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Cytotoxicity of *Acanthus ilicifolius* root endophytes based on acid-induced enhancement and metabolites of *Penicillium javanicum*

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Abstract. *Ainy EQ, Audah KA, Tan MI, Aryantha INP. 2026. Cytotoxicity of* Acanthus ilicifolius *root endophytes based on acid-induced enhancement and metabolites of* Penicillium javanicum*. Biodiversitas 27 (3): d270322. https://doi.org/10.13057/biodiv/d270322*. Mangrove ecosystems are characterized by extreme environmental fluctuations, driving associated endophytic fungi to evolve adaptive metabolic strategies. These microbial communities represent an untapped reservoir of chemical diversity, yet their metabolic response to abiotic stressors, such as acidity, remains underexplored. This study investigates the ecological and chemical plasticity of endophytic fungi isolated from *Acanthus ilicifolius* roots. Using a stress-driven culture approach, we evaluated the effect of acidic fermentation on the metabolic profiles and cytotoxic potential of bioactive isolates. Among the screened strains, *Penicillium javanicum* 2RA6 exhibited a distinct physiological response to acidic stress. While neutral conditions favored vegetative growth, acidic fermentation triggered a fundamental metabolic reprogramming, shifting the biosynthetic output from constitutive polyketides to a specialized stress 'portfolio'. Comparative metabolomics revealed the enrichment of diverse fatty acid amides (including putative 9-oxo-(10E, 12E)-Octadecadienoic Acid (9-oxo-ODA), α-Eleostearic Acid (α-EA), and Palmitoyl Ethanolamide (PEA)) and diketopiperazines. This chemical reorganization correlated with a marked increase in specific cytotoxicity against MCF-7 breast cancer cells, mediated primarily through apoptosis-like pathways. These findings suggest that mimicking environmental stressors is a viable strategy to unlock cryptic biosynthetic gene clusters. This study highlights the importance of mangrove habitats as reservoirs of metabolic diversity and demonstrates that acid-enhanced cultivation can access bioactive fungal metabolites typically overlooked in standard fermentations.

Keywords: *Acanthus ilicifolius*, acidic stress, apoptosis-like, endophytic fungi, microbial communities

INTRODUCTION

Microbial biodiversity in extreme environments represents an immense, yet largely untapped, reservoir of functional chemical diversity. While natural products are the cornerstone of anticancer drug discovery, with over 60% of chemotherapeutic agents derived from or inspired by microbial or plant metabolites. However, the discovery of new compounds is limited by frequent rediscovery of known species and metabolite redundancy. To overcome this challenge, the focus of bioprospecting has shifted toward examining microbial functional diversity in unique ecological niches (Gao et al. 2021). Endophytic fungi, particularly those co-evolving with stress-adapted plants, exemplify this potential. Their ability to synthesize complex secondary metabolites is not merely a pharmacological asset but an ecological trait developed for host defense and competition. The metabolites often mimic or complement the biological activities of their host plants, ranging from antimicrobial to potent anticancer effects (Qiao et al. 2017; Prajapati et al. 2021; Tran et al. 2022).

Mangrove ecosystems are biodiversity hotspots that provide unique habitats for stress-adapted microbes. Situated in the intertidal zones, mangroves are subjected to extreme environmental fluctuations, including high salinity, tidal inundation, intense UV radiation, and oxygen limitations. These severe constraints drive the selection of a unique fungal community producing unusual and biologically active metabolites that are rarely found in terrestrial fungi (Chi et al. 2019; Wang et al. 2022). Within this ecosystem, the root system constitutes a distinctive and biologically complex microhabitat. Unlike plant tissues, mangrove roots are directly exposed to sediment environment, facing challenges such as anaerobic conditions, heavy metal accumulation, and pH fluctuations due to soil chemistry variations (Gao et al. 2021). These stresses may drive fungi to evolve specialized metabolic traits, yielding compounds not typically produced by fungi in leaves or stems. Despite this, the chemical ecology of mangrove root endophytes remains underexplored.

Among environmental stresses, pH variation has a strong influence on fungal metabolism. Acidic culture conditions mimic the natural stress of mangrove sediments-which can be naturally acidic due to organic decomposition -and have been shown to repress pH-responsive regulators such as PacC. This deregulation can activate silent biosynthetic gene clusters, stimulating the production of cryptic metabolites with enhanced bioactivity (Yan et al. 2019). For example, acid-stress induction has previously succeeded in yielding unique anthraquinone derivatives (Jin et al. 2017) and alkaloids with cytotoxic properties (Duy Ngoc et al. 2022; Gao et al. 2021). These reports

highlight acid stress as a strategy to unlock the hidden potential of fungal metabolites and expand the repertoire of bioactive compounds.

Acanthus ilicifolius L. (locally known as "*jeruju hitam*") is a medicinally and ecologically significant mangrove species widely distributed across Southeast Asia. Phytochemical studies have revealed diverse metabolites, including alkaloids, benzoxazinoids, lignans, flavonoids, triterpenoids, and steroids associated with antioxidant, antimicrobial, anti-inflammatory, analgesic, and anticancer activities (Andriani et al. 2020; Karim et al. 2021; Wang et al. 2022). While the pharmaceutical potential of the plant itself is well-documented, studies on its associated endophytes have predominantly focused on aerial tissues. Fungi isolated from the leaves and stems of *A. ilicifolius*, such as *Diaporthe phaseolorum* SKS019, *Lasiodiplodia theobromae* ZJ-HQ1, and *Penicillium* sp. HS-N-27 and HS-N-29, have demonstrated cytotoxic effects against various cancer cell lines (Chen et al. 2016; Cui et al. 2017; Wang et al. 2022).

Despite these reports, most studies have concentrated on aerial tissues, leaving the root fungal community underexplored. Moreover, no study has examined how acid stress influences the cytotoxic metabolite repertoire of *A. ilicifolius* root endophytic fungi. Considering the ecological stresses experienced by roots and the evidence that acidic fermentation enhances metabolite diversity, the root endophytic fungi of *A. ilicifolius* may represent an untapped source of cytotoxic compounds.

Therefore, this study aimed to evaluate the cytotoxic activity of *A. ilicifolius* root endophytic fungi and to determine the influence of acid-induced fermentation on their secondary metabolite profiles. By focusing on a relatively unexplored tissue niche and employing an environmental modulation strategy, this study addresses a critical gap in mangrove endophyte-based drug discovery, thereby contributing to the search for novel anticancer agents.

MATERIALS AND METHODS

Chemicals

Fungal growth media, including Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB), were obtained from HiMedia (India). Dulbecco's Modified Eagle's Medium (DMEM) and Dimethyl Sulfoxide (DMSO) were purchased from Sigma Aldrich, USA. Fetal Bovine Serum (FBS) and trypsin-EDTA were supplied by GIBCO-BRL. Ethyl Acetate (EA) and ethanol were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used were of analytical grade.

Procedures

Fungal material

To document the functional microbial diversity of a threatened ecosystem, samples were collected from the Segara Anakan Mangrove Forest (SAMF), Central Java, Indonesia (7°38'22''-7°42'34.7'S and 108°55'41.5''-109°1'50''E). No specific permissions were required for the study location. This location serves as a crucial habitat for *A. ilicifolius.* A total of 21 endophytic fungal strains were isolated from apparently healthy roots with no visible symptoms of disease or physical damage (Jayatilake and Munasinghe 2020). Fungal identification was based on morphological characteristics and rDNA sequence analysis using an Internal Transcribed Spacer (ITS) (Zhou et al. 2018). Representative sequences were deposited in GenBank under accession numbers PQ358843-PQ358853 and PQ373186-PQ373195. The fungal isolates were as follows: *Penicillium senticosum* 1RB31, *P. chrysogenum* 2RA1, *P. javanicum* 2RA6, *P. shearii* 2RB10, *P. rubens* 2RB12, *Aspergillus aculeatus* 1R21B, *A. sydowii* 2RA3, *A. flavus* 2RB2, *A. versicolor* 2RB5, *Talaromyces fuscoviridis* 1R12, *T. wortmannii* 2RA5, *T. argentinensis* 2RA9, *T. stipitatus* 2RA11, *T. oumae-annae* 2RB6, *T. pinophilus* 2RB13, *Hamigera avellanea* 2RA15, *Fusarium solani* 2RA10, *F. oxysporum* 2RA18, *F. proliferatum* 2RB4, *F. decemcellulare* 2RB14, and *Pilatoporus ostreiformis* 2RA2.

Ecologically, the culturable root fungal endophytes dominated by the phylum Ascomycota, particularly the genera *Talaromyces* (6 isolates), *Penicillium* (5 isolates), and *Aspergillus* (4 isolates). This dominance pattern reflects a typical mangrove root mycobiota, where stress-tolerant Eurotiales and Hypocreales are often selected to survive in saline and anaerobic sediment conditions. All isolates were maintained on PDA at 4°C and subcultured monthly to ensure purity and viability.

Acanthus ilicifolius *root endophytic fungi cultivation and secondary metabolite extraction*

Fermentation was conducted in two distinct phases: (i) preliminary toxicity screening of all 21 isolates, and (ii) acid-stress fermentation of the selected active isolates. A standardized basal condition was applied to both phases: 200 mL PDB prepared with Artificial Seawater (ASW), inoculated with four 6-mm mycelial plugs, and incubated for 15 days at room temperature with intermittent shaking (150 rpm, 1 h day^{-1}). For phase 2 (acid-stress), the selected isolates were cultured in independent biological triplicates under two specific pH regimes: neutral control (pH 7.0) and acidic stress (pH 3.0, adjusted with sterile citric-phosphate buffer) (Gao et al. 2021).

Extraction followed a unified protocol. Cultures were filtered (Whatman No. 1) to separate mycelial mat and broth. Dried mycelia and culture filtrate were exhaustively extracted with ethyl acetate. The organic layers were separated, combined, and evaporated at 45°C using a vacuum rotary evaporator to yield the crude extracts, which were weighed and stored at 4°C until analysis. The extraction yield was calculated using (Ramírez-Villalobos et al. 2023) formula:

$$\text{Yield (\%)} = \frac{mass\ of\ dried\ crude\ extract\ (g)}{mass\ of\ dried\ fungal\ biomass\ (g)} \times 100$$

Fungal extract toxicity screening using the Brine Shrimp Lethality Test (BSLT)

Toxicity screening was conducted using *Artemia salina* (Linnaeus, 1758) larvae to prioritize isolates. Artificial Seawater (ASW) was prepared by dissolving 27 g of sea salt in 900 mL of distilled water. *A. salina* eggs were hatched under continuous illumination, and ten nauplii

were transferred to each vial for testing. A hierarchical two-stage strategy was employed using technical triplicates of pooled extracts. First, all 21 extracts were screened at concentrations of 1,000, 500, 250, and 125 µg mL^{-1} (prepared in a DMSO/ASW mixture). Isolates exhibiting ≥90% larval mortality at 125 µg mL^{-1} were advanced to the second stage. These candidates were re-evaluated at lower concentrations (80, 40, 20, 10, and 5 µg mL^{-1}) alongside a thymol positive control. Mortality was recorded 24 hours. LC_{50} (lethal concentration 50%) values were calculated using Probit regression and classified according to Meyer's and Clarkson's toxicity index (Ntungwe et al. 2020; Niksic et al. 2021).

Cytotoxicity assay of fungal extracts using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

The cytotoxic activity of the selected candidates was validated against MCF-7 breast cancer cells. The cells were obtained from the Medical Parasitology Laboratory at the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada. This assay utilized extracts from three independent biological fermentation replicates to account for biological variability. The cells were cultured in DMEM supplemented with 10% FBS and 2% penicillin-streptomycin in a humidified incubator with 5% CO_2 and 95% air at 37°C. The cells were seeded at 1×10^4 cells/well in 96-well plates (100 µL per well) and allowed to attach for 24 hours. Extracts were dissolved in 2% DMSO, and Doxorubicin, as a positive control, was prepared in DMEM. Cells were then exposed to serial concentrations of the extract (0, 31.25, 62.5, 125, 260, and 500 µg mL^{-1}) or Doxorubicin (0, 6.25, 12.5, 25, 50, and 100 µg mL^{-1}) and incubated for 24 h under the above conditions. After treatment, the culture medium was aspirated, and 10 µL of MTT solution (5 mg mL^{-1} in Phosphate-Buffered Saline (PBS)) was added to each well. The plates were incubated for 4 h to allow for formazan formation. Crystals were dissolved by adding 100 µL of 10% (w/v) Sodium Dodecyl Sulfate (SDS) in 0.01 N Hydrochloric acid (HCl) per well and incubating overnight at room temperature protected from light. Absorbance was measured at 595 nm using a microplate reader. The cell viability was calculated as follows:

$$\text{Viability (\%)} = \frac{(Abs\ of\ test\ group - Abs\ medium)}{(Abs\ cell - Abs\ medium)} \times 100$$

Dose-response curves were generated, and IC_{50} values were obtained by linear regression interpolation (Darmadi et al. 2021; Gemantari et al. 2021; Zhou et al. 2022).

Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) flow cytometry

MCF-7 cells (1×10^5 cells/well) were seeded in 6-well plates and treated with fungal extracts for 24 h. Cells were stained with Annexin V-FITC/PI flow cytometry kit according to the manufacturer's protocol and analyzed using a Fluorescence-Activated Cell Sorting (FACS) verse flow cytometer at excitation/emission wavelengths (λ) of 488/520 and 540/630 nm for Annexin V-FITC and PI, respectively (Alshehade et al. 2024; Dhayanithy et al. 2019). To evaluate the mode of cell death, extracts from three independent biological fermentations were pooled to generate a representative sample for each condition. This pooled extract was then analyzed in technical triplicate to ensure measurement reproducibility.

Mycochemical analysis of potential extracts using Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS)

Crude extracts were analyzed using a Thermo Scientific™ Vanquish™ Ultrahigh-Performance Liquid Chromatography (UPHLC) system coupled with a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer. Separation was performed on an Accucore™ phenyl-hexyl analytical column (100 mm × 2.1 mm × 2.6 µm) using a mobile phase of water (0.1% formic acid) and ethanol (0.1% formic acid) at a flow rate of 0.3 mL min^{-1}. Mass spectrometry was performed in positive ionization mode with a scan range of 66.7-1,000 m/z. The instrument was calibrated weekly to ensure mass accuracy (<5 ppm) (Windarsih et al. 2024).

Compound annotation followed an untargeted metabolomics workflow. Structural features were putatively identified by matching Mass Spectrometry (MS) 1 precursor ions and MS/MS fragmentations against the mzCloud database. As no authentic standards were used, all annotations are reported as putative (Metabolomic Standards Initiative (MSI) Level 2). This analysis focused on qualitative profiling, specifically detecting relative shifts in metabolite cases between neutral and acidic conditions. Peak areas (Area Max.) were used solely to describe relative signal intensities without normalization to fungal biomass, extract mass, or internal standards. Therefore, results should be interpreted as exploratory rather than absolute quantitative comparisons. The analysis was conducted on a pooled representative strain extract to profile metabolites associated with cytotoxicity under different conditions.

Statistical analysis

Data analyses were performed using Python 3.12 (SciPy, Statsmodels). Results are expressed as mean±Standard Deviation (SD). Normality and homogeneity of variances were verified using the Shapiro-Wilk test and Levene's test, respectively. Differences in extraction yields and apoptosis rates evaluated using One-Way ANOVA followed by Tukey's Honestly Significant Difference (HSD) post-hoc test, while Two-Way ANOVA was used to assess pH and fungal strain effects.

For dose-response assays, analyses utilized individual replicate data points (n = 3) to ensure robust parameter estimation. LC_{50} values (BSLT) were calculated via Probit analysis using a Generalized Linear Model (GLM). IC_{50} values (MTT assay) were determined using an Ordinary Least Squares (OLS) regression on log-transformed concentration data. The Delta Method was applied to calculate 95% Confidence Intervals (CIs). Regression models were considered valid for potency estimation if $R^2 \geq 0.70$ and the 95% CIs were not excessively wide. Statistical significance was set at p-value<0.05.

RESULTS AND DISCUSSION

Extracts yields

In total, 21 endophytic fungal isolates were fermented in PDB at room temperature with intermittent agitation until the stationary phase. Prior to the One-Way ANOVA, assumptions of normality and homogeneity of variance were assessed. The global normality test indicated that the data were not normally distributed (p = 0.004); however, the homogeneity of test confirmed that variances were equal across groups (p = 0.792). Despite the violation of the normality assumption, One-way ANOVA was proceeded with, given the robustness of the test. The One-way ANOVA results revealed a highly statistically significant difference between groups (F = 21.79, p<0.001). Specifically, the yields exhibited a wide range of metabolic productivity, with *H. avellanea* and *P. chrysogenum* producing significantly higher crude weights compared to the majority of the endophytic collection. This indicates a high degree of physiological variability in biomass and secondary metabolite accumulation among the different fungal taxa under identical fermentation conditions.

Based on the Tukey HSD post-hoc test, *H. avellanea* produced the highest yield (39.55±10.28% (w/w)), which was statistically significantly different from most other species, with the exception of *P. chrysogenum* (32.67±1.85% (w/w)). Conversely, the lowest yield group was dominated by *F. solani* (9.83±1.25% (w/w)) and *P. rubens* (9.28±1.56% (w/w)), with no significant difference observed between these two. Interestingly, a yield gradation was observed in the middle group (ranging from *T. stipitatus* to *P. shearii*), where differences between adjacent species were not statistically significant (indicated by overlapping notations) (Figure 1).

Toxicity screening of fungal extracts using the Brine Shrimp Lethality test

Preliminary lethality test of Ethyl Acetate (EA) extracts

All EA extracts were screened using BSLT at concentrations of 1000, 500, 260, and 125 µg mL^{-1}, simultaneously reducing the number of fungal isolates that exhibited toxic activity above the 90% larval mortality threshold (Ntungwe et al. 2020; Niksic et al. 2021). Table 1 shows the five extracts with the highest toxicity, even at a concentration of 125 µg mL^{-1}. These extracts were produced by *P. javanicum* 2RA6, *T. stipitatus* 2RA11, *T. oumae-annae* 2RB6, *F. proliferatum* 2RB4, and *A. versicolor* 2RB5. The identification of these highly potent isolates from the broader screen indicates that significant bioactive potential is not uniformly distributed across the endophytic community.

Classification of toxicity and LC_{50} values

The five fungal extracts were further evaluated at expanded concentrations (2.5 to 80 µg mL^{-1}). All the tested compounds showed concentration-dependent toxicity. Probit regression analysis showed that the LC_{50} values of the EA extracts ranging from 15.08 to 35.27 µg mL^{-1}. *P. javanicum* 2RA6 exhibited the highest toxicity with the lowest LC_{50} (15.08 µg mL^{-1}) (Table 2). As expected, the positive control Thymol exhibited higher potency, with an LC_{50} value of 9.86 µg mL^{-1}.

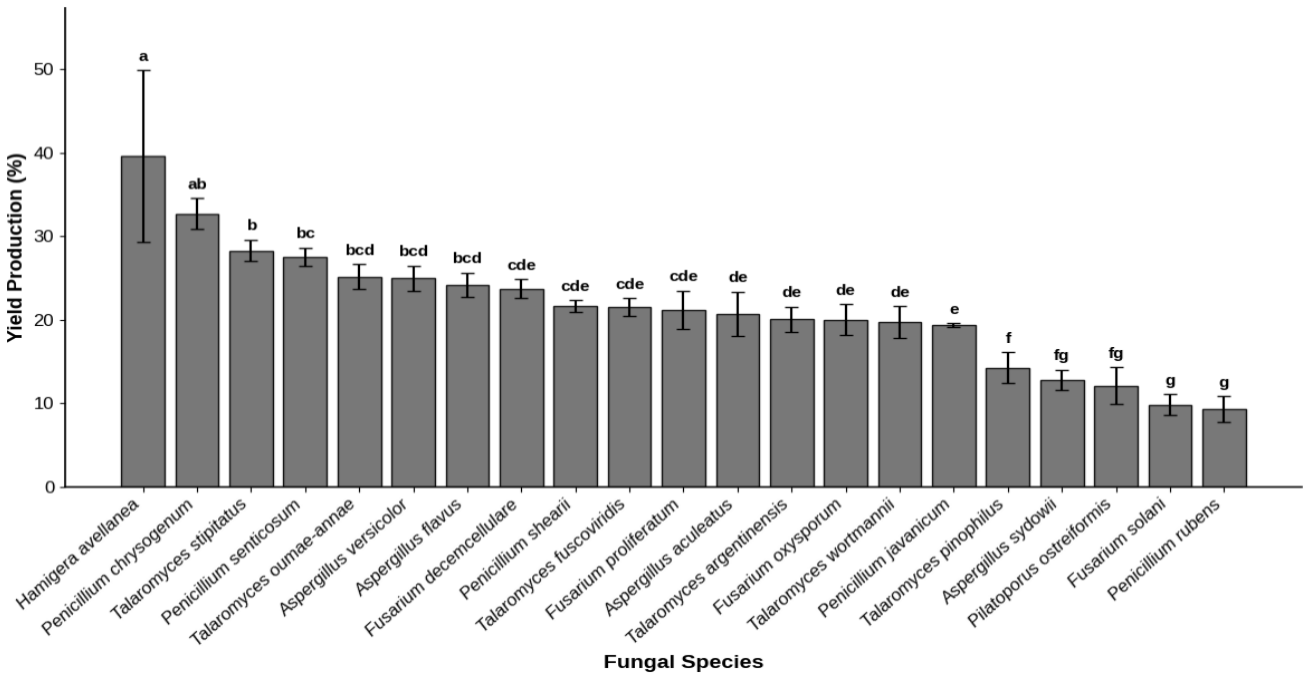


Figure 1. Extraction yield (%) of Ethyl Acetate (EA) extracts from 21 root endophytic fungi of *Acanthus ilicifolius*. Values represent mean±SD (n = 3 independent experiments). Statistical analysis was performed using One-Way ANOVA (F-statistic = 21.79, p<0.001)

Extract yield and effect of pH

The quantitative yield of secondary metabolite extracts varied among the five fungal isolates under different pH conditions (Figure 2). In general, fermentation at neutral pH resulted in nominally higher crude extract yields compared to acidic conditions for the majority of the isolates. Under neutral conditions, *T. stipitatus* 2RA11 produced the highest yield (28.28±1.23)%. Conversely, under acidic conditions, *A. versicolor* 2RB5 recorded the highest yield (24.96±1.48)%. A notable observation was made for *F. proliferatum* 2RB4; while it produced a comparable yield at neutral pH (24.96±1.48)%, it failed to grow (NG) under acidic conditions, indicating a high sensitivity to low pH environments. Despite the observable trend of reduced yields in acidic media, a Two-way ANOVA revealed that these differences were not significant. The *p*-value (>0.05) suggests that neither the variation in pH nor the fungal species significantly influenced the extract weight in this specific dataset.

In vitro cytotoxic activity of endophytic fungal culture extracts against the MCF-7 cell line as assessed by the MTT assay

The cytotoxic potential of fungal extracts cultivated under different pH conditions was evaluated against the MCF-7 cell line. The dose-response relationship was analyzed using linear regression on individual replicate data points (n = 3) to account for experimental variance. The calculated IC_{50} values, along with their 95% CI and coefficients of determination (R^2), are summarized in Table 3.

The results demonstrated a pH-dependent transition in bioactivity. The acid extract *P. javanicum* 2RA6 exhibited the most distinct dose-dependent inhibition, with an IC_{50} of 181.63 [121.74-270.99] µg mL^{-1} and satisfactory regression fit (R^2 = 0.71). Notably, this represents a marked increase in the cytotoxic response compared to neutral conditions. While the 95% CI reflects the inherent biological variability of the essay, the shift toward higher potency under acidic stress suggests that low pH serves as a metabolic trigger for the production of cytotoxic metabolites. In comparison, the positive control, Doxorubicin, demonstrated an IC_{50} of 27.84±1.41 µg mL^{-1}.

In contrast, several isolates displayed weak or indeterminate cytotoxic activity within the tested concentration range (up to 500 µg mL^{-1}). These extracts yielded regression models with low goodness-of-fit (R^2<0.60), and their IC_{50} values are reported as >500 µg mL^{-1} to avoid statistical overprecision. This distinction highlights that the stress-induced cytotoxic profile is a specialized trait of specific isolates like 2RA6, rather than a universal response of the endophytic collection to acidic media.

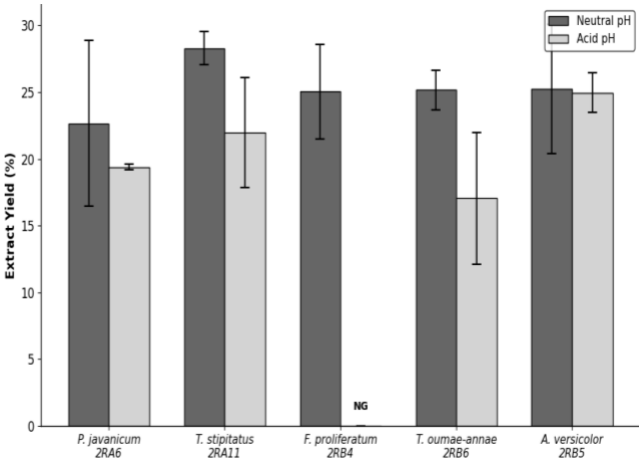


Figure 2. The effect of fermentation pH (neutral vs. acidic (pH 3)) on the crude extract yield (%) of five endophytic fungal isolates. Data are presented as mean±SD. Statistical analysis showed no significant difference between pH treatments across the group (P>0.05, Two-Way ANOVA). NG: No Growth

Table 1. Preliminary mortality of brine shrimp for ethyl acetate extracts at serial concentrations

Extract concentration (µg mL^{-1})	Number of extracts that exhibited toxic activities (mortality ≥90%)
1,000	20
500	18
260	9
125	5 (*Penicillium javanicum* 2RA6, *Talaromyces stipitatus* 2RA11, *Talaromyces oumae-annae* 2RB6, *Fusarium proliferatum* 2RB4, *Aspergillus versicolor* 2RB5)

Table 2. BSLT results for fungal extracts, including LC_{50} estimates, 95% CI, and regression goodness-of-fit (R^2)

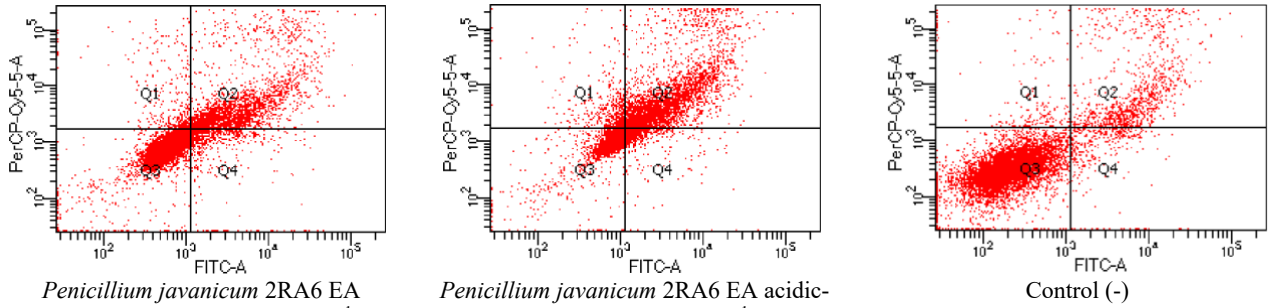
Fungal strain	LC_{50} (µg mL^{-1}) [95% CI][a]	Pseudo R^2	Toxicity (Meyer/Clarkson)*
Penicillium javanicum 2RA6	15.08 [10.57-21.52]	0.908	Highly toxic
Talaromyces oumae-annae 2RB6	33.05 [16.23-67.3]	0.857	Highly toxic
Fusarium proliferatum 2RB4	33.26 [23.79-46.5]	0.895	Highly toxic
Aspergillus versicolor 2RB5	35.48 [21.9-57.8]	0.881	Highly toxic
Talaromyces stipitatus 2RA11	35.27 [21.25-58.54}	0.839	Highly toxic
Thymol	9.86 [5.79-16.80]	0.831	Highly toxic

Note: *: The toxicity class of the extracts is based on Meyer's and Clarkson's toxicity criteria (Niksic et al. 2021)

Table 3. Cytotoxic activity of EA fungal extracts against MCF-7 cells

Fungal strain	pH	IC_{50} (µg mL^{-1}) [95% CI]*	R^2	Cytotoxicity
Penicillium javanicum 2RA6	Neutral	259.39 [139.25-483.18]	0.5809	Weak
	Acid	181.63 [121.74-270.99]	0.7117	Weak
Talaromyces oumae-annae 2RB6	Neutral	ND	-	Inactive
	Acid	374.05 [191.69-729.91]	0.6346	Weak
Fusarium proliferatum 2RB4	Neutral	ND	-	Inactive
	Acid	ND	-	Inactive
Aspergillus versicolor 2RB5	Neutral	ND	-	Inactive
	Acid	ND	-	Inactive
Talaromyces stipitatus 2RA11	Neutral	ND	-	Inactive
	Acid	ND	-	Inactive

Note: *: Values are expressed as mean followed by the 95% CI in brackets based on error propagation from linear regression parameters. ND: Not Determined due to the estimated IC_{50} exceeds the maximum tested concentration, or the upper confidence limit falling outside the valid range, lack of dose-response linearity, or negative inhibition, -: indicates that the R^2 value is not reported due to poor model fit (R^2<0.70) or showing excessive variability



Penicillium javanicum 2RA6 EA neutral-pH extract (315 µg mL^{-1}) | *Penicillium javanicum* 2RA6 EA acidic-pH extract (260 µg mL^{-1}) | Control (-)

Figure 3. Flow cytometric assessment of cell death modes in MCF-7 breast cancer cells treated with *Penicillium javanicum* 2RA6 extracts. Cells were treated with the neutral pH extract and acidic pH extract for 24 h. Representative dot plots of Annexin V-FITC/PI staining show four cell populations: the lower left quadrant ($FITC^-/PI^-$) represents viable cells, the lower right quadrant ($FITC^+/PI^-$) represents early apoptotic cells, the upper right quadrant ($FITC^+/PI^+$) represents late apoptotic cells, and the upper left quadrant ($FITC^-/PI^+$) represents necrotic cells. The distribution highlights extract-induced apoptotic effects

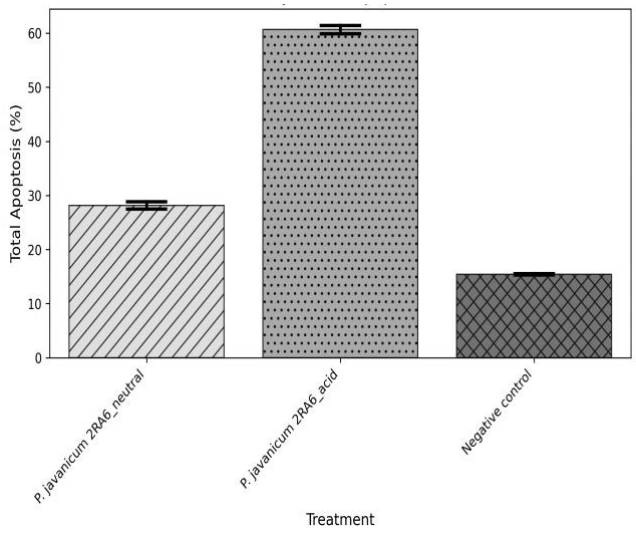


Figure 4. Percentage of total apoptotic MCF-7 cells (early + late) following treatment with *Penicillium javanicum* extracts from different fermentation conditions, analyzed by Annexin V-PI flow cytometry. Data are expressed as mean±SD of technical triplicates (n = 3) derived from a pooled representative extract. Statistical significance was determined by One-Way ANOVA (p<0.001)

Evaluation of EA extracts on cell death modes via Annexin V/PI staining

To further characterize the cytotoxicity of *P. javanicum* 2RA6 extracts, Annexin V-FITC/PI was used to differentiate between apoptotic and necrotic pathways. Quantitative analysis of technical replicates confirmed a distinct apoptotic profile for the acidic fermentation (Figures 3 and 4). The acidic-pH extract induced more than double the total apoptotic cells (60.67%±0.76) compared to the neutral extract (28.12%±0.67; p<0.001). While both extracts increased cell death relative to the control, the 32.54% magnitude of difference confirms that acidic fermentation conditions modulate the production of pro-apoptotic metabolites.

Bioactive compounds in potential MEF extracts based on LC-HRMS analysis

Comparative LC-HRMS profiling of *P. javanicum* 2RA6 revealed distinct metabolic trajectories under neutral and acidic conditions (Figure 5). A comparative summary of the major metabolite classes and their relative abundance under differing fermentation conditions is presented in Table 4. Under neutral pH, the metabolism favored the production of complex aromatic polyketides, specifically

putative flavonoid- and xanthone-like features. In contrast, acidic induction triggered a significant shift toward nitrogenous and lipid-based metabolism. This reprogramming was characterized by the enrichment of fatty acid derivatives (including α-Eleostearic Acid (α-ESA) and fatty acid amides like Linoleoyl Ethanolamide (LEA), and Palmitoyl Ethanolamide (PEA) and a more diverse array of Diketopiperazines (DKPs). Notably, acidic stress also favored the production of iron-chelating compounds (e.g., nocardamine-like features) (Figure 6). These results indicate that the fungal biosynthetic machinery undergoes a fundamental reorganization in response to pH, prioritizing protective and stress-related protective compounds.

Discussion

The significant variability in extract yields observed among the 21 fungal isolates reflects the diverse ecological strategies of mangrove endophytes. While some isolates prioritized rapid biomass accumulation (high yield), others, particularly *P. javanicum* 2RA6, invested in potent chemical defense (high toxicity). The extreme environmental fluctuations characteristic of mangroves likely drive the evolution of adaptive metabolic pathways, yielding structurally diverse bioactive compounds. This aligns with the recognition of mangrove ecosystems as reservoirs of endophytic fungi capable of producing bioactive metabolites with potential anticancer properties (Sajna et al. 2020; Wang et al. 2022; Sandrawati et al. 2023). Consequently, the high lethality observed in the preliminary BSLT screening was not an isolated event but a reliable predictor of cytotoxicity against MCF-7 cells. This relation between BSLT and cytotoxicity tests reinforces the utility of brine shrimp lethality as a cost-effective high-throughput tool for prioritizing fungal isolates that posseses specialized anticancer metabolites.

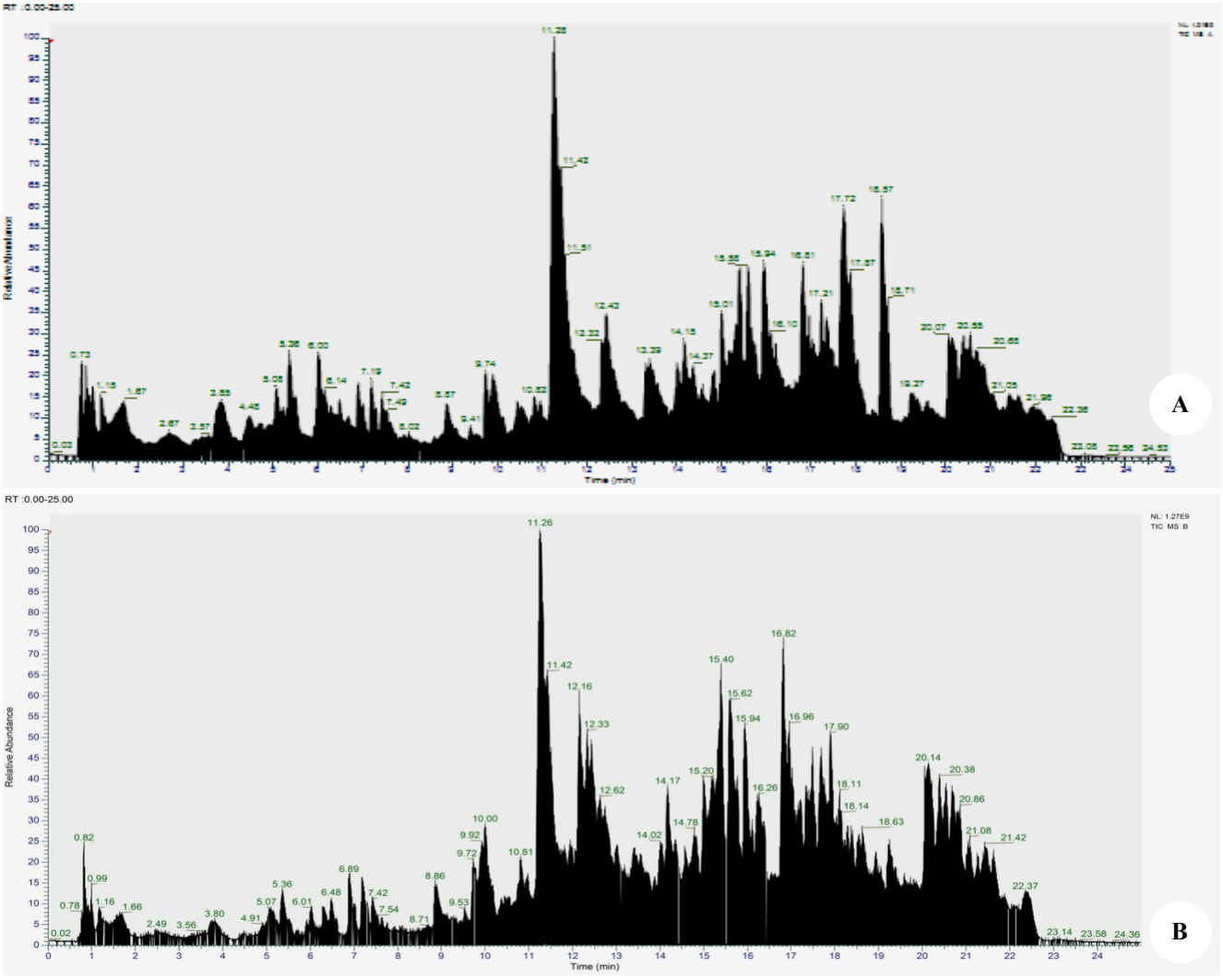


Figure 5. Total Ion Chromatogram (TIC) in positive mode of EA extracts from *Penicillium javanicum* 2RA6 cultured under two conditions: A. Neutral pH, and B. Acidic pH. The chromatographic profiles illustrate pH-dependent differences in metabolite production

A central finding of this study is the capacity of acidic stress to reshape the fungal metabolome. The shift from polyketide-dominant metabolism (neutral pH) to a lipid/ nitrogen-rich profile (acidic pH) highlights the plasticity of *P. javanicum* 2RA6. This transition likely reflects the repression of the pH-responsive transcription factor PacC, which typically governs the expression of acid-induced genes (Keller 2019; Zhang et al. 2024).

Similar pH-driven metabolic modulation has been documented in mangrove-derived *Penicillium* spp. OUCMDZ-4736 produced unique anthraquinone derivatives with potent anti-HBV activity when cultured at pH 2.5, which is note observed under neutral conditions (Jin et al. 2017). Similarly, *P. oxalicum* OUCMDZ-5207 exhibited distinct metabolic profiles at pH 3, generating auroglaucin with strong cytotoxic activity against A549 cells, which was absent under neutral pH (Gao et al. 2021). Collectively, these findings highlight that acid stress activates cryptic metabolic pathways, increasing the diversity of lipid- and nitrogen-based metabolites with potential cytotoxic properties.

As summarized in Table 4, the acid-induced enrichment of fatty acid amides and diketopiperazines correlates with the increase bioactivity. Although non-inoculated medium controls were not included in this specific run, the likelihood that the metabolites are fungal origin is further supported by the recurrence of structurally similar compounds in taxonomically diverse fungi. However, we must transparent about the level of identification achieved. The LC-HRMS annotations in this study represent putatively identified metabolites (MSI Level 2), as structural confirmation using authentic standards or spectroscopic methods was not performed. Although MS1 and MS2 spectral features, molecular formula agreement, and library matches provide strong support for the proposed structures, the identifications can not be considered definitive (MSI Level 1). Therefore, the biochemical interpretation presented here is inferential and based on chemical class-level evidence rather than fully validated molecular structures.

Table 4. Comparison of major metabolite classes in *Penicillium javanicum* 2RA6 extracts and reported bioactivities of putative features

Metabolite class	Putative metabolite	Acid effect	Reported bioactivity and mechanism (Ref.)
Fatty acid and amide	α-Eleostearic acid (α-ESA)	Strongly enriched	Induction of apoptosis via lipid peroxidation: downregukation of Bcl2, Caspase-3 activation (Montecillo-Aguado et al. 2023; Ranasinghe et al. 2021)
	Oleamide Palmitoyl Ethanolamide (PEA) Linoleoyl Ethanolamide (LEA)	Higher abundance and diversity	Antiproliferative effects in MCF-7 cells via modulation of apoptosis-related genes (Rai et al. 2023) Known for anti-inflammatory and potential cytotoxic properties in stress-response contexts (Ferreri et al. 2017)
Organonitrogen	Multiple Diketopiperazines (DKPs)	Diversified	Induction of apoptosis via poly (ADP-ribose) polymerase (PARP) cleavage and mitochondrial pathway disruption
	Nocardamine	More scaffolds detected	(Lalitha et al. 2016; Seo et al. 2020)
Purine derivatives	Adenine	Maintained	Interference with nucleic acid synthesis, promoter of apoptosis in cancer cells, including MCF-7 (Abdelsattar et al. 2025)
Oxo-fatty acids	9-oxo-(10E,12E)-octadecadienoic acid (9-oxo-ODA)	Enriched	Modulation of oxidative stress pathways and induction of apoptosis in various cancer cells (Zhao et al. 2015; Mogi et al. 2023)

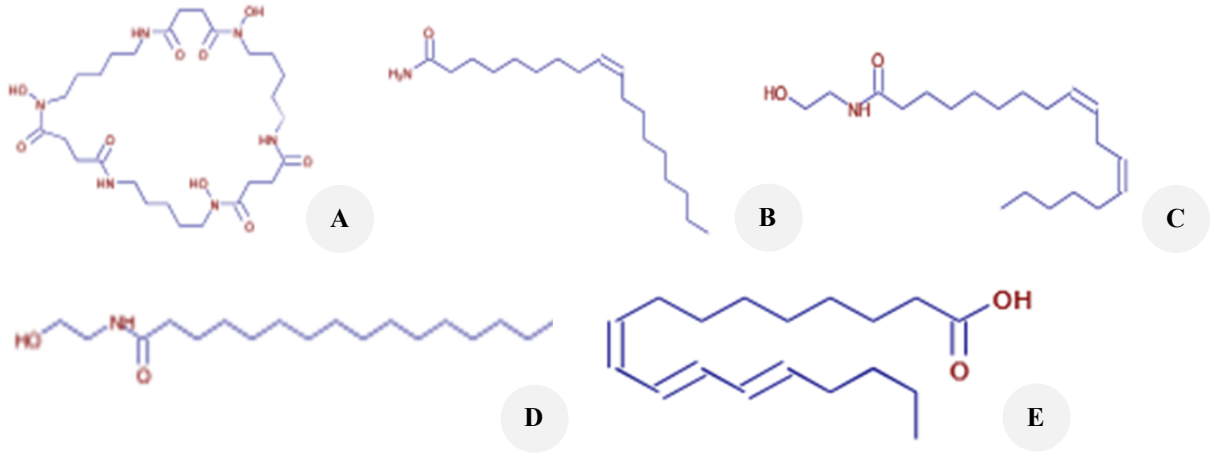


Figure 6. Proposed structures of stress-responsive fungal metabolites putatively identified via untargeted metabolomics. Annotations are classified as MSI Level 2 (based on MS/MS fragmentation patterns) due to the absence of orthogonal validation. A. Nocardamine, B. Oleamide, C. Linoleoyl Ethanolamide (LEA), D. Palmitoyl Ethanolamide (PEA), and E. α-Eleostearic Acid (α-ESA)

Beyond simple cytotoxicity, flow cytometry analysis revealed a distinct mode of action for the acid-induced metabolites. Unlike non-specific toxic agents that typically cause immediate necrotic cell rupture, the acidic extract triggered the externalization of phosphatidylserine (a hallmark of early apoptosis) while initially maintaining membrane integrity. This pattern confirmed that the bioactive compounds, likely the enriched fatty acid amides, activate a regulated programmed cell death pathway rather than causing chaotic necrosis. This mechanism is highly desirable in anticancer drug discovery, as it minimizes the inflammatory response associated with necrotic tissue damage.

While these findings are promising, several limitations must be acknowledged. The present study focused on *P. javanicum* 2RA6 as a representative isolate selected through a bioactivity-guided screening approach. While this enabled in-depth analysis of pH-dependent cytotoxic metabolite production, this selection was data-driven and intended to represent a proof-of-concept demonstration. We acknowledge that focusing on a single isolate may limit the generalizability of the findings to the broader mangrove fungal community.

Furthermore, regarding the association between these metabolite profiles and cytotoxicity, the current evidence is correlative rather than causal. The induction of apoptosis-like cell death suggests a specific mechanism, but molecular confirmation remains to be performed. Future investigations will include comparative metabolomic profiling across multiple bioactive endophytic fungi from *A. ilicifolius* roots to determine whether acid-induced modulation of cytotoxic metabolites is a broader trend among this fungal community. Additionally, future studies integrating bioassay-guided fractionation, molecular docking, isolation, and structural elucidation of this active compounds, along with mechanistic studies in an appropriate in vivo model, are essential to advance these metabolites for therapeutic applications.

In conclusion, this study provides evidence that manipulating abiotic stress is a viable strategy to unlock the cryptic biosynthetic potential of mangrove-derived endophytes. This study demonstrated that acidic fermentation modulates the metabolite profiles of *P. javanicum* 2RA6, triggering a shift from constitutive polyketides to a specialized stress portfolio enriched with fatty acid amides (α-ESA, PEA) and diverse diketopiperazines. This chemical reprogramming resulted in a marked increase in specific cytotoxicity and the induction of apoptosis-like cell death, despite a reduction in overall biomass. These findings suggest that acid-enhanced cultivation is a viable strategy to maximize fungal chemical diversity, accessing bioactive leads that are otherwise overlooked in standard neutral fermentations.

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