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MEDICINE AND PUBLIC HEALTH - RESEARCH PAPER

Anti-mycobacterial, cytotoxic effects of *Penicillium mallochii* ethanol extract, and quantitative detection of its extrolites by lc-q-tof-ms/ms

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Abstract

Tuberculosis and cancer are the most critical global health problems of our time, and the diseases that cause the most deaths globally. This investigation focused on *Penicillium mallochii*, a key component of our fungus collection, which is registered in GenBank under the number MG591446. In this study, the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of *P. mallochii* ethanol extract (EE) against *Mycobacterium* species, as well as their cytotoxic activities on cell lines, were investigated. For this purpose, the anti-mycobacterial activity of *P. mallochii* EE extract was tested against all tuberculosis (TB) strains, including avirulent *Mycobacterium tuberculosis* H37Ra (MT-H37Ra) and virulent *Mycobacterium tuberculosis* H37Rv (MT-H37Rv), and six clinical strains (MT-1 to MT-6). It has also been investigated to explore its anticancer activity on HL-60, K562, and A549 cancer cell lines. Additionally, Liquid chromatography-quadrupole time-of-flight tandem mass spectrometry analysis (LC-Q-TOF-MS/MS) enabled the identification of a total of four compounds in the EE, including harmane, kojic acid, simvastatin, and lovastatin. We have provided information about its extrolite content and antituberculosis and cytotoxic effects. *P. mallochii* is a potential microorganism for future medical drug studies due to its promising antituberculosis activity, cytotoxic effects, and valuable extrolites.

Keywords *Penicillium mallochii* · Ethanol extract · Anti-mycobacterial · Anticancer · Cytotoxic activity, extrolite.

Introduction

Tuberculosis (TB) remains the world's deadliest infectious disease due to its high virulence and increasing prevalence of multidrug resistance (MDR), exacerbated by the escalating threat of MDR. *Mycobacteria* exhibit intrinsic resistance to many antibiotics, disinfectants, and chemical agents [1]. Globally, TB causes nearly one million childhood deaths annually [2, 3], with the World Health Organisation (WHO) reporting 10 million cases in 2019. Approximately 90% of cases occur in adults, predominantly in men; untreated, the mortality rate approaches 50%, and current treatment regimens span 4–6 months [4]. In 2022, TB ranked second only to COVID-19 as a cause of death from a single infectious agent, and case counts have fluctuated, 7.1 million in 1995, dipping to 5.8 million in 2020, then rising to 7.5 million in 2022, mainly due to pandemic-related disruptions [5]. Cancer similarly constitutes a major global health burden, with incidence and mortality climbing steadily. Per GLOBOCAN 2020, there were 19.3 million new cancer cases and nearly 10 million cancer-related deaths, with future projections indicating continued escalation [6]. Therapeutic efficacy is undermined by severe systemic toxicity, organ-specific damage, and MDR, leading to treatment failure and relapse [7, 8]. These limitations highlight the urgent need for novel agents featuring alternative mechanisms and improved safety profiles. Natural products have re-emerged as pivotal sources for drug discovery, with over 60% of anticancer agents derived from or inspired by them [9].

Fungi, in particular, represent an underexplored reservoir of bioactive secondary metabolites with promising pharmacological properties. Fungal-derived compounds

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offer notable advantages, including structural diversity, high biocompatibility, and lower toxicity compared to synthetic drugs [10]. Many of these compounds have demonstrated effectiveness against MDR cancer cells by modulating critical molecular pathways involved in tumor progression, apoptosis, and drug resistance [11]. These attributes support their potential not only in oncology but also in infectious diseases like TB. Fungal metabolites are increasingly recognized for their pharmacological potential, with applications in treating cardiovascular diseases, Alzheimer's disease, cancer, infections, and parasitic conditions such as malaria [12]. Among these, beauvericin a cyclic hexadepsipeptide isolated from Beauveria bassiana and other entomopathogenic fungi exerts strong antimicrobial and cytotoxic effects through ionophoric mechanisms that disrupt membrane integrity [13]. Similarly, simvastatin and lovastatin, traditionally known as HMG-CoA reductase inhibitors, have been reported to suppress the growth of *Mycobacterium tuberculosis* by modulating host lipid metabolism and impairing cell wall biosynthesis [14]. Kojic acid, on the other hand, stands out as a fungal metabolite with well-documented antioxidant and antimicrobial properties, particularly due to its ability to chelate metal ions that are essential for bacterial enzymatic processes [15]. Complementing these mechanisms, harmane, a β-carboline alkaloid, is capable of intercalating into DNA and inhibiting topoisomerase activity, thereby contributing to bacteriostatic effects [16]. Within this context, the genus *Penicillium* has emerged as a prolific producer of bioactive metabolites, and *Penicillium mallochii*, originally isolated from *Rothschildia lebeau* (Lepidoptera: Saturniidae) and *Citheronia lobesis* (Lepidoptera: Saturniidae) [17], has begun to attract attention despite limited biological data. Ethanol extracts of *P. mallochii* have shown insecticidal activity against *Cadra cautella* (Lepidoptera: Pyralidae) and cytotoxicity toward L929 mouse fibroblasts at concentrations ≥ 0.708 mg/mL [18]. Bouhri et al. [19] reported that its pigments possess dyeing capabilities along with antiproliferative activity against glioblastoma (T98G) cells. Zhang et al. [20] isolated two novel sclerothioramine derivatives, isochromophyllon and two known compounds, sclerothioramine and (+)-sclerothiorine, with demonstrated antibacterial and antiyeast effects.

Although pigment production in *P. mallochii* has been partially studied, comprehensive information on its secondary metabolites, ecological adaptations, and host interactions is lacking. The specific compounds responsible for its cytotoxic, antimicrobial, and entomopathogenic effects, as well as their mechanisms of action, remain unclear, and its safety profile and potential environmental risks have not been sufficiently investigated. Furthermore, the limited genomic and transcriptomic data represent a major gap in understanding its biosynthetic pathways and biotechnological potential. Building upon these findings, the present study aimed to investigate the dual therapeutic potential of *P. mallochii*, focusing on its antimycobacterial and anticancer properties. The present study evaluated the antimycobacterial activity of *P. mallochii* EE against *M. tuberculosis* strains (including virulent, non-virulent, and six clinical isolates) and its cytotoxic activity on selected cancer cell lines. These included acute myeloid leukaemia (K562), non-small cell lung cancer (A549), chronic myeloid leukaemia (HL-60), and mouse embryo fibroblasts (3T3) as a healthy control cell line. Furthermore, four important extrolites—harmane, kojic acid, simvastatin, and lovastatin—produced by *P. mallochii* were identified using LC-Q-TOF-MS/MS.

Materials and methods

P. mallochii and media

P. mallochii was isolated from beech tree bark from Balikesir, Türkiye and identified in Charles River lab (USA) via DNA barcoding by using Internal transcribed spacer (ITS) [19]. The ITS region was amplified via PCR by using ITS4 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 5 m (5'-TCC TCC GCT TAT TGA TAT GC-3') primers. The amplification primers were also used for sequencing which accomplished commercially through a biotechnology company (BMLabosis, Ankara, Türkiye). DNA sequences were manually edited and aligned visually using the computer program BioEdit [21] and The ITS sequence data were analyzed separately using PAUP 4.0b10 [22] for the phylogenetic analysis. The sequence was deposited in GenBank under the accession number MG591446 [19]. The specimen was preserved in a lyophilised form at −20 °C in the fungal culture collection of the Department of Biology at Balıkesir University, Türkiye. Malt Extract Agar (MEA) and Sabouraud Dextrose Agar (SDA) were used as media for experimental studies. For spore suspension preparation, *P. mallochii* was cultured on MEA at 28 °C for seven days. Stock cultures were maintained on MEA slants and stored at 4 °C and −20 °C. To prepare the EE extract, *P. mallochii* was cultivated on SDA at 30 °C in the dark for seven days.

Cultivation conditions, preparation of the inoculum and the extract

To prepare the inoculum, all flasks were inoculated with a spore suspension containing 2.5×10^5 conidia/mL, derived from fresh MEA slants of the fungus, and incubated at 28 °C under constant conditions for 4–7 days. *P. mallochii* was incubated on SDA in the dark at 30 °C for 18 days. After the incubation period and colony growth were completed, 2

mL of Tween 80 was added to each Petri dish and the spores were scraped away. The extracellular pigment-containing agar (100 g) from the Petri dishes was cut into small blocks (10 × 10 mm) and transferred into vials. Ethanol was added at a ratio of 1:2 (w/v), and the mixture was agitated on a shaker (ZHWY-211D) at 160–180 rpm for 72 h to extract the compounds into the solvent. Ethanol was used as the extraction solvent due to its ability to efficiently dissolve a wide range of polar and moderately polar bioactive compounds, including pigments, while being safe, biocompatible, easy to remove, and environmentally friendly [23]. The extracts were then filtered using syringe filters with pore sizes of 0.45 µm and 0.20 µm, respectively, and subsequently concentrated using a rotary evaporator (IKA RV 10 basic). The resulting *P. mallochii* EE extract was lyophilised using a freeze-dryer (CHRIST ALPHA 1–2 LD) [18, 19]. A total of 2.8 g of an orange-colored, pasty, and water-soluble substance was obtained.

Anti-mycobacterial activity assay

Microorganisms and preparation of mycobacterial inocula

Six clinical strains (from MT-1 to MT-6) and two standard strains (MT-H37Ra and MT-H37Rv) were used for susceptibility testing. Mycobacteria Growth Indicator Tubes (MGIT), including Middlebrook 7H9 Broth Base (4 mL) by adding OADC (Oleic acid, Albumin, Dextrose, and Catalase) (0.5 mL) and PANTA (Polymyxin-B, Amphotericin-B, Nalidixic acid, Trimethoprim, and Azlocillin) (0.1 mL), were used for the growth of the *Mycobacterium* strains at 37 °C during 8–12 days. Microorganism growth in the tubes was tested daily starting from the second day of incubation using a fluorescence reader (MicroMGIT) equipped with long-wavelength UV light. The inoculum range was between 0.5×10^5 and 2.8×10^5 CFU/mL.

Determination of minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs)

Susceptibility testing of *Mycobacteria* was performed according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS), using the M7-A7 method for broth dilution antimicrobial susceptibility testing of bacteria [24, 25]. The activity of *P. mallochii* EE extract against MT strains was assessed using the Microplate PrestoBlue Assay (MPBA). For this purpose, all wells were filled with 100 µL of Middlebrook 7H9 broth (MBB). Initially, 100 µL of the pigment solution was added to the first column of each row, followed by serial two-fold dilutions. Then, 20 µL of MT inoculum was added to all wells except the negative controls. The stock solution concentration of the extract was 100 mg/mL, and the final test concentrations ranged from 0.09 to 50 mg/mL. Microplates were incubated at 37 °C for 8 days. To monitor bacterial growth, 15 µL of PrestoBlue reagent (Invitrogen, Life Technologies) was first added to the positive control wells and incubated for ten minutes. Subsequently, the same reagent was added to all wells. A positive result (indicating growth) was determined by a color change from blue to pink, whereas a negative result (no growth) was suggested by the blue color remaining unchanged. The MIC was defined as the lowest concentration at which the colour remained blue. To determine the MBC, 20 µL from the wells showing no visible growth was transferred to new wells containing 80 µL of fresh MBB. After an additional two to three days of incubation, MPBA was reapplied, and MBCs were reevaluated using the same method. Anti-mycobacterial activity tests were performed in three series. Rifampicin (RIF, Sigma-Aldrich 557303) and Isoniazid (INH, Sigma-Aldrich 1349706 served as the reference antibiotic in these evaluations (Fig. 1).

Cell Viability/Cytotoxicity assay

Cell culture and WST-1 assay

The cells used in the study are A549 (non-small cell lung cancer, ATCC-CCL-185), HL-60 (acute myeloid leukaemia, ATCC-CCL-240), K562 (chronic myeloid leukaemia, ATCC-CCL-243) and healthy cell lines 3T3 (Mouse embryo fibroblast, ATCC-CRL-1658). All cell lines were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 atmosphere. To evaluate the cytotoxic effects of *P. mallochii* EE, a WST-1 assay was performed. Cells were seeded into 96-well plates at a density of 5×10^3 cells per well. The cells were then treated with different concentrations of the extract: 31.25, 62.5, 125, 250, and 500 µg/mL. After 24 h of incubation with the extract, 100 µL of WST-1 working solution was added to each well. Plates were further incubated for 3–4 h under the same conditions (37 °C, 5% CO_2). Following incubation, the absorbance was measured at 450 nm using a microplate reader (Cytation3, BioTek, USA).The absorbance values, which reflect mitochondrial metabolic activity, were used to determine the percentage of viable cells. Cell viability was calculated using the formula:

$$Cell\ viability\ (\%) = (Absorbance of treated cells / Absorbance of control cells) \times 100$$

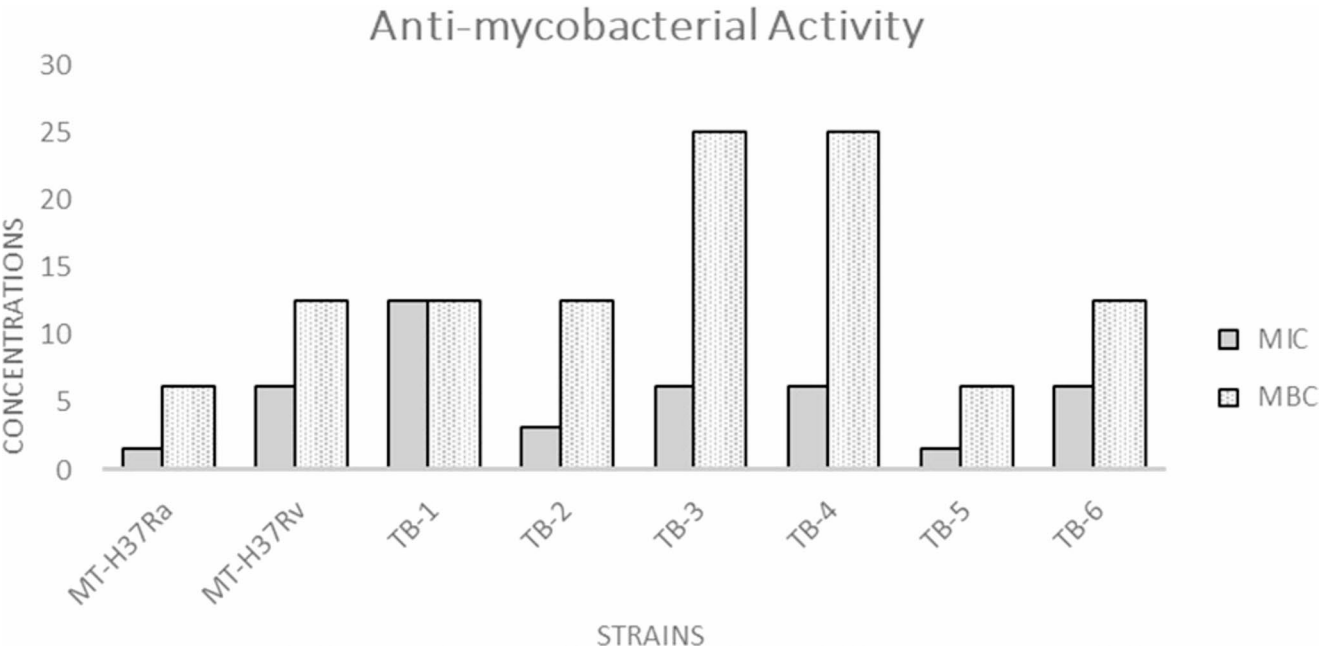

Fig. 1 The anti-mycobacterial activity of the *P. mallochii* EE against MT strains. MT-1 to MT-6: Six clinical strains and MT-H37Ra and MT-H37Rv: two standard strains; MIC and MBC values are expressed in mg/mL

As a positive control, Cyclophosphamide was used. All experiments were conducted in triplicate ($n=7$), and the results are shown in Fig. 2; Table 3.

Statistical analysis

WST-1 assay results were statistically analysed using SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to determine significant differences between groups. P-values less than 0.05 were considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). In addition, GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA) was employed for data analysis and graphical representation.

LC-Q-TOF-MS/MS

LC-Q-TOF-MS/MS conditions

This study conducted qualitative LC-Q-TOF-MS/MS analyses using funds from our project budget at the Ege University Central Research Test and Analysis Laboratory Application and Research Centre, located in Bornova, İzmir, Türkiye. The analytical methods employed are provided here to

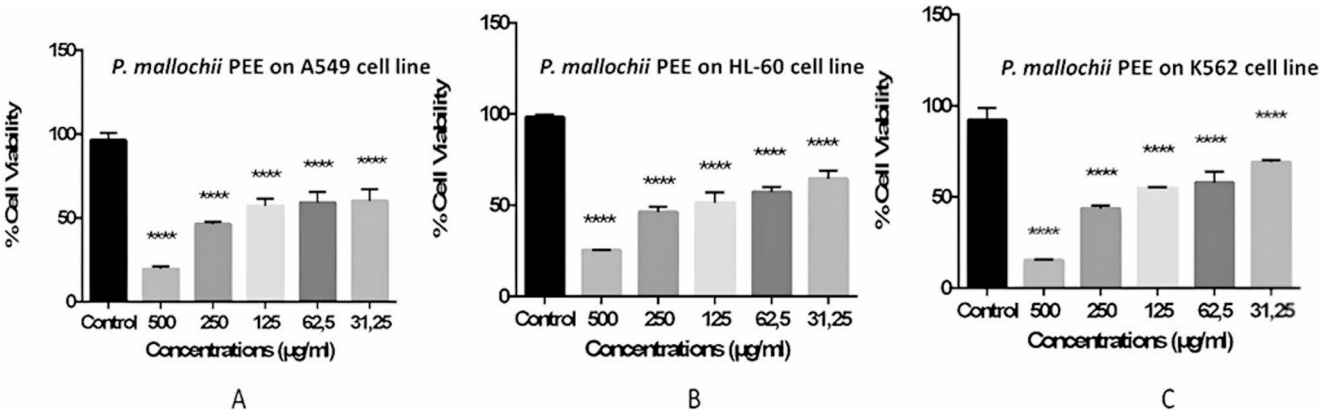

Fig. 2 Cell viability (%) of *P. mallochii* EE against **A**) A549, **B**) HL-60 and **C**) K562 cell line (*$P < 0.05$,**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, n=7)

inform the reader. Chromatographic separation was carried out using an Agilent 1260 Infinity series HPLC system (Agilent Technologies, Santa Clara, CA, USA), equipped with a binary pump, an online degasser, an autosampler, and a Poroshell 120 EC-C18 column (3.0 × 50 mm, particle size 2.7 µm; Agilent Technologies). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B), applied using a gradient elution program as follows: 0–0.5 min, 5% B; 0.5–2 min, 25% B; 2–4 min, 50% B; 4–6 min, 75% B; 6–10 min, 95% B; 10–16 min, 5%B for equilibration of the column. The column temperature was maintained at 35 °C to ensure experimental stability. The injection volume was 10.0 µL, and the flow rate was 0.3 mL/min. For mass spectrometric analysis, an Agilent 6550 iFunnel high-resolution Accurate-Mass QTOF-MS equipped with an Agilent Dual Jet Stream electrospray ionisation (Dual AJS ESI) source was used in positive ion mode. Instrument parameters were set as follows: drying gas flow at 14 L/min, nebuliser pressure at 35 psi, drying gas temperature at 290 °C, sheath gas temperature at 400 °C, and sheath gas flow at 12 L/min. The scan range was m/z 50–2000, with an acquisition rate of 1.5 spectra per second. Data acquisition and processing were performed using the advanced MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

Preparation of the samples and Standarts

Beauvericin, Simvastatin, Kojic Acid, Harmane, and Lovastatin were purchased from Sigma-Aldrich. The samples and standarts were dissolved in a methanol-water (80:20, v/v) mixture and filtered before being injected into the device. The masses and retention times of the compounds are listed in Table 1 below.

Results

Anti-mycobacterial sensitivity assay

The *P. mallochii* EE exhibited variable anti-mycobacterial activity across different MT strains, with MIC values ranging from 1.56 to 12.50 mg/mL and MBC values between 6.25 and 25 mg/mL. The most potent activity was observed against MT-H37Ra and TB-5 strains (MIC: 1.56 mg/mL), while TB-1 showed the highest MIC (12.50 mg/mL), indicating strain-dependent susceptibility. Notably, MT-H37Rv and MT-H37Ra are well-known indicator organisms due to their extensive use in drug susceptibility tests. The results are given in Table 2; Fig. 1.

Table 1 LC-Q-TOF-MS/MS-Based characterisation of detected compounds with corresponding m/z values and retention times

Compound	Mass (m/z)	Retention Time (min)
Beauvericin	784.4159	9.06
Simvastatin	419.2763	8.89
Kojic Acid	143.0817	1.51
Harmane	183.0923	4.13
Lovastatin	405.2633	8.37

Cytotoxicity assay

The cytotoxic effects of *P. mallochii* EE on cancer and normal cell lines were evaluated using the WST-1 assay, and IC_{50} values were determined (Table 3). The extract exhibited moderate cytotoxicity against A549 (IC_{50} = 194.01 µg/mL) and K562 (IC_{50} = 183.90 µg/mL) cell lines, while showing weak cytotoxicity toward HL-60 cells (IC_{50} = 497.71 µg/mL). In contrast, the IC_{50} value for the normal murine fibroblast cell line (3T3) was greater than 500 µg/mL, indicating very low toxicity toward healthy cells. Cyclophosphamide, used as the positive control, also showed IC_{50} values above 500 µg/mL under the tested conditions.

The Selectivity Index (SI) values, calculated as the ratio of the IC_{50} for 3T3 cells to that for each cancer cell line, were ≥2.58 for A549, ≥2.72 for K562, and ≥1.00 for HL-60. According to common cytotoxicity criteria, SI values greater than 2 suggest moderate selectivity toward cancer cells. Thus, *P. mallochii* EE displayed moderate selectivity against A549 and K562 cells, whereas no significant selectivity was observed for HL-60 cells. These findings suggest that the extract may have potential anticancer activity, particularly against lung carcinoma and chronic myeloid leukaemia cell lines, with minimal toxicity to normal fibroblasts.

LC-Q-TOF-MS analyses

P. mallochii EE was characterised by LC-Q-TOF-MS/MS for extrolite profiles (Table 4). The total ion chromatogram (TIC) of the mixture containing standards (Beauvericin, Simvastatin, Kojic Acid, Harmane and Lovastatin) and the extracted ion chromatograms (EIC) and mass spectra of the standards were given in Fig. 3. The biologically essential compounds detected by ESI TIC MS of *P. mallochii* EE in negative and positive ionisation modes and the EIC chromatogram of *P. mallochii* EE for all standards are given below (Fig. 4).

Discussion

Penicillium is one of the most essential genera known for producing biologically active metabolites under various environmental conditions [26]. Extrolite profiling is considered a complementary tool in fungal identification,

Table 2 Anti-mycobacterial activity results for *P. mallochii* EE

Strains	Anti-mycobacterial Activity (mg/mL)			
	MIC		MBC	
MT- H37Ra	1.56		6.25	
MT-H37Rv	6.25		12.50	
TB-1	12.50		12.50	
TB-2	3.12		12.50	
TB-3	6.25		25.00	
TB-4	6.25		25.00	
TB-5	1.56		6.25	
TB-6	6.25		12.50	
	INH*		**RIF***	
	MIC	MBC	MIC	MBC
MT-H37Ra	0.31	0.16	1.24	1.24
MT-H37Rv	0.39	0.78	3.12	6.24

*: Standard drugs concentrations: (µg/mL)

Table 3 IC50 values of the *P. mallochii* EE on A549, HL-60, K562 and 3T3 cell lines were calculated using the WST-1 method

Cell line	IC50 values
3T3	>500
A549	194.01
HL-60	497.71
K562	183.9
Cyclophosphamide (positive control)	>500

particularly since filamentous fungi such as *Penicillium* are difficult to distinguish using traditional microbiological techniques. This profiling encompasses all secondary metabolites produced by a fungus on a given substrate, including toxins, antibiotics, fatty acids, and other bioactive compounds [27]. The expression of specific fungal metabolites is associated with the formation of sexual or asexual reproductive structures, such as cleistothecia, and the differentiation of other specialised cell types [28]. Consequently, the consistent production of certain secondary metabolites in the mycelium and spores of a fungus serves as a distinguishing feature from other species [29]. In this study, the EE of *P. mallochii* was investigated for its anti-mycobacterial activity, anticancer activity against HL-60, K562, and A549 cancer cell lines, and the profiling of several extrolites using LC-Q-TOF-MS/MS analysis.

The EE of *P. mallochii* demonstrated notable inhibitory activity against *M. tuberculosis* reference strains and clinical isolates, with MIC values ranging from 1.56 to 12.50 mg/mL and MBC values between 6.25 and 25.00 mg/mL. Although these values are substantially higher than those of standard first-line drugs such as INH and RIF (MIC range: 0.31–3.12 µg/mL), the broad-spectrum efficacy against multiple clinical isolates, including both drug-sensitive and drug-resistant forms, indicates a promising antimicrobial potential. Notably, TB-5 and MT-H37Ra exhibited the highest susceptibility, while TB-3 and TB-4 displayed relative tolerance, suggesting possible differences in cell wall composition or efflux pump activity that may influence susceptibility [30, 31]. Notably, MT-H37Rv and MT-H37Ra are well-known indicator organisms due to their extensive use in drug susceptibility tests.

In this study, the cytotoxic activity of *P. mallochii* EE was evaluated using the WST-1 assay across a range of concentrations (31.25–500 µg/mL) in both cancerous (HL-60, K562, A549) and non-cancerous (3T3) cell lines. The results clearly demonstrate that the EE exhibited dose-dependent antiproliferative activity, significantly reducing cell viability in malignant cell lines, particularly K562 (IC_{50} = 194.01 µg/mL), A549 (IC_{50} = 183.0 µg/mL), and HL-60 (IC_{50} = 497.71 µg/mL), while showing minimal cytotoxicity toward healthy fibroblast 3T3 cells even at the highest tested concentrations. The observed selectivity suggests that *P. mallochii* EE may exert cancer-specific cytotoxicity, a desirable property in anticancer drug discovery. This selectivity suggests that *P. mallochii* EE may exert anticancer effects via mechanisms such as apoptosis induction, ROS generation, or cell cycle arrest, as reported for other fungal-derived compounds [32]. The relatively moderate IC_{50} values compared to conventional chemotherapeutics indicate that activity optimisation, through fractionation, purification, and potential structural modification, may enhance potency. This selective effect aligns with previous findings where ethanol fractions from endophytic or fungal extracts demonstrated stronger antiproliferative effects compared to aqueous or methanolic fractions, likely due to enrichment of semi-polar bioactive secondary metabolites such as polyketides, terpenoids, and phenolic compounds [33, 34]. The decreased mitochondrial activity, as indicated by WST-1 assay, suggests a potential interference with mitochondrial respiration or induction of apoptosis-related pathways. The WST-1 assay relies on mitochondrial dehydrogenase activity; thus, the reduction in absorbance at higher concentrations implies a compromised

Table 4 LC–Q-TOF–MS/MS data of *P. mallochii* EE

No	Name	Formula	CAS	Mass (m/z)	Retention Time (min)	Quantity (ng/g)	ID Source	(MFG) Algorithm
1.	Beauvericin	$C_{45}H_57N_3O_9$	26048-05−5	784,4159	9.06	nd	DBSearch	Auto MS/MS
2.	Simvastatin	$C_{25}H_{38}O_5$	79902-63−9	419.2763	8.89	7.01	DBSearch	Auto MS/MS
3.	Kojic acid	$C_6H_6O_4$	501-30−4	143.0817	1.51	287.93	DBSearch	Auto MS/MS
4.	Harmane	$C_{12}H_{10}N_2$	486-84−0	183.0923	4.13	8.16	DBSearch	Auto MS/MS
5.	Lovastatin	$C_{24}H_{36}O_5$	75330-75−5	405.2633	8.37	0.91	DBSearch	Auto MS/MS

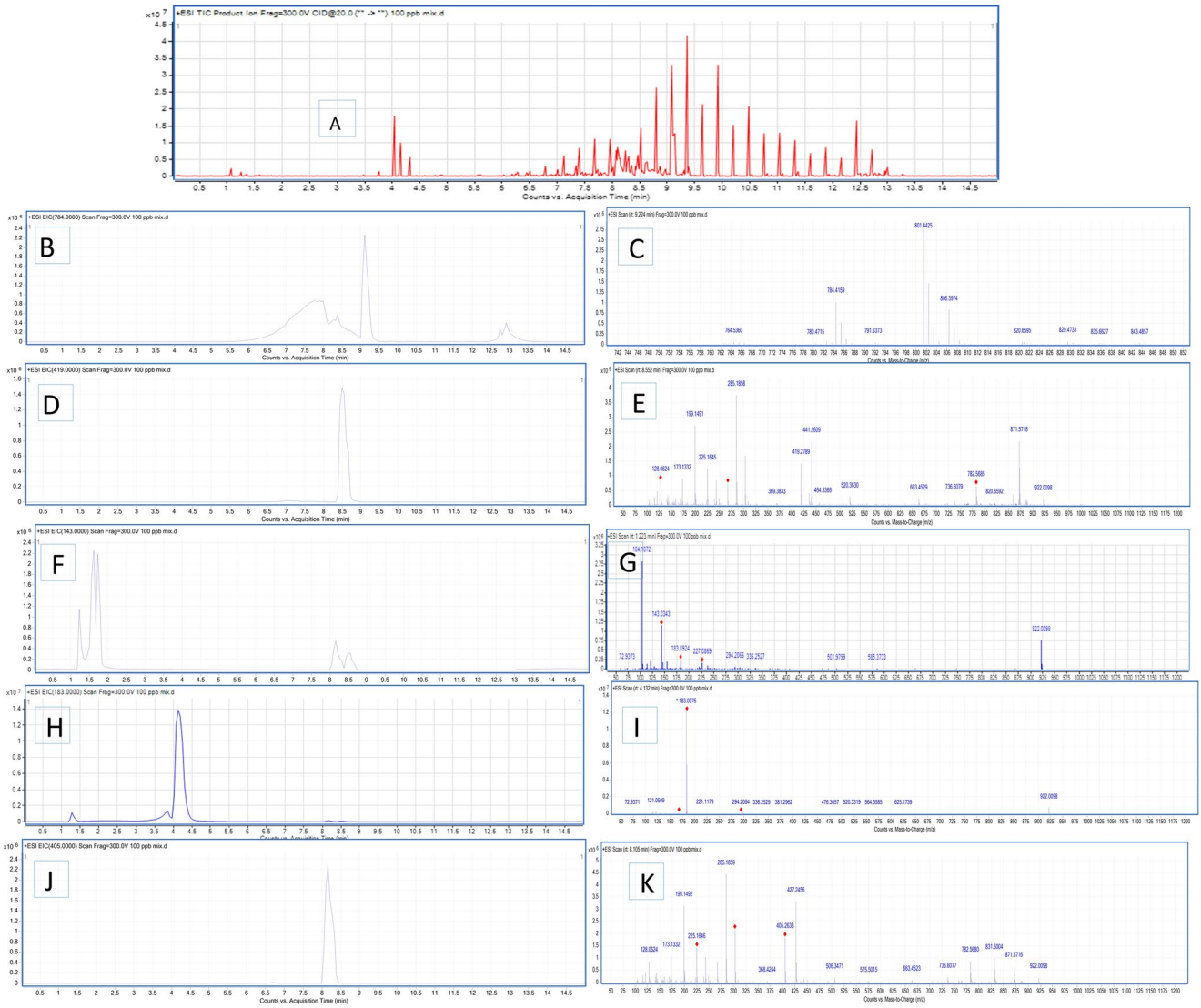

Fig. 3 **A**) TIC of the mixture with standards; **B**-**C**) EIC and mass spectra of Beauvericin; **D**-**E**) EIC and mass spectra of Simvastin; **F**-**G**) EIC and mass spectra of Kojic acid; H-I) EIC and mass spectra of Harmane; **J**-**K**) EIC and mass spectra of Lovastatin.TIC: The total ion chromatogram; EIC: Extracted ion chromatograms

metabolic state in cancer cells. Similar mechanisms have been observed in studies of fungal-derived extracts that induce intrinsic apoptotic pathways via mitochondrial depolarization [35, 36]. Among the tested cancer cell lines, the strongest cytotoxic effect was seen in K562 cells, a chronic myeloid leukemia line, followed by A549 and HL-60. These findings suggest that *P. mallochii* EE may be particularly effective against hematological malignancies. This trend is consistent with other extracts derived from endophytic fungi or actinomycetes that preferentially target leukemia

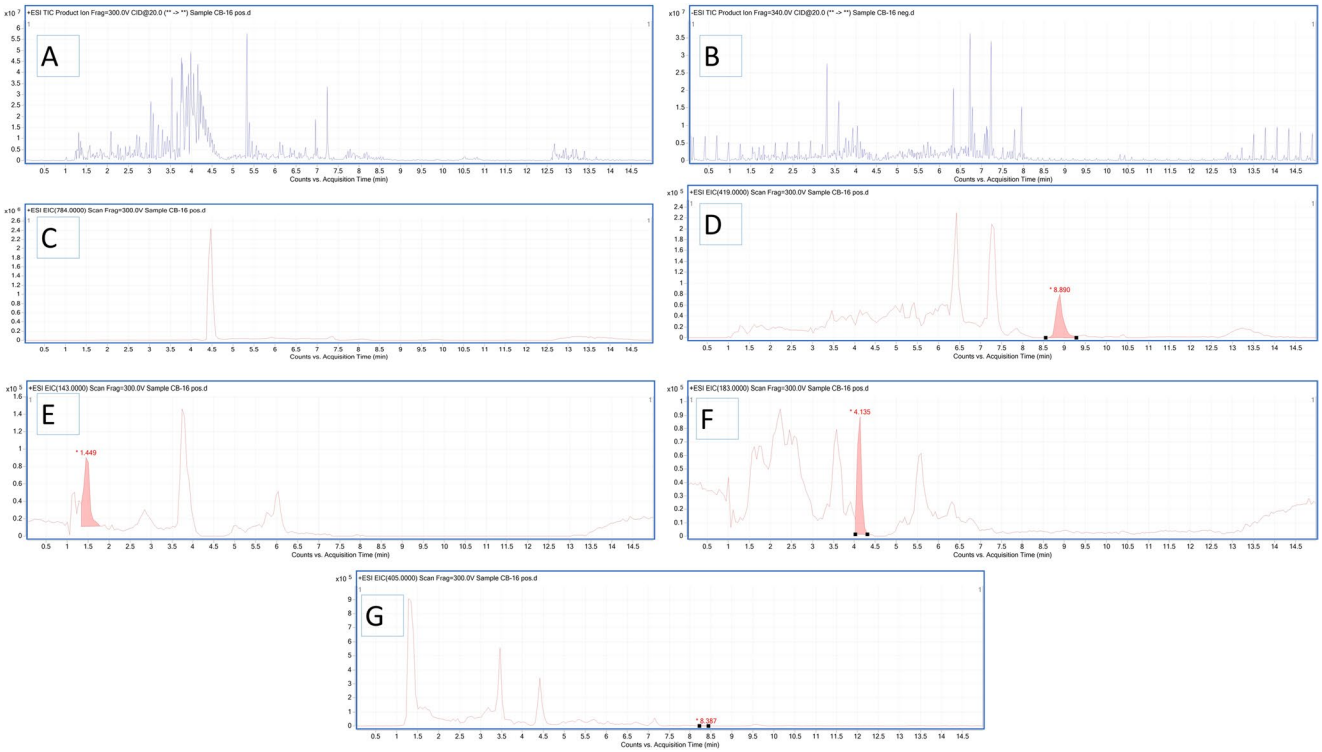

Fig. 4 LC-Q-TOF MS/MS analysis EICs of *P. mallochii* EE (**A**) positive mode TIC; (**B**) negative mode TIC; (**C**) EIC chromatogram of *P. mallochii* EE for Beuavericin; (**D**) EIC chromatogram of *P. mallochii* EE for Simvastatin; (**E**) EIC chromatogram of *P. mallochii* EE for Kojic acid; (**F**) EIC chromatogram of *P. mallochii* EE for Harmane; (**G**) EIC chromatogram of *P. mallochii* EE for Lovastatin. TIC: The total ion chromatogram; EIC: Extracted ion chromatograms

cell lines due to differential uptake or susceptibility to fungal metabolites [37]. Importantly, the extract did not exhibit significant cytotoxicity on 3T3 fibroblast cells, indicating a favorable therapeutic index. The lack of toxicity on normal cells is a critical advantage, as many current chemotherapeutics exhibit narrow safety margins. Selectivity toward cancer cells has been associated with differences in metabolic activity, membrane composition, or redox states between normal and malignant cells [38]. Although the observed decrease in cell viability in the WST-1 assay may hint at mitochondrial involvement, the assay reflects general metabolic activity. It is influenced by multiple factors beyond strictly mitochondrial function. To substantiate a mitochondrial-specific mechanism in A549, HL-60, or K562 cells, additional targeted assessments (e.g., mitochondrial membrane potential assays, oxygen consumption rate measurements) or literature directly correlating WST-1 readouts with mitochondrial alterations in these specific cell lines would be required. For example, studies have reported associations between mitochondrial dysfunction (e.g., dissipation of mitochondrial membrane potential and cytochrome c release) and cytotoxic outcomes in HL-60 and K562 cells, underscoring the importance of direct mitochondrial assays alongside WST-1 data.

LC-Q-TOF-MS/MS is a powerful analytical technique capable of selectively detecting and characterizing compounds by separating, identifying, and interpreting their ion patterns. Its high selectivity and versatility make it a valuable tool for determining a broad spectrum of metabolites [39]. The sensitivity of this method is attributed to the correlation between an analyte's signal intensity and its concentration, allowing for quantification by comparing unknown samples to standards [40]. In our research, LC-Q-TOF-MS/MS analysis of *P. mallochii* extracts revealed the presence of several noteworthy extrolites: harmane, kojic acid, simvastatin, and lovastatin. These compounds are recognised for diverse bioactivities, including antimicrobial, anticancer, immunomodulatory, and cholesterol-lowering effects, underscoring the fungus's multifaceted therapeutic potential. Beauvericin is a cyclic hexadepsipeptide produced by *Fusarium* species, well-known for its strong antimicrobial and cytotoxic activities [41]. It exhibits broad-spectrum antibacterial activity, including against Gram-positive and Gram-negative pathogens, as well as *M. tuberculosis*, by disrupting cellular ion balances rather than targeting the cell wall [42]. Although beauvericin is a well-known fungal metabolite, it was not detected in the EE extract. Therefore, the observed biological activities are likely attributed to other identified compounds.

Statins (lovastatin/simvastatin precursor) are produced industrially, with lovastatin production being classically associated with *Aspergillus terreus*. Other fungi, including *Penicillium citrinum*, *Monascus* spp., and *Pleurotus ostreatus*, also produce it to varying degrees. Thus, a lovastatin-like signal in *P. mallochii* is taxonomically plausible within Eurotiales and aligns with known statin biosynthetic capacity beyond *A. terreus* [43].On the other hand, simvastatin (7.01 ng/g) and lovastatin (0.91 ng/g) are other compounds found in the EE extract. Simvastatin and lovastatin are HMG-CoA reductase inhibitors used for cholesterol lowering, but their bioactivity extends beyond lipid metabolism. They exhibit immunomodulatory and antimycobacterial effects, including inhibition of intracellular *M. tuberculosis* in macrophages by promoting autophagy [44]. Lovastatin can inhibit tyrosine phosphorylation [45] and expression, specifically impairing cholesterol-dependent survival mechanisms in *M. tuberculosis*. Both statins have been noted to have anticancer effects via anti-angiogenic and pro-apoptotic pathways. Recent studies have demonstrated several important applications for lovastatin, including its use as an antimicrobial agent and as a treatment for cancers and bone diseases [46]. Kojic acid is produced mainly by *Aspergillus* (e.g., *A. oryzae*, *A. niger*), but surveys document production by ~58 fungal strains spanning *Aspergillus*, *Penicillium*, and a few others [47, 48]. Harmane shows broad antimicrobial/antifungal activity and has been studied against *Penicillium digitatum* and *Botrytis cinerea* (membrane effects, germination blockade) [49]. Kojic acid is the compound with the highest concentration in the EE extract (287.93 ng/g). Kojic acid and its derivatives can act as antioxidants, antimicrobials, anti-inflammatories, radioprotectors, and anticonvulsants [50]. Kojic acid, a fungal metabolite commonly used in cosmetics [51], inhibits tyrosinase and has recognised antibacterial and antioxidant effects [52]. Its metal-chelating property contributes to antimicrobial activity [53]. Although moderate in cytotoxicity, it is reported that its inclusion alongside other agents in the extract could provide synergistic antimicrobial benefits. Another compound, harmane (8.16 ng/g), exhibits acetylcholinesterase, antioxidant, antibacterial, and antimycobacterial properties, as mentioned above. Harmane, a β-carboline alkaloid, exhibits antimicrobial, antioxidant, acetylcholinesterase, antitumor, transmitter, and neuroactive properties [54, 55]. Its antibacterial activity—demonstrated across plant and animal pathogens—is linked to multiple mechanisms, including DNA intercalation and enzyme inhibition, such as topoisomerase II and DNA gyrase [56]. Lopes-Ortiz et al. [57] evaluated β-carboline derivatives as antituberculosis compounds. Harmane disrupts mature biofilms by reducing exopolysaccharide content and killing embedded bacteria. It damages the bacterial cell wall through interaction with peptidoglycan, causing irreversible membrane disruption, as demonstrated by fluorescent staining and leakage assays. Once inside the cell, harmane disturbs redox balance, inhibits metabolic activity, and targets ribosomes [58]. Four prominent bioactive compounds—simvastatin, kojic acid, harmane, and lovastatin—each showing unique pharmacological potential (LC-Q-TOF-MS/MS data). Their combined presence supports the extract's promise as a source of antimicrobial and anticancer agents.

While notable synergistic effects have been established in specific dermatological and metabolic contexts, no evidence currently supports synergistic interactions among simvastatin, kojic acid, harmane, and lovastatin or between any pair or across this compound set. However, synergistic effects exist between aforamented compounds and other compounds. A study examined the combination of lovastatin and eicosapentaenoic acid (EPA). The synergy between lovastatin and EPA was shown as enhanced modulation of gene expression related to cholesterol metabolism in HepG2 cells [59]. As a result of the synergy between simvastatin and folic acid, superior wound healing results were observed with the formation of a topical chitosan-based film compared to either drug used alone. This synergy was observed to increase collagen expression and reduce inflammation [60]. When combined with agents such as kojic acid, hydroquinone, or glycolic acid, these combinations demonstrate greater efficacy than kojic acid alone, demonstrating synergistic potential in the treatment of hyperpigmentation [61]. Additionally, a synergistic effect of kojic acid, tranexamic acid, and niacinamide formulation was found to provide significant improvements (>60% reduction) in the treatment of melasma and post-inflammatory hyperpigmentation [62]. The dual bioactivity profile of *P. mallochii* EE against both mycobacterial pathogens and cancer cell lines highlights its potential as a source of multifunctional bioactive compounds. Similar findings have been reported for other entomopathogenic fungi, where secondary metabolites such as destruxins, bassianolide, and enniatins exhibited broad-spectrum antimicrobial and antitumor properties [63].

Future studies should focus on bioassay-guided fractionation to isolate individual compounds, determine their specific mechanisms of action, and evaluate synergistic interactions. Moreover, in vivo validation in relevant infection and tumour models will be critical for assessing therapeutic feasibility.

Conclusion

The findings of this study demonstrate that the EE of *P. mallochii* exhibits promising anti-mycobacterial and anticancer activities. The antimycobacterial efficacy of the extract was confirmed against both virulent (MT-H37Rv) and avirulent (MT-H37Ra) MT strains, as well as six clinical isolates, highlighting its potential as a source of novel therapeutic agents. Notably, this is the first report to evaluate the cytotoxic effects of *P. mallochii* on HL-60, K562, and A549 cancer cell lines. Collectively, our findings support the potential of *P. mallochii* as a promising source of selective anticancer agents, particularly for hematologic and lung malignancies. LC-Q-TOF-MS/MS profiling of the extract revealed several bioactive extrolites, including simvastatin, lovastatin, kojic acid, and harmane, which are known for their antimicrobial and anticancer properties. These compounds likely contribute either individually or synergistically to the observed biological effects. Despite these promising findings, the study is limited by the absence of purified compounds and in vivo validation. Future research should focus on the isolation and purification of bioactive constituents from the *P. mallochii* ethanolic extract using chromatographic and spectroscopic techniques, followed by structural elucidation through nuclear magnetic resonance (NMR) and mass spectrometry (MS). Once identified, the cytotoxic potential of individual compounds should be evaluated to pinpoint the agents responsible for the observed effects. Mechanistic studies, including apoptosis detection assays (e.g., Annexin V/PI staining, caspase activation, mitochondrial membrane potential analysis) and cell cycle profiling, will help clarify the pathways underlying their cytotoxicity. In addition, in vivo studies with relevant tumour models are crucial to assess efficacy, safety, pharmacokinetics, and therapeutic potential. Together, these investigations will enable the translation of in vitro findings into preclinical applications and support future drug development efforts. In conclusion, *P. mallochii* emerges as a valuable fungal species with potential applications in developing selective anti-mycobacterial and anticancer agents.

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Declarations

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