Effect of *Plectranthus scutellarioides (L.)* Leaf Extract as Natural Antibacterial Against *Staphylococcus aureus* and *Escherichia coli* Isolated From Dairy Cattle with Subclinical Mastitis

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ABSTRACT

Mastitis is one of the causes of low milk production in dairy cows, and can be caused by bacteria such as *Escherichia coli* and *Staphylococcus aureus*. Plectranthus scutellarioides (L.) leaf has antibacterial compounds such as flavonoids, eugenol, polyphenols, steroids, tannins, and so on. The type of this research is experimental laboratory research that aims to test the antibacterial activity of the *P. scutellarioides* (L.) leaf extract with ethanol pro analysis as the solvent against *E. coli* and *S. aureus* bacteria isolated directly from the sample of dairy cow's milk which CMT score is (+) 3, by measuring its inhibition ability indicated by the presence of clear zone using the agar-well diffusion method. The extract concentrations tested are 5%, 10%, 20% and 40%. The result of the antibacterial test of *P. scutellarioides* (L.) leaf extract with ethanol p.a. as the solvent showed antibacterial activity against *E. coli* and *S. aureus* bacteria isolated directly from cow's milk samples with subclinical mastitis at certain concentrations.

Keywords: *Plectranthus scutellarioides* (L.), Mastitis, Dairy Cattle, *Staphylococcus aureus, Escherichia coli*

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INTRODUCTION

Dairy milk product is a natural food product with high nutrition that is used to meet human animal protein needs. Important compounds found in milk include carbohydrates (lactose), fat, vitamins and minerals (Safitri and Swarastuti, 2013). Based on data from the Directorate General of Livestock and Animal Health in 2021, the population of dairy cattle is 0.5 million, the number of domestic demand for milk is 4.3 million tons per year, while domestic fresh milk production is0.9 million tons per year, so domestic milk products only contribute 22% of the milk demand amount and the rest is fulfilled with milk imports. Dairy farms in Indonesia are dominated by small-scale businesses where farmers have an average of 2-4 adult dairy cattle per family (Surani, 2011). Because small-scale businesses have limited workers, this results in a lack of good cattle management and care, so the production and quality of milk is low. Breeders have an important role in running milk production, lack of knowledge about hygiene management and the correct way of milking can result in disease in cattle followed by decrease in milk production. One of the common diseases that are found in dairy cattle is mastitis (Tewari, 2014).

Mastitis is an inflammatory disease of the mammary glands caused by several factors, including bacterial infection, trauma, and udder injury (Tewari, 2014). Mastitis is known to have 2 types of disease, the subclinical mastitis and clinical mastitis. Subclinical mastitis is the type that does not show clinical symptoms and is usually identified through examination

of mastitis, either by California Mastitis Test (CMT) or laboratory tests. While clinical mastitis shows clinical symptoms, such as swelling and hardening of the udder followed by pain and heat, decrease in udder function, milk production and quality (Nurhayati and Martindah, 2015). Mastitis-causing bacteria are divided into two groups, environmental such as coliforms (*Escherichia coli* andklebsiella*)*, pseudomonas, and *Streptococcus uberis*, as well as contagious pathogens such as *Staphylococcus aureus, Streptococcus dysgalactiae* and they cause acute or chronic subclinical and clinical mastitis in susceptible hosts (Garvey, 2019). Treatment used for mastitis are commonly antibiotics such as streptomycin, ampicillin, cloxacillin, penicillin, and tetracycline, but it also causes antibiotic resistance in pathogenic bacteria (Yang *et al.*, 2019). Plants with bacteriostatic qualities could be used to treat mastitis in dairy cattle as an alternative to antibiotics because they do not cause bacteria to develop resistance, allowing them to be used for a long period (Radzikowski *et al.,* 2020).

Plectranthus scutellarioides (L.) plant is an ornamental plant that comes from the Lamiaceae family. *P. scutellarioides* (L.) plant is 30-150 cm high with leaf shaped similar to heart shape (Tabalubun, 2013), the leaves also have continuous narrow grooves on either edge that are supported by petioles (Mentari, 2018). The size of the leaves are 6-12 cm long, 5-8 cm broad, the structure is hairy and it gives velvety looks, the color of the leaves are maroon in the middle part, green at the edge of the leaves, meanwhile the base of the leaves are wide and rounded, and the tip is sharp (Kalita *et al.,* 2020). *P. scutellarioides* (L.) plant contains anti-inflammatory, antioxidant, antimicrobial and antibacterial properties, as well as the ability to accelerate wound healing (Rahmawati, 2018 in Utami *et al.,* 2020), and also used as antidiabetes, immunodulator, antihistamine, antihelminthic (Novanti and Susilawati, 2017). This research aims to examine the antibacterial effect of *P. scutellarioides* (L.) leaf extractagainst *S. aureus* and *E. coli* isolated from dairy cattle with subclinical mastitis.

METHODS

In order to know the antibacterial activity of the *P. scutellarioides* (L.) leaf extract against *E. coli* and *S. aureus* bacteria isolated directly from dairy cattle with subclinical mastitis, this study used the agar well diffusion method where the presence of clear zone indicating the ability of the *P. scutellarioides* (L.) leaf extract to inhibit the growth of *E. coli* and *S. aureus* isolated from dairy cattle with subclinical mastitis.

The samples used are the *P. scutellarioides* (L.) leaves which chosen based on the characteristics: fresh leaves, no holes, dirt, fungus or other diseases. The bacterial samples used for the test were isolated directly from the cow's milk which had CMT score (+) 3. The equipments used for this study are the California Mastitis Test(CMT) paddle, plastic bottle, styrofoam cool box, autoclave, incubator, ose, light microscope, bunsen burner, blender, petri dish, caliper, test tubes, analytical scale, evaporator, measuring cup, micropipette, funnel, vial bottle, beaker glass, glass jar. Meanwhile, the materials used for this study are The research materials used for this study are CMT reagents, Mannitol Salt Agar (MSA) plate, Eosin Methylene Blue Agar (EMBA) plate, cover glass, object glass, crystal violet, Lugol's solution, alcohol 95%, safranin, immersion oil, H2O2 3%,

rabbit blood plasma, toothstick, peptone water, kovac's reagent, MR/VP broth, MR indicator, alpha naphtol, KOH 40%, simmons citrate agar, *P. scutellarioides* (L.) leaves, ethanol p.a. solvent, filter paper, sterile aquadest, aluminium foil, oxytetracycline powder, ciprofloxacine powder, Nutrient Agar (NA), physiological NaCl, Mueller Hinton Agar (MHA) plate, and micropipette tips.

Research Procedure

There are several procedure for this research. First, the sample collection. The leaf samples are taken from the toga plant shops around Pakal District, Surabaya. Milk samples were taken from a farm in the Mulyorejo sub-district, Surabaya, after the CMT test was carried out on the spot. The next step is the bacterial isolation and identification. The milk sample which tested (+) 3 for mastitis with the CMT were cultured in MSA and EMBA media, then incubated at 37^{o}C for 24 hours. The bacteria identification for the colonies grew on MSA are the catalase and coagulase test. The bacteria identification for the colonies grew on EMBA are the IMViC tests. The Gram staining also done to both colonies.

The next step is the extraction of iler leaves. The collceted leaves washed under flowing water before being dried with oven-drying method. Blend the leaves into smaller particles using a blender to expand the contact and increase the interaction with solvent (Ningsih *et al.,* 2017). Put 60 grams of simplicia into a glass jar that is air-tight, does not leak, and also covered in aluminium foil, then add ethanol p.a. solvent as much as 225 ml (Mpila *et al.,* 2012). Soak the simplicia in the solvent for 3 x 24 hours with occasional stirring at room temperature. After 3 days have passed, filter the *P. scutellarioides* (L.) leaf extract solution, and separate the filtrate and the residue. The residue is remacerated 1 x 24 hours, then the final products are condensed using a rotary evaporator to evaporate the solvent in the extract (Kurnijasanti, 2019).

The antibacterial activity testing is done with several steps. The various concentration of *P. scutellarioides* (L.) R. Br leaf extract that are going to be tested are 5%, 10%, 20%, and 40%. They are made by diluting the extract in each 1 ml CMC solutions (Mpila *et al.,* 2012), as below:

Table 1. Calculation of various extractconcentration making

No.	Extract (gr)	CMC (ml)	Concentration
1.	0,4 gr	1 ml	40%
2.	0,2 gr	1 ml	20%
3.	0,1 gr	1 ml	10%
4.	0,05 gr	1 ml	5%

The positive control stock solution with the concentration 5 mg/ml is made by crushing ciprofloxacin 500 mg tablet in 100 ml of sterile aquadest, then 1 ml are taken from the stock and add 100 ml of sterile aquadest so that the concentration become 50 µg/ml (Hendriana, 2018). A positive control solution of ampicillin was prepared by weighing 50 mg of ampicillin and dissolving it with 100 ml of sterile aquadest, then 2 ml of the solution was pipetted and then added sterile aquadest up to 10 ml, then 1 ml of that solution was pipetted again and the volume was made up to 10 ml (Anggrainy and Darwin, 2017). The negative control used is sterile aquadest.

Preparation of the tested bacterial suspension is carried out by culturing the bacteria on Nutrient Agar and incubate overnight, then the bacteria is diluted in physiological NaCl solution, its turbidity then will be standarized to the McFarland 0,5 or equivalent to 1.5 x 10^{8} CFU/ml so that the bacteria fits the

standard for sensitivity testing (Prayoga, 2013). Streak the bacteria suspension over the entire surface of Muellen Hinton Agar evenly (Rif'an *et al.,* 2014). The inhibition test of *P. scutellarioides* (L.) leaf extract is using the agar well diffusion method. Use the hole puncher with the diameter 6 mm to make the well before filled it with various concentration of *P. scutellarioides* (L.) leaf extract: 5%, 10%, 20%, 40%, ampicillin and ciprofloxacin as positive control and standard antibiotic for comparison, also sterile aquadest as the negative control; incubate at 37^{o}C for 24 hours. The antibacterial activity of *P. scutellarioides* (L.) leaf extract is observed visually and measures the diameter of the clear zone with a caliper.

Data Analysis

The data taken are various sizes of inhibition zones which are observed visually and measured to know the antibacterial activity of the extract of *P. scutellarioides* (L.) leaf in various concentrations against mastitis-causing bacteria then will be explained descriptively.

RESULT AND DISCUSSION

The extract of *P. scutellarioides* (L.) leaf used the ethanol solvent which is a universal solvent that is able to dissolve polar compounds such as tannins, flavonoids, and polyphenolic compounds, as well as semipolar compounds such as alkaloids (Ghani, 2019). The extract olf iler leaf product obtained after evaporation was weighing 2.30 grams. Its color was dark green and the consistency was condensed, it didn't spill out when it was poured. For the bacteria sample isolated from the milk from dairy cattle with CMT score (+) 3, the colonies of that were growing on EMBA looked metallic green, while growing colonies on MSA looked yellowish. The identification of bacteria colonies grew on EMBA was carried out by the IMViC test. The result of the indole test was positive, showing a red ring which indicates a positive reaction.

The MR test showed a positive result with the color of the solution turning red. The VP test result was negative because it did not show any color change in the solution. The result of the citrate test on Simmons Citrate Agar (SCA) showed a negative reaction which was indicated by the absence of a color change on the agar. The Gram staining showed rod shaped bacteria which were pinkish in color. Meanwhile the identification of bacteria colonies grew on MSA was carried out by coagulase and catalase tests. Coagulase test showed the presence of thickening and smooth little particles, while the catalase test result showed the presence of bubbles. On Gram staining, the bacteria appear coccus-shaped with a purplish color. All tests showed positive results.

The method used to test the antibacterial activity of iler leaf extract in this study was the agar well diffusion using Muellen Hinton Agar (MHA) media with four times repetition to minimize data errors in research. According to Nurhayati *et al.* (2020), the agar well diffusion method has the advantage of measure the inhibition zone easily because the bacteria are active not only on the surface of the agar but also active on the lower part.

The positive control of this study are the ampicillin for the *S. aureus* and ciprofloxacin for *E. coli* testing. The sensitive diameter of inhibition zone of ampicillin against *Staphylococcus spp.* according to the CLSI is ≥29 mm, and ciprofloxacin is also noted to have an inhibition zone diameter criteria that is sensitive to *Enterobacteriaceae* bacteria, which is ≥31 mm. Below are the result of the agar well diffusion method which the

diameters have been measured using a caliper.

Based on the results of the agar well diffusion test for iler leaf extract against *E. coli* bacteria, it shows that extracts with concentration 10%, 20% and 40% showed antibacterial activity, while extract with a concentration of 5% did not show antibacterial activity. The largest diameter of the inhibition zone was shown by the extract with a concentration of 40% which had an average inhibition zone of 9,83 mm. Followed by a concentration of 20% which has an average inhibition zone of 8,69 mm. The smallest inhibition zone was shown by the concentration of 10% which had an average inhibition zone of 3,905 mm. Control (+) with ciprofloxacin had an inhibition zone diameter of 44,92 mm. Control (-) did not show any inhibition zone.

Meanwhile, the result against *S. aureus* in table 4.2, the extract showed the presence of antibacterial activity in the concentration of 40% while the extract concentrations of 5%, 10%, and 20% did not form an inhibitory zone. The concentration of 40% had an average inhibition zone of 7,315 mm. Control (+) with ampicillin showed an inhibition zone of 58.12 mm, while control (-) did not show any inhibition zone.

The criteria for assessing the strength of antibacterial agents' activity according to Davis and Stout (1971) in Rahayu (2019) are as follows:

a. Inhibition zone diameter > 20 mm: very strong
b. Inhibition zone diameter 10-20 mm: strong
c. Inhibition zone diameter 5-10 mm: moderate
d. Inhibition zone diameter < 5 mm: weak

Based on the results of the agar well diffusion test and from the categorization above, iler leaf extract against *S. aureus* bacteria atthe concentration of 40% can be categorized in moderate inhibition ability. Control (+) with ampicillin hasvery strong inhibitory strength. *E. coli* have weak to moderate inhibition zones at extract 10%, 20% and 40% when compared to control (+) which has very strong inhibitory ability.

Iler leaf extract with a higher concentration produced a larger inhibition zone for bacterial growth due to differences in substance levels active at each concentration so it affects the inhibition zone or antimicrobial activity of the extract (Isra, 2018). The higher the concentration of the extract, the more it consists active antibacterial compounds (Ningtyas, 2010 in Lingga *et al.,* 2016). In this research, extract with the concentration of 40% which is the highest concentration tested against the *E. coli* showed the largest diameter of inhibition zone compared the concentration of 5%, 10% and 20%. The largest inhibition zone diameter against *S. aureus* is also produced by the extract with the concentration of 40%, which is the highest concentration, compared to the lower concentrations.

The active compounds contained in the ethanol extract of iler leaf are flavonoids, alkaloids and tannins (Rizal *et al.,* 2018). Flavonoids work by inhibiting nucleic acid synthesis, inhibiting cell membrane function and inhibiting energy metabolism (Rijayanti, 2014 in Nurhasanah and Gultom, 2020), tannins causes protein denaturation that causes disturbances on bacterial metabolism (Angelina *et al.,* 2015 in Rahayu 2019), alkaloids work by interfering with the components constituent of peptidoglycan in bacterial cells causing the layer to not completely formed and causes cell death (Ningsih *et al.,* 2016 in Nurhasanah and Gultom, 2020).

Table 2. The result of the antibacterial activity testing of *P. scutellarioides* (L.) extract against *E. coli*

Concentration	Inhibition Zone Diameter (mm)				Mean
	1	2	3	4	
5%	0	0	0	0	0
10%	0	7,98	7,64	0	3,905
20%	9,24	8,92	8,12	8,48	8,69
40%	11,64	9,12	8,84	9,72	9,83
Control (+) Ciprofloxacin			44,92		
Control (-)			0		

Table 3. The result of the antibacterial activity testing of *P. scutellarioides* (L.) extract against *S. aureus*

Concentration	Inhibition Zone Diameter (mm)				Mean
	1	2	3	4	
5%	0	0	0	0	0
10%	0	0	0	0	0
20%	0	0	0	0	0
40%	7,14	7,24	7,22	7,66	7,315
Control (+) Ciprofloxacin			58,12		
Control (-)			0		

The result also showed that *E. coli* is more sensitive to the extract solution of iler leaf more than *S. aureus.* According to Radji (2011) in Mpila *et al.* (2012), Gram negative bacteria have a thin peptidoglycan layer, while Gram positive bacteria have a thick peptidoglycan layer. Due to the difference in the cell wall structure, Gram negative bacteria are more sensitive to physical shocks such as administration of antibiotics or other antibacterial agents compared to Gram positive bacteria. In this study, *E. coli* which is a Gram-negative bacteria is more sensitive to the tested extracts than *S. aureus,* which is a Gram-positive bacteria.

Positive controls with ampicillin for *S. aureus* and ciprofloxacin for *E. coli* showed greater results than the iler leaf extracts at various concentrations. This might be because herbal extracts have many active compounds that can cause various interactions that have the potential to have antagonistic properties so that they are less effective in killing bacteria, while antibiotics only have a single compound (Gupta *et al.,* 2015 in Afdhila *et al.,* 2021). According to Ningtyas (2010) in Lingga *et al.* (2015), an increase in the concentration of antibacterial compounds contained in higher concentrations is suspected to increase the penetration of antibacterial compounds into the interior of bacterial cells which will cause cell death, so the higher the concentration is, the more cell death it will cause. In addition, the small inhibition zone produced by the extract solution can be due to the solvent used is ethanol which is a universal solvent that can dissolve various compounds, even those that don't have antibacterial activity, so the antibacterial components obtained are not maximal enough. When the ethanol extract concentration is high, the concentration of compunds that do not have the antibacterial activity is likewise high, resulting in a lower ability to

suppress bacterial growth (Sinarsih *et al.,* 2016 in Rahayu, 2019).

CONCLUSION

Based on the results of the research that has been done, it can be concluded that *P. scutellarioides* (L.) leaf extract with ethanol p.a. as the solvent showed antibacterial activity against *E. coli* and *S. aureus* bacteria isolated directly from cow's milk samples with subclinical mastitis at certain concentrations.

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