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Research Article

Exploration of Antibiotics-Producing Endophytic Bacteria Isolates from Betel Leaves in Jambi City Forest Park

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Abstract

Background and Objective: Sources of antibiotics that are cheap and easy to obtain are by exploiting endophytic microbes from betel plants because betel plant extracts are known to produce anti-microbial compounds. This research aimed to obtain isolates of antibiotic producer endophilic bacteria from betel leaves in Jambi city forest park. **Materials and Methods:** This research consists of several stages, sterilization of the collected betel leaf surface, isolation and purification of endophytic bacterial isolates, screening of antibiotic-producing bacteria and characterization of endophytic bacteria. **Results:** There were 3 types of betel plants found in the area, namely: *Piper betle* L. (green betel), *Piper crocatum* Ruiz and Pav (red betel) and *Piper aduncum* L. (forest betel). In red betel leaves, 9 types of endophytic bacterial isolates were obtained, while only 1 isolate was found in each green betel and forest betel leaf. Of these 11 isolates, 9 of them produced antibiotics. However, only 6 isolates were potential since they had a halo zone bigger than 10 mm for *E. coli* (gram-negative) and *S. aureus* (gram-positive). Among these 6 isolates, 5 isolates were from red leaves with isolate codes of BESKJ-m2, BESHKJ-m3, BESHKJ-m4, BESHKJ-m5, BESHKJ-m6 and 1 isolate from forest betel leaves with code of BESHKJ-s1. **Conclusion:** From the control positive test using chloramphenicol, it was known that the endophilic bacteria had a broad spectrum. The bacterial colonies were white and had an elevation on the surface of the media with flat to irregular margins. The catalase and Gram stain tests showed that the bacteria were Gram-positive.

Key words: Exploration, bacteria, endophytic, antibiotics, betel leaf, forest park, fermentation

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Betel is a type of natural medicinal plant, which is usually used as an alternative, antiseptic without side effects. The leaf extract can produce anti-bacterial compounds, including chavibetol acetate and safrole. Green betel extract fractions have antibacterial activity against food pathogens[1,2]. The potential of endophytic bacteria in betel leaf bones to produce new antibiotics is very large, so it is necessary to research the isolation, identification, characterization and antibiotic testing of these endodopitic bacteria. The types of endophytic bacteria in betel plants are highly dependent on the environment in which they grow. So in this study, the location of the betel leaf sampling was determined in the Jambi city forest park, known as the Muhammad Sabki City Forest Park, in Kenali Village, Asam Bawah, District New city, Jambi City. With more than 200 species of herbs, trees and shrubs, with the presence of several betel plants, it is possible to find new endophytic bacteria that are different from other endophytes, because climate, nutrition and location, are factors that determine the diversity of betel endophytic bacteria.

Pathogenic microbes that are resistant to antibiotics are quite abundant, this adds to the need for new and more effective antibiotics. The production of antibiotics can be done by chemical synthesis processes, from microbes and plants[3]. The manufacture of antibiotics in Indonesia is highly dependent on imports of medicinal raw materials, now the import value is still high (90%). In efforts to reduce imports, the government has determined that the use of the country's biodiversity is gradually used as raw materials for the manufacture of antibiotics, the production of which is through fermentation[4,5]. Resource search endophytic microbes get a lot of attention because there are still many that have not been characterized, so their potential has not been maximized, especially in our country, Indonesia[6].

Another advantage of endophytic bacteria is that their life cycle is short and produces secondary metabolites on a large scale, which is why endophytic microbes are said to have excellent prospects in the discovery of new compounds, one of which produces antibiotics[7].

The purpose of this study was to obtain bacterial isolates endophytic betel plant, which lives in urban forests as a producer of antibiotics and to determine the character of the endophytic bacterial isolates.

MATERIALS AND METHODS

Time and place: This research has been carried out at the UPT Basic and Central Laboratory andalas University, Padang and the Faculty of Microbiology Laboratory Saintek, Jambi University, Mendalo Indah Village, Subdistrict Jambi Out of Town, Regency Muaro Jambi from June-November, 2021.

Tools and materials: Research using equipment, laminar flow, oven, incubator, autoclave, microscope, microwave, petri dish, test tube, Erlenmeyer, glass beaker, micropipette, glass, loop needle, knife, scalpel, pH meter, thermometer, micrometre, calliper and analytical balance.

The materials used are, medium Nutrien Agar, Bakto Agar, Nutrien Broth, Tryptic Soy Broth, colouring agents, alcohol, lugols, distilled water, coffee paper, labels, permanent markers and chemicals for characterization of endophytic bacteria, aluminium foil and betel leaf.

Sampling: A sampling of leaves from the three types of betel plants studied, (green betel, red betel and forest betel) was conducted by purposive random sampling method, samples were taken from the location Jambi City Forest Park Area. Leaf samples were taken that looked healthy, not young leaves or old leaves, each leaf sample was taken with as many as ten leaves from each clump[4].

Sterilisation of surface forest betel leaf: The surface of the forest betel leaf that has been taken is then carried out by surface sterilization by immersing each forest betel leaf into sterile distilled water for 10 min, then transferred to the second solution, namely 70% ethanol solution for 10 min, then rinsed with sterile distilled water for 5 min. The surface of the sterilized betel leaf is then cut off the leaf part near the leaf bone with a size of 1×1 cm using a sterile scalpel[8].

Isolation and purification of Jambi betel leaf endophytic bacteria: For isolation and purification of endophytic bacteria, sterile plant segments were cut into pieces, ground until smooth, then put into a 0.85% physiological NaCl solution and homogenized, after being homogeneous, 0.1 mL of the solution was inoculated into a Petri dish containing medium. TSA containing the fungicide benomyl as much as 1 L mL^{-1} using the spread plate method and then incubated at room temperature for 24-48 hrs.

Purification of endophytic bacterial isolates of forest betel plants: Bacteria that grew with different colonies were separated and inoculated in nutrient agar on a streak plate and incubated again for 24 hrs, at room temperature. Bacteria with single colonies that were homogeneous were purified and propagated on slanted media and labelled with isolate code.

Screening test of endophytic bacteria isolate leaves of antibiotic-producing forest betel plants

Making sources of endophytic bacterial inoculum: Bacterial inoculum was prepared by inoculating 1-2 oses of endophytic bacterial isolate into sterile Tryptic Soy Broth (TSB) medium in Erlenmeyer with a volume of 25 mL of dium. The medium was shaken using a shaker incubator at a speed of 120 rpm, for 24 hrs at a temperature of 28°C.

Production of antibiotics bacterial endophytic leaves of forest betel plants by fermentation: About 5 mL of inoculum was then transferred to a new sterile TSB medium using an erlenmeyer with a final volume of 100 mL of media. The medium was then shaken with a shaker incubator at a speed of 120 rpm for 24 hrs at a temperature of 28°C.

Preparation of test bacterial suspension: Each of the test bacteria, namely *E. coli* and *S. aureus*, was inoculated with 1 ose isolate into 9 mL sterile 0.85% NaCl physiological solution in a test tube. The test tube was vortexed until homogeneous. This suspension solution was compared with McFarland 0.5 to form the number of test bacterial cells as many as 106 CFU cells.

Endophytic bacteria antibiotic activity test using Disc Diffusion Method: The fermented medium was then taken as much as 1 mL and put into a sterile microtube tube, then the microtube tube was centrifuged at 10,000 rpm for 10 min. The purpose of this disterifus is to separate bacterial cells. The bacterial supernatant that had been formed was then tested by taking as much as 10 L of the supernatant placed on sterile disc paper on top of NA media which had been spread by the test bacteria. The medium was incubated for 24-48 hrs at 28°C. The positive control used was the commercial antibiotic chloramphenicol and the negative control used sterile distilled water.

RESULTS AND DISCUSSION

Isolation of betel leaf endophytic bacteria: Three types of betel plants of different species, namely green betel (*Piper betle* L.), red betel (*Piper crocatum* Ruiz and Pav) and forest betel (*Piper aduncum* L.) were found in Jambi city forest park. These three types of betel plants have leaves that are morphologically very different, in shape, size and colour. Green betel leaf and red betel leaf have a leaf shape that is almost round like a heart and very flat, with flat edges and tapered ends, pinnate leaf bones with a grooved base.

The green betel leaf is wider than the red betel leaf and the colour of the two is also very different, the green betel leaf is bright green, while the red betel leaf is darker in colour, approaching maroon in low light and green in the sunlight. The leaf surface looks rougher than the green betel leaf (Fig. 1a-c).

Taxonomically, this betel plant belongs to the piperaceae family, the order piperales and the class dicotyledoneae. The betel plant has several names, depending on where it comes from, the name betel itself is called by people who come from Minangkabau and Palembang, in Aceh it is called Ranub, in Mandailing it is called Burangir, in Karo, it is called Belo Batak, in Mentawai, it is called Chili, in Sunda, it is called Seureuh, in Madura, it is called Sere, in Flores, it is called Mota and so on.

In the field, this betel plant grows vines that lean on other plants, on house walls, fences and so on. It has a single leaf with a plant length of 10 meters.

Table 1 presents the results of bacterial isolation from 3 kinds of betel leaves. In 4 Petri dishes planted with red betel leaf, the growth of bacterial colonies was more and more varied than in the Petri dishes planted with green betel and forest betel. In Petri dish no. 2, bacterial colonies were found that filled the entire surface of the medium on the petri dish evenly, while in Petri dish no. 1, 3 and 4, which were cultured, 22, 14 and 22 bacterial colonies were obtained, respectively. The results of the isolation of bacteria on red betel leaf found 9 different types of bacteria, while on green betel leaf and forest betel, each of them found only 1 type of bacteria from 4 cultured Petri dishes.

The number of colonies that live from each type of betel leaf is different as shown in Table 1. A large number of bacterial colonies were obtained on a red betel leaf. The interaction of endophytic bacteria and plants is a form of symbiosis. The symbiosis between plants and endophytic bacteria is neutral, mutualism or commensalism[9]. Mutualism symbiosis between endophytic bacteria and plants, in this case, endophytic bacteria get nutrients from plant metabolism and protect plants against the pathogen, sedangkan tanaman obtain nutritional derivatives and active compounds needed for life[10].

The endophytic bacterial colonies isolated from these forest plants showed diversity, in terms of colour, shape and growth speed. This was following[11] which stated that endophytic bacteria in one host plant generally consist of several genera and species. The diversity of endophytic bacteria in a plant is also influenced by plant growth conditions, especially soil conditions. In some cases, plants

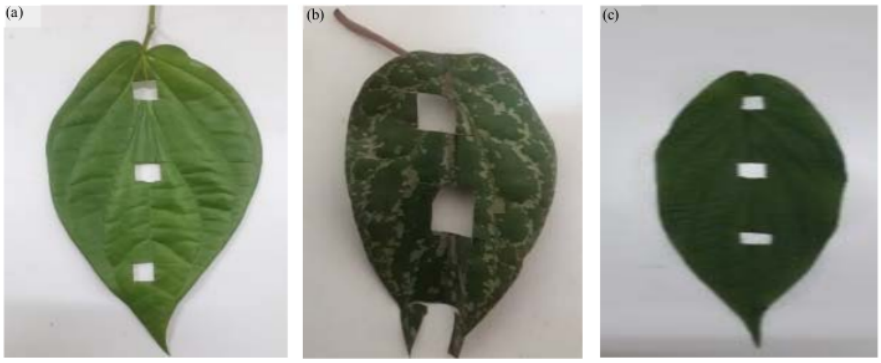

Fig. 1(a-c): Morphology of leave battle forest, (a) Green betel, (b) Red betel and (c) Forest betel

Table 1. Isolation results of endophytic bacteria from forest betel leaf plants

Betel types	Point	Number of colonies	Purified isolate
Green betel (*Piper battle* L.)	I	4	
	II	4	
	III	3	
	IV	11	1
Red betel (*Piper crocatum* Ruiz & Pav)	I	22	3
	II	Full/spread	1
	III	14	2
	IV	22	3
Forest betel (*Piper aduncum* L.)	I	6	1
	II	Full	
	III	Full	
	IV	6	

Table 2: The results of the screening test for endophytic bacterial isolates from 3 types of betel leaf from Jambi city forest park

			Inhibition zone		
mple names	Origin of isolate	Isolate codes	*E. coli* (mm)	*S. aureus* (mm)	Screening test isolate bacteria
Green betel (*Piper battle* L.)	I/1	BESHKJ-h1	0	0	Not have
Red betel (*Piper crocatum* Ruiz & Pav)	I/1	BESHKJ-m1	8	0	Kill G- no G+
	I/2	BESHKJ-m2	11	10	Kill G- and G+
	I/3	BESHKJ-m3	25	25	Kill G- and G+
	II/1	BESHKJ-m4	20	22.5	Kill G- and G+
	III/1	BESHKJ-m5	11.5	13.5	Kill G- and G+
	III/2	BESHKJ-m6	13	17.5	Kill G- and G+
	IV/1	BESHKJ-m7	19.5	0	Kill G- no G+
	IV/2	BESHKJ-m8	0	0	No potential producer of antibiotics
	IV/3	BESHKJ-m9	8	9	Positive kill *E. coli*
Forest betel (*Piper aduncum* L.)	I/1	BESHKJ-s1	16.25	16	Kill G- and G+
Positive control Kloramfenikol			30	27.5	Kill G- and G+ board spectrum
Negative control sterile aqua dest			0	0	No activity

with the same type or species have endophytic bacteria that were not always the same. In some plants, there are specific and distinctive endophytic bacteria that inhabit these plants.

Screening of endophytic bacteria isolates in forest betel leaf produces antibiotics: A total of 11 different endophytic bacterial isolates were then tested for screening as producers of antibiotics, presented in Fig. 2. and Table 2.

The result of Table 2 and Fig. 2 can be seen that 11 isolates had activity as antibiotics, which were able to kill *E. coli* and *S. aureus* bacteria. Of these 11 isolates, six potential isolates were obtained, namely the activity of the inhibition zone above 10 mm. Six of these isolates are indicated by the isolated code BESHKJ m-2, BESHKJ m-3, BESHKJ m-4, BESHKJ m-5, BESHKJ m-6 dan BESHKJ- s1. The six isolates of endophytic bacteria are categorized as strong inhibitors because they have inhibition above 10 mm. In

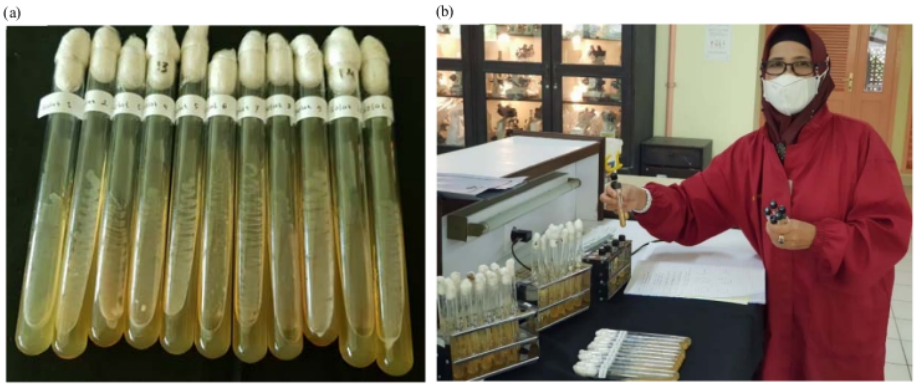

Fig. 2: Pure isolates of endophytic bacteria from 3 kinds of betel leaf from the Jambi city forest

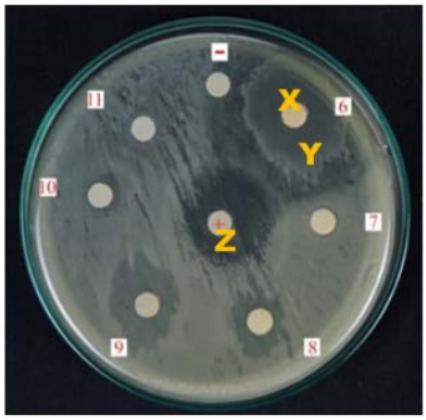

Fig. 3: Killing power of antibiotics and endophytic bacteria from 3 kinds of betel leaf from the Jambi city forest

3x: Endophytic bacteria, 3y: clear zone, 3z: chloramphenicol positive control, (6-11) isolate code number, (+) chloramphenicol as a positive control and (-) distilled water as a negative control

Fig. 3 isolates of endophytic bacteria no. 6-X, forming a halo zone that is larger than the other isolates (3Y). The halo zone formed is larger than the halo zone formed by the antibiotic chloramphenicol as the positive control (3Z).

The formation of a clear area around the colony of endophytic bacterial isolates indicates the possibility of antibacterial compounds capable of killing or at least inhibiting the growth of pathogenic bacteria. This was confirmed by Yahya *et al.*[10] and Sudewi *et al.*[12], who conducted a similar test with endophytic bacteria from Indonesian medicinal plants. Isolated endophytic bacteria showed inhibitory activity against pathogenic bacteria characterized by the formation of a clear area around the endophytic bacterial colonies.

These six potential endophytic isolates were categorized as board spectrum because they could kill Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. The presence of the Slavy zone produced by endophytic bacteria was because endophytic bacteria have antibacterial compounds. The advantage of using these endophytic bacteria have the same compounds as the original plant. Betel plants contain phenolic compounds and their derivatives which can inhibit the growth of Propionibacterium acnes bacteria. The antibacterial mechanism of phenol compounds in killing microorganisms is by denaturing bacterial cell proteins and the presence of these compounds so that endophytic bacteria produce an inhibitory zone against the test bacteria *E. coli* and *S. aureus*[13].

Table 3: Macroscopic characterization of potential endophytic bacteria of leave betel forest

		Macroscopic character			
Isolate sample codes	Isolate codes	Colour	Shapes	Margin	Elevation
Na/SM-1-2	BESHKJ-m2	White	Circular	Undulate	Convex
Na/SM-1-3	BESHKJ-m3	Cream	Bulat	Bergerigi	Convex
Na/SM-2-1	BESHKJ-m4	White	Irregular	Undulate	Convex
Na/SM-3-1	BESHKJ-m5	Cream	Irregular	Undulate	Convex
Na/SM-3-2	BESHKJ-m6	White	Irregular	Undulate	Convex
Na/SR-2-1	BESHKJ-s1	Cream	Irregular	Undulate	Flate

Table 4: Microscopic characterization of isolates of endophytic bacteria producing antibiotic potential from forest betel leaf

		Characteristic microskopis			
Isolate sample codes	Isolate codes	Gram	Cell shape cell	Arrangement	Endospore
Na/SM-1-2	BESHKJ-m2	Positive	Basil	Diplobasil	Have
Na/SM-1-3	BESHKJ-m3	Positive	Basil	Diplobasil	Have
Na/SM-2-1	BESHKJ-m4	Positive	Basil	Streptobasil	Have
Na/SM-3-1	BESHKJ-m5	Positive	Basil	Basil	Have
Na/SM-3-2	BESHKJ-m6	Positive	Basil	Streptobasil	Have
Na/SR-2-1	BESHKJ-s1	Negative	Basil	Streptobasil	Have not

Macroscopic and microscopic characterization of isolates of potential endophytic bacteria in antibiotic-producing forest betel leaf: Macroscopic and microscopic characterization of potential isolates is presented in Table 3 and Table 4. These six potential isolates, it was found that they were very diverse based on their morphological characteristics. Meanwhile, on microscopic observation, five isolates were Gram-positive, one was Gram-negative with rod cell shape and cell arrangement consisting of diplobacillius and streptobacillus and five isolates had endospores.

CONCLUSION

The current study concluded that Muhamad Sabki City Forest Park, Jambi City, has three kinds of betel plants, namely green betel, red betel and forest betel. About 11 types of antibiotic-producing endophytic bacteria from betel leaf were isolated. Endophytic bacteria that produce potential antibiotics have 5 isolates from red betel leaf with isolate code BESKJ-m2, BESHKJ-m3, BESHKJ-m4, BESHKJ-m5, BESHKJ-m6 and 1 isolate from forest betel leaf with isolate code BESHKJ-s1. Six isolates had 5 microscopic characteristics including Gram-positive and one isolate was Gram-negative, with cells composed of diplobacillus, streptobacillus and five isolates formed endospores.

SIGNIFICANCE STATEMENT

Endophytic bacteria can produce antibiotics that are needed by the community because it is necessary to find a source of production, betel leaf is one source of endophytic bacteria, betel leaf is widely found in the forest park of Jambi City. It is hoped that new types of endophytic bacteria with the potential for producing antibiotics will be obtained from betel leaves.

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