



ISOLATION AND CHARACTERIZATION ENDOPHYTIC FUNGAL ISOLATE FROM PERONEMA CANESCENS JACK LEAF AND COPTOSAPELTA TOMENTOSA VAL. K. HEYNE ROOT

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ABSTRACT

Has been done Isolation, Characterization and Secondary Metabolite Endophytic Fungal Isolate from *Peronema canescens* Jack Leave and *Coptosapelta tomentosa* Valetton K. Heyne Root. The aim of this research is to know the number of fungal isolates, chromatogram profile and secondary metabolite group of endophytic fungal isolates from *P. canescens* leaves and *C. tomentosa* root. Characterization of endophytic fungal isolates was done macroscopically and microscopically. Identification of secondary metabolites endophytic fungal isolates were performed by chemical reaction test and TLC (Thin Layer Chromatography) method with specific spray reagents. The data of this study were obtained based on the number of endophytic fungal that can be isolated, observing macroscopic and microscopic morphological profiles, chromatogram profile and secondary metabolites of each endophytic fungal isolated. The results showed that endophytic fungal that can be isolated from *P. canescens* leaves four isolates, and two isolates from *C. tomentosa* root. Morphological profile macroscopic endophytic fungal of the six isolates showed a greenish-colored colony, white gray, clear black. Microscopic profile of each fungal isolate having spores, sprangiosphora, sporangium, conidia, hyphae and stolon. The identified secondary metabolites are: alkaloids, terpenoids, and flavonoids, and polyphenols.

Keywords: *Peronema canescens* Jack, *Coptosapelta tomentosa*, secondary metabolites, endophytic fungal

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INTRODUCTION

Indonesia is a country that has biodiversity that can be processed into drugs various [1]. More than 1000 plant species can be utilized as raw material of

medicine. Various types of bioactive compounds with functions various contained in plants, allegedly can be produced by endophytic microbes. Approximately 300,000 species of plants

are scattered on the face of this earth, each plant contains one or more endophytic microbes consisting of fungal and bacteria. The ability of endophytic microbes to secondary metabolite compounds produce according to their host plants is a tremendous and reliable opportunity to secondary metabolites produce from endophytic microbes isolated from these host plants [2].

Endophytic fungal is it that lives in plant tissue at a certain period and is able to live by forming colonies in plant tissues without endangering its host [3]. Endophytic fungal are present in plant tissue systems, such as leaves, flowers, twigs or plant roots. The ability of endophytic microbes to produce secondary metabolite compounds according to their host plants is a tremendous and reliable opportunity to produce secondary metabolites from endophytic microbes isolated from these host plants [4].

Some of the traditional herbs used for treatment are Sungkai (*Peronema canescens* Jack) and Merung (*Coptosapelta tomentosa* Valeton K. Heyne) roots, young leaves of *P. canescens* by Dayak tribe use as cold medicines, fever, ringworms, used as for women a bath after delivery and as a gum toothache prevention [5]. As for the root of *C. tomentosa* is also a native Indonesian medicinal plant widely used by people Kutai Kartanegara used for the treatment of diseases such as hypertension, pain (analgesic), diabetes mellitus, diuretic and anticancer [6].

The chemical compounds contained in the leaves of *P. canescens* include β -sitosterol, phytol, β - amyryl and several diterpenoids, among others: peronemin compounds A1, A3, B2, B2, B3, C1 and peronemin D1 [7], essential oils, lantaden terpenoid A compounds, lantaden B, lanthanolic acid, lantat acid, and class of lantonin alkaloids [8]. In addition, the crude extracts of methanol and ethyl acetate fractions contain classes of

alkaloids, terpenoids, steroids, flavanoids, phenolics and saponins [9]; [10]. Several studies of the bioactivity of *P. canescens* leaf have been reported, among others, n-hexane extract, ethyl acetate bark of *P. canescens* antioxidant effect [11]. Activities crude extract methanol as antibacterial [9]. Methanol extract and n-hexane fraction of *P. canescens* leaves are active against the shrimp larvae [12].

The secondary metabolite of *C. tomentosa* root contains secondary metabolite classes such as alkaloids, flavonoids, tannins, phenolics, and sapaonins, and its bioactivity to *Artemia salaina* Leach obtained LC₅₀ values of 346,74 $\mu\text{g/mL}$ for ethyl acetate extract, and 933.25 $\mu\text{g/mL}$ for the extract n-butanol [13]. In addition, the antioxidant activity value obtained by LC₅₀ methanol crude extract was 113,60 $\mu\text{g/mL}$, n-hexane fraction 69,02 $\mu\text{g/mL}$, ethyl acetate fraction 97,64 $\mu\text{g/mL}$, and n-butanol fraction 432,30 $\mu\text{g/mL}$ [6].

Although it is well known that the leaves of *P. canescens* and *C. tomentosa* roots contain many secondary metabolite groups and chemical compounds and have many uses, to support and supplement previously reported research results, it is necessary to explore potential *P. canescens* leaf and *C. tomentosa* root through various research methods, one research method that can be done is the search for natural chemical compounds can be done through a biological approach that is the method of endophytic fungal isolation in vitro. The aim of this research is to know the number of endophytic fungus isolated from *P. canescens* leaf and *C. tomentosa* valeton root is to know the morphological profile, chromatogram profile and secondary metabolite group of endophytic fungal isolates from *P. canescens* leaf and *C. tomentosa* roots.

MATERIALS AND METHODS

Plant materials.

Part of the plant used is the leaves of *P. canescens* Jack, obtained from Tanah Merah-Samarinda, and *C. tomentosa* Valeton K. Heyne root obtained from Kutai Kartanegara, both samples has been determined by Dendrology Laboratory Faculty of Forestry, Mulawarman University.

Media and reagent.

The materials used in the study were Aquadestilata, medium PDA (Potato Dextrose Agar), MEA (Malt Extract Agar), PDB (Potato Dextrose Broth) and NA (Nutrient Agar), 0.9% NaCl solution physiological. Reagent Dragendorf, Liebermann-Bouchard, 10% H₂SO₄, FeCl₃, Sitroborat, Thin Layer Chromatography plate.

Research procedure

Isolation of endophytic fungal.

Leaf and root surfaces were cleaned with running water, sterilized for 1 minutes leaf surfaces with 70% alcohol, then immersed for 3 minutes with 5.25% NaOCl (sodium hypochlorite), soaked for 30 seconds with 70% alcohol. Dried prepare with sterile tissue, leaves and roots are cut horizontally or longitudinally with size $\pm 1 \times 1$ cm using a sterile knife. The prepare of leaves and roots were attached to a 100 μ g/mL PDA medium contains chloramphenicol (PDAC) in a Petri dish, incubated for 7-14 days at room temperature (25°C). Observations are done every day until a visible colony of fungal grows.

Endophytic fungal colonies purification.

Fungal colonies grown on PDAC medium, purified by inoculation into a new PDA medium, were incubated for 5 to 7 days at room temperature (25°C) until a

pure endophytic fungal isolate was obtained by viewing the characteristics visually [14].

Endophytic fungal isolates characterization.

Characterization of endophytic fungal isolates was done both macroscopic and microscopic. Macroscopic observation was done by looked at the shape and color of the endophytic fungal colonies on the Petri dish. While the microscopic characterization done direct and indirect observation a camera microscope using. Direct microscopic observations of fungal isolates were inoculated on glass objects which then spilled with methylen blue reagent and observed under a microscope. Indirect observation, on the glass object is added 1 - 2 drops of medium PDA above glass object closed with cover glass, incubated at room temperature (25°C) for 7 days and observed by microscope.

Secondary metabolite production of endophytic fungal isolates.

Seed endophytic fungal isolates were taken 20 mL, and added to 250 mL of PDB media in Erlenmeyer and then incubated for 5-7 days with shaking. Then filtered using Whatmann paper number 41. Miselia collected, dried in oven, then extracted using methanol for one day, evaporated in oven 40°C temperature. The extract was used for the identification of secondary metabolites with direct chemical reagents and TLC method.

RESULT AND DISCUSSION

One effort to find and propagate raw materials of drugs can be done biologically microbiology through the process of isolation of endophytic fungal. Propagating and reseach raw materials of drugs through biological means can be done on natural materials (plants/animals) with various criteria such as: plants have been nearing extinction, very long growth of

development, very little active secondary metabolites produced, very difficult of cultivation, the location of active metabolite on the part that if excessive exploitation can accelerate the extinction so as to affect the environment of ecosystem.

Peronema canescens and *C. tomentosa* are plants that have the above criteria is having a potentially active metabolite but the growth is long enough and if exploited can accelerate extinction. The scientifically these plants, especially the leaves and roots contain secondary metabolites of alkaloid compounds, carotenoid, phenolic compounds, flavonoids, saponins, tannins and steroids, both have high toxicity potential than the potential for development as raw materials and drugs potential compounds.

Endophytic Fungal Isolation

Isolation is a way to separate a microbe from its environment, so that a culture is obtained which is not mixed with other or called pure cultures [15]. Isolation of endophytic fungal was done by using sterilization method of surface stratified. The surface sterilization method was used as a pretreatment to isolate endophytic fungal originating from fresh plant organs [16]. Endophytic fungal can be isolated from plant tissue systems, such as: leaves, flowers, twigs, or plant roots.

P. canescens leaves and *C. tomentosa* roots that have been sterilized surface, opened the tissue by way of horizontally sliced. The sliced tissue is then attached of the medium of PDA to the surface which has been added chloramphenicol antibiotics. The addition of chloramphenicol serves to suppress the growth of contaminant bacteria and endophytic. These antibiotics work by blocking the enzyme peptidyl transferase on the 50s ribosome. As a result of such enzyme blocking the protein synthesis stop process [17]. According to Jawetz [18],

chloramphenicol antibiotic is a broad-spectrum bacteriostatic that is active against positive or negative gram bacteria aerobic and anaerobic.

Based on the result of isolation of endophytic fungal which has been purification, obtained 4 isolate from *P. canescens* leaf which each isolate is given DS21, isolate DS22, isolate DS23 and isolate DS74 code, while isolate of fungal from *C. tomentosa* root obtained 2 isolate and given AM61 and AM102 code.

Profile of purified fungal isolates from *P. canescens* leaf

The purified endophytic fungal isolates were then characterized. This characterization aims to determine the morphology of endophytic fungal isolates through microscopic and microscopic observations. The profiles of each isolate are described as follows:

Morphology fungal isolates of macroscopic

The results of observation of DS21 isolates on macroscopic, showing colonies of greenish black, rounded shapes clustered, irregularly wavy colony edges, smooth fungal surfaces. The DS22 isolate shows a round, grayish white, shaped fibers with thin threads separated, the edges of irregular colonies, smooth and irregular colony surfaces. The DS23 isolate shows white colored colonies, fibrous shape, smooth, wavy colony edges, smooth colony surface, forming air bubbles on yellowish surface medium rather whitish. The DS74 isolate show white colony surface, black colony base, mushroom shape like cotton, filamentous, irregularly shaped root-shaped colony, have black pigment. Profile of macroscopic morphology fungal isolates of DS21, DS22, DS23 and DS74 can be seen in Figure 1.

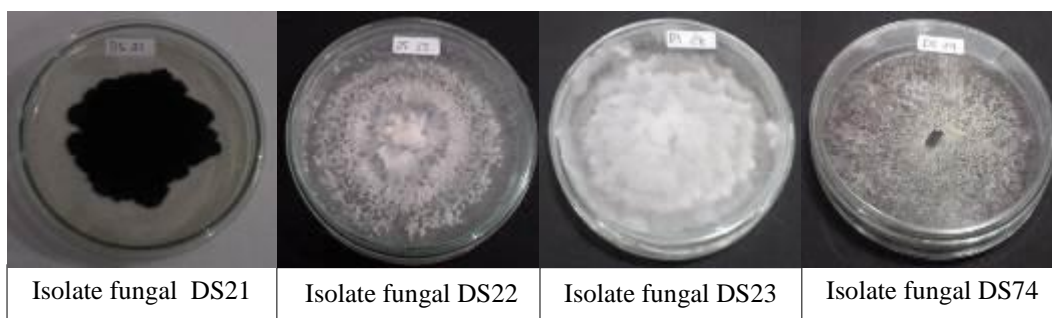


Figure 1. Profile of macroscopic morphology isolates fungal of DS21, DS22, DS23 and DS74.

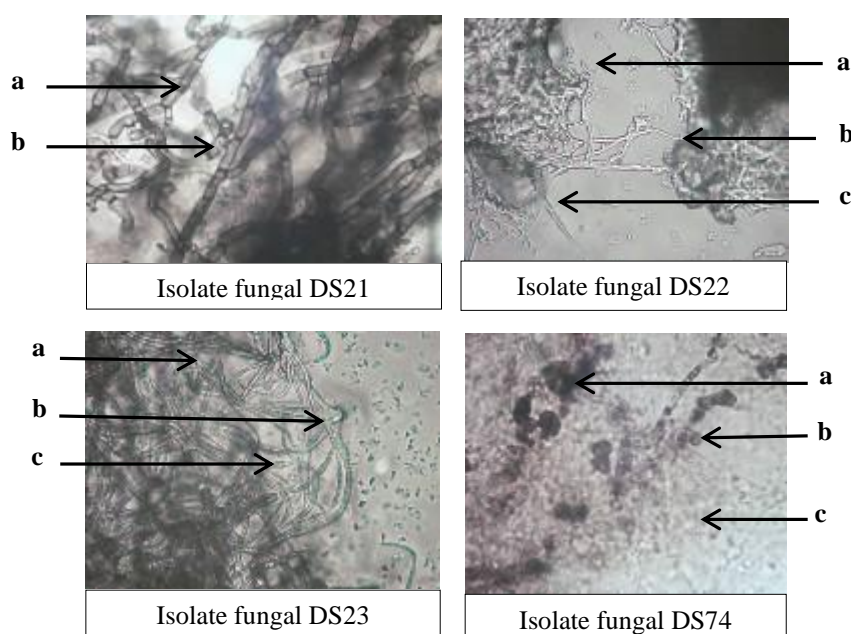


Figure 2. Profile of microscopic fungal isolates DS21, DS22, DS23 and DS74 400x magnification.

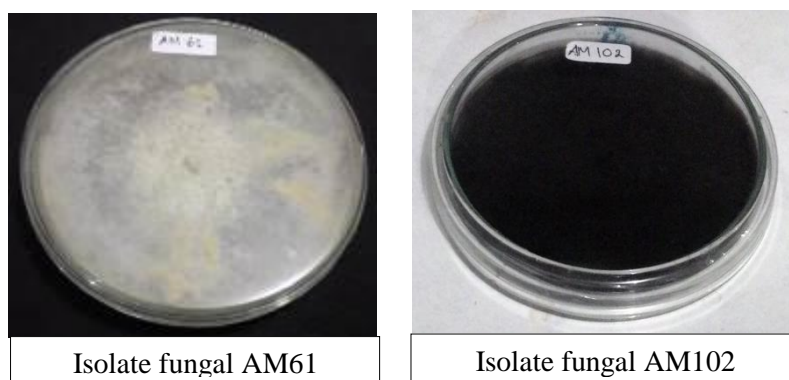


Figure 3. Profile of macroscopic morphology of isolates AM61 and AM102

Morphology isolates fungal of microscopic

The result of observation microscopic isolate of DS21 at 400x enlargement indicates the fungus has (a) sprangiosphora, and (b) sporangium. As for the isolate fungal DS22 there are (a) spores, (b) sprangiosphora and (c) stolon. Microscopic fungal DS23 isolates showing the fungus having (a) sprangiosphora, (b) spores, and (c) hyphae. Isolate of DS74 fungal having (a) sprangiosphora, (b) spores, and (c) conidia. Profile of microscopic fungal isolates DS21, DS22, DS23 and DS74 can be seen in Figure 2.

Profile of purified fungal isolates from *C. tomentosa* root

The purified endophytic fungal isolates were then characterized by endophytic fungal isolates, to determine the morphology of endophytic fungal isolates through macroscopic and microscopic observations. The profiles of each isolate are described as follows:

Morphology fungal isolates of macroscopic

The observations of AM61 isolate are macroscopic, showing colonies of white, and yellow cloudy, smooth surfaces resembling cotton. There air bubbles on the surface of the medium, the edge of irregular colonies, have a pink yellow

pigment. For macroscopically isolated fungal AM102, the colonies are clear black, circularly irregularly shaped with varying sizes, there are mycelium-like roots in medium and blackish-gray attached, the edges of flat colonies, containing black pigments. Macroscopic images isolate of AM61 and AM102 can be seen in Figure 3.

Morphology fungal isolates of microscopic

The observation results of AM61 isolates microscopic, at 400x magnification showed the fungus had (a) sprangiosphora, (b) spores, and (c) conidia. As for AM102 fungus isolates there are (a) sprangiosphora, (b) spores, and (c) Stolon. Microscopic observation of AM61 and AM102 isolates can be seen in Figure 4.

Secondary Metabolite Production

Each plants usually contains several endophytic fungal of producing a secondary metabolite compound similar to that of its host, thought to be the result of genetic recombination or co-evolution from host plant in endophytic fungal. One way the its to adapted is by adopting some genetic information (DNA) from host plant. That matter a characteristic chemical of its to produce component in host plant [19].

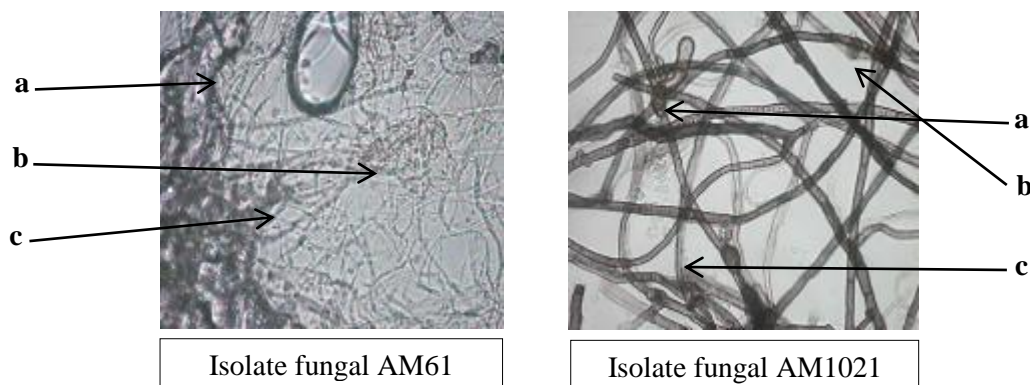


Figure 4. Profile of microscopic morphological of isolates AM61 and AM102 magnification 400x

According to Carlile [20] the secondary metabolism of the endophytic fungal generally occurs at a time when the growth phase to an end and begins to enter its stationary phase. It supports the statement [15], which states that the secondary metabolites of endophytic fungal can be harvested in the stationary phase growth its. The stationary phase can occur when, there is a balance between the number of cells divided by the numbered of cells that are dead.

The endophytic fungal isolates obtained were seeded using a liquid growth medium of PDB (Potato Dextrose Agar), which was then incubated using a rotary shaker. The incubation process is performed with agitation to satisfy the necessary oxygen requirements in the growth of the endophytic fungal and maintain the nutrient homogeneity present in the medium used. The result of the process is a mycelium of fungal isolates which is then dried and extracted by maceration method using methanol solvent. Methanol solvent is a universal solvent which can dissolve polar and nonpolar active compounds. The viscous

extract obtained is then dried and used for secondary metabolite identification.

Profile spot isolate fungal of UV254 nm, UV366 nm and H₂SO₄ 10%

Identification of secondary metabolite groups from methanol extracts of fungal isolates using chemical reaction test method (tube test) and TLC. The principle of the TLC method is based on the separation of chemical components by adsorption and partition, in which the chemical component moves up following the mobile phase due to adsorbent absorption of the chemical components at different rates according to their polarity, causing the separation of active compounds in spot spots on the chromatogram plate [21].

Isolation of fungal spotted to isolates used eluent was hexane: ethyl acetate (3: 1) was further spotting observed using spotting of 10% H₂SO₄, UV254 nm and UV366 nm rays, obtained R_f (rate of flow) values of each fungal isolate. The R_f value of each spot of elution spot can be seen in Table 1.

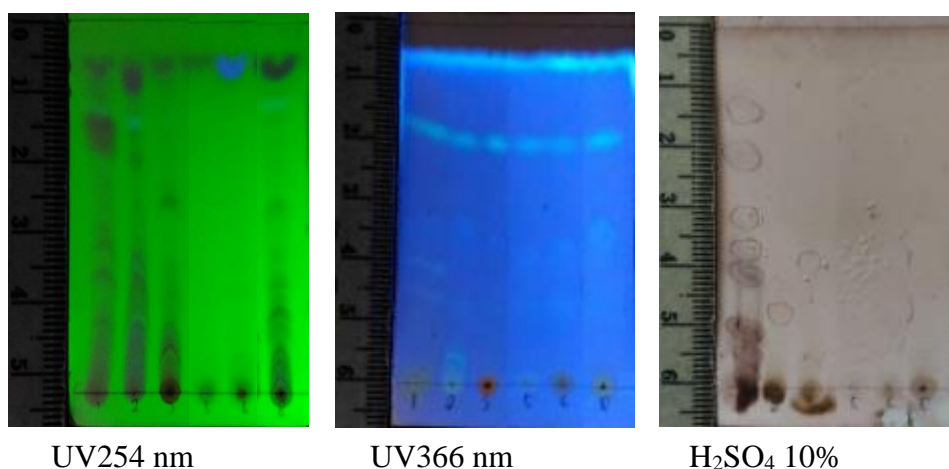


Figure 5. Chromatogram of methanol extract endophytes fungal isolate from *P. canescens* leaf and *C. tomentosa* root

Table 1. Rf value spot extract methanol endophytes fungal isolate from P. canescen leaf and C. tomentosa root.

No.	Sample (Code)	Spotting		
		UV 254 nm Rf value	UV 366 nm Rf value	H ₂ SO ₄ 10% Rf value
1	DS21	0.25	0.25	0.03
		0.33	0.33	0.08
		0.42	0.42	0.62
		0.77	0.72	0.32
				0.38
				0.45
				0.63
				0.78
2	DS22	0.05	0.08	
		0.08	0.12	
		0.12	0.22	0.20
			0.42	
		0.67		
3	DS23	0.12	0.22	
		0.15	0.40	0.33
		0.18	0.65	
5	DS74	-	0.17	
			0.33	-
			0.63	
6.	AM61	-	0.03	
			0.37	-
			0.63	
8.	AM102	0.05	0.42	
		0.10	0.65	-

Table 2. Results of secondary metabolite group identification of endophytic fungal of P. canescen leaf and C. tomentosa root

Secondary Metabolite	Fungal Isolate P.canescens				Fungal Isolate C. tomentosa	
	DS21	DS22	DS23	DS74	AM61	AM102
Alkaloid	+	+	+	+	+	+
Terpenoid-Steroid	+	+	+	-	-	+
Flavanoid	+	+	+	+	+	+
Polifenol	-	+	+	-	-	+

Description: (+) = Secondary metabolites are identified
 (-) = No secondary metabolites are identified

From Table 1 and Figure 5. above, the amount detected of spot on chromatogram for each fungal isolate using

UV254 nm, UV366 nm and 10% H₂SO₄ spotting is for isolate DS21 obtained by 4 spot on UV254 nm and UV 366 irradiation,

and 8 spot on 10% H₂SO₄ visible light. Isolate of DS22 obtained 3 spot on UV254 nm, 5 spot stain on UV366 nm and 1 spot stain on 10% H₂SO₄. Isolate of DS23 obtained each 3 spot on UV 254 nm and 366 nm UV and 1 spot stain on H₂SO₄10%. As for the DS74 only obtained 3 spots on UV 366 nm.

Profile secondary metabolite

Secondary metabolites of identification of four endofite fungal isolates from *P. canescens* leaf and two fungal isolates from *C. tomentosa* root using specific chemical reagents of spray compounds such as Dragendorff reagents for alkaloid, FeCl₃ for polyphenol, Lieberman Bourchard for terpenoids-sterols, and Sitroborat for flavonoid detection. The identification results showed that four isolates of *P. canescens* leaf fungal contain alkaloid that is isolates DS (21, 22, 23 and 74), terpenoid - steroid isolates DS (21, 22, and 23), Flavanoid isolates DS (21, 22, 23 and 74), and polyphenol isolates DS (22 and 23). While the secondary metabolite identification endophytic fungal from *C. tomentosa* root results of two isolates contain alkaloid that is isolates AM (61 and 102), steroids isolate AM102, Flavanoid isolates AM (61 and 102), and polyphenol isolates AM102. The complete result of secondary metabolite group identification endophytic fungal of methanol extract two samples can be seen in Table 2.

In Table 2, fungal isolates of methanol extract from *P. canescens* leaf showed that secondary metabolite contained in fungal isolates showed similarity of secondary metabolite contained in its host (*P. canescens* leaf) such as: alkaloids, terpenoids - steroids, flavanoid, and tannin (polyphenols) [9];[10]. Similarly, secondary metabolites of fungal isolates *C. tomentosa* root also show similar secondary metabolites with those present in their host plants: alkaloids,

flavonoids, tannins (polyphenols), and phenolic [13].

CONCLUSION

1. Endophytic fungal that can be isolated from *P. canescens* Jack leaf amount to 4 types of fungal with isolate codes of DS21, DS22, DS23, and DS74, and two isolates of fungus from *C. tomentosa* root with code isolates AM61 and AM102.
2. Profile of endophytic fungal isolates from *P. canescens* leaf are: greenish-green colony, white gray-black base, rounded clustered shape, microscopically fungal having spores, sprangiosphora, sporangium, conidia, and hyphae. To isolate the fungal from *C. tomentosa* root are: clear black colonies, rounded fibers, there is blackish gray mycelium, flat colony, black pigment. Microscopic there are sprangiosphora, spores, conidia and stolon.
3. The secondary metabolites identified in the six isolates endophytic fungal of the *P. canescens* leaf are: alkaloids, terpenoids, and flavanoids, and polyphenols, and *C. tomentosa* roots are: alkaloids, terpenoids, flavanoids and polyphenols.

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