

METABOLITE PROFILING AND PHARMACEUTICAL POTENTIAL OF ENDOPHYTIC FUNGAL ISOLATE E (*Aspergillus niger*) VIA LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY (LC-MS/MS)

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ABSTRACT

Endophytic fungi represent a rich yet underexploited bioresource of novel pharmaceutical compounds. In this study, untargeted metabolite profiling of endophytic fungal isolate E (*Aspergillus niger*), isolated from the roots of *Borassus flabellifer* L. (palmyra palm), was conducted using liquid chromatography–mass spectrometry (LC-MS/MS) on a Shimadzu LCMS-8040 system equipped with a Shim-pack FC-ODS column. The chromatographic run (50 min, isocratic 90% methanol) resolved 138 peaks that were tentatively annotated as distinct chemical constituents by spectral library matching. The metabolite profile revealed considerable chemodiversity, spanning primary amino acids (glutamic acid 2.90%, valine 2.43%, alanine 1.32%), piperidine alkaloids ((E)-7-(benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)hept-6-en-1-one 2.57%; (2E,4E,6E)-7-(benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)hepta-2,4,6-trien-1-one 2.15%), phenolic acids and flavonoids (naringenin 1.16%, coumestrol 0.97%, apigenin 0.90%, caffeic acid 0.85%), and sterols and triterpenoids (ergosterol 0.21%, lanosterol 0.15%, stigmasterol 0.34%, alpha-amyrin 0.31%); values are expressed as relative peak area. Many of the annotated compounds have reported antioxidant, antimicrobial, anti-inflammatory, and cytotoxic activities, which highlights isolate E as a promising candidate for bioassay-guided investigation, biotechnological exploitation, and pharmaceutical development. Confirmation of compound identities with authentic standards and high-resolution MS/MS, together with bioactivity assays, is required.

KEYWORDS: *Aspergillus niger*; endophytic fungi; LC-MS/MS; metabolite profiling; piperidine alkaloids; flavonoids; pharmaceutical biotechnology.

INTRODUCTION

Antibiotic resistance can spread to human pathogenic bacteria through the horizontal transfer of antibiotic resistance genes (ARGs), which ultimately leads to outbreaks of antibiotic-resistant superbugs that directly threaten human health (Wang et al., 2023). ARGs have been detected in plants, animals, the human gut, and commensal bacteria, as well as in environmental reservoirs such as wastewater, where a shift from single- to multi-antibiotic resistance has been observed (Machado et al., 2023).

In Indonesia, methicillin-resistant *Staphylococcus aureus* (MRSA) is among the most frequently reported resistant bacteria, and its prevalence varies between hospitals and patient groups. A study in Surabaya reported an MRSA colonization rate of 8.1% among inpatients on admission, whereas a prevalence of 0.8% was reported among elective preoperative patients in Jakarta (Kuntaman et al., 2016). In contrast, a much higher prevalence was reported in Malang, averaging 38.2% and peaking at 45.3% (Erikawati et al., 2016). A recent study in Yogyakarta further reported that 83.3% of *S. aureus* isolates from humans and animals were MRSA, with an alarming multidrug-resistance pattern (Fitrandi et al., 2023). These differences reflect variation in local epidemiology, but collectively confirm that antibiotic resistance in *S. aureus* is a serious problem in Indonesia that requires alternative solutions. Because this bacterium is resistant to various β -lactam antibiotics, alternative therapeutic agents are urgently needed (Rasyid et al., 2021).

Controlling infections caused by antibiotic-resistant bacteria requires safer and more environmentally friendly alternatives to conventional therapies. Natural products are one option, because they are generally biocompatible, biodegradable, associated with fewer side effects, and rich in bioactive metabolites with antimicrobial potential (Gani et al., 2022; Newman & Cragg, 2020). In this context, endophytic fungi, which live symbiotically within plant tissues, have received increasing attention because they produce unique secondary metabolites that not only enhance host resistance to pathogens (Akram et al., 2023; Zanudin et al., 2023; Zhang et al., 2024) but also exhibit significant antibacterial activity (Kaul et al., 2012; Wen et al., 2023). Endophytic fungi are also highly diverse, and their ecological roles range from mutualists and saprophytes to latent pathogens and antagonists of other microorganisms (Abdelaziz et al., 2025; Hande & Suradkar, 2025). Therefore, the exploration of endophytic fungi from medicinal plants is a promising strategy for discovering new antimicrobial candidates to address bacterial resistance.

Several recent studies have shown that endophytic fungi can inhibit the growth of various pathogenic bacteria. For example, among 65 endophytic fungal isolates obtained from the leaves of *Indigofera suffruticosa*, 18 isolates showed antibacterial activity in preliminary screening, most of them against both Gram-positive and Gram-negative bacteria. Specifically, all active isolates inhibited *Bacillus subtilis*, whereas *Staphylococcus aureus* and *Klebsiella pneumoniae* were inhibited by approximately 77.8% and 72.2% of the active isolates, respectively, and *Pseudomonas aeruginosa* was inhibited by only one isolate (dos Santos et al., 2015). Another study reported that extracts of endophytic fungi isolated from *Ageratina adenophora* inhibited several multidrug-resistant pathogenic bacteria, including MRSA, with minimum inhibitory concentration (MIC) values varying among isolates (Wen et al., 2023).

Infectious diseases, chronic inflammatory disorders, and cancer remain major global health threats, necessitating the continuous discovery of structurally diverse bioactive natural products (Newman & Cragg, 2020). Fungi, particularly endophytic and soil-associated isolates, are widely recognized as prolific producers of unique secondary metabolites with antibacterial, antifungal, antioxidant, and antitumor properties (Kaul et al., 2012). *Aspergillus niger*, for instance, produces a wide array of secondary metabolites, including naphtho- γ -pyrones, pyranonigrins, bicoumarins, cyclic

peptides, and mycotoxins such as ochratoxin A and fumonisins (Nielsen et al., 2009); however, the metabolite profiles of endophytic *A. niger* strains associated with Indonesian medicinal plants remain poorly documented. Chemical screening of fungal extracts is a crucial preliminary step in identifying lead compounds for drug discovery.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is widely used for untargeted metabolomics because of its high sensitivity, selectivity, and rapid spectral acquisition. In this investigation, endophytic fungal isolate E, isolated from the roots of *B. flabellifer* and identified as *A. niger* by morphological and ITS rDNA sequence analysis, was subjected to metabolite profiling using a Shimadzu LCMS-8040 system to characterize its chemical constitution and evaluate its potential pharmaceutical relevance. Accordingly, the aim of this study was to describe the metabolite profile and pharmaceutical potential of endophytic fungal isolate E (*Aspergillus niger*) using LC-MS/MS.

MATERIALS AND METHODS

Fungal Isolate and Extract Preparation

Endophytic fungal isolate E (*A. niger*) was isolated from surface-sterilized roots of *B. flabellifer* collected from siwalan farmland in Tasikmadu Village, Palang District, Tuban Regency, East Java, Indonesia (6.908964° S, 112.095087° E), between June and September 2025. Root segments were surface-sterilized in 1% NaOCl and 70% ethanol (30 s each), plated on potato dextrose agar (PDA) supplemented with chloramphenicol (100 mg/L), and incubated at 25–27 °C, and emerging colonies were purified on PDA. The isolate was identified from its macroscopic and microscopic characters and by sequencing of the internal transcribed spacer (ITS) rDNA region amplified with primers ITS1 and ITS4; the sequence showed 99.6% identity with reference sequences of *A. niger* and *A. awamori*. Because the ITS region does not reliably discriminate species within *Aspergillus* section Nigri, the name *A. niger* is used here in the broad sense (*A. niger* aggregate). The isolate was cultured in liquid medium, and the culture was extracted with an organic solvent at Universitas Negeri Malang. The crude extract was filtered and redissolved in LC-MS-grade solvent prior to injection.

LC-MS/MS Instrumentation and Chromatographic Conditions

Chemical analysis was carried out using a Shimadzu LCMS-8040 system consisting of a high-performance liquid chromatograph coupled to a triple quadrupole mass spectrometer. Chromatographic separation was performed on a Shimadzu Shim-pack FC-ODS column (150 mm × 2.0 mm i.d., 3 µm particle size) maintained at 35 °C. The analytical parameters used during data acquisition are detailed in Table 1.

Table 1: Operational parameters for LC-MS/MS analysis of the isolate E extract.

Parameter	Specification / Value
Instrument	Shimadzu LCMS-8040 (LC coupled to triple quadrupole MS)
Column	Shim-pack FC-ODS (150 mm × 2.0 mm i.d., 3 µm)
Injection volume	1.0 µL
Mobile phase	Isocratic, methanol–water (90:10, v/v)
Flow rate	0.5 mL/min
Column temperature	35 °C
Ionization mode	Electrospray ionization (ESI), positive ion mode
Capillary voltage	3.0 kV
Sampling cone voltage	23.0 V
Source / desolvation temperature	100 °C / 350 °C
Desolvation gas flow	60 mL/h
Collision energy / method	5.0 V / low-energy CID
Mass scan range	m/z 10–1000 (0.6 s/scan)
Total run time	50 min

Chromatographic peaks were detected and integrated using LabSolutions LCMS Ver. 5.80 SP1 (Shimadzu Corporation, Kyoto, Japan). Compounds were tentatively annotated by matching mass spectra (m/z) and retention times against Shimadzu Library Search system and the Shimadzu mass spectral library, and only matches with a similarity index above 92% were accepted. Relative abundance was expressed as the percentage of each peak area relative to the total peak area; these values indicate relative signal intensity rather than absolute concentration, because ionization efficiency differs among compounds. Annotation confidence was assigned according to the reporting levels of the Metabolomics Standards Initiative (MSI) (Sumner et al., 2007).

RESULTS AND DISCUSSION

Chromatographic Overview and Metabolite Annotation

The LC-MS/MS total ion chromatogram (TIC) of Fungal Isolate Sample E displayed excellent chromatographic resolution across the 50-minute run time. A total of 138 distinct chromatographic peaks were separated and tentatively identified by matching mass spectra (m/z) and retention times against reference mass spectral databases with similarity indices exceeding 92%.

Chemical Classification of Annotated Constituents

The annotated metabolites were classified into four predominant biochemical groups: primary metabolites (amino acids and organic acids), piperidine alkaloids and alkamides, phenolics and flavonoids, and terpenoids/sterols. Representative major compounds from each class are summarized in Table 2.

Table 2: Representative primary and secondary metabolites tentatively annotated in the extract of endophytic fungal isolate E (*Aspergillus niger*).

Peak	Compound name	RT (min)	Relative peak area (%)	Chemical class
1	Glycine	1.049	0.85	Amino acid
2	Piperidine (hexahydropyridine)	1.157	1.90	Piperidine alkaloid
8	Valine	1.241	2.43	Amino acid
21	Coumarin	1.542	0.40	Benzopyrone (coumarin)
36	Lysine	2.228	1.47	Amino acid
37	Glutamic acid	2.276	2.90	Amino acid
42	Eugenol	2.694	0.64	Phenylpropanoid
45	Gallic acid	3.042	1.06	Phenolic acid
51	Caffeic acid	4.643	0.85	Hydroxycinnamic acid
53	Ferulic acid	5.043	0.67	Hydroxycinnamic acid
80	Coumestrol	9.083	0.97	Coumestan
81	Apigenin	9.365	0.90	Flavonoid (flavone)
83	Naringenin	9.732	1.16	Flavonoid (flavanone)
89	(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)penta-2,4-dien-1-one (piperine)	10.111	2.15	Piperidine alkamide
99	(2E,4E,6E)-7-(benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)hepta-2,4,6-trien-1-one (piperettine)	11.526	2.15	Piperidine alkamide
100	(E)-7-(benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)hept-6-en-1-one	11.532	2.57	Piperidine alkamide
130	Ergosterol	13.278	0.21	Fungal sterol
133	Stigmasterol	15.638	0.34	Phytosterol
136	Alpha-amyrin	19.438	0.31	Pentacyclic triterpenoid

Potential Pharmaceutical Relevance of the Annotated Metabolites

Origin of the Annotated Metabolites

Several of the annotated compounds, notably the methylenedioxyphenyl piperidine alkaloids (piperine-type amides), the flavonoids apigenin and naringenin, coumestrol, stigmasterol, and alpha-amyrin, are characteristic plant metabolites; piperine and piperettine in particular are hallmark alkaloids of *Piper* species (Gorgani et al., 2017). In contrast, hallmark *A. niger* metabolites such as naphtho- γ -pyrones, pyranonigrins, malformins, ochratoxin A, and fumonisins (Nielsen et al., 2009) were not among the major compounds annotated in this study (Table 2). The presence of plant-type metabolites may reflect genuine biosynthesis by the endophyte, since endophytes are known to produce compounds originally attributed to their host plants. The presence of plant-type metabolites may reflect genuine biosynthesis by the endophyte, since endophytes are known to produce compounds originally attributed to their host plants (Kaul et al., 2012), but it may also reflect carry-over from plant-derived components of the culture medium or misannotation arising from nominal-mass library matching on a unit-resolution instrument. Analysis of an uninoculated medium blank and confirmation with authentic standards and high-resolution MS/MS are therefore required before these compounds can be attributed to isolate E. Conversely, the detection of ergosterol, the principal membrane sterol of fungi, is consistent with the fungal origin of the extract.

Antioxidant and Anti-inflammatory Potential

The detection of phenolic acids, including gallic acid (1.06%), caffeic acid (0.85%), and ferulic acid (0.67%), together with flavonoids such as naringenin (1.16%), apigenin (0.90%), and genkwanin (0.65%), suggests that the extract of *A. niger* isolate E may possess antioxidant and anti-inflammatory activity. Compounds of these classes are known to scavenge reactive oxygen species (ROS) and inhibit key inflammatory mediators such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Kumar & Pandey, 2013). However, these activities have not yet been demonstrated for the extract itself and should be confirmed, for example, by DPPH/ABTS radical-scavenging and cell-based anti-inflammatory assays.

Antimicrobial and Cytotoxic Potential

A notable finding in *A. niger* isolate E is the relatively high abundance of piperidine-derived alkaloids bearing methylenedioxyphenyl moieties (peaks 89, 99, and 100; 6.87% of the total peak area). Piperine-type alkaloids have been reported to exhibit a broad range of pharmacological activities, including antimicrobial and anti-inflammatory effects (Gorgani et al., 2017). Furthermore, coumarin (0.40%), eugenol (0.64%), and triterpenoids such as alpha-amyrin (0.31%) belong to compound classes with reported antibacterial activity, which for eugenol and several terpenoids has been linked to disruption of the bacterial cytoplasmic membrane (N. A. Mahizan et al, 2019, A. Marchese et al, 2017, J. Sharifi-Rad et al, 2021). Nevertheless, the antibacterial activity of the extract against MRSA and other resistant strains, which motivated this study, has not yet been tested and should be evaluated using agar diffusion and broth microdilution (MIC/MBC) assays.

Nutritional and Primary Metabolic Foundation

The extract of *A. niger* isolate E also contained essential and non-essential amino acids (glutamic acid 2.90%, valine 2.43%, lysine 1.47%, alanine 1.32%). Such primary metabolites support fungal biomass growth and serve as precursor substrates for secondary metabolite biosynthesis; for example, lysine is the biosynthetic precursor of the piperidine ring of piperidine alkaloids (Dewick, 2009). However, because these polar compounds eluted close to the column void

volume under isocratic 90% methanol conditions and may partly originate from the culture medium, their annotation and origin should be interpreted with caution. Limitations.

This study has several limitations. The profile was obtained from a single extract analysed in positive ion mode only, which under-represents acidic compounds such as phenolic acids that ionize more efficiently in negative mode, as well as sterols, which ionize poorly by ESI. Annotation relied on library matching of nominal-mass spectra from a triple quadrupole instrument without authentic standards, corresponding at best to MSI level 2 (putatively annotated compounds) (Sumner et al., 2007). Relative peak areas were used to describe differences in metabolite abundance and should not be interpreted as absolute concentrations. In addition, the present study focused on metabolite profiling and did not include bioactivity assays; therefore, potential biological activities of the tentatively annotated metabolites cannot be inferred directly from the LC-MS profiles.

CONCLUSION

Untargeted LC-MS/MS metabolite profiling of endophytic fungal isolate E (*A. niger*) tentatively annotated 138 chemical constituents, revealing a chemically diverse extract that contains several classes of compounds with reported pharmacological activity. The co-occurrence of piperidine alkaloids, antioxidant flavonoids, phenolic acids, and fungal sterols underscores the biotechnological promise of *A. niger* isolate E. Future work should confirm compound identities using authentic standards and high-resolution MS/MS, verify their fungal origin against uninoculated medium blanks, and evaluate the antibacterial (particularly anti-MRSA), antioxidant, and cytotoxic activities of the extract through bioassay-guided fractionation to identify specific drug development candidates.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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