

## ENDOPHYTIC BACTERIA DIVERSITY FROM ZINGIBERACEAE AND ANTICANDIDAL CHARACTERIZATION PRODUCED BY *Pseudomonas helmanticensis*

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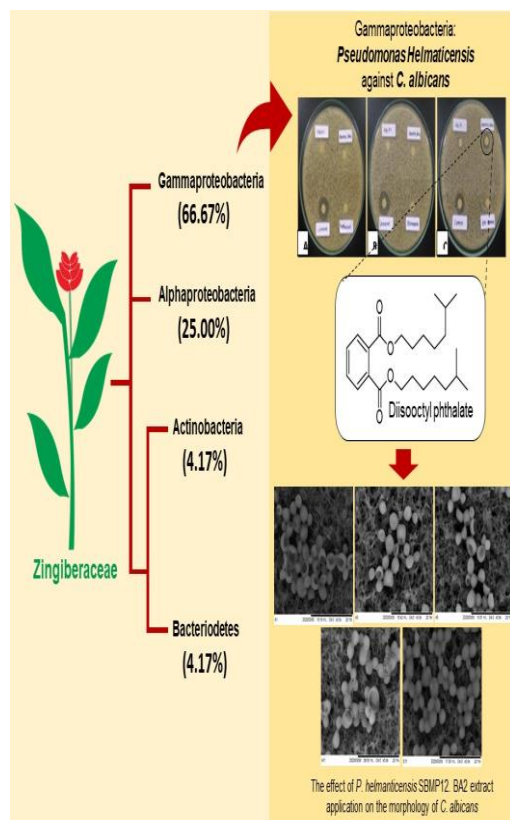
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### Graphical abstract



### Abstract

Endophytic microbes are sources for the novel antibiotic. We isolated endophytic bacteria from Zingiberaceae collected from West Sulawesi, Indonesia, and investigated their anticandidal activity. Molecular identification of the isolates was done using 16S rRNA gene sequence analysis. The antimicrobial activity was tested against four bacteria and one yeast. The anticandidal compound of selected bacteria was extracted using three different solvents (chloroform, ethyl acetate, and methanol), and each fraction was tested for their anticandidal activity. Anticandidal minimum inhibitory concentration (MIC) was determined with concentration ranging from 300 to 18.75 µg/mL, and the morphology of the *Candida* cells after treatment was confirmed by scanning electron microscope (SEM). The identification of anticandidal compounds was conducted using GC-MS. A total of 24 isolates were collected from Zingiberaceae plants. There were 14 genera and 19 species belonging to Gammaproteobacteria (66.67%), Alphaproteobacteria (25.00%), Actinobacteria (4.17%), Bacterioidetes (4.17%), and a new record for *Lelliottia aquatilis* as an endophytic bacteria. One of 24 isolates identified as *Pseudomonas helmanticensis* isolated from *Alpinia melichroa* showed anticandidal activity. Ethyl acetate was the appropriate solvent to extract the anticandidal compounds. Diisooctyl phthalate was found as the most abundant compound in the extract for the anticandidal activity. An increase in extract concentration did not reduce the *Candida* cell number. The extract treatment showed membrane disruption of *Candida albicans* cells. We propose that active compounds from *P. helmanticensis* are potential as anticandidal sources and could be explored more for the pharmaceutical industry.

**Keywords:** Anticandidal, diversity, endophytic, identification, Zingiberaceae

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## 1.0 INTRODUCTION

Bacteria and microfungi, such as *Candida*, *Malassezia*, and *Pityrosporum* are normal flora in the human body, and each has different functions. These normal floras can be harmful and become pathogenic when there is an imbalance. The Profusion of *Candida* in the mucosa and vagina can cause the infection and is commonly called candidiasis. Disregarding prescribed or overconsumption of broad-spectrum anticandidal has given rise to the increasing of anticandidal resistance. Moreover, the limited number of anticandidal agents has generated effort in the exploration of new active anticandidal. Microorganisms including endophytic bacteria known as the best sources of active compounds [1].

In the last two decades, many studies have explored endophytic microbes (bacteria and fungi) associated with medicinal plants. Endophytic bacteria refers to the bacteria residing inter- or intracellularly in healthy plants or come out from the part of the plant, without causing any symptom or negative effect on the host plant [2, 3]. Even though only a few of the plant species have been studied, almost all plant species are believed to have an association with at least one type of endophytic microbe [2, 4, 5].

Sulawesi island, located in the central part of Indonesia is a biodiversity hotspot of Wallacea with unique flora and fauna that cannot be found in another area. At least 500 endemic plant species are well known. According to ethnobotanical knowledge, endemic plants growing in unique locations are generally associated with a promising novel endophyte and often produce unique bioactive compounds [5]. The chemical profile and biological activity of bioactive compounds produced by endophytes might be similar to that present in the host plants. Endophytes release bioactive compounds that might be responsible for the medicinal value of these plants. Several bioactive compounds serve as a potential antimicrobial, anticancer, and many more properties drugs such as alkaloids, phenolic acids, quinones, steroids, saponins, tannins, and terpenoids [2, 6].

Plants belonging to the Zingiberaceae family are well known contained the active compound, particularly in the rhizome and leaves. *Alpinia*, *Amomum*, and *Etilingera* are several genera having valuable active compounds useful in the pharmaceutical industry. *Alpinia* is the largest clan of the Zingiberaceae family, and *Alpinia melichroa* is one of the endemic plant species in Sulawesi. The rhizome of *A. melichroa* can be used as a tea and potential as an anti-infection agent. The rhizome extract from *Amomum nilgircum* showed antimicrobial activity against 7 bacteria and 5 microfungi including *Candida albicans* [7]. Another report also stated that seed extracts of *Amomum subulatum* Roxb. showed activity against microbes

such as bacteria and yeast [8]. Ud-Daula et al. (2016) [9] reported that the essential oils from leaves, stem, and rhizomes have antioxidant and antimicrobial activities. While secondary metabolites from *Etilingera pavieana* rhizomes showed weak activity against *Mycobacterium tuberculosis* [10].

Through an exploration of endophytic microbes, particularly from endemic and ethnobotanical plants, it is possible to gain novel endophytic bacteria that carry the characteristics of the host plant without destroying it [5]. Our main purpose was to find a new candidate for an anticandidal compound from bacteria, especially endophytic bacteria from Zingiberaceae. The overall goal of the study was to investigate the species diversity of endophytic bacteria associated with Zingiberaceae and search for a new biological compound potential as anticandidal.

## 2.0 METHODOLOGY

### Sample Collection

The wild Zingiberaceae plants were collected from Mamasa, West Sulawesi, Indonesia (Table 1). The plant samples did not show any disease symptoms. The samples were identified based on morphological characteristics at the Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, Indonesia.

**Table 1** Zingiberaceae plants collected from Mamasa, Sulawesi

Sample voucher code	Sample plant	Local name
SBMP4	<i>Alpinia melichroa</i>	Katimbang balao
SBMP9	<i>Amomum</i> sp.	Kapulaga
SBMP10	<i>Etilingera</i> sp.	Kecombrang
SBMP11	<i>Alpinia melichroa</i>	Katimbang balao
SBMP12	<i>Alpinia melichroa</i>	Katimbang balao
SBMP14	<i>Alpinia melichroa</i>	Katimbang balao

### Surface Sterilization and Isolation of Endophytic Bacteria from Zingiberaceae Plants

The samples were washed using running tap water for 5-10 min. Surface-sterilization was carried out using stepwise washing, consisting of 70% ethanol for 3 min, 3% sodium hypochlorite solution for 5 min, and 70% ethanol for 30 s. This was followed by washing with sterile distilled water for three times 2 min each. This was performed by shaking the bottle up contained sample and distilled water, and the last dried using a sterilized tissue towel.

Endophytic bacteria isolation followed the Chowdhury et al. method [11] with some modification on the media used R2A medium (1/10 concentration and supplemented with

cycloheximide 50 µg/mL). Bacteria appearing on the surface of the plant tissue were picked up and purified. After getting a pure culture, endophytic bacteria were selected based on their morphological characteristics and grouping of gram-positive and gram-negative bacteria with the KOH test. Following stored at -80°C in 10% (v/v) glycerol for further study.

Validation of the surface sterilization process was evaluated by as much as 100 µL of the last washing distilled water was spread on the surface of R2A medium and incubated at 28°C for 2-15 days. Microbial growth on the medium indicated that surface sterilization failed.

### DNA Extraction and Amplification of 16S rDNA

DNA extraction was done following the method described by Packeiser et al. [12]. The isolated colony was picked up using a sterile toothpick and suspended in 20 µL of nuclease-free water. The suspension was vortexed and next lysis by incubation at 98°C for 5 min. This was followed by micro-centrifugation, and the supernatant was collected as a DNA template. PCR Amplification of 16S rDNA was performed using the primer pair of 27F (5'-AGAGTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTGTACGACTT-3') [13]. The composition of 16S rDNA amplification was Ultrapure water, GoTaq Green Master Mix, ten µM of each primer, dimethyl sulfoxide (DMSO), and DNA template, in a total volume of 25 µL. The PCR program was an initial denaturation at 95°C for 90 sec, followed by 30 cycles of denaturation at 95°C, 30 sec; annealing at 50°C, 30 sec; elongation at 72°C, 90 sec and a final extension at 72°C 5 min, finally at 4°C for 20 min. The amplified DNA was checked on 1% agarose gel and soaked using an ethidium bromide solution (5 mg/mL). The results were visualized using a UV transilluminator.

### DNA Sequencing and Phylogenetic Analysis

The amplified DNA was partially sequenced using forward and reversed primers by automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The sequenced data were processed using the Bioedit program. The homology of the 16S rDNA sequence was searched online using EzBioCloud database ([www.ezbiocloud.net](http://www.ezbiocloud.net)) [14], and the reference sequence was obtained from the GenBank/DDBJ/EMBL. Construction of a phylogenetic tree was done using a neighbor-joining tree method (NJ) implemented in MEGA 6.0 software [15]. p-distance was selected as the model for the analysis. The strength of internal branches of the phylogenetic tree was tested with bootstrap analysis using 1000 replications.

### Antimicrobial Activity Screening

The antimicrobial activity of endophytic bacteria was screened against four bacteria strains; *Bacillus subtilis* InaCC B1, *Pseudomonas aeruginosa* InaCC B3, *Staphylococcus aureus* InaCC B4, *Escherichia coli* InaCC B5, and one yeast test strain *Candida albicans* InaCC Y116. Bacteria were grown on Nutrient Broth (NB) at 37°C for 20 hours, and yeast was grown on Potato Dextrose Broth (PDB) at 30°C for 20 hours. Bacteria cultures were diluted to 10<sup>6</sup> cells/mL, and yeast was diluted to 10<sup>5</sup> cells/mL on semisolid agar, then overlaid on NA or PDA as basal media, and those media were called seeded agar plates.

The assay of antimicrobial activity was carried out by Agar well diffusion method [16]. Endophytic bacteria were cultured on NB for five days with shaking and centrifuged at 4°C, 16400xg for 15 min (Tomy, KITMAN-T24). The supernatant of endophytic bacteria was placed on the well (6 mm of diameter) of seeded agar plates. The plates were refrigerated for 2 hours to allow the supernatant diffuse to the media, then incubated at 37°C for 24–48 hours. Antimicrobial activity was indicated by a clear zone formed around the well and measured it. Each assay was done in three replicates.

### Bacterial Cultivation and Extraction of Metabolites

The selected bacteria SBMP12.BA2, which formed a clear zone around the well on seeded agar plates of *C. albicans* was cultivated in 500 mL Erlenmeyer flasks containing 200 mL of the King'S B medium. The flasks were incubated at 28°C for 72 hours with periodical shaking at 120 rpm. After the incubation period, the bacterial cells were separated from the cultures by centrifugation at 2718xg for 10 min (Thermo Scientific, Sorvall Lynx 6000) [17]. The bacterial metabolites were extracted using organic solvent; chloroform, ethyl acetate, and methanol [18]. An equal volume of filtrate and solvent were taken in a separating funnel and shaken vigorously for 45 min. The organic phase was collected and evaporated using a vacuum evaporator at 40 °C. The extract was stored below 5 °C for further tests. The best solvent for metabolites extraction was chosen for further extraction.

### Anticandidal Activity Assay

To investigate which is an appropriate solvent for anticandidal compound extraction was carried out by the Agar-disk diffusion method [16]. The extract was dropped on a sterilized paper disc (6 mm) and allowed to dry, then placed on seeded agar plates. The plates were refrigerated for 2 hours to allow the compounds to diffuse to the media, and followed by incubation at 37 °C for 24–48 hours. The anticandidal activity was indicated by a clear zone formed around the paper disc. Each assay was done in three replicates. Itraconazole 1 mg/mL was used as a positive control.

### Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration was determined by the microdilution method using a 96 well plate, according to NCCLS (2008) [16]. Micro dilution methods involve the use of plastic microtiter plates (96 well). The two-fold serial dilution of the antibiotics was prepared from 300 to 18.75 µg/mL range (final concentration in Sabouraud dextrose broth (SDB). Briefly, overnight cultures of yeast were suspended to a density of  $1-5 \times 10^6$ , and 100 µL of the cell were added to each well of 96-well plates containing media. The plates were incubated at 30°C for 48 hours. The MIC is the lowest concentration of the agent, which inhibits the growth of microorganisms tested. The control containing no agent should be turbid (negative control). Each concentration was carried out in triplicate.

### Scanning Electron Microscopy (SEM) Analysis

The morphology of *C. albicans* treated with extract was evaluated by SEM. *C. albicans* with or without treatment (control), was grown on pre-sterilized plastic coverslips (1 cm), fixed in the cellulose acetate filter membrane 0.2 µm, and dried. The specimens were coated with gold prior to using MC1000 ion sputter coater (Hitachi High-Tech, Japan) before being observed under the low vacuum tabletop microscope Hitachi TM3030 (Hitachi High-Tech, Japan) at 15 kV [19].

### Identification of Anticandidal Compound

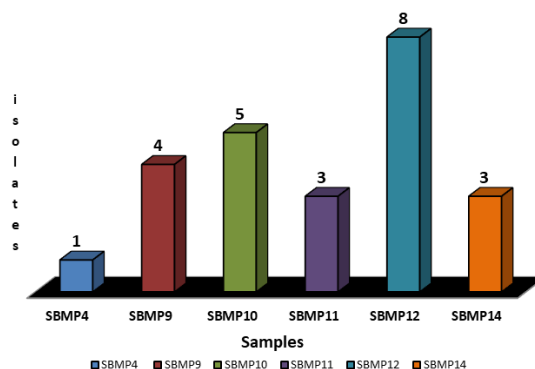
The anticandidal compound of selected endophytic bacteria was analyzed using Gas Chromatography-Mass Spectrometer (GC-MS) QP2010 ultra, Shimadzu. It was equipped with a silica column (5% diphenyl/95% dimethyl polysiloxane) 30 m in length and 0.25 mm in diameter). The GC condition was heated from 60 °C to 150 °C and held for 3 min. The temperature was increased to 270 °C at a rate of 5 °C per min for 3 min. Helium was used as a carrier gas. The sample was injected in the injection temperature 230 °C; the mass spectral range was set up as 80-450 m/z, ion source temperature was 200°C with interface 250 °C. The identification of the compounds was based on a 90 % similarity between the MS spectra of unknown and reference compounds in an MS spectra library [20].

## 3.0 RESULTS AND DISCUSSION

### Isolation of Endophytic Bacteria Associated with Zingiberaceae Plants

We isolated 24 isolates of endophytic bacteria from Zingiberaceae plants of Mamasa, West Sulawesi (Figure 1). Most isolates showed phenotypic

characteristics; circular, elevation was pulvinate, the margin was entire, opaque, shiny, and most of the isolates are white, yellow, and yellowish color. Rapid KOH test results in about 95.83% of endophytic bacteria were Gram-negative and 4.17% Gram-positive bacteria, which implies culturable endophytic microbes associated with Zingiberaceae plants are majority Gram-negative bacteria.



**Figure 1** The number of selected endophytic bacteria associated with Zingiberaceae plants

Endophytic bacteria were found in leaves and stems of Zingiberaceae plants collected from Mamasa, West Sulawesi. The result of this study is in line with Jalgaonwala & Mahajan study [21], which found endophytic bacteria in almost all parts of the host plant, including roots or rhizomes, stems, leaves, seeds, fruits, tubers, and nodules of legume also.

The recovery number of endophytic bacteria isolated from Zingiberaceae was less than another study. This was presumably caused by the isolation method and medium. By using the plant piece method, bacteria were present inside the sample. While the isolation process by spreading method could increase the recovery number of isolates, the sample first homogenized, and the colonies-dispersed on the isolation medium [22]. Besides the isolation method, the isolation medium influenced the recovery number of isolates. The utilization of low nutrient R2A medium (1/10 concentration) suppressed the fast-growing bacteria, and it perhaps could be the potential bacteria in the pharmaceutical industry. R2A medium is a nutrient-poor medium that is suitable for the growth of diverse plant-associated bacteria. Kumar *et al.* (2016) reported as much as 14 endophytic bacteria isolates collected from the rhizome of *Curcuma longa* L using nutritionally rich medium Nutrient Agar [23]. However, our endophytic isolation technique was succeeded in isolating most potential endophytic bacteria on 1/10 R2A medium supplemented with cycloheximide 50 µg/mL.

We found that the diversity of the endophytic bacterial community associated with Zingiberaceae is species-as well as part-of-issue-dependent (Table 2 and Figure 2). Chowdhury *et al.* (2017) [11] stated that microbial communities associated with plants



are established through several factors, i.e. variety of hosts (genotypes of plants) and environmental factors, including geographic location, climates, and soil chemistry. Moreover, part of the tissue could influence the abundance and distribution of endophytic bacteria, presumably due to different parts of the plant having different anatomical structures and physiological characteristics [24]. Generally, specific habitat shows low diversity and a low abundance of microorganisms compared to soil [25]. According to Chow & Ting (2015) [26], the highest number of endophytes in medicinal plants was found in leaves, followed by stems and roots. Leaves are generally chosen by endophytes because of their wide surface area, they are rich in nutrients and have thin cell walls that facilitate endophytic colonization. In line with that result, 14 of 24 isolates were collected from leaves. Furthermore, a report by Putra et al. (2015) [27] and Sulistiyani et al. (2014) [28] showed that domestication activity toward wild Zingiberaceae plants affected their endophytic bacterial diversity in various parts of the plants.

#### Identity of Endophytic Bacteria Based on 16S rDNA Sequence

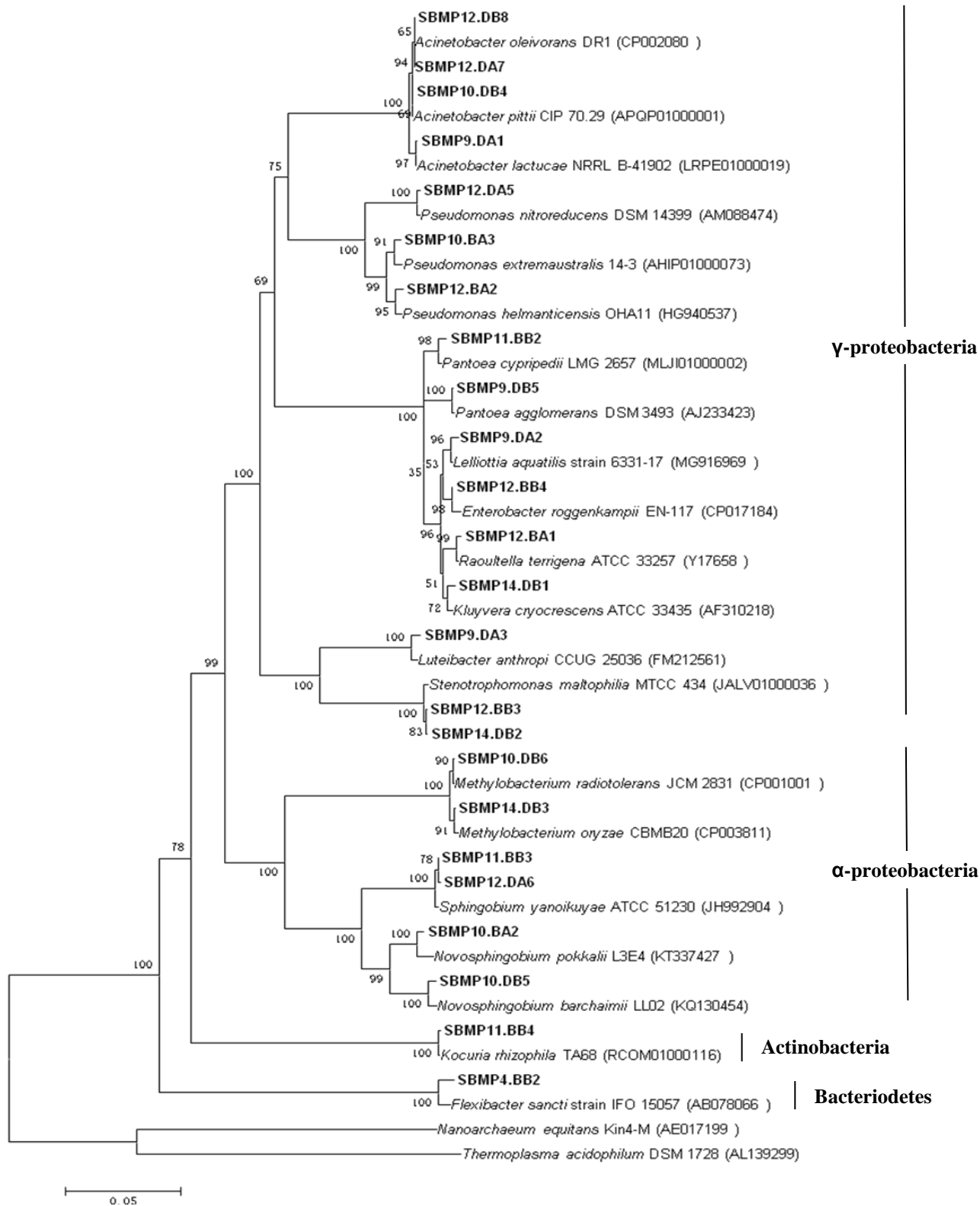
Based on molecular analyses of the 16S rDNA sequence, endophytic isolates associated with Zingiberaceae plants showed high similarity, between 98% to 100% with the reference database in Genbank/DDBJ/EMBL. The molecular identification of 24 of the isolates consists of 14 genera and representing 19 species (Table 2). The fourteen different genera include *Acinetobacter*, *Flexibacter*, *Enterobacter*, *Kocuria*, *Kluyvera*, *Lelliottia*, *Luteibacter*, *Pantoea*, *Methylobacterium*, *Novosphingobium*, *Sphingobium*, *Pseudomonas*, *Raoultella*, and *Stenotrophomonas*. Thirteen out of 14 genera, (*Acinetobacter*, *Flexibacter*, *Enterobacter*, *Kocuria*, *Kluyvera*, *Luteibacter*, *Methylobacterium*, *Novosphingobium*, *Pantoea*, *Pseudomonas*, *Raoultella*, *Sphingobium*, and *Stenotrophomonas*) were reported previously as endophytic bacteria; while according to Kämpfer and Glaeser (2013), *Lelliottia aquatilis* was not reported as an endophytic bacteria, but rather was from drinking water [29].

Each sample showed a different isolate number. From six samples of the Zingiberaceae plant, the most diverse was SBPM12 (*A. melichroa*). It consisted of 9 isolates and represented eight species. SBMP4, SBMP11, SBMP12, and SBMP 14 were the same species of *A. melichroa*, but collected from a different site in the same region, and the number and

composition of isolated bacteria were different. This implies that the composition of microbes in the host plants is influenced by ecological conditions. Another reason may be the age of the host plant. Vendan et al. (2010) [30] reported that the composition of the endophytic community could be affected by the age of the ginseng plants.

**Table 2** The identity of endophytic bacteria based on 16S rDNA sequence

Isolate code	Hit taxon name	Hit strain name	% identity
SBMP4.BB2	<i>Flexibacter sancti</i>	IFO15057	98.23
SBMP9.DA1	<i>Acinetobacter lactucae</i>	NRRL B-41902	99.78
SBMP9.DA2	<i>Lelliottia aquatilis</i>	6331-17	99.42
SBMP9.DA3	<i>Luteibacter anthropi</i>	CCUG 25036	98.91
SBMP9.DB5	<i>Pantoea agglomerans</i>	DSM 3493	99.93
SBMP10.BA2	<i>Novosphingobium pokkali</i>	L3E4	99.01
SBMP10.BA3	<i>Pseudomonas extremaustralis</i>	14-3	99.27
SBMP10.DB4	<i>Acinetobacter pittii</i>	CIP 70.29	100.00
SBMP10.DB5	<i>Novosphingobium barchaimii</i>	LL02	98.96
SBMP10.DB6	<i>Methylobacterium radiotolerans</i>	JCM 2831	99.93
SBMP11.BB2	<i>Pantoea cyripedii</i>	LMG 2657	98.82
SBMP11.BB3	<i>Sphingobium yanoikuyae</i>	ATCC 51230	99.69
SBMP11.BB4	<i>Kocuria rhizophila</i>	TA68	99.85
SBMP12.BA1	<i>Raoultella terrigena</i>	ATCC 33257	99.27
SBMP12.BA2	<i>Pseudomonas helmanticensis</i>	OHA11	99.12
SBMP12.BB3	<i>Stenotrophomonas maltophilia</i>	MTCC 434	99.63
SBMP12.BB4	<i>Enterobacter roggenkampii</i>	EN-117	99.48
SBMP12.DA5	<i>Pseudomonas nitroreducens</i>	DSM 14399	99.71
SBMP12.DA6	<i>Sphingobium yanoikuyae</i>	ATCC 51230	99.70
SBMP12.DA7	<i>Acinetobacter oleivorans</i>	DR1	100.00
SBMP12.DB8	<i>Acinetobacter oleivorans</i>	DR1	100.00
SBMP14.DB1	<i>Kluyvera cryocrescens</i>	ATCC 33435	98.66
SBMP14.DB2	<i>Stenotrophomonas maltophilia</i>	MTCC 434	99.71
SBMP14.DB3	<i>Methylobacterium oryzae</i>	CBMB20	99.78



**Figure 2** Phylogenetic tree of endophytic bacteria isolated from Zingiberaceae plants using the Neighbor-Joining tree method computed with the p-distance model. Bootstrap value 1000 replicates are shown next to the branches

According to 16S rRNA gene sequence similarity, twenty-four of the selected isolates belonged to Actinobacteria, Alpha-proteobacteria, Bacteroidetes, Gamma-proteobacteria. Gamma-proteobacteria was the most dominant (66.67%), followed by Alpha-proteobacteria (25.00%), Actinobacteria (4.17%), and Bacteroidetes (4.17%). Corresponding to the previous report by Chowdhury *et al.* (2017) [11] that Proteobacteria were dominant in ginseng plants from Korea. It can be assumed that the endophytic bacteria from the medicinal plants' majority was dominated by the Proteobacteria group.

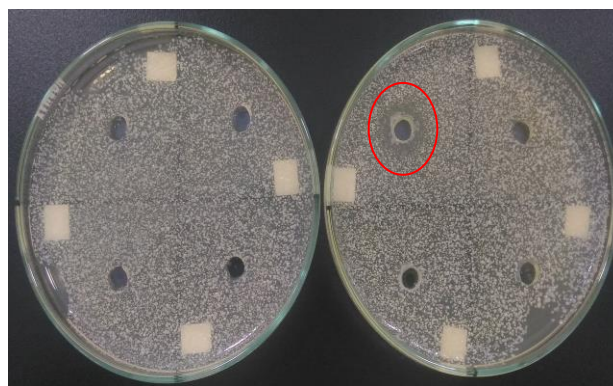
Gamma-proteobacteria were dominant phyla (n=16), consisting of 9 genera, including *Acinetobacter*, *Pseudomonas*, *Pantoea*, *Lelliottia*, *Enterobacter*, *Raoultell*, *Kluyvera*, *Luteibacter*, and *Stenotrophomonas*. The second dominant phyla was Alpha-proteobacteria (n=6) comprised of *Methylobacterium*, *Sphingobium*, and *Novosphingobium* genera. This was followed by Actinobacteria (n=1), identified as *Kocuria rhizophila*, and Bacteroidetes (n=1) as *Flexibacter sancti* with 100% of the bootstrap analysis, respectively. *Nanoarchaeum* and *Thermoplasma* belonging to Archaea were chosen as the outgroup in the phylogenetic tree construction. Proteobacteria is a big group, consisting of Alpha, Beta, Delta, Epsilon, Gamma-proteobacteria, and commonly isolated from a variety of samples. However, Actinobacteria and Bacteroidetes group were minor culturable endophytic microbes of Zingiberaceae (Figure 2).

### Screening of Antimicrobial Activity

One out of 24 isolates of endophytic bacteria showed antimicrobial activity against a pathogen test strain of *C. albicans*, which was indicated by the formation of a clear zone around the well on a seeded agar plate (Figure 3). The selected isolate was collected from the stem of *A. melichroa* (SBMP12) and coded as SBMP12.BA2 has the closest identification with *Pseudomonas helmanticensis*. The number of endophytic bacteria producing antimicrobial activity was low. This might be because the isolates were collected from the leaves and stem, while the bioactive compound is more distributed in the rhizome or root. Li *et al.* (2012) [20] reported that the rhizome of Zingiberaceae plants in Taiwan exhibited antimicrobial activity against *S. aureus*.

Medicinal plants with an intensified ethnobotanical record have a higher chance of discovering the endophytic bacterial species carry the characteristics of the host plant. The profile and biological activity of bioactive compounds from endophytes might be related to the bioactivity produced by the host plants. According to Tan & Zou (2001) [2], several endophytic bacteria producing bioactive compounds are typically associated with the characteristics of the host plant. Our result is in agreement with the study of Banisalam *et al.* (2011)

[31] that found rhizome extract of white turmeric has antibacterial pharmacological effects against pathogenic bacteria *E. coli*, *P. aeruginosa*, *B. cereus*, and *S. aureus* [38]. We expected to find new antimicrobial compounds relatively similar to the active compounds produced by the Zingiberaceae as a prospective antimicrobial producer, to be applied in the health sector. An earlier study by Tan & Zou found endophytic bacteria carry the characteristic of the host plant [2]. In our result, we found that *P. helmanticensis* SBMP12.BA2 isolated from *A. melichroa* carried the characteristics of the host plant as an antimicrobe (or antifungal) producer. *A. melichroa* itself is a plant species narrowly distributed in Sulawesi island according to the report by Sharp *et al.* (2012) [32], and the plant, particularly the rhizome part, has been traditionally used by the locals to combat various infections. However, to the best of our knowledge, no scientific data is available yet to support this claim.

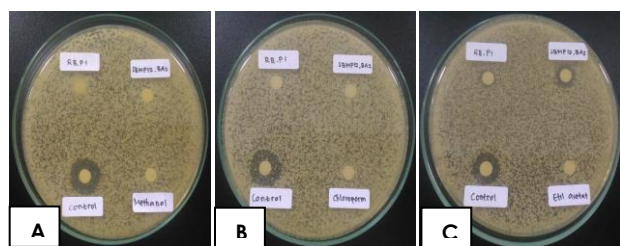


**Figure 3** Antimicrobial activity of SBMP12.BA2 against *C. albicans*

The selection of solvent is a critical step in the extraction of the active compound and in determining a specific target compound. We found ethyl acetate was the appropriate solvent to extract the anticandidal compound produced by selected endophytic bacteria *P. helmanticensis* SBMP12.BA2 and was more effective compared to methanol and chloroform. The extraction of hydrophilic compounds commonly use polar solvents such as methanol, ethanol, or ethyl acetate [33], and we found the anticandidal compound from *P. helmanticensis* SBMP12.BA2 is a hydrophilic compound.

Based on the anticandidal activity indicated by the clear zone around the well on the seeded agar plate, the crude extract of *P. helmanticensis* SBMP12.BA2 showed ability against *C. albicans* compared to itraconazole as a positive control (Figure 4). It was verified by the visual clarity of the inhibition zone by crude extract of *P. helmanticensis* SBMP12.BA2. In contrast to the itraconazole, *C. albicans* still be able to grow in the inhibition zone. Itraconazole inhibits the growth or reproduction of *C.*

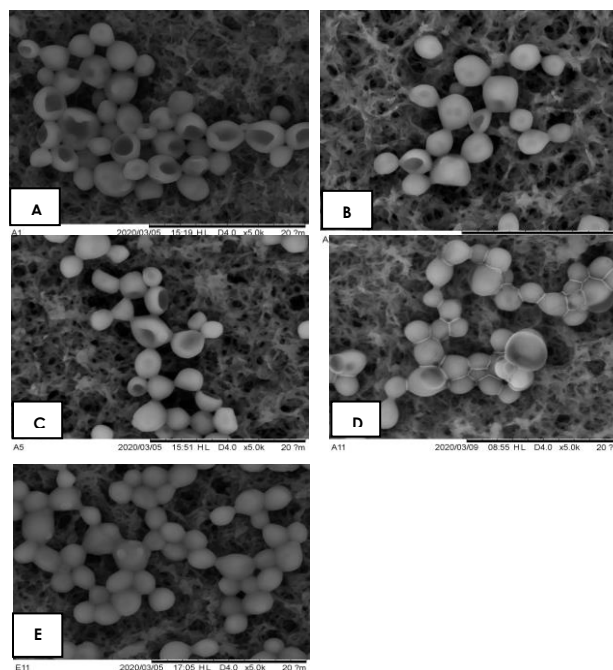
*albicans*, while *P. helmanticensis* extract kills *C. albicans*. Sole application of the organic solvents did not interfere with the *C. albicans* cell growth. Itraconazole is one of the broad-spectrum antifungal substances and effective to treat the various cause of fungal infections, including *Aspergillus* spp, *Blastomyces dermatitidis*, *Candida* (*Candida albicans* and *Candida tropicalis*), *Histoplasma capsulatum* [34, 35]. Similar to other azole antifungal groups, itraconazole inhibits lanosterol 14 $\alpha$ -demethylase thus blocking ergosterol synthesis in fungi [36]. The diameter inhibition of *P. helmanticensis* SBMP12.BA2 extract was smaller than the positive control. It might be because the Itraconazole was in a purified active compound, while *P. helmanticensis* extract was a crude extract that needed further purification.



**Figure 4** The anticandidal activity of *P. helmanticensis* SBMP12.BA2 when extracted using three solvent (A: methanol; B: chloroform; C: ethyl acetate; sample: extract of SBMP12.BA2; control: itraconazole)

### Scanning Electron Microscope

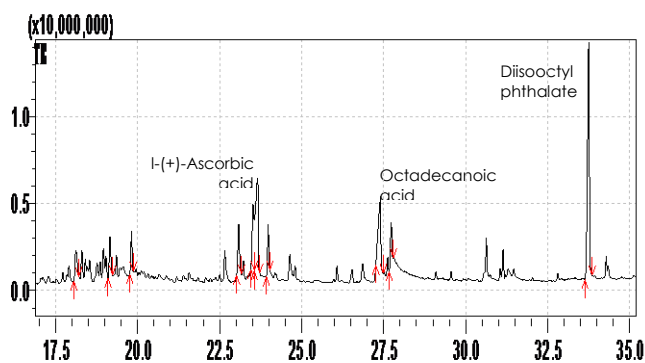
Figure 5 shows the SEM micrographs of the *C. albicans* cells, which were treated with *P. helmanticensis* SBMP12.BA2 extract. Extract application of different concentrations showed that the treated cells alter *Candida* cells as compared to the control cells. The treated cells were getting indentation on the mother cells. The morphology of control cells was smooth and there was no indentation found in the mother cells. The extract affected cell morphology and followed by cell death. We found that the dose of the extract was related to the rate of cell deformation. This suggests that the bacterial extract might be contributing to the altered permeability of the outer layer of *C. albicans*. The same result was obtained with Seyedjavadi et al. (2020) study, the morphology of *C. albicans* cells that had been exposed with an antimicrobial peptide produced by chamomile, the treated yeast cells showed deformation and shrinkage that lead to cell death [37].



**Figure 5** The effect of *P. helmanticensis* SBMP12. BA2 extract application on the morphology of *C. albicans*. A. 300  $\mu\text{g/ml}$ , B. 75  $\mu\text{g/ml}$ , C. 18.75  $\mu\text{g/ml}$ , D. E Negatif control. The Magnification of all Images is 5000X

### Identification of Anticandidal Compound

Figure 6 shows the representative result of GC-MS profile analysis from *P. helmanticensis* SBMP12.BA2 extract. Based on the abundance, the top three major compounds in ethyl acetate extract were Diisooctyl phthalate (RT 33.7), l-(+)-Ascorbic acid 2,6-dihexadecanoate (RT 23.6), and Octadecanoic acid (RT 27.4), with the main compound being Diisooctyl phthalate as much as 32.06%.



**Figure 6** GC-MS chromatogram of *P. helmanticensis* SBMP12. BA2 ethyl acetate extract



The presence of ten different active compounds from *P. helmanticensis* SBMP12.BA2 extract is presented in Table 3. According to the study, five out of ten compounds have been identified as antimicrobial compounds with the most active compound being Diisooctyl phthalate. Diisooctyl phthalate is a phthalate derivative, with synonyms bis-(2-Ethylhexyl) Phthalate (DEHP). Despite its wide utilization as a plasticizer in many materials and often associated with water pollution, many studies have persistently found Diisooctyl phthalate as a natural product [38]. Several phthalate derivatives from natural resources such as microorganisms, plants, animals, marine macroalgae have been reported to have pharmacological activities as well as antimicrobial activity [39]. *Bacillus pumilus* MB40 isolated from marine can metabolize DEHP, which plays a key role in inducing apoptosis by inhibiting human erythroleukemic K562 with an IC50 value of 21.0  $\mu$ M [40]. Rajammanikyam et al. (2017) [41] reported that *Brevibacterium mcbrellneri* produced bis-(2-Ethylhexyl) phthalate and dibutyl phthalate having antibacterial activity and mosquito larvicidal activity. Other studies found that Diisooctyl phthalate from *Streptomyces parvus* isolated from marine sediment had an inhibitory effect on MCF-7 cell lines [42]. The report of Abd-elnaby et al. (2018) [42] strengthens the results of this study that Diisooctyl phthalate plays an important role in inhibiting or deformation of *Candida* cells.

**Table 3** Biologically active chemical compounds of ethyl acetate extract of *P. helmanticensis*

Name of compounds	% Area	Activity
Allyldimethylphenylsilane	4.40	-
Phenol, 4-(1,1,3,3-tetramethylbutyl)-E-15-Heptadecenal	4.65	Antioxidant, anticancer
	3.79	Antimicrobial, antioxidant [43]
1H-Indene-4-acetic acid, 6-(1,1-dimethylethyl)-2,3-dihydro-1,1-dimethyl-	6.13	-
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	8.42	-
l-(+)-Ascorbic acid 2,6-dihexadecanoate	18.78	Antioxidant, anticancer [45][46]
Octadecyl trifluoroacetate	4.25	-
Octadecanoic acid	13.28	Antimicrobial [47]
Eicosyl heptafluorobutyrate	4.24	-
Diisooctyl phthalate	32.06	Antimicrobial [39, 43]

The second potential antimicrobial compound was Octadecanoic acid and E-15-Heptadecenal. The active compound E-15-Heptadecenal from *Halimeda discoidea* caused cell deformations of *Klebsiella pneumoniae* ATCC 13883 [48]. The third potential compound contained in *P. helmanticensis* SBMP12.BA2 extract was l-(+)-Ascorbic acid 2,6-

dihexadecanoate. The compound has several biological activities, such as antibacterial, anticancer, anticarcinogenic, anti-inflammatory, antimalarial, antioxidant, antiproliferant, antiprotozoal, antiseptic, antistroke, and antiviral activity [44].

Beside five antimicrobial compounds, the *P. helmanticensis* SBMP12.BA2 extract also consisted of 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (8.42%), 1H-Indene-4-acetic acid, 6-(1,1-dimethylethyl)-2,3-dihydro-1,1-dimethyl-(6.13%), Allyldimethylphenylsilane (4.40%), Octadecyl trifluoroacetate (4.25%), and Eicosyl heptafluorobutyrate (4.24%).

## 4.0 CONCLUSION

This is the first report on species diversity and metabolite activity of culturable endophytic bacteria associated with Zingiberaceae, particularly *A. melichroa*. The result demonstrates that many genera of bacteria reside in the Zingiberaceae plants, and *P. helmanticensis* from *A. melichroa* (SBMP12) have the ability to produce Diisooctyl phthalate among other metabolites that have a role as the anticandidal candidate. These compound destroyed the *Candida* cells.

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