

Antibacterial and antioxidant activity of Endophytic Fungal Extract Isolated from the Root of *Sonneratia caseolaris*

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Abstract

Sonneratia caseolaris is a mangrove species of considerable pharmacological importance; however, its natural populations are increasingly threatened by habitat degradation and overexploitation. Endophytic biotechnology provides a sustainable alternative for exploring the bioactive potential of this plant without imposing additional pressure on its fragile ecosystem. This study aimed to evaluate the antibacterial and antioxidant activities of endophytic fungal extracts isolated from the roots of *S. caseolaris*. Morphological and molecular techniques were employed for fungal identification, while antibacterial activity was assessed using the disc diffusion assay, and antioxidant capacity was evaluated through the DPPH radical scavenging assay. The selected fungal isolate, RSC2, exhibited strong antibacterial and antioxidant activities. Thin Layer Chromatography (TLC) analysis confirmed the presence of flavonoid compounds, and molecular identification verified the isolate as *Aspergillus niger*. This study reports, for the first time, the isolation of *A. niger* from *S. caseolaris* roots with the ability to produce flavonoid metabolites exhibiting notable bioactive potential. These findings provide a scientific basis for future in vivo investigations aimed at developing natural compounds from mangrove-associated endophytes as promising pharmaceutical candidates.

Keywords: Antibacterial, Antioxidant, Endophytic fungi, *Sonneratia caseolaris*

Introduction

Sonneratia caseolaris is a mangrove species that grows naturally along tropical and subtropical coastlines (Audah, 2024; Pratiwi et al., 2024). Conserving mangrove biodiversity, particularly *S. caseolaris*, is strategically important for both ecological stability and human well-being (Arifanti et al., 2022; Pratiwi et al., 2024). Ecologically, *S. caseolaris* provides habitat for numerous marine organisms and serves as a natural barrier against coastal erosion. Beyond its environmental significance, this plant also shows considerable potential as a traditional medicinal resource. Its roots, leaves, and bark are known to contain a wide range of bioactive compounds, including alkaloids, flavonoids, and terpenoids, which exhibit anti-inflammatory, antimicrobial, and antioxidant properties (Kartikaningsih et al., 2025; Melki et al., 2009). Several studies have further reported the therapeutic use of *S. caseolaris* extracts for treating microbial infections and digestive disorders (Kalor et al., 2025).

However, habitat loss, climate change, and overexploitation pose increasing threats to the survival of this species (Alongi, 2015; Ward et al., 2016). Protecting and preserving *S. caseolaris* is

therefore essential to prevent local extinction and maintain ecosystem resilience. To utilize its therapeutic potential in an environmentally sustainable manner, alternative strategies that reduce the need for direct harvesting are required (Chen et al., 2016). One promising approach involves the use of endophytic fungi, symbiotic microorganisms that inhabit plant tissues without causing disease symptoms. These endophytes are capable of producing diverse secondary metabolites with biological activities such as antioxidant and antibacterial effects (Koche et al., 2016; Yu et al., 2025). Endophyte-derived metabolites not only represent valuable sources of novel pharmaceutical compounds but also contribute to mangrove conservation by reducing destructive collection of plant materials (Nasution et al., 2024; Noviyanto et al., 2025).

Although the bioactive potential of *S. caseolaris* endophytic fungi has been reported in previous studies, the diversity of root-associated isolates and their specific biological functions remain poorly understood. Therefore, this study aims to isolate, morphologically and molecularly characterize, and evaluate the bioactivity of endophytic fungi derived from the roots of *S. caseolaris*. This research seeks

to explore the secondary metabolites produced by these fungi as potential sources of new bioactive compounds, while simultaneously supporting mangrove biodiversity conservation.

Materials and Methods

Sampling and Isolation of Endophytic Fungi

Fresh and healthy roots of *S. caseolaris* were collected from Pulau Payung, Sungsang IV, Banyuasin II Subdistrict, Banyuasin Regency, South Sumatra, Indonesia (30971). The samples were washed under running tap water to remove surface debris. Endophytic fungi were isolated from the root segments following surface sterilization with 70% ethanol for 1 minute and sodium hypochlorite solution (1%) for 1 minute, followed by rinsing three times with sterile distilled water. The sterilized roots were cut into approximately 0.5 cm segments and placed on potato dextrose agar (PDA) plates. The plates were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for 3–7 days. Hyphal tips emerging from the plant tissues were sub-cultured onto fresh PDA to obtain pure isolates (Budiono et al., 2019; Oktiansyah et al., 2023; Permatasari et al., 2023).

Morphological Characterization and Identification

Morphological identification of the fungal isolates was conducted through both macroscopic and microscopic observation. Macroscopic features such as colony color, texture (cottony, granular, or mucoid), and radial or concentric growth patterns on PDA were documented. Microscopic characteristics, including hyphal and spore morphology, were examined under $1000\times$ magnification using the slide culture method. Species-level identification was carried out by comparing morphological data with standard taxonomic keys and literature (Watanabe, 2010). Isolates showing notable bioactivity were selected for molecular identification.

Cultivation and Extraction of Endophytic Fungal Metabolites

Pure isolates were first grown on PDA plates for 5–7 days and then transferred into 250 mL Erlenmeyer flasks containing potato dextrose broth (PDB). Cultures were incubated statically for 30 days at room temperature to allow the production of secondary metabolites. After incubation, the cultures were filtered, and the filtrates were extracted three times with equal volumes of ethyl acetate. The combined organic phase was

concentrated using a rotary evaporator to obtain crude extracts (Fadhillah et al., 2019; Habisukan et al., 2024).

Antioxidant Activity Assay (DPPH Method)

The antioxidant potential of the fungal extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay following Molyneux (2003). Extracts at concentrations of 1000, 500, 250, 125, 62.5, 31.25, and 15.625 $\mu\text{g/mL}$ were each mixed with 3.8 mL of 0.5 mM DPPH solution in methanol and incubated in the dark for 30 minutes. Absorbance was measured at 517 nm using a UV–Vis spectrophotometer. The percentage of free radical scavenging activity was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{A_k - A_s}{A_s}$$

Where A_k is the absorbance of the control and A_s is the absorbance of the sample.

The IC_{50} value (concentration required to scavenge 50% of DPPH radicals) was determined from the linear regression equation of inhibition percentage (Y) versus extract concentration (X).

Antibacterial Activity Assay (Disc Diffusion Method)

Antibacterial activity was evaluated using the disc diffusion method. Sterile paper discs (6 mm diameter) impregnated with 400 $\mu\text{g/mL}$ of fungal extract were placed on Mueller–Hinton agar (MHA) plates inoculated with bacterial cultures: *Salmonella typhi* (IPCCCB.11.669), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Bacillus subtilis* (ATCC 6633). Plates were incubated at 37°C for 24–48 hours. Antibacterial activity was determined by measuring the diameter of the inhibition zones around the discs. Tetracycline (30 $\mu\text{g/disc}$) served as the positive control, and DMSO was used as the negative control (Aini et al., 2022; Hapida, 2021; Syarifah et al., 2021). The antibacterial activity index (AI) was calculated according to Mossie (2024) and Rathnayake et al. (2020).

$$\text{AI} = \frac{\text{Inhibition Zone diameter of the sample}}{\text{Inhibition Zone diameter of the standard}}$$

Thin Layer Chromatography (TLC) Analysis

Crude ethyl acetate extracts were dissolved in the same solvent and spotted onto silica gel TLC plates using a capillary tube. A solvent system of ethyl acetate: n-hexane (1:1) was used for elution. The plates were allowed to air-dry and examined under UV light at 254 nm and 365 nm. Subsequently, the

plates were heated at 100°C to visualize color spots indicative of secondary metabolites. Plates were sprayed with 20% H₂SO₄ in methanol to enhance spot visibility. The retention factor (R_f) values and spot characteristics were recorded as indicators of compound diversity (Aisyiyah et al., 2023; Nasution et al., 2024).

Molecular Identification of Endophytic Fungi

Genomic DNA was extracted using a commercial DNA extraction kit or the cetyltrimethylammonium bromide (CTAB) method. The internal transcribed spacer (*ITS*) region of ribosomal DNA was amplified by PCR using universal primers *ITS1* and *ITS4*. The amplified products were purified and

sequenced, and the resulting sequences were analyzed using BLAST and MEGA software to identify the isolates and determine their phylogenetic relationships (Budiono et al., 2019).

Results and Discussion

Isolation and Morphological Identification

A single endophytic fungal isolate, designated RSC2, was successfully obtained from the root segments of *S. caseolaris*. Morphological identification was performed based on both macroscopic and microscopic observations (Figure 1 and Table 1).



Figure 1. Macroscopic and Microscopic Characteristics of RSC2 isolate grown on Potatoes Dextrose Agar (PDA) after 3 days. a: front view, b: reverse view, c: hyphae, d: vesicle, e: conidiophore, f-g: conidia

Macroscopically, the fungal colony exhibited a black surface with a white to cream reverse, irregular margins, and a slightly uneven texture. The colony displayed concentric growth zones and radial lines, indicative of active mycelial expansion. Microscopically, the isolate consisted of transparent, septate hyphae with small spherical spores arranged

in chains. The presence of upright conidiophores bearing spherical vesicles covered with phialides that produced black conidia is characteristic of the genus *Aspergillus*. Based on these morphological traits, the RSC2 isolate was provisionally identified as an *Aspergillus species*.

Table 1. Macroscopic and Microscopic Characteristics of RSC2

Observation	Parameter	Description
Macroscopic	Surface colony	Black in color (a).
	Reverse colony	White to pale cream in color (b).
	Structure	Dense colony with irregular margins.
	Elevation	Raised with an uneven surface.
	Pattern	Shows concentric rings and radial growth.
	Exudate drops	No distinct exudate droplets were observed on the colony surface.
	Radial line	Fine radial lines are visible in the colony growth.
	Concentric line	Concentric rings were observed due to different growth zones.
Microscopic	Shape	Small, spherical (g), consisting of conidia arranged in chains.
	Hyphae	Hyaline (transparent) hyphae, septate (c).
	Spora	Conidia (g) borne on conidiophores.
	Distinctive characteristics	Erect conidiophores (e) ending in a round vesicle (d) covered with phialides forming black conidia (f).

Antibacterial and Antioxidant Activities

The bioactivity assays for the RSC2 extract are summarized in Table 2. The ethyl acetate extract exhibited strong antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Bacillus subtilis*, as indicated by prominent inhibition zones.

Antioxidant potential, evaluated using the DPPH radical scavenging assay, yielded low IC₅₀ values, confirming a strong free-radical scavenging capacity comparable to that of ascorbic acid. These results suggest that the endophytic fungus associated with *S. caseolaris* possesses substantial antioxidant and antibacterial properties, aligning with previous reports of *Aspergillus*-derived metabolites exhibiting multifunctional bioactivities.

Table 2. Antioxidant and Antibacterial Activity of RSC2

Sample	IC ₅₀ (μg/mL)	(mm) Antibacterial Activity			
		<i>E. coli</i>	<i>S. typhi</i>	<i>S. aureus</i>	<i>B. subtilis</i>
RSC2	21.437***	18.70 ± 0.25	18.28 ± 0.50	21.52 ± 0.04	82.1 ± 0.41***
Positive control	Ascorbic acid 10.083****	Tetracycline 24.9	Tetracycline 24.5	Tetracycline 26.8	Tetracycline 28.2

Note: Antioxidant activities IC₅₀ (μg/mL): ****very strong <20 μg/mL; ***strong <100 μg/mL; **moderate 100–500 μg (Budiono et al., 2019).

Classification of bacterial growth inhibition zone responses based on the diameter of the clear zone includes weak responses (diameter 5 mm), moderate responses (diameter 5-10 mm), strong responses (diameter 10-20 mm), and very strong responses (diameter > 20 mm) (Putri et al., 2023).

TLC and Phytochemical Characterization

Thin-layer chromatography (TLC) of the RSC2 extract (Figure 2 and Table 3) revealed two major spots with R_f values of 0.21 and 0.28 after spraying with 20% H₂SO₄. Under UV light (254 nm), both spots exhibited quenching, and upon heating, they turned brown, features consistent with conjugated aromatic compounds such as flavonoids. The low R_f values further indicate polar characteristics, supporting their classification as flavonoid-type compounds.

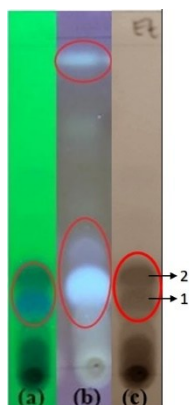


Figure 2. Chromatogram profiles of the endophytic fungus extract of RSC2. (a) UV light at λ 254 nm; (b) UV light at λ 366 nm. (b); (c) after spraying with 20% H₂SO₄ (c).

The presence of flavonoids strongly correlates with the extract's observed bioactivity since these metabolites are well-documented for their potent antioxidant and antimicrobial properties. Thus, the

TLC results confirm the involvement of flavonoid metabolites in the biological effects of the RSC2 extract.

Table 3. Determination of Secondary Metabolite

Sample	Number of spots	R _f Value (cm)	Color after H ₂ SO ₄ 20%	Secondary Metabolite
Extract of RSC2	1	0.21	Brown	Flavonoid
	2	0.28	Brown	Flavonoid

Molecular Identification and Bioactivity Implications

Molecular analysis based on *ITS* region sequencing confirmed the RSC2 isolate as *Aspergillus niger* (Figure 3). *A. niger* is a widely distributed opportunistic species capable of surviving in diverse environments (Mousavi et al., 2016; Yu et al., 2021). Although this species can act as an opportunistic pathogen, its adaptability, rapid spore dispersal, and frequent plant associations suggest that it can also function as an endophyte (Dias et al., 2021; Gurgel et al., 2023). Biosafety considerations are essential when exploring its pharmaceutical applications; however, endophytic strains of *A. niger* have repeatedly demonstrated the ability to produce bioactive secondary metabolites similar to those found in their host plants (Khan et al., 2020; Yang et al., 2023; Muteeb et al., 2023).

In this study, the ethyl acetate extract of *A. niger* exhibited strong antioxidant and antibacterial

activities, with an IC_{50} value comparable to ascorbic acid and inhibition against all tested bacterial strains. This biological potency can be attributed to the presence of flavonoids and other secondary metabolites, as confirmed by TLC analysis. The fungus's extensive biosynthetic gene clusters (BGCs) enable the production of diverse metabolites such as amides, cyclopeptides, polyketides (e.g., aspergillone), flavonoids, alkaloids (e.g., purolles, pyridones), and sterols (He et al., 2022; Yu et al., 2021). Among these, flavonoids are particularly notable for their broad-spectrum antioxidant and antimicrobial properties (Alam et al., 2021; El-Zahar et al., 2022; Ndezo Bisso et al., 2022).

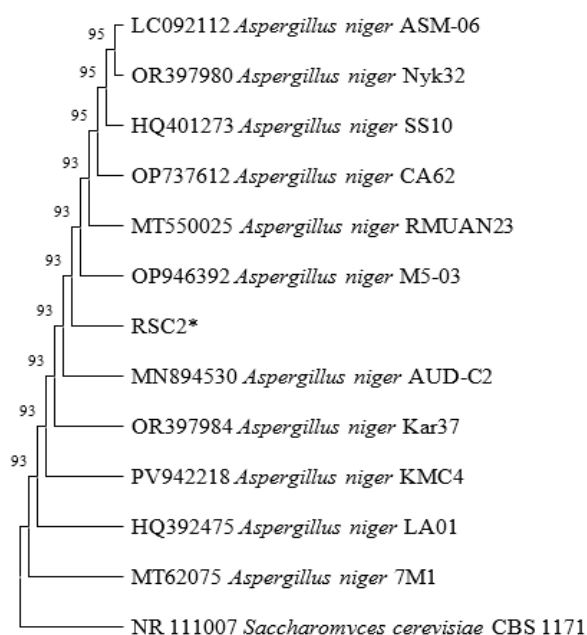


Figure 3. Phylogenetic tree of the RSC2 isolate (marked with an asterisk) constructed using the Neighbor-Joining method (bootstrap value = 1000)

The use of ethyl acetate, a semi-polar solvent, proved effective in recovering a wide range of metabolites due to its ability to dissolve both polar and nonpolar compounds. Overall, these findings highlight the remarkable potential of the *A. niger* endophyte from *S. caseolaris* as a natural source of pharmacologically active compounds.

Future work should focus on compound purification using LC-MS/MS or NMR spectroscopy and in vivo validation to assess pharmacological efficacy and safety in model organisms. This study thus provides a valuable foundation for future bioprospecting and sustainable drug discovery from mangrove-associated endophytic fungi.

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