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Extract Bee Propolis (*Trigona* sp) for Preventive Increase Protease Activity and Defect of Trachea Histology in Rats (*Rattus norvegicus*) Exposed to Cigarette Smoke

E N Indasari^{1,2}, A P W Marhendra³, and A W Wardhana⁴

¹Magister student of Animal Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, Airlangga University, Surabaya, East Java 60115, Indonesia

²Bachelor of Veterinary Medicine, Brawijaya University, Malang, East Java 65151, Indonesia*

³Biology Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, East Java 65145, Indonesia

⁴Veterinary Anatomy Laboratory, Faculty of Veterinary Medicine, Brawijaya University, Malang, East Java 65151, Indonesia

Email: analis_wardana@gmail.com

Abstract. Environmental tobacco smoke (ETS) refers to exposure to tobacco smoke, not from our smoking but from being exposed to someone else's cigarette. Breathing in ETS is known as passive smoking. Cigarette smoke contains dangerous components, include *Radical Oxygen Species*. Exposure free radical from cigarette result unbalance antioxidant in our body, it can increase protease activity and cell defect. Free radical was inhibited by exogen antioxidants in derivate caffeic acid phenetyl ester (CAPE) propolis *Trigona* sp. Propolis extract is honey bee product that has many active ingredients with many important roles, as antioxidant, anti-inflammation, DNA protection, and immunomodulator. This research to learn about potential extract propolis *Trigona* sp for inhibiting increased protease activity and defect of trachea histology in rats exposed to cigarette smoke. It is used five groups, they are negative control, positive control exposed to 2 cigarettes/day, and three preventive groups given by propolis, dose 10, 20 and 30 mg/200gBW/day. We conclude that propolis has the effect to prevent increase protease activity and defect of trachea histology from exposure to cigarette smoke.

Keywords: Cigarette smoke, ETS, Propolis, Protease, Trachea

1 Introduction

Smoking is a habit that can endanger yourself and the environment. Smoking does not cause death directly but can trigger various diseases (Sitepoe, 2000). The number of cigarettes smoked by Indonesian people increase from 182 billion cigarettes in 2001 to 260.8 billion cigarettes in 2009 (WHO, 2008). Indonesia is the third country with the highest number of smokers in the world with a prevalence reaching 36.1%. The number of cigarettes smoked by the Indonesian population over the age of 10 years is 12.3 cigarettes per day (Menkes, 2013).



Animals in the environment around humans also feel the negative effects of cigarette smoke. Environmental tobacco smoke (ETS) refers to exposure to tobacco smoke, not from our smoking but from being exposed to someone else's cigarette. Breathing in ETS is known as passive smoking. According to Tu (2009), owners are known to often play with their pets accompanied by activities that can interfere with the animal's health. One of them is smoking. Cigarette smoke that comes out of the cigarette and exhaled by smokers spreads into the air, then enters inhaled into the respiratory tract of animals called Secondhand. Cigarette smoke can also in the carpet, hair, and other objects around the animal so that it enters the body orally such as through licks called Thirdhand. Based on the research of Bertone et al. (2002) that cats with a smoker environment have a 2.4x risk of developing malignant lymphoma within a period of two years, even can increase the risk to 3.2x in a period of five years or more.

One cigarette that burned produce about 5,000 mg of gas (92%) and solid particle (8%) (Britton and Edwards, 2007). The content of cigarette smoke is classified as Radical Oxygen Species (ROS) (Cahyani et al., 2014). ROS (Reactive Oxygen Species) are highly reactive oxygen derivative oxidizing compounds consisting of free radical groups and non-radical groups. Free radical groups include superoxide anion (O₂⁻), hydroxyl radicals (OH⁻), and peroxy radicals (RO₂⁻). Non-radical compounds such as hydrogen peroxide (H₂O₂), and organic peroxides (ROOH) (Halliwell and Whiteman, 2004). Free radicals in cigarette smoke can cause increasing protease enzyme (Kumar and Vats, 2010) because macrophages activity to inhibit free radicals triggers neutrophils to increase the production of protease.

Tissue damage is the result of ROS attacks is known as oxidative stress, while factors that can protect tissues against ROS are called antioxidants. Tissues that can be damaged due to ROS include Deoxyribose Nucleic Acid (DNA), lipids, and protein (Bender, 2009). Increasing free radicals that unbalanced with increasing antioxidants will be a phenomenon that underlies the pathophysiology of various diseases (Armentis et al., 2010). Propolis is one source of natural antioxidants found in Indonesia. Propolis has the most powerful antioxidant activity against free radicals compared to other bee products. Caffeic acid phenethyl ester (CAPE) is the active side of flavonoids in propolis which has high antioxidant activity. CAPE has antioxidant activity 4-6 times stronger against free than vitamin C (Nakajima, 2009). Lethal Dosage (LD₅₀) propolis in rats reaches 8,000-40,000 mg/kg BW (Burdock, 1998). The effect of long-term propolis consumption does not cause damage to the blood, liver, kidney, and does not affect body weight or disturb liver and kidney function (Sarto and Saragih, 2009).

2 Materials and Methods

2.1 Study area

Research in may-june 2016 at Biomedics Laboratory, Faculty of Medicine, University of Muhammadiyah Malang. Protease activity tested in Biochemistry Laboratory, Faculty of Mathematics and Natural Sciences Brawijaya University, and tracheal histology preparations in Anatomy Pathology Laboratory, Faculty of Medicine, Brawijaya University. Phytochemical *Trigona sp* tested in Chemistry Laboratory, State Polytechnic Analytical in Malang. The study was approved by the Ethics Committee of Brawijaya University (Approval No. 532-KEP-UB).

2.2 Sampling

Rats model is male *Rattus norvegicus*, Wistar strain, age 8-12 weeks (Epstein, 2004). Bodyweight 150-200 grams. This experimental using a completely randomized design (CRD). According to Kusningrum (2008), the estimated sample size is calculated by the formula:

$$\begin{aligned} p(n-1) &\geq 15 \\ 5(n-1) &\geq 15 \\ 5n-5 &\geq 15 \\ 5n &\geq 20 \\ n &\geq 20/5 \end{aligned}$$

$$n \geq 4$$

p = number of groups

n = number of repetitions

Based on the calculation, for 5 groups, a minimum number of repetition is four times in each group, so that need a minimum of 20 experimental animals.

2.3 Extract of propolis *Trigona sp*

Extraction of propolis with 70% ethanol. Propolis is obtained from Lawang. The instrument are thermostirer 150 rpm for 4 hours and rotated with 5 cm magnetic stirrer. Propolis filtered with filter paper to obtain propolis filtrate. The filtrate is separated from the solvent by evaporation in a rotary evaporator temperature 70°C at 2-3 rpm. Ethanol propolis extract is a paste. The results of ethanol extract of propolis that has been evaporated, diluted to be easily given to experimental animals through oral methods. Ethanol extract of propolis is calculated to make a dose, then tween 80 is added as an emulsifier and diluted with distilled water (Radiati et al., 2008).

Phytochemical tests were carried out to determine the bioactive components contained in the ethanol extract of propolis *Trigona sp*. LCMS test continued, found several spots showing various types of flavonoids that exist in ethanol extract of propolis. Further tests using infrared spectrophotometry revealed that there was a CAPE uptake with a molecular weight of 283 grams/mol. The dose of ethanol extract of propolis *Trigona sp* refers to the research of Radiyati et al. (2008), which is 100 mg/kg to increase the immune system. In this experiment used 3 dosage variations based on the calculation of propolis dosage which is 10mg/200gBW/day, 20mg/200gBW/day, and 30 mg/200gBW/day.

2.4 Rats Model Cigarette smoke exposure

Acclimatize rats on days 1-7 in the maintenance cage. Days 8-28 were given ethanol extract of propolis (EEP) to increase antioxidants in the body. Exposure to cigarette smoke on days 15-28 with non-filter clove cigarettes using a smoking pump. Cigarette smoke is exhaled with a smoking pump into a glass-enclosed enclosure of two cigarettes/day for each group for two weeks. Exposure to cigarette smoke to increase free radicals in the body. In this experiment, rats were divided into 5 groups randomly. The first group was negative control (C-), each rat was not given any treatment starting from day 1-28. The second group was positive control (C+), exposed to cigarette smoke on days 15-28 of 2 cigarettes/day/cage without EEP. The third group was treatment 1 (T1), exposed to 2 cigarettes/day on days 15-28 and given EEP 10 mg/200gBW/day on days 8-28. The fourth group was treatment 2 (T2), exposed to 2 cigarettes/day on days 15-28 and given EEP 20 mg/200gBW/day on days 8-28. The fifth group was treatment 3 (T3) exposed to 2 cigarette smoke/day on days 15-28 and given EEP 30mg/200gBW/day on days 8-28. Exposure to cigarette smoke for 15 minutes. EEP given orally (PO) using a sonde.

2.5 Protease Activity

Rats that have been dissected, tracheas are taken in PBS azide. Protease isolation from trachea by cutting into small pieces 0.5 gram, added PBS-tween: PMSF (9: 1) 1 mL and quartz sand, crushed with mortar, added PBS-Tween solution: PMSF (9: 1) 2 mL, transferred to polypropylene tube that has been sterilized by autoclave. Then homogenized with vortex 10 minute, sonicated 10 minute, and centrifuged 15 minutes (6000 rpm). After that, the supernatant is taken and 1:1 absolute ethanol is added and allowed 24 hours to form a precipitate. Centrifuged for 15 minutes (10,000 rpm), the sediment is taken, and dried until the aroma of ethanol disappears. Finally, the sediment was added with a solution of 0.02 M Tris-HCl pH 6.5 cold with a volume ratio of 1:1 (Aulani'am, 2005).

Measurement of protease activity by mixing casein 500 ppm 200 μ L, phosphate buffer solution pH 7 300 μ L, and protease enzyme 100 μ L, then allowed 60 minutes at 37°C in the incubator. Then added TCA (trichloroacetic acid) solution 4% 400 μ L, allowed to stand 30 minutes at 27°C. Played with centrifuge 4000 rpm 10 minute. The supernatant was taken 100 μ L and diluted 5 times the sample

volume with PBS, then measured its absorbance value at a maximum tyrosine of 275 nm (Aulani'am, 2005). One unit of protease activity is defined as the amount of the enzyme that catalyzes the conversion of one micromole of substrate casein per minute under the specified conditions of the assay method.

Measurement of protease activity based on the walter method (1984) using the formula:

$$\text{Protease Activity} = \frac{[\text{Tyrosine}]}{\text{Mr Tyrosine}} \times \frac{v}{p \times q} \times f_p$$

Mr Tyrosine = molecular weight of tyrosine (181 $\mu\text{g} / \mu\text{mol}$)

v = volume sample (mL)

p = volume enzyme (mL)

q = incubation time (minutes)

f_p = dilution factor

2.6 Trachea Histology

Making histological preparations with hematoxylin-eosin (HE) staining. The principle of HE staining is acidic cell nuclei will attract alkaline substances (hematoxylin) so they will turn blue. Cytoplasm which has alkaline will attract acidic substances (eosin) that are red. First, tracheal fixation in 10% formalin and washing. Trimming is organ cut \pm 3 mm and put into a tissue cassette. Dehydrated with 70% alcohol 30 minutes, 96% alcohol 30 minutes, 96% alcohol 30 minutes, 60 minutes absolute alcohol, and xylol alcohol 1: 1 30 minutes. Furthermore, clearing to clean the remaining alcohol with xylol I and II, 60 minutes respectively. Impregnation with paraffin 60 minutes in a 65°C oven. Embedding is cleaning the remaining paraffin. Liquid paraffin is poured into tissue blocks. Paraffin containing tracheal pieces is released from the tissue block by inserting at 4–6°C. Cutting is done, the best cut sheets are chosen. The tissue sheets are moved in a 60°C water bath, placed on a slide, placed in a 37°C incubator 24 hours until the tissue is fully attached.

Staining with hematoxylin-eosin is done after the tissue is fully attached to the slide. Deparaffinized in 5 minutes xylol I solution, 5 minutes xylol II, 60 minutes absolute ethanol, 96% alcohol hydration 2 minutes, 70% alcohol 2 minutes, and water 10 minutes. Core staining is made with 15 minutes hematoxylin. Rinse with running water. Stained with eosin for a maximum of 1 minute. Hydrated with 70% alcohol for 2 minutes, 96% alcohol for 2 minutes, and absolute alcohol for 2 minutes. Purification is done with xylol I 2 minutes and xylol II 2 minutes. Mounting with entelan and covered with glazed decks, drops entelan, and covered with glass cover. Slides were examined under a light microscope at 400x magnification.

2.7 Statistical analysis

Data were analyzed with Microsoft Office Excel and SPSS using various ANOVA analyzes. If there is a difference in treatment, it will be continued using the Honestly significant Difference Test - Beda Nyata Jujur (BNJ) $\alpha = 5\%$. Then proceed with Tukey test for giving notation to each treatment (Kusriningrum, 2008).

3 Results

Rats exposed to cigarette smoke showed that increasing protease activity in trachea. This step to increasing amount of free radicals in the body. This experiment divided into five groups. They are positive control group (C +), treatment I (T1) with EEP dose of *Trigona sp* 10mg/ 200gBW/day, treatment II (T2) 20mg/200gBW/ day, treatment III (T3) 30mg/200gBW/day, and negative control (C-) which are healthy rats without treatment.

Negative control had protease activity $0.020979028 \pm 0.002243474 \mu\text{mol/mL}\cdot\text{minute}$. Protease activity in negative group used as a standard to reference increasing that occurred. According to Pratiwi et al. (2013), proteases are normally present in body tissues which play a role in cellular defense to breakdown of foreign proteins that enter in the body.

Table 1 The average of protease activity in tracheal

Groups	The average of protease activity in tracheal ($\mu\text{mol/mL}\cdot\text{menit}$)	Protease Activity (%)	
		Increasing to C-	Decreasing to C+
Positive Control (C+)	$0.112708988 \pm 0.002243474^d$	81.39 %	-
Treatment I (T1) (10mg/200gBW/day)	$0.097663068 \pm 0.008283763^c$	-	13.35 %
Treatment II (T2) (20mg/200gBW/day)	$0.050443955 \pm 0.00686859^b$	-	55.24 %
Treatment III (T3) (30mg/200gBW/day)	$0.020928875 \pm 0.003028487^a$	-	81.43 %
Negative Control (C-)	$0.020979028 \pm 0.002243474^a$	-	-

Note: The notations a, b, c, and d indicate differences between the treatment groups

Positive control group showed increasing protease activity in 81.39%. Increasing ROS in the body, triggering neutrophils to increase protease activity to inhibit free radicals. Protease enzyme can create balance of antioxidants and free radicals in the body by controlling the activity, synthesis, and development of proteins. Protease activity can inhibited by antioxidants from exogen antioxidants.

Treatment I with EEP *Trigona sp* dose 10 mg/200gBW/day showed decreasing protease activity in tracheal 13.35% compared with positive control. Antioxidant in propolis can inhibit free radicals in the body, thereby reducing protease activity. However, it has different in notation in the treatment.

Treatment II with EEP *Trigona sp* dose 20mg/200gBW/day showed decreasing protease activity in tracheal 55.24% compared with positive control. This indicates that increasing the dose of EEP can decrease in tracheal protease activity. The treatment dose II gives quite significant results, due to the role of antioxidants contained in EEP. But it still shows the difference between notation in negative control, which means this dose is still less effective than expected.

Treatment III with EEP *Trigona sp* dose 30mg/200gBW/day had significant decrease in protease activity that was 81.43%. The measurement results obtained tracheal prosthetic activity of $0.020928875 \pm 0.003028487 \mu\text{mol/mL}\cdot\text{minutes}$ that have the same notation as negative control (healthy rats). In negative control, the resulting protease activity was $0.020979028 \pm 0.002243474 \mu\text{mol /mL}\cdot\text{minutes}$. This third treatment dose can provide the expected effect of tracheal protease activity produced in normal amounts so that it can inhibit tissue damage. In this study, treatment III showed significant differences compared to positive control and not significantly different from negative control. So that in this study, the most effective dose to inhibit protease activity in rats from exposure to cigarette smoke was in treatment III with an EEP dose of 30mg/200gBW/day.

Decreasing protease activity of rats resulting from exposure to cigarette smoke that was given EEP *Trigona sp* due to the content of CAPE which is the active side of flavonoids. CAPE has an antioxidant effect that will break down ROS by capturing free radicals, reducing, donating hydrogen atoms and binding to singlet oxygen. It also induces the G6PD gene which is an antioxidant coding gene. This condition will prevent chain reaction and stabilize the level of free radicals in the body (Nakajima, 2009).

Balance between free radicals and antioxidants in the body can be achieved because treatment of EEP *Trigona sp* one week before exposure to cigarette smoke to increase antioxidants in the body. Based on research by Li et al. (2016), CAPE is stored in plasma in the albumin section for a long period. CAPE has the strongest bond compared to other bioactive components in propolis. Furthermore, CAPE will be distributed throughout the body through blood vessels, including the trachea.

Treatment EEP *Trigona sp* continued for two weeks during exposure to cigarette smoke to maintain antioxidant levels in the body so that it can inhibit oxidative stress. This mechanism will prevent increasing protease activity that are produced in excess due to free radicals. Normal protease

activity will prevent protease inhibitor deficiency in the body. So that proteolysis of tissue proteins does not occur and prevents damage to the tracheal histological structure.

Epithelial erosion is damage due to free radical activity. Erosion is characterized by the release of epithelial cells from the basement membrane (Vegad, 2007). Erosion of the cilia and tracheal epithelium, caused by irritation of the tracheal mucosal layer composed of epithelial cells (Febrianti and Suryati, 2014). Erosion of tracheal epithelial cells exposed to cigarette smoke is caused by the presence of free radicals contained in cigarette smoke (Krisnabudhi, 2004).

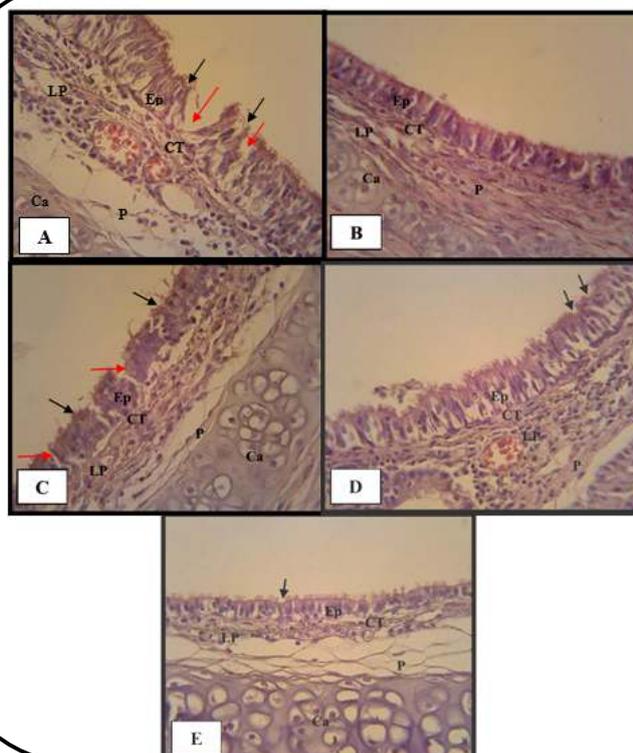


Figure 1

Tracheal Histology with Hematoxylin Eosin (HE) Staining, magnification 400x.

Note : (A) Positive control, (B) Negative control (C) Treatment I, (D) Treatment II, (E) Treatment III.

(Ep) = Epithelium, (CT) = Connective Tissue, (LP) = Lamina Propria, P = Perikondrium, Ca = Hyaline cartilage.

↖ = Ciliary erosion, ↗ = Epithelial erosion.

Based on the results of the study showed that in male rats (*Rattus norvegicus*) exposed to 2 cigarettes/group/day for 15 minutes caused high damage to the tracheal histology in epithelial and cilia (**Figure 1A**). Negative control showed trachea histological in normal condition, there was no tissue damage (**Figure 1B**). The structure of normal trachea consists of ciliated pseudostratified column (cylindrical) epithelium, connective tissue, lamina propria, perichondrium, and C-shaped hyaline cartilage accompanied by smooth muscle (Mescher, 2010). Negative control was used as a reference to compare with treatment in other groups. Treatment I with EEP dose 10mg/200gBW /day showed a lot of damage in the form of cilia and epithelium erosion on the entire surface of the trachea (**Figure 1C**). EEP in this dose has not significantly improved the damage of tracheal histology. This is because free radicals originating from cigarette smoke can not be inhibited, so it will bind to cellular components

that cause damage to the cilia and tracheal epithelium. According to Wadworth et al. (2012), epithelium in the respiratory tract is the first barrier that interacts directly with foreign body exposure so that damage to the epithelium can be used as an indication of antigen exposure in the respiratory tract in the form of free radicals from cigarette smoke.

Treatment II with EEP dose 20mg/ 200gBW/day showed increasing repair of histological trachea, absence of epithelial damage but there was still much erosion in the cilia (**Figure 1D**). This is because the antioxidants in EEP are capable of binding to exogenous free radicals that enter the body of rats, thereby inhibiting free radicals from binding to cellular components. This antioxidant prevents damage to the tracheal epithelium, but it is still not too strong to inhibit ciliary erosion in the tracheal epithelium. The decrease in damage is influenced by hydrogen atoms from the hydroxyl (OH) group in the CAPE structure found in EEP *Trigona sp.* Hydrogen atoms will be released to bind to free radicals and form more stable compounds. Treatment III with EEP dose 30mg/200gBW/day had histological results almost normal condition, without epithelial erosion. However, there is little erosion in the cilia (**Figure 1E**), it is normal response of biochemical processes in the body. According to Lauretta et al. (2013), the damage to tracheal cilia without exposure to exogenous free radicals is a response to biochemical processes that occur in the body. Based on this study, a dose 30mg/200gBW/day provides the least damage to the histological structure of the trachea compared to others.

4 Discussion

Exposure to cigarette smoke in rats can increase exogenous free radicals, namely ROS (Radical Oxygen Species) in the body. ROS comes from cigarettes that have been burned and undergoes two phases namely the particle and gas phases. The particle phase in cigarette smoke contains free radicals found in tar. Tar is identified as containing free radicals in the form of quinone/hydroquinone (Q/QH₂). This hydroquinone will react actively in the redox system that binds with oxygen molecules to produce reactive free radicals, namely ROS in the form of superoxide (O₂⁻) and hydroxyl radical (OH⁻). In the gas phase, nitrogen dioxide (NO₂) can bind to other cigarette components, such as isoprene. This bond will produce carbon radicals that quickly bind with oxygen (O₂) so that from this reaction ROS will form peroxy radicals (RO₂⁻) (Daniel et al., 1985). It cause an accumulation of high levels of ROS in the body. Resulting in an imbalance of ROS and antioxidants in the body.

ROS from cigarette smoke will increase the accumulation of free radicals in the body. ROS in cigarette smoke is superoxide (O₂⁻), hydroxyl radicals (OH⁻), and peroxy radicals (RO₂⁻) (Daniel et al., 1985). The high ROS in the body causes oxidative stress. When oxidative stress occurs, neutrophils are drawn by cytokines in large quantities so that protease secretion increases and is out of control. As a result, there is a protease inhibitor deficiency that causes proteolysis of tissue-forming components so that tissue damage occurs (Greenlee, 2007). ROS will also attack the PUFA (Polyunsaturated Fatty Acid) chain on the cell membrane causing lipid peroxidation and tissue damage. Lipid peroxidation is a chain reaction that provides a continuous supply of free radicals that initiate further peroxidation. The body has several defense mechanisms against free radicals. The defense is carried out by antioxidants in the body as an effort to prevent cell damage (necrosis), some of the active antioxidants include glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase, superoxide dismutase (SOD), catalase, glutathione synthetase (Valko, 2007). However, if free radicals in the body is too high, the body is unable to inhibit endogenous antioxidants so exogenous antioxidants are needed by using EEP *Trigona sp.*

Under normal circumstances, there is a balance between antioxidants and free radicals in the body. Exposure to cigarette smoke triggers oxidative stress, which is an imbalance between antioxidants and free radicals in the body, triggering the activation of macrophages to secrete cytokines, which in turn activate neutrophils. Neutrophils will produce protease enzymes in large numbers due to high free radicals in the body. According to Segal (2005), the protease enzyme involved in tissue damage is serine protease (neutrophil elastase) stored in neutrophil granules, functioning for phagocytosis of foreign bodies, including free radicals.

Based on the results of research through observation of tracheal histology with hematoxylin-eosin (HE) staining, proved that rats exposed to cigarette smoke given extract of ethanol propolis (EEP) *Trigona sp* can inhibit tracheal histological damage. According to Cadenas and Parker (2002), propolis can become an exogenous antioxidant in the body caused by the ability of propolis to express Glucose-6-Phosphate Dehydrogenase (G6PD), known as an antioxidant gene. CAPE contained in propolis extract is known to inhibit ROS by preventing lipid peroxidation, which can increase glutathione peroxidase activity. In addition, CAPE can reduce the adverse effects of free radicals, by inhibiting lipid peroxidation through the mechanism of peroxidase. Peroxidase is useful to prevent the accumulation of H₂O₂, which becomes dangerous if together with O₂⁻ because it can form OH radicals which are the most reactive and most dangerous free radicals, which can damage cell membranes by causing the breakdown of unsaturated fatty acids in cell membranes.

The mechanism of CAPE as an antioxidant is by preventing the initiation process, through filtering OH⁻ in reactions that involve electron transfer and possibly the elimination of heavy metals. Furthermore, it will break the peroxidative chain reaction by stabilizing the peroxy radicals and forming them into peroxides by donating hydrogen atoms (H). Then regeneration of α -tocopherol by reducing α -tocopherol radicals (Hardiyanti, 2011). In addition, CAPE is known to increase the activity of antioxidant genes that produce Glucose-6-Phosphate Dehydrogenase (G6PD). G6PD will reduce NADP⁺ to NADPH so that it will release H atoms to bind to free radicals. The high activity of the

G6PD gene will produce a lot of H atoms which will be used to bind free radicals so that it inhibits the binding between free radicals and cellular components to prevent tissue damage.

Cell damage caused by free radicals is preceded by damage to cell membranes consisting of phospholipid components and proteins, through three mechanisms namely free radicals and membrane components will form covalent bonds resulting in changes in the structure and function of the receptors. Furthermore, the process of transportation across the membrane will be disrupted due to the oxidation of the thiol group in the cell membrane component. Then free radicals will bind to unsaturated fatty acids (PUFA) that are in the membrane so that lipid peroxidation occurs. PUFA chain has a carbon double bond that is easily decomposed and reacts with other compounds to get a stable composition of saturated fatty acids. The more double the number of bonds will be easy to react. The lipid peroxidation process begins with the withdrawal of hydrogen atoms containing PUFA double bonds to form lipid radicals. Reaction with oxygen will cause the formation of peroxy lipid radicals which will attract more hydrogen atoms from other PUFA double bonds so that the next lipid radicals are formed. Lipid peroxy radicals will decompose into lipid peroxide which is unstable so that a chain reaction occurs which causes damage to the cell membrane. In proteins, free radicals can cause fragmentation, thus accelerating proteolysis. In nucleotides will cause changes in the structure of DNA and RNA resulting in mutations and cytotoxicity (Gitawati, 1995). This is what affects tissue damage due to free radicals in cigarette smoke.

5 Conclusion

Based on the results of the research that has been carried out, it can be concluded that the administration of ethanol extract of *Trigona sp* in rats model of exposure to cigarette smoke can reduce the activity of tracheal proteases and inhibit tracheal histological defect.

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