Endophytic Fungi Isolated from *Jasminum sambac* L.: Identification, Histological Observation, and Content Analysis of Secondary Metabolites

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Abstract. *Jasminum Sambac* L. plant, especially the flower used for ritual ceremony, i.e.: the wedding ceremony. Commonly medicinal plants have an interaction with endophytic fungi species that lived in their tissues, the interaction form is mutualism symbiotic. The research was done to: (1) identify the endophytic fungi species in the *J. sambac* flower petal, leaf, and twig tissues; (2) determine the position of endophytic fungi in the *J. sambac* flower petal, leaf, and twig tissues (3) analyze the contents of secondary metabolite produced by the endophytic fungi liquid cultures. The endophytic fungi mycelium position in the flower petal, leaf, and twig tissues was determined by microscopic observation. The flower petal, leaf, and twigs of *J. sambac* plant was taken for endophytic fungi isolation and determination their position by microscopic observation. These plant parts cut and inoculated on PDA plate medium. Each endophytic fungi species colonies that grow on PDA plate medium cut into 5x1 cm in size and inoculated in PDB medium, centrifugated and shooked at the speed of 120 rpm at 27 °C for a week. The supernatant use for content analysis of tannin, flavonoid, alkaloid, saponin, and terpenoid. The research results are: (1) There are ten endophytic fungi species found in *J. sambac* tissues: Mycelia sterilia, *Colletotrichum kahawae*, *Chaetomium reflexum, Colletotrichum alienum, Nigrospora gorlenkoawa, Nigrospora oryzae, Cladosporium allienum, Geotrichum candidium, Nigrospora musae,* and *Alternaria tennuis*; (2) the position of endophytic fungi is on the flower petal epidermis cell wall, the leaf neighbor cell wall and stomata guard cell wall, and the twig epidermis and parenchyma cell wall; (3) The secondary metabolites contents of some endophytic fungi are tannin content ranged: 56,09–131,77 mg/kg; flavonoid content ranged: 904,06–1661,88 mg/kg; alkaloid content ranged: 27,34–56,37 mg/kg; terpenoid content ranged: 116,23–222,59 mg/kg; and saponin content ranged: 1, 05–5,63 mg/kg.

Keywords: endophytic fungi · identification · histological observation · secondary metabolites · *Jasminum sambac* L.

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1 Introduction

Jasminum sambac flower has a fragrant scent, that's why people use these for traditionally ceremonies, i.e.: the wedding, the funeral etc. This leaf extract contains some compounds including alkaloid, tannin, glycoside [1]. The flower extract contains some compounds based on the phytochemical screening result are steroid, flavonoid, terpenoid, and saponin [2]. This plant commonly used in some infectious disease treatment, by the antimicrobial compound content a medicinal plant.

Commonly the medicinal plant lives with endophytic fungi species in mutualism symbiotic relationship. The host plant was protected by antimicrobial secondary metabolites produced by endophytic fungi. Besides that, the host plant protects the endophytic fungi from the high temperature or low humidity [3, 4]. There is no damage on the host plant tissue caused by the endophytic fungi. The location of endophytic fungi in the host plant tissue could be determined by histological observation. Based on the previous research, the endophytic fungi position in the host plant tissue, i.e.: in the leaf, twig, and bark tissues of *Tripterygium wilfordii* plant [5]; in the flower petal, leaf, and twig tissues of *Hedycium acuminatum* plant [6]; in the leaf and twig of *Physalis angulata* plant [7]. The identification research result about endophytic fungi in *Switenia mahagony* plant twigs and leaves were found eight species: *Mycelia sterilia*, *Hansfordia biophila, Rhizoctonia* sp., *Colletotrichum asianum, Colletotrichum siamense, Colletotrichum kahawae, Colletotrichum theobromicola,* and *Colletotrichum gloeosporioides* [8]. Some antibacterial secondary metabolites produced by the endophytic fungi. Another alternative besides to take the plant parts for infection diseases natural medicine treatment is use the antibiotic source obtained from the endophytic fungi. So, we can protect the medicinal plant from extinction. The research aimed to: (1) identify the endophytic fungi position in the *J. sambac* flower petal, leaf, and twig tissues; (2) to determine the position of the endophytic fungi in the *J. sambac* plant tissues; (3) analyze the contents of some secondary metabolite from the endophytic fungi liquid cultures.

2 Methodology

2.1 Preparation of *J. sambac* Flower Petal, Leaf, and Twig for Determine Endophytic Fungi Position from *J. sambac* Plant Tissues

The *J. sambac* flower petals, leaves, and twigs was rinsed with sterile distilled water, then sliced peridermal and transversal section for several microscopic slides. By histological observation, the endophytic fungi position in the *J. sambac* flower petals, leaves, and twigs plant parts could be determined.

2.2 Fungi Isolation and Identification from *J. sambac* Plant

J. sambac flower petals, twigs, and leaves was rinsed and soaked in 1% NaOCl during one min., then washed in sterile distilled water. Afterwards the plant parts were dipped within 70% alcohol during a minute, then rinsed with sterile distilled water [9]. The leaves and flower petals part cut in 1x1 cm^2 in size, the twigs part cut with the twig

of 0.5 cm thickness. Afterwards that plant part pieces of each sample inoculated on the PDA plate medium which contained 100 mg/L chloramphenicol, then incubated in 25 °C–27 °C for 7 days. The ten endophytic fungi species were seeded onto PDA slant media and incubated at 27 °C for three consecutive days to be observed under a microscope. Each endophytic fungi isolate's culture slide was created for microscopic examination, and the fungi's characteristics were documented to aid in identification. The OLYMPUS microscope CX 21 FS 1 was the instrument we employed for microscopical examination. The slide culture for fungi description and identification were prepared. The identification books for fungi are Pitt, John. I. and Hocking, Ailsa D. 1985. *Fungi and Food Spoilage.* Tokyo: Academic Press; Watanabe, Tsuneo. 2002. *Pictorial Atlas of Soil and Seed Fungi Morphologies of Cultured Fungi and Key to Species.* Florida: CRC Press; Barnett, H. L. and Hunter, Barry. B. 1972. *Illustrated Genera of Imperfect Fungi.* Minnesota: Burgess Publishing Company.

2.3 The Endophytic Fungi Liquid Culture Preparation

The endophytic fungi liquid culture was prepared to obtain to determine the secondary metabolite contents. The colonies of each Endophytic fungi species on PDA plate medium were sliced into 5 × 1 cm2 squares, inoculated onto PDB medium, and then incubated at 27 °C for 7 consecutive days while being shaken at 120 rpm speeds. Then the liquid culture centrifugated at 3000 rpm speeds within ten minutes. Detection of secondary metabolites contents used the supernatants, i.e.: tannin, alkaloid, terpenoid, flavonoid, and saponin produced by each endophytic fungi species.

2.4 The Secondary Metabolite Detection from the Liquid Culture of Each Endophytic Fungi Species

The spectrophotometric method was used to analyze the levels of tannin, alkaloid, flavonoid, saponin, and terpenoid in each endophytic fungi species' liquid culture. Following is an explanation of how these secondary metabolites were determined.

2.4.1 The Alkaloid Content Determination [10]

2.4.1.1 Preparation of Standard Solution

Atropine solution 100 mg/L is used as the standard solution. Ten milligrams of the atropine solution were dissolved in 100 mL of chloroform. Several concentrations, including 0, 1, 5, 10, 25, and 50 mg/L, were prepared.

2.4.1.2 Preparation Standard Solution and Sample Solution

The flower petals, leaves, and twigs sample were weighed in 0,1 g, crushed, and then dissolved in ten mL Dimethyl Sulfoxide (DMSO) added with a mililiters HCL 2N and Five litters of phosphate buffer and five litters of brom cresol green solution, homogenized, and allowed to stand for 60 min. Ten milliliters of chloroform were added to the solution in a separatory funnel, which was then shaken while the layers formed. The upper phase of the solution was then taken out for the subsequent process. Then,

five milliliters of atropine solution, one milliliter of HCL 2N, five milliliters of brom cresol green solution, and five milliliters of buffer phosphate were combined to create the standard solution, which was then homogenized and used for the next process.

2.4.1.3 The Determination of Alkaloid Content

The upper phase or the standard solution was taken 11 ml in volume. Look for the solution color, the solution color will be yellowish orange, if the sample contains an alkaloid. The solution dissolved with chloroform to five milliliters in volume. The measurement of the absorbance was done at $\lambda = 470$ nm. The determination of alkaloid content uses the regression standard equation.

2.4.2 The Flavonoid Content Determination [11]

2.4.2.1 Preparation of Standard Solution

The standard solution use quercetin 100 mg/L. The quercetin standard solution was prepared by dissolve 100 mg in distilled water to 100 ml. Some concentrations were prepared: 0; 0.5; 1; 10; 25 and 50 mg/L.

2.4.2.2 Preparation Standard Solution and Sample Solution

The sample of flower petals, leaves, and twigs was weighed in grams, crushed, and then dissolved in methanol to a concentration of ten milliliters before being homogenized and left to stand for 30 min. After that, it was filtered using a vacuum filter and centrifuged for 10 min at 3000 rpm. After that, the supernatant was removed, a liquid sample containing five microliters of methanol was added, and the mixture was homogenized for five minutes. Using a vacuum filter, the solution was filtered. The filtrate is then used in the following step.

2.4.2.3 The Determination of Flavonoid Content

The standard solution or sample solution as much as 0.1 ml add with 0.1 ml 2%AL2CL3, homogenized and let stands for 60 min. Then distilled water added a milliliter in volume. Look for the solution color, the solution color will be becoming red, if the sample contains a flavonoid. The measurement of the absorbance was done at $\lambda = 420$ nm. The determination of flavonoid content uses the regression standard equation.

2.4.3 The Saponin Content Determination [12]

2.4.3.1 Preparation of Standard Solution

The saponin standard solution 100 mg/L. The saponin standard solution were prepared by dissolve ten milligrams saponin in 20% ethanol to 100 mililiters. Some concentrations were prepared: 0; 1; 5; 10; 25; and 50 mg/L.

2.4.3.2 Preparation of Standard Solution and Sample Solution

The sample of flower petals, leaves, and twigs was weighed at 0.1 g, crushed, and dissolved in 10 ml of 90% ethanol, then homogenized and heated in a water bath at

55 °C for 90 min. After that, filter paper was used to purify the solution. The sludge was then reextracted with up to 10 mL of 90% ethanol. Extracts of the flower petals, leaves, and twigs were combined, heated to 90 °C, and allowed to cool until only half of the solution remained. After that, the solution was removed from the separation funnel, 40 mililiters of diethyl ether were added, it was shaken, and it was allowed to stand until the solution separated. 60 ml of butanol and ten milliliters of 5% sodium chloride (NaCl) were placed in the bottom phase, which was then filtered. Then used a dry oven set at 60 °C to dry the filtrate. Once saponin has been produced, it is diluted in five milliliters of 20% ethanol.

2.4.3.3 The Determination of Saponin Content

Five milliliters of standard solution or sample solution should be used, along with 0.5 ml of 0.1 N ferric chloride (FeCl3) and 0.5 ml of 0.008 M K3Fe(CN)6. The solution should then be homogenized and allowed to stand for 30 min before being used. If the sample contains a saponin, look for the solution color, which will be blue. The solution was then diluted with chloroform to a volume of 10 ml. At a λ of 470 nm, the absorbance was measured. Regression standard equation is used to determine saponin content.

2.4.4 The Tannin Content Determination [10]

2.4.4.1 Preparation of Standard Solution

The standard tannic acid solution is 50 mg/L. Tannic standard solution was made by dissolving five milligrams of tannic acid in ten milliliters of 20% ethanol. There were some concentrations prepared: 0, 1, 5, 10, 25, and 50 mg/L.

2.4.4.2 Preparation of Sample and Standard Solution

The sample of flower petals, leaves, and twigs was measured at 0.1 g, crushed, and then dissolved in 10 ml of methanol before being homogenized and allowed to stand for 30 min. The solution was filtered using a vacuum filter and centrifuged for 10 min at 3000 rpm. The supernatant is then used for the subsequent process.

2.4.4.3 The Determination of Tannin Content

Take five milliliters of the standard solution or a sample solution, add 0.1 ml of ferric chloride (FeCl3) and 0.008 ml of K3Fe(CN)6, homogenize it, and leave it stand for 30 min. Distilled water was used to dilute the solution to a volume of 10 ml. At a λ of 620 nm, the absorbance was measured. Regression standard equation is used to determine tannin content.

2.4.5 The Terpenoid Content Determination [13]

2.4.5.1 Preparation of Standard Solution

The linalool standard solution 0.5 mg/100 mL. The linalool standard solution was prepared by dissolve 0.5 mg linalool in chloroform to 100 mililiters. Some concentrations were prepared: 0; 0.01; 0.05; 0.1; 0.25 and 0.5 mg/L.

2.4.5.2 Preparation of Sample and Standard Solution

The sample of flower petals, leaves, and twigs was divided into two portions and weighed at ten grams. It was then crushed, dissolved in methanol to a volume of 25 ml, homogenized, and left to stand for 30 min. For five minutes, the solution was filtered and centrifuged at 3000 rpm. After adding a milliliter of chloroform to the supernatant, the top phase (non-polar) was extracted, yielding up to five milliliters. The solution is applicable to the next process.

2.4.5.3 The Determination of Terpenoid Content

Take standard solution or sample solution as much as five milliliters added with 3 ml concentrated sulfuric acid (H_2SO_4), homogenized, and let stands for a while. Look for the solution color, the solution color will be brownish red color, if the sample contains a terpenoids. The solution diluted with chloroform to ten milliliters in volume. The measurement of the absorbance was done at $\lambda = 538$ nm. The determination of terpenoids concentration determined by regression standard equation.

2.5 Data Analysis

The position of endophytic fungi hyphae in *J. sambac* shown in Table 1 and Fig. 1. Each endophytic fungi microscopical characteristics shown in Fig. 2. Description data of ten endophytic fungi species from *J. sambac* shown in Table 2. The endophytic fungi species secondary metabolite content shown in Table 3.

3 Result and Discussion

3.1 The Endophytic Fungi Position in *Jasminum sambac* Description

The endophytic fungi hyphae in *J. sambac* tissues position were obtained from microscopic observation result. The hyphae location in *J. sambac* flower petal, leaf, and twig by histological observation result are shown in the Table 1 and Fig. 1.

3.2 The Endophytic Fungi Species Isolated from *J. sambac* Plant Identification Result

The identification result of the endhophytic fungi from *J. sambac* flower petal, leaf, and twig was found ten species, consist of: *Colletotrichum kahawae*, *Chaetomium reflexum, Colletotrichum alienum, Mycelia sterilia, Nigrospora gorlenkoawa, Nigrospora oryzae,*

Table 1. The Endophytic Fungi Hyphae Position in *J. sambac* Flower Petal, Leaf, and Twig Tissues

No.	The Plant Parts	Slice section	Location
1	The flower petals	Peridermal	Epidermis cell wall
2	The leaves	Peridermal	Epidermis cell wall Stomatal cell wall
			Guard cell wall and neighbor cell wall
		Transversal	Epidermis cell wall
3	The twigs	Transversal	Parenchyma cell wall
			Epidermis cell wall

Cladosporium allienum, Geotrichum candidium, Nigrospora musae, and *Alternaria tennuis*. The photomicrograph of 10 endophytic fungi species from *J. sambac* shown in Fig. 2.

The endophytic fungi hyphae were found in the epidermal cell walls of flower petals, in the epidermal cell wall, neighbor cell wall, and guard cell wall of leaves, in the epidermis cell wall and parenchyma cell wall of twigs based on the histological observations. The description results of each endophytic fungi isolate characters shown in Table 2.

The ten fungi species found in flower petals, leaves and twigs from *J. sambac* tissues have different in characters each other (Fig. 2). The previous research also found some of these endophytic fungi species. *Colletotrichum alienum* and *Colletotrichum kahawae* was also found as endophytic fungi in *Cananga odorata,* a sort of plant that the flower also used for ritual ceremony as well as *J. sambac* [6]. *Colletotrichum kahawae* also found in *Cordilyne fratiosa* plant [14] and *Switenia mahagoni* [8] The research about endophytic fungi in *Hedychium acuminatum* also reported that *Colletotrichum alienum* and *Rhizoctomia* sp. Also found in this plant [14] and found in *Physalis angulata* [7].

The endophytic fungi position based on histological observation is found on the cell wall. There are no hyphae insert into the host plant cell. It is proved that there is no damage in the host plant tissues caused by the endophytic fungi.

3.3 The Secondary Metabolite Contents from Ten Endophytic Fungi Species Isolated from *J. Sambac* Analysis

The analysis result of secondary metabolite contents especially flavonoid, alkaloid, tannin, saponin and terpenoid are shown in Table 3. Each endophytic fungi species has potential to produce secondary metabolites with different contents. *Alternaria tennuis* produce the five of secondary metabolites with the highest number.

Jasminum sambac commonly stay healthy and spared from infectious disease caused by bacteria. This research result has been proved that this plant has symbiotic mutualism interaction with some endophytic fungi that lived in the host plant tissues. Ten endophytic fungi species have been isolated from *J. sambac* flower petal, leaf, and twig.

All fungi liquid culture has potentially produced tannin, flavonoid, saponin, alkaloid, and terpenoid. These antimicrobial secondary metabolites protect the *J. sambac* from microbial infection. On the other way, the endophytic fungi could obtain benefit, since it lived inside the host plant tissues which could protect the fungi from the harmful abiotic factors, for instance the extreme air temperature, the low humidity, and lack of water [15].

Based on the histological observations, it is evident that none of the hyphae penetrate the host plant cells. This fact indicates that the endophytic fungi does not make any damage to the host plant tissues. The fungi take nutrition that does not take by the host plant in the intercellular space. Based on the results of this study shows that the liquid culture of endophytic fungi can be used as an eco-friendly antibiotic source. This is another alternative way besides taking part of the plant for natural medicine.

The research result were: (1) the position of endophytic fungi is on the flower petal epidermis cell wall, the leaf neighbour cell wall and stomata guard cell wall, and the twig epidermis and parenchyma cell wall; (2) There are ten endophytic fungi species found in *J. sambac* tissues: Mycelia sterilia, *Colletotrichum kahawae*, *Chaetomium reflexum, Colletotrichum alienum, Nigrospora gorlenkoawa, Nigrospora oryzae, Cladosporium allienum, Geotrichum candidium, Nigrospora musae,* and *Alternaria tennuis*; (3) Each species of endophytic fungi can produce secondary metabolites, which was the tannin content ranged: 56,09–131,77 mg/kg; the flavonoid content ranged: 904,06–1661,88 mg/kg; the alkaloid content ranged: 27,34–56,37 mg/kg; the saponin content ranged: 1, 05–5,63 mg/kg; the terpenoid content ranged: 116,23–222,59 mg/kg.

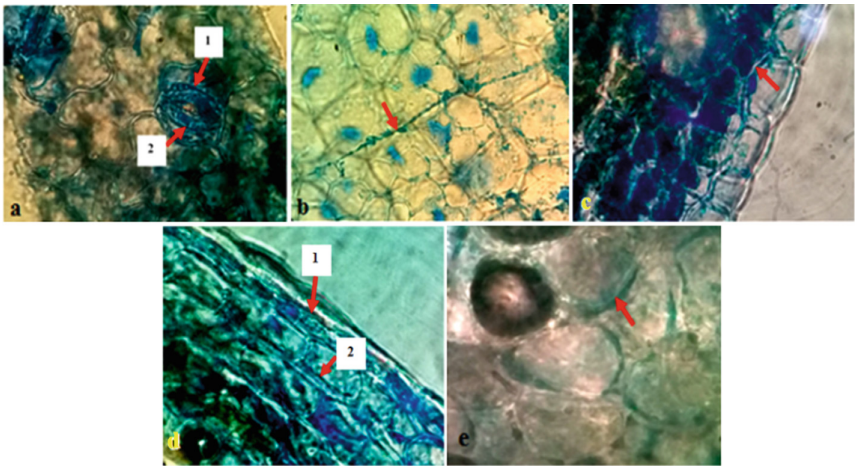

Fig. 1. Histological Observation Photomicrograph of Endophytic Fungi in *J. sambac* Plant. Description: (a) endophytic fungi hyphae in the leaf neighbor cell wall (1) and guard stomatal cell wall (2). (b) endophytic fungi hyphae in the leaf epidermis cell wall peridermal section. (c) endophytic fungi hyphae in the leaf epidermis cell wall transversal section. (d) endophytic fungi hyphae in the twig epidermis cell wall (1) and parenchyma cell wall (2) on transversal section. (e) endophytic fungi in the flower petal epidermis cell wall peridermal section.

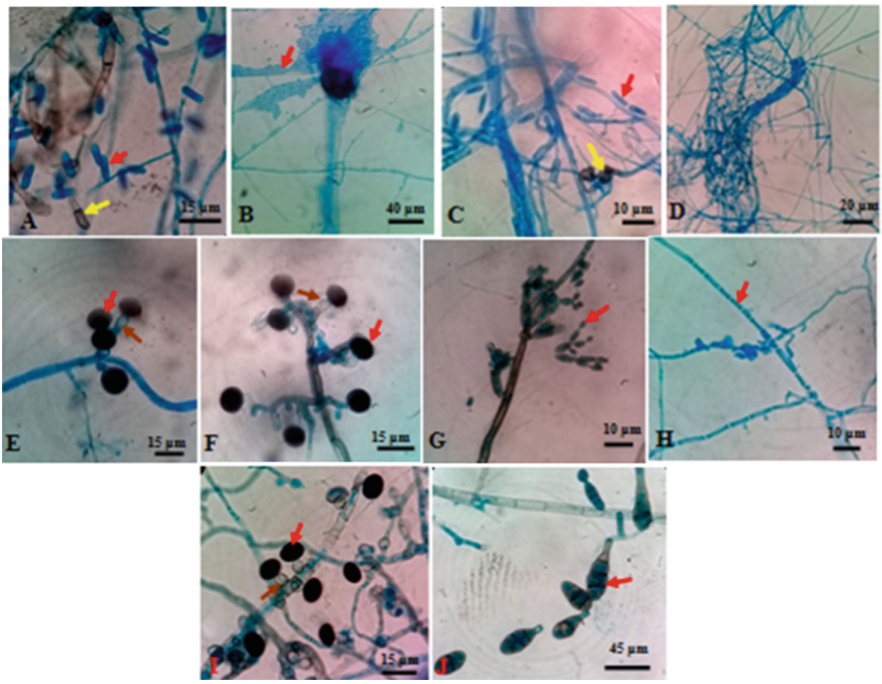

Fig. 2. Photomicrograph of Each Endophytic Fungi Species from *J. sambac*. (A) *Colletotrichum kahawae*; (B) *Chaetomium reflexum*; (C) *Colletotrichum alienum*; (D) *Mycelia sterilia*; (E) *Nigrospora gorlenkoawa*; (F) *Nigrospora oryzae*; (G) *Cladosporium allienum*; (H) *Geotrichum candidium*; (I) *Nigrospora musae*; (J) *Alternaria tennuis.* Conidia = red arrow; appressorium = yellow arrow; conidiogenous cell = brown arrow

Table 2. The Microscopic Characteristics of Each Endophytic Fungi Isolates from *J. sambac* Plant

Species	Isolate Code	The Plant Part	Colony Morphology	Appresorium (Shape, Size)	Conidia (Colour, Shape, Size, Wall Character)	Conidiogenous Cell (Colour, Shape, Size)	Peritechium (Colour)
Colletotrichum kahawae	A	Leaf	Color: greyish brown Reverse: brown, cottony	Fusoid and irregular Size: 6x10 μm × 13–22 μm	Color: brownish, Smooth wall Size: 4,5 μm × 11–14 μm	–	–
Chaetomium reflexum	B	Leaf	Color: white Reverse: yellowish white, cottony	–	–	–	Colour: black Size: 50x40 μm Terminal hair: dichotomy, 2 μm diameter
Colletotrichum alienum	C	Leaf	Color: white, orange Reverse: greyish white, cottony	Ellipse, irregular Size: 6–8 μm × 10 μm	Color: hyalin Shape: Cylindric Size: 3 μm × 10–17 μm Smooth wall	–	–
Mycelia sterilia	D	Twig	Color: light brown Reverse: brownish black cottony with intercalary chlamydospore, hyalin	–	–	–	–

(*continued*)

Table 2. (*continued*)

Species	Isolate Code	The Plant Part	Colony Morphology	Appresorium (Shape, Size)	Conidia (Colour, Shape, Size, Wall Character)	Conidiogenous Cell (Colour, Shape, Size)	Peritechium (Colour)
Nigrospora gorlenkoawa	E	Twig	Color: grey Reverse: blackish grey, cottony	–	Color: black Shape: solitary globose Size: 15 μm in diameter Smooth wall	–	–
Nigrospora oryzae	F	Twig, Flower petal	Color: grey Reverse: blackish white, cottony	–	Color: black Shape: solitary globose Size: 14 μm in diameter Smooth wall	–	–
Cladosporium allienum	G	Flower petal	Color: brownish grey Reverse: black, cottony	–	Color: brownish Shape: subglubose and ovoid in chain Size: 2–3 μm × 3–8 μm Smooth wall	–	–

(*continued*)

Table 2. (*continued*)

Species	Isolate Code	The Plant Part	Colony Morphology	Appresorium (Shape, Size)	Conidia (Colour, Shape, Size, Wall Character)	Conidiogenous Cell (Colour, Shape, Size)	Peritechium (Colour)
Geotrichum candidium	H	Flower petal	Color: yellowish white Reverse: yellowish, cottony	–	Color: hyalin Shape: cylindric in chain Size: 3 µm × 6–9 µm Smooth wall	–	–
Nigrospora musae	I	Flower petal	Color: white Reverse: brownish white Cottony	–	Color: black Shape: globose Size: 15 µm in diameter	–	–
Alternaria tennuis	J	Leaf	Color: white Reverse: brownish white Cottony	–	Color: brownish Shape: Clavate, pyriform with small beak, 1–7 segment Size: 8–15 µm × 20–45 µm Smooth wall	–	–

Table 3. The Secondary Metabolite of Each Endophytic Fungi Isolates from *J. sambac*

Isolate Code	Isolates	Secondary Metabolites Content (mg/kg)				
		Flavonoid	**Tannin**	**Saponin**	**Alkaloid**	**Terpenoid**
A	*Colletotrichum kahawae*	1169.69	82.62	2.09	37.34	153.51
B	*Chaetomium reflexum*	1533.75	118.98	4.71	51.05	204.61
C	*Colletotrichum alienum*	1329.06	98.53	3.23	43.34	175.88
D	*Mycelia sterilia*	904.06	56.09	1.05	27.34	116.23
E	*Nigrospora gorlenkoana*	1125.94	78.25	1.77	35.70	147.37
F	*Nigrospora oryzae*	1358.75	101.50	3.44	44.46	180.04
G	*Cladosporium allicinum*	1635.31	129.12	5.44	54.88	218.86
H	*Geotrichum candidum*	1188.44	84.49	2.22	38.05	156.14
I	*Nigrospora musae*	1374.38	103.06	3.56	45.05	182.24
J	*Alternaria tenuis*	1661.88	131.78	5.63	55.87	222.59

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