Screening of Endophytic Bacteria Producing Antifungal Isolated from Indonesia Medicinal Plant, Java Ginseng (Talinum triangulare)(Jacq)

by Alimuddin Ali
SCREENING OF ENDOPHYTIC BACTERIA PRODUCING ANTIFUNGAL ISOLOG FROM INDONESIA MEDICINAL PLANT, JAVA GINSENG (TALINUM TRIANGULARE) (JACQ.) WIIILD

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ABSTRACT

Objective: The objective of this research was to isolate and characterize endophytic bacteria from Talinum triangulare having antifungal activity.

Methods: The endophytic bacteria were isolated from roots tissue of Talinum triangulare by surface sterilization method. The isolates were cultured in Tryptic Soybean agar and antagonist activities were evaluated by the dual culture assay against Fusarium oxysporum, Trichoderma reesei, and Candida albicans. For metabolite antifungal activities, bacterial isolates were grown for 4 d in TS broth at 35 °C under shaking condition. The antifungal Activities of the supernatant extract were determined by using the disk agar diffusion. Polyketide synthase (PKS) I and NRPS genes fragments of all isolates were amplified.

Results: The result reveals that 4 of 23 endophytic bacterial isolates demonstrated great antifungal potentiality against many tested fungi. Polyketide synthase (PKS) I and NRPS genes amplification were showed 10 and 4 of endophytic isolates detected harboring K5 type and NRPS genes, respectively. In general, high frequencies of positive PCR amplification were obtained for PKS I (43.47%). Phylogentic analysis based on the 165 rRNA gene sequence, morphological, physiological and biochemical showed that the isolates were identified as a member of genus Bacillus and Brevibacillus.

Conclusion: These results indicated that the endophytic bacteria from java ginseng could be used as an alternative source of antifungal agents.

Keywords: Endophytic bacteria, Antifungal, Talinum triangulare, PKS I, NRPS, 165 rRNA

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INTRODUCTION

Endophytic microorganisms are defined as those microorganism that show endosymbiosis relationship with their host plants. These can be isolated from tissues or plant organ. Plants and microorganisms shows a complex interaction in the environment through produce a natural compounds for survival benefit of them. Because the role of these compounds is high biological activity, so it can be searched for drug discovery [1]. These interaction occur by the environmental recognition follow by transition of molecular. Therefore, plant find a benefit such as improve tolerance to various environmental stress, plant growth stimulation or prevent of the disease against pathogens [2-4]. Many reports in literature that diseases caused by fungi, bacteria, viruses and even damage caused by insects or nematodes can be prevented by inoculation of endophytic bacteria into plant tissue. [5-8].

Association of microorganisms and plants has been reported, among others the group of Actinobacteria such as Saccharopolyspora endophytica sp. and isolated from Myrtaceae mucrosporumus roots plant, a traditional medicinal plant in China [9]. Endophytic bacteria confer benefit extensively to plant such as enhanced resistance to various pathogen [10, 11]. Actinobacteria isolates from plants like grapes (Vitis vinifera) which produce coronarvines and show inhibitory activity against fungi Pythium. This compound is also known to inhibit the growth of the human fungal pathogen, Cryptococcus neoformans [12].

The study of endophytic microorganisms commonly reported on plants from temperate and tropical regions. Many of researchers were began to focused on the plant tropical regions [13, 14]. The research of tropical endophytic microorganisms was triggered by the important role of microorganisms endophytic against of global diversity. Moreover, the dynamics of plant communities as a source of new bioactive compounds and biological control agent were used for tropical agroforestry [15, 16].

Talinum triangulare (Jacq.)Willd., commonly found in Indonesia and is widely distributed throughout the tropical and subtropical regions of Southeast Asia. In Indonesia, it is known as "Java Ginseng". Traditionally, particularly of South Sulawesi people has been used in herbal formulations for treatment of fatigue and backache. Moreover, there are no previous literature reports on isolation endophytic bacteria and fungi from these plants.

Isolation of antifungal compounds from various plant sources, especially endophytic plants have difficulty both chemical and physical. A new technique to find a novel compound has been improved to obtain the compounds in a short time. Molecular approaches were used to discover the novelty of compounds by exploration of genes that coding formation of substance metabolites [17]. One of the substance was atracting attention is PKS (polyketide synthases), it is a multifunctional enzyme which responsibility for polyketides biosynthesis. Polyketides is a group of important secondary metabolites that produced by microorganisms. These metabolites has various structure and many biological active substances such as antibacterial, antifungal, anticancer and immunosupressant [18, 19]. The present study involved the isolation of endophytic bacteria from the tissue of healthy plants (Talinum sp.), detection of pkS and arps genes and evaluation of the antifungal activity of their secondary metabolites.

MATERIALS AND METHODS

Collection of the plant material

Healthy plants of T. triangulare was collected from Sidrap district, South Sulawesi provinces of Indonesia at five different location (Fig. 1). The distances between the selected site sampling is 10 km. Roots were collected by digging the soil adjacent of the main stem. The roots sample were cutted about 3-4 cm in length and collected in zip lock plastic. All sample were brought into the laboratory and isolation were done within in 48 h. A herbarium specimen as been
preserved in Laboratory of Botany, Department of Biology, Universitas Negeri Makassar, Indonesia. The authentication of the sample was done by Dr. St. Fatma Hola (voucher specimen no LE/FMIPA/UNIMA/2001/2015).

Isolation of endophytic bacteria

Plant roots were washed with running tap water to remove soils or particles that attached on the roots surface and cut into small pieces of 0.3 cm x 2 cm. They were then surface-sterilized in 70% ethanol for 1 min and air-dried in a laminar flow chamber. These pieces roots tissues were rinsed in 0.1% Tween 20 for 30 s, then in 0.5% sodium hypochlorite for 2 min. Finally, pieces roots were washed in sterilized distilled water for 5 min, then dried using sterile filter paper. Roots were cut into small pieces by scalpel, then subsequently crushed with a pestle in a mortar under sterile conditions [20, 21]. Approximately 0.1 ml of suspension were spread on the surface of TSA media plate amended by nystatin 100 μg/ml of medium. The plate was incubated at 35 °C for 7 d until showing growth of colonies. Colonies on the medium were independently transferred onto freshly prepared TSA medium plate until a single colony isolates showed purity. The pure isolates were streaking on Nutrient agar slant as isolates stock for further studies.

![Fig. 1: Map showing the location of the sampling sites. Sidrap district, South Sulawesi, Indonesia](image)

Characterization of isolate

The potent selected isolates were characterized by morphological and biochemical methods. Morphology of the isolate colonies was determined in TSA media. Morphological methods consist of macroscopic and microscopic methods. The micro-morphological characteristics were studied by light microscopy on the 3 d cultures in TSA media. The biochemical characterization was done by casein and starch hydrolysis, and growth temperature range. The observed structure was compared with Bergey's Manual of Determinative Bacteriology [22].

Detection of antifungal activity

Test organism

Three test fungi, viz., Fusarium oxysporum KFCC 11363P, Trichoderma reesei NRRL 31329 and Candida albicans ATCC 90026 were used for antifungal activities. These test organisms were procured from the Research Center for Biotechnology, Gadjah Mada University, Yogyakarta, Indonesia.

Antifungal activity

Detection of antifungal activity of isolates was evaluated by using dual culture assay method against the test fungi [23]. The isolates were streaked on the edge of the TSA plate, while the test fungi were inoculated at the centre of the plate on the same media. The plate was incubated for 7 d at 35 °C. Endophytic bacteria isolates were showed the growth inhibition against test fungi (clear zone around the endophytic bacterial isolates) considered as isolates producing antifungal.

Fermentation and extraction of metabolites

The extraction of the antifungal metabolite was conducted to ensure that the mechanism of inhibition is not due to nutrient competition. The pure culture of endophytic isolates was grown on 100 ml of TS broth media in Erlenmeyer flask 500 ml and fermented for 4 d at 35 °C in a shaker incubator. After the fermentation liquid was centrifuged for 15 min at 7500 rpm and the supernatant was used to evaluate antifungal activity. Subsequently, the supernatant was extracted by ethyl acetate, chloroform and n-hexane solvent (1:3 v/v) and the extract was subjected to rotary evaporator at 40 °C to remove the excess of solvent. The extract was obtained used as antifungal inhibition by paper disc diffusion method after dissolved in 10% dimethyl sulfoxide (DMSO).

Determination of MIC value

The MIC (Minimum Inhibitory Concentration) value of extracts was evaluated using Candida albicans ATCC 90026 as a test fungi. The serial two-fold dilutions of the crude extracts were made in a concentration which ranged from 0.5 μg/ml to 25 μg/ml. Microbial suspension (50 μl) containing 10^6 cfu/ml of C. albicans was poured into each well of the 96-well microplate. One well contained 50 μl of microbial suspension which was added a 50 μl of 1% DMSO and Ketotifen were used as a negative and positive control, respectively. The plates were incubated with agitation at 35 °C for 48 h. The MIC was defined as the lowest concentration of extract that inhibited the growth of test fungi after streaked on Sabouraud Dextrose agar and incubated under appropriate conditions.

Amplification of PKS type I, NRPS and 16S rRNA genes

The DNA genome of selected isolates were isolated from cells grown in TS broth according to the method as described by [24]. Isolates were grown on TS broth medium for 24 h at 35 °C in an incubator shaker. Biomass cell was harvested by centrifuging at 5000 rpm for 20 min. Pellet was washed twice with TE pH 8.0 (Tris EDTA buffer). Subsequently the pellet was used for DNA extraction by following the steps as follows: cells were lysed with 800 μl of lysis solution (100 mmol/l Tris-HCl, pH 7; 20 mmol/l EDTA; 250 mmol/l NaCl, 2% m/v SDS; 1 mg/ml lysozyme), and added 5 μl of 50 μg/ml RNase solution. The suspension was incubated at 37 °C for 60 min. After was added of 10 μl of protease K (50 mg/ml) and lysis solution, suspension was incubated at 65 °C for 20 min. Lysate was extracted with an equal volume of phenol and centrifuged at 15,000 rpm for 10 min. Supernatant were re-extracted with phenol (50%-50% v/v), then with chloroform (50%-50% v/v). DNA was obtained from the aqueous phase by the addition of NaCl (150 mmol/l, final concentration) and 2 times volumes of cold ethanol 95% v/v before centrifugation. Precipitates of DNA was cleaned with 50 μl of 70% ethanol and resuspended with TE buffer (10 mmol/l Tris-HCl pH
Table 1: Morphological and physiological characteristic of endophytic bacteria

| Isolate ID | Physiological characteristics | Cells shape |
|------------|------------------------------|-------------|
|            | Gram staining | Spore-forming | Casein hydrolysis | Starch hydrolysis | Growth temperature range (°C) |
| GJ2.1      | positive       | +            | -               | 20-50            | Rod shape             |
| GJ2.2      | positive       | -            | +               | 20-50            | Coccos                |
| GJ2.3      | positive       | +            | +               | 20-50            | Rod shape             |
| GJ2.6      | positive       | -            | +               | 20-50            | Coccos                |
| GJ2.7      | negative       | -            | +               | 20-45            | Rod shape             |
| GJ2.9      | negative       | -            | +               | 15-45            | Coccos                |
| GJ3.1      | positive       | +            | +               | 20-50            | Curved rod            |
| GJ3.4      | negative       | -            | -               | 20-45            | Short rod             |
| GJ3.6      | negative       | -            | -               | 20-45            | Coccos                |
| GJ3.7      | negative       | +            | +               | 20-50            | Rod shape             |
| GJ4.1      | negative       | -            | +               | 15-45            | Rod shape             |
| GJ4.2      | negative       | +            | +               | 15-45            | Short rods            |
| GJ4.3      | positive       | +            | +               | 20-50            | Rod shape             |
| GJ4.5      | negative       | -            | -               | 15-45            | Rod shape             |
| GJ4.6      | positive       | -            | +               | 20-50            | Rod shape             |
| GJ4.7      | negative       | +            | +               | 20-50            | Coccos                |
| GJ4.9      | negative       | -            | -               | 15-45            | Short rods            |
| GJ5.1      | positive       | +            | +               | 20-45            | Coccos                |
| GJ5.3      | positive       | +            | +               | 20-50            | Rod shape             |
| GJ5.6      | negative       | -            | +               | 15-45            | Short rods            |
| GJ5.7      | positive       | +            | +               | 20-50            | Rod shape             |
| GJ5.9      | positive       | +            | -               | 15-45            | Rod shape             |

a+, present; a-, absent; b+, active; b-, no activity

The antifungal activity of isolate

Four isolates of endophytic bacteria were designated as GJ2.1, GJ3.1, GJ4.3 and GJ5.3 show growth inhibition against test fungi by the formation of a clear zone around the block agar (a zone is not covered by test fungi colony) (Fig. 2). It is suggested that the isolate producing antifungal substances.

However, the inhibitory mechanism of isolate on microorganism not always caused by antifungal compounds produced by bacterial isolate but may also be due to other mechanisms. Therefore, to determine of inhibition mechanism, the fermentation broth were extracted using non-polar solvent. The results of diffusion agar showed the antifungal activity against test fungi. Three of solvent with different level polarity showed different activities against test fungi. The ethylacetate extract was showed activities while weak to no detect by chloroform and n-hexane extract. Subsequently, the MIC value of each ethylacetate extract were various values (Table 2).

Amplification of PKS1 and NRPS gene

These isolates were carried out detection of PKS1 and NRPS gene by PCR amplification for P. sian and N. pseudonitrosozyma. All isolates are detected of PCR amplification with bands 1400bp and 700bp, respectively bp. These results indicated that the endophytic bacteria isolates were...
positive of polyketide biosynthesis system. Four of 28 isolates were able to inhibit growth two species of test fungi based on halo zone forming around the bacterial isolates colony. Five of isolates were inhibited against test fungi detected of PKS amplios, whereas three isolates of NRPS amplios were detected but two isolates, (GJ2.3 and GJ3.3) show antifungal produce (table 3).

**Phylogen of endophytic bacteria isolates**

Preliminary characterization of selected isolates by profile matching approach as a key character for a basic reference with related genera. The isolates showed high similarities with Bacillus sp. genus character e.g. Gram-positive, rod-shaped and endospores-forming. These characters was enough to reveals that the isolates are members of the group of Bacillus.

Nevertheless, it is necessary to evaluate determination of related species with other species based on database analysis of ribosomal gene. Analysis of 16S rRNA gene sequences of the isolates was used to perform phylogenetic construction by compare of the 16S rRNA gene sequences; all isolates are members of the Bacillus species (GJ2.1, GJ5.3, GJ4.3) or Broviacillus (GJ2.3 and GJ3.3) as shown in fig. 3.

**Table 2: Antifungal activities of endophytic bacterial and MIC value of extract crude**

| Isolate ID | Erythyl acetate | Chloroform | n-hexane |
|------------|-----------------|-------------|-----------|
| GJ2.1      | +++             | +           | -         | 128.04 |
| GJ2.3      | +++             | ++          | -         | 57.32  |
| GJ5.3      | +++             | +           |           | >256.11|
| GJ3.3      | +               |             |           | 64.24  |
| GJ4.3      | +               |             |           | >256.67|
| Ketocanazole|                |             |           | 0.50   |

*Estimated by measuring the diameter of the clear zone of growth inhibition, each isolate was tested using 3 replications; +++, ≥ 15 mm; ++, 10-15 mm; +, 5-10 mm; -, <5 mm (no antifungal activity).

**Table 3: In vitro anti microbes activity of all isolates and metabolite biosynthetic genes**

| S. No | Isolate ID | F. oxysporum KFCC 11363P | T. reesei NRBC 31329 | C. albicans ATCC 90026 |
|-------|------------|--------------------------|-----------------------|--------------------------|
|       |            |                          | (-dual culture assay) |                          |                          |
| 1     | GJ2.1      | Active                  | Active                | Active                   |
| 2     | GJ2.2      | ND                       | ND                    | ND                       |
| 3     | GJ2.3      | Active                  | Active                | Active                   |
| 4     | GJ2.6      | ND                       | ND                    | ND                       |
| 5     | GJ2.7      | ND                       | ND                    | ND                       |
| 6     | GJ2.8      | ND                       | ND                    | ND                       |
| 7     | GJ3.1      | Active                  | Active                | Active                   |
| 8     | GJ3.4      | ND                       | ND                    | ND                       |
| 9     | GJ3.6      | ND                       | ND                    | ND                       |
| 10    | GJ3.7      | ND                       | ND                    | ND                       |
| 11    | GJ4.1      | ND                       | ND                    | ND                       |
| 12    | GJ4.2      | ND                       | ND                    | ND                       |
| 13    | GJ4.3      | Active                  | Active                | Active                   |
| 14    | GJ4.5      | ND                       | ND                    | ND                       |
| 15    | GJ4.6      | ND                       | ND                    | ND                       |
| 16    | GJ4.7      | ND                       | ND                    | ND                       |
| 17    | GJ4.9      | ND                       | ND                    | ND                       |
| 18    | GJ5.1      | ND                       | ND                    | ND                       |
| 19    | GJ5.3      | Active                  | Active                | Active                   |
| 20    | GJ5.4      | ND                       | ND                    | ND                       |
| 21    | GJ5.6      | ND                       | ND                    | ND                       |
| 22    | GJ5.7      | ND                       | ND                    | ND                       |
| 23    | GJ5.9      | ND                       | ND                    | ND                       |

*ND = not detected; +, present; -, absent.
Fig. 3: Neighbour-joining phylogenetic tree inferred from 16S rRNA gene sequences. The phylogenetic relationship of endophytic bacteria isolates with related genera. Bootstrap values are expressed as percentages of 1000 replications.

Bootstrap values, ≥50% are shown at branch points. Score bar represents 1 nucleotide substitution per 100 nucleotides.

DISCUSSION

All of the plant compartments including seeds were obtained by endophytic bacteria. These bacteria generally colonize and form a community in intracellular spaces both monocotyledous and dicotyledous plants [29]. Endophytic bacteria were obtained from disease-free Talinum pumilum, several site sampling in South Sulawesi, Indonesia yielded 23 isolates. In this study, we explored the plant roots as an alternative source for screening of endophytic microorganisms. Our results of the research have shown that roots of jawa ginseng inhabited of diverse of bacteria. This work and other reports support that plant roots are the habitat of microorganisms. It has an important role with regard to plant health through nutrient assimilation or the in situ secretion many secondary metabolites that affected of plant growth [30, 31].

All isolates were obtained considered as endophytic bacteria because they had the different characteristic of colonies growing on control media plates. The effectiveness of surface sterilization was carried out to eliminate the epiphytic microorganisms were enough good. The colonies were growth on the control media plate from final rinse of the specimen when sterilization was different of colony characters between colony endophytic than non-endophytic. Based on the criteria, colonies grew on plate expressed as endophytic bacteria. The key of success to isolate endophytic microorganisms are surface sterilization guarantee of plant specimen or organ [32].

The existence of endophytic bacteria was obtained from the root of jawa ginseng suggests that the plant is one of a good habitat for endophytic bacteria. Some microorganisms such as bacteria, fungi and Actinomycetes have been isolated from ginseng rhizosphere [33]. Endophytic fungi were isolated from different parts of medicinal plant Jasminum nudiflorum Hance, was shown antibacterial and antioxidative [34]. The presence of abundant microorganisms in around of the plant roots area by specific mechanisms are moving entrance into the root tissue to form an association with host plants. These mechanisms are influenced by many biological and environmental factors such as cultivar of the plant, age, type of tissue and sampling time [35, 36].

Endophytic microorganisms on plant tissues organ as an alternative source of microorganism's exploration become an interesting object. Various methods have been developed to identify a potential of isolates producing bioactive substances such as detection of genes which responsible for biosynthesis of compound. Many primers have been reported used to amplify of region encoding of ketosialase module, acylase transferase and adenylase [24]. A result of gene amplification was shown that five of endophytic isolates detected harboring PKS type I genes. In general, high frequencies of positive PCR amplification were obtained for PKS I (63.47%). The high detection level of PKS-I biosynthetic genes observed in the isolates confirm the diversity of distribution of these sequences in this genera on jawa ginseng plant. These genes play an important role to produce secondary metabolites, especially of polyketide compounds structures. Therefore, although was not the known structure of bioactive compounds produced of endophytic isolates, it could be stated that these isolates synthesized polyketide compounds. Meanwhile, the isolat CE31 (closely related to Brevibacillus brevis) was antifungal production show harboring NRPS gene (17.39%). Although the substances were not determined of chemical structure, however, the study was reported revealed that Brevibacillus brevis was coding tyrosine synthase 2 tylc and gramicidin S synthetase [37].

Results of these studies indicate that endophytic bacteria which producing bioactive metabolites established the benefits interaction with the host [2]. Microorganisms often act as an antagonistic community to protect of the host against pathogenic fungi. Other competitions may be also caused by essential element competition process such as the formation of siderophore to binding Fe ions (chelating). Deficiency of the essential element caused a metabolism and reproduction process was disturbed [38].

Our results showed that the ethyl acetate extract of selected bacteria was detected inhibit the growth of fungi test while other extract solvent had no activities. A similar finding was observed in the report of antimicrobial activity of endophytic microbes isolated from medicinal plant Cardiopeuma hirtum [38]. The productivity of bioactive substances isolation depends upon the type of solvent used in extraction procedure [40]. Moreover, endophytic microorganisms have been known to produce antifungal substances extractable with ethyl acetate. Therefore, it was suggested that inhibition factor against fungal test growth by isolates was not caused by nutrients competition, but antifungal compounds produced by the endophytic bacterial isolates.

Molecular approaches of isolation and characterization of endophytic bacteria were reported. Microorganisms colonizing in the roots, stems and tubers tissues of plant different varieties have been analyzed by 16S rRNA gene. The diversity of bacteria colonizing agronomic crops such as C. fruticosus, C. clavibacter,
Curtobacterium, Pseudomonas and Microbacterium had reported [35].

Phylogenetic analysis of 165 rRNA gene sequences showed that isolates were clustering into Bacillus and Brevibacillus genera. Our results concluded that the selected endophytic bacteria are a member of the Bacillus genus and closely related to Bacillus and Brevibacillus brevis. B. brevis was first described in 1990 [33] and reclassification as a new species of Brevibacillus brevis and a new genus Brevibacillus. Subsequently, there are many isolates identified as a new species such as Brevibacillus mojavensis [41] and Brevibacillus subtilis [42]. Based on the phylogenetic distance from recognized Brevibacillus species, and relatively low 16S rRNA gene sequence similarities (97 %; see fig 3), it is apparently that GJ-1 and GJ-2 isolates could be determined by the novelty species of the genus Brevibacillus. However, limitation of presented data makes it difficult to represent isolates as a novel species.

CONCLUSION

Java ginseng plant is one of the alternative sources of endophytic bacterial producing antitumor. The endophytic bacterial isolate that was described in this study had potential to produce antitumor agent because of promoting due to their ability to produce endosperm. The selected isolate was detected harboring a PKS I gene and NRP for screened a polyketide biosynthesis peptide non ribosome system, respectively. Analysis of 165 rRNA gene showed that the isolate producing anti fungal closely related to Bacillus and Brevibacillus genera.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICTS OF INTERESTS

All authors declare no conflict of interest.

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