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# Preliminary Antibacterial Activity of Crude Extracts of Endophytic Fungi from a Medicinal Plant against Four Multidrug-Resistant Bacterial Strains

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**ABSTRACT**

*Multidrug-resistant (MDR) bacteria pose significant challenges to infection treatment, highlighting the need for new antibacterial agents. This study isolated endophytic fungi from the leaves, stems, and roots of Zingiber zerumbet collected in North Sumatra, Indonesia, and evaluated their antibacterial activity against four MDR bacterial strains: methicillin-resistant Staphylococcus aureus (MRSA), ESBL-producing Escherichia coli, MDR Klebsiella pneumoniae, and MDR Pseudomonas aeruginosa. Eight fungal isolates (EF1–EF8) were obtained and tentatively identified through morphological and microscopic characteristics. Ethyl acetate extracts were screened using agar well diffusion, while minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by broth microdilution. Isolate identity significantly influenced inhibition zones against all tested bacteria ($p < .001$). EF1, tentatively identified as Aspergillus sp., demonstrated the strongest activity, followed by EF7 (Nigrospora sp.). EF1 exhibited MIC values of 12.5–50 µg/mL and MBC values of 25–100 µg/mL, with the strongest activity against MRSA. Phytochemical screening indicated the presence of alkaloids, flavonoids, terpenoids, saponins, and steroids. These findings suggest that endophytic fungi associated with Z. zerumbet represent potential sources of antibacterial metabolites. However, molecular identification, compound characterization, cytotoxicity assessment, and additional experimental controls are required to confirm their antibacterial potential.*

***Keywords*** *: Endophytic Fungi, Zingiber Zerumbet, Multidrug-Resistant Bacteria, Antibacterial Activity*


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## INTRODUCTION

Antimicrobial resistance (AMR) is one of the most pressing global health challenges of the twenty-first century. The Antimicrobial Resistance Collaborators (2022) estimated that bacterial AMR was directly responsible for 1.27 million deaths and associated with 4.95 million deaths worldwide in 2019. The GBD 2021 Antimicrobial Resistance Collaborators (2024) estimated 1.14 million attributable and 4.71 million associated deaths in 2021, and forecast that 1.91 million deaths attributable to AMR and 8.22 million deaths associated with AMR could occur globally in 2050. The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are of particular concern because they combine high virulence with a capacity to acquire resistance to multiple antibiotic classes (Miller & Arias, 2024). The World Health Organization (WHO) Bacterial Priority Pathogens List 2024 places carbapenem-resistant *A. baumannii* and third-generation cephalosporin-resistant and carbapenem-resistant Enterobacterales in the critical priority group, and methicillin-resistant *S. aureus* (MRSA) and carbapenem-resistant *P. aeruginosa* in the high priority group (WHO, 2024b). At the same time, the antibacterial development pipeline remains small and contains few innovative agents (WHO, 2024a), so the search for structurally diverse antibacterial compounds remains an urgent scientific priority.

Endophytic fungi are microorganisms that colonize living plant tissues internally without causing apparent harm to the host, and they are widely studied as producers of bioactive secondary metabolites (Fadiji & Babalola, 2020; Bansal et al., 2026). Because endophytes live in close association with their hosts, they have been reported to produce metabolites with antimicrobial, antioxidant, or anticancer properties (Fadiji & Babalola, 2020). It has been proposed that the endophytes of medicinal plants may be an especially productive source of such metabolites (Mamangkey et al., 2024). This proposal motivates the present work but was not tested here, because no non-medicinal comparator plant and no host-plant extract were included.

Recent studies illustrate the antibacterial potential of endophytic fungi. Ababutain et al. (2021) isolated six endophytic fungi from the stems of *Artemisia sieberi* and tested them against nine pathogenic bacteria; the extract of the most active isolate was more active against Gram-positive than Gram-negative bacteria. Elkady et al. (2022) isolated an endophytic *Aspergillus niger* from *Opuntia ficus-indica* fruit peels and identified benzaldehyde derivatives and cristatumin B as active compounds against multidrug-resistant (MDR) bacteria. Fanele and Ndlovu (2023) reported that *Alternaria alternata* and *Nigrospora oryzae* isolated from the medicinal plant *Sclerocarya birrea* were active against Gram-positive and Gram-negative MDR clinical isolates, and Nouh et al. (2024) reported that tannic acid produced by *Penicillium oxalicum*, isolated from *Opuntia ficus-indica* cladodes, inhibited virulence and quorum sensing in extensively drug-resistant *P. aeruginosa*. Reviews have also summarized the classes of endophytic fungal metabolites that are active against MDR bacteria, including MRSA (Bansal et al., 2026; Mamangkey et al., 2024). Studies published in 2024–2026 have further expanded this evidence base, including several from Indonesia. Yulianis et al. (2024) isolated 19 endophytic fungi from the medicinal tree fern *Cyathea contaminans* collected in Kerinci, Jambi, Indonesia; four isolates (CBK3, CDK1, CDK4, CAK2) inhibited *S. aureus*, MRSA, and

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*Escherichia coli* with zones of 15.08–23.52 mm, and were identified molecularly as *Paecilomyces subglobosus*, *Penicillium citrinum*, and *Aspergillus terreus*. Satriawan et al. (2025) obtained 11 endophytic fungi from *Orthosiphon aristatus*; isolate OAD1, identified as *Aspergillus niger*, produced a 12.57 ± 1.09 mm inhibition zone against MRSA. Putri (2026) reported that 23 of 24 endophytic fungal isolates from *Zingiber zerumbet* rhizomes inhibited MRSA with zones of 12.55–27.91 mm, and identified isolate JRT5 as *Phanerochaete* sp. Beyond Indonesia, Swandiny et al. (2025) identified dicerandrol C from *Diaporthe longicolla* associated with *Salacia intermedia*, with a minimum inhibitory concentration (MIC) of 1.04 μM against MRSA outperforming tetracycline and broad-spectrum activity against MDR *P. aeruginosa*, *K. pneumoniae*, and *E. coli*. Banji-Onisile et al. (2026) reported that *Aspergillus fumigatus* from *Terminalia phanerophlebia* had an MIC of 15.6 μg/mL against MRSA. Mishra et al. (2026) reviewed the chemical diversity of endophytic fungal secondary metabolites—alkaloids, terpenoids, steroids, polyketides, and flavonoids—and emphasized that many endophytic fungi remain unexplored for antimicrobial drug discovery.

The four test strains were chosen as representatives of resistant pathogens of clinical concern: MRSA, *K. pneumoniae*, and *P. aeruginosa* are ESKAPE organisms, and extended-spectrum β-lactamase (ESBL)-producing *E. coli* represents third-generation cephalosporin-resistant Enterobacterales, a critical-priority group in the WHO list (WHO, 2024b). *E. coli* is not an ESKAPE organism, and carbapenem-resistant *A. baumannii*, a critical-priority pathogen, was not tested.

Despite this accumulating evidence, the endophytic fungi of *Zingiber zerumbet* have not been examined against the four MDR clinical strains used in the present study (MRSA, ESBL-producing *E. coli*, MDR *K. pneumoniae*, and MDR *P. aeruginosa*). The only available endophyte study on *Z. zerumbet* (Putri, 2026) tested activity against MRSA only, did not include Gram-negative MDR strains, and did not report MIC, minimum bactericidal concentration (MBC), or dose–response data. No study has compared the potency of *Z. zerumbet* endophytes across Gram-positive and Gram-negative MDR pathogens or profiled their phytochemistry in relation to activity. This gap is addressed here. We hypothesized that (i) the isolates differ in antibacterial potency and (ii) the Gram-positive strain (MRSA) is more susceptible than the three Gram-negative strains. The objectives were to (1) isolate endophytic fungi from the leaf, stem, and root of *Zingiber zerumbet* (L.) Roscoe ex Sm. (Zingiberaceae) and characterize them morphologically; (2) determine which isolate is most potent against four MDR bacterial strains by agar well diffusion; (3) assess whether susceptibility differs between the Gram-positive strain and the three Gram-negative strains; and (4) determine the dose–response relationship, MIC, MBC, and preliminary phytochemical profile of the most active isolate(s).

## METHODS

Healthy leaf, stem, and root samples of *Zingiber zerumbet*(L.) Roscoe ex Sm. (Zingiberaceae) were collected from a local cultivation site in Medan, North Sumatra, Indonesia, and a voucher specimen (no. ZR-2024-017) was deposited at Herbarium Bogoriense. Tissues were cut into 0.5 × 0.5 cm segments, surface-sterilized sequentially in 70% ethanol (1 min), 5.3% sodium hypochlorite (3 min), and 70% ethanol (30 s), rinsed three times in sterile distilled water, plated onto Potato Dextrose

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Agar (PDA) supplemented with chloramphenicol (100 mg/L), and incubated at 27–28 °C for 5–14 days; emerging hyphal tips were sub-cultured onto fresh PDA to obtain pure isolates, and eight morphologically distinct isolates (EF1–EF8) were retained and stored in 20% glycerol at −80 °C. Isolates were tentatively identified to genus level on the basis of macroscopic colony characteristics and microscopic features using standard mycological keys (Barnett & Hunter, 1998; Pitt & Hocking, 2009); because identification was morphology-based only and internal transcribed spacer (ITS) sequencing was not completed, all genus names are tentative.

Each isolate was fermented in Potato Dextrose Broth (150 mL) at 27 °C and 120 rpm for 14 days, and the culture filtrate was extracted three times with an equal volume of ethyl acetate; the combined organic layer was dried over anhydrous $Na_2SO_4$ and concentrated under reduced pressure at 40 °C to yield the crude extract, which was dissolved in dimethyl sulfoxide (DMSO) for bioassay (stock solution 100 mg/mL; two-fold dilutions of the stock were prepared for the dose–response test).

Four MDR clinical isolates were used as test organisms: methicillin-resistant *Staphylococcus aureus*(MRSA), extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, MDR *Klebsiella pneumoniae*, and MDR *Pseudomonas aeruginosa*, one isolate per species, with inocula prepared at 0.5 McFarland standard. Antibacterial screening was performed by agar well diffusion on Mueller–Hinton agar; wells of 6 mm diameter were loaded with 50 µL of crude extract (100 mg/mL in DMSO, i.e., 5 mg of extract per well), DMSO alone served as the negative control, vancomycin (MRSA) or meropenem (Gram-negative strains) discs served as positive controls, plates were incubated at 37 °C for 24 h, and the inhibition zone diameter was recorded in millimeters as the net zone (total clear-zone diameter minus the 6 mm well diameter, so that 0 mm denotes no inhibition), with each isolate–bacterium combination tested in triplicate.

EF1 and EF7, the two most active isolates in the screening, were selected for MIC and MBC determination, and EF1 was used for the dose–response test; dose–response testing by agar well diffusion used extract concentrations of 6.25, 12.5, 25, 50, and 100 mg/mL (50 µL per well) against all four strains, referring to the stock solution loaded into the well, while the MIC was determined by broth microdilution in 96-well plates using two-fold serial dilutions of the extract over the range 0.78–100 µg/mL (µg of crude extract per mL of broth) and the MBC was determined by sub-culturing clear wells onto Mueller–Hinton agar; because the agar-well concentrations (mg/mL, stock solution) and the broth MIC/MBC values (µg/mL, in broth) are expressed on different bases, they are not directly comparable (1 mg/mL = 1,000 µg/mL).

The EF1 and EF7 crude extracts were qualitatively screened for alkaloids, flavonoids, terpenoids, saponins, tannins, and steroids using standard reagents, and results were recorded as present (+) or absent (−). Inhibition zone data ($n$= 3) are expressed as mean ± standard deviation (SD); one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test ($\alpha$ = .05) was used to compare isolates for each bacterium, and linear regression of zone diameter on $\log_2$ concentration was fitted for MRSA, with all analyses performed in Python (SciPy and statsmodels) and significance set at $p < .05$.

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## RESULTS

### 1. Isolation and Morphological Characterization

Eight endophytic fungal isolates (EF1–EF8) were obtained from leaf (EF1, EF2, EF6), stem (EF3, EF4, EF7), and root (EF5, EF8) tissues (Table 1). Based on colony and microscopic morphology, the isolates were tentatively assigned to eight different genera that have been reported as plant endophytes: *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, *Colletotrichum*, *Curvularia*, *Nigrospora*, and *Chaetomium*. Genera such as *Aspergillus* and *Penicillium* (Mamangkey et al., 2024) and *Nigrospora* and *Alternaria* (Fanele & Ndlovu, 2023) have been reported as endophytes of medicinal plants in earlier studies. The eight isolates were retained as distinct morphotypes based on consistent differences in colony appearance and microscopic features. Because colonization frequency, relative abundance, and diversity indices were not determined, no conclusion is drawn on community composition or on differences among tissues.

**Table 1. Tissue source and tentative genus (morphology only) of the eight endophytic fungal isolates (EF1–EF8)**

| Isolate Code | Tissue Source | Tentative Genus (morphology) |
|---|---|---|
| EF1 | Leaf | *Aspergillus* sp. |
| EF2 | Leaf | *Penicillium* sp. |
| EF3 | Stem | *Trichoderma* sp. |
| EF4 | Stem | *Fusarium* sp. |
| EF5 | Root | *Colletotrichum* sp. |
| EF6 | Leaf | *Curvularia* sp. |
| EF7 | Stem | *Nigrospora* sp. |
| EF8 | Root | *Chaetomium* sp. |

### 2. Antibacterial Screening

All eight isolates produced inhibition zones against at least one of the four MDR bacterial strains, although potency varied substantially among isolates (Table 2, Figure 1). Inhibition zone diameters are net values, i.e., the total diameter of the clear zone minus the 6 mm well diameter (see Methods), so that 0 mm denotes no inhibition; the 6 mm well size therefore does not represent an activity threshold and is not drawn in Figure 1. Isolate EF1 (tentatively *Aspergillus* sp.) produced the largest mean inhibition zones against all four bacteria (10.97–18.47 mm), followed by EF7 (*Nigrospora* sp., 9.97–15.87 mm) and EF4 (*Fusarium* sp., 7.70–13.07 mm). Isolate EF8 (*Chaetomium* sp.) produced some of the smallest inhibition zones (4.20–6.67 mm). Across all isolates, inhibition zones against the Gram-positive MRSA were larger than against the three Gram-negative species, a pattern consistent with a previous report that endophytic fungal extracts are more active against Gram-positive than Gram-negative bacteria (Ababutain et al., 2021).

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**Table 2. Inhibition zone diameter (mm, mean ± SD, *n* = 3) of the crude extracts of isolates EF1–EF8 against four multidrug-resistant bacterial strains by agar well diffusion**

| Isolate | MRSA (mm) | ESBL *E. coli* (mm) | MDR *K. pneumoniae* (mm) | MDR *P. aeruginosa* (mm) |
|---|---|---|---|---|
| EF1 | 18.47 ± 0.25 | 12.53 ± 0.64 | 12.77 ± 0.40 | 10.97 ± 0.40 |
| EF2 | 10.33 ± 0.55 | 8.67 ± 0.74 | 6.33 ± 0.55 | 6.87 ± 0.55 |
| EF3 | 6.97 ± 0.75 | 6.03 ± 0.64 | 5.87 ± 0.40 | 4.53 ± 1.20 |
| EF4 | 13.07 ± 0.76 | 9.33 ± 0.15 | 8.53 ± 0.35 | 7.70 ± 1.10 |
| EF5 | 6.10 ± 0.10 | 5.00 ± 0.82 | 5.37 ± 0.58 | 4.20 ± 0.75 |
| EF6 | 10.13 ± 0.57 | 7.23 ± 0.59 | 7.77 ± 0.06 | 5.97 ± 0.23 |
| EF7 | 15.87 ± 0.46 | 10.53 ± 0.64 | 10.83 ± 0.61 | 9.97 ± 0.21 |
| EF8 | 6.67 ± 0.59 | 4.80 ± 0.78 | 4.20 ± 0.46 | 4.23 ± 0.15 |
| Positive control | 18.70 | 16.40 | 15.10 | 13.80 |
| Negative control (DMSO) | 0.00 | 0.00 | 0.00 | 0.00 |

*Table note: Values are mean ± SD inhibition zone diameter (mm) of triplicate assays (100 mg/mL extract, 50 µL per well). Zone diameters are net values (total clear-zone diameter minus the 6 mm well diameter); 0 mm = no inhibition. MRSA, methicillin-resistant S. aureus; ESBL, extended-spectrum β-lactamase; MDR, multidrug-resistant. Positive control: vancomycin disc for MRSA, meropenem disc for the three Gram-negative strains; negative control: DMSO.*

**3. Statistical Comparison of Isolate Potency (ANOVA)**

One-way ANOVA showed that isolate identity had a significant effect on inhibition zone diameter for every tested bacterium (Table 3), indicating that the differences among isolates in Table 2 are unlikely to be due to assay variability alone. The effect sizes were large (partial $\eta^2$ = .989 for MRSA, .959 for ESBL-producing *E. coli*, .981 for MDR *K. pneumoniae*, and .949 for MDR *P. aeruginosa*).

**Table 3. One-way ANOVA of inhibition zone diameter among the eight isolates for each bacterial strain (*n* = 3 per isolate)**

| Bacterium | df (between) | df (within) | *F*-value | *p*-value | Conclusion |
|---|---|---|---|---|---|
| MRSA (*S. aureus*) | 7 | 16 | 205.34 | < .001 | Significant |
| ESBL-producing *E. coli* | 7 | 16 | 53.17 | < .001 | Significant |
| MDR *K. pneumoniae* | 7 | 16 | 121.11 | < .001 | Significant |
| MDR *P. aeruginosa* | 7 | 16 | 42.64 | < .001 | Significant |

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Tukey's HSD post hoc test on the MRSA dataset showed that EF1 differed significantly from all seven other isolates ($p < .001$ for each comparison; the largest value, EF1 vs. EF7, was $p = .0005$). EF7 was significantly more active than EF4 ($p = .0002$) and than all other isolates except EF1, so EF1, EF7, and EF4 formed three distinct potency tiers. In contrast, several isolate pairs with similar low potency showed no significant difference, notably EF2 vs. EF6 ($p = .9998$), EF3 vs. EF5 ($p = .5477$), and EF3 vs. EF8 ($p = .9968$), indicating a cluster of weakly active isolates. The full post hoc comparison matrices for all four bacterial strains are provided in Table A1 (Appendix).

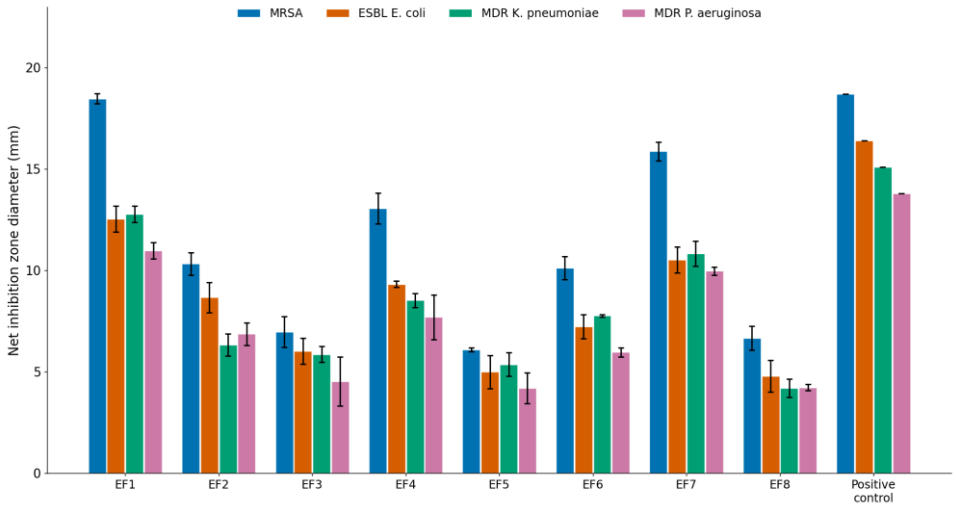



**Figure 1. Inhibition zone diameter of eight endophytic fungal isolates against four MDR bacterial strains by agar well diffusion. Bars are means and error bars are SD ($n = 3$). Zone diameters are net values (total clear-zone diameter minus the 6 mm well diameter), so 0 mm denotes no inhibition. Positive control: vancomycin (MRSA) or meropenem (Gram-negative strains) disc; negative control (DMSO): 0 mm for all strains**

### 4. Dose–Response Relationship of the Most Active Isolate

For isolate EF1, inhibition zone diameter increased with extract concentration against all four bacteria, from 5.51–10.14 mm at 6.25 mg/mL to 11.82–20.16 mm at 100 mg/mL (Figure 2). The dose–response experiment was performed on a separate fermentation batch from the screening assay, which accounts for the small numerical differences in zone diameters at 100 mg/mL between Figure 2 (11.82–20.16 mm) and Table 2 (10.97–18.47 mm); the same concentration-dependent trend was observed in both experiments. For MRSA, linear regression of inhibition zone diameter on $\log_2$-transformed concentration (five concentrations; $n = 5$ means; df = 3) was significant ($F(1, 3) = 239.6$, $p = .0006$), with $R^2 = .988$, indicating that 98.8% of the variance in the five mean zone diameters was explained by $\log_2$ concentration. With only five points, this fit should be interpreted cautiously, and dose-dependence was tested for MRSA only.

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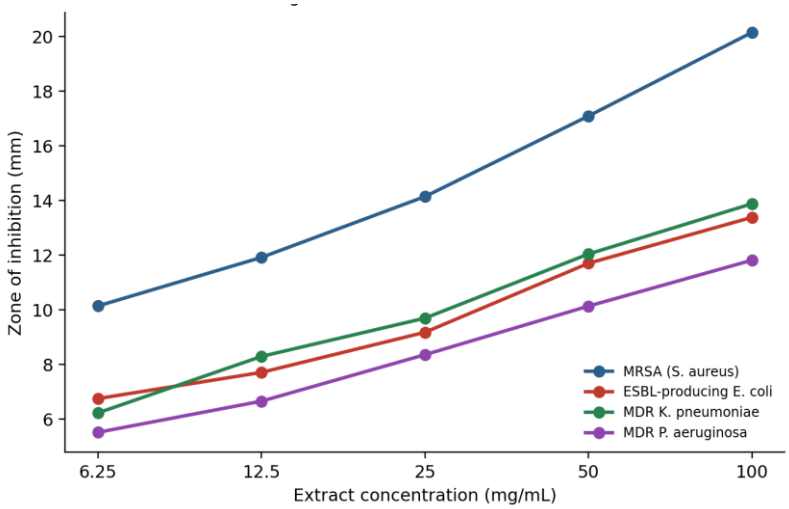




**Figure 2. Dose–response relationship of isolate EF1 crude extract against four MDR bacterial strains**

**5. Minimum Inhibitory and Bactericidal Concentrations**

MIC and MBC values for the two most active isolates, EF1 and EF7, are presented in Table 4. MIC and MBC values are expressed in µg of crude extract per mL of broth, whereas the concentrations used for agar well diffusion in the dose–response test (6.25–100 mg/mL) refer to the stock solution loaded into the wells (1 mg/mL = 1,000 µg/mL); the two scales are therefore not directly comparable. EF1 showed the lowest MIC against MRSA (12.5 µg/mL), followed by ESBL-producing *E. coli* and MDR *K. pneumoniae* (25 µg/mL each), and the highest MIC against MDR *P. aeruginosa* (50 µg/mL). EF7 required consistently higher concentrations, with an MIC of 25 µg/mL against MRSA and 50 µg/mL against all three Gram-negative species. For both isolates, the MBC was one dilution step higher than the corresponding MIC (MBC/MIC ratio = 2 in all cases), which suggests bactericidal rather than purely bacteriostatic activity under these test conditions. The MIC of a reference antibiotic was not determined, so these values cannot be benchmarked.

**Table 4. MIC and MBC (µg/mL, i.e., µg of crude extract per mL of broth) of the crude extracts of isolates EF1 and EF7 against four multidrug-resistant bacterial strains by broth microdilution; MBC/MIC is the ratio of the two values**

| Isolate | Bacterium | MIC (µg/mL) | MBC (µg/mL) | MBC/MIC ratio |
|---|---|---|---|---|
| EF1 | MRSA (*S. aureus*) | 12.5 | 25 | 2 |
| EF1 | ESBL-producing *E. coli* | 25 | 50 | 2 |
| EF1 | MDR *K. pneumoniae* | 25 | 50 | 2 |
| EF1 | MDR *P. aeruginosa* | 50 | 100 | 2 |
| EF7 | MRSA (*S. aureus*) | 25 | 50 | 2 |

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| Isolate | Bacterium | MIC (μg/mL) | MBC (μg/mL) | MBC/MIC ratio |
|---|---|---|---|---|
| EF7 | ESBL-producing *E. coli* | 50 | 100 | 2 |
| EF7 | MDR *K. pneumoniae* | 50 | 100 | 2 |
| EF7 | MDR *P. aeruginosa* | 50 | 100 | 2 |

**6. Phytochemical Screening**

Qualitative phytochemical screening of the EF1 and EF7 crude extracts is summarized in Table 5. The EF1 extract, which showed the strongest and most consistent antibacterial activity, tested positive for alkaloids, flavonoids, terpenoids, saponins, and steroids, and negative for tannins. The EF7 extract tested positive for alkaloids, flavonoids, and tannins, but negative for terpenoids, saponins, and steroids.

**Table 5. Qualitative phytochemical screening of the EF1 and EF7 crude extracts**

| Phytochemical Group | EF1 Extract | EF7 Extract |
|---|---|---|
| Alkaloids | + | + |
| Flavonoids | + | + |
| Terpenoids | + | − |
| Saponins | + | − |
| Tannins | − | + |
| Steroids | + | − |

*(+) = detected; (−) = not detected.*

## DISCUSSION

All eight isolates produced inhibition zones at a high extract loading (5 mg of crude extract per well), and isolate identity significantly affected zone diameter for every strain (one-way ANOVA). Because crude extracts at this loading are expected to show some activity, the activity of all isolates is consistent with, but does not by itself demonstrate, the potential of endophytic fungi as antibacterial sources described in earlier studies (Fadiji & Babalola, 2020; Mamangkey et al., 2024). The superior activity of isolate EF1 (tentatively *Aspergillus* sp.) is in line with reports of antibacterial metabolites from endophytic *Aspergillus* species; for example, Elkady et al. (2022) isolated an endophytic *A. niger* from *Opuntia ficus-indica* fruit peels that produced benzaldehyde derivatives and cristatumin B active against MDR bacteria, and Mamangkey et al. (2024) reviewed antibacterial metabolites from endophytic *Aspergillus* and *Penicillium* species. Because the genus assignment of EF1 is tentative and only a few examples are compared, no conclusion is drawn that *Aspergillus* is a particularly bioactive endophytic genus.

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The larger inhibition zones against the Gram-positive MRSA than against the three Gram-negative species mirror the pattern reported by Ababutain et al. (2021), who found that an endophytic fungal extract from *Artemisia sieberi* was more active against Gram-positive than Gram-negative bacteria. A possible explanation is the outer-membrane permeability barrier of Gram-negative bacteria, although this was not tested here. EF1 gave a zone against MRSA (18.47 mm) similar to that of the vancomycin disc (18.70 mm), whereas a larger gap remained against MDR *P. aeruginosa* (10.97 mm versus 13.80 mm for the meropenem disc). These comparisons are indicative only, because the extract was loaded into a well and the antibiotic was applied as a disc, and the positive-control values have no replicate statistics.

The increase in EF1 zone diameter with concentration against MRSA ($R^2$ = .988; five concentration means) is consistent with a concentration-dependent effect of the extract, but five points give limited information on potency. The MIC values (12.5–50 μg/mL) and MBC/MIC ratios of 2 suggest bactericidal activity in vitro. These MIC values are expressed per mL of broth and lie far below the lowest stock concentration used in agar well diffusion (6.25 mg/mL = 6,250 μg/mL); because the extract diffuses and is diluted in the agar, the two assays are on different bases and cannot be converted into one another, and the low MIC values of a crude extract should be confirmed by repeat determination alongside a reference antibiotic. MIC values of crude extracts depend on the medium, inoculum, solvent, and extract composition and therefore cannot be benchmarked against other studies on an equal basis without a reference-antibiotic MIC determined under the same conditions.

The phytochemical screening offers a plausible, though preliminary, explanation for the difference in potency between EF1 and EF7. The presence of terpenoids, saponins, and steroids in the EF1 extract, which are metabolite classes often implicated in membrane-disrupting and antibacterial activity, together with alkaloids and flavonoids shared by both extracts, suggests that the activity of EF1 may reflect a broader secondary metabolite profile rather than a single dominant compound class. Antibacterial activity of endophytic fungi is often attributed to a combination of interacting metabolites (Fadiji & Babalola, 2020). These qualitative tests, however, have limited specificity; extraction with ethyl acetate alone favors medium-polarity metabolites, which makes the detection of saponins and tannins in these extracts worth confirming; and compounds from the culture medium could react in the tests. Bioassay-guided fractionation, as used by Elkady et al. (2022) and Nouh et al. (2024), would be required to identify the metabolites responsible for the activity of EF1.

These findings are preliminary: agar diffusion and MIC data on crude extracts against four strains cannot establish therapeutic potential, and no cytotoxicity or selectivity data are available. They support further screening of endophytic fungi from *Zingiber zerumbet* and other locally available plants, followed by identification of the active compounds. MRSA, *K. pneumoniae*, and *P. aeruginosa* are ESKAPE pathogens (Miller & Arias, 2024), whereas *E. coli* is not an ESKAPE organism, and *A. baumannii* was not tested.

This study has several limitations. First, fungal identification relied on morphology alone; ITS sequencing was not completed, so all genus assignments are tentative and the isolates should be

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confirmed molecularly before further use. Second, epiphytic contaminants cannot be entirely excluded because no dedicated surface-sterilization control was included, and the absence of a medium-blank control means that a contribution from culture-medium components to the phytochemical tests cannot be formally excluded. Third, only the culture filtrate was extracted, with a single solvent, so mycelium-bound metabolites were not sampled. Fourth, the extracts are crude mixtures, the responsible metabolites are unidentified, and no cytotoxicity or time-kill data were collected. Fifth, no reference-antibiotic MIC was determined, the positive control was a disc rather than a well, and only one clinical strain per species was tested. Finally, the host plant was documented by voucher specimen and sampling site, but no non-medicinal comparator plant or host-plant extract was included, so the proposal that medicinal-plant endophytes are an especially productive metabolite source was not tested here.

## CONCLUSIONS

Eight endophytic fungal isolates were obtained from the leaf, stem, and root tissues of *Zingiber zerumbet*. Their crude ethyl acetate extracts produced inhibition zones against four multidrug-resistant bacterial strains, and isolate identity significantly affected zone diameter for every strain. Isolate EF1, tentatively identified as *Aspergillus* sp. on morphology, was the most potent isolate, with MIC values of 12.5–50 μg/mL, MBC/MIC ratios of 2, and the strongest activity against MRSA; MRSA was more susceptible than the three Gram-negative strains in agar well diffusion. These results are preliminary evidence of antibacterial activity in crude extracts. They are limited by morphology-only identification, the absence of dedicated sterilization-check and medium-blank controls, the use of four strains, and the lack of compound identification and cytotoxicity data.

ITS-based molecular confirmation of isolate identity, together with sterilization-check and medium-blank controls, is a prerequisite for further work. Future research should then include chromatographic fractionation and structural elucidation (e.g., by liquid chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy) of the EF1 extract, testing across additional clinical MDR isolates of each species, cytotoxicity testing, and in vivo evaluation of efficacy and safety before any translational application is considered.

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