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Original article

The effect of Ethanolic extract of Indonesian propolis on endothelial dysfunction and Multi Organ dysfunction syndrome in anthrax animal model

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1. Introduction

Anthrax is a common zoonotic infection caused by Bacillus anthracis that often appears as an extraordinary event in an area (Doganay and Demiraslan, 2015). Indonesia is a tropical country with an increasing number of extraordinary cases of anthrax (Indonesian Health Ministry, 2017). There was an anthrax outbreak in Pati Regency in 2007 and then in Boyolali Regency in 2009. Anthrax outbreak then appeared in 2011–2015 in Boyolali, Sragen and Pacitan Regencies (Redhono et al., 2018). In 2019–2020, there were similar outbreak in Gunungkidul district, Yogyakarta. The increasing incidence of anthrax has made this disease one of the priorities of the 14 zoonotic diseases elimination program in Indonesia (Redhono & Dirgahayu, 2016).

Anthrax transmission to humans occurs after direct or indirect contact with animals infected with anthrax through abraded skin, respiratory tract or digestive tract. (Olani et al., 2020). Contact with an infected animal causes B. anthracis endospores to enter the host (Emanuele et al., 2019; Savransky et al., 2020). After entering the host, B. anthracis will turn into a vegetative form which can replicate and produce toxins (Heninger et al., 2006; Moayeri et al., 2009). The host will initiate a response that triggers the expression of pro-inflammatory cytokines, including Tumor Necrosis Factor-alpha (TNF- α), Interleukin 1β (IL-1β), Interleukin 18 (IL-18), and Interleukin 6 (IL-6), that will increase the production of Reactive Oxygen Species (ROS), which can be assessed by serum Malondialdehyde (MDA) levels (Kalns et al., 2002; Ayala et al., 2014; Cherian et al., 2019). Excessive production of ROS can lead to endothelial dysfunction as characterized by increasing E-selectin production (Doganay & Demiraslan, 2015). This could result in apoptosis marked by an increase in Caspase-3 and Multi Organ Dysfunction Syndrome (MODS) in the skin, lung, kidney, and liver (Cherian et al., 2019; Liu et al., 2018).

Antibiotic is widely used to prevent target organ dysfunction in individuals exposed to anthrax. However, antibiotic therapy has various adverse effects such as nausea, vomiting, and antibiotic resistance (Savransky et al., 2020). Thus, it is necessary to find an alternative therapy with fewer side effects, non-toxic, and natural. Propolis is a potential option in MODS on the bacterial infection (Silveira, et al., 2019). Propolis is a resinous product that is collected by bees from various plant sources (Salatino et al., 2005). Ethanolic Extract of Propolis (EEP) is a local product with active Caffeic Acid Phenethyl Ester (CAPE) that has an antioxidant (Swamy et al., 2014; Bazmandegan et al., 2020) anti-inflammatory effect (Pickering & Merkel, 2004; Shang, et al., 2020) and antibacterial infection (Wright, et al., 2015). The propolis was taken from the slopes of Mount Lawu containing CAPE and Quercetin (Sarsono et al., 2012). A study by Prasetyo et al., (2013) showed that propolis isolate has the potential as an antioxidant at a dose of 200 mg/kgBW used for 30 days, by reducing MDA levels and healing wounds on the diabetic feet of Balb/C mice (Prasetyo et al., 2013)

This study aims to identify the role of EEP to prevent endothelial dysfunction and multiple organ dysfunction syndrome in animal models induced with anthrax spores.

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2. Methods

2.1. Research design and data collection techniques

This is a true experimental, post-test only, control group study that was performed in male *Rattus norvegicus* (Twenhafel et al., 2010) which is known to have Anthrax Toxin Receptors (ANTXR2) (Modlinska and Pisula, 2020). This study was conducted in Veterinary Research Centre , Bogor, University Research Centre of Universitas Gajah Mada, Yogyakarta and Anatomical 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, given a short-term EEP at a dose of 200 mg/kg, corresponding to 140% polyphenol (Prasetyo et al., 2013; Curti et al., 2019). We excluded sick rats with dimmed eyes, dull fur and lethargic, as well as decreased weight at the end of the study.

Propolis is a product of bees that functions to cover for holes and cracks in honeycomb reconstruction, smoothen the inner surface of the honeycomb, maintain the internal temperature of the hive and prevent weathering and predator invasion. The propolis will become soft and sticky when heated and has a distinctive aroma (Pasupuleti et al., 2017). Propolis contains resins, waxes and volatile compounds (Bankova et al., 2002). Wax comes from bees and plants produce resins and volatile compounds. The quality of propolis is highly determined by the origin of the plant. Resins are found in alcoholic extracts which are often used as alternative medicine (Salatino et al., 2005). The largest compound of propolis is resin (50%) and followed by wax (30%), essential oil (10%), pollen (5%) and other organic compounds (5%) with a maximum carbohydrate of 1 g/100 g, maximum protein of 1 g/100 g and maximum fat of 1 g/100 g (Gomez-Caravaca et al., 2006; Fatahinia et al., 2012). Generally, propolis is divided into three groups of flavonoids, cinnamic acid and terpenoids (Khalil, 2006; Adewumi and Ogunjinmi, 2011). Phenolic acids and flavonoids are natural ingredients in propolis, which have the effect of reducing oxidative stress (Kurek et al., 2013). The biological factors, geographical areas, environment, seasons and the type of extraction solution can affect the quality and quantity of propolis production (Kocot et al., 2018).

Caffeic Acid Phenethyl Ester has antioxidant (Nanaware et al., 2017), anti-inflammatory (Kanbur et al., 2009; Salmas et al., 2017), antiviral, antifungal effects (Capoci et al., 2015) and can inhibit the activation of potent and specific NFκB (Fitzpatrick et al., 2001) and reduce the production of ROS in human neutrophils in xanthine oxidase with a concentration of 10 mol/L (Irmak et al., 2003). Another active ingredient of propolis is quercetin which can reduce the production of Nitric Oxide (NO) by lipopolysaccharides, the expression of inducible Nitric Oxide Synthase (iNOS) and the release of IL-6 and TNF-α. Quercetin can inhibit the activation of MAPK and NFκB, complex transcription factors that play a role in the expression of pro-inflammatory genes (Liu et al., 2018). The active substance contained in EEP Mount Lawu, namely CAPE was $30.24 \pm 3.53 \times 10^{-6}$ g/ml and Quercetin $4.42 \pm 0.50 \times 10^{-6}$ g/ml (Sarsono et al., 2012). The anti-inflammatory effect was demonstrated in a study by Prasetyo et al. (2013) in which propolis from Mount Lawu can reduce HMGB-1 levels in male infertility mice models and the antioxidant effect at a dose of 200 mg/kg BW/day given for 30 days, reduce MDA levels and heal diabetic foot wounds in Balb/C mice. The CAPE content in propolis from Mount Lawu was higher than from other areas . Meanwhile, in Sragen and Wonogiri Regencies, it reached $12.40 \pm 0.77 \times 10^{-6}$ g/ml and $17.00 \pm 1.84 \times 10^{-6}$ g/ml respectively (Prasetyo et al., 2013).

These anthrax animal models were randomly assigned into 5 groups : anthrax animal model receiving 200 mg/kg BW EEP 7 days before induction for 14 days (P1), anthrax animal model receiving 200 mg/kg BW EEP for 7 days (P2), anthrax animal model receiving 200 mg/kg BW EEP for 14 days (P3), anthrax animal model receiving 200 mg/kg BW EEP and Amoxicillin 9 mg/ 200 mg (P4) and anthrax model animal without any treatment (K) as control. Each group consisted of 8 male animal models. All animal models were inoculated subcutaneously (SC) with B. anthracis spore diluted in 10 ml of normal saline on their back in the dose of 0.2 ml, equivalent to 2×10^{11} CFU spore (Kalns et al., 2002). The animal model was then sacrificed 14 days after B. anthracis inoculation (Goossens, 2009). Blood was then collected for E-selectin, SGPT and creatinine measurement. Lung tissue was taken for histopathology and immunohistochemistry examination.

E-selectin was expressed in endothelial cells stimulated by cytokines that recognize some glycoproteins in leukocytes (Cummings and McEver, 2009). Anthrax infection produced a toxin, called a lethal toxin, which is the main virulence factor of B. anthracis, which can increase Vascular Cell Adhesion Molecule 1 (VCAM-1) on human endothelial cells and cause vasculitis (Warfel et al., 2008). E-Selectin is used as a marker in assessing the presence of endothelial dysfunction by examining serum and immunohistochemistry. Serum E-selectin levels are a ratio scales, whereas immunohistochemistry E-selectin are an ordinal scale assessed with scoring system, ranging from 0 (no E-selectin positive area), 1 (1–25% E-selectin positive area), 2 (26–50% E-selectin positive area), 3 (51–75% E-selectin positive area) and 4 (76–100% E-selectin positive area).

Organ dysfunction is marked by SGPT level for liver, creatinine level for kidney and inflammation in a histopathological examination for lung. SGPT and creatinine level are ratio scale, whereas histopathological examination of lung tissue is an ordinal scale. The histopathological examination of lung tissue was assessed by the scoring system, ranging from 0 (no inflammation), 1 (mild inflammation), 2 (moderate inflammation), 3 (severe inflammation with necrotic tissue and suppurative granulomatous inflammation).

The histopathological examination was carried out by two anatomical pathologists using a scoring system as explained earlier. Then, the Kappa coefficient was calculated to determine the level of appropriateness of the assessment between 2 readers. The Kappa test is a reliability test to determine the consistency of measurements made by two assessors. The Kappa coefficient ranges from 0 to 1.00, with 0 for very weak consistency and 1.00 for very strong consistency (Tang et al., 2015).

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 No. 015/UN27.06.6.1/KEPK/EC/2020.

2.2. Statistical analysis

The E-selectin, SGPT, and creatinine level, as well as lung tissue histopathological examination scores were recorded and analyzed with SPSS for Windows Release 25.0. Categorical data were tested with non-parametric test and the results would be considered to have a significant difference if $p < 0.05$. This study use the Kruskal Wallis test to determine the correlation between groups and the Mann-Whitney test to determine the mean difference between groups (Jan and Shieh, 2014).

Numerical data were analyzed using ANOVA test with a significant value of $p < 0.05$. (Wang et al., 2017). Further, it used Shapiro Wilk test to test the normality of the data distribution, Levene's

test to test the homogeneity of the data, ANOVA test to test the differences in the five groups and Post-hoc Tuckey test to test the mean difference between groups (Kim and Cribbie, 2018).

3. Results

3.1. Serum E-Selectin level

Table 1 shows the highest average of E-selectin levels in the K (control) group levels after 14 days (34.307 pg/ml) and the lowest average of E-selectin levels in the P1 group (17.646 pg/ml). The results of the ANOVA and Post Hoc test (Fig. 1) show a significant relationship in all groups, with $p < 0.05$, except for the relationship between P2 and P4 ($p = 0.770$).

3.1.1. Immunohistochemistry E-Selectin examination

Fig. 2 shows E-selectin expression in areas of lung tissue inflammation. The K (control) group shows a score of 2 (A, F), P1 group shows a score of 2 (B, G),P2 group shows a score of 2 (C, H), P3 group shows a score of 2 (D, I) and P4 group shows a score of 1 (E, J).

Table 2 shows that the highest mean E-selectin immunohistochemistry score is in the K (control) group (2.25 ± 0.463), while the P4 group shows the lowest score (1.5 ± 0.535). The Kappa coefficient value of the lung tissue E-selectin histopathological examination is 0.88 suggesting strong consistency from 2 Anatomical Pathologists. Table 3 shows a significant difference between the P4 and control groups ($p = 0.010$) based on the T-test results.

3.2. Serum SGPT level

Table 4 showed that the highest mean serum SGPT level is in the K (control) group 3720.250 ± 49.280, while the P1 group shows the lowest mean score (1826.625 ± 57.477). Fig. 3 shows a significant difference between the P1 and control groups ($p \leq 0.001$), the P2 and control groups ($p \leq 0.001$), the P3 and control groups ($p \leq 0.001$) and the P4 and control groups ($p \leq 0.001$) based on the results of the ANOVA and Post Hoc Tuckey Test.

3.3. Serum creatinine level

Table 5 shows that the highest mean serum SGPT level is in the K (control) group (316.875 ± 5.986), while the P1 group shows the lowest mean score (75.000 ± 1.851). Fig. 4 shows a significant difference between the P1 and control groups ($p \leq 0.001$), the P2 and control groups ($p \leq 0.001$), the P3 and control groups ($p \leq 0.001$) and the P4 and control groups ($p \leq 0.001$) based on the results of the ANOVA and Post Hoc Tuckey Test.

3.4. Lung tissue histopathological examination

The K (control) group shows severe inflammatory cells in necrotic tissue (A), P1 group shows moderate inflammatory cells (B), P2 group shows moderate inflammatory cells with a score of 2 (C),P3 group shows moderate inflammatory cells (D) and P4 group shows mild inflammation of the peribronchial and interstitial tissues of the lung Fig. 5.

Table 6 shows that the highest mean rank is in the K (control) group (25.75 from Anatomical Pathologist 1 and 25.00 from

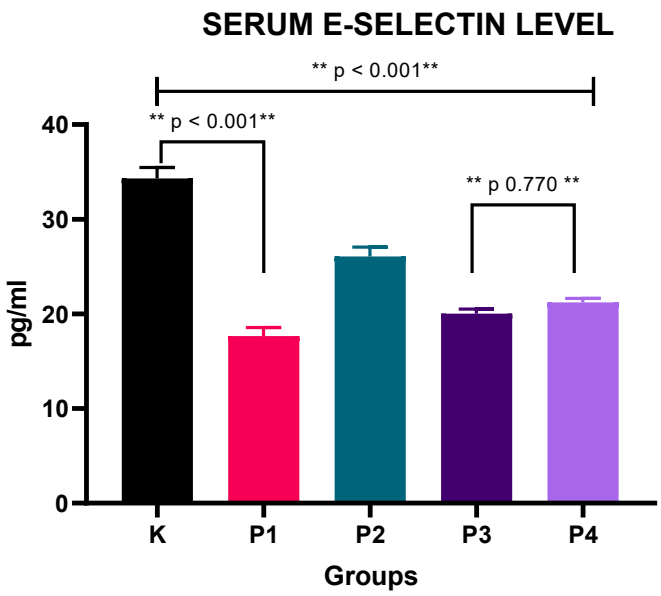

Fig. 1. ANOVA test and Post Hoc Test of E-selectin Levels.

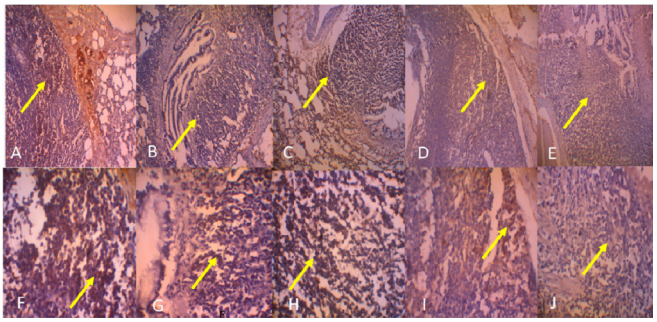

Fig. 2. E-selectin expression in lung tissue. A, B, C, D, and E at 100x magnification; F, G, H, I, J at 400x magnification. Yellow arrows indicate a positive E-selectin area.

Table 1
Mean and Standard Deviation of Serum E-selectin Levels.

Group	N	Mean ± SD	p
K	8	34.307 ± 1.160	
P1	8	17.646 ± 0.909	
P2	8	26.057 ± 0.999	**0.001**
P3	8	20.017 ± 0.500	
P4	8	21.208 ± 0.426	

Table 2
Mean and Standard Deviation of E-selectin Immunohistochemistry Score.

Group	N	Mean ± SD	p
K	8	2.25 ± 0.463	
P1	8	2.00 ± 0.926	
P2	8	1.75 ± 0.707	**0.103**
P3	8	1.88 ± 0.835	
P4	8	1.50 ± 0.535	

Table 3
T-test results of E-selectin Immunohistochemistry Examination.

Group	P
P1 – K	0.506
P2 – K	0.116
P3 – K	0.285
P4 – K	0.010

Table 4
Mean and Standard Deviation of Serum SGPT Level.

Group	N	Mean ± SD	p
K	8	3720.250 ± 49.280	
P1	8	1826.625 ± 20.325	
P2	8	2864.375 ± 15.781	**0.001**
P3	8	2272.251 ± 19.343	
P4	8	2360.750 ± 15.782	

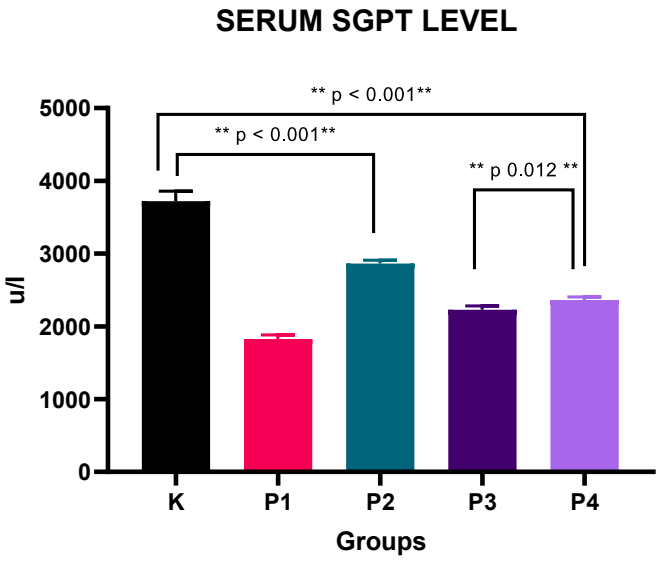

Fig. 3. ANOVA test dan Post Hoc Tuckey Test of Serum SGPT Level.

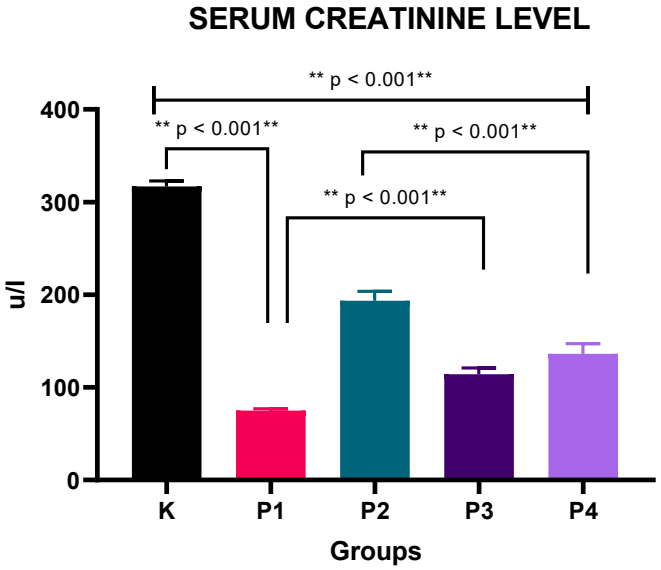

Fig. 4. ANOVA test dan Post Hoc Tuckey Test of Serum Creatinine Level.

Anatomical Pathologist 2). The lowest mean rank is in the P4 group (15.00 from Anatomical Pathologist 1 and 15.50 from Anatomical Pathologist 2). The Kappa coefficient value of the lung tissue histopathological examination is 0.86 suggesting strong consistency from 2 Anatomical Pathologists. Fig. 6 shows a significant difference between P4 and control groups (p = 0.015)

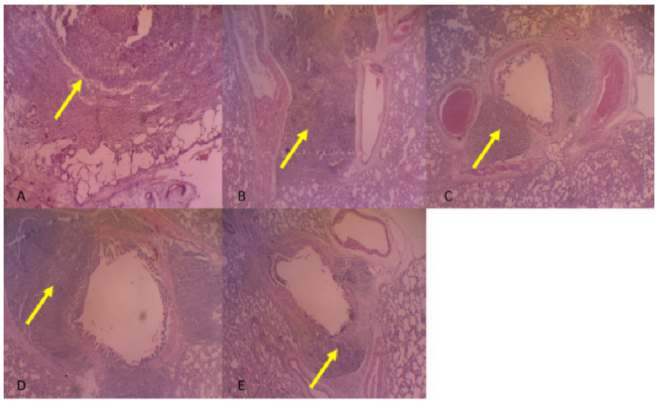

Fig. 5. Lung tissue histopathological examination at 400x magnification. Yellow arrows indicate inflammation area.

Table 5
Mean and Standard Deviation of Serum Creatinine Level

Group	N	Mean ± SD	p
K	8	316.875 ± 5.986	
P1	8	75.000 ± 1.851	
P2	8	193.500 ± 10.184	**0.001**
P3	8	114.125 ± 6.937	
P4	8	136.250 ± 10.951	

Table 6
Mean Rank of Lung Tissue Histopathological Examination

Group	N	*Mean rank* 1	*Mean rank* 2
K	8	25.75	25.00
P1	8	23.63	22.75
P2	8	21.38	22.75
P3	8	19.25	20.50
P4	8	12.50	11.50

4. Discussion

Anthrax is a zoonotic disease caused by gram-positive spores, B. anthracis (Doganay and Demiraslan, 2015; Savransky et al., 2020). Transmission to human occurs through direct or indirect contact with infected animals or animal products, through the abraded skin, by injection, inhalation and ingestion (Savransky et al., 2020). One of the markers used in assessing the presence of an inflammatory process in anthrax infection in this study was TNF-α, where there is a significant increase after infection (Warfel et al., 2008). After the spores enter, within a few hours they change to the vegetative form of B. anthracis, which then multiply and produce toxins. This causes an initial response that triggers the expression of pro-inflammatory cytokines, including TNF-α that lead to dysfunction on endothelial and organ targets, such as lungs (Cherian et al., 2019; Jeon et al 2014).

E-selectin is a biomarker used in assessing endothelial damage. Anthrax infection has the potential to damage the endothelium of blood vessels and tissues since there is major inflammation (Warfel et al., 2005). During inflammation, the presence of high TNF-α can stimulate the endothelium to express E-selectin which binds PMN leukocytes to secrete lysozyme, which is a strong proteolytic that can cause cell necrosis (Purwanto, 2012). EEP is a promising product that may inhibit the damage to these organs (Korish and Arafa, 2011).

Anthrax infection has the potential to damage the endothelium of blood vessels and tissues (Xie, Auth and Frucht, 2011). Adminis-

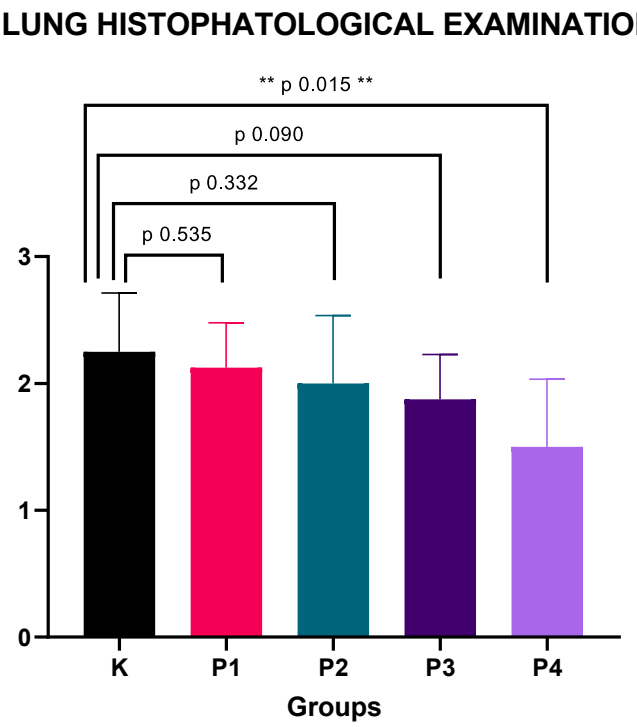

Fig. 6. Mann Whitney Test of Lung Tissue Histopathological Examination.

tration of propolis can reduce E-selectin levels, in cases of infection, according to a previous study by Franchin et al. (2018). This study shows that the lowest E-selectin levels were 17.646 ± 0.909 in the P1 group compared to the other groups. This proves that the administration of EEP 200 mg/kg BW before anthrax induction can reduce E-selectin levels better than the administration of EEP just after exposure. EEP administration shows a significant difference in serum E-selectin level compared to groups without EEP administration ($p \leq 0.01$). This finding shows that EEP administration before and after B. anthracis spore induction can reduce serum E-selectin levels and prevent endothelial dysfunction, which is the cause of the multiple organ system to necrosis.

Based on the E-selectin immunohistochemistry examination of the lung, there was a significant difference between the control and P4 groups, whereas there was no significant difference between the control and P1, P2, P3 groups. It shows that the combination of antibiotics and EEP generates better results than EEP only. This is different from the results of serum E-selectin level, which is a significant difference in all groups given EEP, especially in the P1 group. This may be caused by the levels of E-selectin in the tissues have not increased at the time of termination or the induction of B. anthracis spores by subcutaneous injection have not been reached the lung tissue.

Anthrax infection could lead to organ dysfunction, especially liver, lung, and kidney damage (Coggeshall et al., 2013). Liver is a detoxification place of toxins in the body and if an infection occurs, it will spur an increase in SGPT levels (Minemura, Tajiri and Shimizu, 2014). Anthrax infection can cause dysfunction in some organs, especially liver, lungs, kidneys and gastrointestinal system (Coggeshall et al., 2013). Liver function examination was measured study with SGPT level parameters at the end of the study after anthrax spore induction by SC injection. Propolis has been used in traditional medicine and proven to have hepatoprotective effects (Lofty et al., 2006) and as an antibacterial against Gram-positive Bacillus subtilis (Kumar et al, 2008; El-Bassuony and AbouZid, 2010).

In this study, the lowest average SGPT level (1826.625 ± 57.477) was found in the P1 group. This shows that EEP administration before anthrax induction can reduce SGPT levels compared to EEP administration after anthrax induction. The administration of EEP before anthrax induction was also better than the combination of EEP and antibiotics administered after anthrax induction. It is in line with the research done by Korish et al. (2011). Rat that received CAPE pretreatment showed significantly reduced liver damage. These rat had intact liver lobules and normal hepatocyte form, with a definite boundary between nucleus and cytoplasm. Bleeding, inflammation and necrosis are also lower in mice that received CAPE pretreatment (Korish and Arafa, 2011). This study also shows that EEP administration before and after induction gives a significant difference with the control group ($p \leq 0.01$). It indicates that EEP administration can prevent the occurrence of liver dysfunction in anthrax.

One of the markers of decreased kidney function is serum creatinine levels (Jafar, Schmid and Levey, 2005). The presence of anthrax infection can cause damage to the kidneys due to an inflammatory reaction and the formation of ROS, causing endothelial dysfunction which results in necrosis of kidney nephron cells. It can be assessed by measuring serum creatinine. If it continues, it will cause kidney failure (Nankivell, 2001). Based on the results of this study, the lowest average serum creatinine level was found in the P1 group (75.000 ± 1.851). This finding suggests that EEP administration for 14 days, starting 7 days before anthrax induction is beneficial in preventing kidney damage. This is in line with the study done by Silveira et al. (2019), which found the benefit of propolis administration in preventing kidney inflammation. This finding shows that propolis is beneficial to reduce creatinine levels in anthrax, especially when given as prophylaxis to anthrax spores (Silveira et al., 2019).

The P2 group, which was administered EEP for 14 days shortly after anthrax induction showed a lower mean serum creatinine level (114.125 ± 6.937) compared to the P3 and P4 groups. Study of Trumbeckaite et al. (2017) show that giving CAPE 1.5 h before

kidney ischemia could protect the part of mitochondria from injury caused by ischemia, which may reduce the incidence of kidney necrosis (Trumbeckaite et al., 2017). This study also shows a significant difference ($p \leq 0.01$) of creatinine level between the control group and P1, P2, P3, P4 groups. This finding shows that EEP administration can prevent kidney dysfunction in anthrax infection. It is in line with a study related to propolis of Pakistan (El-Sayed et al., 2009).

In anthrax infection, the cell is damaged due to free radicals caused by the presence of lipid peroxidation. The formation of free radicals can take place during the inflammatory process caused by a bacterial infection. Phagocytic cells form and free these toxic oxygen radicals to eliminate the pathogen, a process known as the respiratory burst. However, in prolonged infection, phagocytes tend to die and free these toxic radicals that will affect the surrounding cells. The lipid peroxidation activity in anthrax can lead to damage to cell components, including lipids, proteins and DNA, thereby disrupting the metabolism of lung cells, which in turn could result in cell damage (Duong et al., 2006). In this study, a microscopic analysis was classified based on a scoring system, ranging from 0 (normal histopathology), 1 (mild inflammation), 2 (moderate inflammation) and 3 (severe inflammation of the peribronchial and pulmonary interstitial tissue accompanied by necrotic tissue and suppurative granulomatous inflammation). This is in line with the microscopic analysis of lung specimens by Ozdulger et al. (2003) who made 4 grades classification in which grade 1 for normal histopathology results, grade 2 for a small amount of neutrophil leukocyte infiltration, grade 3 for moderate neutrophil leukocyte infiltration, the formation of perivascular edema and partial damage to lung architecture and grade 4 for solid neutrophil leukocyte infiltration, the formation of abscess and complete damage to lung architecture (Ozdulger et al., 2003).

Based on the results of the histopathological examination of the alveolar and lung parenchymal tissue, it shows that 50% of the samples have mild to moderate inflammation in the peribronchial and pulmonary interstitial tissues with the highest mean rank of the control group. These results are consistent with the study of Koksel et al. (2006) which show lung necrosis in mice induced with gram-negative LPS through intraperitoneal injection (Koksel et al., 2006).

5. Conclusions

This study found that the administration of EEP significantly results in lowered serum E-selectin, SGPT and creatinine levels. EEP administration also results in lowered E-selectin expression and inflammation area in histopathological and immunohistochemistry examination. These findings are associated with the protective effect of propolis on endothelial, liver, kidney and lung tissue. These findings can imply the potential of propolis as a complementary therapy in anthrax infection.

6. Funding Statement

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

7. Data Availability Statement

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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