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EFFECT OF ETHANOLIC EXTRACT *PROPOLIS TRIGONA* SPP. MALANG INDONESIA ON ISOLATE *STAPHYLOCOCCUS AUREUS* BIOFILM ARCHITECTURE FROM CHRONIC RHINOSINUSITIS A CONFOCAL LASER SCANNING MICROSCOPIC STUDY

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Keywords:

CRS, EEP, *Staphylococcus aureus* biofilm, CLSM

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ABSTRACT: Biofilm in chronic rhinosinusitis (CRS) is the infection by bacteria, which is difficult to overcome. It has recurrent infections, mucosal inflammation, and postoperative symptoms. Propolis is a natural product that is potential as an anti-biofilm choice. The purpose of this study was to determine the ethanolic extract propolis (EEP) effect on the morphology of *Staphylococcus aureus* biofilm. The purpose of this study was to determine the effect of EEP on the morphology of Staphylococcus aureus biofilm. Isolate *Staphylococcus aureus* was taken from meatus medius of CRS patients in endoscopy sinus surgery at PHC Hospital Surabaya, Indonesia. Identification of Staphylococcus aureus uses Mannitol Salt Agar, Gram Staining, Catalase test, and Coagulase test. Biofilm produced from congo red agar culture. EEP was macerated from alcohol 70%, and after that the biofilm formed put in 24 well culture plate for 48 h using EEP solution dosages of 0.0%, 0.2%, 0.4%, 0.8%, 2.0%, 8.0%, 10.0% and negative control. Measurement intensity of expression Syto9 and the depth of biofilm using Confocal Laser Scanning Microscopy (CLSM) magnification is 400x. There were observed for 3 times field of view well. Morphology of *Staphylococcus aureus* biofilm was assessed by a decrease in the intensity of expression Syto9 and depth of biofilm. Based on the Kruskal Wallis test, the results showed that there were significant differences in the intensity of Syto9 expression p 0.001 <α 0.05 and depth of biofilm p 0.001 <α 0.05. In the Post Hoc test, EEP *Trigona sp.* 2.0% -10.0% inhibits biofilm growth.

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].

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Biofilms have shown affect treatment outcomes in CRS patients [2, 3]. Persistent inflammation of the sinonasal tissues and is known that cause significant physical symptoms, negatively the quality of life, and substantially impair daily functioning [2, 3, 4, 5].

Bacteria embedded in biofilms were often difficult to eradicate with standard antibiotic regimens [6, 7]. The treatment of resistant bacteria requires doses of 10-1000 times of an antibiotic than planktonic bacteria [1]. One of CRS etiology and pathogenesis are biofilms [8].

A study of 33 patients was divided into 2 groups, 26 CRS patients and 7 control group patients who underwent septoplasty. Biofilms were evaluated by scanning electron microscopy. Biofilm was detected in 14 (42.4%) of 33 patients. Biofilm was present in 13 (50%) of 26 patients in the CRS group, but only one (14.3%) of the seven patients in the control group [9]. *Staphylococcus aureus* has 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 from 15 patients. Biofilm formation is one of the defense mechanisms of *Staphylococcus aureus* [10]. Singhal (2011) in the study of 39 CRS patients, 30 patients, were caused by bacterial biofilm, and 70% involve *Staphylococcus aureus* [11]. Also, the difficulty of treating biofilms with the standard antibiotic is the alternative treatment that has to play their role in the treatment of biofilms [12].

Propolis is the natural product produced by honeybees in the form of sap (resin) is collected from shoots of trees, gums, bushes, and other plant sources. Various studies have shown that the propolis has an antimicrobial effect [13, 14]. Propolis was known as an effective product in the fighting of gram-positive bacteria especially *Staphylococcus aureus* and gram-negative bacteria such as Salmonella sp evaluated the purification of antibacterial activity of the propolis extract against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) [14, 15, 16]. The propolis has anti-biofilm activity against biofilm produced *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated *in-vitro* from urine catheter [12].

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 hours of exposure [17]. CLSM is an ideal tool for monitoring at micro-spheric size spatial resolution and enables the study of non-destructive biofilms through an examination of all layers in different depths, making it possible to reconstruct biofilm morphology, three-dimensional structure, characteristics of biofilm growth, quantification of biofilms, the study of biofilm edges [18]. Cerca (2010) performed an analysis using CLSM against the biofilm *Staphylococcus epidermidis* gave farnesol, vancomycin, and rifampicin obtained reduced biomass biofilms [19].

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

MATERIALS AND METHODS:

Preparation Ethanolic Extract of Propolis (EEP): One-kilogram Propolis put in a glass container and given 70% ethanol, stirring several times. It allowed standing for 24 h. The screening has done after 24 h separate of the extract. The dregs are squashed by immersion in 70% ethanol and stirred several times and then stand for 24 h. Filtering was 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%, 2.0%, 8.0% and 10.0%. Measurement intensity of expression Syto9 and biofilms profile use CLSM magnification 400 times.

Preparation of Microorganisms: Isolate was taken from middle meatus discharge CRS patients who undergo functional endoscopic sinus surgery at PHC Hospital 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 used congo red agar. The biofilm was formed micro titered on 24 culture plate at 48 h used EEP solution dosages of 0,0%, 0.2%, 0.4%, 0.8%, 2.0%, 8.0%, 10.0% 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 λ625 nm to obtain 10^8 bacteria/ml. After that placed into the well of a polystyrene microtiter plate, including negative control and incubated for 48 h at 37 °C. In the sample added propolis extract according to the dose. Incubated for 48 h at 37 °C [20].

CLSM Staining: The biofilms formed on the 24 culture plates were carefully rinsed with 2 times pH 7.4 solution of Phosphate Buffered Saline (PBS) for 5 min while it was shaking. Stained with Syto 9 fluorescent nucleic acid marker 1: 500.

Incubate in a dark room at 30 °C for 45 min, then wash with 2 times PBS for 5 min while shaking. The CLSM (Olympus) type FV1000 dan dianalisa dengan Olympus Fluoview Software version 1.7a examination uses 400 times magnification. There were observed for 3 times field of view [21].

RESULTS: Biofilm culture results examined after 48 h. Staining biofilms used syto9 green fluorescent nucleic acid. Measurement intensity of expression Syto9 usedCLSM magnification 400 times can be viewed on **Fig. 1**.

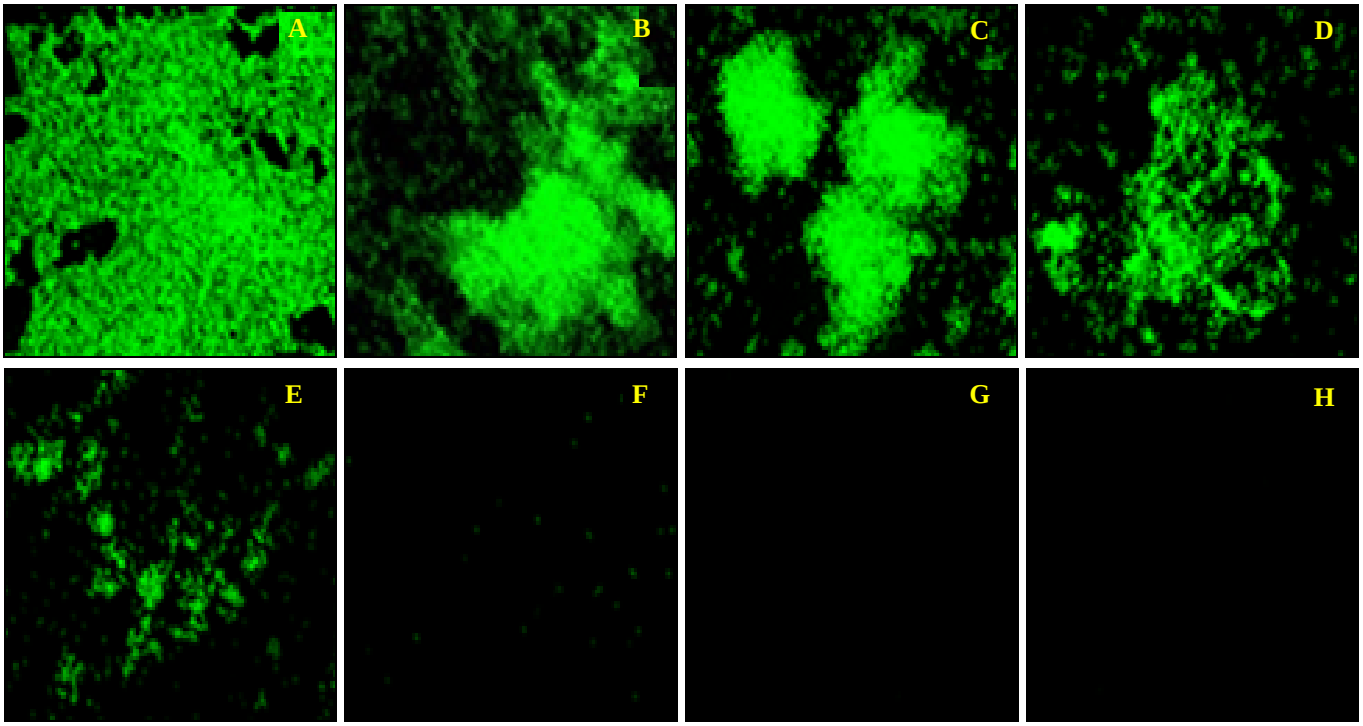

FIG. 1: THE ARCHITECTURE OF *STAPHYLOCOCCUS AUREUS* BIOFILM ON ADMINISTRATION OF DIFFERENT EEP WAS EXAMINED BY USING CLSM (OLYMPUS) TYPE FV1000 WITH 400 TIMES MAGNIFICATION AND ANALYZED BY USING OLYMPUS FLUOVIEW SOFTWARE VERTION 1.7A. DOSAGES: (A) 0.0%, (B) 0,2%, (C) 0,4%, (D) 0,8%, (E) 2.0%, (F) 8.0%, (G) 10.0% (H) NEGATIVE CONTROL.

Note: A. The architecture of *Staphylococcus aureus* biofilm on the administration of EEP 0.0% are1098.95 au (arbitrary unit) for the Intensity of Expression Syto9 and 15.7 µm (micro meter) for the depth of biofilm. B. The architecture of *Staphylococcus aureus* biofilm on the administration of EEP 0.2% is 928.75 au for the Intensity of Expression Syto9 and 18 µm for the depth of biofilm. C. The architecture of *Staphylococcus aureus* biofilm on the administration of EEP 0.4% are 913.32 au for the Intensity of Expression Syto9 and 14.3 µm for the depth of biofilm. D. The architecture of *Staphylococcus aureus* biofilm on the administration of EEP 0.8% are 305.80 au for the Intensity of Expression Syto9 and 15.7 µm for the depth of biofilm. E. The architecture of *Staphylococcus aureus* biofilm on the administration of EEP 2.0% are 106.37 au for the Intensity of Expression Syto9 and 11 µm for the depth of biofilm. F. The architecture of *Staphylococcus aureus* biofilm on the administration of EEP 8.0% are 7.52 au for the Intensity of Expression Syto9 and 8.8 µm for the depth of biofilm. G. The architecture of *Staphylococcus aureus* biofilm on the administration of EEP 10% are 0.12 au for the Intensity of Expression Syto9 and 8.3 µm for the depth of biofilm. H. The architecture of *Staphylococcus aureus* biofilm on negative control are 0.02 au for the Intensity of Expression Syto9 and 7.2 µm for the depth of biofilm.

TABLE 1: CORRELATION DOSAGES EEP WITH THE INTENSITY OF EXPRESSION SYTO9

S. no.	EEP dose	n	Mean	Std. deviation	Minimum	Maximum
1	0.0%	3	1098.9490	568.29614	447.14	1490.54
2	0.2%	3	928.7480	239.55995	652.13	1067.06
3	0.4%	3	913.3160	331.39747	555.94	1210.49
4	0.8%	3	305.8043	181.10176	128.64	490.60
5	2.0%	3	106.3680	21.39301	84.26	126.97
6	8.0%	3	7.5133	7.65214	2.04	16.26
7	10.0%	3	0.1240	0.16215	0.00	0.31
8	Control	3	0.0233	0.02774	0.00	0.05

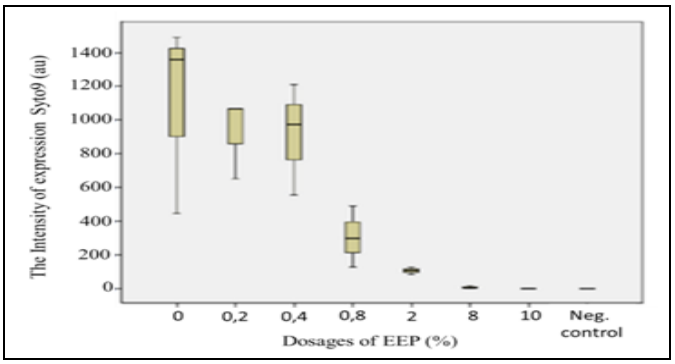

FIG. 2: BOXPLOT CORRELATION DOSAGES EEP WITH THE INTENSITY OF EXPRESSION SYTO9

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 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: CORRELATION EEP DOSAGES WITH THE DEPHT OF BIOFILM (µm)

S. no.	Dosis Propolis	n	Mean	Std deviation	Minimum	Maximum
1	0,0%	3	15.6667	1.52753	14.00	17.00
2	0.2%	3	18.0000	1.00000	17.00	19.00
3	0.4%	3	14.3333	1.04083	13.50	15.50
4	0.8%	3	15.6667	1.44338	14.00	16.50
5	2,0%	3	11.0000	0.50000	10.50	11.50
6	8,0%	3	8.8333	1.25831	7.50	10.00
7	10,0%	3	8.3333	2.51661	6.00	11.00
8	Control	3	7.1667	0.76376	6.50	8.00

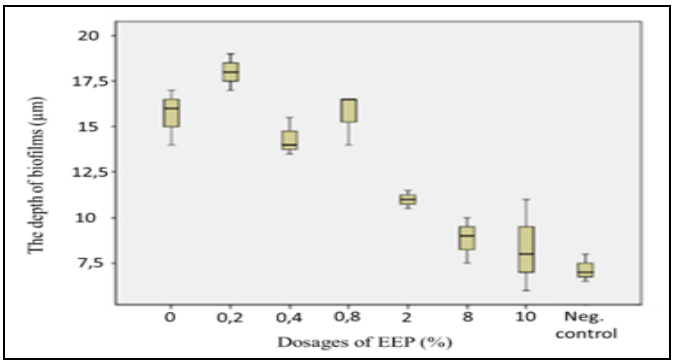

FIG. 3: BOXPLOT CORRELATION EEP DOSAGES WITH THE DEPHT OF BIOFILM

Table 2 and **Fig. 3** show the mean, standard deviation, minimum value, and maximum value of the depth biofilm *Staphylococcus aureus*. The mean, standard deviation, minimum, and maximum values of the depth of biofilm in the 2.0% - 10.0% dose groups tended to decrease. In the post hoc test, depth of biofilm showed that the dosage of 0.0%-0.8% propolis was significantly different compared to negative control and 2.0% - 10.0% did not found significant differences.

TABLE 3: KRUSKAL WALLIS TEST ON INTENSITY OF EXPRESSION SYTO9 AND DEPTH OF BIOFILM

Variables	Kruskal Wallis Test Sig. (p)	
Intensity of expression Syto9	0.001	α (alfa) = 0.05
Depth of biofilm	0.001	

Table 3 shows the results of an intensity of expression Syto9 from Kruskal Wallis test and depth of biofilm. Syto9 gave the significance (p) were 0.001. So, the study found that there was a difference in intensity of expression Syto9 results in the treatment dose group ($p < \alpha$ (alfa) = 0.05). In the depth of biofilm, the result of significance (p) was 0.001. It can be concluded that there was a different result in dose group ($p < \alpha$ (alfa) = 0.05).

This study observed the biofilm *Staphylococcus aureus* had been given EEP Trigona sp Malang Indonesia different doses of 0.0% to 10.0%. Sample staining used Syto 9 green-fluorescent nucleic acid dye. The intensity of expression Syto 9 signifies the number of bacteria *Staphylococcus aureus* present in the biofilm architecture. **Table 1** shown the higher dose in the expression of Syto 9.

The dose of EEP Trigona Sp reduces the number of bacteria *Staphylococcus aureus* in the biofilm. The statistical analysis results in **Fig. 2** show that the significant difference in the decrease in the expression of intensity Syto 9 gave EEP Trigona Sp 2% or more. **Table 2** shown the higher dose to the less depth biofilm. Then the statistical analysis results in **Fig. 3** show that the significant difference in the decrease of the expression intensity of Syto 9 gave EEP Trigona Sp 2.0% or more.

DISCUSSION: Infect due to the MRSA still as a problem in the hospital, including Indonesia. The almost organ in human can be infected by MRSA. A study from Karthoum and Shagra found that all of the *Staphylococcus aureus* strains were resistant to methicillin antibiotic 100% [22]. Therefore our study used the MRSA as a bacterial model. The bacteria were isolated from the patient suffer CRS from PHC Surabaya Indonesia. Propolis has been long known as a popular drug among people in various countries and widely prepared as healthy food and beverage [13, 23, 24]. Propolis has known as a quality healing method since Egyptian and Greek civilization [25]. Hippocrates, an inventor of modern medical science, uses propolis to cure diseases, including pain and wounds [25, 26]. Clinically, propolis was known effective as antibacterial, antifungal and anti-inflammatory, antiviral, antioxidant, anti-tumor, antiprotozoal, local anesthetics, immunostimulating, cytostatic, and hepatoprotective [27, 28, 29, 30, 31, 32]. The antimicrobial activities of propolis, have been researched over recent years as alternatives for new therapeutic agents for the treatment of bacterial biofilm infections [10, 33].

CRS with biofilms has recurrent infections, mucosal inflammation, and postoperative symptoms [11, 34]. 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 [35]. In this study, the *Staphylococcus aureus* biofilm had been given EEP Trigona sp with different doses of 0.0% to 10.0%. In the post hoc test, the intensity of expression Syto 9 and the depth of biofilm **Table 1** and **2**; **Fig. 1** and **2** that the dose of EEP 0.0-0.8% was significantly different if this test compares to the negative control and 2-10% was not found significantly different. The result shows that 2.0-10.0% EEP inhibited the growth of *Staphylococcus aureus* biofilms. Propolis inhibitory capability to bacteria is different depending on the type of propolis, geographic origin, the plant source of the main component.

Kruskal Wallis test results both the intensity of expression Syto 9 and the depth of biofilm are significant $p = 0.001$ ($\alpha = 0.05$), so EEP Trigona Sp Malang Indonesia inhibited the production of *Staphylococcus aureus* biofilm from CRS isolate. Aissat (2016) 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* prevent invasive biofilm formation [10]. Bryan (2015) 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. The combination of cranberry and propolis has a strong impact on the motility and the biofilm formation of a collection of uropathogenic *Escherichia coli* (UPEC) [17, 36]. Wojtyczka (2013) showed that the biofilm formation ability of the all tested *Staphylococcus epidermidis* strains inhibited at EEPP (Polandia) concentrations ranging from 0.39 to 1.56 mg/ml [37].

Various antibacterial mechanisms in propolis have proposed by researchers [38]. Cushnie and Lamb (2005) reported the presence of other flavonoids as galanin also has antibacterials [39]. 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 [40]. Ajuha (2011) found that propolis was known as a product that has ability of antimicrobial activity by inhibiting bacterial mobility and altering the deeper permeability of bacterial membranes [41]. The ability of propolis as an antimicrobial was known as an effective in gram-positive bacteria such as *Staphylococcus aureus* than in gram-negative bacteria. Propolis affects the cytoplasmic membrane and is 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 [42].

CONCLUSION: Ethanolic extract of *Propolis trigona* Sp Malang Indonesia inhibited the 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.

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

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