Screening Endophytes of Neem Leaf that Potential Anti-Anthrax through Tests of Anti Staphylococcus aureus

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Screening Endophytes of Neem Leaf that Potential Anti-Anthrax through Tests of Anti *Staphylococcus aureus*

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INTRODUCTION

Anthrax is an infectious disease caused by *B. antrachis*. Spores of this type of bacteria can survive in the extreme temperatures and chemicals. The bacteria are potentially infectious agents for biowarfare, usually acute in different types of livestock (ruminants, horses, pigs and so on) characterized by tissue changes which are septicemia, serohemorrhagic infiltration of the subcutaneous and subserous tissues, as well as the acute swelling of the spleen. In Indonesia anthrax causes many deaths in livestock. This fact proves that anthrax is so dangerous and detrimental that a new breakthrough is needed to prevent the case efficiently and effectively through neutralization mechanisms, as well as by safe research methods (Vaithiyanathan *et al.,* 2016). Handling the cases of anthrax disease in livestock that have been done, so far proved to be less effective and has many shortcomings such as the use of vaccines sometimes toksigenik cause pain and necrosis at the injection site, subcutaneous edema and death of livestock post vaccination (Siregar, 2002). *Azadirachta indica* leaf extract (neem) can fight bacterial pathogen *Bacillus anthracis* (Vaithiyanathan *et al.,* 2016). Based on these facts, the handling of anthrax disease in livestock needs to be developed with the utilization of endophytic bacteria-based biomaterials. The use and utilization of endophytes or microbial symbionts in neem trees can replace inefficient extraction methods, since they require large numbers of plants to be extracted on a large scale, thus causing ecological imbalances (Wensing, 2014). In addition, microbial endophytes in neem plants have the potential to resist vegetative growth of anthrax agents.

From the facts, will be made an innovation for the handling of anthrax cases in Indonesia with the use of herbal endophytic biomaterials. Screening of neem leaf endophytes is potentially anti-anthrax through the Anti-*Staphylococcus aureus* MRSA test which is an invention by utilizing endophytic extraction biomaterials that have the potential to counteract the vegetative growth of anthrax agents.

This research is expected to provide solution to anthrax threat in Indonesia through neem endophyte which has mechanism of action anti *S. aureus* which is pathogen same with anthrax bacteria. The aim of this research is to obtain the active biomaterials from selected neem leaf endophytes to be tested in *S. aureus* MRSA as bacteria with Anthrax pathogens. In addition, to examine and analyze the effects of active biomaterials in *Staphylococcus aureus* MRSA.

METHODS

The Making of Nutrient Agar (NA) and Nutrien Broth (NB)

Nutrient agar (NA) and Nutrient Broth (NB) media were prepared by dissolving each of the nutrient powders to 20 g and NB into 1 liter of aquadest. The solution was sterilized by autoclave at 121°C. for 2 hours. This medium is used as a growth medium for MRSA bacteria.

Isolation and Identification of Endophytes of Neem Leaf (*Azadirachta indica*)

The methods used are isolation, selection, endophytic extraction and screening methods of *S. aureus* MRSA culture medium. More specifically, this research used a method with a material that had a specific dose with special stages. The leaves of neem were washed with water for 5 minutes, then distrerilized the surface by inserting it into a 70% alcohol solution for 5 minutes, proceeded into sodium hypochlorite solution 5 minutes then continued into sterile distilled 3 times for 5 minutes, then the leaves were cut longitudinally as many as 4 pieces but not broken and then planted in petri dish contained NA media. Endophytes used are endophytes that grow on the inner hemisphere. Endophytes are cultured in liquid medium 37^0C 48 hours 200 rpm. The extraction of endophytic cultures was centrifuged 1000 rpm to obtain the active compound of its secondary metabolite.

Test of Pathogenicity and In Vitro of Biomaterial Active of Neem Endophytes

Pathogenicity tests of pathogenic bacteria *Staphylococcus aureus* MRSA and endophytic bacterial isolates were performed by culturing bacteria on the blood medium. Furthermore, In Vitro Test is done, the initial step is carried out minimum inhibitory test to determine the effective concentration of neem endophytic bioactive in inhibiting the growth of MRSA bacteria. First, Nutrient agar media sterilized in autoclave and then prepared MRSA culture. The MRSA bacteria were suspended into the NA medium then incubated at the 37^0C incubator for 24 hours. Positive results obtained MRSA colonies on petri dishes. Then, paperdisk that is given a neem endophytic bioactive with a concentration of 200, 400, and 600 µl placed on each MRSA culture. Then the MRSA culture was incubated again at 37^0C incubator for 24 hours. the positive results obtained inhibition zone. The parameters observed were the inhibitory zone diameter in the each treatment.

RESULTS AND DISCUSSION

Based on this research, the neem endophytic biomaterials contained the anti-anthrax compound were a*zadirachtin* compounds. The used of endophytic bacteria is more effective than extracting neem leaves on a large scale. These endophytic bacteria lived within the neem leaf tissues that produced secondary metabolite compounds including *azadirachtin* compounds. So to produced biomaterial active anti antraks on a large scale, simply by multiplying the pure culture of endophytic bacteria from the neem leaves. The ability of endophytic microbes to produced secondary metabolite compounds in according with the host plant was very beneficial, especially in producing secondary metabolites in higher quantities (Shimarmata *et al.,* 2007) . In addition, the used of endophytic microbes in the production of active compounds had several advantages, among others, more quickly produced a product with a uniform quality and can be produced on a large scale (Diniyah, 2010). The used of neem endophytic endemic biomaterials for livestock didn't cause side effects such as synthetic drugs or vaccines for anthrax disease so far that cause pain and necrosis at the injection site, subcutaneous edema and post-vaccination livestock deadly (Purwanto, 2011). The *azadirachtin* content of the active neem leaf endophytic biomaterial was able to inhibit and kill bacteria with anthrax pathogenicity and stoped the vegetative growth from the pathogen deficiency.

The isolation and selection stage of endophytic bacteria from neem leaves was done by growing it on Nutrien Agar (NA) medium and incubated for 37^0C 48 hours. After that, purification was done to obtain a culture of pure endophytic bacteria. Isolation was a way to separate a microorganism from its environment, so a culture is obtaine that was not mixed with other cultures or called pure culture. Before to isolation, an initial treatment was required for the success of the isolation process. Pretreatments performed depend on the characteristics of the substrate or host in which the endophytic shell lies. surface sterilisation was used as a pretreatment to isolate endophytes from plant organs still fresh (Darminto et al., 2009).

Medium agar was an proper substrate for separated the mixture of microorganisms. The technique used allowed bacteria to grow at a great distance from each other and form colonies. All cells in the colony were considered to be a derivative or progeny of a microorganism that called a pure culture. The extraction of the endophytic cultures was centrifuged 1000 rpm to obtain the active compound of its secondary metabolite and the result obtained by the active biomaterial from the neem leaf endophytes in Figure 1. The centrifugation principle was based on the molecular separation of subcellular cells or organelles. The separation was based on the suspended particles in a container will settle (sedimentate) to the bottom of the container due to the force of gravity. Thus the rate of precipitation of a suspended particle can be adjusted by increased or decreased the gravitational effect on the particles. Setting the rate of precipitation can be done by placed a container containing the particle centrifugation particle suspension precisely on the part of the rotor which rotate with a certain speed. This is reinforced by the opinion of Ferbiyanto et al (2013). That centrifugation was a separation technique used to separate a small amount of suspension. This suspension was inserted into the test tube and then diffused. Rapid centrifugation produces a larger centrifugal force so that the suspended particles settle at the bottom of the tube and then decant.

Furthermore, pathogenicity test of pathogenic bacteria and endophytic bacteria used blood medium. In order for blood was the most widely used media planting bacteria that were difficult to growth due to blood agar sheep contained the nutrients the bacteria needed (Yeh *et al.,* 2009). Media on essentially composed of protein sources (peptone), soy protein, preparations (containing KH), NaCl, Lamb agar and blood 5%. Extracellular enzyme-producing bacteria that can lyse cells red blood of sheep on agar (hemolysis) (Heindl *et al.,* 2012). There were three forms blood hemolysis, Alpha-hemolysis that formed a greenish to brown zone young around the colony, the bacteria partially hemolize hemoglobin thus leaving the green pigment biliverdin. Beta-hemolysis that formed a transparent or clear zone around the colony, the bacterium produces β-hemolysin (Streptolysin O and S), which lyse red blood cells in the medium perfect. Gamma-hemolysis that didn't hemolize the blood so it didn't form hemolysis zone around the bacterial colony (Zomorodian *et al.,* 2011).

The pathogenicity test carried out by transferring bacteria into the blood medium. Pathogenic bacteria used was *Staphylococcus aureus* MRSA which had pathogenicity similar to anthrax tested its pathogenicity by using blood medium and in Figure 2 it was proved that the bacteria were actually pathogenic with marked occurrence of lysis on blood medium. It included Alpha-hemolysis. *S. aureus* had hemolisin and

leukosidin toxin that were dengerous. Hemolisin in *S. aureus* was a toxin that can form a zone of haemolysis around bacterial colonies, consists of α-hemolysin, β-hemolysin, and δ-hemolysin. α-hemolisin was the toxin responsible for formation of hemolysis zone around the *Staphylococcus aureus* MRSA colony at medium for blood. This toxin can cause necrosis on the skin of animals and humans. β-hemolysin was a toxin mainly produced Staphylococci isolated from animals, which caused lysis of red blood cells of sheep and cattle. While delta hemolisin was a toxic that can lyse human red blood cells but their lysis effects less against sheep red blood cells. Leukosidin was toxin that can killed white blood cells in some animal. But his role in human pathogenesis is not obviously, because *Staphylococcal* pathogens can not kill cells human white blood and can be phagocytosed. *Staphylococcus aureus* MRSA produced coagulase, lecosidine, and toxic toxic shock syndrome (Heindl *et al.,* 2012).

Koagulase iwas a protein resembling an enzyme capable of agglomerating plasma supplemented with oxalate or citrate in the presence of a factor present in the serum, whereas lecosidine was a toxin that can kill white blood cells in various animals (Yuwono, 2012). This *Staphylococcus aureus* MRSA also produced a variety of other toxins that were clustered according to their mechanism of action, included cytotoxins, superantigens of pyogenic toxins, enterotoxins, and exfoliative toxins. Cytotoxins were 33-kd protein-alpha toxins, causing changed in core formation and stimulating proinflammation in mammalian cells (Yeh *et al.,* 2009). These changed will cause cellular damaged and played a role in the manifestation of sepsis syndrome. Superantigen pyogenic toxin was structurally similar to cytotoxin, bound to a protein class of histocompatibility complex (MHC) class II. This toxin caused T cell proliferation and cytokine released. Enterotoxin molecules can caused disease resulting from its proteins, namely toxic shock syndrome and food poisoning (Gordon & Lowy, 2008).

Pathogenicity test in endophytic bacteria was done because to know that endophytic bacteria was not pathogenic. Figure 3 shows no lysis of the blood medium when endophytic bacterial isolates were cultured on the blood medium. It included Gamma-hemolysis.

In vitro tests were performed to determine the ability of active neem leaf endophytic biomaterials to inhibit and stoped the growth of *Staphylococcus aureus* MRSA bacteria with anthrax-like pathogenicity. Antibacterial activity test can be done by diffusion and dilution method (Clorinda, 2012). Disc diffusion test or disk diffusion test was done by measuring clear zone diameter which was indicative of the response inhibition of bacterial growth by an antibacterial compound in the extract. In this test, a plate containing pathogenic bacteria grown in the medium was tested for its inhibitory zone using a paperdisk that had been administered an active biomaterial of neem leaves (Jagtap & Chavan, 2016).

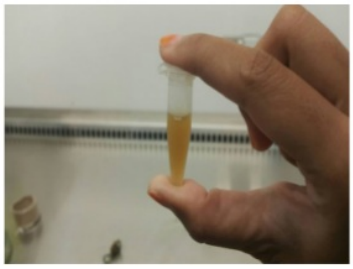

Figure 1. Isolate of centrifuge result

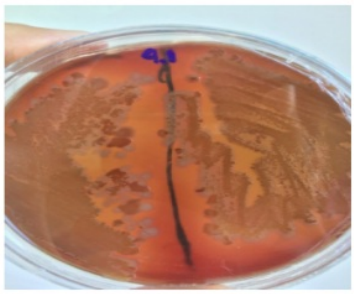

Figure 2. Pathogenesis Test of Pathogenic Bacteria

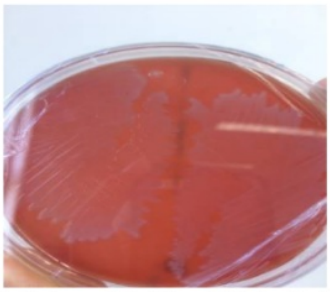

Figure 3. Pathogenicity Test of Endophytic Bacteria

The method that used in this research is KirbyBauer disc method. Parameters that used to determine the ability of antibacterial power was to measure the area of inhibit zone that occured around the paper disc. Tests of antibacterial activity were positive when around of the paper discs had clear zones that were free from bac-

terial growth. This is reinforced by the opinion of Putra (2015) that inhibition of the growth of microorganisms, the obstacle zone will be seen as clear areas around the paper disk contained antibacterial substances. Boundary zone diameter of bacterial growth indicated bacterial sensitivity to antibacterial substances. The treatment is given by giving concentration concentration 200, 400, and 600 µl paperdisk. Then incubated and calculated the diameter of the inhibit zone.

Table 1. Inhibition Zone Test Results

Treatment (Concentration) (µl)	Diameter of inhibition zone (mm)
Control	-
200	18
400	23
600	27.5

Based on in vitro inhibition zone test, it was known that the largest inhibitory zone diameter of 27.5 mm on the treatment of active neem endophytic biomaterial concentration of 600 µl. From the above data, it was known that the higher concentration of active neem leaf endophytic concentration was given the ability to inhibit and kill the growth of *Staphylococcus aureus* MRSA bacteria with pathogenicity of anthrax. The mechanism of inhibition of bacterial growth by the compound antibacterial can be the destruction of cell walls by inhibited forming it or changed it once it was formed, changed in permeability cytoplasmic membrane that caused the removal of food from the cell, changed in protein molecules and nucleic acids, inhibition of enzyme action, and inhibition of synthesis of nucleic acids and proteins (Alvarez *et al.,* 2010). Bacteriocidal effect by killing the cells but not lysis cell or cell rupture. This was indicated by the addition of antimicrobials to microbial cultures in the logarithmic phase. After addition of the substance antimicrobials in the logarithmic phase obtained total fixed cell count while the number of living cells decreased. This is reinforced by the opinion of Gursky et al (2011) that the content of *azadirachtin* compounds of neem endophytes was known to inhibit the growth of *B. antrachis* bacteria that caused anthrax disease.

In the future, the development of biofuel coctail endofit from natural materials was expected to inhibit the growth of anthrax bacteria more effectively and efficiently. According to Diniyah (2010) bioactive endophytes of natural materials were believed to inhibit the growth of pathogenic bacteria effectively without causing harmful residues to the environment.

The renewal of this research was that anthrax disease can be solved without having to isolate *B. antrachis* bacteria which was dangerous because it had vegetative spores. This research was sufficient to use *S. aureus* MRSA isolates that had pathogenicity similar to *B. antrachis*. This research used the biomaterials were derived from bacteria growing in plant tissues that capable of produced anti-anthrax compounds. Thus, this research had the latest methods of dealing with anthrax disease more effectively and safely.

This research was very useful for research and social progress. In the field of research, the present of invention was the first discovery of the method of handling anthrax cases that had not been done previously by using pathogenic bacteria that have anthrax-like pathogenicity. The used of endophytic herbs in anthrax cases was also the first discovery compared to previous antecedent handling which was more risky and had adverse effects after it was done. So this research became a good start of the development of safe handling methods of anthrax cases. Further research with this method was also needed, such as using in vivo method and experimenting with biomaterials from endophytes to livestock infected by anthrax disease. The benefit of this research to the people was to help the handling of anthrax cases that had been used as bioterorrism so that the community feel safe because it had found a solution of the handling of anthrax disease with a safe method and had no adverse impact to the living thing and their environtment.

CONCLUSION

Active biomaterials anti-antrax can be isolated from neem leaf endophytes by isolation, cetfugation, pathogenic and invitro methods of effect of neem endophytic biomaterials on growth of MRSA *Staphylococcus aureus*. Active biomaterials of antibacterial from neem leaf endophytes can inhibit the growth of MRSA *Staphylococcus aureus* that had pathogenicity similar to *B. anthracis* bacteria. Inhibitory zone testing results that have been done, it was known that the droplet of endophytic active biomaterial at most able to inhibit the growth of *S. aureus* MRSA bacteria effectively.

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