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Investigation of Antioxidant and anti-melanogenic Activities from Secondary Metabolites of Endophytic Fungi Isolated from *Centella asiatica* and *Syzygium polyanthum*

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Abstract

This study explores the potential of natural-based and environmentally friendly ingredients for functional cosmetics by investigating the antioxidant and anti-melanogenic activities of endophytic fungi associated with *Centella asiatica* (CA) and *Syzygium polyanthum* (SP). Three endophytic fungi were isolated from leaves and stems of CA and SP collected from Bogor and Serpong, Indonesia using Rose Bengal agar medium. The fungal isolates were cultivated through submerged fermentation in malt extract broth medium. After separating the mycelial biomass with Whatman filter paper, the supernatant was extracted using ethyl acetate. The ethyl acetate fraction of the mycelial biomass extract from these three fungal isolates was screened for antioxidant and anti-tyrosinase activities. In the preliminary screening using the DPPH method by thin-layer chromatography (TLC) with a mobile phase of ethyl acetate-methanol (9:1, *v/v*) and toluene-ethyl acetate (9:1, *v/v*), strong yellow spots indicating antioxidant activity were observed from each isolate after being sprayed with DPPH reagent. Screening for anti-tyrosinase activity was conducted using a colorimetric assay with button mushroom tyrosinase, kojic acid as a positive control and 3,4-dihydroxyphenylalanine (L-DOPA) as a substrate. One isolate with higher antioxidant and anti-tyrosinase activities was further identified based on sequence data of the internal transcribed spacer (ITS) rDNA (including ITS1, 5.8S rDNA, and ITS2). The identification of the selected isolate showed 99.45% similarities with *Colletotrichum sojae*.

Keywords: Endophytic fungi, *Centella asiatica*, *Syzygium polyanthum*, Secondary metabolite, Antioxidant, Anti-tyrosinase, *Colletotrichum sojae*

1. Introduction

Endophytic fungi living symbiotically within their plant host have been known as sources of various types of biologically active compounds secreted as an adaptive response to environmental stresses (Aly et al., 2013). These compounds exhibit diverse biological activities such as antioxidant, cytotoxic, antimicrobial, anti-inflammatory, anticancer, herbicidal, and anti-leishmanial properties (Anand et al., 2023). One particular interest in recent times is the evaluation of endophytic fungi for their antioxidant and anti-melanogenic activities in the cosmetic industry. This interest stems from the growing demand for natural and eco-friendly ingredients in functional cosmetics, including anti-aging, skin-lightening, and sunscreen products (Burger et al., 2016). Concerns regarding the safety of synthetic raw materials commonly used in cosmeceuticals, such as hydroquinone and retinoids, have also contributed to this shift toward natural alternatives (Barbaud, Lafforgue, 2021).

Despite the potential benefits, research on cosmetic ingredients derived from endophytic fungi remains limited. A previous study examined the skin-whitening characteristics of comoclathrin, a chemical generated by endophytic Comoclathris strains, and proved its efficacy using a tyrosinase inhibitory assay (Georgousaki et al., 2022). While metabolites' antioxidant properties are often considered as the primary mechanism for anti-aging effects, another approach for screening new skin-

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lightening agents is to inhibit the tyrosinase enzyme. Tyrosinase is crucial in the initial steps of melanin biosynthesis, and its dysregulated expression or hyperactivity can lead to pigmentation disorders (Pillaiyar et al., 2017; Slominski et al., 2004).

Antioxidants play a vital role in cellular homeostasis by neutralizing reactive oxygen species (ROS), which contribute to oxidative stress and various health disorders (Al-Ghamdi et al., 2020). Melanogenesis, the process of skin pigmentation, is influenced by oxidative stress, and excessive melanin production can result in hyperpigmentation disorders such as melasma and age spots (Lee et al., 2021; Liu et al., 2018). Two ethnomedicinal plants, *Centella asiatica* (L.) Urban. (CA) and *Syzygium polyanthum* (SP), have long been used in South and Southeast Asia to treat various ailments. CA is known for its anti-inflammatory and wound-healing properties, while SP is used traditionally to treat diabetes, hypertension, gastritis, ulcers, and skin diseases (Abdulrahman, 2022). The high antioxidant activity of bioactive compounds from CA and SP may contribute to their therapeutic effects (Gohil et al., 2010; Hartanti et al., 2019).

Previous studies have shown that the interaction between endophytic fungi and their host plants significantly influences the production of bioactive compounds and their biological activities (Jia et al., 2016). For example, endophytic fungi associated with *Nerium oleander L*. (Apocynaceae) increased the production of phenolic compounds in the host plant with potent antioxidant activity (Huang et al., 2007). Therefore, investigating endophytic fungi within medicinal plants presents a novel approach to discovering bioactive substances with enhanced characteristics and potential therapeutic applications. This study aims to evaluate the potential of endophytic fungi from CA and SP as new skin-lightening agents through screening for antioxidant and anti-tyrosinase activities. Four isolates from CA leaves and SP stems with optimal biological activities were further identified using sequence data from internal transcribed spacer (ITS) rDNA (including ITS1, 5.8S rDNA, and ITS2) and 18S.

2. Materials and methods

2.1. Materials

The study utilized the following materials: 1,1-diphenyl-2-picryl hydrazyl (DPPH), methanol, HPLC-grade methanol, Triton X-100, and ammonium sulphate, purchased from Merck (Darmstadt, Germany), ascorbic acid (vitamin C) from Kalbe Farma (Jakarta, Indonesia), fresh button mushrooms (*Agaricus bisporus*) from local market, L-DOPA from Sigma-Aldrich (Steinheim, Germany), thin-layer chromatography plates Silica gel 60 F_{254}, from Merck (Darmstadt, Germany), kojic acid from Sigma-Aldrich (Steinheim, Germany), C_{18} column chromatography XBridge 5 µm (4.6x150 mm) from Waters (Milford, Massachusetts, Amerika) equipped with a PDA detector and HPLC apparatus.

2.2. Isolation of endophytic fungi

Samples of CA and SP were collected from Serpong and Bogor, West Java, Indonesia, on March 21st, 2022. Healthy mature living leaves and twigs from randomly selected mature plants were obtained and transported to the laboratory in sterile plastic bags. Processing of the samples commenced within a few hours after the collection. The surface of the leaves and twigs was sterilized by washing with 70% ethanol followed by sterile distilled water. Subsequently, all plant samples were air-dried in a laminar airflow cabinet on sterile tissue paper for an hour (Abraham et al., 2015). The effectiveness of the sterilization procedure was evaluated following the method developed by Schulz et al (Schulz et al., 1993).

The sterilized plant tissue samples with a size approximation of 1 cm^2, were pressed onto the surface of the Rose Bengal agar medium (Himedia, Mumbai, Maharashtra, India) supplemented with chloramphenicol antibiotic. The absence of mycelia growth on the surface of the medium confirmed that the sterilization procedure was effective in removing the surface fungi from the plant tissue (Abraham et al., 2015). The combination of direct plating using Rose Bengal agar medium supplemented with chloramphenicol and a moist chamber method was applied to isolate endophytic fungi from plant samples (Ananda, Sridhar, 2002). For the direct plating method, the fragment of plant samples, approximately 0.5 cm x 0.5 cm, were placed on the surface of agar media and incubated in the incubator at 28°C. The plant samples were placed on the moist sterile towel tissue in the sterile plastic container. The growth observation of fungal mycelia in the plant sample's internal tissue was conducted daily (Polishook et al., 1996). The growth mycelial were then isolated aseptically in potato dextrose agar medium (Himedia, Mumbai, Maharashtra, India), purified and preserved in a 2 mL CryoTube containing 1 mL of 10% glycerol solution (*v/v*) and 5% lactose monohydrate (Merck, Darmstadt, Germany) (*w/v*) at −80°C (Abraham et al., 2015).

2.3. Fermentation and extraction of fungal secondary metabolites

The submerged fermentation process was conducted in a 1 L flask containing 500 mL of malt extract medium (30 g of malt extract, 5 g of peptone in 1 L of distillate water) for 14 days in a shaker incubator at 110 rpm and 28°C (Abraham et al., 2015). The mycelial biomass from each fermentation flask was separated by filtration using Whatman filter paper (no. 1), and the obtained supernatant was collected. Each filtrate was extracted by ethyl acetate (1/1, *v/v*) in the separation funnel. The water fraction on the upper layer was collected and then re-extracted three times with ethyl acetate. The ethyl acetate fraction on the bottom layer was eventually collected and concentrated using a rotary evaporator.

2.4. Screening for anti-oxidant activity

The evaluation of anti-oxidant activity was performed by using a radical scavenging assay of 2-diphenyl-1-picrylhydrazyl (DPPH). The DPPH solution (0.1 mM) was first dissolved in methanol. A series concentration of vitamin C solution (1.25; 2.50; 5; 25; 50; and 100 ppm) was then prepared in methanol as a standard solution. Each extract sample was prepared by dissolving it in methanol at the concentration of 10 mg/mL. 20 µL of the sample volume was mixed with 100 µL DPPH solution in each well of the 96-well plate and was further incubated for 15 minutes in the dark. Following the incubation, the

absorbance of the mixture was measured at 520 nm in triplicate against a blank methanol without DPPH radical (as control). The antioxidant activity was determined as an inhibition percentage of DPPH radical discoloration, and calculated using the following Eq. (1) (Guil-Guerrero et al., 2006):

$$\%\ Inhibition = \frac{A_{control} - A_{sample}}{A_{control}}\ x\ 100$$

(1) (Nurzaman et al., 2022)

where, $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of the extract. To indicate the antioxidant capacity, the IC_{50} value of each extract was measured using linear regression analysis of the standard solution.

2.5. Screening for anti-tyrosinase activity

The evaluation of anti-melanogenic activity was conducted using the tyrosinase colorimetric method (Hsu et al., 2018). The tyrosinase enzyme used in this bioassay was previously extracted from fresh button mushrooms (*Agaricus bisporus*), which was purchased from a local supermarket. To extract the enzyme, 200 grams of mushroom slices were mixed with 300 mL of chilled phosphate buffer (pH 5.8) and homogenized for 10 minutes at 19,500 rpm using an Ultra Turrax homogenizer (IKA, T50 digital, Selangor, Malaysia) in an ice bath. Subsequently, the mushroom slurry was centrifuged (Hitachi, Himac CR 22N, Ibaraki, Japan) for 20 minutes at 4°C and 9,000 rpm. For enzyme precipitation, firstly the supernatant was collected, and then 80 grams of ammonium sulphate (was added. The mixture was further stirred in an ice bath using a magnetic stirrer (Thermo Fisher Scientific, Singapore) for 15 minutes to dissolve the components, which was then followed by centrifugation for 20 minutes at 4°C and 9,000 rpm to separate the tyrosinase enzyme.

The anti-melanogenic screening was done using the tyrosinase-based thin layer chromatography (TLC) assay similar to the method previously explained with some minor changes (Almeda et al., 2015). In this protocol, 3,4-dihydroxyphenylalanine (L-DOPA) was used as a reaction substrate by dissolving 2 mM L-DOPA in phosphate buffer (50 mM, pH 6.5) and followed by the addition of 1% Triton X-100. Tyrosinase (3 mg) was dissolved in 10 mL phosphate buffer. Kojic acid was used as a positive control in this assay by preparing 10 mg/mL kojic acid solution in phosphate buffer. The tested extract (10 mg/mL) was dissolved in the mixture of DMSO and phosphate buffer (1:9, *v/v*). Each extract solution was then spotted in the TLC plate and eluted with ethyl acetate and methanol (9:1, *v/v*) for separating its corresponding components. L-DOPA solution was sprayed onto the TLC surface followed by incubation of the plat at 25°C for 10 minutes. In addition, tyrosinase solution was also sprayed on the same TLC surface and further incubated at 30°C for 30 minutes. Eventually, the presence of brownish-purple spots on the plate indicates the tyrosinase inhibition activity of the extract. The inhibitory activity of the sample was expressed as the percentage of tyrosinase inhibition using the following Eq. (2) (Casañola-Martín et al., 2007):

$$\%\ Tyrosinase\ inhibition = \frac{A_{control} - A_{sample}}{A_{control}}\ x\ 100$$

(2) (Govindappa et al., 2016)

where, $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of the extract.

A sample with high tyrosinase inhibition activity was further analyzed to determine its IC_{50} value using colorimetry technique. The extract solution was prepared in phosphate buffer at concentrations ranging from 7 to 1000 ng/mL. The positive control solution of kojic acid was also prepared with the concentration range of 0.39 – 50 ng/mL (Hamed, El-Sharkawy, 2020). The assay was started by mixing 70 µL sample solution with 70 µL tyrosinase solution in each well of the 96-well plate. L-DOPA solution (110 µL) was subsequently added into the mixture and followed by incubation of the well plate at 37°C for 30 minutes. The absorbance intensity of the color change after the incubation due to the formation of the DOPA chrome was measured at 450 nm using a microplate reader.

2.6. Detection of kojic acid by HPLC analysis

Sample with high anti-oxidant and anti-tyrosinase activity was further assessed to measure the kojic acid content (as a positive control) by HPLC analysis. The HPLC protocol used in this study followed a previously validated method by Rovira and coworkers (Galimany-Rovira et al., 2016). Briefly, kojic acid and ascorbic acid were separately dissolved in a mixture of methanol:water (15:85, v/v). These two standard solutions and used eluent (methanol:water, 15:85, v/v) were filtered using PTFE filter with a pore size of 0.45 µm. Both sample and standard solution (10 µL) were injected into the HPLC (C18 column) under isocratic condition at a flow rate of 1.0 ml/minute. Detection was performed using a PDA detector at 260 nm. Kojic acid was quantified using calibration curve obtained from kojic acid measurement.

2.7. Fungal identification

The rDNA of the selected fungal isolate was extracted and purified using Genomic DNA extraction with Quick-DNA Magbead Plus Kit (Zymo Research, California, USA). The fungi rDNA was amplified with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Each 50 µL reaction mixture consisted of 25 MyTaq™ HS Red Mix (Meredian Bioscience, Ohio, USA), 1 µL for each primer, 1 µL DNA template, and 22 µL nuclease-free water. Amplification was performed in T100 Thermal Cycler (Bio-Rad Laboratories Inc, Foster City, USA). PCR thermal cycle using ITS primers was conducted with the following parameters: initial denaturation at 94°C for 1 minute, denaturation at 95°C for 15 seconds, annealing at 52°C for 15 seconds, extension at 72°C for 45 seconds, and final extension at 75°C for 5 minutes. The total number of cycles was 35 (Wirya et al., 2020). The expected size of the PCR products was verified on a 1% TBE agarose gel using an electrophoresis apparatus (Mupid-exU, Tokyo, Japan). The DNA of the sample that exhibited higher anti-oxidant and anti-tyrosinase activity was analyzed using 3130 AB1 Applied Biosystem sequencer.

Purified PCR products were sequenced using an automated DNA sequencer (Applied Biosystems 3130 Genetic Analyzers, California, USA). The sequence data

of ITS rDNA of the fungal strains were deposited in GenBank at NCBI. The fungal isolates were identified based on sequence homology with fungal sequences obtained from the GenBank DNA database hosted by NCBI (http://blast.ncbi.nlm.nih.gov) using the BLAST search tool. The isolates were identified based on the sequence similarity cut-off point for fungal species delimitation of at least ≥ 97%, according to Brock et al. and with an E value cut-off of 0.01 (Brock et al., 2009). The sequences of ITS rDNA of fungal isolates were aligned with other sequences retrieved from GenBank using ClustalX (Thompson et al., 1997). The fungal ITS sequences were analyzed for phylogenetic evolutionary relationships using the Molecular Evolutionary Genetic Analysis program (MEGA X). A phylogenetic tree was constructed using the neighbor-joining method with bootstrap values based on 1,000 replications (Felsenstein, 1985; Saitou, Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980).

3. Results

3.1. Isolation of endophytic fungi

As shown in Table 1, 12 endophytic fungi isolates were obtained from CA and SP plant samples cultivated in 2 different cities in Indonesia (Serpong and Bogor). In this study, 7 endophytic fungi were successfully isolated from plant stems, and 5 isolates were obtained from plant leaves. Ten fungal isolates were obtained from direct planting method, and the other two isolates were obtained from moist chamber method. For easier discussion, specific sample denotations will be used here onwards for every isolated endophytic fungus based on their place of origin, plant source as well as its corresponding isolation method, such as Spg for Serpong, Bgr for Bogor, L for leaves, S for stem, Dp for direct planting and Mc for moist chamber.

Table 1. Fungal isolates obtained from the plant samples and the corresponding information.

No.	Plant samples	Sampling location	Source of isolate and isolation method	Sample code	Biomass yield from fermentation (gram)
1.	CA	Serpong	5 isolates from leave by direct planting; 1 isolate from stem by direct planting	CA-Spg-LDp 1.2.2	5.18
				CA-Spg-LDp 2	4.34
				CA-Spg-LDp 3.1	3.61
				CA-Spg-LDp 3.2	2.19
				CA-Spg-LDp 3.3	3.77
				CA-Spg-SDp 2.1	1.86
		Bogor	4 isolates from leave by direct planting; 2 isolates from stem by direct planting	CA-Bgr-LDp 1.1	5.46
				CA-Bgr-LDp 2.1	5.88
				CA-Bgr-LDp 2.2.2	4.83
				CA-Bgr-LDp 3.1	3.68
				CA-Bgr-SDp 3.1	2.70
				CA-Bgr-SDp 3.1	0.40
2.	SP	Serpong	1 isolate from leave by direct planting; 5 isolates from stem (3 isolates by direct planting, 2 isolates by moist chamber)	SP-Spg-LDp 3.1	2.50
				SP-Spg-SDp 1.2	3.91
				SP-Spg-SDp 3.1	1.28
				SP-Spg-SDp 3.3	4.57
				SP-Spg-SMc 1.4	4.61
				SP-Spg-SMc 2.2	2.32
		Bogor	2 isolates from leave by direct planting; 4 isolates from stem (3 isolates by direct planting, 1 isolate by moist chamber)	SP-Bgr-LDp 1.1	3.59
				SP-Bgr-LDp 2.1	4.81
				SP-Bgr-SDp 1.3	1.38
				SP-Bgr-SDp 3.1	0.31
				SP-Bgr-SDp 3.2	3.18
				SP-Bgr-SMc 1.1	5.06

The morphological characteristics of each isolate, including colony color, elevation, and texture, were observed. The representative images of the isolated fungi are shown in Figure 1. Six distinct endophytic fungi from CA were identified, and Figure 1a illustrates the range of their morphological features. These features included colony color (black, grey, and brownish with white border), colony elevation (umbonate and rugose), and colony texture (powdery and velvety). On the other hand, the endophytic fungi from SP showed different morphological characteristics compared to those from CA, including colony color (brownish white, white, blackish white, and yellowish brown), colony elevation (umbonate and rugose), and colony texture (velvety and cottony) (Figure 1b).

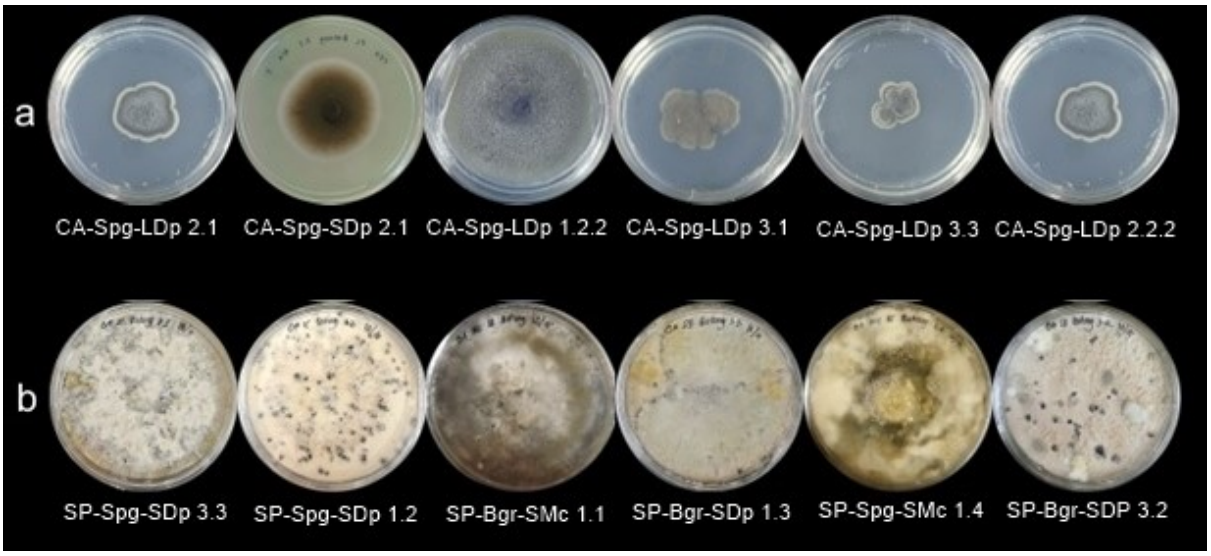

Figure 1. Colonies of endophytic fungi isolate from CA (a) on Potato Dextrose Agar Medium and SP (b) on Oatmeal Agar Medium.

3.2. Fermentation and extraction of fungal secondary metabolites

A fermentation shaker was used to ferment liquids at 110 rpm for 14 hours (28°C). Table 1 shows that fungal fermentation yield biomass range from 2.84 gr (CA-Spg-SDp 2.1) to 5.67 gr (CA-Spg-LDp 2.1).

3.3. Screening for anti-oxidant activity

The results of the screening for anti-oxidant activity are presented in Table 2. The anti-oxidant values vary widely, ranging from 29.32% (A: CA-Bgr-LDp 2.1) to 83.96% (C: CA-Spg-LDp 1.2.2). Notably, the extract with the highest inhibition value exhibits anti-oxidant activity which is comparable to that of vitamin C as a positive control (85.17%). Additionally, 70.83% of the total isolates display more than 50% DPPH radical inhibition, indicating the potential of the fungal endophytes isolated from CA and SP as anti-oxidant resources. Table 2 also reveals that most fungi obtained from CA exhibit higher anti-oxidant activity compared to those isolated from SP. To determine the concentration of extract required for 50% free radical scavenging activity, three isolates with the highest DPPH radical inhibition, isolated from the stem and leaf of CA, were tested for IC_{50} measurement. As shown in Table 3, isolate C (CA-Spg-LDp 1.2.2) exhibits an IC_{50} value of 590.19 ppm for anti-oxidant activity, whereas vitamin C has an IC_{50} value of 23.84 ppm.

Table 2. Anti-oxidant activity of the extracts.

No.	Sample code	Inhibition %
1	A (CA-Bgr-LDp 2.1)	29.32 ± 1.40
2	B (CA-Bgr-SDp 2.1)	54.55 ± 1.91
3	C (CA-Spg-LDp 1.2.2)	83.96 ± 1.08
4	D (SP-Spg-SDp 3.3)	43.94 ± 1.44
5	E (SP-Spg-SDp 1.2)	56.48 ± 0.79
6	F (SP-Bgr-SMc 1.1)	39.52 ± 4.22
7	G (SP-Bgr-SDp 1.3)	53.22 ± 5.46
8	H (CA-Spg-LDp 3.1)	73.35 ± 7.79
9	I (CA-Spg-LDp 3.3)	78.03 ± 2.60
10	J (SP-Spg-SMc 1.4)	61.82 ± 6.96
11	K (CA-Bgr-LDp 2.2.2)	79.62 ± 1.27
12	L (SP-Bgr-SDp 3.2)	60.07 ± 0.87
13	M (CA-Spg-LDp 3.2)	79.10 ± 0.76
14	N (SP-Spg-SDp 3.1)	73.58 ± 0.61
15	O (CA-Spg-SDp 2.1)	45.68 ± 8.42
16	P (SP-Bgr-SDp 3.1)	68.05 ± 1.54
17	Q (CA-Bgr-LDp 1.1)	43.01 ± 3.35
18	R (CA-Spg-LDp 2)	44.30 ± 7.09
19	S (SP-Spg-LDp 3.1)	77.81 ± 1.02
20	T (SP-Bgr-LDp 1.1)	48.10 ± 6.86
21	U (CA-Bgr-LDp 3.1)	67.36 ± 4.02
22	V (SP-Spg-SMc 2.2)	59.84 ± 4.39
23	W (CA-Bgr-SDp 3.1)	79.53 ± 0.56
24	X (SP-Bgr-LDp 2.1)	68.65± 6.28
25	Vitamin C	85.17 ± 3.80

Results are expressed as the mean ± RSD (n = 3). Vitamin C was used as positive control. The IC_{50} of the selected samples (no. 3, 11, and 23) were further analyzed.

Table 3. The IC_{50} value of the antioxidant capacity from the three selected extracts.

No.	Sample code	IC_{50} value (ppm)
1	C (CA-Spg-LDp 1.2.2)	590.19 ± 1.91
2	K (CA-Spg-LDp 2.2.2)	2943.00 ± 5.20
3	W (CA-Spg-SDp 3.1)	2143.18 ± 8.16
4	Vitamin C	23.84 ± 47.34

Results are expressed as the mean ± RSD (n = 3). Vitamin C was used as positive control.

3.4. Screening for anti-tyrosinase activity

The screening result of anti-tyrosinase activity by TLC method is shown in Figure 2. The positive result was indicated by the presence of a white zone on the black background of the TLC plate. It can be seen from the TLC result that there are two samples of C (CA-Spg-LDp 1.2.2) and R (CA-Spg-LDp 2) that actively inhibit tyrosinase activity. The active compound in sample C appears at a retention time of 5.5, while sample R appears at a retention time of 6.5. Kojic acid as a positive control in this test also appears at a retention time of 5.5. Therefore, the active compound in sample C could likely be kojic acid. Both samples C and R were then further analyzed to determine their IC_{50} values, which were 341.02 and 570.40 ppm, respectively (Table 4). This result suggests that both samples may be exploited as whitening agents.

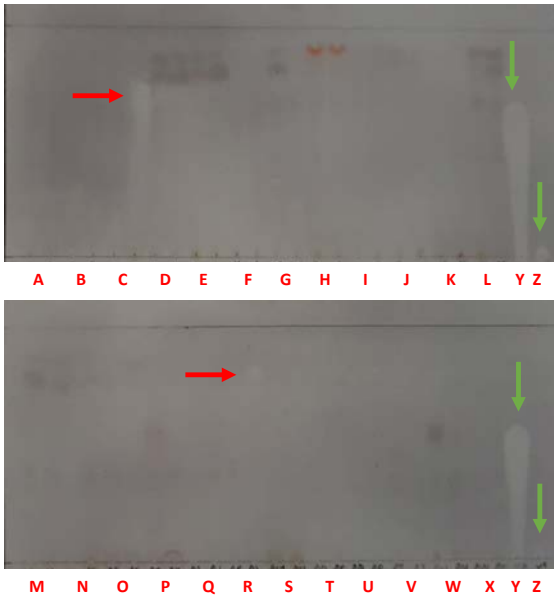

Figure 2. Tyrosinase-based TLC bioautography of the extracts. A (CA-Spg-LDp 2.1); B (CA-Spg-SDp 2.1); C (CA-Spg-LDp 1.2.2); D (SP-SPg-SDp 3.3); E (SP-Spg-SDp 1.2); F (SP-Bgr-SMc 1.1); G (SP-Bgr-SDp 1.3); H (CA-Spg-LDp 3.1); I (CA-Spg-LDp 3.3); J (SP-Spg-SMc 1.4); K (CA-Spg-LDp 2.2.2); L (SP-Bgr-SDp 3.2); M (CA-Bgr-LDp 3.2); N (SP-Spg-SDp 3.1); O (CA-Spd-SDp 2.1); P (SP-Bgr-SDp 3.1); Q (CA-Spg-LDp 1.1); R (CA-Spg-LDp 2); S (SP-Spg-LDp 3.1); T (SP-Bgr-LDp 1.1); U (CA-Spg-LDp 3.1); V (SP-Spg-SMc 2.2); W (CA-Spg-SDp 3.1); X (SP-Bgr-LDp 2.1); Y (Kojic acid); Z (Vitamin C). The red arrow indicated the expected samples with tyrosinase inhibition activity and the green arrow indicated positive control.

Table 4. The IC_{50} value of the tyrosinase inhibition activity from the selected extracts.

No.	Sample code	IC_{50} value (ppm)
1	C (CA-Spg-LDp 1.2.2)	341.02
2	R (CA-Spg-LDp 2)	570.40
3	Kojic acid	5.36

Results are expressed as the mean (n = 3). Kojic acid was used as positive control.

3.5. Detection of kojic acid by HPLC analysis

Detection of kojic acid by HPLC analysis involves extracting kojic acid from the sample exhibiting high antioxidant and anti-tyrosinase activity using an appropriate solvent. Before injecting the sample, a series of standard solutions of kojic acid and ascorbic acid at known concentrations in the mobile phase is prepared. The HPLC analysis generates retention time data for each analyte; for example, ascorbic acid and kojic acid were detected at 1.53 and 2.19 minutes, respectively. The obtained R^2 values for the standard curves are 0.9779 for ascorbic acid and 0.9945 for kojic acid (data are not shown), indicating a good linear regression. Importantly, the chromatogram of sample C (CA-Spg-LDp 1.2.2) in Figure 3 shows a significant peak at approximately 2.2 minutes, which closely resembles the kojic acid peak in the

standard solution. This data verifies the presence of kojic acid in the sample with a concentration of 9.61 ppm.

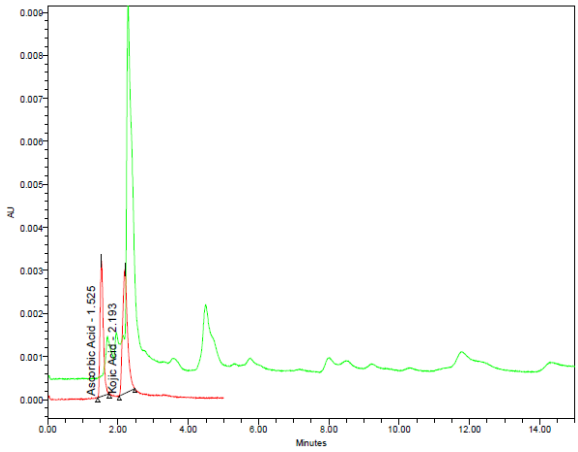

Figure 3. HPLC chromatogram of the sample C (CA-Spg-LDp 1.2.2) (green line) and positive controls (red line) of ascorbic (retention time of 1.53 minutes) acid and kojic acid (retention time of 2.19 minutes).

3.6. Fungal identification

The DNA isolated from selected fungal isolates was amplified using Internal Transcribed Spacer (ITS) primers. Figure 4 depicts the PCR results of the ITS region, indicating successful amplification of DNA samples by the appearance of DNA bands with expected gene sizes of approximately 550 bp.

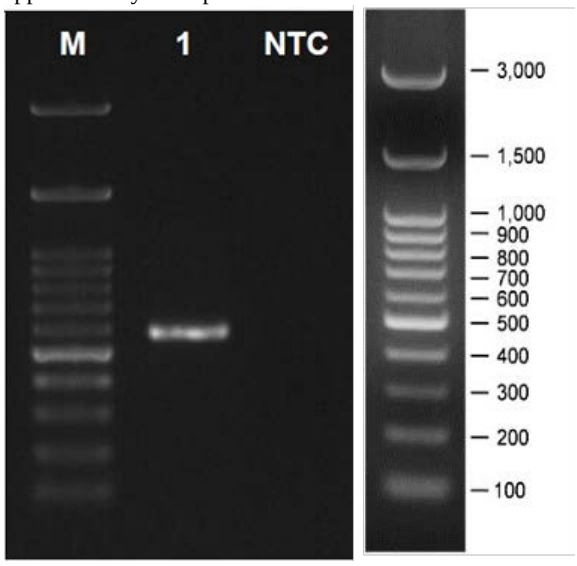

Figure 4. Electropherograms of the amplified ITS sequence of CA-Spg-LDp 1.2.2. Isolates assessed by electrophoresis with 1 % TBE agarose

Table 5. The BLAST search result of CA-Spg-LDp 1.2.2 isolates from NCBI Database.

No.	Species	Query cover	Percent identification (%)	Length (bp)	Accession number
1	*C. sojae* Strain ATCC 62257	98%	99.45	548	KC110794.1
2	*C. plurivorum* CBS 125474	96%	99.81	539	NR_160828.1
3	*C. syngoniicola* strain LC8894	100%	98.75	567	MZ595863.1
4	*C. cliviicola* CBS 125375	96%	99.63	539	NR_137097.1
5	*C. cattleyicola* CBS 170.49	96%	99.44	539	NR_160832.1
6	*C. sojae* ATCC 62257	96%	99.44	539	NR_158358.1
7	*C. musicola* CBS 132885	96%	98.89	539	NR_160830.1
8	*C. brevisporum* BCC 38876	100%	97.85	578	NR_111637.1
9	*C. brevisporum* strain LC0600	99%	97.85	592	KC790943.1
10	*C. orchidearum* CBS 135131	96%	98.70	538	NR_160831.1

The amplified sequence of CA-Spg-LDp 1.2.2 isolates was submitted to Genebank at NCBI under the accession number of PP345512.1, and BLAST analysis was performed to compare the sequence to numerous references in the NCBI database. The result showed higher homology with Colletotrichum sojae strain ATCC 62257 with a percent identification of 99.45% and gene length of 548 bp (Table 5). This finding is consistent with the electropherogram of the CR product, which indicates that the sequence is approximately 550 bp in length.

Following the analysis of the BLAST results, a phylogenetic tree was constructed to compare the isolate sequences with the reference sequences retrieved from the NCBI database. The phylogenetic analysis involved comparing one isolate from this study with ten Colletotrichum reference isolates from the Gene Bank and two fungal strains used as an outgroup. The phylogenetic tree (Figure 5) indicated that CA-Spg-LDp 1.2.2 belongs to the Colletotrichum genus and is closely related to Colletotrichum sojae strain ATCC 62257, with a bootstrap value of 73%. A bootstrap proportion of >70% often indicates a likelihood of >95%, highlighting that the clade is real.

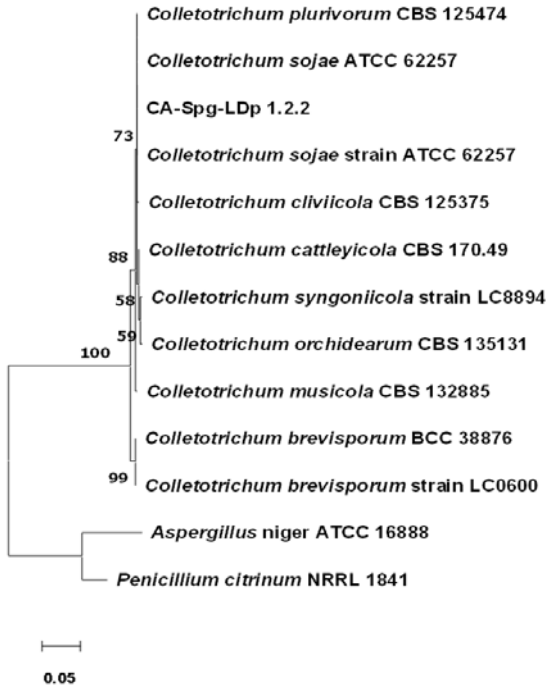

Figure 5. Phylogenetic tree of CA-Spg-LDp 1.2.2 isolates based on ITS.

4. Discussion

In this study, the number of fungi isolates obtained from the direct planting method was higher than those of direct moist chamber method. This result indicated that direct planting is more suitable for obtaining endophytic fungi from plant samples. This data is indeed consistent with the previous research groups that used direct planting techniques to isolate 85 and 18 endophytic fungi from CA and SP plants, respectively (Radiastuti et al., 2019; Widjajanti et al., 2023). The obtained endophytic fungi from CA are similar to those of other studies, displaying a variety of morphological characteristics, including colony color (ranging from black, white, brownish white, yellow, and yellowish white) and colony texture (either cottony or velvety) (Radiastuti et al., 2019). Similar morphological traits of fungi from the SP's stem are also shown by the endophytic fungi from SP, demonstrating similarity in surface colony color (yellowish brown, white yellow, white, and pale brown), colony elevation (umbonate and rugose), and colony texture (powdery and cottony) (Widjajanti et al., 2023). The distribution of the obtained fungi was influenced by the exact location of plant tissues. Most fungi were found in leaves of CA, likely due to the proximity of the leaves to the ground and soil, which supports the penetration and colonization of endophytes. Conversely, in SP, most fungi were obtained from stems. This distribution may be attributed to the varying abilities of each endophyte species to utilize substrates or tissues to acquire resources from different parts of the plant.

Based on the previous report, the fermentation method adopted in this study was used for effectively extracting secondary metabolites with antioxidants and anti-melanogenic activity (Kim et al., 2021). According to Boukaew and coworkers, the preferred medium for biomass production is malt extract medium, particularly malt extract broth (MEB) which contains carbon and nitrogen sources, minimal salts, trace elements, and vitamins, with a suitable pH (Boukaew, Prasertsan, 2014). The obtained fungal biomass correlates with the mycelial morphology, the fermentation substrate, and the species of the fungal itself, while the type of fermentation might affect fungal morphology. For instance, in submerged fermentation, filamentous fungi may exhibit freely dispersed hyphae or form spherical agglomerates of hyphae known as pellets (Quintanilla et al., 2015).

The mechanism of DPPH antioxidant screening is linked to the ability of anti-oxidants to transfer hydrogen. The odd electrons from the nitrogen atom on DPPH are reduced by accepting hydrogen atoms from anti-oxidant agents, forming 2,2-diphenyl-1-picrylhydrazine (Kedare, Singh, 2011; Liu et al., 2015). The disappearance of the deep purple color indicates the presence of anti-oxidants, as they dampen DPPH free radicals (Nath et al., 2014). Furthermore, the anti-oxidant activity is affected by methods, polarity of the solvent, extraction procedure, and purity of extracted compounds (Waisundara, Watawana, 2014). The IC_{50} value of CA-Spg-LDp 1.2.2 is quite similar to the IC_{50} value of those obtained from the extract of CA plant as a host (Waisundara, Watawana, 2014). It indicates that this isolated endophytic fungal strain could be a potential agent in scavenging free radicals (Wang et al., 2023). The anti-oxidant activity of CA endophytic fungi is attributed to their secondary metabolites, such as flavonoids, tannins, coumarins, alkaloids, and steroids (Bhavana et al., 2020). These secondary metabolites donate electrons due to the presence of a chemical structure (β-ring conjugated) with several hydroxyl groups, facilitating the hydrogenation mechanism and reacting with oxidative free radicals. Consequently, these secondary metabolites are exploited by the host plant of CA to prevent oxidative stress and to protect biochemical functions from natural oxidants (Choudhary et al., 2011).

The anti-tyrosinase activity screening finding is consistent with other study that optimized the extraction of kojic acid from fermentation of different species of fungi isolated from California, including *Penicillium* and *Aspergillus* (Couteau, Coiffard, 2016). Previous studies have shown that CA contains some significant components, including asiatic acid, asiaticoside, madecassic acid, and madecassoside which belong to the pentacyclic triterpenoids known as centelloids (Bylka et al., 2014; Kwon et al., 2014; Kwon et al., 2012). These substances have been recognized as effective skin moisturizers, antioxidants, whitening, agent, wound healers, anti-inflammation, anti-psoriatic, and anti-aging. Previous studies revealed that the water and ethanol (95%) extract of CA displayed tyrosinase inhibitory activity with inhibition values of 53.22% and 31.25%, respectively (Seo, Kim, 2019; Sungthong, Phadungkit, 2015). The active compound of asiaticoside in CA may be responsible in lowering the amount of melanin during the melanocyte formation by blocking the expression of tyrosinase mRNA (Kwon et al., 2014).

The fungal identification result is consistent with prior publications that successfully amplified the ITS region using primers ITS1 and ITS4, with an expected size of 542 bp (Wirya et al., 2020). Several researches showed endophytic fungi from *Colletotrichum* group produced bioactive compounds with anti-microbial, anti-oxidant (Gurgel et al., 2023), antidepressant, anti-inflammatory,

antioxidative, and anticancer activities (Chithra et al., 2014). In addition to some of the activities mentioned previously, *Colletotrichum* sp. AP12 has been reported to promote growth andrographolide biosynthesis of medicinal plant *Andrographis paniculata* (Burm. f.) Nees (Xu et al., 2023). Another species of *Colletotrichum gloeosporioides* isolated from *Sonneratia apetala* can produced secondary metabolites such as kojic acid which exhibited anti-microbial properties (Nurunnabi et al., 2018) and also anti-melanogenic activity (Happi et al., 2015).

5. Conclusion

We successfully isolated four endophytic fungi from CA and SP with high anti-oxidant and anti-tyrosinase activity from this result. Furthermore, one isolated with high anti-oxidant and anti-tyrosinase activity was molecularly identified using ITS rDNA, and the result showed that the selected isolate belonged to genus *Colletotrichum* and species *Colletotrichum sojae*.

6. Conflicting interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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