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SHORT COMMUNICATION

Isolation and Screening of Endophytic Microbes from *Morinda citrifolia* and their Ability to Produce Anti-Microbial Substances

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Assaying for, and isolation and screening of endophytic microbes from the *Morinda citrifolia* plant and their ability to produce anti-microbial substances was carried out. Endophytic microbes are microorganisms that live asymptomatically within the living tissue of host plants. Microorganisms such as bacteria, fungi, and yeast can be associated with the host and produce secondary metabolites. These secondary metabolites may be enzymes and other bioactive substances with medicinal activity such as anti-arthritic, anti-cancer, and anti-microbial compounds. The aims of these experiments was to investigate the ability of endophytic microbes isolated from *M. citrifolia* to produce secondary metabolites which can act as anti-microbial agents. A direct seed inoculating technique was used by planting the plant sample onto the surface of Nutrient Agar and Potato-Dextrose Agar. Assessment of their ability to produce anti-microbial substances was conducted by growing the endophyte isolates in Muller Hinton Broth for bacterial isolates, and in Potato-Dextrose Yeast Broth for fungal isolates. The agar diffusion method using paper disk was applied to assay the anti-microbial activity of each substance. The results of the endophyte isolation in these experiments gave five bacterial isolates and eleven fungal isolates. All of the bacterial isolates showed a broad anti-microbial spectrum while ten of the fungal isolate demonstrated broad anti-microbial activity and four out of the ten fungal isolates had activity towards *Candida albicans.*

Key words: anti-microbial, endophytic microbe, *Morinda citrifolia* (*mengkudu*), secondary metabolite

Infectious disease is one of the main health problems in the developing countries including Indonesia. According to Household Health Survey 1997, the main cause of death are as follows: 28.1% are caused by infectious and parasitic diseases, 18.9% are due to vascular diseases, and 15.7% are caused by respiratory diseases (Depkes 1997). This evidence demonstrates that the prevalence of infectious disease in Indonesia is still high, and to solve this problem new anti-infection drugs that are potent and affordable must be available especially for those in the low to middle economic society classes. This encourages research to find new natural low cost medicinal sources with potent anti-microbial activity.

The microbes existing in plants are called endophytic microbes. These microbes spent part or their entire lifetime in the living tissue of the host plant without causing any harm (Petrini *et al*. 1992). Microbes such as fungi, yeast, and bacteria associated with the host plant assist in the metabolism of the host plant, and produce secondary metabolites with potent medicinal activity such as anti-tumor, anti-bacterial, and anti-fungal compounds as decomposing enzymes, and also plant growth hormones (Petrini *et al*. 1992; Strobel *et al*. 1996; Strobel 2002).

Mengkudu is a tropical plant that has been used to treat a variety of diseases since thousands of years ago. Studies showed that parts of the *mengkudu* plant (*Morinda citrifolia*) such as the fruit, leaf, root, and bark apparently have a therapeutic effect. Some of these effects are the lowering of high blood pressure. Improving body resistance, pain relief, anti-tumor and anti-cancer, anti-inflammatory and anti-bacterial activity.

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Up to now there have been few studies, which focus on the medicinal activity of endophytic microbes as anti-microbial substances. The objective of this study is to identify the endophytic microbes in the *mengkudu* plant and their capacity to produce secondary metabolites that have anti-microbial effect.

The twig of the *mengkudu* plant which was used in this study had been identified at the Herbarium Bogoriense, Balitbang Botani, Puslitbang Biologi LIPI, Bogor (The Bogor Herbarium, Botanical Research and Development Agency). The test microbes used in this assay were *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Salmonella typhimurium,* and *Candida albicans*. These microorganisms were derivates of ATCC and were obtained from laboratory of microbiology, medical faculty, University of Indonesia. Twigs from the second and the third branches of the *mengkudu* plant (diameter of 0.3-0.6 cm) were selected for use in this study. Leaves were removed and the twigs were cut into lengths of 2 cm and washed with under running water (tap water) for about 10 min. The sections of the twigs were sterilized by soaking in 75% v/v ethanol for 1 min in the Laminar Air Flow Cabinet (Gelman Sciences, Australia), and then in a 5.3% w/v solution of NaOCl for 5 min. Following the sterilization step, samples were air-dried on sterile tissue paper for 1 min. After drying, they were placed on a sterile glass slide and cut longitudinally with a scalpel into two equal parts. One part was placed on an nutrient agar (NA) medium that had been treated with Nystatin (0.01% w/v) as an antifungal agent and the other part was on Potato Dextrose Agar that had been treated with Chloramphenicol (0.005% w/v) as antibiotic. Each sample was positioned longitudinally such that the surface of the cut was placed in contact with the medium. Samples were then placed in an incubator for 5-7 days at 27-29 °C (Bacon 1988).

Bacterial colony identification was through observation and was carried out based on the following criteria: color, surface, the margin of the colony as well as Gram staining. Fungal colony identification was through observation based on the color of colonies and color contrast. Sample that matched with this observation criteria were considered to be the same isolate while those that did not match with the criteria were considered as a different isolate. Furthermore, individual colonies with different morphology were separated and considered as individual isolates. Observation of the morphology was again conducted after 5-7 days of incubation and each isolate was transferred into two different types of culture media (stock and working culture). Each stock culture was kept in paraffin lig, while each working culture was kept in slant agar.

The 24 h bacterial sample in the NA slant was suspended by adding 5 ml of muller hinton broth (MHB). The opacity of bacteria is made equal to the opacity of 0.5 McFarland standard. Then, 1 ml of bacterial suspension was put into a 50 ml Erlenmeyer flask containing 9 ml of MHB medium. The fermentation process was done using a shaking incubator for 48 h with a speed of 120 rpm. The secondary metabolites, as product of fermentation, were centrifuged with a speed of 3 000 rpm for 20 min. The supernatant was used for anti-microbial assay (Rosana *et al*. 2001).

A section of the fungal isolate that had sporulated (aged 7-10 days) was taken out using a sharp forceps, (dimension of 1 x 1 cm) and was placed into 10 ml liquid fermentation medium of Potato Dextrose Yeast Extract Broth in a 50 ml Erlenmeyer flask. The fermentation process was conducted by using a shaker for 7 days with a speed of 170 rpm. The product of fermentation was centrifuged with a speed of 3 000 rpm for 20 min, and the supernatant was used for anti-microbial assay (Rosana *et al*. 2001).

The assay was performed using a paper disk for the fungi and using stainless cylindrical tubes for the bacteria (Kumala *et al*. 2006). The Gram positive test bacterial were *S. aureus* and *B. subtilis*, the Gram negative test bacterial were *E. coli* and *S. typhimurium*, and the yeast test microbe was *C. albicans*. Nutrient Agar was used as the medium for *S. aureus, B. subtilis, E. coli,* and *S. typhimurium* bacterial test and PDA was used as the medium for *C. albicans* yeast test microbe. Disks were dipped into the suspension until it was saturated, and then placed on the agar medium that had been inoculated with test microbes. The test microbe was adjusted with 0.5 McFarland standard. For the bacteria, 100 ml of suspension of the product of fermentation was put into the stainless cylinder that had been placed on the agar medium that had been inoculated with the test microbe. Then, it was incubated at a temperature of 37 °C and assayed for two days. Assay was by observation which was carried out to determine a clear zone around the disk and stainless cylindrical tubes.

Table 1 Data of endophytic bacteria

Part of plant	Isolate code	Shape	Gram stain
Second branching	NATgPc 5A	Cocco bacil	Gram negative
First branching	NATgPc 3B	Cocco bacil	Gram positive
Second branching	NATgPc 2A	Cocco bacil	Gram positive
Second branching	NATgPc 1B	Cocco bacil	Gram positive
Second branching	NATgPc 1C	Cocco bacil	Gram negative

The endophytic bacteria isolated from the piece of twig were grown in NA medium that had been added with Nystatin as an anti-fungal agent. Three Gram positive endophytic bacteria and two Gram negative endophytic bacteria were obtained (Table 1).

Endophytic fungi isolated from the piece of twig were grown in PDA medium that had been added with chloramphenicol as an anti-bacterial agent to grow the fungi. Eleven endophytic fungi were obtained (Table 2).

In this study, we screen endophytic microbes that can produce secondary metabolite that has potential anti-microbial substances. The *in vitro* testing showed that the secondary metabolite of the endophytic microbes produced a zone of inhibition of the test microbes.

Of the 5 bacteria, all of them (100%) had activity against the test bacteria (*S. aureus, E. coli, S. typhimurium, B. subtilis*) and on the yeast (*C. albicans*). The diameter of the zone of inhibition and the illustration of the zone of inhibition can be seen in Table 3 and Fig 1. Table 4 shows the inhibition zone of the endophytic fungi.

This study is a preliminary study to find a source of anti-microbial compounds from endophytic microbes. The isolates obtained were fungal and bacterial endophytic microbes.

From the isolations carried out, we obtained five endophytic bacterial isolates and eleven endophytic fungal isolates. Of the five endophytic bacterial isolates obtained, all of them had the same cell shape, that is rod-shaped, in which 3 isolates were Gram positive and 2 isolates were Gram negative. Identification of endophytic bacterial and fungal isolates from the *mengkudu* plant was only carried out based on observed morphology and grouping by Gram staining. These results showed that endophytic microbes living in the *mengkudu* plant contained more Gram positive than Gram negative. The products of bacterial fermentation were tested for anti-microbial activity. The test microbes used were

Table 2 Macroscopic morphology of endophytic fungi

Part of plant	Isolate code	Morphology and color of colony on PDA medium at 27-29 °C
First branching	PDAPgPc 5A1-1	white undulate, reverse white, aerial concentric
First branching	PDAPgPc 4A1-A2	green to black velvety, reverse black
Second brancing	PDATgPc 3A2	brown to withish,, reverse white, aerial concentric
Second brancing	PDATgPc 3A	white hyphae, reverse white, with aerial concentric
First branching	PDAPgPc 1C	black at center, reverse white milk
Second brancing	PDATgPc 5A	white undulate, reverse white, aerial concentric white milk
First branching	PDAPgPc 1D	grey velvety, edge of colony entire, reverse white
Second brancing	PDAPgPc 4-C2	black velvety, reverse grey
First branching	PDATgPc 2	yellow, reverse white milk, aerial concentric
First branching	PDAPgPc 4A1	white, reverse white
Second brancing	PDATgPc 5A1-2	white edge of colony undulate, reverse white

Table 3 Data for anti-microbial assay of endophytic bacteria using a 1.0 cm cylindrical tube

Isolate code	Diameter of zone inhibition (cm)				
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Salmonella typhimurium*	*Candida albicans*
NATgPc 5A	++	+	+	+	+
NAPgPc 3B	+	+	+	+	+
NATgPc 2A	+	+	+	+	+
NATgPc 1B	+	+	+	+	+
NATgPc 1C	+	+	+	+	+

+: < 1.2, ++: > 1.2.

Table 4 Data for anti-microbial assay of endophytic fungi using 0.6 cm paper disk

Isolate code	Diameter of zone inhibition (cm)				
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Salmonella typhimurium*	*Candida albicans*
PDAPg Pc 5A1-1	+	+	+	+	-
PDAPg Pc 4-A2	+	+	+	+	-
PDATg Pc 3A2	++	+	++	+	-
PDATg Pc 3A	+	+	+	+	-
PDAPg Pc 1C	-	-	-	-	-
PDATg Pc 5A	+	+	+	++	-
PDAPg Pc 1D	+	+	+	+	-
PDAPg Pc 4-C2	++	+	+	++	++
PDATg Pc 2	++++	++	+++	+++	++
PDA Pg Pc 4A1	+	+	+	+	+++
PDATg Pc 5A1-2	+	+	+	+	+

-: no inhibition, +: < 0.80, ++: > 0.80 - < 1.00, +++: > 1.00 - < 1.20, ++++: > 1.20.

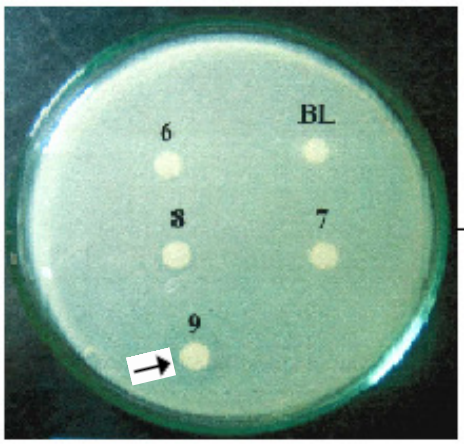

Fig 1 Anti-microbial assay of endophytic fungi towards *Staphylococcus aureus.* ➔No. 9 shows inhibition zone.

S. aureus, B. subtilis, E. coli, and one yeast *C. albicans.* The results of the anti-microbial activity test showed that the five endophytic bacterial isolates of the *mengkudu* plant produced anti-microbial substances. This was demonstrated by the formation of a clear zone surrounding the paper disk, indicating that there was a zone of inhibition of growth of the test microbe. Of the five test microbes, *S. aureus* was the most sensitive to the anti-microbial substances produced by the endophytic bacteria. Shaking was used in this assay as Kumala *et al*. (2005) stated that isolated fungal or bacterial endophytes produced more secondary metabolites than if still fermentation methods were used.

The product of fungal fermentation in the PDY medium showed that ten endophytic fungal isolates from the *mengkudu* produced anti-microbial substances. This was demonstrated by the formation of a clear zone surrounding the cylindrical tube, indicating that there was a zone of inhibition of the growth of the test microbe.

The four types of test bacteria used, which were *S. aureus, B. subtilis, E. coli, S. typhimurium*, and the test yeast used, which was *C. albicans*, were sensitive to the anti-microbial compounds produced by the endophytic fungi. Of the five test microbes, *S. aureus* was the most sensitive to the anti-microbial substance produced by the endophytic fungi. There was one endophytic fungal isolate that was not capable of producing anti-microbial substances. This showed that no zone inhibition of growth of the test microbes.

Using cylindrical tubes not paper disks, the bacteria isolate needs to obtain a clear zone. For the smalles quantities of anti-microbial agent it is easier to assay using cylindrical tubes rather than paper disks (Kumala *et al*. 2006).

The result of this anti-microbial assay showed that the anti-microbial substances produced by the endophytic bacteria and fungi had a broad spectrum because it was capable of inhibiting the growth of Gram positive bacteria, Gram negative bacteria, and a yeast. Furthermore, the number of isolated fungi which were obtained in this study were more than for the bacteria endophytes. These results agree with a previous investigation (Kumala *et al*. 2006).

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