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Effect of Ethanolic Extract of Propolis Trigona Spp. Malang Indonesia on Isolate *Staphylococcus aureus* Biofilm Architecture from Chronic Rhinosinusitis: A Confocal Laser Scanning Microscopic Study

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Abstract: The biofilm in chronic rhinosinusitis due to the infection by bacteria is a difficult problem to overcome. Bacteria in side embedded in biofilms are difficult to treat. CRS with biofilms has recurrent infections, ongoing mucosal inflammation and persistent postoperative symptoms. Propolis is a natural product that has the potential as an anti-bacterial choice. The purpose this study was to determine the effect of propolis on the growth of bacteria *Staphylococcus aureus* biofilm from isolate chronic patient rhinosinusitis, using CLSM with staining of green fluorescent nucleic acid Syto9. Isolate *Staphylococcus aureus* taken from swab discharge of meatus medius of chronic rhinosinusitis patients in functional endoscopy of sinus surgery at PHC Hospital Surabaya, Indonesia. Identification of *Staphylococcus aureus* using medium Mannitol Salt Agar, Gram Staining, Catalase Test and Coagulase Test. Biofilm produced bacterial cell cultured in congo red agar. EEP macerated of alcohol 70% and after that the biofilm formed put in 24 well culture plate 48 h using EEP solution dosages of 0%, 0.2 was %, 0.4%, 0.8%, 2%, 8%, 10% and negative control. Measurement intensity of expression Syto9 and biofilm depth using CLSM magnification 400x. Each well checked for 3x field of view. Ethanolic Extract of Propolis Trigona sp. solution dosages of 0, 0.2, 0.4, 0.8, 2, 8, 10% and negative control, biofilm *Staphylococcus aureus* obtained decreased intensity of expression Syto9 and biofilm depth. Kruskal Wallis test, Syto9 the result of significance (p) was 0.001. The result showed there was a difference of intensity of expression Syto9 results in the treatment dose group ($p<\alpha = 0.05$). In the biofilm depth, the result of

significance (p) was 0.001. The study showed that there was a difference of biofilm results in treatment dose group ($p<\alpha = 0.05$). In Post Hoc test the EEP Trigona sp. 2-10% dose can inhibit the growth of biofilms.

INTRODUCTION

The majority of human bacterial infections are biofilm-related. According to the centers for disease control and prevention, at least 65% of all bacterial infections in humans are caused and accompanied by biofilms included chronic rhinosinusitis[1]. Biofilms have shown affect treatment outcomes in CRS patients. Persistent inflammation of the sinonasal tissues and is known to cause significant physical symptoms, negatively the quality of life and substantially impair daily functioning[2].

Bacteria embedded in biofilms were often difficult to eradicate with standard antibiotic regimens[3]. The treatment of resistant bacteria requires doses 10-1000 times of an antibiotic than planktonic bacteria[4, 5]. Tatar *et al*.[6] in the studying of biofilm found 32 patients CRS without polyp, 24 (75%), 14 of 19 (73.7%) of CRSwNP (chronic rhinosinusitis with nasal polyps) and 11 of 15 (73.3%) of CRSsNP (chronic rhinosinusitis without nasal polyps), respectively. Danielsen *et al*.[1] *Staphylococcus aureus* have in the play of the persistence of chronic infections included Chronic Rhinosinusitis. His research examined in mucosal specimens of 15 patients. The results found seven biofilms the 15 patients. Biofilm formation is one of the defense mechanisms of *Staphylococcus aureus*[3]. Singhal[2] in the study of 39 CRS patients, 30 caused bacterial biofilm and 70% involving *Staphylococcus aureus*. In addition to the difficulty of treating biofilms with the standard antibiotic, recently the alternative treatment has to play their role in the treatment of biofilms[7, 8]. Propolis is a natural product produced by honeybees in the form of sap (resin) collected from shoots of trees, gums, bushes and other plant sources[9, 10]. Various studies have shown that the propolis has an antimicrobial effect. Propolis was known effective in the fighting of gram-positive bacteria, especially, *Staphylococcus aureus* and gram-negative bacteria such as Salmonella sp.[11]. Astani etc., evaluated the purified antibacterial activity of the propolis extract against Methicillin-Susceptible *Staphylococcus aureus* (MSSA) and methicillin-Resistant *Staphylococcus aureus* (MRSA), *Streptococcus pyogenus* and vancomycin-susceptible and Vancomycin-Resistant *Enterococcus faecalis* (VRE). Aissat *et al*.[8] showed that the propolis has anti-biofilm activity against biofilm produced *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated *in-vitro* from urine catheter. Bryan *et al*.[12] exposure the Russian of propolis extracts to the *Staphylococcus aureus* biofilm led to the degradation of the extracellular polymer matrix and killed more 99.9% *Staphylococcus aureus* after 12 h of exposure.

Confocal Laser Scanning Microscopy (CLSM) is an ideal tool for monitoring at micro-spheric size spatial resolution and enables the study of non-destructive biofilms through examination of all layers in different depths, making it possible to reconstruct a three-dimensional structure[13]. Cerca[7] performed an analysis using CLSM against the biofilm *Staphylococcus epidermidis* gave farnesol, vancomycin and rifampicin obtained reduced biomass biofilms.

This research is intended to analyze Trigona sp. different doses of Malang Indonesia on the isolated biofilm *Staphylococcus aureus* from CRS. Analysis using CLSM with Syto9 green nucleic acid staining.

MATERIALS AND METHODS

Preparation Ethanolic Extract of Propolis (EEP): About 1 kg Propolis put in a glass container and given 70% ethanol, stirring several times and allowed to stand for 24 h. The screening does after 24 h separate the extract. The dregs are squashed by immersion in 70% ethanol and stirred several times and then stand for 24 h. Filtering is done to separate the extract. Do the same thing for up to 3 days. The collected ethanol extract was evaporated over the water-bath at a temperature of 60°C until all ethanol evaporated. Made EEP solution with dose 0,2, 0,4, 0,8, 2, 8 and 10%. Measurement intensity of expression Syto9 and biofilms profile using CLSM magnification 400x.

Preparation of microorganisms: Isolate taken from middle meatus discharge CRS patients who undergo functional endoscopic sinus surgery at RS PHC Surabaya Indonesia. The isolates were cultured on Mannitol Salt Agar to obtain *Staphylococcus aureus*. Identification of *Staphylococcus aureus* examined for gram staining, catalase test and coagulase test. The test of biofilm culture using congo red agar. The biofilm formed was micro-titered on a 24 well culture plate 48 hour using EEP solution dosages of 0, 0.2, 0.4, 0.8, 2, 8, 10% and negative control, respectively.

Microtiter: *Staphylococcus aureus* biofilms are grown in Tryptic Soy Broth-Glucose (TSB-G) medium and incubated for 24 h at 37°C. Spectrophotometry was performed on a wave of 625 nm to obtain

108 bacteria mL^{-1}. After that placed into the well of polystyrene microtiter plate, including negative control. Incubated for 48 h at 37°C. In the sample added propolis extract according to the dose. Incubated for 48 h at 37°C.

CLSM staining: The biofilms formed on the 24 well culture plates were carefully rinsed with a 2x pH 7.4 solution of Phosphate Buffered Saline (PBS) for 5 min while shaking. Stained with Syto9 fluorescent nucleic acid marker 1: 500. Incubate in a dark room at 30°C for 45 min, then wash with 2x PBS for 5 min while shaking. The CLSM examination uses 400x magnification. Each well checked for 3x field of view.

RESULTS

Biofilm culture results examined after 48 h. Staining biofilms using Syto9 green fluorescent nucleic acid. Measurement intensity of expression Syto9 using clam magnification 400x dipegted in Fig. 1.

From Fig. 1 shows a decrease in the intensity of Syto9 and biofilm depth on each slide along with increasing dose of EEP.

Table 1 and Fig. 2 shown the mean, standard deviation, minimum and maximum values of the EEP dosages group in the Syto9 expression. Based on the table it is shown that the greater the dose of EEP given the smaller the average value on the intensity Syto9. In the post hoc test, the expression result in Syto9 showed that the dosage of propolis was 0-0,8% significantly different compared to negative control and 2-10% did not found significant differences.

Table 2 and Fig. 3 shown the mean, standard deviation, minimum value and maximum value in the depth of the biofilm *Staphylococcus aureus*. The mean, standard deviation, minimum and maximum values of biofilm depth in the 2-10% dose groups tended to decrease. In the post hoc test, depth of biofilm showed that the dosage of 0-0,8% propolis was significantly different compared to negative control and 2-10% did not found significant differences.

Table 3 shows the results of Kruskal Wallis test an intensity of expression Syto9 and depth of biofilm. By using Syto9 give the significance (p) were 0.001. So, the study finds that there was a difference of intensity of expression Syto9 results in the treatment dose group ($p<\alpha = 0.05$). In the biofilm depth, the result of significance (p) was 0.001. After that had found, there was a difference of result in dose group ($p<\alpha = 0.05$).

In this study, we observed the biofilm *Staphylococcus aureus* had been given EEP Trigona sp. Malang Indonesia different doses of 0-10%. Sample staining using Syto9 green-fluorescent nucleic acid dye. The intensity of

Table 1: Correlation dosages EEP with intensity of expression Syto9

EEP dose (%)	n	Mean	SD	Minimum	Maximum
0	3	1098.9490	568.29614	447.14	1490.54
0.2	3	928.7480	239.55995	652.13	1067.06
0.4	3	913.3160	331.39747	555.94	1210.49
0.8	3	305.8043	181.10176	128.64	490.60
2	3	106.3680	21.39301	84.26	126.97
8	3	7.5133	7.65214	2.04	16.26
10	3	0.1240	0.16215	0.00	0.31
Control	3	0.0233	0.02774	0.00	0.05

Table 2: Correlation EEP dosages with biofilm depth (μm)

Dosis propolis (%)	n	Mean	SD	Minimum	Maximum
0	3	15.6667	1.52753	14.00	17.00
0.2	3	18.0000	1.00000	17.00	19.00
0.4	3	14.3333	1.04083	13.50	15.50
0.8	3	15.6667	1.44338	14.00	16.50
2	3	11.0000	0.50000	10.50	11.50
8	3	8.8333	1.25831	7.50	10.00
10	3	8.3333	2.51661	6.00	11.00
Control	3	7.1667	0.76376	6.50	8.00

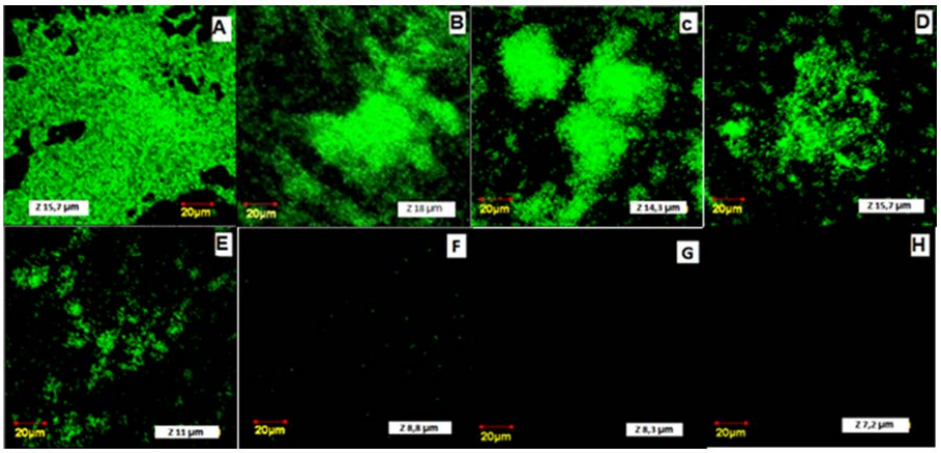

Fig. 1(A-H): Intensity of expression Syto9 and biofilm depth. Dosages EEP: (A) 0%, (B) 0,2%, (C) 0,4%, (D) 0,8%, (E) 2%, (F) 8%, (G) 10% and (H) negative control

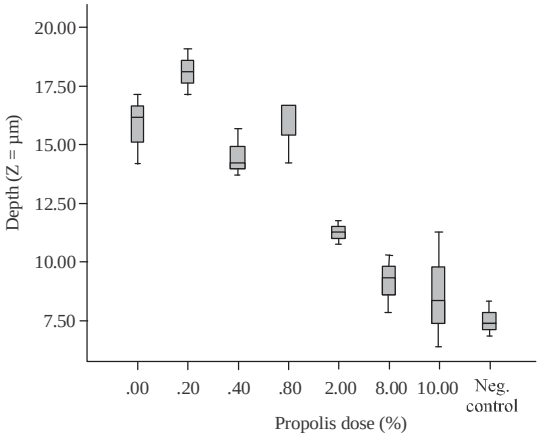

Fig. 2: Boxplot correlation dosages EEP with intensity of expression Syto9

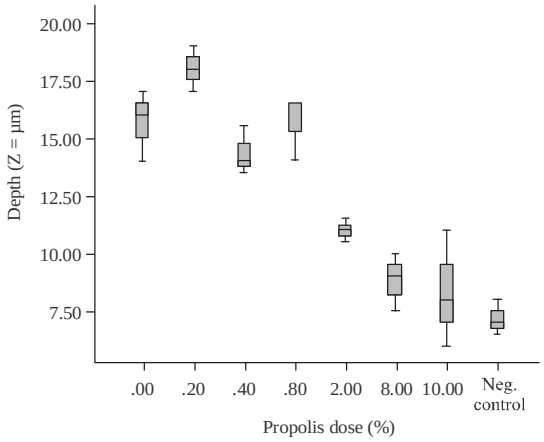

Fig. 3: Boxplot correlation EEP dosages with biofilm depth

Table 3: Kruskal Wallis test on intensity of expression Syto9 and depth of biofilm

	Kruskal Wallis test	
Variables	Sig.	(p-value)
Intensity of expression Syto9	0.001	$\alpha = 0.05$
Depth of biofilm	0.001	

expression Syto9 signifies the number of bacteria *Staphylococcus aureus* present in the biofilm architecture. Table 1 shown the higher dose in the expression of Syto9. May be the dose of EEP Trigona sp. reduces the number of bacteria *Staphylococcus aureus* in the biofilm. The statistical analysis results in Fig. 2 shown that the significant difference in the decrease in the expression of intensity Syto9 giving EEP Trigona sp. 2% or more. Table 2 shown the higher dose to the less the depth biofilm. Then the statistical analysis results in Fig. 3 shown that the significant difference in the decrease of the expression intensity of Syto9 giving EEP Trigona sp. 2% or more.

DISCUSSION

Infect due to the MRSA still as a problem in the hospital including Indonesia. The almost organ in human can infected by MRSA. A study from Karthoum and Shagra found that all of the *Staphylococcus aureus* strains were resistant to methicillin antibiotic 100%[14]. There for our study used the MRSA as a bacterial model. The bacteria were isolated from the patient suffer CRN from PHC Surabaya Indonesia.

Propolis has been long known as a popular drug among people in various countries and widely prepared as a healthy food and beverage[15]. Propolis has known as a quality healing method, since, Egyptian and Greek civilization. Hippocrates, an inventor of modern medical science, uses propolis to cure diseases including pain and wounds[11, 10]. Clinically, propolis was known effective as antibacterial, antifungal and anti-inflammatory, antiviral, antioxidant, anti-tumor, antiprotozoal, local anesthetics, immunostimulating, cytostatic and hepatoprotective[9, 15, 10]. The antimicrobial activities of propolis have been researched over recent years as alternatives for new therapeutic agents for the treatment of bacterial biofilm infections[8].

CRS with biofilms has recurrent infections, ongoing mucosal inflammation and persistent postoperative symptoms[2]. One of the defense mechanisms of *Staphylococcus aureus* is the capacity to produce biofilms. Bacteria that embedded in the biofilms are often difficult to eradicate with standard antibiotic regimens and inherently resistant to host immune responses. In this study, the *Staphylococcus aureus* biofilm had been given EEP Trigona sp. with different doses of 0-10%. In the post hoc test, the intensity of expression Syto9 and the depth of biofilm (Table 1 and 2; Fig. 1 and 2) that the dose of EEP 0-0,8% was significantly different if this test compares to the negative control and 2-10% was not found to be significantly different. The result shows that a 2-10% EEP can inhibit the growth of *Staphylococcus aureus* biofilms. Propolis inhibitory capability to bacteria is different depending on the type of propolis, geographic origin, plant source of the main component.

Kruskal Wallis test results both the intensity of expression Syto9 and the depth of biofilm are significant $p = 0.001$ ($\alpha = 0.05$), so, EEP Trigona sp. Malang Indonesia can inhibit the production of *Staphylococcus aureus* biofilm from CRS isolate. Aissat *et al*.[8] propolis Sahara honey against *Staphylococcus aureus* with the dose of 16-47%, *Pseudomonas aeruginosa* with dose 17-57% and *Escherichia coli* 16-65% *in-vitro* can prevent invasive biofilm formation. Bryan *et al*.[12] exposure to Russian propolis extracts of the *Staphylococcus aureus* biofilm led to the degradation of the extracellular polymer matrix and killed 99.9% more *Staphylococcus aureus* after 12 h of exposure. Wojtyczka *et al*.[16] showed that the

biofilm formation ability of the all tested *Staphylococcus epidermidis* strains inhibited at EEPP (Polandia) concentrations ranging from 0.39-1.56 mg mL^{-1}.

Various antibacterial mechanisms in propolis have proposed by researchers. Cushnie and Lamb[17] reported the presence of other flavonoids as galanin also has antibacterials. Mechanisms involved in overcoming bacterial cytoplasmic membranes by removing potassium ions and causing damage from autolysis cells. Quercetin also found in honey that serves to increase membrane permeability and eliminate its potential, allowing bacteria to lose the ability to synthesize ATP, transport membranes and motility[18]. Ajuha and Ajuha[19] found that propolis was known to have the ability of antimicrobial activity by inhibiting bacterial mobility and altering the deeper permeability of bacterial membranes. The ability of propolis as an antimicrobial were known to be effective in gram-positive bacteria such as *Staphylococcus aureus* than in gram-negative bacteria. Propolis affects the cytoplasmic membrane and able to inhibit bacterial motility, enzyme activity, cell division and protein synthesis. After that, propolis also inhibits RNA-polymerase which partially explains the synergism of propolis with drugs that act to inhibit protein synthesis[20].

CONCLUSION

Ethanolic Extract of Propolis Trigona sp. Malang Indonesia can inhibit production of *Staphylococcus aureus* biofilm from isolate secret CRS of starch. Propolis has a variety of bacteria anti-bacterial mechanisms. The ability of anti-biofilms depends on the concentration of the propolis.

We declare no conflict interest in this study and also passed for examination by ethical clearance our institutional team.

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