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Endophytic fungus isolated from *Zingiber officinale* Linn. var. *rubrum* as a source of antimicrobial compounds

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ABSTRACT

In Indonesia, red ginger (*Zingiber officinale* Linn. var. *rubrum*) is an essential traditional medicine used to treat various ailments. Red ginger plants may have an endophytic fungus capable of producing several bioactive chemicals. This research aimed to identify endophytic fungi and evaluate their antimicrobial efficacy. The fungal strains were isolated from red ginger leaves, stems, and rhizomes by direct planting and pour methods using fungus culture medium and cultivated on rice medium. In the secondary metabolite extraction process, ethyl acetate (EtOAc) is used as a solvent. The agar diffusion procedure examined the EtOAc extract for antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. This study succeeded in isolating 10 fungal strains. The result of antimicrobial screening revealed that the fungal strain JMR4 has a high ability to suppress the development of pathogenic bacteria *S. aureus* and *E. coli*, with an inhibition zone of 14.38 ± 1.34 and 16.88 ± 0.69 mm, respectively. The fungus was later identified using the molecular approach, which stated that this fungus is *Aspergillus terreus*. This research demonstrates that endophytic fungus on red ginger plants may synthesize antibacterial substances crucial for developing novel antibiotics.

INTRODUCTION

Antibiotic requirements have lately increased. The global burden of bacterial illnesses is very high, aggravated by antibiotic resistance. Antibiotic resistance leads to failed infection treatment, which can ultimately lead to death (WHO, 2017). Thus, research related to the development of new drugs must continue.

One potential source is red ginger, a traditional medicinal plant that the community has long used for treatment (Germplasm Resources Information Network *et al*., 2017). Ginger comes from the flowering plant *Zingiber officinale* of the Zingiberaceae family. The rhizome of red ginger was reported to contain sesquiterpenes, such as beta-bisabolene and zingiberene, zingerone, shogaols, and gingerols, with [6]-gingerol as the significant pungent compound primarily (An *et al*., 2016). On the other hand, Wang *et al*. (2020) reported that ginger essential oil could inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* with a minimum inhibitory concentration of 1.0 and 2.0 mg/ml, respectively.

Endophytic fungi live on plant tissue with a beneficial symbiotic relationship without causing any adverse (Schulz *et al*., 2002). It has been known that endophytic fungi are essential sources of bioactive secondary metabolites, such as antimicrobial compounds (Schulz *et al*., 2002; Strobel, 2003a, 2003b). It was hypothesized that 51% of bioactive metabolite taken from endophytic fungus had not been found before (Schulz *et al*., 2002). Endophytic fungi can create bioactive secondary metabolites with anticancer and antimicrobial effects, including alkaloids, phenolic acids, quinones, steroids, saponins, tannins, and terpenoids (Gouda *et al*., 2016).

Research reports on fungi originating from red ginger plants are still very limited in number. To investigate the

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endophytic fungus as a source of antimicrobial compounds, this study will report fungus isolated from the red ginger plant and explore its antimicrobial activity against *S. aureus*, *E. coli*, and *Candida albicans*.

MATERIALS AND METHODS

Identification of sample material

Stems, rhizomes, and leaves of red ginger (*Zingiber officinale* Linn. var. *rubrum*) were fresh from Mukomuko Regency, Bengkulu Province, Indonesia. Geographically, it is located at 101˚01′15.1″–101˚51′29.6″ east longitude and 02˚16′32.0″–03˚07′46.0″ south latitude. The sample has been identified in the Herbarium of Universitas Andalas, Padang, Indonesia.

Sample preparation

Sterilization of samples was accomplished through two methods: direct planting and pouring. In direct planting, sample pieces were sequentially sterilized by dipping them in 70% ethanol for 1 minute to sterilize the surface, drying them with a sterile cotton cloth (or rinsing them with distilled water) waiting until the ethanol evaporated. The cleavage surface of each plant organ was put facing the sabouraud dextrose agar (SDA) media. The SDA (g/l) composition is peptone from casein 5.0; peptone from meat 5.0; D(+)glucose 40.0; agar-agar 15.0 (European Pharmacopeia 5.6, 2006). The samples were cultured at 25°C for 5–7 days (room temperature). Each sample used in the pouring method was cleaned with distilled water and cut into little pieces, and then a sample of 10 g was collected and placed in the Erlenmeyer, followed by 100 ml of distilled water. After that, it was diluted to a concentration of 10^{-6}, inoculated on SDA medium, and incubated for 5–7 days at a temperature of 27°C–29°C. Colonies that differ from other colonies in terms of their shapes and colors are known as isolates. Purification was performed until pure isolates were obtained (Kjer *et al*., 2010).

Cultivation of pure fungi isolates in the medium of rice and extract preparation

The colony of fungus growing in a Petri dish was cut into small pieces and then put into solid rice media in a 1,000 ml Erlenmeyer flask at room temperature. The cultivation process could be done when the fungal is overgrown on the rice after 4–6 weeks. The fungus develops rapidly to cover the rice's entire top surface. After the maximum growth of the fungus, the cultivation results were extracted using ethyl acetate (EtOAc) solvent. This solvent was used to obtain both semipolar and nonpolar compounds that might be responsible for antimicrobial activity. The fungus's EtOAc extract was evaporated using a rotary evaporator.

Antimicrobial activity screening

Agar well diffusion was conducted to investigate the antimicrobial activity of EtOAc extract of endophytic fungus from red ginger against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *C. albicans*. Petri dishes were prepared by adding Nutrient Agar (Merck®) containing a bacterial suspension (0.5 McFarland). Nutrient agar contains (mass/volume): 0.5% peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% sodium chloride, and pH adjusted to neutral (6.8) at 25°C (77°F) (American Public Health Association *et al*., 1920). Chloramphenicol disks (Oxoid®) in a concentration of 30 µg/ml and nystatin disks (Oxoid®) in a concentration of 100 unit/disk were used for positive control against bacterial and fungus pathogenic. As a negative control, paper discs were used, which were added with 20 µl dimethyl sulfoxide (DMSO). For 48 hours, fungi were incubated at 25°C–27°C, whereas bacteria were incubated at 37°C for 24 hours. The EtOAc extract was 5 mg/ml (in DMSO). The antimicrobial screening was carried out using the previously described method (Handayani *et al*., 2018; 2019; 2020; 2021; Handayani and Aminah, 2017; Handayani and Artasasta, 2017; Sandrawati *et al*., 2020). The presence of bacterial and fungal inhibition activity is indicated by the clear zone on the Petri disk (Bauer, 1959). Triplicate experiments were conducted to test for antimicrobial activity. The mean diameter of the inhibition zone and the standard deviation were calculated for each extract with an inhibition zone more significant than 10 mm.

Determination of the content of secondary metabolites

Secondary metabolites/phytochemical screening for EtOAc extracts of endophytic fungi with antibacterial activity (inhibition zone >10 mm) was performed. This method detects the content of terpenoid, alkaloid, phenolic, and steroid compounds from the extract (Handayani *et al*., 2019; Tiwari *et al*., 2011).

Molecular identification

Using the ITS primer, molecular identification was performed. The DNA extraction was performed using the modified Atashpaz *et al*. (2010) approach. The first BASE, located in Malaysia, received the polymerase chain reaction products for sequencing. The National Center for Biotechnology Information's MycoBank database and the Basic Local Alignment Search Tool aligned the sequences pairwise.

RESULTS AND DISCUSSION

Bioactive compounds produced by endophytic fungi from sea sponges, mangrove trees, and medicinal plants from West Sumatra, Indonesia, are the focus of our further research. The findings of this study indicate that endophytic fungi can serve as a surrogate source for new drug discovery (Aminah *et al*., 2020; Artasasta *et al*., 2017; Handayani *et al*., 2016; 2018; 2019; 2021, Handayani and Aminah, 2017; Handayani and Artasasta, 2017; Sandrawati *et al*., 2020). Medicinal plants are one source of the wide variety of endophytic fungi, and these plants' organs have been found to contain endophytic fungi. Thirty endophytic fungi from the red ginger's leaves, stems, and rhizomes show antifungal action against the plant's pathogenic fungus, *Fusarium oxysporum*, according to Ginting *et al*. (2013). The living conditions of red ginger in different and unique locations make this plant a host for various types of endophytic fungi, which can produce chemical metabolites with new chemical structures.

This study discovered 10 endophytic fungi from the leaves, stems, and rhizomes of *Z. officinale* Linn. var. *rubrum* (Fig. 1). Based on the characteristics of different colonies obtained from red ginger leaves, rhizomes, roots, and stems, isolates of endophytic fungi were obtained. Leaves and stems have almost

the same number of endophytic isolates. Endophytic fungi from leaves were designated with codes JMD, stems by JMB, and rhizomes by JMR. The rhizome yielded five fungal strains, a more significant number than the other plant organs studied (Fig. 2).

The findings of the antimicrobial activity screening are displayed in Table 1. The clear zone on the Petri disk indicates the presence of bacterial and fungal inhibition activity. Four fungal extracts were effective against *S. aureus*, five against *E. coli*, and two against *C. albicans* at a concentration of 5%. The two isolates with the highest inhibition diameter of all the fungi extracts were JMR4 and JMD1 (>10 mm). With an inhibition zone of 14.38 ± 1.34 mm for *S. aureus* and 16.88 ± 0.69 mm for *E. coli*, the JMR4 had the largest diameter of inhibition (Fig. 3). As a result of its capacity to prevent the growth of Gram-negative and Gram-positive pathogenic bacteria, this fungal extract may be characterized as broad-spectrum (Table 2). JMR4 EtOAc extract exhibited a small impact on *C. albicans* growth suppression, with an inhibition zone of 7.17 mm.

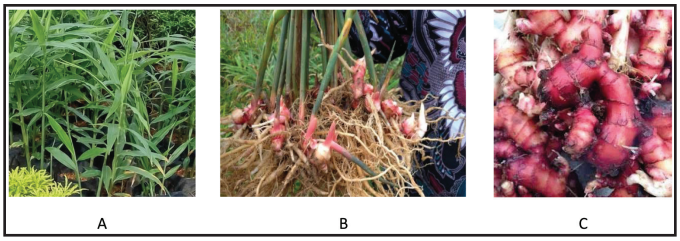

Figure 1. The pictures of red ginger (*Z. officinale* var. *rubrum*): (A) leaves, (B) stems, and (C) rhizomes.

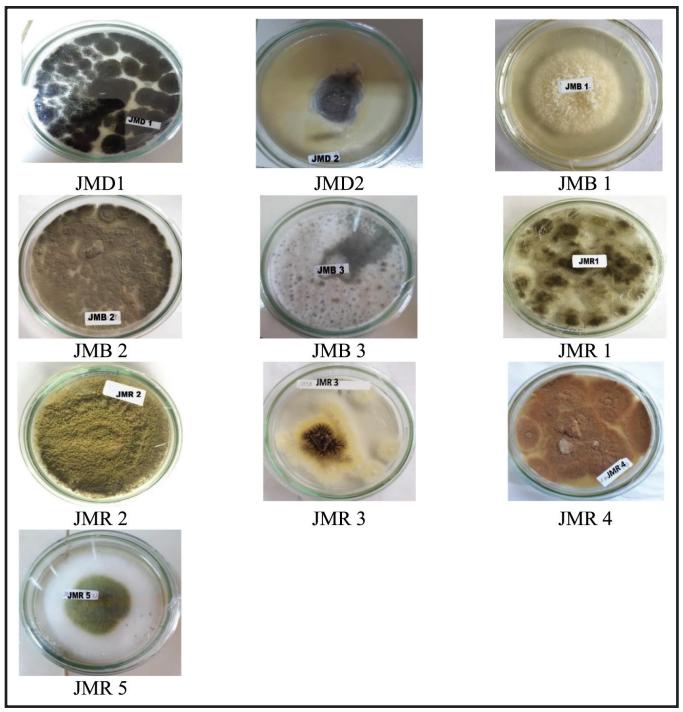

Figure 2. The pictures of isolated fungal endophytes from *Z. officinale* var. *rubrum* growing on SDA.

Furthermore, the phytochemical screening of extracts was tested for the JMR4 and JMD1 extracts with the highest antimicrobial activity. This screen observes the color changes after The JMR4 extract produced a positive red color after being tested with the Wilstater test. In the alkaloid content test, JMR4 and JMD1 gave positive results forming a white precipitate after being given Meyer's reagent. In the phenolic identification test, a blue-black color is formed after phenol reacts with $FeCl_3$. Only JMD1 shows a positive result for the phenol test. The Lieberman–Burchard reagent is used to identify terpenoids and steroids, which gives a green-blue color indicating the presence of steroids. In contrast, a violet ring indicates the presence of terpenoids. The identification of terpenoids and steroids in the EtOAc extract of endophytic fungi JMR4 and JMD1 were positive for the presence of terpenoids with the formation of a violet ring (Table 3).

Macroscopically, the colony of JMR4 shows brown with fading margins, large rounded, flat surfaces, and edges.

Table 1. Antimicrobial activity of endophytic fungi from *Z. officinale* var. *rubrum* plant against the pathogenic microbe.

Fungal code	Organ	Average of inhibition zone (mm)		
		S. aureus	*E. coli*	*C. albicans*
JMD1	Leaf	7.39	9.32	-
JMD2	Leaf	-	-	-
JMB1	Stem	7.64	7.20	-
JMB2	Stem	-	-	-
JMB3	Stem	7.87	7.13	7.21
JMR1	Rhizome	-	-	-
JMR2	Rhizome	-	-	-
JMR3	Rhizome	-	7.50	-
JMR4	Rhizome	12.05	14.55	7.17
JMR5	Rhizome	-	7.22	-

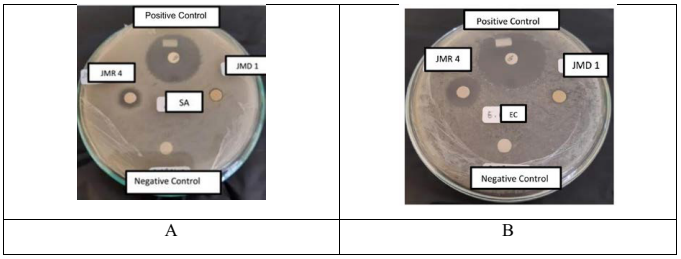

Figure 3. The agar plate pictures of the inhibition zone of fungal isolate JMR4 extract against the growth of (A) *S. aureus* and (B) *E. coli*.

Table 2. Antimicrobial activity of fungal endophyte JMR4 and JMD1.

Fungal code	Zone of inhibition ± standard deviation (SD)		
	S. aureus	*E. coli*	*C. albicans*
JMR4	14.38 ± 1.34	16.88 ± 0.69	8.28 ± 0.07
JMD1	7.35 ± 0.04	8.18 ± 0.16	7.51 ± 0.27

The value is expressed as the mean ± standard deviation; $n = 3$.

JMR4 was seen under a microscope, revealing vehicles, conidiophores, and spherical conidia (Fig. 4). Molecular identification showed that JMR4 had identical to *Aspergillus terreus* with sequence identities of 100%. The phylogenetic tree was constrained using the neighbor-joining method with a bootstrap value of 1.000 (Fig. 5). JMR4 is clustered and identic with *Aspergillus terreus* EUR1 with accession number MF590163.1. The Kimura two-parameter model shows no genetic difference between JMR4 and *A. terreus* EUR1 (Table 4). An overview to summarize the experimental design of this research is shown in Figure 6.

The fungus *A. terreus* was shown to have tetracyclic acid A, a member of the sesquiterpene antibiotic family having antibacterial and anticancer properties. (Goutam *et al*., 2017; Hirota *et al*., 1982). Lovastatin, a medication used to treat high cholesterol in humans, is produced by the fungus *A. terreus*. This fungus makes more than just lovastatin, and it also makes sulochrins, terretonins, asterriquinones, and butyrolactones, all bioactive compounds (Chen *et al*., 2018). Liu *et al*. (2018) discovered three novel compounds: a prenylated tryptophan derivative, luteoride E, a butenolide derivative, versicolactone G, and linear aliphatic alcohol (3E,7E)-4,8-dimethyl-undecane-3,7-diene-1,11-diol and nine known compounds from the coral-associated fungus *A. terreus*. Versicolactone G had a significant α-glucosidase inhibitory activity with an IC_{50} value of 104.8 ± 9.5 µM, less than the positive control acarbose (IC_{50} = 154.7 ± 9.5 µM). Furthermore, practically all substances reported considerable anti-inflammatory efficacy against nitric oxide production, with IC_{50} values ranging from 5.48 to 29.34 µM. The discovery of bioactive compounds produced by *A. terreus* has shown the great potential of endophytic fungus for producing structurally unique and pharmacologically active natural products.

Table 3. Phytochemical screening of EtOAc extract of JMR4 and JMD1.

Fungal code	Chemical constituents				
	Alkaloid	Flavonoid	Terpenoid	Steroid	Fenolik
JMR4	+	+	+	−	−
JMD1	+	−	+	−	+

Figure 4. Macroscopic (A) and microscopic (B) pictures of fungal isolate JMR4: (1) conidia, (2) vesicles, and (3) conidiophore.

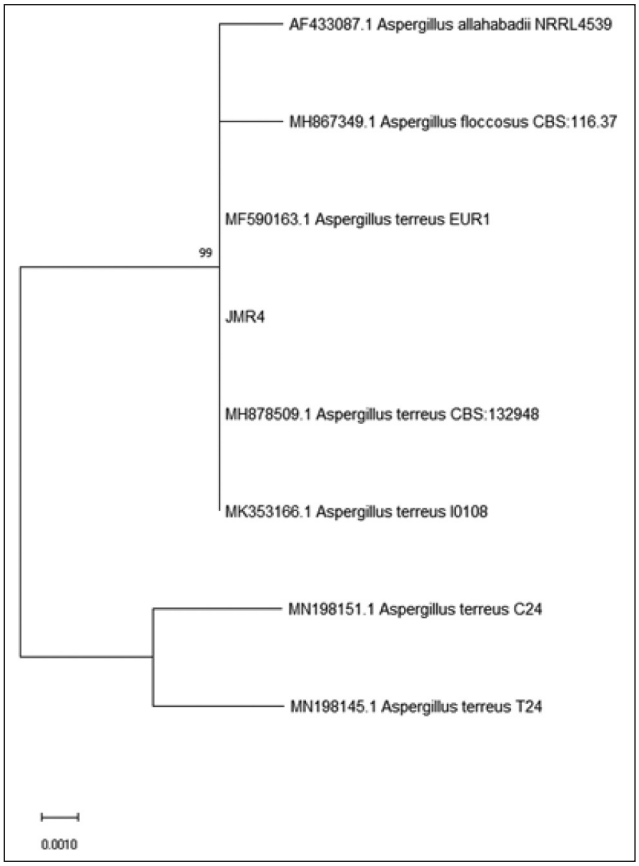

Figure 5. The phylogenetic tree inferred using the neighbor-joining method of ITS sequence of fungus JMR4 derived from *Z. officinale* and its allied taxa.

Table 4. Estimation of evolutionary divergence between sequences using the Kimura two-parameter model.

Accession number	1	2	3	4	5	6	7	8
JMR 4								
MF590163.1	0.000							
MK353166.1	0.000	0.0051						
MH878509.1	0.000	0.000	0.000					
AF433087.1	0.0016	0.0016	0.0016	0.0017				
MH867349.1	0.0016	0.0016	0.0016	0.0016	0.0024			
MN198145.1	0.0051	0.0051	0.0051	0.0051	0.0056	0.0055		
MN198151.1	0.012	0.012	0.0051	0.012	0.0146	0.0146	0.0146	

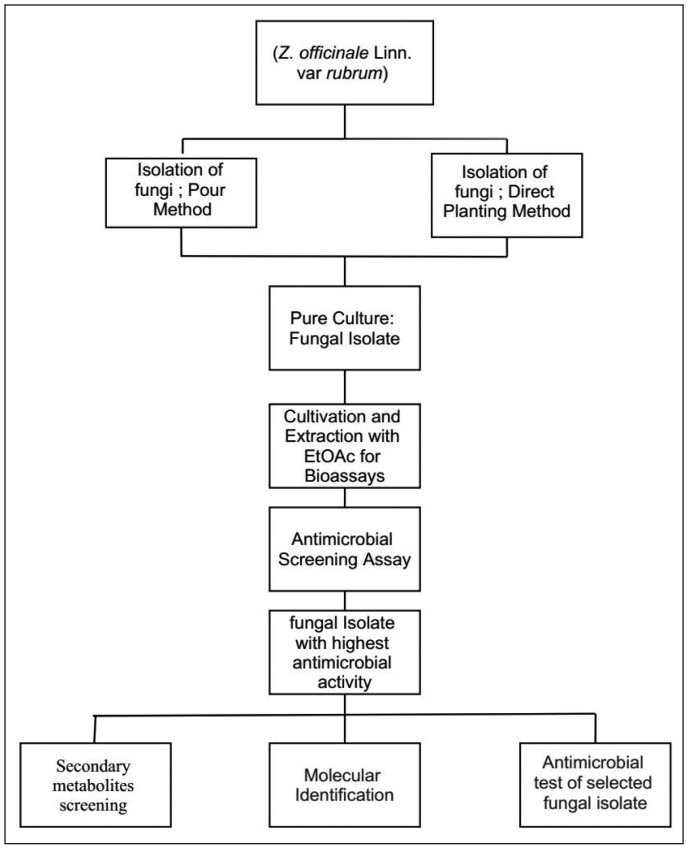

Figure 6. The experimental design of endophytic fungus isolation from *Z. officinale*.

CONCLUSION

Endophytic fungus is an important source of secondary metabolites with fascinating bioactivities and diverse chemical structures. As a result of this study, 10 fungi from red ginger (*Z. officinale* Linn. var. *rubrum*) have been successfully isolated. Based on the results of antimicrobial activity screening, the fungal strain JMR4 has a high ability to suppress the development of pathogenic bacteria such as *S. aureus* and *E. coli*. The JMR4 is identical to *A. terreus*. It is a big challenge to isolate the bioactive chemicals from this fungus for further studies and to develop new antibiotic candidates.

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AUTHORS' CONTRIBUTIONS

All authors contributed significantly to the concept and design, data gathering, data analysis and interpretation, and paper revision.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ETHICAL APPROVAL

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

DATA AVAILABILITY

This study article contains all of the data that was generated and examined.

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