The potency of endophytic fungi isolated from Hippobroma longiflora (L) G. Don as an antioxidant sources

by Hary Widjajanti

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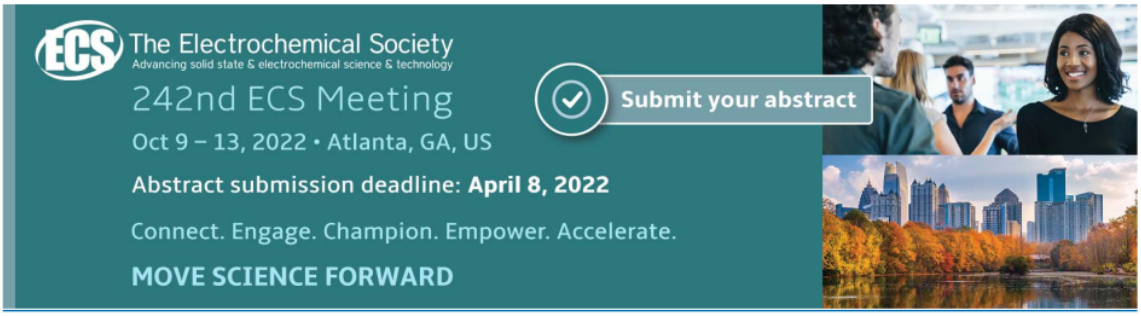

The potency of endophytic fungi isolated from *Hippobroma longiflora* (L) G. Don as an antioxidant sources

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Abstract. Endophytic fungi are friendly microbes colonizing in plants and play an effective role in plant-environment interactions. They produce valuable secondary metabolites that both plants and human beings can benefit from such products. In this study, an antioxidant-producing endophytic fungi were screened and identified from the leaves of *Hippobroma longiflora* (L.) G.Don which is one of the traditional medicinal plants. The objective of this study to evaluate the antioxidant activity of ethyl acetate extracts of 6 endophytic fungi isolated from *Hippobroma longiflora* (L.) G.Don. The qualitative and quantitative antioxidant activity was screened by scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH). Qualitatively 6 extracts from the endophytic fungi isolates (AOL_1, AOL_2, AOL_3, AOL_4, AOL_5, and AOL_6) from *Hippobroma longiflora* (L.) G. Don showed antioxidant activity, but quantitatively the extracts that showed very strong activity was extracts from AOL_1 isolate with IC_{50} values of 28.50 μg/mL. Based on phenotypic and molecular identification AOL_1 isolate identified as *Phyllosticta* sp and produce alkaloid, flavonoid, and terpenoid.

Keywords: endophytic fungi, *Hippobroma longiflora*, antioxidant, DPPH.

1.Introduction

The increase in free radicals that cause various degenerative diseases cannot be avoided, but free radicals that enter the body can be reduced by taking preventive measures. Antioxidants are needed to counteract and protect the body from free radicals [1]. Antioxidants are compounds that are able to capture or reduce the negative effects of oxidants in the body. Antioxidants work by donating one electron to compounds that are oxidant so that the activity of oxidant compounds can be inhibited [2]. The antioxidant activity of a compound can be measured by its ability to reduce free radicals [3,4]. The free radical commonly used as a model for measuring antioxidant attenuation is DPPH (2,2-diphenyl-1-picrylhydrazyl) because it is fast, simple and easy to use [5].

Hippobroma longiflora (L.) G. Don is a type of medicinal plant found in tropical and subtropical areas, which belongs to the Campanulaceae family. *Hippobroma longiflora* has the potential as a producer of antioxidants. Research by Zarta [6] proved that *Hippobroma longiflora* contains secondary metabolites in the form of flavonoids, tannins, saponins, steroids, and alkaloids which have high antioxidant activity with an IC_{50} value of 8.08 g/mL.

Endophytic fungi are fungi that interact with plant tissues and can produce the same secondary metabolites as their host [7]. The similarity of secondary metabolites is thought to be the result of genetic recombination between endophytic fungi and their hosts [8]. Endophytic fungi can be used to

obtain more efficient bioactive compounds. Utilization of endophytic fungi is very beneficial, because it has a shorter life cycle [9].

The ability of endophytic fungi to produce secondary metabolites according to their host plants is a very large and reliable opportunity to produce secondary metabolites from endophytic microbes isolated from their host plants. According to Stierle *et al*., [10], the use of endophytic microbes in producing active compounds has several advantages, including (1) faster production with uniform quality, (2) can be produced on a large scale, and (3) the possibility of obtaining new bioactive components by providing favorable conditions.

The objectives of this research were: 1. To obtain isolates of endophytic fungi from *Hippobroma longiflora* (L.) G. Don which produces antioxidant secondary metabolites, 2. To conduct in vitro tests to verify the antioxidant of the endophytic fungi from *Hippobroma longiflora* (L.) G. Don, 3. To identify endophytic fungal isolates from *Hippobroma longiflora* (L.) G.Don plants which have high potential in producing antioxidant.

2. Materials and Methods

2.1. Sample collection

Sample collected from Palem Raya Village, Ogan Ilir Regency, North Indralaya District, South Sumatra. Geographically, it is located at coordinates 3°12'7.14"LS and 104°39'23.72"BT. The leaves that are collected must be in fresh and healthy condition, have no spots on the parts used.

2.2. Isolation and purification of endophytic fungi

Surface sterilization of *Hippobroma longiflora* (L.) G. Don leaf was carried out according to the method of Radji *et al*. [11]. Subsequently, sample was with 70% alcohol for 1 minute, dried and soaked in 1% NaOCl for 5 minutes, dried and was soaked n in 70% alcohol for 30 sec, then rinsed with steriled destilled water for 1 to 3 sec. Samples were cut to size 2 cm x 1 cm. Two pieces of sterile *Hippobroma longiflora* (L.) G. Don leaves with a size of 1x2 cm were aseptically placed on the surface of potato dextrose agar (PDA) medium which was added with chloramphenicol as an antibacterial in a petri dish and incubated at room temperature (28°C) until fungi were grown. Each colony with different morphological characteristics were purified into a new PDA medium, and incubated for 5-7 days.

2.3. Cultivation and extraction of secondary metabolites of endophytic fungi

A total of 10 pieces of agar plug with a mm diameter from pure culture of fungi were put into a cultivation bottle containing 500 mL of Potato Dextrose Broth (PDB) medium, incubated at room temperature for ± 30 days under static conditions. The change of the color of the medium indicates the formation of secondary metabolites. The fungal biomass was filtered and dried, the medium was extracted with ethyl acetate solvent with a ratio between medium and solvent of 1: 1 and evaporated using a rotary evaporator to obtain a concentrated extract.

2.4. Qualitative test of antioxidant activity

Each extract from six isolates and ascorbic acid as control were spotted on the TLC plate as much as ±5 µL. To determine an antioxidant activity was conducted by spraying TLC plate with DPPH 0,1 mM. Isolates selection was done by comparing the closest color spots of the isolates to the yellow spot of ascorbic acid.

2.5. Quantitative test of antioxidant activity by DPPH radical scavenging activity

The free radical scavenging activities of extracts were measured by using 1, 1- diphenyl-2-picryl-hydrazyl (DPPH). Quantitative test of antioxidant activity was carried out using the DPPH method [12]. DPPH solution was made by 5 mg of 0.05 mM DPPH and dissolved in 250 ml of methanol. Endophytic fungi extract was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 1000 g/mL then the extract was diluted to 200; 100; 50; 25; 12.5; and 6.25 g/mL. A total of 0.2 ml of extract was added with 3.8 ml of 0.05 mM DPPH solution, homogenized and left for 30 minutes in a dark place, then the

absorbance was measured using a spectrophotometer. Ascorbic acid was used as the positive control, and DMSO as negative control. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = [(Abs control-Abs sample)/Abs control] × 100

The extract concentration providing 50% inhibition (IC50) was calculated was obtained by interpolation from linear regression analysis.

2.6. Thin Layer Chromatography (TLC) analysis of endophytic fungi extracts

The endophytic fungi extracts were analyzed by thin layer chromatography using plate silica gel 60 F_{254} (Merck). The extract with ethyl acetate solvent was taken using a capillary tube and plotted on the TLC plate. Then the TLC plate was eluted into a chamber containing the eluent solvent in the form of ethyl acetate and n-hexane with a ratio of 2:1, until a chromatogram pattern was obtained. The chromatograph pattern formed was then sprayed with 10% H_2SO_4 and heated on a hot plate to see the color spots. From the color spots formed and then measured the migration distance of the extract and the migration distance of solvent to determine the Rf value [13].

2.7. Characterization and identification of endophytic fungi

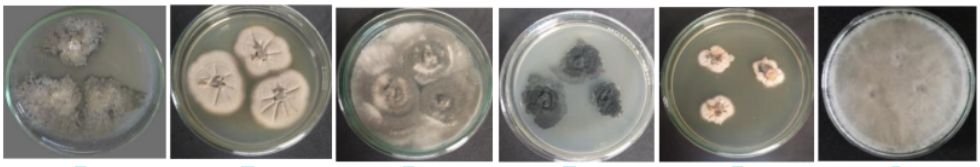
Endophytic fungi that have the potential to produce the highest antioxidant compounds were characterized and identified phenotypically based on their macroscopic and microscopic morphology and molecularly. Macroscopic characterization was carried out by growing endophytic fungi isolates on czapex dox agar (CDA), malt extract agar (MEA), and potato dextrose agar (PDA) media in petri dishes, then incubated at room temperature (± 28°C) for approximately 5 days. Characteristics of fungal isolates observed included colony growth, colony diameter, colony color, and colony reverse color [14]. Microscopic characterization was carried out by making preparations using the Henrici's slide culture (HSC) method and using lactic acid preparation. Fungi were taken aseptically using a loop needle and inoculated on PDA medium which was dropped on a sterile glass slide, then incubated for approximately 1-2 days at room temperature. The microscopic morphological characters observed included cells (unicellular/multicellular), hyphae (septat or aseptat, dark pigmented or hyaline), reproduction (sexual/asexual), branching hyphae, and asexual spores (shape, color, surface, and diameter) and other characters that characterize the special character of a type of fungus [14, 15].

Molecular identification of endophytic fungi was carried out by amplification of the fungal ITS region was performed using universal primer set ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS 4 5'TCCTCCGCTTATTGATATGC 3') [16]. The PCR products were sent to 1st BASE. The sequences were combined by Bioedit before analyzed using the BLAST algorithm (www.ncbi.nlm.nih.gov). The evolutionary tree for the dataset was carried out by Mega 7.

3.Results and Discussion

3.1. Isolation and purification of endophytic fungi

Six isolates of endophytic fungi were obtained from *Hippobroma longiflora* leaves, namely AOL_1, AOL_2, AOL_3, AOL_4, AOL_5 and AOL_6.The macroscopic morphological character of the six isolates of endophytic fungi obtained showed variation in form, size, and color of colonies (Figure 1).



a b c d e f

Figure 1. Pure isolate of endophytic fungi from *Hippobroma longiflora* leaves
a. AOL_1 b.AOL_2 c.AOL_3 d.AOL_4 e.AOL_5 f.AOL_6

3.2. Cultivation and extraction of secondary metabolites of endophytic fungi

The results of cultivation of each endophytic fungi isolates in 300 mL PDB medium for ±30 days obtained extract weights ranging from 0.042 to 0.261 grams and biomass weights ranging from 0.33 to 1.86 g (Figure 2). The highest ethyl acetate extract obtained from AOL_2 isolate extract (0.261 grams) and the highest of biomass obtained from AOL_4 isolate extract (1.86 grams). Fungal biomass is not directly related with the amount of extract. This is because between biomass and secondary metabolite extracts produced through different metabolism. According to Srikandace *et al*. [17], endophytic fungi produce secondary metabolites in the stationary phase, where the cells become old, the rate of reproduction decreases and some cells die due to the shrinking of nutrients in the medium. However, metabolism will continue which causes an abundance of secondary metabolite production in the medium.

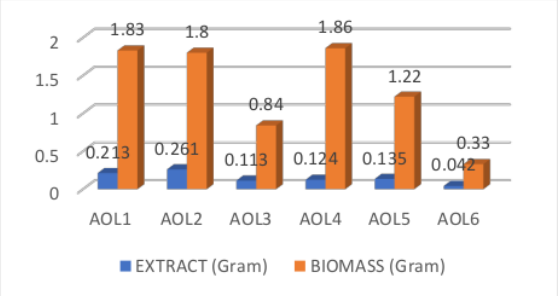


Figure 2. Etyl acetate extract and biomass of endophytic fungi *Hippobroma longiflora*.

3.3. Qualitative test of antioxidant activity

The results of the qualitative test of the antioxidant activity of endophytic fungi extract are presented in Fig 3. The presence of antioxidants is indicated by a color change from purple to yellow when it is sprayed with DPPH. All extracts obtained from six endophytic fungi isolates qualitatively all showed antioxidant activity with varying intensity of color changes.

The spot results shown in Fig 3. show that all extracts have antioxidant activity, although there are still impurities in some isolates so that it is continued by elution of the extracts to separate compounds that have the potential as antioxidants.

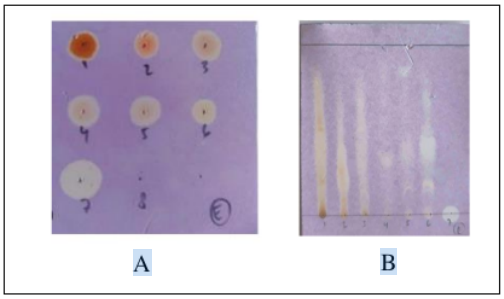


Figure 3. TLC profile extract of endophtyic fungi *H Longiflora* after being sprayed with DPPH. A. Spotted extract on TLC plate B. Extract eluted with ethyl acetat: n-hexane (2:1)1.AOL_5, 2.AOL_2, 3.AOL_4, 4.AOL_1, 5AOL_3, 6.AOL_6, 7.Ascorbic acid, 8.Etyl acetate

Yellow spot formed indicate the activity of antioxidant compounds. The ability of endophytic fungal extracts to donate hydrogen atoms to the DPPH solution which acts as a free radical to become a more stable compound, causing DPPH to be reduced and absorbance reduced, indicated by a change in the

color of the DPPH solution from purple to whitish-yellow. It is also explained by Prakash [18] that compounds containing antioxidants give a yellow color with a purple background on the silica plate.

3.4. Antioxidant activity of endophytic fungi extract

Antioxidant activity was indicated by the IC_{50} value, which is a parameter that indicates the effectiveness of a compound in inhibiting 50% of free radical activity. As a comparison, ascorbic acid was used as a control.

Table 1. Antioxidant activity of endophytic fungi extract

No.	Isolate	IC50(μg/mL)	Antioxidant activity*)
1.	AOL1	28,50	Very strong
2.	AOL2	168	Weak
3.	AOL3	385,29	Very weak
4.	AOL4	64,09	Strong
5.	AOL5	115,15	Moderate
6.	AOL6	257,09	Very weak
7.	Ascorbic acid	15,14	Very strong

*) Criteria: IC50 >200 (very weak); IC50 150-200 (weak); IC50 100-150 (moderate); IC50 50-100 (strong); IC50 ≤50 (very strong) [19].

Extracts that have antioxidant activity are able to inhibit an oxidation reaction from free radicals. According to Karim *et al*. [20] (2015) antioxidant activity is the ability of an extract or compound to inhibit the oxidation reaction which is expressed by the percentage of inhibition or the percentage of inhibition. Based on Table 1, the antioxidant activity of the AOL_1 extract was very strong because they had IC_{50} values of 28.50 g/mL. The smaller the IC_{50} value, the stronger the antioxidant activity of the compound. The difference in IC_{50} value can be caused by the amount of antioxidants contained in the extract.

3.5. TLC of AOL_1 endophytic fungi extract

Based on the TLC analysis of AOL_1 extract presented in Figure 4, showed several different color pattern on the TLC plate. The color differences are affected by different types of compound inside each extract hence when separated by eluted TLC it will separated. The extract of AOL_1 isolates contained alkaloids, flavonoid and terpenoid according to Harborne [21]. The secondary metabolite extract compound detected from the color that were formed on the plate. According to research by Normansyah *et al*. [22]. that the detection of compounds can be observed from the color of the stain formed. Alkaloids form orange or brick red color, brown tannins and yellow stains on the plate prove the presence of phenolic compounds. Phenolic compounds such as flavonoids are natural antioxidants [23]. The content of alkaloid and terpenoid compounds in AOL_1 isolate was able to act as a natural antioxidant. Reda [24] explained that the antioxidant activity of flavonoids is based on their ability to donate hydrogen atoms which can neutralize the toxic effects of free radicals. The phenolic hydroxyl groups of flavonoids have the ability to capture free radicals and their activity as metal chelators causes antioxidant activity in flavonoids. Yuhernita and Juniarti [25], explained that alkaloid compounds have the ability to efficiently stop free radical chain reactions by acting as hydroxy radical absorbers. The research of Graßmann [26], explained that terpenoids have been shown to have potential antioxidant activity and protective effects against oxidative stress in mitochondria, especially lipophilic terpenoids.

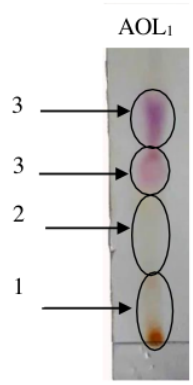


Figure 4. Chromatogram extract of AOL1 endophytic fungi isolate 1.Alcaloid, 2. Flavonoid, 3. Terpenoid

Table 2. TLC analysis and *Rf* of AOL_1 extract from endophyitic fungi

Isolate	*Rf*	Color	Active Compound group [21]
AOL_1	0,250	Brownish orange	Alkaloid
	0,575	Yellow	Flavonoid
	0,700	Pink	Terpenoid
	0,875	Pink	Terpenoid

3.6. Endophytic Fungus Characterization and Identification

Based on the results of macroscopic and microscopic characterization of endophytic fungi, isolates of AOL1 which were incubated at room temperature for 7 days on PDA medium had a colony diameter of 4.6 cm, the colonies were greyish green. Colonies in MEA medium had a diameter of 4.5 cm, the color of the colonies was greyish green. Colonies on CDA medium had a diameter of 4.3 cm and the color of the colonies was greyish-green (Figure 6).

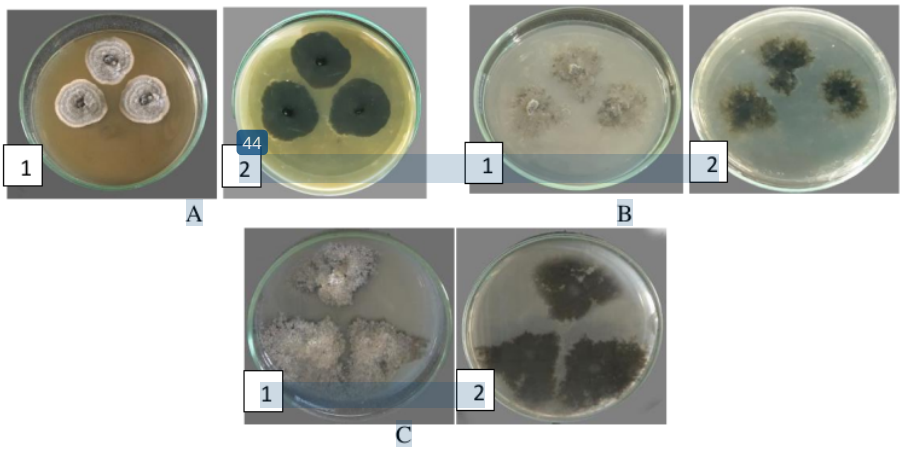


Figure 5. Colony of AOL_1 isolates on different media A.CDA, B. MEA, C.PDA,1.Colony, 2. Reverse colony.

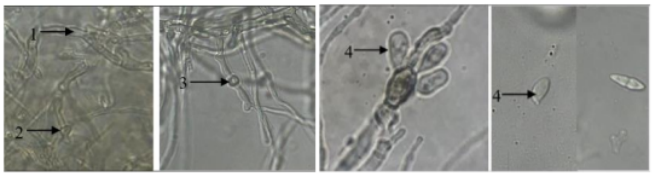


Figure 6. Microscopic morphology of AOL_1 endophytic fungi isolate: 1. Hypha 2. Swollen hypha 3. Chlamydospore 4. Conidia

Table 3. BLAST analysis of ITS region AOL_1 fungi isolate

Isolate	BLAST result	Accession Number	Identity (%)
AOL_1	*Phyllosticta rhizophorae* isolate NCYUCC 19-0-352	MT360030.1	99.54
	Phyllosticta fallopiae MUCC 0113	NR_147316.1	99.84
	Guignardia musicola CBS 123405	NR_137716.1	99.84
	Guignardia alliacea isolate MUCC0014	AB454263.1	99.68
	Phyllosticta capitalensis CPC 18848	NR_144914.1	99.83
	Phyllosticta paracapitalensis CBS 141353	NR_153303.1	99.48

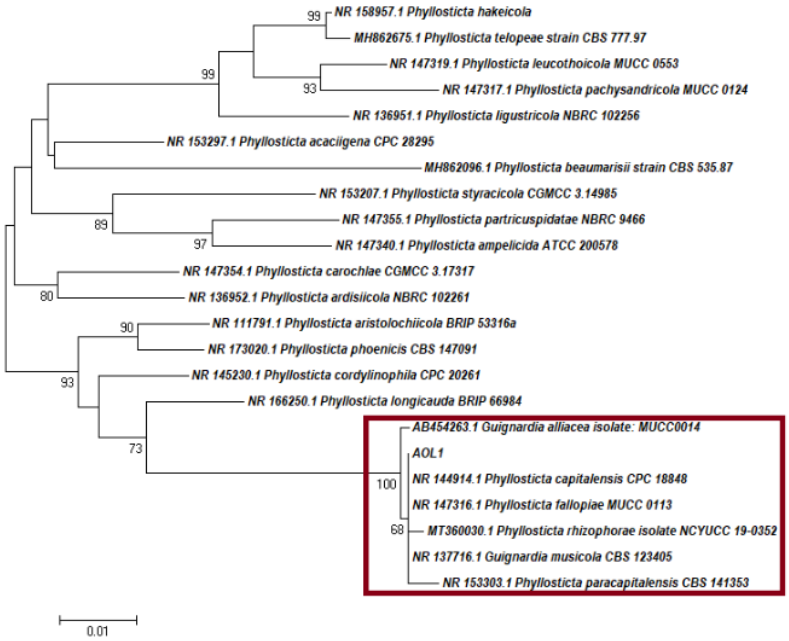


Figure 7. Phylogeny tree of AOL_1 fungal isolates together with the reference strain constructed based on the Neighbor-Joining algorithm. The numbers in each branch indicate the bootstrap. The scale indicates a substitution of 1 per 10 nucleotides in the ITS rDNA region sequence.

Based on the BLAST results, it was found that AOL_1 isolates had similarities with members of the Phyllosticta genus with identity values above 99% (Table 3). This means that the ITS rDNA region sequence of AOL_1 isolates has a homology of at least 99% with the fungal strain sequence in the same region in Genbank. The construction of the AOL_1 fungal isolate phylogeny tree with the reference strain

(Figure 7) showed the same results as the BLAST results, namely the AOL1 strain was in the same cluster as the BLAST reference strain. However, in the cluster there were still several species so that the isolate AOL1 was identified as a member of the *Phyllosticta* genus. Further identification down to the species level can be done using other, more specific primers. According to Srinivasan et al [27] the ethanolic extract of *Phyllosticta* sp. hat isolated from *Guazuma tomentosa* showed potent antioxidant activity against both ABTS and DPPH radicals with the EC50 value of 580.02 ± 0.57μg/ml and 2030.25±0.81 μg/ml respectively. Acording to Smita et al [28] the endophytic fungi *Phyllosticta sp* from *Andrographis paniculata* showed significant *in vitro* free radical scavenging activity as compared to other extracts. The IC_{50} values for ethyl acetat fungal extract was found to be 86.59 μg/ml.

4. Conclusion

Based on the research qualitatively 6 extracts from the endophytic fungi isolate (AOL_1, AOL_2, AOL_3, AOL_4, AOL_5, and AOL_6) from *Hippobroma longiflora* (L.) G. Don showed antioxidant activity, but quantitatively the extracts that showed very strong activity was extracts from AOL_1 isolate with IC_{50} values of 28.50 μg/mL. Based on phenotypic and molecular identification AOL_1 isolate identified as *Phyllosticta* sp and produce alkaloid, flavonoid, and terpenoid.

Aknowledgment

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