ENDOPHYTIC FUNGI FROM *PERSICARIA HYDROPIPER* PRODUCE METABOLITES WITH SELECTIVE ANTIBACTERIAL AND CELL-PROLIFERATIVE ACTIVITIES

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Abstract. *Six endophytic fungal isolates were obtained from healthy tissues of Persicaria hydropiper L., and the root-derived isolate 8i3k was selected for detailed biological investigation. The crude ethyl acetate extract exhibited selective antibacterial activity against Gram-positive bacteria, with inhibition zones of 17.0 ±0.2 mm against Bacillus subtilis and 12.0 ±0.1 mm against Staphylococcus aureus, and no activity against Gram-negative bacteria or Candida albicans. Minimum inhibitory concentrations were determined as 312.5 µg/mL (B. subtilis) and 1250 µg/mL (Escherichia coli) by broth microdilution. MTT cytotoxicity evaluation at 100 µg/mL revealed a pro-proliferative rather than cytotoxic effect, with cell viability reaching 132.1 ± 6.9% in HEp-2 and 117.5 ± 6.0% in A549 cells. These findings demonstrate that isolate 8i3k produces secondary metabolites with selective antibacterial and cell-stimulatory activities, potentially linked to the traditional wound-healing properties of the host plant. Further compound purification and molecular identification of the isolate are warranted.*

Keywords: *endophytic fungi; Persicaria hydropiper; secondary metabolites; antimicrobial activity; cell proliferation; MTT assay; natural products.*

1. Introduction

Medicinal plants represent an important reservoir of natural compounds with diverse and biologically significant activities. Nevertheless, the accumulation of these metabolites in plant tissues is often limited, making large-scale extraction challenging. In this context, endophytic microorganisms associated with medicinal plants have gained increasing scientific attention. Medicinal plants possess a distinct microbiome, and their endophytes are known to produce unique, structurally diverse, and biologically active secondary metabolites. Consequently, endophytes of medicinal plants are considered promising sources of novel bioactive compounds for pharmaceutical and agricultural applications and provide valuable opportunities for the discovery of new microorganisms with significant biotechnological potential. Therefore, the isolation of endophytic fungi associated with the medicinal plant *Persicaria hydropiper* and the evaluation of the biological activities of secondary metabolites produced by these fungal isolates are of significant scientific and practical importance.

Endophytic fungi are recognized as important components of plant-associated microbiomes, contributing to plant growth, metabolic regulation, and defense against pathogens, herbivores, insects, and other environmental challenges. Their ability to synthesize diverse secondary metabolites with potential biological activities has stimulated increasing global interest in their study and utilization, particularly in pharmaceutical and agricultural biotechnology.

Comprehensive studies have demonstrated that endophytic fungi are promising reservoirs of biologically active compounds with significant therapeutic and protective potential for both human health and plant protection under adverse environmental conditions. Secondary metabolites obtained from endophytes exhibit a broad spectrum of biological activities, including antimicrobial, antioxidant, and cytotoxic properties. In particular, the screening of antimicrobial compounds from endophytic fungi offers a promising approach to overcoming the growing global challenge of drug-resistant pathogenic microorganisms in medical and agricultural contexts.

Infectious diseases continue to represent one of the most serious global health challenges [1–3]. In 2019, 13.66 million people died globally because of infection-related causes. Most of these infection-related deaths (4.95 million) are associated with antibiotic-resistant pathogens [4]. Projections indicate that by 2050, deaths caused by antibiotic-resistant bacteria may rise substantially compared with present-day mortality rates [5]. Furthermore, various alternative strategies are increasingly being employed in the search for novel antibacterial compounds. These strategies include the exploration of uncultured microorganisms [6], *in silico* screening of chemical compound libraries through deep machine learning approaches [7], and the investigation of insect [8] and animal microbiota [9] as promising reservoirs of new antibacterial substances. Endophytic microorganisms are increasingly recognized as a promising source of new antibiotics. Their high species diversity and adaptability to diverse environmental conditions make them valuable reservoirs of biologically active metabolites [10,11]. In addition, endophyte-derived secondary metabolites have been reported to possess a wide range of biological properties, including antibacterial, antifungal, anticancer, antiviral, antioxidant, anti-inflammatory, immunomodulatory, and hepatoprotective activities [12–14]. Endophytes represent a taxonomically diverse group of microorganisms that colonize the internal tissues of plants and maintain symbiotic relationships with their hosts without inducing apparent disease symptoms. They constitute the plant endosphere, comprising the endorhizosphere and endophyllosphere, and are recognized as an integral component of the plant microbiome alongside the phyllosphere and rhizosphere. Owing to their broad biological activities and promising pharmacological potential, endophytic microorganisms, including fungi, bacteria, and archaea [15], have long attracted the interest of researchers in biology and pharmacology [16–18].

2. Materials and methods

2.1. Plant material and isolation of endophytic fungi

Healthy tissues (leaves, stems, flowers, fruits, and roots) of *Persicaria hydropiper* L. were collected from the Academician F.N. Rusanov Tashkent Botanical Garden on May 18, 2025. Samples were processed within 24 hours. Tissue segments (~0.5 cm in diameter) were surface-sterilized by sequential immersion in 70% ethanol for 1 min and 2% sodium hypochlorite for 3 min, followed by rinsing with sterile distilled water for 2 min. The sterilized segments were placed on Potato Dextrose Agar (PDA) supplemented with chloramphenicol (100 mg/L) and incubated at 26 °C for 7 days. Pure fungal cultures were obtained by subculturing actively growing mycelial tips onto fresh PDA plates and maintained in 15% glycerol at −20 °C for long-term storage.

2.2. Cultivation and extraction of secondary metabolites

Fungal isolates were initially grown on PDA at 26 °C for 7 days. Mycelial agar plugs (approximately 5 mm in diameter) were inoculated into Erlenmeyer flasks containing Potato Dextrose Broth (PDB). The cultures were incubated on an orbital shaker at 120 rpm and 26 °C for 21 days. Following incubation, the culture broth was extracted with an equal volume of ethyl

acetate. The organic phase was separated and concentrated using a rotary evaporator at 40 °C to yield the dry crude extract.

2.3. Antimicrobial activity assay

The test microorganisms included *Bacillus subtilis* (RKMUz-5), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27879), *Escherichia coli* (RKMUz-221), and *Candida albicans* (RKMUz-247). Antimicrobial activity was evaluated using the agar disk diffusion method. Standardized bacterial suspensions were inoculated into Nutrient Agar, while *C. albicans* (1×10^6 CFU/mL) was inoculated into Mueller-Hinton agar. Sterile filter paper discs (6 mm) were impregnated with 20 µL of each extract. Ampicillin, gentamicin, and fluconazole were used as positive controls. Discs were placed on the inoculated plates and pre-incubated at 4 °C for 3 h, followed by incubation at 37 °C for 24 h for bacteria, and 29 °C for 48 h for fungi. The zones of inhibition were measured in millimeters, and all tests were performed in triplicate.

2.4. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the crude ethyl acetate extract of isolate 8i3k was determined using the broth microdilution method in 96-well microtiter plates. Briefly, 2 mg of the crude extract was accurately weighed, and a stock solution was prepared in DMSO at an initial concentration of 5000 µg/mL. Mueller-Hinton Broth (MHB) was prepared and sterilized by autoclaving at 121 °C for 15 min; all equipment and consumables were sterilized prior to use.

Bacterial inocula of *Bacillus subtilis* (RKMUz-5) and *Escherichia coli* (RKMUz-221) were prepared by suspending a single colony of each organism in 2 mL of sterile physiological saline. The turbidity of each suspension was adjusted to be equivalent to the 0.5 McFarland standard. Working inocula were subsequently prepared by adding 50 µL of each standardized bacterial suspension to 5 mL of MHB (1:100 dilution ratio).

For the microdilution assay, 50 µL of MHB was dispensed into each well of columns 1–11. A 50 µL aliquot of the stock solution (5000 µg/mL) was added to the wells of column 1, yielding an initial extract concentration of 2500 µg/mL. Two-fold serial dilutions were performed sequentially from column 1 through column 10 by transferring 50 µL from each well to the next; the excess 50 µL was discarded from column 10 to maintain uniform volumes throughout the dilution series. This procedure generated a concentration gradient of 2500, 1250, 625, 312.5, 156.25, 78.13, 39.06, 19.53, 9.77, and 4.88 µg/mL in columns 1 through 10, respectively. Subsequently, 50 µL of the standardized bacterial inoculum was added to each well of columns 1–10. The assay was performed in three technical replicates per bacterial strain.

Column 11 served as the growth control (positive control), containing bacterial inoculum in MHB without the test extract. Column 12 served as the sterility control (negative control), containing only sterile MHB without bacteria or extract. Following inoculation, the microtiter plates were incubated at 37 °C for 18 h. Optical density was subsequently measured using a microplate reader, and the MIC was defined as the lowest concentration of the extract that completely inhibited visible bacterial growth.

2.5. Cytotoxicity Assay

The cytotoxic potential of the crude ethyl acetate extract of isolate 8i3k was evaluated at a single concentration of 100 µg/mL against two human cancer cell lines: HEp-2 (human laryngeal carcinoma) and A549 (lung adenocarcinoma), using the MTT assay. Cells were seeded in 96-well plates at a density of 2.5×10^3 cells/well in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution, and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. Following the initial incubation, the medium was replaced with extract-

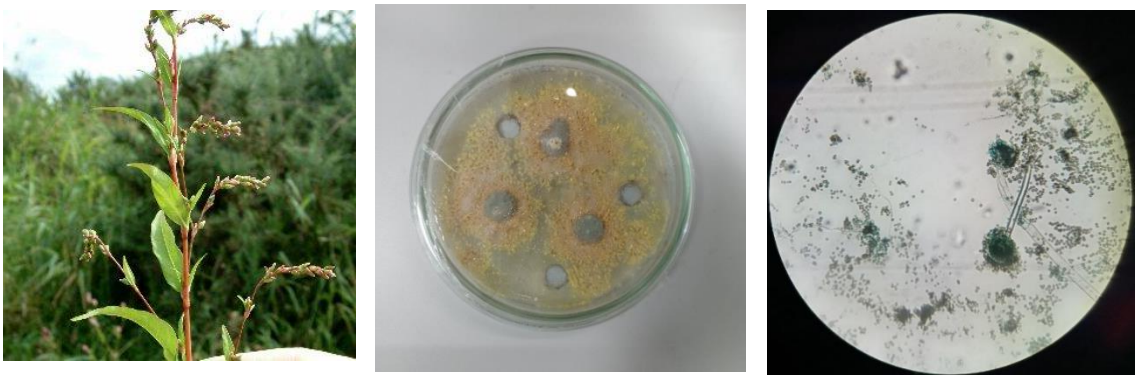
supplemented medium containing the 8i3k crude extract at 100 µg/mL (prepared from a stock solution in DMSO; final DMSO concentration ≤ 0.1%), and cells were incubated for an additional 24 h. Subsequently, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline) was added to each well and incubated for 4 h at 37 °C. The resulting formazan crystals were dissolved in 50 µL of DMSO, and absorbance was measured at 630 nm using a microplate reader. Cisplatin served as the positive (cytotoxic) control, and untreated cells served as the negative control. Cell viability was expressed as a percentage relative to untreated controls. All experiments were performed in triplicate, and statistical analysis was conducted using Origin 2024 software.

3. Results

3.1. Isolation and Morphological Characterization of Endophytic Fungi

Persicaria hydropiper L., commonly known as water pepper, is a medicinal plant belonging to the Polygonaceae family (Figure 1A). A total of six endophytic fungal isolates were successfully obtained from various healthy tissues of this plant: two isolates from flowers, one from leaves, and three from roots. Among these, the root-derived isolate designated as 8i3k was selected for detailed investigation based on its distinct morphology and subsequently confirmed antibacterial activity.

Morphological evaluation on Potato Dextrose Agar (PDA) after 18–21 days of incubation revealed that isolate 8i3k initially produced pale lemon-colored colonies, which transitioned to orange after several days, eventually developing a distinctive purple pigmentation in older cultures (Figure 1B). The isolate demonstrated limited aerial mycelial development, forming a very thin growth layer on the solid medium surface. Microscopic examination revealed non-septate hyphae (Figure 1C), a morphological trait characteristic of certain early-diverging fungal lineages.



A ***B*** ***C***

Figure 1. Morphological appearance of the medicinal plant Persicaria hydropiper L.(A), Macroscopic morphology of the root-derived endophytic fungal isolate 8i3k cultured on Potato Dextrose Agar (PDA) medium(B), Microscopic characteristics of endophytic isolate 8i3k under a compound light microscope, demonstrating non-septate hyphae(C).

3.2. Antimicrobial Activity and Minimum Inhibitory Concentration (MIC)

3.2.1. Disk Diffusion Assay

The crude ethyl acetate extract of isolate 8i3k was evaluated for antimicrobial activity using the agar disk diffusion method against a panel of pathogenic microorganisms. The results demonstrated selective antibacterial activity against Gram-positive bacteria, producing distinct inhibition zones of 17 mm against *Bacillus subtilis* (RKMUz-5) and 12 mm against *Staphylococcus aureus* (ATCC 25923). In contrast, no inhibitory activity (inhibition zone < 6 mm) was observed against the Gram-negative bacteria *Escherichia coli* (RKMUz-221) and

Pseudomonas aeruginosa (ATCC 27879), or against the yeast *Candida albicans* (RKMUz-247) under the tested conditions. Ampicillin, gentamicin, and fluconazole served as reference controls and produced inhibition zones consistent with established breakpoints (Table 1).

Isolated fungal isolates	Inhibition zone (mm, ± SE, P≤0.05)				
	Gram-positive bacteria		Gram-negative bacteria		*Candida albicans*
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	
8i3k	17.0 ±0.2	12.0 ±0.1	N/A	N/A	N/A
Ampicillin* (10 µg/disk)	23.01 ±0.2	22.02 ±0.3	Not tested	Not tested	Not tested
Gentamicin* (10 µg/disc)	Not tested	Not tested	19.04 ±0.3	26.05 ±0.1	Not tested
Fluconazole* (25 µg/disc)	Not tested	Not tested	Not tested	Not tested	26.01 ±0.3

Table 1. Antimicrobial activity of the crude ethyl acetate extract from endophytic isolate 8i3k (inhibition zone diameter, mm, mean ± SE, P ≤ 0.05). Ampicillin (10 µg/disc): positive control for Gram-positive bacteria; Gentamicin (10 µg/disc): positive control for Gram-negative bacteria; Fluconazole: positive control for yeast. N/A: no activity detected (zone < 6 mm); —: not tested.

3.2.2. Minimum Inhibitory Concentration (MIC)

Following confirmation of antibacterial activity by disk diffusion, the MIC of the 8i3k crude extract was determined against Bacillus subtilis (RKMUz-5) and Escherichia coli (RKMUz-221) using the broth microdilution method in Mueller-Hinton Broth. A stock solution (5000 µg/mL) was prepared, and the extract was introduced into column 1 of the microtiter plate at an initial concentration of 2500 µg/mL, followed by two-fold serial dilutions across columns 1 through 10, yielding a concentration gradient ranging from 4.88 to 2500 µg/mL. Columns 11 and 12 were reserved for growth and sterility controls, respectively.

The 8i3k extract demonstrated differential antibacterial potency against the two test organisms. Against Bacillus subtilis (RKMUz-5), complete growth inhibition was observed at a MIC of 312.5 µg/mL, indicating strong activity consistent with the pronounced inhibition zone recorded in the disk diffusion assay. Against Escherichia coli (RKMUz-221), the MIC was determined to be 1250 µg/mL, reflecting a markedly reduced susceptibility of this Gram-negative organism to the crude extract under the tested conditions. This difference in MIC values between the two species further corroborates the selective Gram-positive antibacterial activity observed in the disk diffusion assay. The positive growth control (column 11) confirmed bacterial viability throughout the assay, and the sterility control (column 12) remained turbidity-free, validating the experimental conditions. The MIC values are summarized in Table 2.

Table 2. Minimum Inhibitory Concentration (MIC) of the crude ethyl acetate extract from endophytic isolate 8i3k against *Bacillus subtilis* and *Escherichia coli*, determined by broth microdilution in Mueller-Hinton Broth (MHB; 37 °C, 18 h, n = 3).

3.3. Cytotoxic Activity of Isolate 8i3k

The cytotoxic potential of the crude ethyl acetate extract of isolate 8i3k was assessed at a concentration of 100 µg/mL against HEp-2 and A549 human cancer cell lines using the MTT assay. The results are summarized in Table 3.

Microorganism	Strain	Concentration range tested (µg/mL)	MIC (µg/mL)
Bacillus subtilis	RKMUz-5	4.88 – 2500	312.5
Escherichia coli	RKMUz-221	4.88 – 2500	1250

At the tested concentration, the 8i3k extract did not exhibit cytotoxic activity against either cell line. Instead, it produced a pronounced pro-proliferative effect, increasing cell viability to 117.5 ± 6.0% in A549 cells and 132.1 ± 6.9% in HEp-2 cells relative to the untreated control (100%). Cisplatin, used as a positive cytotoxic control, reduced viability to 48.3 ± 3.4% and 57.1 ± 3.0% in A549 and HEp-2 cells, respectively, confirming the validity of the assay conditions. These findings indicate that the secondary metabolites present in the crude extract of isolate 8i3k stimulate mammalian cell proliferation rather than inhibiting it under the tested conditions.

Cell Line / Sample, µg/mL	Number of viable cells, %	
	A549	HEp-2
8i3k	117,5±6,0	132,1±6,9
Cisplatin (positive control)	48,3±3,4	57,1±3,0
Control (untreated)	100	100

Table 3. Effect of crude fungal extract (100 µg/mL) on viability of HEp-2 and A549 cancer cell lines (MTT assay, mean ± SE, $n = 3$).

4. Discussion

The selective antibacterial activity of the 8i3k extract against Gram-positive bacteria is consistent with findings commonly reported for ethyl acetate extracts of endophytic fungi [11, 12, 13]. The lack of activity against Gram-negative organisms likely reflects the permeability barrier imposed by their outer membrane, which limits the uptake of hydrophobic compounds enriched in organic solvent extracts [11, 18]. The MIC of 312.5 µg/mL against *B. subtilis* compares favorably with values reported for crude endophyte-derived extracts in related studies [12, 13], indicating the presence of bioactive metabolites worthy of further isolation. Given the growing global burden of antimicrobial resistance, with 4.95 million deaths linked to resistant pathogens in 2019 alone [4], endophytic fungi represent a valuable and underexplored source of novel antibacterial leads [10, 11].

Rather than cytotoxicity, the 8i3k extract produced a pronounced pro-proliferative effect on both tested cancer cell lines, which contrasts with the cytotoxic properties frequently reported for endophyte-derived secondary metabolites [12, 13]. This stimulatory activity may be linked to the traditional wound-healing and regenerative properties of *P. hydropiper* [14]. It has been proposed that endophytes contribute to host plant bioactivities through the biosynthesis of functionally related metabolites [10, 14, 17], and the observed cell-stimulatory effect is consistent with this hypothesis, since active cell proliferation is a central component of tissue repair. The absence of cytotoxicity at the tested concentration further indicates low mammalian toxicity, which is favorable for potential therapeutic development.

Several limitations should be acknowledged. Biological activities were assessed using crude extracts at a single concentration, precluding dose-response analysis and IC_{50} determination. The molecular identity of isolate 8i3k remains unestablished, limiting comparison with related taxa and known metabolite profiles. Future work should focus on ITS-based molecular identification, compound isolation, and structural characterization by LC-MS and NMR spectroscopy.

5. Conclusion

This study demonstrates that the root-derived endophytic fungal isolate 8i3k from *Persicaria hydropiper* produces secondary metabolites with selective Gram-positive antibacterial activity and a notable pro-proliferative effect on human cancer cell lines. The low mammalian cytotoxicity and cell-stimulatory properties of the extract suggest a possible microbiological contribution to the traditional wound-healing properties of the host plant, and highlight the biotechnological potential of endophytic fungi associated with *P. hydropiper*. Compound purification, structural elucidation, and molecular identification of the isolate are the priority for future investigations.

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