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Endophytic fungi from red ginger (*Zingiber officinale var. rubrum*) as promising source of antimicrobial and cytotoxic secondary metabolites

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ABSTRACT

In the present study, we reported eight endophytic fungi isolated from stems, rhizomes, and roots of red ginger (*Zingiber officinale* var. *rubrum*), collected from Bali, Indonesia. Molecular biology protocol through amplification of internal transcribed spacer and LSU region led to the identification of six fungal isolates as *Microdochium colombiense* ZOR-S1-1*, Phlebiopsis flavidoalba* ZOR-S1-3, *Penicillium citrinum* ZOR-S1-4.1, *Dactylonectria anthuriicola* ZOR-Rh1-3, *Setophoma terrestris* ZOR-Br1-1, and *Xylaria cubensis* ZOR-Rh1-1. Meanwhile, two fungal isolates, ZOR-S1-4 and ZOR-Br1-2, are remain unidentified. Following rice fermentation of all isolated endophytes, all fungal extracts were subjected to antimicrobial, toxicity, and cytotoxicity assays. In the antimicrobial assay, *S. terrestris* ZOR-Br1-1 extract showed the most pronounced activity against *Staphylococcus aureus* ATCC 6538 and *Candida albicans* ATCC 10231, with MIC values of 31.3 and 15.6 µg/ml. Meanwhile, *D. anthuriicola* ZOR-Rh1-3 extract revealed the most potent activity in toxicity screening employing the brine shrimp lethality test (BSLT), with an LC_{50} value of 6.8 µg/ml. When tested further for cytotoxicity against breast cancer cells, MCF-7 and 4T1, extracts of *D. anthuriicola* ZOR-Rh1-3, *P. citrinum* ZOR-S1-4.1, unidentified isolates ZOR-S1-4 and ZOR-Br1-2, showed strong to moderate inhibition against both tested cell lines with IC_{50} values ranging from 14 to 74 µg/ml. In light of the bioactivity of endophytic fungal extracts from red ginger found in this study, investigation on secondary metabolites and their pharmacological action on antimicrobial and cytotoxicity of endophytic *S. terrestris* ZOR-Br1-1, *D. anthuriicola* ZOR-Rh1-3, and *P. citrinum* ZOR-S1-4.1 are of scientific interest for further research. Moreover, this result highlights the bioprospecting opportunity of endophytic fungi associated with medicinal plants as a source of bioactive secondary metabolites.

INTRODUCTION

Endophytic fungi are microorganisms that asymptomatically live in the plant tissues during the entire or certain part of their life cycle. Many studies have shown the capability of these microorganisms to produce structurally diverse secondary metabolites with various pharmacological activities, including antimicrobial and anticancer [1]. Endophytic fungi also attracted attention due to their potential to produce valuable bioactive compounds originally derived from medicinal plants, as exemplified by the isolation of anticancer agents, paclitaxel and camptothecin, among others from endophytic fungi [2,3]. It is estimated that only around 1–2% of 300,000 known plant species have been studied for their fungal endophytes [4], indicating that exploration of endophytic fungi from plants offers great opportunities in the search for bioactive molecules.

In particular, medicinal plants have been repeatedly reported in many previous studies as one of the promising sources of talented endophytic fungal strains capable of producing bioactive compounds. For instance, an investigation on endophytic *Fusarium* sp. BZCB-CA isolated from Chinese

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medicinal plants *Bothriospermum chinense* led to the isolation of lateropyrone, which exhibited antibacterial activity toward several bacterial strains with MICs ranging from 3.1 to 25 µM. In addition, rubrofusarin isolated from this fungal strain displayed cytotoxicity against various cancer cells with IC_{50} values ranging from 6.2 to 7.7 µM [5]. Our recent investigation on methanolic extracts of endophytic fungi *Penicillium steckii* AAB-01 and *Daldinia eschscholtzii* AAB-05, associated with medicinal plants *Antidesma bunius*, revealed their cytotoxicity against breast cancer cells MCF-7 and 4T1 [6].

Continuing our endeavor to explore the antimicrobial and cytotoxic metabolites from endophytic fungi, in the present study we investigated fungal endophytes from *Zingiber officinale* var. *rubrum*, which is commonly known as red ginger. Red ginger is renowned for its ethnopharmacological uses and diverse therapeutic properties, such as antifungal [7], antihyperglycemic [8], antihypertensive [9], analgesia [10], and antibacterial [11]. Rhizomes of red ginger are rich in essential oil which mainly consists of monoterpenes and sesquiterpenes, and showed anti-biofilm activity [12]. Moreover, gingerol and shogaol derivatives, along with cinnamic acids, ethyl cinnamate, and ethyl *p*-methoxycinnamate were reported from the red ginger extract in a previous study [13]. Secondary metabolites produced by medicinal plants might be influenced by the interaction between the host plant and their associated fungal endophytes [14,15]; therefore, investigating the endophytic fungi associated with red ginger holds the potential to discover bioactive compounds.

A previous study on endophytic fungi isolated from red ginger, collected in Bogor, Indonesia, led to the isolation of endophytic *Curvularia affinis*, *Fusarium solani*, and *Glomerella cingulata* which could inhibit the growth of plant pathogenic fungus *Fusarium oxysporum* [16]. More recently, ethyl acetate extract of fungal endophyte *Aspergillus terreus* isolated from the rhizome of red ginger growing in Bengkulu, Indonesia, was reported to possess antimicrobial properties against *Candida albicans, Staphylococcus aureus,* and *Escherichia coli* with the inhibition zone of 8.3, 14.4, and 16.9 mm, respectively [17]. Herein, we reported eight fungal endophytes isolated from stems, rhizomes, and roots of red ginger, collected from Bali, Indonesia. Following the fermentation procedure, the resulting fungal extract was tested for antimicrobial, toxicity, and cytotoxicity against breast cancer cells 4T1 and MCF-7, as well as noncancer cells Vero.

MATERIALS ND METHODS

Fungal material

The endophytic fungi were isolated from the healthy and fresh stems, rhizomes, and roots of the red ginger plant growing in Tabanan Regency, Bali, Indonesia. The host plant was collected in December 2021. Authentication of the host plant, red ginger (*Zingiber officinale* var. *rubrum*), was done in the Herbarium Biologi Udayana, Udayana University, and was deposited under voucher specimen no. PY-ZOR09 (HBU) top of form. The isolation of fungal endophytes was carried out as previously described [6]. Each of the collected plant parts was thoroughly washed under running tap water for 3 minutes. Surface sterilization was then performed by immersing the samples in 70% EtOH for 2 minutes and allowed to dry. As the negative control, sterilized samples were pressed onto a petri dish containing an isolation medium, consisting of malt extract (Merck), Bacto agar (Difco BD), and chloramphenicol (Nalgene) in demineralized water. The same sample was then aseptically cut into smaller pieces before being placed into the second isolation medium. The inoculated samples were incubated in ambient conditions for several days to allow the growth of fungal mycelia. Once the fungal mycelia were observed, they were transferred into a new agar plate to obtain the pure fungal isolate. For long-term preservation, pure fungal isolates were cultured on media containing malt extract, Bacto agar, yeast extract, and glycerol (Vivantis) in demineralized water.

Identification of endophytic fungi

The pure fungal isolates were then identified through the comparison of the sequence of the internal transcribed spacer (ITS) region. The fungal genomic DNA was extracted using Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research) following the manufacturer's instructions. PCR procedure was done to amplify the region of ITS1-5.8S-ITS2 rDNA from the extracted fungal DNA utilizing forward primer, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) (IDT), and reverse primer, ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (IDT) according to the procedure described by Putra *et al*. [18]. Meanwhile, for those that were unable to be identified through ITS region analysis, their D1/D2 domain of the large subunit (LSU) region was analyzed by amplifying it using forward NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′′) (IDT) and reverse NL4 (IDT) (5′-GGTCCGTGTTTCAAGACGG-3′) primers. Sequencing analysis of the PCR products was done by 1st BASE. The resulting sequence was compared to the deposited sequence in the database of NCBI GenBank through the Basic Local Alignment Search Tool (BLAST) for nucleotides program, to search its closest homologous taxon. The sequence of the samples and their related homologous taxon were aligned using the MUSCLE algorithm and the phylogenetic tree was reconstructed using MEGA version 11.0.11 by a neighbor-joining algorithm with replication of 1,000 bootstraps.

Fermentation and extraction

To produce a sufficient amount of extract, each fungal isolate was subjected to fermentation on rice media, following the procedure described before [6]. Briefly, each pure fungal isolate in Petri dish agar was excised into small blocks and seeded onto two conical flasks (each 1,000 ml) containing a medium of autoclaved 100 g rice in 110 ml distilled water. Each flask was incubated at ambient temperature for 3–4 weeks until the rice media were completely covered by the fungal mycelia. Fermentation was terminated by pouring 500 ml ethyl acetate into each fermentation flask, then agitated on a shaker at 150 rpm for 6–8 hours. The mixture was filtered under reduced pressure and the filtrate was concentrated *in vacuo* in a rotary evaporator to obtain the crude extract. The crude extract was partitioned between methanol consisting of 10% water and n-hexane. The aqueous phase was concentrated *in vacuo* to dryness and the resulting methanolic extract was subjected to antimicrobial, toxicity, and cytotoxic assays, in addition to phytochemical analysis.

Preliminary identification of phytochemicals

Identification of phytochemicals was done to detect the presence of alkaloids, terpenoids, polyphenols, flavonoids, and saponins in fungal methanolic extracts using the procedure described earlier [18].

Antimicrobial assay

Evaluation of the antimicrobial properties of the fungal extracts was carried out utilizing the broth microdilution method under the protocol described by the Clinical and Laboratory Standards Institute (CLSI) [19]. Briefly, each fungal methanolic extract was pre-dissolved in 20 µl DMSO (Merck) before the assay. Next, in the 96-microwell plates (Iwaki), each methanolic extract solution was serially diluted to afford a final concentration from 1,000 to 1.95 µg/ml. Four bacterial strains, i.e., *S. aureus* ATCC 6538, methicillin-resistant *S. aureus* (MRSA) ATCC 3351, *Staphylococcus epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC 9027, along with a fungal strain *C. albicans* ATCC 10231 were selected for the antimicrobial assay. Two-fold dilution of chloramphenicol with concentrations ranging from 0.0625 to 32 µg/ml and ketoconazole with concentrations ranging from 0.5 to 256 µg/ml were included as positive controls in the assay against the tested bacteria and fungus, respectively. All the experiment was conducted in triplicates. The minimum inhibition concentration (MIC) value was determined from the lowest concentration of extract that can inhibit the growth of the tested microorganism.

Toxicity

Toxicity screening was performed employing the brine shrimp lethality test (BSLT) as described earlier [6,20]. Each fungal methanolic extract was dissolved in DMSO and then diluted in artificial seawater resulting in a series of concentrations ranging from 1 to 1,000 µg/ml. To prepare the brine shrimp larvae, 20 g of *Artemia salina* eggs (Supreme Plus) were incubated in a brine incubator loaded with 300 ml artificial seawater [9.5 g artificial sea salt (Himedia) in 300 ml distilled water]. The eggs were maintained under a continuous light regime and aeration for 24 hours until they hatched to be mature nauplii. Active nauplii free from eggshells in the illuminated side of the incubator were drawn for toxicity assay. Toxicity screening was conducted by transferring 10 nauplii using a Pasteur pipette to the vials supplemented with a serial concentration of fungal extract. Nauplii were also placed in vials containing artificial seawater consisting of 0.5% DMSO as a negative control. Each test was carried out in triplicates, and the vials were kept at room temperature under constant light for 24 hours. Afterward, the number of dead nauplii in each vial was counted, and the percentage of mortality was calculated with the following equation [21]:

$$\text{Mortality (\%)} = \frac{\text{Number of dead nauplii}}{\text{Initial number of live nauplii}} \times 100$$

With SPSS v.26., the value of median lethal concentration (LC_{50}) of each methanolic extract was estimated using probit analysis of concentration versus mortality.

Cytotoxicity assay

The 4T1, MCF-7, and Vero cell lines were maintained by the Cancer Chemoprevention Research Center, Faculty of Pharmacy UGM, Indonesia. Sub-confluent cultures of 4T1, MCF-7, and Vero were harvested and re-suspended in a Dulbecco's Modified Eagle Medium (Gibco) at a 1.0×10^5 cells/ml density. A 100 µl aliquot of this cell suspension was seeded in 96-well cell culture plates (Iwaki) at a final density of 3.0×10^3, 2.5×10^3, and 1.0×10^4 cells/well, respectively. Cells were incubated for 24 hours to allow adherence and further incubated with fresh culture medium as the control or treated at increasing concentrations (5–500 µg/ml) of the fungal extracts for 24 hours. The cell viability of the extracts was assessed using the CCK-8 kit assay (Dojindo, Japan). The absorbance of each well was measured using a Micro-titer Plate Reader (BioRad) at 490 nm wavelength. The percent viability of cells was calculated using the following equation:

$$\text{Cells viability (\%)} = \frac{A_{treatment} - A_{blank}}{A_{control} - A_{blank}} \times 100\%$$

where A is the absorbance.

RESULTS

Isolation and identification of endophytic fungi

Eight endophytic fungal strains were isolated from various parts of red ginger. Fungal isolates designated as ZOR-S1-1, ZOR-S1-3, ZOR-S1-4, and ZOR-S1-4.1 were isolated from the stems. Two fungal isolates ZOR-Rh1-1 and ZOR-Rh1-3 were obtained from the rhizomes, while isolates ZOR-Br1-1 and ZOR-Br1-2 were isolated from the roots of red ginger. The macroscopic appearance of each fungal isolate is shown in Figure 1.

Species determination of the endophytic fungi was primarily done by the sequence comparison of ITS1-5.8S-ITS2 rDNA. For the isolate ZOR-Rh1-1, since no amplified DNA band was observed in the gel electrophoresis after PCR on its ITS region, this fungal isolate was further identified through the analysis of the D1/D2 domain of its LSU region.

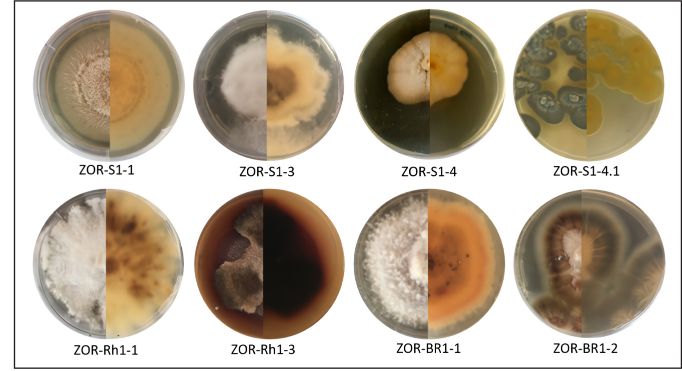

Figure 1. The front (half left) and reverse (half right) views of each endophytic fungal colony isolated from various parts of red ginger grown on an agar dish containing malt extract, yeast extract, glycerol, and Bacto agar in demineralized water.

However, fungal isolates ZOR-S1-4 and ZOR-Br1-2 remained unidentified as we were unable to amplify this fungal DNA either using amplification of the ITS region or the D1/D2 domain of the LSU region.

Amplification of the analyzed region employing PCR protocol yielded a single band of DNA segment with a size ranging from 500 to 750 base pairs, visualized on gel agarose electrophoresis as depicted in Figure 2. To predict the taxon of each endophytic fungus, the obtained sequences were compared to the fungal sequences deposited in the GenBank database utilizing the BLAST nucleotide algorithm of NCBI. As a result, the fungal sequences comparison indicated high sequence homology to six distinct species with good similarity percentage (>99%) and *E* values (Table 1). In addition, the phylogenetic tree construction showed that each isolate lineage differentiated into six primary clades with good bootstrap support, as shown in Figures 3 and 4.

Alignment analysis of the sequences suggested that isolate ZOR-S1-1 belonged to the species of *Microdochium colombiense* with 99.8% similarity to this species (OP855525.1), which was in line with its phylogenetic analysis with 99% bootstrap support. Whereas, isolate ZOR-S1-3 showed 99.8% similarity to *Phlebiopsis flavidoalba* (MZ087901.1), supported with high bootstrap support (100%). In addition, isolate ZOR-S1-4.1 was identified as *P. citrinum* proven by its similarity of 99.8% with this species (MN249873.1) and bootstrap support of 99% on phylogenetic tree. Moreover, sequence alignment showed that the ZOR-Rh1-3 isolate possessed high similarity (100%) with *Dactylonectria anthuriicola* (KP942924.1), confirmed with bootstrap support of 95% on the phylogenetic study. Isolate ZOR-Br1-1 had high sequence homology (99.8%) with the species of *Setophoma terrestris* (MN522036.1) which was supported by 99% bootstrap support on phylogenetic analysis. Finally, isolate ZOR-Rh1-3 displayed significant sequence similarity (99.8%) to the *Xylaria cubensis* (AB376701.1) species, which was confirmed by a 95% bootstrap support in the phylogenetic analysis.

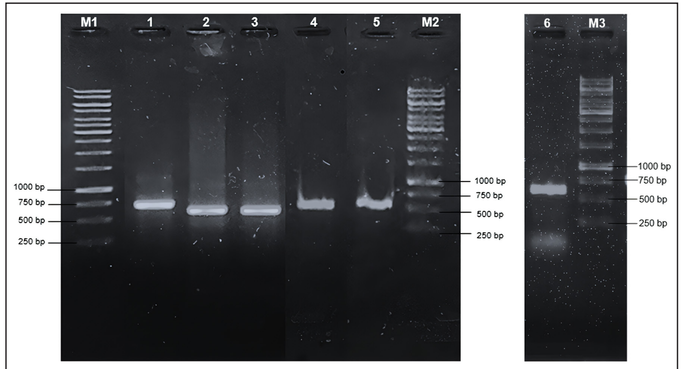

Figure 2. Electrophoregram of amplified ITS rDNA from (1) ZOR-S1-3; (2) ZOR-S1-4.1; (3) ZOR-S1-1; (4) ZOR-BR1-1; (5) ZOR-Rh1-3; and amplified LSU region of (6) ZOR-Rh1-1. M1: 1 kb DNA marker (lane 1-3); M2: 1 kb DNA marker (lane 4-5); M3: 1 kb DNA marker (lane 6).

Preliminary identification of phytochemicals

Phytochemical screening revealed the presence of alkaloids and terpenoids in all fungal extracts, except for *X. cubensis* ZOR-Rh1-1 extract which showed positive results only for alkaloids (Table 2). Polyphenols were detected in four fungal extracts produced by *P. citrinum* ZOR-S1-4.1, *D. anthuriicola* ZOR-Rh1-3, *S. terrestris* ZOR-Br1-1, and isolate ZOR-Br1-2.

Antimicrobial activity

The antimicrobial activity of each methanolic extract produced by the isolated fungal endophytes from red ginger was evaluated. The MIC values of each fungal methanolic extract toward the tested microbial strains are displayed in Table 3. Among the tested extracts, methanolic extract from *S. terrestris* ZOR-Br1-1 was found to show pronounced antimicrobial activity against *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228, and *C. albicans* ATCC 10231 with MIC values of 31.3, 125, and 15.6 µg/ml, respectively. Meanwhile, the methanolic extract of *D. anthuriicola* ZOR-Rh1-3 along with isolate ZOR-Br1-2 showed weaker inhibition against the tested *S. aureus* and *S. epidermidis* with MIC values of 62.5 and 500 µg/ml. In addition, weak inhibition was found in the assay of methanolic extract of *P. citrinum* ZOR-S1-4.1 against *S. aureus* ATCC 6538, MRSA ATCC 3351, *S. epidermidis* ATCC 12228, and *P. aeruginosa* ATCC 9027 with MIC values of 500 µg/ml. Likewise, *P. flavidoalba* ZOR-S1-3 possessed weak inhibition against *S. aureus* ATCC 6538 with a MIC value of 500 µg/ml. Extracts of *M. colombiense* ZOR-S1-1, isolate ZOR-S1-4, and *X. cubensis* ZOR-Rh1-1 were found inactive toward all the tested microbial strains up to the tested concentration of 1,000 µg/ml.

Brine Shrimp Lethality Test

The result of toxicity screening employing BSLT as displayed in Figure 5, showed that extracts of *D. anthuriicola*

Table 1. BLAST-N result of sequenced PCR products from endophytic fungi associated with red ginger.

Isolate Code	Fungal identity/GenBank accession number[a]	References of GenBank accession number used	Maximum score	Similarity (%)	Query coverage (%)	*E* value
ZOR-S1-1	*M. colombiense* (OR437987)	OP855525.1	891	99.8	96	0.0
ZOR-S1-3	*P. flavidoalba* (OR437979)	MZ087901.1	1072	99.8	97	0.0
ZOR-S1-4.1	*P. citrinum* (OR437983)	MN249873.1	928	99.8	99	0.0
ZOR-Rh1-1	*X. cubensis* (OR438033)	AB376701.1	987	99.8	98	0.0
ZOR-Rh1-3	*D. anthuriicola* (OR437978)	KP942924.1	911	100.0	97	0.0
ZOR-BR1-1	*S. terrestris* (OR437972)	MN522036.1	920	99.8	98	0.0

[a]The accession numbers were acquired upon the submission of the fungal DNA sequences to GenBank.

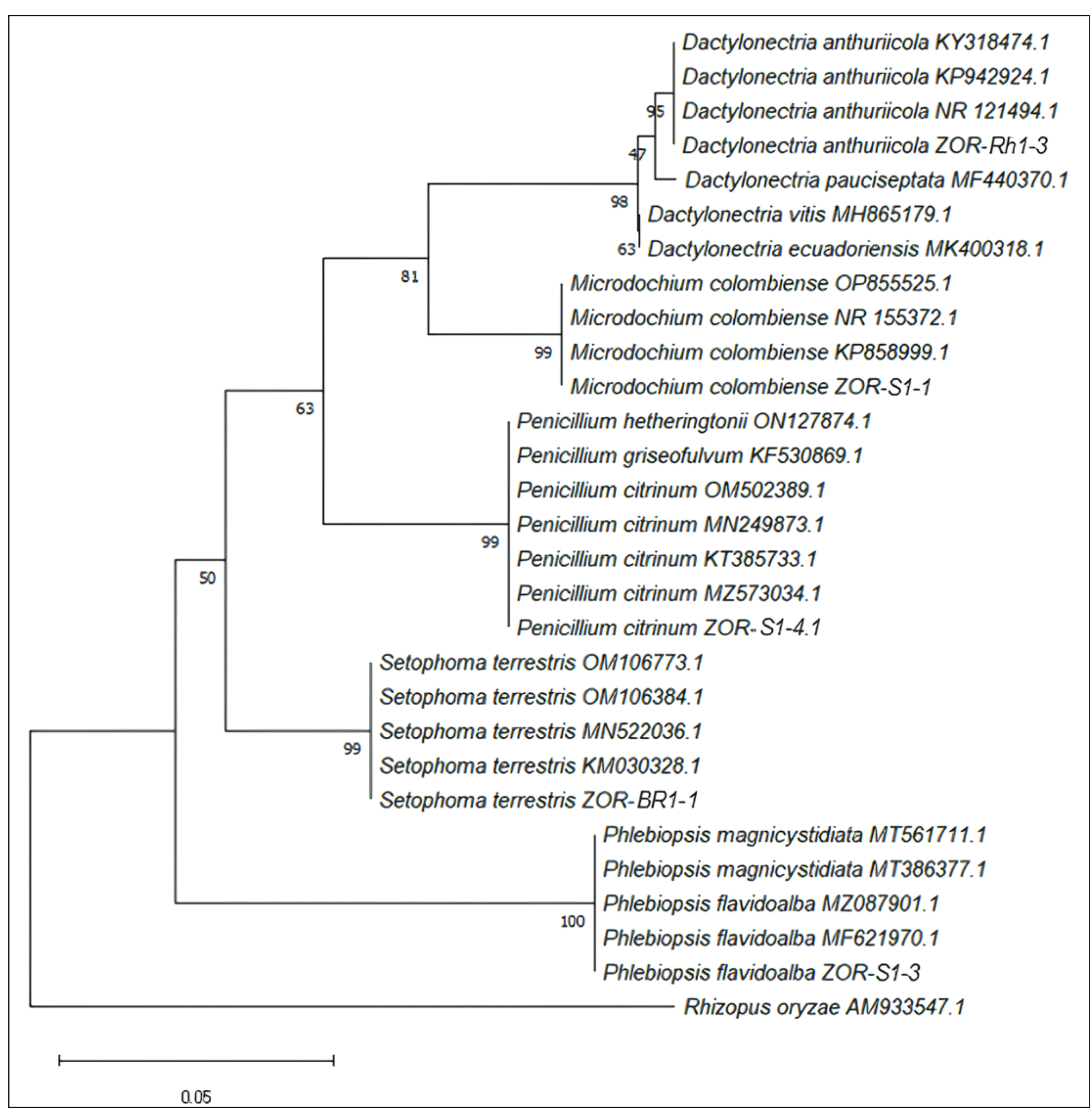

Figure 3. Phylogenetic analysis of *M. colombiense* ZOR-S1-1, *P. flavidoalba* ZOR-S1-3, *P. citrinum* ZOR-S1-4.1, *D. anthuriicola* ZOR-Rh1-3, and *S. terrestris* ZOR-BR1-1 through the ITS region comparison, using the neighbor-joining algorithm, employing 1,000 bootstraps for support.

ZOR-Rh1-3, *X. cubensis* ZOR-Rh1-1, isolate ZOR-Br1-2, and *M. colombiense* ZOR-S1-1 were found to have strong toxicity against *A. salina* nauplii with LC_{50} values ranging from 6.8 to 70.2 µg/ml. The remaining methanolic extracts exhibited moderate toxicity with LC_{50} values starting from 114 to 770 µg/ml.

Cytotoxicity potency against breast cancer cells and selectivity of tested extracts

We further explored the cytotoxicity of the fungal extracts against breast cancer cells. We used 4T1 and MCF-7 cell lines which represent triple-negative breast cancer (TNBC) and non-TNBC subtype breast cancer cells, respectively. The selectivity of samples was evaluated by employing a normal cell line, Vero. We treated the cells with the fungal extracts in a series of 5–500 µg/ml concentrations. In the TNBC cell lines, 4T1, all the treatments with the fungal extracts decreased cell viability (Fig. 6A) in a dose-dependent manner, where isolate ZOR-Br1-2 showed the highest toxicity with IC_{50} values of 38 ± 5.6 µg/ml. Extracts with IC_{50} values of 2–89 µg/ml are categorized as a moderate cytotoxic agent [22]. Based on this category, in addition to isolate ZOR-Br1-2, endophytic isolate ZOR-S1-4, *D. anthuriicola* ZOR-Rh1-3, and *P. citrinum* ZOR-S1-4.1 also have moderate cytotoxicity (Table 4). We observed similar results when we treated the non-TNBC cells, MCF-7 cells, with the fungal extracts at the same series of concentrations. Samples treatment showed a dose-dependent manner toward cell viability (Fig. 6B), and isolate ZOR-Br1-2 was also the most potent sample with an IC_{50} value of 14 ± 3.9 µg/ml**.** It was followed by the activity of isolate ZOR-S1-4,

D. anthuriicola ZOR-Rh1-3, *S. terrestris* ZOR-Br1-1, and *P. citrinum* ZOR-S1-4.1 as shown in Table 4. However, the MCF-7 cells were more sensitive than 4T1 cells as indicated by the lower IC_{50} value of samples on those tested cells. Other samples showed lower cytotoxicity with IC_{50} values of more than 100 µg/ml. Moreover, we also checked the samples' effect on normal epithelial cells using the Vero cell lines, a normal kidney cell, to evaluate the selectivity of the samples. The top four potent samples, fungal isolates ZOR-Br1-2 and ZOR-S1-4, *D. anthuriicola* ZOR-Rh1-3, and *P. citrinum* ZOR-S1-4.1, have a selectivity index (SI) of 12, 8, 3, and 3, respectively (Fig. 6C), indicating that they are selective to cancer cells. A SI of more than three indicates high selectivity in cancer cells compared to noncancerous cells [23].

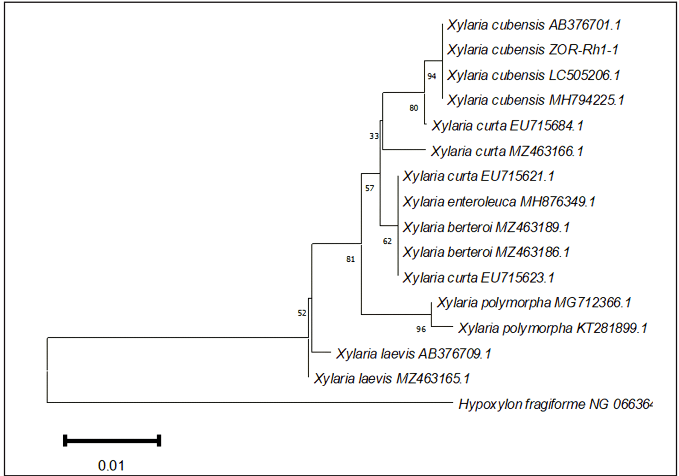

Figure 4. Phylogenetic analysis of *X. cubensis* ZOR-Rh1-1 through the region of LSU comparison, using the neighbor-joining algorithm with 1,000 bootstraps.

DISCUSSION

Medicinal plants serve as a valuable reservoir of endophytic fungi with the ability to produce a wide variety of bioactive substances. Diversity and species richness of endophytic fungi among the same host plant species are primarily shaped by the geographical site and environmental factors of the plant's habitat, such as humidity, temperature, and sunlight intensity [2]. This implies that distinct endophytic fungi can be isolated even from the same medicinal plants that have been investigated before. In our study, we identified different endophytic fungal species in contrast to earlier studies that also isolated endophytic fungi from red ginger. Several endophytic fungi across various genera were reported to be associated with red ginger, for instance: *Acremonium, Cochliobolus,*

Table 2. Preliminary detection of phytochemical groups from methanolic extracts of endophytic fungi isolated from red ginger.

Fungal methanolic extract	Phytochemical groups				
	Alkaloid	Terpenoid	Polyphenol	Flavonoid	Saponin
M.colombiense ZOR-S1-1	+	+	−	−	−
P. flavidoalba ZOR-S1-3	+	+	−	−	−
Isolate ZOR-S1-4	+	+	−	−	−
P. citrinum ZOR-S1-4.1	+	+	+	−	−
X. cubensis ZOR-Rh1-1	+	−	−	−	−
D. anthuriicola ZOR-Rh1-3	+	+	+	−	−
S. terrestris ZOR-BR1-1	+	+	+	−	−
Isolate ZOR-BR1-2	+	+	+	−	−

+/- = denote whether the corresponding phytochemical group is present or absent.

Table 3. Antimicrobial activity of methanolic extracts of red ginger-derived endophytic fungi.

Methanolic extracts of endophytic fungi	MIC[a] (µg/ml)				
	S. aureus ATCC 6538	MRSA ATCC 3351	*S. epidermidis* ATCC 12228	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231
M. colombiense ZOR-S1-1	1,000	>1,000	>1,000	>1,000	>1,000
P. flavidoalba ZOR-S1-3	500	>1,000	>1,000	>1,000	>1,000
Isolate ZOR-S1-4	1,000	>1,000	>1,000	>1,000	>1,000
P. citrinum ZOR-S1-4.1	500	500	500	500	>1,000
X. cubensis ZOR-RH1-1	1,000	>1,000	>1,000	>1,000	>1,000
D. anthuriicola ZOR-RH1-3	62.5	>1,000	500	>1,000	>1,000
S. terrestris ZOR-BR1-1	31.3	>1,000	125	>1,000	15.6
Isolate ZOR-BR1-2	62.5	>1,000	500	>1,000	1,000

[a]MIC = minimum inhibitory concentration.

Colletotrichum, Curvularia, Fusarium, Glomerella [16], and *Aspergillus* [17].

In the present study, we isolated eight fungal strains, six of which were identified as *M. colombiense*, *P. flavidoalba, P. citrinum, D. anthuriicola, S. terrestris*, and *X. cubensis*. *Xylaria cubensis* was identified through the analysis of domain D1/D2 of the LSU region. While the ITS region is regarded as the standard barcode in fungal identification [24], the level of variability of this region is not uniform across all fungal species [25]. Apart from the ITS, the D1/D2 domain is also useful in the determination of fungal species based on DNA sequences [26]. It is proven in this study that fungal identification could be achieved by analyzing the D1/D2 region, particularly for the molecular identification of isolate ZOR-Rh1-1.

Endophytic fungi isolated from red ginger in the present study have been also previously reported from different host plants. *Microdochium colombiense* was formerly investigated for being an endophyte in *Musa sapientum* [27]. *Phlebiopsis flavidoalba* was reported as an endophyte associated with *Gastrodia elata* (Orchidaceae) [28]. Endophytic *P. citrinum*

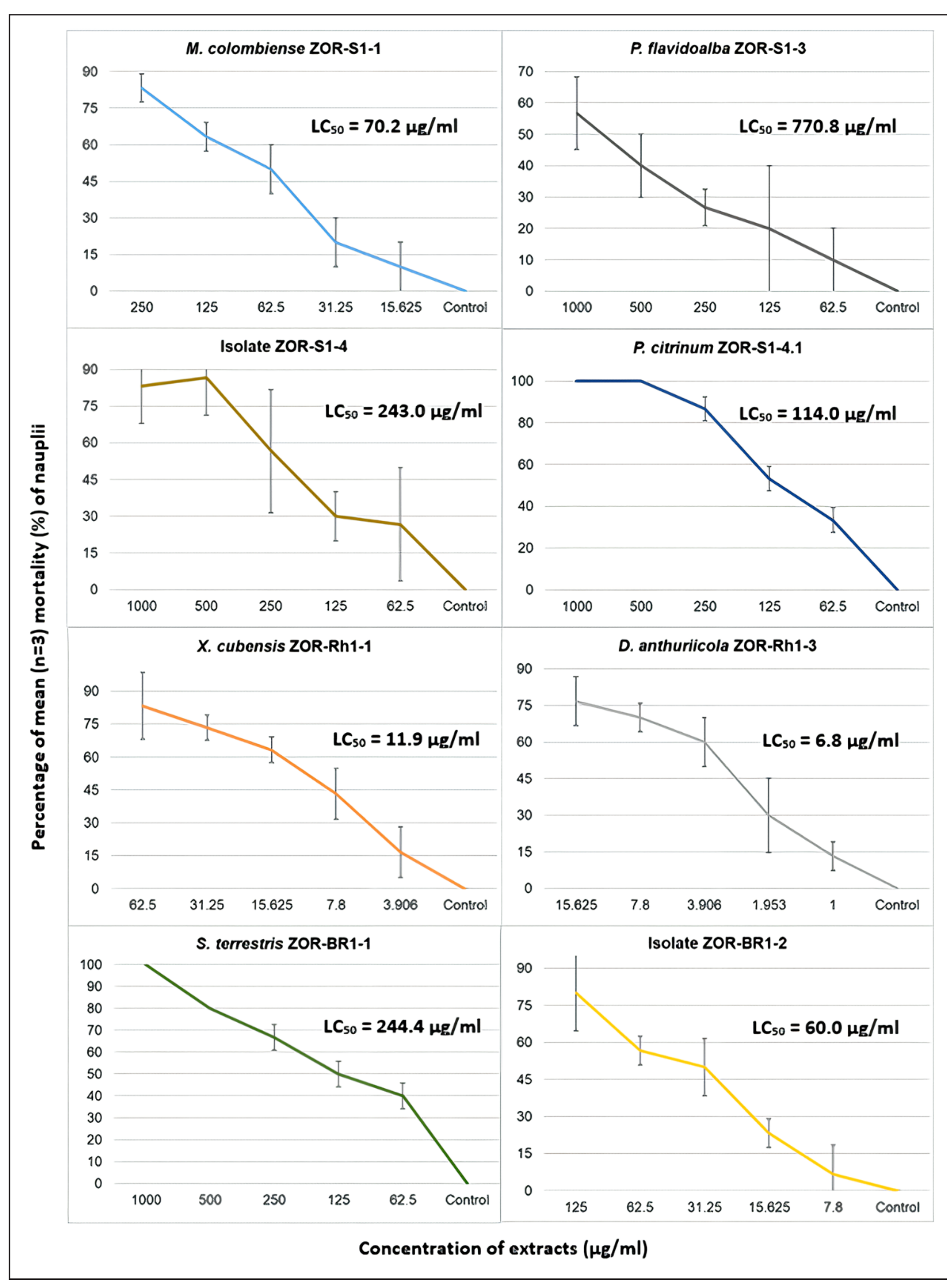

Figure 5. The result toxicity assay through brine shrimp lethality test, showing the relationship between mean mortality percentage ($n = 3$) of nauplii (y axis) at each tested concentration of fungal methanolic extracts (µg/ml) (x axis). LC_{50} (µg/ml) of each methanolic extract is included in each graph.

Table 4. IC_{50} values (µg/ml) of the tested fungal extracts against 4T1, MCF-7, and Vero cell lines along with their SI.

Fungal methanolic extract	IC_{50} (µg/ml, average ± SD)			SI
	4T1	MCF-7	Vero	
M. colombiense ZOR-S1-1	405 ± 10.4	357 ± 10.9	>500	1
P. flavidoalba ZOR-S1-3	121 ± 12.0	103 ± 10.8	>500	3
Isolate ZOR-S1-4	54 ± 6.3	33 ± 6.2	>500	8
P. citrinum ZOR-S1-4.1	74 ± 6.4	73 ±1.6	201 ± 3.8	3
X. cubensis ZOR-Rh1-1	256 ± 10.5	250 ±7.7	>500	1
D. anthuriicola ZOR-Rh1-3	56 ± 7.3	37 ± 1.1	231 ± 2.6	3
S. terrestris ZOR-BR1-1	>500	39 ± 0.3	>500	1
Isolate ZOR-BR1-2	38 ± 5.6	14 ± 3.9	>500	12

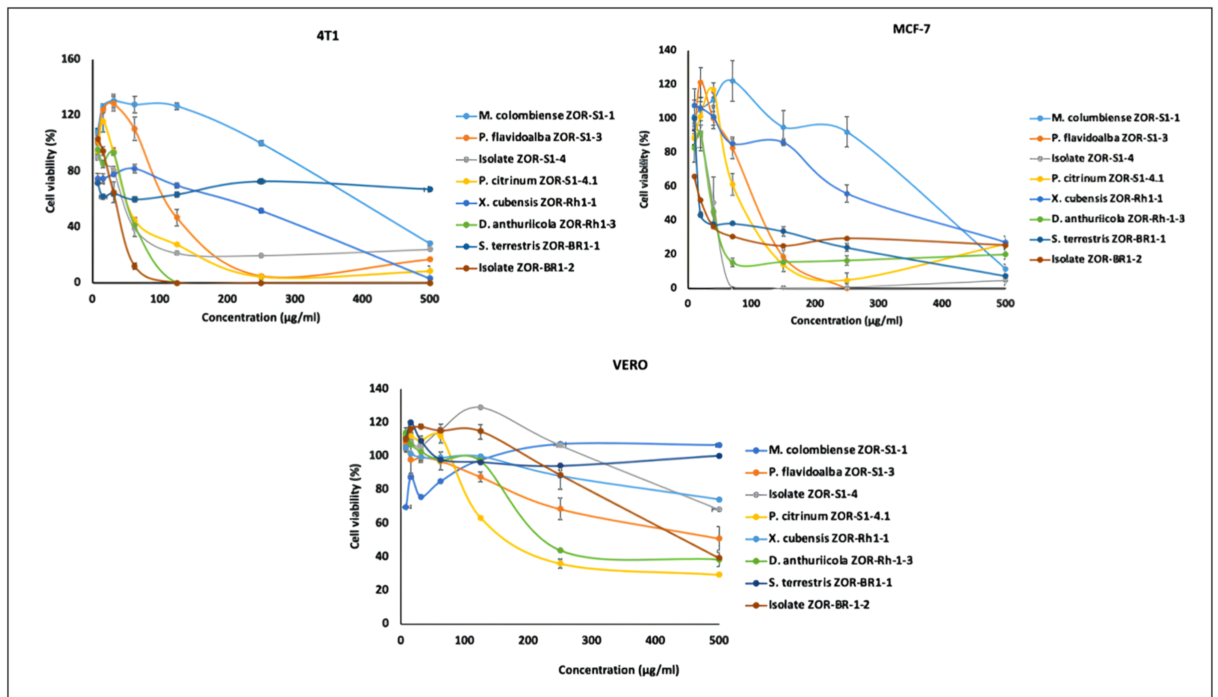

Figure 6. Cytotoxicity of the metabolites against 4T1, MCF-7, and Vero cells. Cells were grown in a 96-well plate as described in the Materials and Methods. Cells were treated with metabolites at 5–500 µg/ml and incubated for 24 hours. The availability of the cells was analyzed using CCK-8 kit assay and then calculated based on their absorption at 490 nm.

was repeatedly isolated from a wide range of hosts, such as *Triticum aestivum* [29], *Jatropha heynei* [30], *Cephalotaxus mannii* [31], and *Rhodomyrtus tomentosa* [32]. *Dactylonectria anthuriicola* was previously isolated from *Anthurium* sp. [33]. *Setophoma terrestris* was found to be associated with the inner tissue of *Brassica oleracea* var. acephala [34], *Psidium guajava* [35], and *Armoracia rusticana* [36]. Meanwhile, *X. cubensis* was reported as an endophytic fungus found in *Litsea akoensis* [37], *Lychnophora ericoides* [38], and *Asimina triloba* [39]. It is the first record of these endophytic fungi being isolated from red ginger.

Some isolated fungi in this study were previously shown to have antimicrobial and cytotoxic potential. *Setophoma terrestris* obtained from the leaves litter in the mangrove ecosystem produced secalonic acid A, penicillixanthone A, and hypothemycin, which displayed remarkable cytotoxic activities against colon and melanoma cell lines with IC_{50} values starting from 0.2 to 2.1 µM. In addition, secalonic acid G and blennolide J were also isolated from this fungal strain and exhibited antimicrobial activity against *S. aureus* with MIC values of 39 and 43 µg/ml, respectively [40]. Moreover, co-cultivation of *S. terrestris* with *Bacillus amyloliquifaciens* led to the isolation of a novel compound, blennolide K, which was found to be active against MCF-7 cells with an IC_{50} value of 4.8 µM [41]. *Penicillium citrinum* isolated from a hydrothermal vent was revealed to produce citrinin together with its derivatives, which served as promising leads in the development of α-glucosidase and ATP-citrate lyase inhibitory agents [42]. New citrinin derivatives, peniciriols A and B, were isolated from *P. citrinum* of *Rhodomyrtus tomentosa,* but did not show any cytotoxic

and antibacterial activity [32]. Penicitrinols C and E from *P. citrinum* found in a sediment estuary in China, showed weak cytotoxic activities toward HL-60 cancer cells [43]. Secalonic acid A from *P. citrinum* of *Tapiscia sinensis*, was revealed to have cytotoxicity against an array of cancer cell lines with IC_{50} values ranging from 11–25 µg/ml [44].

Several investigations on *Microdochium* revealed that fungal strains from this genus could produce brocaeloids, cillifuranone, leptosphaeric acid, 15β-hydroxyl-(22E,24R)-ergosta-3,5,8,22-tetraen-one, (22E,24R)-6β-methoxyergosta-7,22-diene-3β,5α-diol [45], isocoumarins [46], and sesquiterpenes [47], with broad-ranging bioactivities, including anticancer, antimycobacterial [45], antifungal, antibacterial, and antialgal [46]. Furthermore, some bioactive compounds were formerly reported for being isolated from the species of *Phlebiopsis*, including hydroxystrepantibin D, *p*-terphenyls derivatives [48], phlebiopsin A–C, methyl-terfestatin A, and *o*-orsellinaldehyde [49], which showed antioxidant [48] and antifungal [49] activities.

Dactylonectria was reported to produce brefeldins A and C, 7-dehydrobrefeldin A, methyl tetradecanoate, anthraquinone ZSU-H85, and (3β,5α,6β,22E)-ergosta-7,22-diene-3,5,6-triol [50]. Moreover, *X. cubensis* was previously reported to produce polyhydroxyanthraquinones [39], succinic acid derivatives, mellein derivatives, cytochalasin D, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, isosclerone [51], sesquiterpenoids, aliphatic derivative, alkaloids, and isocoumarins [37]. Cytochalasin D showed cytotoxicity toward KB cells with an IC_{50} value of 3.99 µg/ml. Meanwhile, compound 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene1,4-dione displayed mild growth inhibition against *S. aureus* and MRSA with MIC values of 128 µg/ml [51].

Based on the cytotoxicity assay of the fungal extracts in our study, we observed that although 4T1 cells and MCF-7 cells showed similar responses toward samples, the non-TNBC cells (MCF-7) cells were more sensitive than the TNBC cells (4T1 cells). In cancer therapy, TNBC and non-TNBC, which express estrogen receptors (ERs) and progesterone receptors (PRs), have gained much attention. Recent clinical studies have demonstrated the effectiveness of anti-endocrine drugs in treating HR-positive breast cancer. However, TNBC lacks an appropriate therapeutic target, and chemotherapy is the primary treatment option [52]. Conventional approaches to combating those problems have proven inadequate. Chemotherapy has drawbacks, especially when it comes to side effects that may reduce a patient's quality of life. Lack of appetite, nausea, vomiting, constipation, and hair loss are a few of the side effects that chemotherapy patients frequently encounter [53].

It is well-known that the TNBC cells were characterized by lacking expression or no amplification of estrogen receptor, progesterone receptor, and HER-2. Therefore, TNBC also lacks targeted therapy. In the clinic, TNBC exhibits more aggressive cells, often relapses, short progression-free survival, and overall survival (OS) compared to non-TNBC. In addition, certain mutations including *p53* and *PIK3CA*, increased expression of *HIF1-α* and *MYC*, amplification of *cyclin E1*, and loss of *PTEN* and *RB1* have been associated with chemo-resistance of the TNBC patients [54]. On the other hand, MCF-7 is a model of ER^+/PR^+ cells that can be targeted via those receptors. The different sensitivities of 4T1 and MCF-7 cells (Table 4) in our study may indicate that the samples' mechanism of action may involve more of the ER signaling. However, the IC_{50} values of the top four of the samples (unidentified fungal isolates ZOR-Br1-2 and ZOR-S1-4, *D. anthuriicola* ZOR-Rh1-3, and *P. citrinum* ZOR-S1-4.1) against 4T1 cells are relatively low (less than 100 µg/ml), giving a promising indication to be developed further. This includes the isolation of the main compound and characterization of the compound to seek a new candidate for breast cancer therapy, particularly the TNBC type.

CONCLUSION

Eight endophytic fungal strains, identified as *M. colombiense* ZOR-S1-1, *P. flavidoalba* ZOR-S1-3, *P. citrinum* ZOR-S1-4.1, *Dactylonectria anthuriicola* ZOR-Rh1-3, *S. terrestris* ZOR-Br1-1, *X. cubensis* ZOR-Rh1-1, including two unidentified isolates ZOR-S1-4 and ZOR-Br1-2, were isolated from red ginger. Extract of *S. terrestris* ZOR-Br1-1 was found to have the most substantial inhibition toward the growth of *S. aureus* and *C. albicans* with MIC of 31.3 and 15.6 µg/ml. Meanwhile, in the toxicity screening, *D. anthuriicola* ZOR-Rh1-3 extract revealed the most potent toxicity with the LC_{50} value of 6.8 µg/ml. When subjected to cytotoxicity testing on breast cancer cells MCF-7 and 4T1, extracts of *D. anthuriicola* ZOR-Rh1-3, *P. citrinum* ZOR-S1-4.1, unidentified isolates ZOR-S1-4 and ZOR-Br1-2, showed strong to moderate activity with IC_{50} values starting from 14 to 74 µg/ml. Given their bioactivity potentials, further studies of *S. terrestris* ZOR-Br1-1, *D. anthuriicola* ZOR-Rh1-3, and *P. citrinum* ZOR-S1-4.1 extracts on their antimicrobial and cytotoxic metabolites hold significance in the bioprospecting of endophytic fungi.

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AUTHORS CONTRIBUTIONS

Conceptualization and research design are done by NPA. Data acquisition, analysis, and interpretation are done by NPA, IPYAP, NPEK, NN, RIJ, and EM. Drafting and revision of the manuscript, as well as a review of the final version of the manuscript, are done by NPA, IPYAP, NPEK, NN, RIJ, and EM. Funding acquisition is done by NPA, NPEK, and RIJ. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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