**BIOVALENTIA: BIOLOGICAL RESEARCH JOURNAL**
***e*-ISSN: 2477-1392**
**Vol.5 No.2, November 2019**

# Exploration of Endophytic Fungi of Dragon Scale's Fern (*Pyrrosia Piloselloides* L. M.G. Price) as an Antibacterial Sources

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*Peer review under responsibility of Biology Department Sriwijaya University*

## Abstract

*Endophytic fungi are fungi which live inside the host plant tissue and have been undergone a horizontal gene transfer process. Endophytic fungi are able to synthesize the same bioactive compounds which synthesized by their host plants. The host plant used in this research was dragon's scales fern (Pyrrosia piloselloides (L.) M.G. Price). Dragon's scales fern produces various of bioactive compounds which used as antibacterial agents such as polyphenols. This research was aimed to obtain endophytic fungi isolates from trophophyll fronds and sporophyll fronds of dragon's scales fern, to determine the antibacterial activity of the secondary metabolite extracts of endophytic fungi, to determine the Minimum Inhibitory Concentration (MIC), to determine the characteristics of the endophytic fungi isolates which potentially as antibacterial source. Based on the research, 13 endophytic fungi isolates were obtained from dragon's scales fern fronds consist of 5 isolates from trophophyll fronds and 8 isolates from sporophyll fronds. The antibacterial activity test showed that the extract of secondary metabolites of the isolate DTP2 had the highest inhibition zone diameter against E.coli 14.82 ± 4.05 mm, DTP4 against S.aureus 8.80 ± 0.03 mm and DSP4 against S.dysentriae 10.15 ± 0.36 mm. MIC of ethyl acetate extracts of secondary metabolites of isolate DTP2 against E.coli was 125 µg/mL, DTP4 against S.aureus was 125 µg/mL and DSP4 against S.dysentriae was 31.25 µg/mL. The endophytic fungi isolate DTP2 identified as Aureobasidium melanogenum, DTP4 identified as Penicillium allii-sativi and DSP4 identified as Aspergillus flocculosus.*

*Keywords: Dragon's Scales Fern, Endophytic Fungi, Secondary Metabolites, Antibacterials, Minimum Inhibitory Concentration.*

*Received: 21 July 2019, Accepted: 12 October 2019.*

## 1. INTRODUCTION

Endophytic fungi are a group of fungi that spend their life cycle by colonizing the living tissue of host plants without causing a negative impact on their hosts. Endophytic fungi have been co-evolved with their host plant and have undergone a Horizontal Gene Transfer process (Deepika *et al*., 2016). Endophytic fungi have a mutualism symbiotic with its host plants. Endophytic fungi are able to produce various kinds of bioactive compounds that can be used to increase host resistance to pathogenic attacks (Akmalasari *et al*., 2013).

The use of endophytic fungi to produce various antibacterial compounds will reduce the exploitation of host plants. It can thus preserve the host plant from the threat of extinction. The secondary metabolites produced by endophytic fungi are the right choice to overcome the resistance of pathogenic bacteria (Murdiyah, 2017).

One of the host plant that can be used as a source of endophytic fungi isolates is dragon's scales fern (*P.piloselloides* (L.) M.G. Price). Dragon's scales fern is a fern of Polypodiaceae family. This fern has been used by the community as traditional medicines for diarrhea and vaginal discharge (Oktavia *et al*., 2017). Dragon's scales fronds contain various kinds of secondary metabolites which potentially as antibacterial such as flavonoids, polyphenols, tannins, monoterpenoids, saponins, triterpenoids and steroids (Saptarini *et al*., 2018).

This research was aimed to obtain endophytic fungi isolates from trophophyll and sporophyll fronds of dragon's scales fern, to determine the antibacterial activity of secondary metabolites extracts of of the endophytic fungi, to determine the Minimum Inhibitory Concentration (MIC), and to determine the characteristics of endophytic fungi which are potentially as an antibacterial sources.

## 2. Materials And Method

This research conducted in August 2018 until January 2019 at the Microbiology Laboratory, Laboratory of Genetics and Biotechnology, Department of Biology, Faculty of Mathematics and Natural Science, Sriwijaya University. Dragon's scale fern fronds were collected from tea plantations on mounth Dempo, Pagaralam City, South Sumatra Province. The collected dragon's scale fern fronds were young, green and uniform size (Desiyana *et al*., 2016). The bacteria used in this research were *Escherichia coli*, *Staphylococcus aureus* and *Shigella dysentriae*.

### 2.1 Tools and Materials Sterilization

The media includes Czapek Dox Agar (CDA), Czapek Yeast Agar (CYA), Malt Extract Agar (MEA), Mueller Hinton Agar (MHA), Nutrient Agar (NA), Potato Dextrose Agar (PDB), and Potato Dextrose Broth (PDB) sterilized along with the glassware using an autoclave at a pressure of 15 lbs at 121 ºC for 15 minutes

### 2.2 Sample Preparation

Dragon's scales fronds washed using water for 5 minutes, soaked in 70% alcohol solution for 3 minutes and 5% NaClO solution for 5 minutes. The fronds are soaked back in 70% alcohol for 30 seconds, rinsed with sterile distilled water 2 times and dried on sterile tissue. The samples of dragon's scales fronds were cut 1 cm length and crushed using mortar (Ramadhani *et al*., 2017).

### 2.3 Isolation of Endophytic Fungi

The pieces and extract of dragon's scales fronds were placed on the surface of the PDA medium, incubated for 3-7 days at room temperature (Pratiwi *et al*., 2014). Fungal colonies that have the same shape, color and size are considered as the same isolates. Every colony which has different characteristics were purified (Suciatmih *et al*., 2011).

### 2.4 Purification and Stock Preparation of Endophytic Fungi

The mycelium of Endophytic fungi that grew in the surrounding area of fronds samples in the media was taken using sterile osseous needles and transferred aseptically to new petri dishes filled with sterile PDA, incubated at room temperature for 48-72 hours. Endophytic fungi stocks were made by inoculating pure endophytic fungi isolates into the oblique PDA medium (Widowati *et al*., 2016).

### 2.5 Cultivation and Extraction of Secondary Metabolites of Endophytic Fungi

7 days old pure endophytic fungi culture was taken 10 pieces using a cork borer (1 cm in diameter), then put into a culture bottle containing 500 mL of sterile PDB medium (Kumala and Pratiwi, 2014), incubated at room temperature for 4 weeks. After 4 weeks, each cultivated endophytic fungi biomass was determined the wet biomass, then dried using an oven at 60 ºC to obtain the dried biomass. The media of each endophytic fungi isolates were mixed using 500 mL of ethyl acetate. The secondary metabolites layer (at the top) was concentrated using a rotary evaporator at 73 ºC to obtain the extract of endophytic fungi (Desmara *et al*., 2017).

### 2.6 Testing of the Antibacterial Activity using Kirby-Bauer Method

The suspension of the bacteria (*E.coli*, *S.aureus* and *S.dysentriae*) which was equivalent to the Mc Farland 0.5 standard was taken as much as 0.2 mL and poured into a sterile petri dish. Sterile MHA medium was poured into a petri dish containing bacterial suspension and waited until solid. Secondary metabolite extracts made into concentrations of 4000 µg/mL using DMSO. Paper discs saturated with 10 µl extracts of secondary metaboites concentrations of 4000 µg / mL, then placed on the MHA medium surface (Jamal *et al*., 2008).

Positive control used saturated paper discs with 10 µL tetracycline antibiotic with a concentration of 4000 µg/mL, while the negative control used saturated sterile paper discs with 10 µL DMSO. Then incubated in incubator for 24 hours at 37 ºC. The diameter of the inhibitory zone formed includes the diameter of paper discs measured using a caliper (Sulaiman *et al*., 2017).

### 2.7 MIC Determination of the Secondary Metabolite Extracts of Endophytic Fungi

The secondary metabolites extracts of the the endophytic fungi which have the largest inhibitory zone diameter for each bacteria were carried out by MIC testing. MIC testing was carried out to determine the smallest concentration of the secondary metabolite extracts which still formed the inhibition zone to each bacteria. The concentrations of extracts used were 4000, 2000, 1000, 500, 250 and 125, 62.5, 31,25, 15.625, and 7.8125 µg/mL (Salni *et al*., 2016). The diameter of the inhibitory zone formed includes the diameter of paper discs measured using a caliper (Sulaiman *et al*., 2017).

### 2.8 Thin Layer Chromatography (TLC)

The secondary metabolites extracts of endophytic fungi which were tested by MIC were carried out by thin layer chromatography (TLC) analyses to determine the group of chemical compounds contained in the extracts. Secondary metabolite extracts were dissolved with ethyl acetate solvents, then taken using capillary pipes and

dripped onto the TLC plate. Elution is carried out by the mobile phase of n-hexane: ethyl acetate (6:4). The plate was sprayed by 2% $H_2SO_4$ and heated on a hot plate until the spot colors appears (Hidayati, 2012). The Rf value was calculated based on the formula (Sumarto *et al*., 2011):

$$Rf = \frac{Distance\ traveled\ by\ component}{distance\ traveled\ by\ solvent}$$

### 2.9 Characterization of Endophytic Fungi Isolates

One isolate of endophytic fungi which has the largest inhibiton zone to each bacteria based on antibacterial activity test was characterized based on morphological characters.

### 2.10 Macroscopic Characterization of Endophytic Fungi

Endophytic fungi were inoculated into petri dishes containing sterile CDA, CYA, MEA and PDA media, incubated at room temperature for 72 hours, observed for up to 7 days. The macroscopic characters of endophytic fungi were observed in colony color, colony diameter, and color opposite the colonies (Widowati *et al*., 2016).

### 2.11 Microscopic Characterization of Endophytic Fungi

Glass objects dripped with lactic acid. The endophytic fungal mycelium was taken using sterile osseous needle and scratched on the object glass and covered with a cover glass. Microscopic characters include hyphae, spores or conidia and producing bodies of spores or conidia (Sanjaya *et al*., 2010).

### 2.12 Identification of Endophytic Fungi Isolates

Identification of endophytic fungi isolates was using:

- Compendium of Soil Fungi Volume 1. 1980. K.H. Domsch, W. Gams & T-H. Andersen. 1980. Academic Press. London.
- Training Course for the Identification of *Aspergillus*, *Penicillium* and *Talaromyces*. Robert A. Samson. Westerdijk Fungal Biodiversity Institute, Utretch, the Netherlands.

## 3. Results and discussion

### Isolation and Purification of Endophytic Fungi of Dragon's Scales Fern

Based on the research, 13 isolates of endophytic fungi were obtained from dragon's scale fern fronds. The isolates obtained from trophophyll fronds were 5 isolates include DTP1, DTP2, DTP3, DTP4 and DTG1. The endophytic fungi isolates obtained from sporophyll fronds were 8 isolates which included DSP1, DSP2, DSP3, DSP4, DSP5, DSP6, DSG1 and DSG2.

The amount of endophytic fungi isolates from sporophyll fronds were more than trophophyll fronds. Sporophyll fronds contain more carbon sources than trophophyll fronds. According to Watkins *et al*. (2016), photosynthesis in trophophyll fronds is carried out through a $CO_2$ fixation process that produces glucose as an energy source. The results of photosynthesis will be circulated to all parts of the body of the fern including the sporophyll fronds. Sporophyll fronds will accumulate glucose which produced in photosynthesis by trophophyll fronds. Thus sporophyll fronds have higher carbon source than trophophyll fronds, its respiration rate 4 times higher than trophophyll fronds.

### Antibacterial Activity Test of Secondary Metabolite Extracts of Endophytic Fungi

The result of antibacterial activity test of secondary metabolites extracts of endophytic fungi are presented in Figure 1. Based on Figure 1. Secondary metabolites extracts of DTP2 isolate had the largest inhibitory zone diameter againts *E.coli* compared to other isolates which was 14,82 ± 4,05 mm. The diameter of the inhibitory zone formed on the medium was due to the diffusion of active compounds from secondary metabolites extract on paper discs to the medium. According to Erlyn (2016), the inhibition of the growth of bacteria is characterized by the presence of inhibition zones around the paper discs which are not overgrown by the bacteria, because of the paper discs containing antibacterial compounds.

The secondary metabolites extract of DSP4 isolate had the highest inhibition zone diameter against *S.dysentriae* which was 10,15 ± 0,36 mm. The diameter of the inhibitory zone of an extract against the bacteria can be influenced by several factors. Factors that affect the diameter size of the inhibition zone are the concentration of extract, the content of secondary metabolites extract and the type of bacteria. According to Lestari *et al*. (2016), an extract of secondary metabolites containing antibacterial compounds will form an inhibition zone to the bacteria. The diameter of the inhibitory zone depend on the concentration of the extract used. Bacterial resistance also affects the presence or absence of the inhibitory zone.

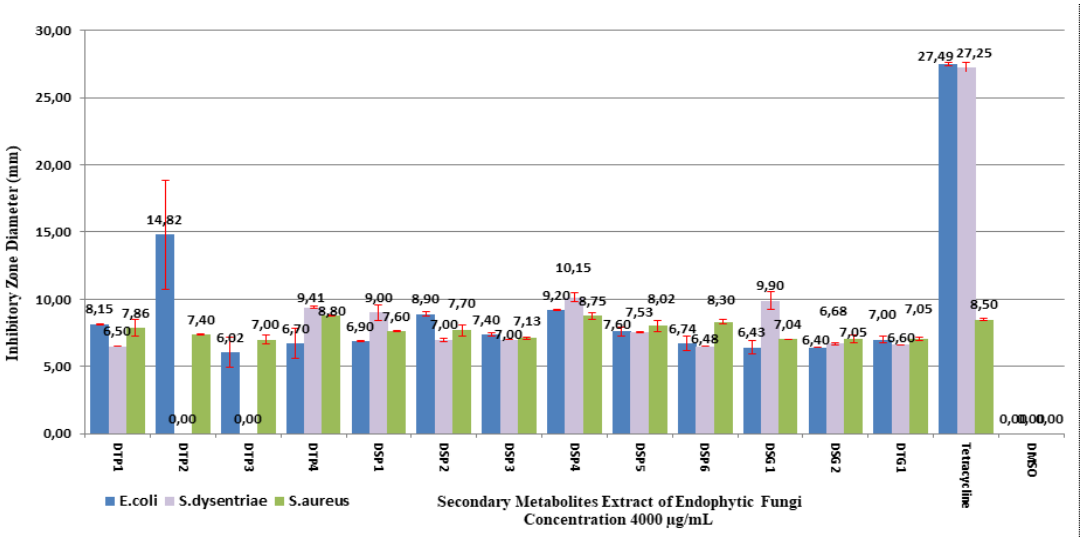



Figure 1. Antibacterial activity of the secondary metabolite extracts of endophityc fungi from dragon's scale fern fronds against *E.coli*, *S.aureus* and *S.dysentriae*

The inhibitory zone diameter of tetracycline against *S.aureus* based on Figure 1. was 8,50 ± 0,06 mm, was lower than the inhibitory zone of secondary metabolites extract of isolate of DTP4 with 8,80 ± 0,03 mm. This might be caused by the resistance of *S.aureus* to tetracycline. According to Chudlori *et al*. (2012), *S.aureus* are pathogenic bacteria that cause infectious diseases which have the ability to develop rapid resistance to various types of commonly used antibiotics. *S.aureus* are able to transfer antibiotic resistance plasmid through an intrinsic resistance mechanism.

**MIC Determination of Secondary Metabolite Extracts of Endophytic Fungi**

Based on MIC results, the smallest concentration of secondary metabolites extracts of isolate of DTP2 which still formed the inhibitory zone to *E.coli* was 125 µg/mL with diameter of inhibitory zone was 6.10 ± 0.00 mm. Based on the results of the MIC determination value, antibacterial activity of the extract of DTP2 against *E.coli* was classified as strong. MIC determination result of the extract of secondary metabolites of DSP4 against *Shigella dysentriae* was 31.25 µg/mL with diameter 6.20 ± 0.01 mm, the antibacterial activity was classified as very strong. MIC value of the secondary metabolites extract of DSP4 against *S.aureus* was 125 µg/mL with inhibitory zone diameter 6.90 ± 0.00 mm, and the antibacterial activity was classified as strong.

The antibacterial activity of an extract based on the MIC determination value classified into five categories includes very strong, strong, medium, weak and inactive. According to Saraiva *et al*. (2011), antibacterial compound which has MIC value less than 100 µg/mL classified as very strong antibacterial. Antibacterial compound which has MIC value 100-500 µg/mL classified as strong. MIC value of an antibacterial between 1000-2000 µg/mL classified as weak and the MIC value of an extract more than 2000 µg/mL classified as inactive compound.

The decrease of an extract concentration will affect the inhibitory zone diameter formed. The lower the concentration of the secondary metabolites extract used, the smaller inhibitory zone formed. Based on Lingga *et al*. (2015), the decrease of an extract concentration influences the inhibitory zone diameter. The lower the concentration of the secondary metabolites extract used, the smaller inhibitory zone formed, because of the amount of active compounds of an extract depends on the concentration of the extract.

**Thin Layer Chromatography (TLC) Analyses**

The results of TLC analyses of this research are presented on Figure 2. Based on Figure 2. Secondary metabolites extract of endophytic fungi of isolate DTP2 contains chemical compounds includes tannins, terpenoids and phenolics. Secondary metabolites extract of endophytic fungi of isolate DTP4 identified contains chemical compounds includes tannins, terpenoids, steroids and phenolics. Secondary metabolites extract of endophytic fungi of isolate DSP4 contains alkaloids, terpenoids, phenolics and tannins.

Chemical compounds of a secondary metabolite extract can be detected by the spots color which are appeared on TLC plates. Each spot color indicate the secondary metabolite compounds of the extract. According to Normansyah *et al*. (2013), terpenoids detected by the purple spot color after sprayed by $H_2SO_4$ and heated. The alkaloids compounds are purplish or brick red, tannins have brownish spots color and steroids are green. Yellow spots on the TLC plate prove the presence of phenolics compounds.

Alkaloids are able to damage the bacterial cell components. According to Erlyn (2016), alkaloids play role as an antibacterial agent by damaging the constituent components of peptidoglycan on bacterial cell walls. Based on Afni *et al*. (2015), tannins, able to shrink cell walls or bacterial cells thereby changing the permeability

of bacterial cells. Based on Lestari *et al.* (2016), steroids will damage the bacterial cell plasma membranes.

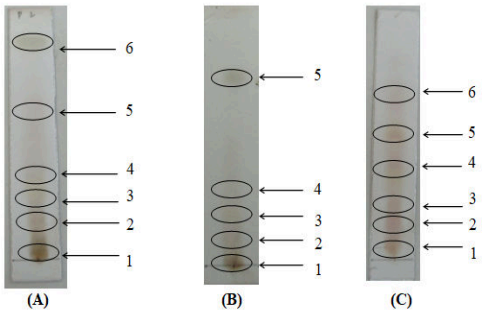



Figure 2. Thin layer chromatography analyses of the secondary metabolite extracts of endophytic fungi from dragon's scale fern fronds
Explanation: A. Isolate DTP2 3. Spot 3
B. Isolate DTP4 4. Spot 4
C. Isolate DSP4 5. Spot 5
1. Spot 1 6. Spot 6
2. Spot 2

Based on Awanis and Mutmainnah (2016), low concentration of phenolics compounds able to form phenol-proteins weak bond. According to Haryati *et al.* (2015), terpenoids bind the transmembrane protein on the outer membrane of bacterial cells.

**Characterization and Identification of Endophytic Fungi Isolates**

Based on the results of the macroscopic morphological characterization, the endophytic fungal isolate DTP2 on the PDA has 2 cm, greenish black colony. Colonies of the isolate of DTP2 which incubated for 7 days in the MEA were black, on the CDA greenish gray with a colony diameter of 3.35 cm. The colonies of endophytic fungi isolate DTP2 in CYA were dark green, 2.26 cm in diameter.

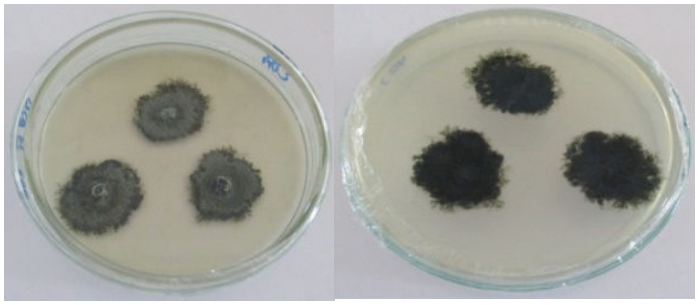
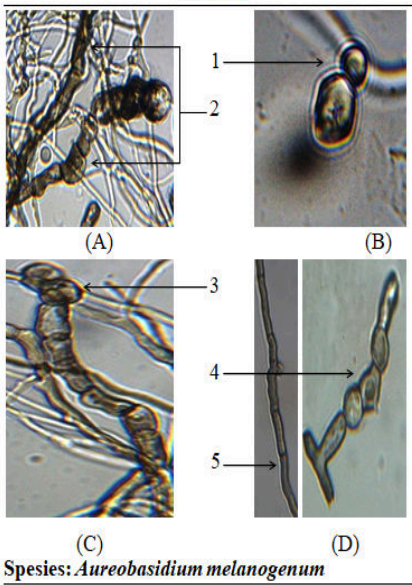


Figure 3. Isolate DTP2 on CDA (7 days incubation at room temperature)

The microscopic morphological characteristics of endophytic fungal isolate DTP2 as shown on the Table 1. has chlamydospore, septate hyphae with a blackish brown color. Based on these morphological characters, the isolate of DTP2 are similiar to *Aureobasidium melanogenum*. The similiar characters of DTP2 was compared to the characters of *A.melanogenum* presented by Castiglia and Kuhar (2015). According to Castiglia and Kuhar (2015), *A.melanogenum* has blackish brown hyphae, thin and insulated walls, ellipses conidia which has varying sizes. Vegetative hyphae was blackish brown, then develop to form dark brown, thick-walled chlamydospore, consisting of one or two cells.

Table 1. Microscopic characters of isolate DTP2

| Picture | Characters |
|---|---|
|   | a. Has *Chlamydospore*<br>b. *Arthroconidia*: Black<br>c. Conidia: *Ellipsoidal*<br>d. Hypae : Septate<br>e. Hypae Color: Brownish Black<br><br>Explanations:<br>1. *Yeast-like Conidia*<br>2. *Conidial aparatus*<br>3. *Chlamydospore*<br>4. *Arthroconidia*<br>5. Hypae<br><br>A-D: 400x Zoom |
| **Spesies: *Aureobasidium melanogenum*** | |

Based on the results of the macroscopic morphological characterization, the endophytic fungal isolate DTP4 after 7 days incubation on the PDA has white colonies, 2,1 cm in diameter and colonies reverse were yellow. The colonies of DTP4 on MEA were grayish green with a colony diameter 2.6 cm and colonies reverse were pale yellow. The colonies color of DTP4 on CDA were greenish gray, the colonies diameter were 1.34 cm, the medium color around the colonies was white and the reverse medium was pale yellow. While on the CYA, colonies of DTP4 have a dark green color, 2.56 cm in diameter, the medium color around the colonies was pale yellow, on the colonies reverse were yellow.

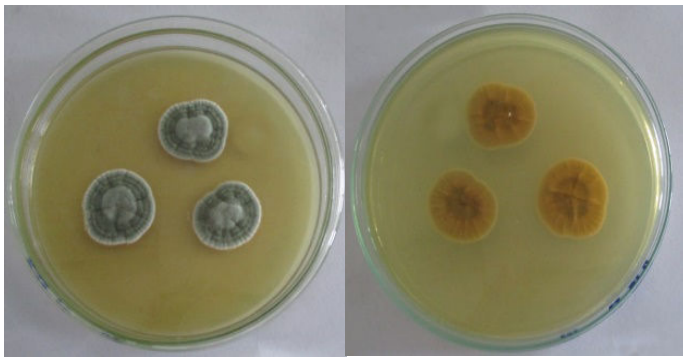
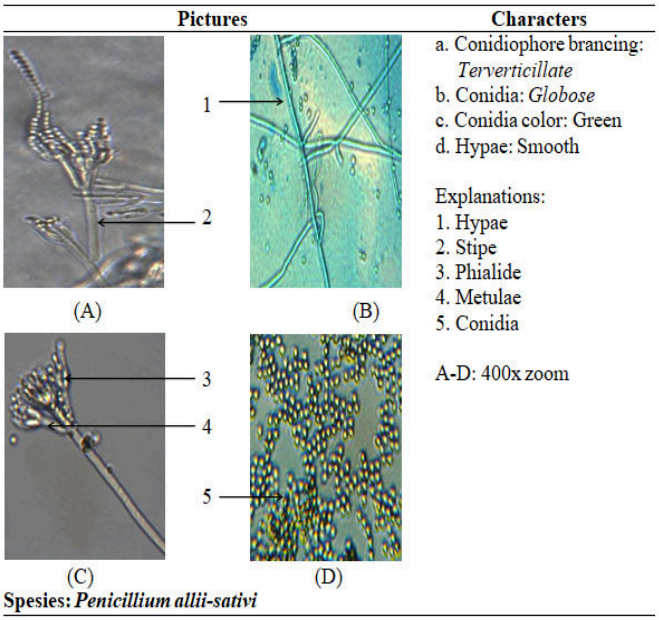


Figure 4. Isolate DTP4 on MEA (7 days incubation at room temperature)

The microscopic morphological characteristics of the isolate of DTP4 as shown on Table 2. were septate hyphae, terverticillate branched conidiophores, conidia

globose and shaped flask phialide. The microscopic morphological characters of DTP4 similiar to *Penicillium alli-sativi* as described by Houbraken *et al*. (2012). According to Houbraken *et al*. (2012), *P.alli-sativi* has terverticillate or quarterverticillate branched conidiophores. Smooth walls stipe, each metula has the same length, ampulliform phialide, and soft textured globose or subglobose conidia.

Table 2. Microscopic characters of isolate DTP4




Morphological characters of endophytic fungi isolate DSP4 on PDA after 7 days incubation was white colonies, 2.7 cm in diameter. The colonies of DSP4 on MEA were yellowish white, 2.6 cm in diameter. The colonies were white on CDA, 1,4 cm. While on the CYA the colonies were white orange, 3.62 cm.

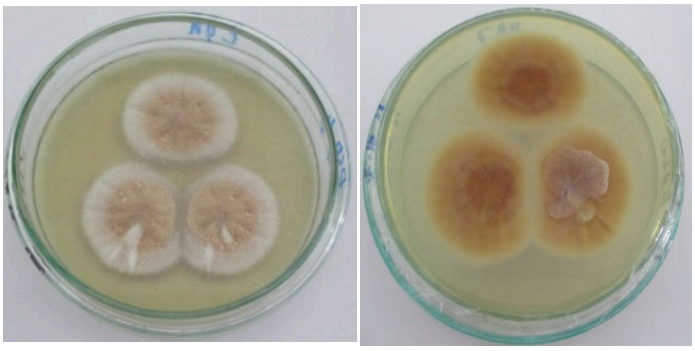
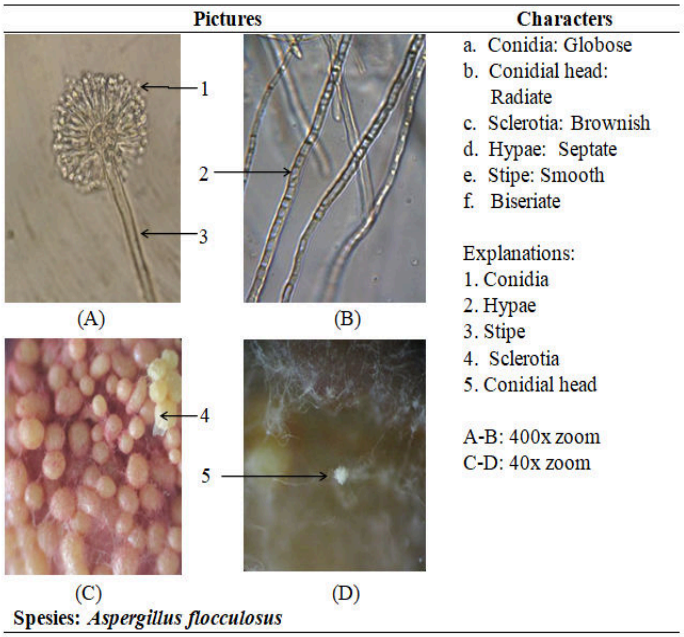


Figure 5. Isolate DSP4 on CYA (7 days incubation at room temperature)

The microscopic morphological characters of DSP4 as shown on the Table 3 were radiate conidial head, globose conidia, septate hypae, and brownish sclerotia. The microscopic morphological characters of DSP4 similiar to *Aspergillus flocculosus* as described by Frisvad *et al*. (2004). According to Frisvad *et al*. (2004), *A.flocculosus* has radiate conidial head, globose vesicles covered by metulae, globose, smooth, and tiny conidia. Globose to subglobose sclerotia, covered by mycelium.

Table 3. Microscopic morphological characters of isolate DSP4




Microscopic morphological characters of DSP4 as shown on Table 3. were radiate conidial head, globose conidia, septate hypae and brownish sclerotia. The characters have the similiar characters to characteristics

Antibacterial activity of *A.melanogenum* was reported by Sepcic *et al*. (2011), the secondary metabolites extract of *A.melanogenum* was able to inhibit the growth of *Bacillus subtilis*. According to Houbraken *et al*. (2012), *P.alli-sativi* are able to produce secondary metabolites which are potentially as antibacterial agents such penicillins. Based on Trinh *et al*. (2017), *A.flocculosus* has antibacterial activity by producing some antibacterial secondary metabolites including *ochratoxin A* and *B*, *penicillic acid*, *asteltoxin*, *xanthomegnin*, *viomellin* and *vioxhantin*.

## 4. Conclusion

1. The isolated endophytic fungi isolate from dragon's scales fern fronds were 13 isolates consisting of 5 isolates from trophophyll fronds and 8 isolates from sporophyll fronds.
2. The secondary metabolites extract of endophytic fungi from dragon's scales fern which potentially as an antibacterial agent were DTP2 against *E.coli*, DTP4 against *S.aureus* and DSP4 against *S.dysentriae*.
3. MIC value of secondary metabolites ethyl acetate extracts of endophytic fungi isolate DTP2 against *E.coli* was 125 µg/mL, DTP4 against *S.aureus* was 125

µg/mL and DSP4 against *S.dysentriae* was 31,25 µg/mL.

4. Endophytic fungi isolate DTP2 identified as *Aureobasidium melanogenum*, DTP4 identified as *Penicillium allii-sativi* and DSP4 identified as *Aspergillus flocculosus*.

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