

Isolation of endophytic fungi with antimicrobial activity from medicinal plant *Rhodomyrtus tomentosa* (Aiton) Hassk

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ABSTRACT

Medicinal plants are an abundant source of natural substances used to treat various diseases; thus, they serve as the foundation for new drug discovery. The interaction between endophytic microorganisms and host plants has been extensively studied because they can produce a wide range of secondary metabolites, including those with significant biological functions from their hosts. This study aimed to isolate the endophytic fungi from the roots, barks, and leaves of the rose myrtle (*Rhodomyrtus tomentosa* (Ait) Hassk) plant. Endophytic fungi were isolated by direct planting and pour methods. From this plant, 13 endophytic fungi were obtained. Each fungus was grown for 3–4 weeks on rice media. Ethyl acetate was used to extract secondary metabolites from the cultivation results. The antimicrobial activity screening of the extracts was done using the disk diffusion method against microbial pathogens *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. The results showed that three fungus extracts, RTKB3, RTKB4, and RTKB7, had antimicrobial activity against *S. aureus* and *E. coli*. However, the extracts did not show inhibition toward the growth of *C. albicans*. These fungal isolates were identified molecularly as *Aspergillus heteromorphus*, *Aspergillus niger*, and *Paecilomyces subglobosus*. In conclusion, the endophytic fungi from *R. tomentosa* (Aiton) Hassk were potential producers of antibacterial compounds that need to be investigated further.

INTRODUCTION

Medicinal plants have efficacy as medicine in healing and preventing disease. One of the plants used by the people of West Sumatra, Indonesia, as medicine, is myrtle (*Rhodomyrtus tomentosa*). Traditionally, this plant has been used as a medicine to treat wounds, scabies, headaches, stomachaches, and diarrhea, preventing bleeding and infection after childbirth (WHO, 1989). Based on previous research, the *R. tomentosa* extract has a potent growth-inhibiting effect against pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Shigella dysenteriae*, and *Salmonella typhi* (Dachryanus *et al.*, 2002; Salni *et al.*, 2002; Salni and Marisa, 2019; Sinulingga *et al.*, 2018).

Some microorganisms, such as bacteria and fungi, live as endophytes and inhabit the interior of plants. According to Petrini (1991), they colonize healthy tissue at some point in their life cycle without causing significant damage. Endophytic fungi that inhabit healthy living tissue without causing side effects also produce certain biological activities of secondary metabolites structurally unique (Strobel, 2003). To colonize the host and nutrition, it can synthesize metabolites for competence with co-occurring endophytes, hosts, and pathogens (Strobel and Daisy, 2003). There are approximately 1.5 million species of fungi on Earth, 1 million of which are endophytic. They primarily inhabit the interiors of plants' stems, leaves, flowers, and seeds, which can be transferred in horizontal or vertical directions (Bacon and White, 2000; Hartley and Gange, 2009). They produce potent antifungal, antiviral, antibacterial, cytotoxic, and immunosuppressive metabolisms (Ruma *et al.*, 2013).

This research continues our investigation of natural products from several mangrove plants and marine sponges (Artasasta *et al.*, 2017; Aminah *et al.*, 2020; Handayani *et al.*,

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2016, 2018, 2019, 2020a, 2021; Handayani and Aminah, 2017; Handayani and Artasasta, 2017; Sandrawati *et al.*, 2020). From the marine sponge-derived fungus *Cochliobolus geniculatus* WR12, radicinin was obtained. This compound shows cytotoxic activity with IC_{50} values of 60.68; 30.89; and 87.89 $\mu\text{g/ml}$ against WiDr, T47D, and Hela cells but is not toxic to Vero cells (IC_{50} value of 607.31 $\mu\text{g/ml}$). Radicinin showed higher cytotoxic activity against T47D cell cultures (IC_{50} = 25.01 ppm) than doxorubicin (IC_{50} = 33.49 ppm). This compound showed a minimum inhibitory concentration (MIC) of 125 $\mu\text{g/disc}$ against methicillin-resistant *S. aureus* (Handayani *et al.*, 2020b). From fungus *Fusarium proliferatum* AED3, derived from the mangrove plant *Ardisia elliptica*, beauvericin was obtained. The cytotoxic activity of the compounds was tested using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide test on T47D cancer cells with an IC_{50} value of 112.2 $\mu\text{g/ml}$ (Handayani *et al.*, 2021).

In this study, we isolated endophytic fungi from the medicinal plant *R. tomentosa* and evaluated their potential for antimicrobial activity. Based on literature studies, research related to the endophytic fungi of *R. tomentosa* or the antimicrobial potential of this microorganism is still very limited.

MATERIALS AND METHODS

Identification of sample material

The material used was ± 100 g of leaves, bark, and roots of *R. tomentosa* obtained from the Bukik Nago Sungkai, Lambung Bukit Village, Padang, West Sumatra, Indonesia, (Geographic coordinates: $^{\circ}56'57.3''$ S $100^{\circ}21'15.4''$ E). Plant identification was carried out at the Andalas University Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Padang, West Sumatra, Indonesia. The letter number for plant authentication was 119/K-ID/ANDA/III/2021, and the voucher number was HF0012021.

Isolation of endophytic fungi from leaves, bark, and roots of *R. tomentosa*

The surfaces of the leaves, bark, and roots were surface-sterilized by rinsing under running water for 10 minutes, followed dipped in 70% ethanol for 1–2 minutes. The samples were then rinsed again with ethanol 70% for 30 seconds after being soaked in bleach solvent (NaOCl 5.3%) for 5 minutes. To stop the sterilization with EtOH, dry the tissue with a sterile cotton cloth or rinse it with sterile artificial seawater (Kjer *et al.*, 2010).

Isolation by direct planting

Cleavage surface attached to the agar medium. Incubation was carried out at 25°C (room temperature) for 3 days. The agar dish was labeled, parafilm-sealed, and stored at 20°C–25°C. Cultures are maintained at 20°C–25°C under daylight. 2–3 days after the experiment's start, fungal growth from the cut segments starts and lasts until 14 days later (Kjer *et al.*, 2010).

Isolation by pouring method

The sample was dispersed in aqua dest sterile for about 10 g until the volume on Erlenmeyer reached 100 ml. The mixture was then diluted until it contained 10^{-6} ml. One milliliter of the sample was poured aseptically onto sabouraud dextrose agar

(SDA) medium in the Petri dish. The plate was incubated at 25°C–27°C for 5–7 days (Kjer *et al.*, 2010).

Pure fungi isolate cultivation in rice medium

Pure Fungi isolate in a Petri dish was sliced 1×1 cm, then cultured in rice medium and incubated for 4–6 weeks at room temperature. Maximum growth of the fungi occurs when the rice is completely covered by the fungi isolate (Kjer *et al.*, 2010).

Extraction of the secondary metabolites

Pure fungus isolates that have been further cultivated were extracted by maceration with ethyl acetate (EtOAc) at a ratio of 1:1 for 24 hours and evaporated using a rotary evaporator to produce fungus extract. The extract is used for antimicrobial screening (Kjer *et al.*, 2010).

Screening for antimicrobial activity

The disk diffusion method was used to screen the EtOAc extracts of endophytic fungi for antimicrobial activity against several pathogenic microbes: *S. aureus* the American Type Culture Collection (ATCC) 25923, *E. coli* ATCC25922, and clinical isolate *Candida albicans*. Nutrient Agar (Merck®) and SDA (Merck®) containing a microbial suspension were added to Petri dishes (0.5 and 0.7 McFarland for bacteria and fungus, respectively). Positive controls against bacterial and fungal pathogens were performed using chloramphenicol (Oxoid®) and nystatin disk. A paper disk was used as a negative control containing 20 μl of dimethyl sulfoxide (DMSO). The plate of fungi was incubated at 25°C–27°C for 48 hours and 37°C for 24 hours for bacteria. The extract was diluted to a concentration of 5% with DMSO. The clear zone on the disk indicates the fungal extract's bacterial and fungal inhibition activity (Bauer *et al.*, 1959). Testing for antimicrobial activity was done in triplicates. For each fungus extract with an inhibition zone larger than 10 mm, the mean diameter of the inhibition zone and the standard deviation were computed.

Determination of the secondary metabolite content

EtOAc extracts of endophytic fungi with an inhibition zone >10 mm were screened for secondary metabolites. As previously described, the phytochemical examination was conducted to determine the extract's terpenoid, alkaloid, phenolic, and steroid content (Handayani *et al.*, 2019; Tiwari *et al.*, 2011)

Microscopic examination

Endophytic fungi showing antibacterial activity were identified using a microscope with 2000 \times magnification. The fungus specimen was placed on an object glass. Then lactophenol was drizzled and combined. Glass cover was placed over the specimen. The morphology of the specimens was examined after the results of molecular identification were obtained.

Molecular identification

DNA extraction

Three endophytic fungal strains (RTKB3, RTKB4, and RTKB7) with the most significant antibacterial activity were identified using DNA amplification and sequencing of the internal transcribed spacer (ITS) region, as per the molecular biological protocol. The DNA extraction was performed using a modified

Atashpaz *et al.* (2010) approach. After the fungus had grown for 24 hours in the SDA medium, its cell mass was moved to the microtube with 500 μ l of lysis buffer. At 4°C and 18,000 rpm, the mixture was spun in a centrifuge for 5 minutes. The filtrate was removed, and 50 μ l of TE buffer pH 8.0 was used to dissolve the DNA in the pellet.

Amplification by polymerase chain reactions (PCRs) and the sequence of the ITS-coding gene

A KOD FX Neo machine from Toyobo, Japan, was employed for PCR amplification. ITS1 (F 5'-TCC GTA GGT GAA CCT GCG G-3') and primer ITS4 (R 5'-TCC TCC GCT TAT TGA TAT GC-3') were used in this PCR, which was conducted following the PCR master mix protocol. The following is the PCR amplification reaction: One cycle at 95°C for 5 minutes, then 35 cycles with steps of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, followed by one cycle at 72°C for 6 minutes, were used in the reaction (Ferrer *et al.*, 2001). The products were sent to Malaysia. The DNA sequences were aligned pairwise on the National Center for Biotechnology Information (NCBI) and MycoBank databases using Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was created in Molecular Evolutionary Genetics Analysis 7.0 using the neighbor-joining method and a bootstrap value of 1,000 replications (Kumar *et al.*, 2016).

RESULT AND DISCUSSION

Rhodomyrtus tomentosa plants belong to the Myrtaceae family. It is a kind of wild plant with woody trees. In the open plains, they are almost as tall as adults (they can get up to 4 m). The leaves are rigid, about 5–7 cm long and 2–3.5 broad cm, oval in shape, the tip from blunt to sharp, at the top glossy green and grayer below. The leaves can be seen in Figure 1a. The flowers are single or in groups (clusters) of 2–3 flowers, 2.5–3 cm in diameter, pink to purple with many stamens and odorless. The flower of *R. tomentosa* can be seen in Figure 1b. The fruit is an oval-shaped berry, about 1–1.5 cm long and about 1 cm wide, with persistent calyx. The young fruit is green and tastes tart (bitter), while the ripe fruit is red–purple to black and tastes sweet—hairy fruit skin like velvet. A persistent calyx is at the top of the fruit (Fig. 1c) (Burkill, 1993).

Thirteen fungal isolates were obtained from *R. tomentosa* (Aiton) Hassk based on their different morphological characteristics and coded according to the organ of their host plant (coded as RTA1-3, RTKB1-7, and RTD1) (Fig. 2). The weight of EtOAc extract ranged from 1 to 6.2 g (Table 1). The antimicrobial activity of EtOAc extracts of individual fungal isolates was tested. The results of antibacterial activity screening of fungus extract with a concentration of 5% against *S. aureus*, *E. coli*, and *C. albicans* are presented in Table 2. The EtOAc extract that had the highest antibacterial activity against the growth of *S. aureus* and *E. coli* was RTKB7 isolate with an average inhibition zone diameter of 19.90 ± 0.34 and 18.61 ± 0.87 mm, respectively. The RTKB3 isolate had an average inhibition zone diameter of 11.29 ± 1.17 and 10.69 ± 0.56 mm. In contrast, the RTKB4 isolate had an average inhibition zone diameter of 9.71 ± 0.56 and 10.15 ± 0.61 mm (Table 3, Fig. 3). According to the diameter of the zone inhibition, Davis and Stout (1971) divided inhibition strength into four categories: very strong (20 mm), strong (10–20 mm), medium

(6–10 mm), and weak (6 mm). All extracts tested negative for antifungal activity against *C. albicans*. It is suspected that this is due to the inability of the EtOAc extract to penetrate the cell wall of the *C. albicans* because this fungus has a complex cell wall, 100–400 nm thick. The sterol membrane in the cell wall plays a vital role as an antifungal target (Brunton, 2006). Thus, endophytic fungi extracts generally have a small or negative inhibition zone against *C. albicans*.

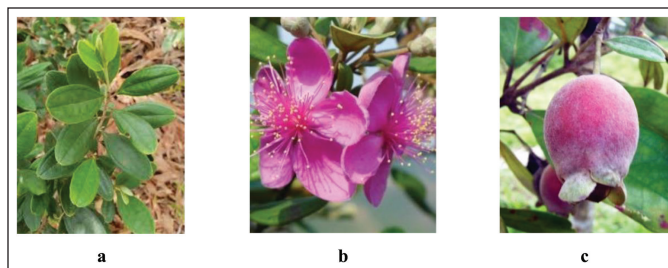


Figure 1. The pictures of (a) leaves, (b) flowers, and (c) fruit of *R. tomentosa* (Aiton) Hassk.

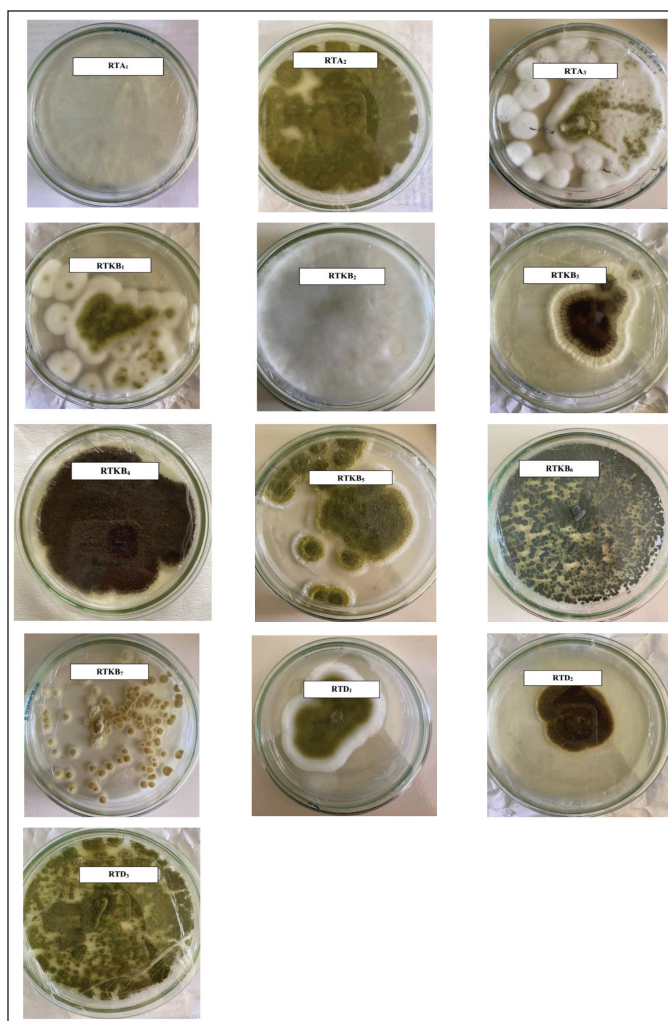


Figure 2. The pictures of isolated fungal endophytes from *R. tomentosa* (Aiton) Hassk growing on SDA.

Based on the test results for the content of secondary metabolites, the EtOAc extract of selected fungus from the stem bark of *R. tomentosa* (Aiton Hassk) contains flavonoid, steroid, and terpenoid (Table 4). Furthermore, fungus isolates of RTKB3, RTKB4, and RTKB7, which have antibacterial activity, were

Table 1. EtOAc extract weight (g) of endophytic fungi from *R. tomentosa* (Aiton) Hassk.

Fungus code	EtOAc extract weight (g)
RTA ₁	2.1
RTA ₂	4.3
RTA ₃	6.2
RTKB ₁	1
RTKB ₂	4.8
RTKB ₃	3.5
RTKB ₄	2.9
RTKB ₅	3.8
RTKB ₆	2.7
RTKB ₇	3.9
RTD ₁	4.4
RTD ₂	4.4
RTD ₃	3.1

Table 2. Antimicrobial activity of endophytic fungi from *R. tomentosa* (Aiton) Hassk against pathogenic microbes.

Fungus code	Organ	Zone of inhibition (mm)		
		<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
RTA1	Root	6.76	6.55	-
RTA2	Root	6.78	6.39	-
RTA3	Root	-	-	-
RTKB1	Bark	-	6.73	-
RTKB2	Bark	-	6.77	-
RTKB3	Bark	8.87	7.51	-
RTKB4	Bark	7.02	7.73	-
RTKB5	Bark	-	-	-
RTKB6	Bark	-	-	-
RTKB7	Bark	11.83	14.41	-
RTD1	Leaf	-	-	-
RTD2	Leaf	8.12	6.75	-
RTD3	Leaf	7.23	-	-

Table 3. Antibacterial activity of fungus isolate RTKB3, RTKB4, and RTKB7.

Fungus code	Zone of inhibition (mm) ± standard deviation (SD)	
	<i>S. aureus</i>	<i>E. coli</i>
RTKB3	11.29 ± 1.17	10.69 ± 0.56
RTKB4	9.71 ± 0.56	10.15 ± 0.61
RTKB7	19.90 ± 0.34	18.61 ± 0.87

The value is expressed as the mean ± standard deviation (SD); *n* = 3.

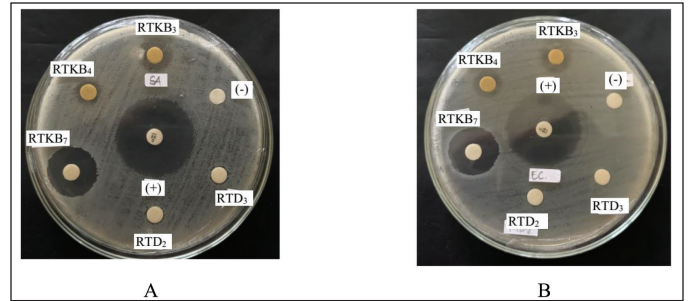


Figure 3. The agar plate pictures of the inhibition zone of fungal isolates RTKB3, RTKB4, and RTKB7 extract against the growth of (A) *S. aureus* and (B) *E. coli*.

Table 4. Phytochemical screening of EtOAc extracts of RTKB3, RTKB4, and RTKB7.

Fungus code	Chemical constituents				
	Alkaloid	Flavonoid	Terpenoid	Steroid	Fenolic
RTKB3	-	+	-	-	-
RTKB4	-	-	+	-	-
RTKB7	-	-	-	+	-

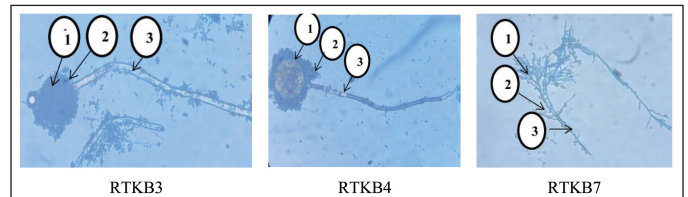


Figure 4. The microscopic pictures of fungal isolate RTKB3 (1. Vesicle, 2. Conidia, 3. Conidiophore), RTKB4 (1. Vesicle, 2. Conidia, 3. Conidiophore), and RTKB7 (1. Conidia, 2. Sterigmata, 3. Conidiophore)

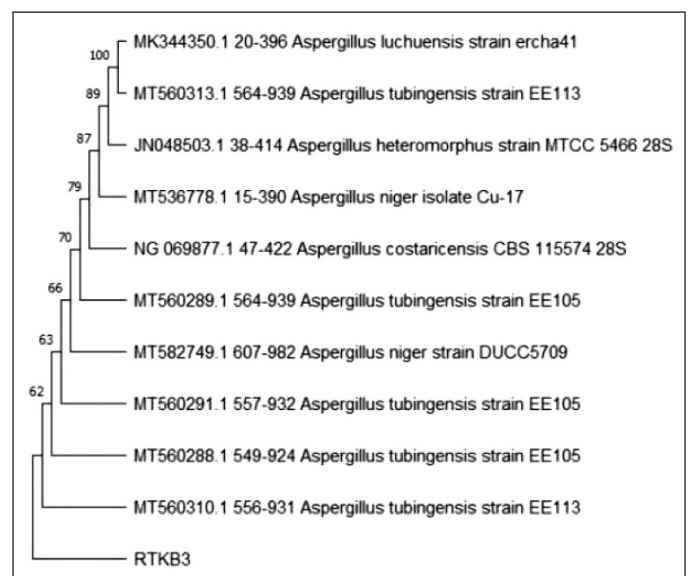


Figure 5. The phylogenetic tree inferred using the neighbor-joining method of ITS sequence of fungus RTKB3 derived from *R. tomentosa* (Aiton) Hassk and its allied taxa.

identified macroscopically, microscopically, and molecularly. The identification of fungi requires both morphological and molecular methods. Morphologically, fungi were classified according to their colony, color, spore size, texture, shape, growth rate, and conidial attachment (Domsch *et al.*, 2007). The fungus RTKB3 had brown, rough, round conidia ranging in size from 3.5–4 μm and an inconspicuous white mycelium at the colony's edge (Fig. 2). The fungus isolate of RTKB4 microscopically had round-shaped vesicles with diameters ranging from 17.52 to 23.4 μm . The conidia are globose with a diameter ranging from 3.5 to 4.5 μm . The conidiophores are long, cylindrical, and colorless (hyaline) (Fig. 4).

The fungus RTKB7 has oval-shaped conidia of varying sizes, septate hyphae, branched conidiophores, and filialids at the ends. Some of them have chlamydospores (Fig. 4) (Domsch *et al.*, 2007; Samson *et al.*, 1988).

The molecular identification was performed at the Indonesian Center for Biodiversity and Biotechnology. A BLAST scan in NCBI-GenBank and MycoBank revealed that isolate RTKB3 and *Aspergillus heteromorphus* strain MTCC 5466 28S have a 99.25 percent identity. Isolate RTKB4 and RTKB7 were 100% identical with *Aspergillus niger* isolate DGGE gel band def2 and *Paecilomyces subglobosus* strain Centraalbureau Voor Schimmelcultures 125145, respectively (Figs. 5–7). The sequence data of RTKB3, RTKB4, and RTKB7 were submitted to GenBank with accession no OQ592160, OQ592159, and OQ592158, respectively. Microscopic observations of fungi have been carried out to complete the results of molecular identification.

Endophytic fungi were reported can produce secondary metabolites of various groups, such as aliphatic metabolites, alkaloids, flavonoids, glycosides, lactones, phenyl propanoids, quinones, steroids, terpenoids, and xanthenes (Zhang *et al.*, 2006). *Aspergillus heteromorphus* has been reported to produce gallic acid with bioactivities such as antibacterial, antiviral, analgesic, and antioxidant (Bakheet *et al.*, 2014; Prasad *et al.*, 2011).

Sadananda *et al.* (2011) reported *A. niger* and *Alternaria alternata* (Host *Tabebuia argentea*) produces lapachol, anticancer agent. *Aspergillus niger* PN2, an endophytic fungus isolated from *Taxus baccata* produced lovastatin (Raghunath *et al.*, 2012). Wei *et al.* (2022) obtained five compounds from *A. niger* xj spore powder. These compounds are ergosterol (1), β -sitosterol (2), 5-pentadecylresorcinol (3), 5-hydroxymethyl-2-furancarboxylic acid (4), and succinimide (5). All compounds showed antibacterial activity against three plant pathogens; *Agrobacterium tumefaciens* T-37, *Erwinia carotovora* EC-1, and *Ralstonia solanacearum* RS-2. Compound 4 showed the strongest antibacterial activity against the tested bacteria, and RS-2 was the most sensitive to compound 4, showing the lowest MIC of 15.56 $\mu\text{g/ml}$.

Little is known about a chemical constituent produced by *P. subglobosus*. However, secondary metabolites produced by *Paecilomyces* vary greatly, including terpenoids, alkaloids, sterols, fatty acids, polyketides, peptides, and others (Salem *et al.*, 2022). One of the most representative metabolites of this fungus is paecilquinones. This compound is a tyrosine kinase inhibitor with various biological activities such as antimicrobial, antiviral, insecticidal, and cytotoxic (Sun *et al.*, 2004).

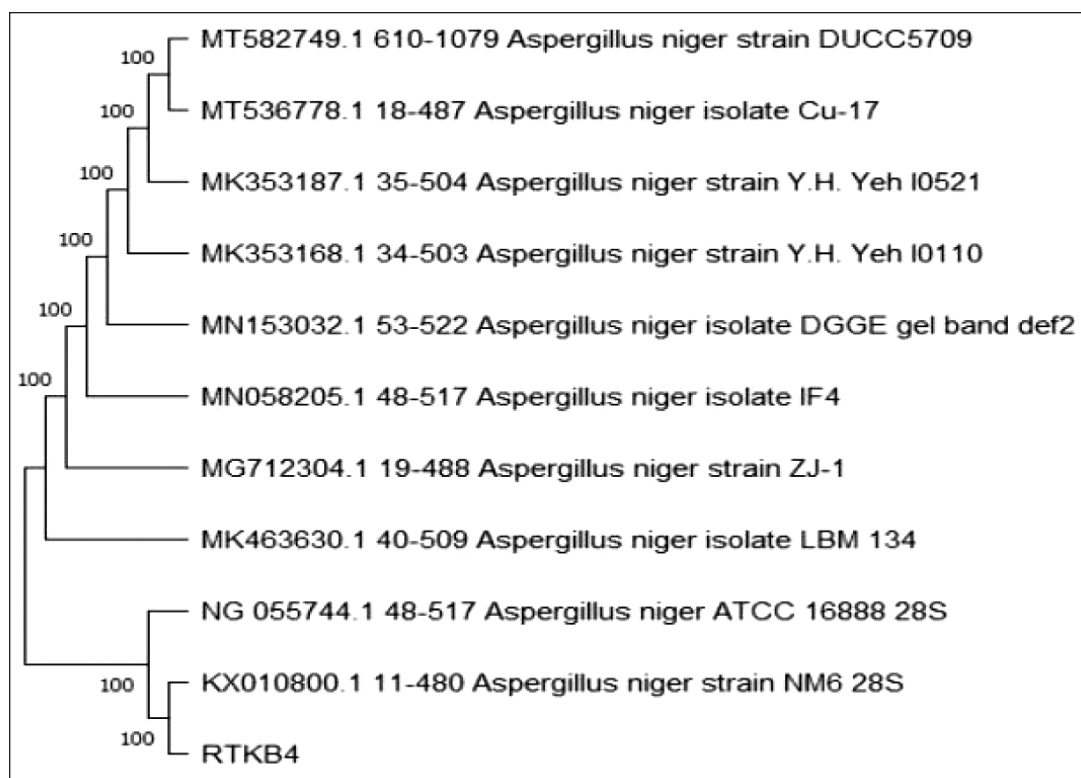


Figure 6. The phylogenetic tree inferred using the neighbor-joining method of ITS sequence of fungus RTKB4 derived from *R. tomentosa* (Aiton) Hassk and its allied taxa.

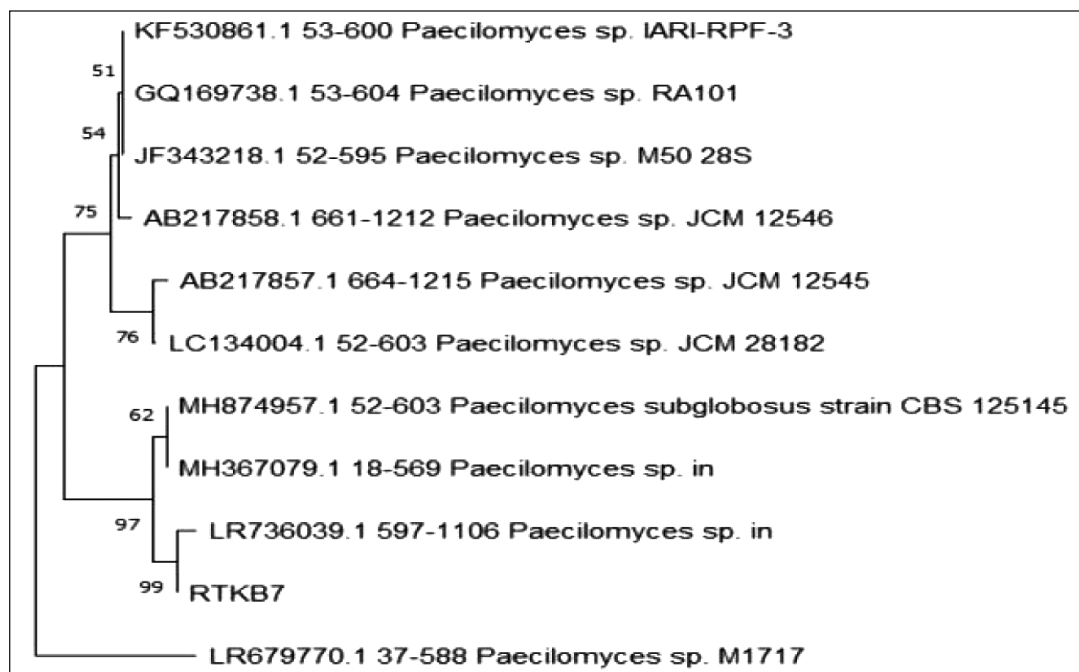


Figure 7. The phylogenetic tree inferred using the neighbor-joining method of ITS sequence of fungus RTKB7 derived from *R. tomentosa* (Aiton) Hassk and its allied taxa.

CONCLUSION

Endophytic fungi have been isolated from the rose myrtle's roots, barks, and leaves (*R. tomentosa* (Ait) Hassk). This study found three endophytic fungi capable of producing antibacterial compounds: *Aspergillus heteromorphus*, *A. niger*, and *P. subglobosus*. Advanced research of these findings and ongoing efforts to purify, identify, and optimize fermentation is necessary. Due to their capacity to synthesize the same compound originating from their host plant, endophytic fungi isolated from plants have drawn numerous researchers' attention in basic and applied research.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICT OF INTERESTS

The authors declare that they do not have any conflicts of interest.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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