

Original Research Article

<https://doi.org/10.20546/ijcmas.2017.612.457>

## Screening of Endophyte *Piper betle* Bacteria from the Forests of HPPB University Andalas as Antibiotics Producer

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### ABSTRACT

#### Keywords

Screening,  
Endophytic  
bacteria, *P. betle*,  
Antibiotics,  
Inhibitory zone.

#### Article Info

Accepted:  
28 October 2017  
Available Online:  
10 December 2017

Today livelihood exploration of endophytic microorganisms as a new source of antibiotics is very intensive, especially from medicinal plants. *Piper betle*, a green betel plant, is a medicinal plant. So that bacterial isolates from *Piper betle* are likely to produce antibacterial compounds. This study aims to screen the endophytic bacteria of green betel plants that live wild in HPPB forest, Andalas University. The leaf sampling was done by purposive random sampling, bacterial isolation was done by spread plate method on tryptic soy agar (TSA). Screening of antibiotic-producing endophytic bacteria was done by paper dish method. The results showed that isolation of endophytic bacteria using TSA medium obtained 86 isolates. Screening of antibiotic-producing bacteria was obtained by eleven isolates with six isolates having antibiotic activity against *E. coli* and *S. aureus* test bacteria; one isolate was only active against *E. coli* alone and four isolates were only active against *S. aureus*. Endophytic bacterial, isolates BES-2 has the largest inhibitory zone diameter (18.58 mm) and includes strong category antibiotics.

### Introduction

Betel plants is one type of medicine from nature that can be used as an alternative as an antiseptic, without side effects. The betel extract contains several active components that have antibacterial activity, such as safrol and kavibetol acetate (Arambewela *et al.*, 2005). Green betel extract fractions have antibacterial activity (Hoque *et al.*, 2011) and antifungals of the genus *Aspergillus* and *Candida* (Ali *et al.*, 2010). Endophytes isolated from betel plants are highly potential for the livelihood of new natural products. In theory, endophytic microbes isolated from a plant can produce secondary metabolites

similar to those of original plants or even in relatively high numbers (Radji, 2005). In addition, the advantages of endophytic microbes are short life cycle and secondary metabolites can be produced on a large scale, therefore endophytic microbes have good prospects in the discovery of new compounds one of them as antibiotic producers (Strobel and Daisy, 2003). As host plants, the endemic plants in the subtropical or tropical regions are particularly important because of their richness in species. Utilization of endophytic bacteria to produce bioactive compounds has several advantages such as can be produced in

large scale, possibly obtained new bioactive compounds with different characteristics (Tanaka *et al.*, 1999).

The HPPB forest area of Andalas University has many types of wild life wild betel which is generally dominated by *P. betle* green betel. This allows the discovery of different betel endinocytes from different endophytes, due to environmental factors such as climate, nutrients and location, a factor that greatly determines the diversity of green betel endophytic microbes. The high diversity of high endophytic microbial diversity, also predicted to be found in various antibiotics and the possibility of finding new antibiotic compounds is very large. This study aims to obtain bacterial endophytic bacterial isolates of green betel plants in HPPB forest areas, Andalas University antibiotic producer.

## **Materials and Methods**

### **Collection of leaf samples from betel plants**

Leaf sampling from wild green betel plant was conducted by purposive random sampling method from four locations in HPPB Unand forest area (Fig. 1). Healthy leaf samples, not young leaves or old leaves, each taken ten pieces of betel leaf from each clump. Leaf collecting from plants refers to Melo *et al.*, (2009) that has been modified, the leaves to be collected are cut with a sterile knife then washed with sterile distilled water, then the green betel leaf is placed in a plastic bag and placed in a cooler. Leaves of plants that have been collected immediately brought to the laboratory.

### **Sterilization of leafy plants leaf surface**

Plant leaf samples that have been collected, cut approximately 1 cm<sup>2</sup>. The leaves were then disinfected with 70% ethanol for 1 minute, then immersed with 2% sodium

hypochloride for 1 minute, then fed into a container containing 70% ethanol and incubated for 30 seconds to remove sodium hypochloride and last washed with three sterile distilled water time.

### **Isolation and purification of endophytic bacteria**

The sterile plant segment was finely sliced and then fed into 0.85% physiologic NaCl solution and homogenized, after homogeneous then 0.1 ml of the solution was inoculated in a spread plate on Petridish containing TSA medium containing benomyl fungicide of 1  $\mu\text{L mL}^{-1}$ . It was then incubated at room temperature for 76 hours. Different bacterial colonies were inoculated on a Nutrient Agar medium streak plate and incubated for 24 hours at room temperature. Single colonies are inoculated on slant agar and labeled.

### **Screening of endophytic bacteria of green betel producing antibiotics**

Screening of endophytic bacteria of betel-producing plant antibiotics is done by means of income stages and antibiotic isolation according to Djamaan *et al.*, (2012) that has been modified. Prepared medium for the production of inoculum and antibiotic production with formula in every 1 L: 30 ml of soaking water corn, 30 g sucrose, 5 g CaCO<sub>3</sub>, 2 g MgSO<sub>4</sub>, 1 g FeSO<sub>4</sub> and 0.1 g ZnSO<sub>4</sub> with sterile pH 7.4 in Erlenmeyer. Rejuvenated endophytic bacterial isolates were each administered 1-2 ose in Erlenmeyer containing 50 mL of medium. Bacterial culture was incubated at room temperature, 150 rpm for 24 hours. A 1 ml bacterial culture was inoculated on Erlenmeyer containing 100 mL of antibiotic production medium. The culture was incubated at room temperature, at 150 rpm for 48 hours. Bacterial culture was centrifuged at 5000 rpm for 15 min. The

supernatant obtained was a crude extract of antibiotics to further test its antibiotic activity.

### **Testing of antibiotics from endophytic bacteria *in vitro***

Testing of antibiotic activity of each endophytic bacterial was done by paper disc method. The disc paper is made by gluing 3 layers of Whatman no. 42, then perforated with a piercing paper to obtain discs with diameter of 6 mm, sterilized by autoclave. Poured 15 ml medium NA on Petridish. Furthermore, each medium in a petri dish was applied to *E. coli* and *S. aureus* test bacteria. The disc paper is immersed in a crude antibiotic solution of each endophytic bacterial isolate, then aseptically the disc paper is removed in a sterile container and waited until dry. Aseptic disc paper is placed on a NA medium that has been applied with test bacteria. Subsequently incubated at room temperature for 24-48 hours. The diameter of the barrier formed around the disc paper is measured with the help of the slide term. Endophytic bacteria indicate antibiotic producers if clear zone is formed around the disc paper. Records of endophytic bacterial isolates may produce antibiotics along with the inhibitory zone diameter.

### **Results and Discussion**

Isolation of endophytic bacteria from wild green betel plants that live in HPPB forest using TSA medium, obtained by bacteria colony isolate endofitik. Endophytic bacterial isolates generally have moderate growth rates ie at 48 hours of incubation with a variety of colonial morphological characteristics such as shape, color, edges, colony elevation. This means that TSA medium can be used as a medium to isolate endophytic bacteria of wild betel plant that live wild in forest area, while Nutrient agar medium can be used as a

medium for purification of endophytic bacteria. Endophytic bacterial characteristics generally grow in isolation medium after two days of incubation (Jalgaonwala *et al.*, 2010). The presence of endophytic bacteria in plants with a high diversity of colonies indicates that the environmental factors in which green betel plants are alive and evolved have a very important role, this is because HPPB forest area of Andalas University is including tropical rain forest with high moisture and forest land covered by litter that is thick enough to contain nutrients and minerals that are ideal for growth and development for soil microorganisms, including bacteria.

Endophytic bacteria originating from the environment around the plant such as the area of rhizosphere and plant phiosphere capable of penetrating into the plant tissue through stomata, lentikula or area of the emergence of lateral roots that cause the difference of the type of endophytic bacteria obtained and secondary metabolites it produces (Mano and Morasaki, 2008). Endophytic bacteria live in the vascular vessels or in the intercellular space, entering into the plant tissue in general through the roots, but also through the leaf stomata (Zinniel *et al.*, 2002).

The results of screening of eighty six green betel endophytic isolates showed that eleven bacterial isolates indicated antibiotics, indicated by the inhibition zone of *E. coli* and *S. aureus* test bacteria, while seventy-five other endophytic bacterial isolates were likely to have other roles in betel plants or have the ability to produce the same secondary metabolites as those of green betel plants such as chemical compounds that have antimalarial activity, inhibitory agents against xanthine oxidase, anti-clotting or bacterial isolates each have the ability to produce other expressed secondary metabolites of the gene that had existed in the bacterial cell (Fig. 2).



Fig.1 Leaf of *Piper betle*



Fig.2 Inhibitory zone on *E. coli*

Table.1 Antibiotic test of isolates on *E. coli* and *S. aureus*

Isolate code	Inhibitory zone (mm)	
	<i>E. coli</i>	<i>S. aureus</i>
BES-1	8.66	6.33
BES-2	18.58	10.33
BES-3	10.66	11.66
BES-4	-	10.00
BES-5	7.66	-
BES-6	10.46	7.66
BES-7	-	7.33
BES-8	9.46	8.66
BES-9	-	9.33
BES-10	-	9.00
BES-11	10.53	11.33

Note: - (no inhibition zone is formed)

Exploration of betel endophytic microbes in the north-eastern part of the Indian state, found *Muscodor albus* which is a beta endophytic betel *Piper nigrum* that has strong antibiotic activity (Banerjee *et al.*, 2014). Endophytic bacteria from betel *P. colubrinum* and *P. nigrum* are also antibiotic producers (Kulkarni *et al.*, 2007). Fourteen green betelitic endophytic bacteria or *P. betle* live in Bogor district with the highest number of isolates from leaf organs with one potentially antibiotic-producing BS1 (*Pseudomonas* sp.) Isolates which can inhibit the growth of three pathogenic bacteria (Purwanto, 2014). Table 1 shows that eleven bacterial endophytic bacteria of green betel produce antibiotics

with different inhibitory zones with each other, six isolates of endophytic bacteria with code BES-1, BES-2, BES-3, BES-6, BES-8 and BES 11 have zone inhibition against both test bacteria, it shows that the six isolates of endophytic bacteria are antibiotics that resulted are broad spectrum. Five other endophytic isolates are narrow spectrum, one isolate of BES-5 endophytic bacteria have antibiotic activity only in Gram negative bacteria *E. coli* and four isolates of endophytic bacteria BES-4, BES-7, BES-9 and BES-10 only on Gram bacteria positive *S. aureus*. Endophytic bacterial isolates BES-2 highest antibiotic producing zones, 18.58 mm against *E. coli* test bacteria, and BES-3

endophytic bacterial isolates with the highest inhibitory zone (11.66 mm) in *S. aureus* test bacteria. Category of antibiotic activity strength based on inhibit zone, showed that five isolates of antibiotic-producing endophytic bacteria with strong category (BES-2, BES-3, BES-4, BES-6 and BES-11). Inhibitory diameter 10 - 20 mm including strong category, diameter more than 20 mm including very strong category and inhibition diameter between 5 - 10 mm belong to medium category (Davis and Stout, 1971).

### Acknowledgments

Acknowledgments are presented to Faculty Mathematics and Natural Science, Andalas University which has provided research funding.

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**How to cite this article:**

Anthoni Agustien, Putra Santoso, Nesity Permata Sari, Fathya Annisa, Nasril Nasir, Yetria Rilda and Akmal Djamaan. 2017. Screening of Endophyte *Piper betle* Bacteria from the Forests of HPPB University Andalas as Antibiotics Producer. *Int.J.Curr.Microbiol.App.Sci*. 6(12): 3970-3975. doi: <https://doi.org/10.20546/ijcmas.2017.612.457>