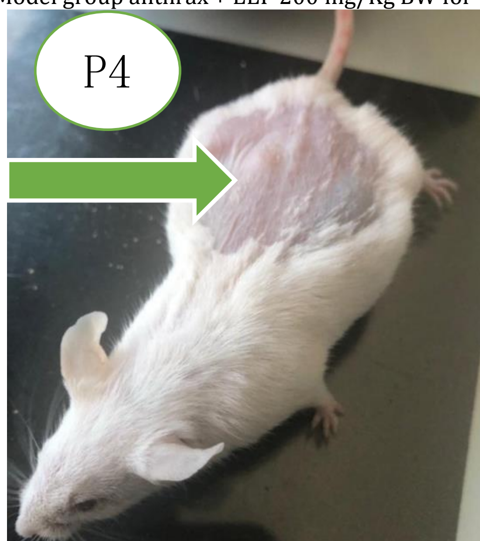

Figure 4. Model group anthrax + EEP 200 mg / KgBW + Amoxicillin 9 mg / 200 mg

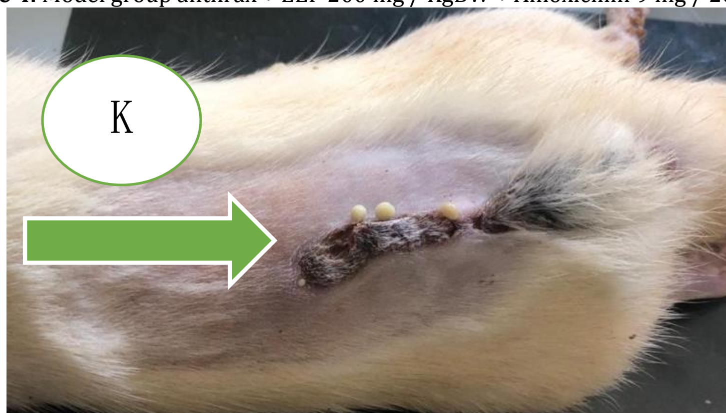

Figure 5. The anthrax model group

The clinical skin manifestation in all groups was assessed by scoring, ranging from 0 (no cutaneous manifestation), 1 (macule), 2 (mild, papule), 3 (moderate, nodule), and 4 (eschar). Skin tissue necrosis was evaluated histologically from a skin biopsy sample using Hematoxillin-Eosin (HE) staining. The score ranging from 0 (histologically normal), 1 (infiltration of lymphocytes, histiocytes, plasma cell, leukocytes without granulomatous inflammation), 2 (suppurative granulomatous inflammation), and 3 (suppurative granulomatous inflammation with necrosis area). This histopathological examination was carried out by two anatomical pathologists, with a scoring assessment as above, then the Kappa coefficient was calculated, to determine the level of appropriateness of the assessment between reader 1 and reader 2. The Kappa test is a reliability test to determine the consistency of

measurements made by two assessors, namely the strength value of the Kappa coefficient.[13]

The experimental animals in this study treatment have fulfilled the 3R principle according to the provisions of the National Center for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and have received approval from the health research ethics committee of Universitas Sebelas Maret, Surakarta.

Statistical Analysis

The clinical skin manifestation and skin tissue necrosis were scored, recorded, and analyzed with SPSS for Windows Release 22 program. The categorical data were analyzed with a non-parametric test Kruskal Wallis/ Mann Whitney) and p-value below 0.05 was considered statistically significant. The numerical data were analyzed with ANOVA test and p< 0.05 was considered significant.

RESULTS

Clinical Manifestation on Skin

Skin clinical manifestation was measured with the score as follows: (0) normal or no skin abnormality, (2) mild with papules, (3) moderate nodules, and (4) eschar. Table I showed that the highest mean skin clinical manifestation abnormalities occurred in the control group (2.75 ± 0.463) and the lowest observed in the P1 group (0.63 ± 0.518). Table II showed a significant difference between the control group and the P1 group, the control group with P2, the P3 group with the control group, and the control group with the P4 group, with p <0.05.

Table I. Mean Scores of Skin Clinical Manifestation

Groups	N	Mean ± SD	*p*
P1	8	0.63 ± 0.518	
P2	8	0.88 ± 0.354	
P3	8	1.25 ± 0.463	***p*≤0.001**
P4	8	1.13 ± 0.354	
K	8	2.75 ± 0.463	

Table II. Intergroup Relation of Skin Clinical Manifestation

Groups	*p*
P1 – KP2 – KP3 – K P4 – K	0.001 0.001 0.001 0.001

Histopathological Examination

Inflammation status in skin tissue or skin necrosis assessed through histopathological examination with Hematoxylin-Eosin (HE) staining. Scoring was used to determine the result of histopathological examination in skin tissue as follow; (0) normal, (1) lymphocyte, histiocyte, plasma cell, and polymorphonuclear cells infiltration without granulomatous inflammation, (2) suppurative granulomatous inflammation, and (3) suppurative granulomatous inflammation with necrosis area.

Table III showed the highest mean of inflammation status on histopathological examination with HE is staining was in the control group (3.00 ± 0.00), while the lowest mean was observed in the P2 group (1.38 ± 0.518). Moreover, Table IV showed significant differences between the control group and the P1, P2, P3, and P4 groups with p <0.05.

Table III. Mean and SD of Inflammation Status in Skin Tissue

Groups	N	Mean ± SD	*p*
P1	8	1.50 ± 0.535	
P2	8	1.38 ± 0.518	
P3	8	2.13 ± 0.354	**0.001**
P4	8	1.88 ± 0.641	
K	8	3.00 ± 0.00	

Table IV. Intergroup Relation of Inflammation Status in Skin Tissue

Groups	*p*
P1 – KP2 – KP3 – K P4 – K	0.001 0.001 0.001 0.001

DISCUSSION

Cutaneous anthrax is often referred to as black eschar. Patients have contact with infected animals or animal products. The first lesions occur within three to five days after spore inoculation and are generally found in open areas, such as the extremities, especially the hands and fingers, head, and neck.[14] Clinically, anthrax appears as painless erythematous or pruritic papules similar to insect bites and develops into ulcerated lesions with central eschar.[14,15] Within 12-36 hours after infection there will be papules, then within 24-36 hours, the papules turn into vesicles filled with dark blue fluid and form a ring of vesicles followed by ulceration of the central papule which dries and leaves a black eschar mark on the typical center of the lesion around the ulcer is accompanied by erythema and edema.[16,17] Complications of cutaneous anthrax are multiple bullae accompanied by severe edema and shock.

This study reported the highest mean of skin clinical manifestation abnormalities occurred in the control group (2.75 ± 0.463) and the lowest mean observed in the P1 (received EEP 7 days before induction) group (0.63 ± 0.518). This is in accordance with previous findings that reported 3 sub-units of toxins produced after the entry of anthrax endospores, i.e edema factor (EF), protective antigen (PA), and lethal factor (LF) that triggers the expression of pro-inflammatory cytokines, such as interleukin (IL-8) and Caspase-1.[18,19] If left untreated, this condition will lead to necrosis of the skin.[18,20,21]

This study also reported the highest mean of inflammation status on histopathological examination with HE is staining was in the control group (3.00 ± 0.00), while the lowest mean was observed in the P2 (received EEP for 14 days) group (1.38 ± 0.518). This is related to a previous study that showed on histologic examination, skin anthrax shows necrosis, severe edema, and lymphocyte infiltration.[17]

Propolis is a natural product that contains caffeic acid phenethyl ester (CAPE) which has been shown to inhibit inflammatory and infectious activity due to bacteria or viruses.[22] Earlier study showed on the content of the main active substance in propolis from Mount Lawu, Indonesia obtained CAPE levels of 30.24 ± 3.53 x 10-6 grams and quercetin levels of 4.42 ± 0.50 x 10-6 grams.[23] In this study, propolis was administered in two conditions: before and after exposure to *Bacillus anthracis* spores in cutaneous anthrax animal models.

This study findings also related to a previous study that showed unsolved acute infection process can lead to tissue damage and vascular degeneration which causes vasculitis and characterized by the recruitment process of leukocytes, monocytes, macrophages, and T lymphocytes in the blood vessels wall.[24] Propolis has an acute and chronic anti-inflammatory effect. The complex anti-inflammatory substance present in propolis contains bioactive components that have an immunomodulating effect through inhibition of neutrophil adhesion and transmigration of leukocyte adhesion molecules such as ICAM-1, VCAM-1, and E-selectin.[24] Thus, these findings explain the reason for better results on histopathological examinations could be observed in groups receiving propolis compared to the control group.

A previous study by Wen-Chien (2016) reported that propolis administration downregulates mRNA and miRNA encoding of pro-inflammatory cytokines such as IL-1β, IL-6, COX-2, and TNF-α. Moreover, TNF-α-induced NF-κB translocation was reduced with propolis administration. The inflammatory process can be inhibited if the NF-κB activity is also suppressed. Propolis suppresses the inflammatory response induced by TNF-α, including the expression of IL-1β, IL-6, COX-2, and other pro-inflammatory cytokines. So it can be concluded that propolis significantly inhibits the upregulation of inflammatory mediators induced by TNF-α.[25] This could explain the lower mean on both skin's clinical manifestation and inflammation status in the histopathological examination within groups receiving EEP compared to the control group.

CONCLUSION

This study found that an administration of ethanolic extract of propolis significantly results in a lower score of skin clinical manifestation abnormalities and inflammation status of the skin tissue assessed through histopathological examination. These findings are associated with the anti-inflammatory effect of propolis. These findings could imply the potential of propolis as a complementary therapy in cutaneous anthrax infection.

FUNDING STATEMENT

This study received Grant Funding from Universitas Sebelas Maret, Surakarta, Indonesia.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Data Availability Statement

The data used for this study are available from the corresponding author upon request.

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