Antibacterial Activity of Bidara Leaf Extract (*Ziziphus mauritiana*) Against *Staphylococcus aureus* Isolated from Mastitis Case In Vitro

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ABSTRACT

This research aimed to know the antibacterial activities of bidara leaf extract against *Staphylococcus aureus* isolated from mastitis cases. The isolate was identified before the antibacterial test. This study was done by five times repetitions on Mueller Hinton Agar. The concentrations used in this study were 30%, 40%, and 50% of bidara leaf extract then a blank disc was dipped into each concentration. Tetracycline disc was used as a positive control and aquadest was used as a negative control. The diameter of the clear zone was measured using calipers after incubation for 24 hours at 37℃. The results showed significant differences ($p<0.05$) between the positive control (K+), negative control K (-), and the concentration group. However, each concentration of bidara leaf extract showed insignificant differences in the diameter of the clear zone. It could be concluded that bidara leaf extract has antibacterial activities against *Staphylococcus aureus* isolated from mastitis cases.

Keywords: *Staphylococcus aureus*, mastitis, bidara leaf, antibacterial

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INTRODUCTION

Mastitis is a disease that affect the mammary gland due to intramammary infection by pathogenic bacteria (Harijanti et al., 2018) such as *Streptococcus agalactiae*, *Str. disgalactiae*, *Str. uberis*, *Str. zooepidermicus*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa* also *Mycoplasma sp.*, *Candida sp.*, *Geotrichum sp.* and *Nocardia sp.* (Riyanto et al., 2016) that can be differentiated into two group which are clinical mastitis and subclinical mastitis. *Staphylococcal mastitis* is the commonest mastitis to be found and has the greatest impact to economy deficit when the dairy farm is affected (Abera et al., 2010). The treatment for mastitis that caused by *Staphylococcus aureus* infection can be use the combination of streptomycin and penicillin (Riyanto et al., 2016). The increase in consumption of antibiotics might be overuse and led the resistance of bacteria ensuring that antibiotic will be the most prevalent cause of death (Khan et al., 2019; Zhang et al., 2022).

One of the alternatives for treatment due to *Staphylococcus aureus* isolated from mastitis case infection is the use of medicinal plants. The leaves of the *Ziziphus mauritiana* plant are reported to contain, among others,

tannins, saponins, glycosides, and phenols (Najafi, 2013). Tannins are part of secondary metabolites found in plants (Hartzfeld, 2002). Tannins contained in plants are reported to have the ability to precipitate microbial proteins which result in these proteins can no longer be utilized (Sodipo et al., 1991).

METHODS

Research Design

Research design that used in this research is experimental study using a completely randomized design (CRD) with 5 different treatments. The isolate of *Staphylococcus aureus* was obtained from Mastitis cases in the Dairy farm in Surabaya, Indonesia. This research was conducted at Bacteriology and Mycology Laboratory, Faculty of Veterinary Medicine Universitas Airlangga and BPKI Laboratory Ketintang for extraction from May - June 2023.

Research Materials

Materials used for the study included *Staphylococcus aureus* isolated from mastitis cases, Mueller-Hinton Agar, Mannitol Salt Agar, Gram-staining, H_2O_2 3%, Rabbit plasma, bidara leaves, 96% ethanol, Tetracycline, Aquadest, CMC-Na.

Sterilization of Tools and Equipments

Equipment such as reaction tube, erlenmeyer and measuring glass, with paper and alumunium foil. Then sterilized using autoclave on 121°C for 15 minutes. While the other equipment made from metal such as ose heated on fire for ±1 minute. For medium sterilization, it is able to use autoclave on 121°C. The sterilized medium placed in the autoclave for 15-20 minutes (Dwidjoseputro, 2005).

Preparation of Bidara Extract

Fresh bidara leaves (*Ziziphus mauritiana*) were washed and then dried in the sun, after dry the leaves are ground to get bidara leaf powder. The powder was weighed 500g and then extracted by maceration method using 96% ethanol as a solvent. Bidara leaf powder is soaked for 2 days and it is ensured that the powder is completely immersed. After the maceration process, the immersion was filtered using Watman filter paper No. 1, then the filtrate was evaporated using a rotary evaporator, so that a thick extract of bidara leaves is obtained. The extract was left at room temperature so that all the solvent (ethanol) evaporates. The extract was weighed in sterile glass bottles and stored at 40°C before use.

Isolation and Identification of *Staphylococcus aureus*

Staphylococcus aureus isolated and identified using microbiological test using sample from dairy cattle that shows clinical sign from mastitis disease. The isolation and identification test is to observe the growth characteristic of *Staphylococcus aureus* which inoculated in Mannitol Salt Agar (MSA) showing discoloration of media from red color to yellow. Moreover, the growth characteristic is also observed at Gram staining test showing Gram positive and grape like shape. *Staphylococcus aureus* identified by catalase test showing foamy like bubble when added a drop of H_2O_2 3%, and observing coagulase on rabbit plasma after mixed with suspension of *Staphylococcus aureus* (Gebremedhin et al., 2022).

Preparation of Bacterial Suspension

The bacteria that have been identified are then made a suspension and diluted with the Mc Farland 0,5 standard equal to 1.5 x 108 CFU/ml. A sterile test tube was prepared, each test tube was filled with 9ml of PBS. In the first tube, 1 ml of bacterial suspension

added using a sterile pipette and then homogenized using a vortex. From the first tube, 1 ml is taken using another sterile pipette and inserted into the second tube to obtain a bacterial density of 1,5 x 108 CFU/ml (Hermawan, 2007).

Antibacterial Testing

The test was carried out on MHA media. The suspension that in accordance with Mc Farland 0.5 standards was put into Mueller-Hinton media with a 0.2 ml spread method using a pipette then flattened using a spatula. Paper disc was immersed to the extract that has been dissolved according to the concentration then placed into the media that has been made. Tetracycline as a positive control and distilled water as a negative control were also placed in the media. The Mueller-Hinton Agar media was then incubated at 37^{o}C for 24 hours.

Data Analysis

Data in the form of inhibition zone diameter were tabulated and then analyzed using the program Statistical Packed for Social Science (SPSS) 25.0 for windows using the ANOVA (Analysis of Variance). If there is difference within treatment will be followed by Duncan's Multiple Range test with $p < 0.05$.

RESULT AND DISCUSSION

Identification of *Staphylococcus aureus*

The sample was identified by cultured on Mannitol Salt Agar Media, Gram-staining, and biochemical test by catalase and coagulase test. The Microscopic examination by using Gram staining test was observed in 100x magnification showed form a round cells shaped like grape with violet color in indicated the bacteria confirmed as a gram positive.

The catalase test was done in a object glass by giving a drop of H_2O_2 3% solution to a drop of bacteria suspension which showed a positive result by presence of foamy bubbles resulted from the reaction of *Staphylococcus aureus* with H_2O_2 3% solution.

The coagulase test showed a positive result by presence of coagulase of rabbit's plasma. The coagulase is occured because *Staphylococcus aureus* produce protein like an enzyme that can coagulate the plasma. The bacteria that coagulate plasma is considered as potential pathogen.

Disc Diffusion Test

The disc was dipped in the bidara leaf extract with concentration 30%, 40%, 50%. For the negative control, the disc was dipped in the aquadest. Tetracycline antibiotic disc was used as positive control. The discs were placed in the media that has been inoculated with *Staphylococcus aureus* then incubated for 24 hours at 37^{o}C.

The result of clear zone of bidara leaf extract against *Staphylococcus aureus* was analyzed using SPSS. Based on the table 1, there is differences between diameter of clear zone. There is significant difference ($p < 0.05$) between K- (Aquadest) with bidara leaf extract at concentration 30%, 40%, 50%, and K+ (Tetracycline) by the absence of clear zone (0 mm) meanwhile for the bidara leaf extract at concentration 30%, 40%, 50% showed significant difference ($p < 0.05$) compared with Tetracycline. There was formed a clear zone each sized 20,7 mm at 30% leaf extract; 23,6 mm at 40% leaf extract; 23,2 mm at 50% leaf extract, and 31,1 mm at K+ (Tetracycline). Based on the data it is showed that K- (Aquadest) has smaller diameter of clear zone compared to bidara leaf extract at 30%, 40%, 50%, and K+ (Tetracycline). Based on the data it is showed that K+ (Tetracycline) has wider diameter of clear zone compared to bidara leaf extract at 30%, 40%, 50%,

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Figure 1. *Staphylococcus aureus* cultured on the Mannitol Salt Agar media showed discoloration of the media due to its ability to ferment mannitol.

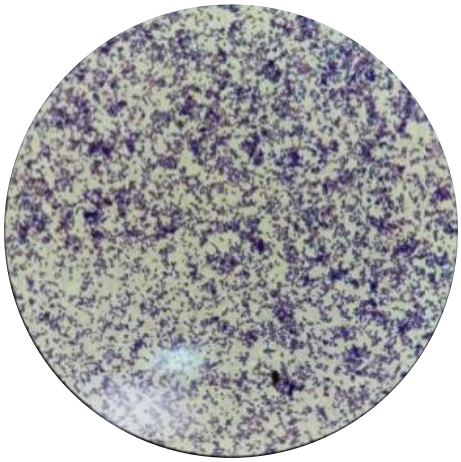

Figure 2. Gram staining of *Staphylococcus aureus* observed by microscope shows a round cells shaped like grape with violet color.

Figure 3. Catalase test showed a positive result by presence of foamy bubbles resulted from the reaction of *Staphylococcus aureus* with H_2O_2 3% solution.

Figure 4. Positive result of *Staphylococcus aureus* by presence of coagulase of rabbit's plasma.

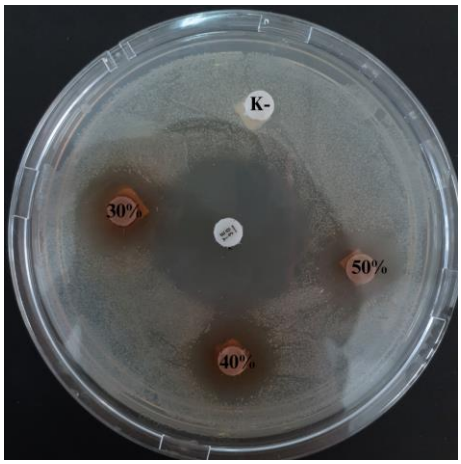

Figure 5. Clear zone of bidara leaf extract at concentration of 30%, 40%, 50%, tetracycline and aquadest.

Table 1. Mean and Standard deviation (SD) of Clear Zone Diameter

Clear Zone Diameter (Mean ± SD)	
K- (Aquadest)	$0^{a} \pm 0$
K+ (Tetracycline 30 **µg**)	$31,1^{c} \pm 0.89$
P1 (30%)	$20,7^{b} \pm 4,04$
P2 (40%)	$23,6^{b} \pm 4,05$
P3 (50%)	$23,2^{b} \pm 4,25$

and K- (Aquadest). As listed on the table, there is no significant difference ($p > 0.05$) between bidara leaf extract at concentration 30%, 40%, and 50% but there is significant difference ($p < 0.05$) between bidara leaf extract with K- (Aquadest) and K+ (Tetracycline).

Disc that has been dipped in K- (Aquadest) showing absence of clear zone (0 mm) indicating that Aquadest can not inhibit the growth of *Staphylococcus aureus* isolated from mastitis case. The absence of clear zone is related to research by Aisyah (2021) about antibacterial activity that there is no clear zone was formed for negative control.

Tetracycline are broad-spectrum antibiotics that disrupt the addition of amino acids to polypeptide chains during protein synthesis of organelle that can be use both for Gram-positive

and Gram-negative bacteria (Sánchez et al., 2004; Etebu and Arikekpar, 2016).

Disc that has been dipped in the 30%, 40% and 50% of bidara leaf extract showed that all of disc are sensitive to *Staphylococcus aureus* isolated from mastitis case. Related study of antibacterial activity ethanolic extract of *Ziziphus mauritiana* against *Staphylococcus aureus* by Muharrami also formed a clear zone at 10% (0,92±0,056 mm), 20% (1,22±0,021 mm), 30% (1,36±0,017 mm) and 40% (1,68±0,03 mm) indicating there is antibacterial activity against *Staphylococcus aureus*.

The antibacterial activity from bidara leaf extract as result of bidara leaf extract contained antimicrobial compound in it. Bidara leaf extract contain phytochemical active element such as saponins, alkaloids, phenolic compound, flavonoid also active compounds of polyphenols (Kurniawan et al, 2020). Saponins antibacterial mechanism is lysing the cell by binding with cholesterol inside of the bacteria forming saponin-cholesterol complex and also by adhering to outer membrane of bacteria cell leading to disruption of cell permeability (Khan et al., 2018). Alkaloid prevent the growth of bacteria in number of ways, including preventing the production of nucleic acids and proteins, altering cell permeability, damaging cell membranes and walls, limiting bacterial metabolism and blocking efflux pumps (Yan et al., 2021). Phenol compounds and its derivatives will damage the cell membrane due to the content of H+ ion will attack the phosphate groups made the phospholipid molecule break down leading to inhibition of cell growth or cell death because the phospholipid unable to maintain the shape of cell membrane. Phenol compound and it derivatives also act as enzyme inhibitors, damaging the cytoplasmic membrane and totally precipitate cell protein after binding to non-specific proteins (Afifi et al., 2017).

CONCLUSION

Based on the result of this research, it can be concluded that there are antibacterial activity of bidara leaf extract (*Ziziphus mauritiana*) at 30%, 40% and 50% concentration against *Staphylococcus aureus* isolated from mastitis case with 30% as the effective dose. Further research needs to be done to ito determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of bidara leaf extract (*Ziziphus mauritiana*) against *Staphylococcus aureus* that isolated from mastitis case.

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