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The potential of endophytic bacteria isolated from atung fruit seeds as antibacterial against skin pathogens

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ABSTRACT

Antibiotic resistance is one of the biggest threats to global health today. Estimated by 2050, antibiotic resistance will cause around 10 million deaths per year if alternative treatments have not been identified. This study was aimed to determine the potential of endophytic bacterial isolates from the seeds of atung fruit (*Parinarium glaberrimum* Hassk) as an antibacterial against skin pathogens *Cutibacterium acnes* and MDR *Staphylococcus aureus*, and *Staphylococcus epidermidis*. This research was a descriptive observational research with laboratory experimental methods. This study used isolates of endophytic bacteria isolated from the seeds of atung fruit which were tested with pathogenic bacteria. The results showed that 2 of 7 endophytic bacterial isolates were able to inhibit the growth of *C. acnes* with clear zone diameter of 4 mm and 9 mm, respectively, yet couldn't able to inhibit MDR *S. aureus* and *S. epidermidis*. This study concluded that the 2 isolates (isolate BBA5 and BBA9) could have potential as antibacterial against *C. acnes*. While 5 other isolates had not been able to inhibit the whole pathogenic bacteria tested. An optimization is necessary in an effort to develop these isolates as antibacterial.

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INTRODUCTION

Infectious diseases are one of the most common causes of morbidity and mortality in health care facilities in the world. Several organisms that can cause infections include viruses, bacteria, fungi, and parasites (Murray et al., 2021). The skin is one of the organs most often attacked by pathogens, because the skin is the main barrier to protect the body. The incidence of skin infections caused by bacteria occupies the third position among other etiologies (Djuanda, 2018). The incidence is increasing due to old age accompanied by chronic disease, use of immunosuppressive drugs, and wound infections due to surgery (Hidayati, 2019).

The most common bacteria that cause skin infections are *Staphylococcus* Genus and *Cutibacterium acnes* (Murray et al., 2021b). *Staphylococcus* is a normal flora that has 30 species, two of which can cause clinical manifestations as opportunistic infections, including *S. aureus* and *S. epidermidis*. (Murray et al., 2021a). Of the several pathogens that cause skin infections, the use of antibiotics is the main option in treating infections (Kurniawati et al., 2015). However, due to inappropriate and prolonged use of antibiotics, the sensitivity to several types of antibiotics begins to decrease over time so that some of these pathogens develop into new strain that leads to Multidrug Resistance Organisms (MDROs) (Calfee, 2022). Considering the high risk of resistance from the use of antibiotic drugs that have been described, the use of endophytic bacterial isolates as antimicrobials has various advantages, apart from avoiding the side effects of using drugs, the ability to protect the host it is attached to is an advantage possessed by endophytic bacteria, including fight pathogens, by producing secondary metabolites in the form of bioactive compounds that function as antimicrobials by inhibiting the growth of other organisms (Sirri et al., 2022; Yunita et al. 2016).

Various methods have been used to treat infectious diseases, one of which is by using traditional medicines from plants (Hehanussa et al., 2019; Yunita et al., 2022a). One of the plants that has the potential as a natural antibacterial is the seeds of the atung plant (*Parinarium glaberrimum* Hassk) whose fruit seed extract has been reported to have antibacterial activity against pathogen bacteria *S. aureus, Escherichia coli*, and *Salmonella enteriditis* (Hehanussa et al., 2019). Interestingly, endophytic bacteria associated with atung fruit seeds can be a promising source in treating skin infections. According to the research conducted by Zulkifli et al. (2018), endophytic bacteria isolated from sugar apple stem bark (*Annona squamosa*) has the potential as an antibacterial against the growth of *S. aureus, E. coli,* and *B. cereus* with an inhibition zone diameter ranging from 7-48 mm. Another research conducted by Yunita et al., (2022b) found that endophytic bacteria isolated from nutmeg plant were able to inhibit *E. coli* and *S. aureus* with the largest inhibition zone around 22.5 and 23.8, respectively. Unfortunately, there is no publication that can be accessed regarding the ability of endophytic bacteria from atung fruit seeds against skin pathogens, particularly MDR bacteria. Previously, Monaten (2022) had isolated endophytic bacteria from atung fruit seeds with the isolate codes of BBA3, BBA4, BBA5, BBA6, BBA7, BBA9, and BBA10, and those isolates were reported to be able to inhibit the growth of pathogenic bacteria including *S. aureus* and *E. coli* with inhibition zone diameters ranging from 11-25 mm (Unpublished Monaten, 2022).

However, the study had not determined the inhibition activity against specific skin pathogens. Therefore, in this study, we conduct further research to determine the potential of 7 isolates of endophytic bacteria from atung fruit seeds (*P. glaberrimmus* Hassk) which have been previously isolated as an antibacterial against specific skin pathogens. The aim of this study was to know and measure the antibacterial activity of isolates BBA3, BBA4, BBA5, BBA6, BBA7, BBA9, and BBA10 in inhibiting the growth of skin pathogens including *C. acnes* and the MDR (*S aureus,* and *S. epidermidis*).

RESEARCH METHODS

Research Design

The study was conducted in January 2023 at the Microbiology Laboratory, Faculty of Medicine, Pattimura University. This research is a descriptive observational research with laboratory experimental methods. Using the bacterial diffusion method. Ethical clearance was issued by Ethical Committee, Department of Medical Education, Faculty of Medicine, Pattimura University with the number: 018/FK-KOM.ETIK/VIII.2023.

Population and Samples

The population and samples used in this study were 7 isolates of endophytic bacteria from atung fruit seeds (*Parinarium glaberrimum* Hassk) obtained from Monaten (2022), including isolates BBA3, BBA4, BBA5, BBA6, BBA7, BBA9, and BBA10, which would be tested against skin pathogens (*C. acnes*) and the MDR (*S. aureus and S. epidermidis*). Pure cultures of skin pathogenic bacteria were obtained from the Maluku Province Health Laboratory Center with the ATCC certificate number (*American Type Culture Collection*) on each bacterium was *Staphylococcus aureus* ATCC 33591, *Staphylococcus epidermidis* ATCC 12228, *Cutibacterium acnes* ATCC 11828.

Instruments

The tools used in this study were, test tube racks, micropipette, ca stirrer, spatula, autoclave, analytical balance, incubator, vortex, petric dish, ose needle, paper disk, spreader rod, tweezers, label paper, cotton, thread, sterile tissue, brown paper, aluminium foil, plasticwrap, spirit, clear plastic size 2 kg, pillcrusher, magnetic stirrer, sterile gauze, 100 ml measuring cup, 500 ml Erlenmeyer, and 1 liter Erlenmeyer, Bunsen lamp.

The materials used in this study were 7 isolates of endophytic bacteria isolated from atung fruit seeds (*Parinarium glaberrimum* Hassk) with isolate codes of BBA3, BBA4, BBA5, BBA6, BBA7, BBA9, and BBA10 obtained from (Monaten, 2022), pure cultures of pathogenic bacteria *S. aureus, S. epidermidis,* and *C. acnes*. Nutrient Agar (NA), disc paper, 70% alcohol, 96% alcohol, aquadest, and Tetracycline (positive control).

Procedures

Preparation of *Nutrient Agar* (NA) Media

Preparation of Nutrient Agar (NA) media was made for the isolation and purification of endophytic bacteria, as well as reculturing pathogenic bacteria. This media was made in the following way: A total of 2 gr Nutrient Agar (NA) was weighed and dissolved in distilled water until the volume reached 100 milliliters. The solution was then heated on hot plate and stirred with magnetic stirrer until homogeneous. Media was sterilized by autoclaving for 15 minutes at 121^{o}C. Furthermore, 10 ml of NA was poured into a sterile petri dish. Sterile media is stored in the refrigerator to prevent contamination and can be used later (Fatim et al., 2018).

Reculturing Endophytic Bacteria

The endophytic bacteria used were obtained from previous studies. Reculturing endophytic bacterial isolates was carried out by taking one loop of bacterial colonies and streaking them on the media. The isolated were incubated for 24-48 hours at 37^{o}C (Saraswati, 2015).

Reculturing Pathogenic Bacteria

Reculturing pathogenic bacteria was prepared by inoculating pure culture of skin pathogens namely *C. acnes* and MDR *S. aureus*, and *S. epidermidis* on NA media, and incubated for 24 hours with a temperature of 37^{o}C. Bacteria that grow were put in the refrigerator as working stock (working culture) (Fatin et al., 2018).

Inhibition Test of Endophytic Bacteria from Atung Fruit Seeds Against the Growth of Pathogenic Bacteria

The inhibition test was carried out by streaking pathogenic bacteria over the entire surface of the *Nutrient Agar* (NA) media. Inhibition activity was measured by *Kirby-Bauer* test method using disc paper. Aseptically, the sterilized paper discs were immersed in the liquid culture of endophytic bacteria for 60 minutes. The disc paper was taken using sterile tweezers and placed on the NA. Distilled water as used as a negative control and tetracycline as a positive control. Furthermore, all treatments were incubated using an incubator at 30°C for approximately 3 days (Saraswati, 2015).

The inhibition zone formed was observed around the bacterial colony. The inhibition zone was measured using a ruler to determine the diameter of the clear zone using the following formula as shown in Figure 1 (Tjiptoningsih, 2021).

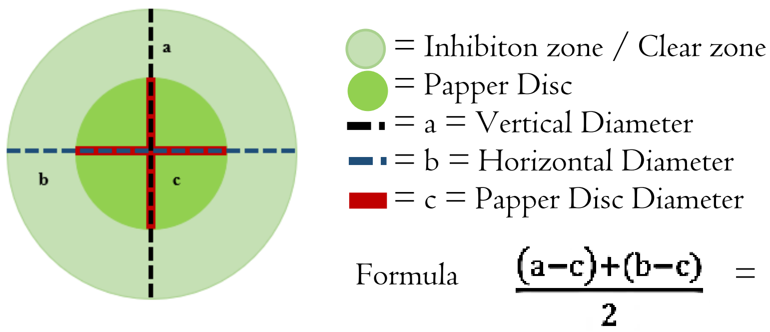

Figure 1. Illustration of the inhibition test in the NA Media (Tjiptoningsih, 2021).

Data Analysis

Data processing was carried out by tabulation by observing the diameter of the inhibition zone of the independent variables (endophytic bacterial isolates) on *S. aureus, S. epidermidis,* and *C. acnes* using a ruler from each petri dish. The results of inhibition zone were then categorized as strong, moderate and weak which referred to the Table 1.

Table 1. Category of antibacterial inhibition based on the diameter of the clear zone (Davis & Stout, 1971).

Clear Zone Diameter (mm)	Category of Inhibition
>20	Very strong
11-20	Strong
6-10	Medium
<5	Weak

RESULTS

The study began with reculturing endophytic bacterial isolates from atung fruit seeds (*Parinarium glaberrimum* Hassk) and skin pathogens *Cutibacterium acnes* and MDR (*Staphylococcus aureus* and *Staphylococcus epidermidis*) on slants NA media as working culture (Figure 2). The inhibition test ware carried out from 7 isolates of endophytic bacteria from the seeds of atung fruit (*P. glaberrimum* Hassk) on the growth of three skin pathogenic bacteria that is *C. acnes* and MDR *S. aureus* and *S. epidermidis* on NA media. The results of the inhibition test are presented in Table 2. The results showed that 2 of 7 endophytic bacterial isolates were able to inhibit the growth of *C. acnes* that is isolate BBA5 and BBA9 (Figure 3) with clear zone diameter of 4 mm and 9 mm, respectively, yet could not able to inhibit MDR *S. aureus* and *S. epidermidis.*

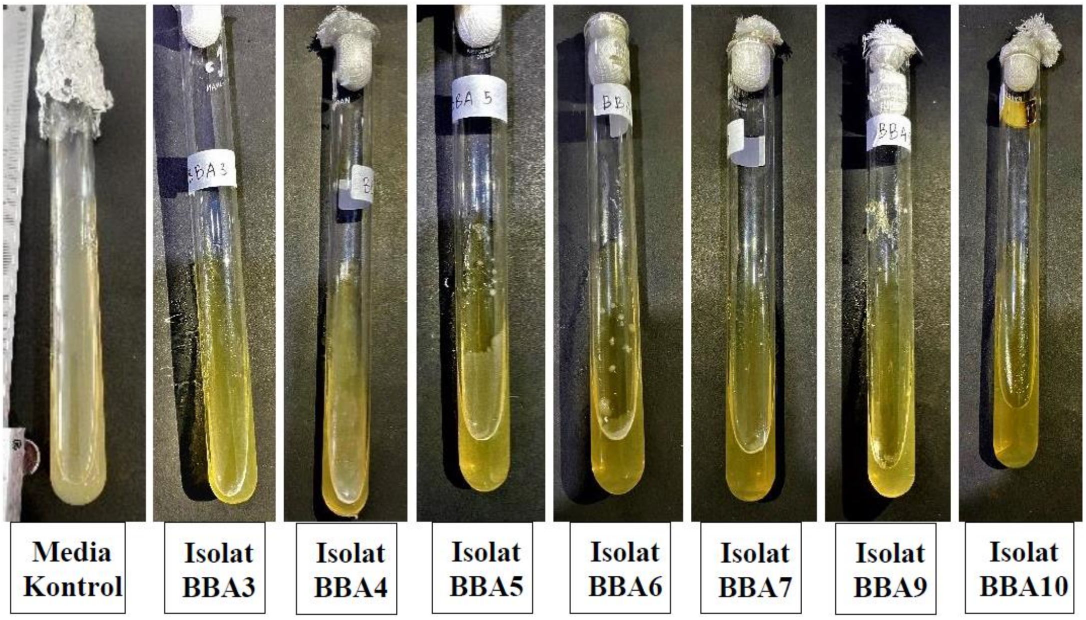

Figure 2. Reculturing of endophytic bacterial isolates from atung fruit seeds (*P. glaberrimum* Hassk)

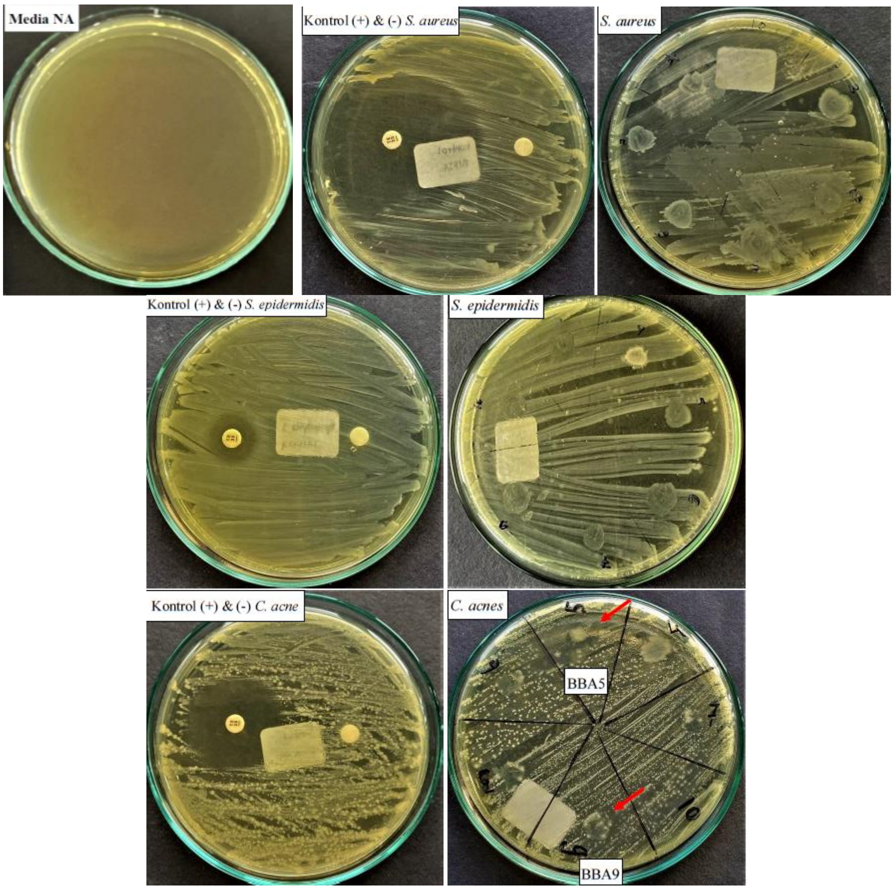

Figure 3. The result of inhibition test of endophytic bacterial isolates against the growth of skin pathogenic bacteria *Cutibacterium acnes* and MDR (*Staphylococcus aureus* and *Staphylococcus epidermidis*)

Table 2. The diameter of the inhibition zone formed on endophytic bacterial isolates from atung fruit seeds (*P. glaberrimum* Hassk) against pathogenic bacteria

Isolate Code	Pathogenic Bacteria					
	Staphylococcus aureus		*Staphylococcus epidermidis*		*Cutibacterium acnes*	
	Inhibiton Zone Diameter (mm)	Category	Inhibiton Zone Diameter (mm)	Category	Inhibiton Zone Diameter (mm)	Category
BBA3	0	-	0	-	0	-
BBA4	0	-	0	-	0	-
BBA5	0	-	0	-	9	Moderate
BBA6	0	-	0	-	0	-
BBA7	0	-	0	-	0	-
BBA9	0	-	0	-	4	Weak
BBA10	0	-	0	-	0	-
K (+)	42	Very Strong	11	Strong	27	Very Strong
K (-)	0	-	0	-	0	-

DISCUSSION

The results of the antibacterial activity test of 7 isolates of endophytic bacteria from atung fruit seeds showed quite different activity This is indicated by the varied diameter of the inhibition zone obtained. Two out of 7 isolates of endophytic bacteria with isolate codes BBA5 and BBA9 formed clear zones against *C. acnes*. The results is in accordance with Baraga's research *et al.* (2022) who tested the antibacterial activity of secondary metabolites of turmeric endophytic bacteria isolates (*Curcuma longa* L.) against *C. acnes* with an inhibition zone diameters ranging from 8.4 to 21.2 mm which indicates that endophytic bacterial isolates has strong potential as antibacterials (Baraga et al., 2022). These results were also reinforced by previous research conducted by Monaten (2022) which characterized isolates of endophytic bacteria from atung fruit seeds (*Parinarium glaberrimum* Hassk) on the growth of *E. coli* and *S. aureus*, where the results showed that isolates BBA5 and BBA9 also had an inhibitory effect on *E. coli* with an inhibition zone diameter ranging from 11 mm to 15 mm, and 15 mm to 16 mm against *S. aureus*. This indicates that these isolates have potential as antibacterial.

According to the microscopic characteristics of the two endophytic bacterial isolates that have been identified previously in Monaten' research (2022), isolate BBA5 and BBA9 have the same cell characteristics, which are bacilli-gram-negative bacteria (Monaten, 2022). This is reinforced by Astari's research et al. (2021) who tested the antibacterial activity of endophytic bacterial of the turmeric plant (*Curcuma longa* L.) on the growth *S. aureus*, found that the endophytic bacteria with the greatest inhibitory activity had microscopic characteristics of bacilli-gram-negative bacilli, and the results of cell characterization of these 2 isolates are similar to *Pseudomonas* sp. The genus *Pseudomonas* are endophytic bacteria that are commonly found in almost all plant samples (Astari et al., 2021). This bacterium is easy to grow and has the potential as a biocontrol agent. In addition, *Pseudomonas* is also known as antagonist bacteria due to the secondary metabolites produced including saponin and terpenoid groups. Saponins as antibacterial have a mechanism to disrupt the permeability of cell membranes. Saponins react with lipopolysaccharides to lower the surface tension of the membrane, which will eventually lead to cell destruction. Terpenoids as antibacterial cause damage to cell membranes by lipophilic compounds.

Terpenoids can react with porins (transmembrane proteins) on the outer membrane of the bacterial cell wall to form strong polymer bonds and damage the porins resulting in reduced permeability of the bacterial cell wall so that the bacterial cells lack of nutrition, inhibit bacterial growth or even die (Leonita et al., 2016; Astari et al., al. 2021). This study was also reinforced by a research that examined the content of atung fruit seeds conducted by Hehanussa (2019) which states that atung seeds have antimicrobial properties. The higher the concentration of atung seed powder, the higher the concentration of secondary metabolites such as flavonoids, tannins, saponins, and alkaloids which act as antibacterials. Higher concentrations of antibacterial compounds may inhibit more bacteria (Hehanussa et al., 2019).

On the other hand, the 7 isolates of endophytic bacteria did not show any clear zones against MRSA and MDR *S. epidermidis*. Reculturing endophytic bacteria in this study was not carried out using atung seed extract-containing NA media as used in the previous study (Monaten, 2022). In addition, the isolates have been stored for months, thus it is suspected that the ability of endophytic bacterial isolates to produce compounds secondary actives with antibacterial effects will also decrease significantly to even nothing (Yuwita, 2021). The combination of the several reasons above could answer why endophytic bacterial isolates have absolutely no inhibition against the MDR bacteria, coupled with other factor that supports the absence of inhibition of endophytic bacterial isolates against the pathogenic bacteria, that is, MDR *S. aureus* and *S. epidermidis* as pathogenic bacteria in this study are resistant bacteria and have been proven by the result of susceptibility analysis by Maluku Province Health Laboratory Center, thus allowing their cell walls to become resistant (Monowat & Bhore, 2014; Govindarajan et al., 2021). The MDR *S. aureus* and *S. epidermidis* are a group of Gram-positive bacteria that are resistant to more than one antibiotic (Dross-Anderson & Elhassanny, 2018; Monowar & Bhore, 2014). It is similar with the findings of Nurzakiyah (2016) that tested the inhibition of endophytic bacteria from *Caulerpa racemosa* against *S. aureus* and *Methicillin Resistant S. aureus* (MRSA), it reported that endophytic bacteria had the antibacterial activity against *Staphylococcus aureus,* yet couldn't able to inhibit MRSA. This is allegedly that MRSA cell walls are resistant to antibacterial compound (Nurzakiyah, 2016). Therefore. an effective treatment for skin pathogenic MDR bacterial infections was still performed by tetracycline as shown in this study. However, the use should be appropriate and under control to prevent the incidence of resistance to the tetracycline group (Carrol et al., 2021; Hetem et al., 2017; Yunita et al. 2022c).

CONCLUSION

This research concluded that antibacterial activity test showed that 2 (isolate BBA 5 and BBA9) out of 7 endophytic bacterial isolates were able to inhibit the growth of *C. acnes* with clear zone diameter of 4 mm and 9 mm, respectively, yet couldn't able to inhibit MDR bacteria *S. aureus* and *S. epidermidis*. This study implies that the two isolates could have potential as antibacterial against *C. acnes* with a quite moderate category, thus it needs to be improved and optimized in an effort to develop antibacterial in the future.

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