

The Effects of Green Betel Leaf (*Piper betle*) Extract Eye Drops on The Number of *Staphylococcus aureus* Colonies in Conjunctivitis Wistar Rats Model (*Rattus norvegicus*)

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ABSTRACT

Irrational use of antibiotics can cause resistance to some diseases such as bacterial conjunctivitis caused by *Staphylococcus aureus*. Previous research said that green betel leaf (*Piper betle*) contains antimicrobial compounds such as eugenol, cavalry, tannins, saponins, and flavonoids which also have antiseptic power as well as antibiotics. This study aims to prove the decrease in the number of *Staphylococcus aureus* colonies after giving green betel leaf eye drops (*Piper betle*) to conjunctivitis Wistar rats (*Rattus norvegicus*). This research was true experimental with a pretest-posttest control group design. 25 male white Wistar rats as objects were selected by simple random sampling and grouped into 5 groups randomly. The rats were given *Staphylococcus aureus*, control group K+ was given 0.5% levofloxacin eye drops, and K- was given aquadest as a comparison. Treatment groups P1, P2, and P3 were given different concentrations of betel leaf eye drops. Swab pretest was done 3 days after the rats were inoculated with *Staphylococcus aureus* and swab post-test was done 5 days after the rats were given green betel leaf eye drops, the calculation of *Staphylococcus aureus* bacteria colonies used the Total Plate Count (TPC) method. There was a significant decrease ($p < 0.05$) in the number of bacterial colonies for K-, K+, P1, P2, and P3 groups. In addition, there was a significant difference ($p < 0.05$) between K+ with P1 and K+ with P3. There was a decrease in the number of *Staphylococcus aureus* for all groups of conjunctivitis Wistar rats (*Rattus norvegicus*)

Keywords: Bacterial conjunctivitis; *Staphylococcus aureus*; *Piper betle*; Total Plate Count

INTRODUCTION

Conjunctivitis is an inflammatory process caused by both infection and non-infection of the conjunctiva, which is characterized by vascular dilatation, cellular infiltration, and exudation (Vaughan *et al.*, 2016). In Indonesia, conjunctivitis cases is ranked in the top 10 most outpatient diseases in 2009. Out of 135,749 patients who visited the eye clinic, 73% among them were conjunctivitis cases (Kemenkes RI, 2010). Conjunctivitis is the most common eye disease in the world. Conjunctivitis is ranked number 3 in the world after cataracts and glaucoma, especially conjunctivitis, which spreads very quickly. This disease varies from mild hyperemia with watery eyes to severe with thick purulent secretions. In general, the most common causes of bacterial conjunctivitis are gram-positive microorganisms, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Staphylococcus epidermidis* (Afjeiee *et al.*, 2013). Mild conjunctivitis is usually benign and self-limited and can be monitored without therapy or easily treated with antibiotics (Epling, 2010). *Staphylococcus aureus* is a normal

bacterial flora of human skin, nose, and throat (Warsa, 2011).

Part of the betel plant, such as betel leaf, was believed to have benefits to strengthen the teeth, heal small wounds in the mouth, stop bleeding gums, and act as a mouthwash. However, there are still few people who knew about the antibacterial properties of the betel leaf (Inayatullah, 2012). Most of the antibacterial effect of betel leaf comes from its essential oil content that consists of phenol compounds and some of their derivatives such as eugenol and chavicol which have the potential to denature bacterial cell proteins (Devi *et al.*, 2010). Eugenol compounds have bactericidal properties by increasing bacterial membrane permeability, while chavicol compounds have five times stronger bactericidal properties compared to other phenol compounds (Haq *et al.*, 2013).

According to the high number of conjunctivitis cases and high rate of antibiotic resistance toward *S.aureus*, and also the antibacterial potential of betel leaf extract content, researchers are interested to investigate the effect of the administration of betel leaf extract (*Piper betle*) eye drops on conjunctivitis wistar rats model caused by *Staphylococcus aureus*.

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METHODOLOGY

This research was true experimental research with a pretest-posttest control group design. The research object was 25 male white rats with pure Wistar strain selected by simple random sampling and grouped into 5 groups. Group K + as comparison group, where the rats received levofloxacin 0.5% eye drops + *Staphylococcus aureus* 1.5×10^8 CFU / ml (n = 5), Group K- as comparison group, where the rats received drops of distilled water + *Staphylococcus aureus* 1.5×10^8 CFU / ml (n = 5), group P1 was given green betel leaf extract eye drops with a concentration of 50% + *Staphylococcus aureus* 1.5×10^8 CFU / ml (n = 5), group P2 was given green betel leaf extract eye drops with a concentration of 75% + *Staphylococcus aureus* 1.5×10^8 CFU / ml (n = 5) and the P3 group was given 100% concentration of green betel leaf extract eye drops + *Staphylococcus aureus* 1.5×10^8 CFU / ml (n = 5). We used 0.5 McFarland standard for 1.5×10^8 CFU / ml of *Staphylococcus aureus*. The eye drops were applied by opening the eyelids of rats wide to make sure all the surfaces got wrapped by betel leaf extract and then dropped the extract carefully. This research already got ethical approval with number 91/EC/H/FK-UNDIP/IX/2020.

Preparation of Green Betel Leaf Extract and Eye Drops

The method used in extracting green betel leaves is maceration using 70% ethanol as solvent. Green betel leaf extract formulated in various concentrations, 50%, 75%, and 100% which are dissolved in distilled water and packaged in the form of eye drops. Dose 50% : $50/100 \times 10 \text{ ml} = 5$ ml of betel leaf extract (then added 5 ml of distilled water until it reached 10 ml in total); Dose 75% : $75/100 \times 10 \text{ ml} = 7,5$ ml of betel leaf extract (then added 2,5 ml of distilled water until it reached 10 ml in total); Dose 100% : $100/100 \times 10 \text{ ml} = 10$ ml of betel leaf extract (no need for distilled water).

Green betel leaf eye drops were manufactured for replacing 0.5% levofloxacin eye drops with green betel leaf extract. Other additional compositions on betel leaf extract eye drops are adjusted according to standard additional compositions of antibiotic eye drops. Because green betel leaf extract is only prone to decay at room temperature and has acidic pH properties, an additional component is needed in purpose to keep the product lasting and raise the pH to suit the eye. The additional composition chosen was 0.18% nipagin and 0.1 N NaOH. Besides being used to extend product lifetime, nipagin was chosen because it has weak

antibacterial properties, so it can increase the antibacterial effect of green betel leaf extract. After mixing the green betel leaf extract with its additional composition, the sterilization process can be carried out by boiling the solution at 98 °C - 100 °C temperature for 30 minutes. After the sterilization process was completed, a sterilization test was performed by adding green betel leaf eye drops to the Nutrient Agar (NA) medium.

Staphylococcus aureus Colony Counting

Using a sterile cotton swab, the discharge or the crust of rats' eyes with conjunctivitis was taken without hurting their eyes. A pretest swab was performed 3 days after the rats were inoculated by *Staphylococcus aureus* and posttest swabs were carried out 5 days after the rats were given green betel leaf eye drops, the calculation of *Staphylococcus aureus* bacteria colonies was carried out using the *Total Plate Count* (TPC) method.

Data analysis, and presentation processed using computer software. Furthermore, Wilcoxon test, Paired Sample T test, and Kruskal Wallis were performed to determine the difference in the number of *Staphylococcus aureus* colonies, data is stated to be significant if the value of $p < 0.05$.

RESULT AND DISCUSSION

This study used 25 male Wistar white rats as a sample and the sample selected according to the inclusion criteria with the simple random sampling method and was divided into 5 groups: K (+) : Positive control, as a comparison group, where the rats received 0.5% levofloxacin eye drops + *Staphylococcus aureus* $1,5 \times 10^8$ CFU / ml; K (-) : Negative control, as a comparison group, where the rats received drops of distilled water + *Staphylococcus aureus* $1,5 \times 10^8$ CFU / ml; P1 : The rats received green betel leaf eye drops with a concentration of 50% + *Staphylococcus aureus* $1,5 \times 10^8$ CFU / ml; P2 : The rats received green betel leaf eye drops with a concentration of 75% + *Staphylococcus aureus* $1,5 \times 10^8$ CFU / ml; P3 : The rats received green betel leaf eye drops with a concentration of 100% + *Staphylococcus aureus* $1,5 \times 10^8$ CFU / ml.

All samples were reared in the animal cage of the Sultan Agung Islamic University Biology Laboratory under the lamp lighting and given standard food and drink ad libitum in the morning. This research was conducted for 17 days, consist of 7 days of experimental animal adaptation and 10 days of treatment, there were no exclusion criteria or animal mortality, so that all experimental animals could be swabbed. Termination is carried out using chloroform in

Table I. The Amount of *S. aureus* Colonies of Pretest Group

PRE						
Group	1 st Rat	2 nd Rat	3 rd Rat	4 th Rat	5 th Rat	Mean
K-	3.0	4.0	5.0	3.0	6.0	4.2
K+	4.0	4.0	6.0	5.0	4.0	4.6
P1	7.0	5.0	9.0	8.0	6.0	7.0
P2	4.0	5.0	3.0	6.0	5.0	4.6
P3	5.0	8.0	4.0	6.0	5.0	5.6

of 10⁻⁵ dilution

Table II. The Amount of *S. aureus* Colonies of Post Test Group

POST						
Kelompok	1 st Rat	2 nd Rat	3 rd Rat	4 th Rat	5 th Rat	Mean
K-	2.0	3.0	1.0	3.0	3.0	2.0
K+	1.0	2.0	1.0	2.0	2.0	1.2
P1	1.0	2.0	3.0	1.0	1.0	1.6
P2	1.0	1.0	1.0	1.0	1.0	1.0
P3	1.0	1.0	0	0	1.0	0.6

of 10⁻⁵ dilution

Table III. Differences in *Staphylococcus aureus* Colonies Between Pretest, Post-Test and Differences Among Them Based on Treatment

Measurement	Group					P
	K+ (n=5)	K- (n=5)	P1 (n=5)	P2 (n=5)	P3 (n=5)	
	Mean±SB;	Mean±SB;	Mean±SB;	Mean±SB;	Mean±SB;	
	Median	Median	Median	Median	Median	
	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	
Pretest	4,2±1,3; 4(3-6)	4,6±0,8; 4(4-6)	7,0±1,5; 7(5-9)	4,6±1,1; 5(3-6)	5,2±1,5; 5(3-9)	0,061 [#]
Posttest	2,0±1,0; 2(1-3)	1,2±0,8; 1(0-2)	1,6±0,8; 1(1-3)	1,0±0,0; 1(1-1)	1,2±0,8; 1(1-3)	0,091 [#]
P	0,02 ^{†*}	0,041 ^{§*}	0,042 ^{§*}	0,002 ^{†*}	0,039 ^{§*}	
Difference	-2,2±1,3; -2(-4-(-1))	-3,4±1,5; -3(-5-(-2))	-5,4±1,5; -6(-7-(-3))	-3,6±1,1; -4(-5-(-2))	-3,9±1,7; -4(-7-(-1))	0,030 ^{**}

(*) : Significant (p < 0.05); (‡): Wilcoxon; (#) : Paired-Samples T test; (-) : Kruskal Wallis

a closed glass container, then cremated with a diesel fuel incinerator.

The green betel leaf used in this study was found to be stored in a plastic container, at room temperature and tightly closed. As soon as the green betel leaves are obtained and dried, the preparation of extracts and eye drops was performed at the Biology Laboratory of the State University of Semarang according to the method mentioned. Here are the results of the difference between pre and post test groups that are showed by table I and table II.

The normality test consists of 2 types, the *Kolmogorov - Smirnov* test and *Shaphiro - Wilk* test. *Kolmogorov - Smirnov* test is performed when the

number of samples is more than 50, while *Shaphiro - Wilk* test is used when the number of samples is less than 50. In this research, the number of samples used was less than 50, so the normality test used was *Shaphiro - Wilk*.

Table III shows the difference in the number of *Staphylococcus aureus* bacteria colonies calculated from the results of rat conjunctival secretions with cotton buds swabs measured using the *Total Plate Count* (TPC) method by pour plate using a colony counter.

Based on table III, the lowest number of bacterial colonies on the pretest was found in the K + (control +) group, as amounted as (4.2 ± 1.3) and the highest number of colonies on the pretest was

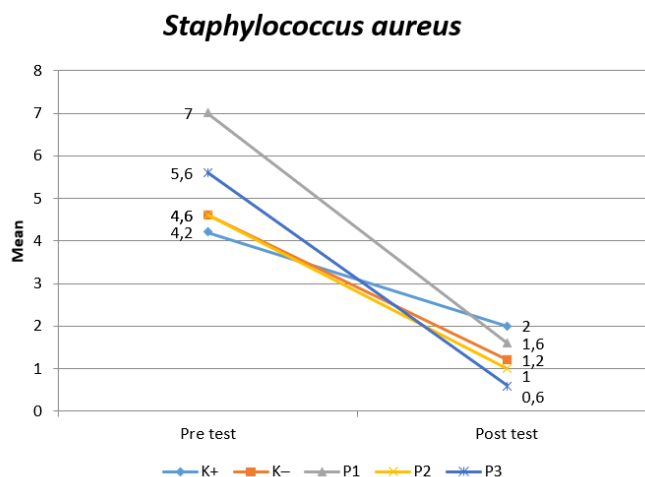


Figure 1. The Average Number of Bacterial Colonies Count at Pretest and Posttest

found in the P1 group (treatment 1) of (7.0 ± 1.5) . This difference stated to be insignificant ($p = 0.061$) according to the Kruskal Wallis test.

At the time of the post-test, the lowest number of colonies was found in the P2 group (treatment 2) as amounted as (1.0 ± 0.0) , and the highest number of colonies was found in the K + group (control +) of (2.0 ± 1.0) . The Kruskal Wallis test results showed that the difference was not significant ($p = 0.091$).

Based on the difference, the lowest number of colonies was obtained in the P1 group (treatment 1) of (-5.4 ± 1.5) and the highest number of colonies was found in the K + group (control +) of (-2.2 ± 1.3) . However, based on the results of the statistical test the difference was stated to be significant ($p < 0.05$; Kruskal Wallis test).

Table III also shows that there was a decrease in bacterial colony's number in the K-, K +, P1, P2, and P3 groups. The results of statistical tests showed that the decrease of bacterial colony number among the groups was significant ($p < 0.05$). Figure 1 shows a decrease in the number of bacterial colonies in the K-, K +, P1, P2, and P3 groups.

Based on data of table IV, it was found that the difference in the number of *Staphylococcus aureus* colonies was statistically significant ($p < 0.05$; Mann Whitney test) between the K + (control +) group with P1 (treatment 1) group and K + (control +) group with P3 (treatment 3) group.

Discussion

This research result shows that there were significant differences between groups. The results

of this research are following the major and minor hypotheses, which stated that green betel leaf (*Piper betle*) eye drops effectiveness against Wistar rats (*Rattus norvegicus*) conjunctivitis model by *Staphylococcus aureus* can be seen from the decrease in bacterial colony's number in each treatment group.

On the 8th day of the experiment, the research objects in all groups were given *S. aureus* bacteria, which is a normal bacterial flora causing acute bacterial conjunctivitis.⁸ All mice had conjunctivitis by letting them incubated for 3 days after dropping *S. aureus*, the clinical signs could be seen from the mice infected *S. aureus* was a discharge which appears in both eyes. On the next day, the rat conjunctival pretest swab was done gently and completely and implanted on MSA media and henceforth, the calculation of the number of bacterial colonies is carried out. On day 12, all groups were given different treatments as the first treatment group was given green betel leaf eye drops with a concentration of 50% + *Staphylococcus aureus* 1.5×10^8 CFU / ml, treatment group 2 was given green betel leaf eye drops with a concentration of 75% + *Staphylococcus aureus* 1.5×10^8 CFU / ml and treatment group 3 was given green betel leaf eye drops with a concentration of 100% + *Staphylococcus aureus* 1.5×10^8 CFU / ml. Whereas for the control treatment as a comparison group, both negative and positive control groups were given different treatments, in the positive control group, the rats received 0.5% levofloxacin eye drops + *Staphylococcus aureus* 1.5×10^8 CFU / ml and in the negative control group, the rats received distilled water drops. + *Staphylococcus aureus* 1.5×10^8 CFU / ml.

Table IV. Difference Between The *Staphylococcus aureus* Colonies According to Mann Whitney Test

<i>Staphylococcus aureus</i>	Category	K-	P1	P2	P3
Difference	K+	0,200	0,020*	0,109	0,017*
	K-	-	0,055	0,829	0,167
	P1	-	-	0,072	0,667
	P2	-	-	-	0,147

* Significant ($p < 0,05$)

Previous research conducted by Gama Wisnu Sanjaya stated that mulberry leaf extract has an antimicrobial activity that can reduce the number of *S. aureus* bacteria colonies.⁹ The mulberry leaves content such as flavonoids and polyphenols is also found in green betel leaves which contain other chemicals such as saponins, flavonoids, and polyphenols. Another research conducted by Seila Inayatullah found that green betel leaf extract can significantly inhibit the growth of *S. aureus* bacteria with strong effectiveness. It is known that more high green betel leaf concentration, produces a stronger inhibition effect toward the growth of *S. aureus*.⁷ Saponin compounds in the betel plant have antimicrobial action where it will damage the cytoplasmic membrane so that it can destroy the cells. Meanwhile, flavonoid compounds are thought to have a working mechanism of denaturing bacterial cell proteins and irreparably damaging cell membranes (Putri, 2010).

This research result shows that treatment groups given green betel leaf eye drops with different concentrations gave a significant reduction of bacteria colonies where the treatment groups 1 and group 3 provided the highest effectiveness in reducing the number of *S. aureus* bacteria compared to a positive control group. Although the decrease in the number of bacteria between groups 1 and 3 was not that much, the results confirmed that group 1 was the treatment group that showed better effectiveness than group 3.

Comparison between one another groups shows that there was no significant difference between one another, where each group had the same effectiveness. So it can be concluded that all groups can reduce the number of *S. aureus* colonies. The reason that might be possible according to this unsuitable result towards the existing theory is caused by the inadequate amount of secretions during swab taking. In addition, uncontrollable object activity such as scratching the eye can bias the results of the study.

If groups 1 and 3 were compared with other groups there was no significant difference where each group had the same effectiveness. So it can be

concluded that all groups can reduce the number of *S. aureus* colonies. The reasons that may have resulted in an unsuitable result were the inadequate amount of secretions during swab taking and in addition, uncontrollable object activity such as scratching the eye which could bias the results of the study.

The limitation of this research is that the researcher cannot control all the activities of the object of research, such as scratching the eye, so that the result of the swab is not optimal and is susceptible to contamination by other bacteria. In addition, the researchers could not control the immunity each mouse had, so the final result was not theoretical.

CONCLUSION

Both green betel leaf eye drops (*Piper betle*) and sterile distilled water and 0.5% levofloxacin eye drops were effective in reducing the number of *Staphylococcus aureus* bacterial colonies against conjunctivitis model Wistar rats. The suggestion that can be given is that the maintenance of Wistar rats needs to be paid more attention so that there are no abnormalities that cause bias in the study, giving treatment to experimental animals needs to be more careful regarding the doses of administration whether it's excessive or not. And it is necessary to change gloves every time you perform a treatment from one group to another.

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