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Research Article

Isolation and Identification of *Penicillium herquei*: Antibacterial, Antioxidant and Cytotoxic Properties of *Penicillium herquei* Ethyl Acetate Extract

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ABSTRACT

Background: Fungi are organisms with wide diversity and are an essential source of bioactive compounds. **Objectives:** Genotypic characterization and analysis of antibacterial and antioxidant activities of *Penicillium herquei* ethyl acetate extract. **Materials and Methods:** In the present study, *Penicillium herquei* Bainier and Sartory-A2 type strains were isolated and characterized from environmental sources (*Phyllanthus niruri* plant leaf). *Penicillium herquei* was selected for genomic identification by sequencing the ITS regions. Fungal DNA was isolated and ITS regions were amplified using primers ITS5 and ITS4. Phylogenetic analysis was performed using the NCBI server and the fungal sequence submitted on the NCBI website (NCBI accession number: MG909554.1). Fungal cultures were grown in a solid medium using potato dextrose agar. Fungal mats were extracted using ethyl acetate and evaluated for antibacterial activity, antioxidant activity (DPPH) and cytotoxicity evaluations. **Results:** Fungal DNA sequenced NCBI BLAST analysis showed 99% similarity with *Penicillium herquei*. *Penicillium herquei* mat extract of ethyl acetate showing the zone of inhibition *E. coli* (13 mm), *B. cereus* (5 mm), *S. aureus* (3 mm) and *K. pneumoniae* (12 mm). The ethyl acetate extract demonstrated strong free radical scavenging activity by donating hydrogen atoms in the DPPH assay. Additionally, it displayed marked cytotoxic effects against SK-MEL-3 human skin cells, with an inhibitory concentration of 16 µg/mL. **Conclusion:** *Penicillium herquei* with bioactive secondary metabolites has good antibacterial, antioxidant and cytotoxic properties.

Keywords: *Penicillium herquei*, ITS5 and ITS4, NCBI, Antibacterial, Antioxidant, Cytotoxicity.

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INTRODUCTION

Filamentous fungi belonging to the *genus Penicillium*, which are part of the Ascomycetes group, are commonly found in various environments. They have been instrumental in medical advancements ever since penicillin was discovered. Penicillin, a β-lactam antibiotic produced by *Penicillium chrysogenum*, marked the beginning of recognition of the therapeutic potential of antibiotics.[1,2] Beyond penicillin, the *genus Penicillium* boasts a rich diversity of over 354 acknowledged species, many of which are known to produce bioactive compounds with pharmacological activities.[3,4] Endophytic fungi, specifically filamentous members of the Penicillium genus, have emerged as valuable sources of low molecular weight bioactive substances. For instance, Chrysogenum is known to produce essential bioactive secondary metabolites. The genus has significantly contributed to the development of various bioactive molecules, with some species producing antibiotics such as Penicillium brevicompactum, which yields mycophenolic acid used in immunosuppressive treatments.[5] Exploration of Penicillium species has revealed a plethora of bioactive compounds, showing their diverse pharmacological potential. These compounds exhibit antimicrobial, anticancer and antifungal activities.[6,7] As a result, an increasing number of Penicillium species have been subjected to bioactive chemical evaluation from various environmental sources. Among Penicillium species, *Penicillium herquei* strains have been identified and secondary metabolites extracted from these strains have been evaluated for their biological activity. This research biological potential of *Penicillium herquei* and exploring its secondary metabolites for potential applications. This research not only contributes to our understanding of the bioactive potential within the Penicillium genus, but also highlights the significance of these fungi in drug discovery and

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pharmaceutical development. The diverse ecological compounds identified within this genus exhibit antiviral, anti-inflammatory and cytotoxic activities.[8-10]

MATERIALS AND METHODS

Sampling site and environmental data

Penicillium herquei Bainier and Sartory A2 was isolated from *Phyllanthus niruri* leaves collected from Palakkad, Kerala. The leaves were rinsed with tap water to obtain a fine powder. Adequate powder was transferred to PDA medium and incubated at room temperature for three days. Fungal cultures were separated and purified using the streaking method.

Isolation and Identification of Fungi

An alternative approach for distinguishing closely related fungal species involves amplifying specific regions of ribosomal RNA gene clusters using universal primers in PCR. In this molecular analysis, fungi were identified through amplification of the rDNA ITS region using the universal primers ITS4 and ITS5 (NCBI Accession number MG909553). The resulting fungal isolates were deposited in the National Fungal Culture Collection of India (NFCCI, accession no: 4233).[11,12]

Microscopic study of *Penicillium herquei*

Fungal morphology was observed using a microscope and morphotaxonomy was observed in the conidial heads, colonies, sterigmata, chlamydospores, conidiophores and vesicles.[13]

Subculturing of the *Penicillium* spp.

The *Penicillium* spp. Subcultured onto PDA plates prepared by dissolving 39 g of PDA in 1000 mL of distilled water and sterilizing at 121ºC for 15 min. After inoculation to the plate, this incubated at 27ºC for 7-10 days the well-grown sample used for further study.

Preparation of production media

Two different media were used to optimize the compounds (Media 1 and 2). Medium 1 contain K_2HPO_4 1.0 g, KCl 0.5 g, $NaNO_3$ 3.0 g, $FeSO_4$ 0.01 g, $MgSO_4·7H_2O$ 0.5 g, yeast extract 5.0 g, sucrose 30.0 g and NaCl 20.0 g, dissolved in distilled water 1,000 mL. Medium 2 contain $NaNO_3$ 3.0 g, beef extract 5.0 g, KH_2PO_4 1.0 g, $FeSO_4$ 0.01 g, $MgSO_4·7H_2O$ 0.5 g, glucose 30.0 g and NaCl 15.0 g in 1000 mL of distilled water. After preparing the media, sterilization was allowed and a 5 mm thick plugged fungal culture using a cork borer and incubated at 30ºC for seven days.

Extraction and purification of compounds

media were prepared separately and incubated at 29ºC for 7-10 days. The mycelial mat was partially grown within 7 days and after obtaining the mycelial mat, the compounds were extracted. First, the broth medium was filtered and the mat was crushed with ethyl acetate at 1:3 (w/v). After mixing, the mixture was incubated for 3 hr with intermediate mixing to obtain an extract. Finally, the aqueous phase was separated and stored to obtain a purified sample, which was used for further studies.[14]

Identification of secondary metabolites

The purified solution was used for the identification of the compounds and TLC was performed. A 4 cm wide and 8 cm high TLC plate was marked from the bottom of 1 cm to load the sample loaded with nearly 50 µL. The dried leaf was allowed to run in a solvent system of chloroform: methanol: 25% of ammonium hydroxide (89.95: 9.95: 0.1), the plate was allowed to dry and the R_f value calculated using the formula.

Antibacterial activity of *Penicillium herquei* ethyl acetate extracts

The isolated fraction of the fungal extract was studied for its antibacterial activity by using the well plate method. The tested bacteria (*E. coli, B. cereus, S. aureus, and K. pneumoniae*) were collected from the authorized center in Coimbatore. The collected bacteria were prepared as an inoculum and used for the study of antibacterial activity analysis.[15,16]

Antioxidant activity of *Penicillium herquei* ethyl acetate extracts

Stock solutions of ascorbic acid and ethyl acetate extracts were each prepared at 1.0 mg/mL, and varying concentrations of ascorbic acid (5, 10, 15, 20, and 25 µg/mL) were combined with the ethyl acetate extract in a methanol solution containing 0.5 mM DPPH. Each mixture consisted of 0.5 mL ethyl acetate extract and the respective ascorbic acid solution, and following a 30-min incubation at room temperature in the dark, absorbance readings were taken at 517 nm using a Stat Fax 4200 ELISA reader. All experiments were repeated twice, and the percentage of inhibition was determined using the formula:[17]

% inhibition = [(mean absorbance of the control – mean absorbance of the sample)/mean absorbance of the control] × 100

Cytotoxicity activity of *Penicillium herquei* ethyl acetate extracts

SK-MEL-3 cells were harvested, counted using a diluted hemocytometer in DMEM (1×104 cells/mL) and seeded into individual wells of 96-well plates. After 24 hr of incubation for cell attachment, the wells were treated with either control or various concentrations of the *Penicillium herquei* ethyl acetate extract. After treatment, SK-MEL-3 cells were incubated for another 24 hours at 37°C in a humidified environment with 95% air and 5% CO_2. The cells were then rinsed with fresh culture medium and incubated for another 4 hours at 37°C with MTT solution (5 mg/mL in PBS). The resulting purple formazan crystals were dissolved in 100 µL of concentrated DMSO, and cell viability was

assessed by measuring absorbance at 540 nm using a multi-well plate reader. The percentage of viable cells compared to the control was calculated using the formula:

% cell proliferation inhibitory = [(mean absorbance of the control – mean absorbance of the sample) / mean absorbance of the control] × 100

Study of apoptotic induction properties of *Penicillium herquei* ethyl acetate extract

SK-MEL-3 cells were seeded in six-well plates at a density of 5×10^4 cells per well and incubated for 24 hr. After exposure to the ethyl acetate extract of Penicillium herquei for 24 hr, cells were collected, rinsed with cold PBS, and stained for 5 min at room temperature with a 1:1 mixture of Acridine Orange and Ethidium Bromide, each at 100 µg/mL. Stained cells were then examined under a fluorescence microscope at 40x magnification to assess apoptotic changes. Following treatment, cells were detached, washed three times with PBS, stained again with the AO/EB mixture under the same conditions, and observed microscopically.

Statistical analysis

All research data are presented as mean values, and statistical analysis was conducted using Windows SPSS software version 12.0. A *p*-value of less than 0.05 was regarded as statistically significant.

RESULTS AND DISCUSSION

Morphological and Genotypic analysis.

Two Penicillium strains were collected from plant leaves and enriched with PDA. Isolates purified by a single spore isolation technique are characterized by microscopic observation and measurement, including penicillin. *Penicillium herquei* Bainier and sartory SCOPS-A2 colonies on CYA fast-growing, velutinous, reverse pale yellow or yellow-brown, 16×18 mm in 7 d. Penicilli strictly verticillate, rarely terverticillate, terminally produced. Metulae 3-5 in number, smooth, hyaline, 9.63x2.88 µm. Phialides ampulliform, cellular short 2-5 per matulae, hyaline, smooth-walled, up to 10.15x2.5 µm. Conidia globose to oval light oluvaceous, 1.95-2.67x2.67-2.94 µm (Figures 1).

Molecular identification of *Penicillium herquei*

The isolates were identified according to standard procedures. The raw sequences generated by the ABI 3100 automated DNA sequencer were manually checked and edited for any discrepancies. Sequence quality was assessed through BLAST analysis on the NCBI server. The fungal isolate exhibited 99% sequence identity with *Penicillium herquei*. Comparison with NCBI accession number MG9095543 and *Penicillium herquei* isolate P14910 yielded the following alignment details: query length of 523, score of 935 bits (1036), expect value of 0.0, 521 out of 523 identical bases (99%), no gaps detected, and alignment on the Plus/Plus strand (Figure 2).

Large scale cultivation

The fungus *Penicillium herquei* was cultivated in solid-enriched media. The mycelial mat was isolated and homogenized with ethyl acetate/aqueous in a ratio of 1:3 (w/v) and from the extraction of bioactive secondary metabolites isolated using chromatography techniques (see the experimental methods).

Antibacterial activity

Investigation of the antibacterial activity of *Penicillium herquei* ethyl acetate extract, assessed by the disc diffusion method against various clinical bacteria, yielded noteworthy results. The extract demonstrated significant zones of inhibition, particularly against *E. coli* (13 mm) and *K. pneumoniae* (12 mm), suggesting its potent antibacterial effects. While *B. cereus* showed a moderate response (5 mm), *S. aureus* exhibited comparatively weaker inhibition (3 mm). In contrast, the standard antibiotic Cefixime-30 displayed varied inhibitory zones. These findings underscore the potential therapeutic applications of the extract, especially against *E. coli* and *K. pneumoniae*, warranting further exploration through additional studies, including Minimum Inhibitory Concentration (MIC) determination, to elucidate its specific applications and

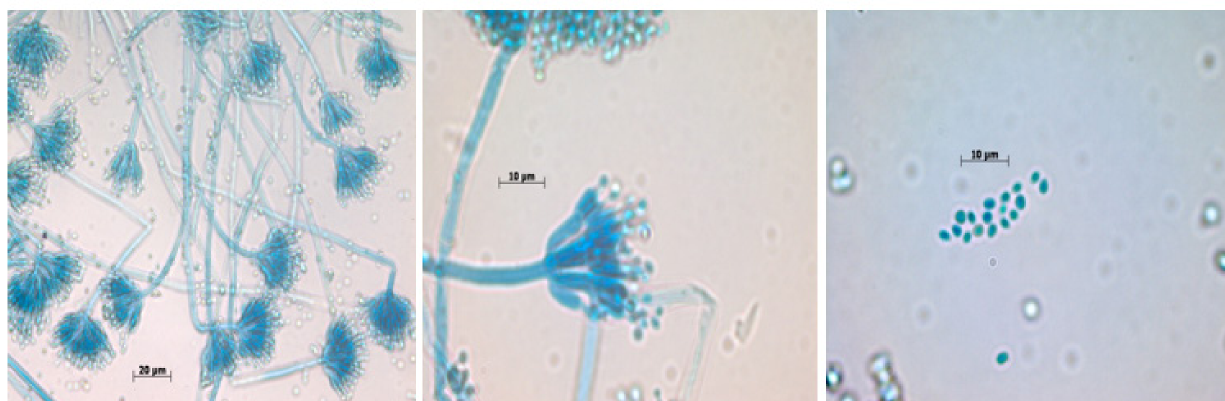

Figure 1: Microscopic observation of *Penicillium herquei.*

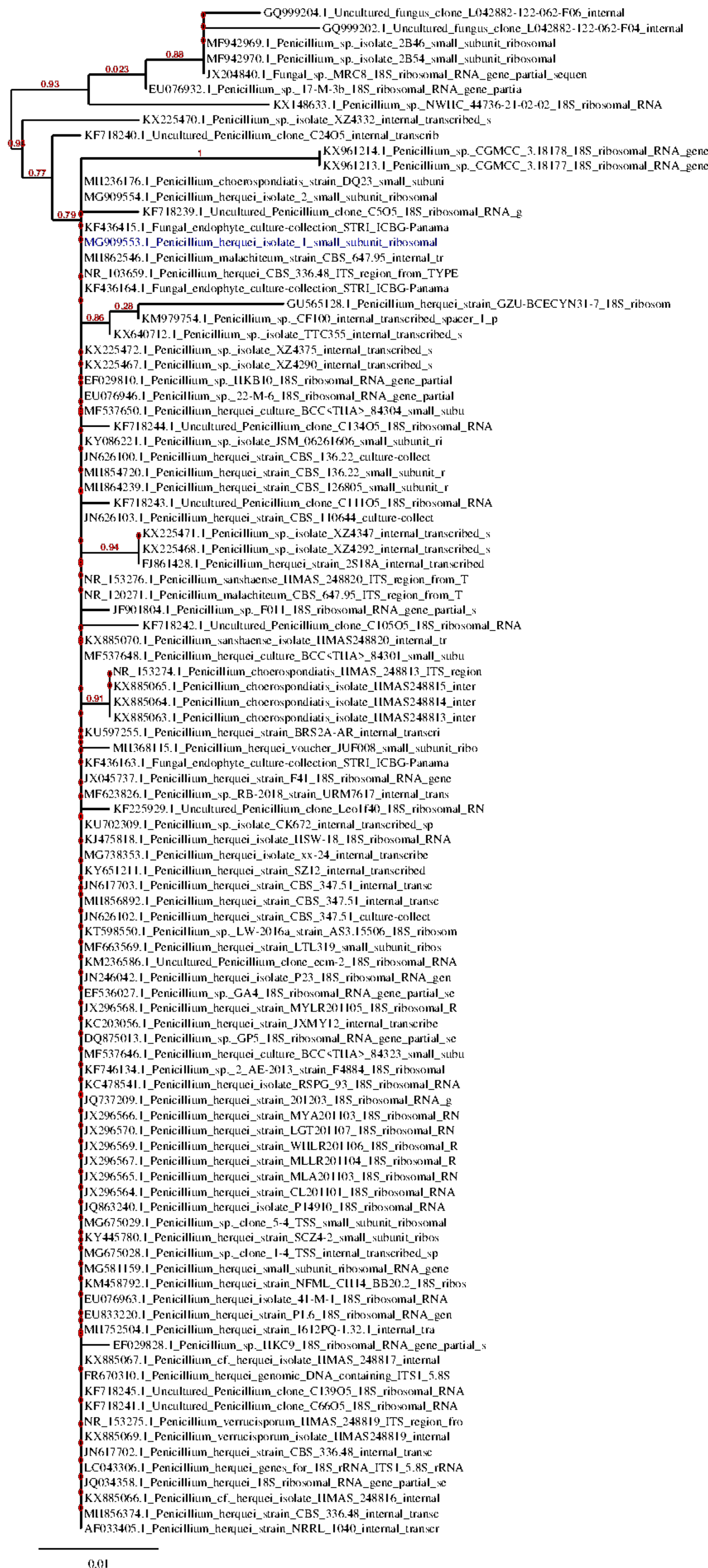

Figure 2: Phylogenetic tree displays the relation of strain *penicillium herquei* to other relevant fungal species recovered from NCBI GenBank (18S ribosomal ribonucleic acid).

mechanisms of action in combating bacterial infections (Table 1 and Figure 3).

Determination of antioxidant activity *Penicillium herquei* ethyl acetate extract by using DPPH method

Determination of antioxidant activity of *Penicillium herquei* ethyl acetate extract using the DPPH method yielded significant insights into its potential as a radical scavenger. The antioxidant capabilities of the extract were compared to those of ascorbic acid standard, a well-known antioxidant. The percentage of inhibition by *Penicillium herquei* ethyl acetate extract was measured at various doses. The observed color change from purple to yellow during the inhibition process was indicative of the reduction of DPPH radicals by the extract. This alteration suggests the addition of hydrogen atoms to the DPPH radicals, resulting in a visible color shift. This phenomenon signifies strong scavenging activity, as the extract effectively reduced the concentration of free radicals. These results imply that *Penicillium herquei* ethyl acetate extract of *P. herquei* possesses antioxidant properties, as evidenced by its ability to neutralize DPPH radicals. The comparison with ascorbic acid served as a reference point, highlighting the efficacy of the extract in scavenging free radicals. This antioxidant potential is crucial for mitigating oxidative stress-related damage in biological systems (Figure 4).

Cytotoxic activity (MTT assay)

Examination of *Penicillium herquei* ethyl acetate extract for its cytotoxic effect against SK-MEL-3 cancer cells at various concentrations, ranging from 10 μg/mL to 35 μg/mL, yielded noteworthy results after a 48 hr incubation period. The *in vitro*

Table 1: Antibacterial activity of *Penicillium herquei* ethyl acetate extract against clinical bacteria by disc diffusion method.

Sl. No.	Test organism	Zone of Inhibition (mm) (*n*=2)	
		Ethyl acetate extract	Standard (Cefixime-30)
1	*E. coli*	13	Nil
2	*B. cereus*	5	7
3	*S. aureus*	3	8
4	*K. pneumoniae*	12	5

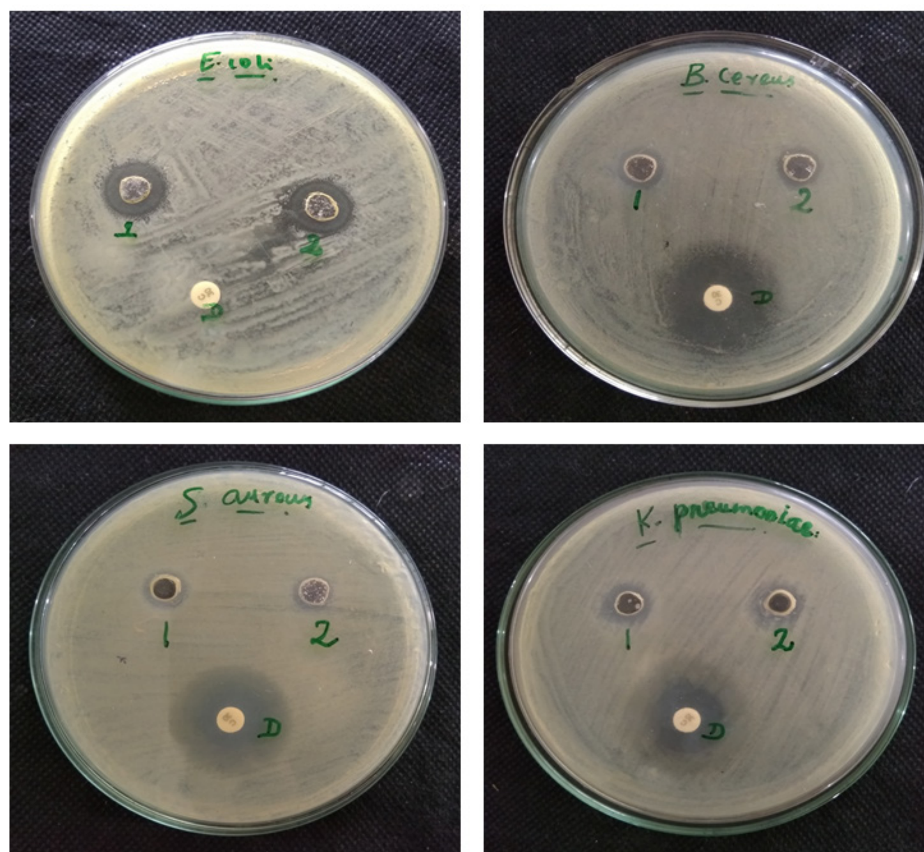

Figure 3: Antibacterial activity of *Penicillium herquei* ethyl acetate extract against clinical bacteria by disc diffusion method.

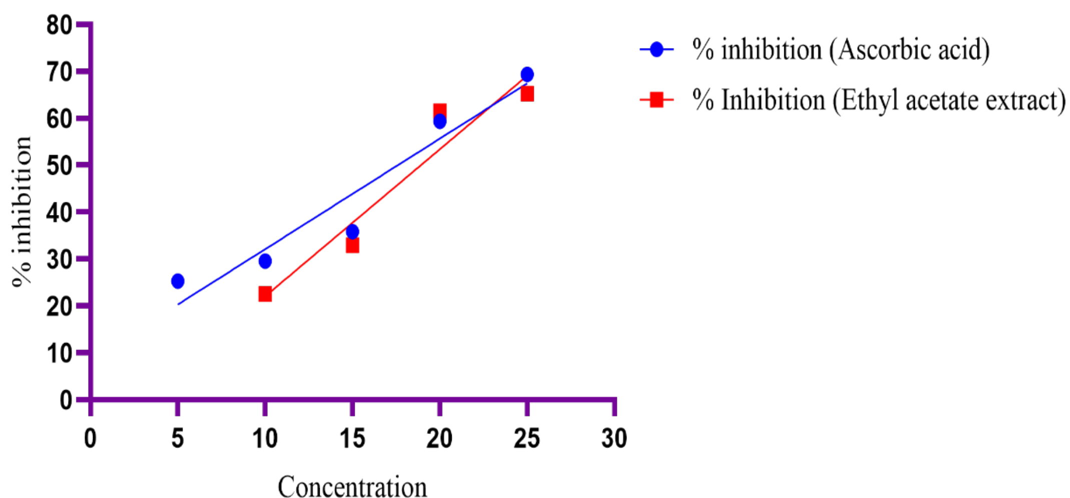

Figure 4: Antioxidant activity *Penicillium herquei* ethyl acetate extract.

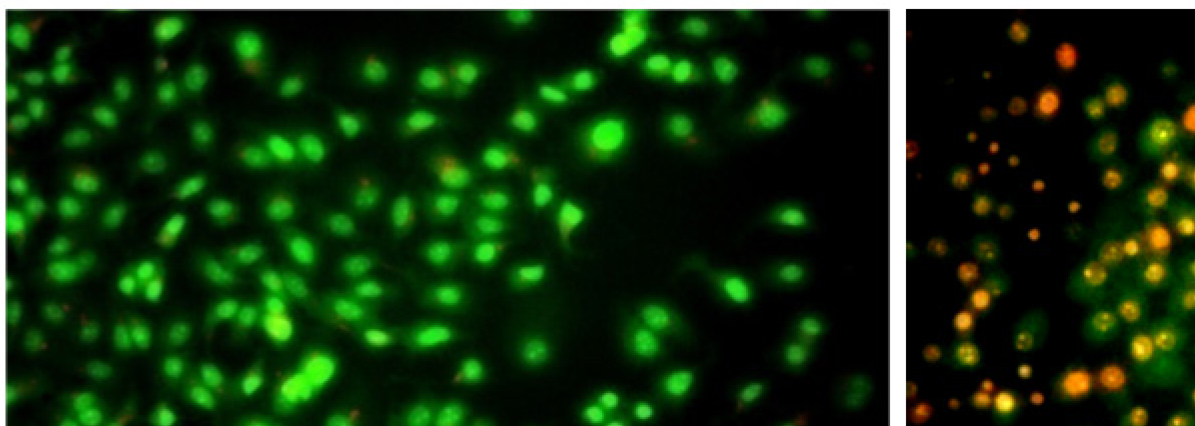

Figure 5: Fluorescence microscopy was used to investigate the dual dye AO/EB-stained *Penicillium herquei* ethyl acetate extract (20 µg) and regulated SK-MEL-3 cells after 24 hr. The standard green nucleus first emerged in living cells; later, necrotic cells and chromatin condensation or fragmentation of orange nuclei and yellow nucleus with chromatin occurred.

cytotoxicity assessment revealed a dose-dependent response, as demonstrated by the modified shape of the SK-MEL-3 cells, as illustrated in Figure 5. In particular, at a concentration of 20 µg/mL, *Penicillium herquei* ethyl acetate extract exhibited a significant inhibitory effect on the growth of SK-MEL-3 cells in comparison to the control cell viability. *Penicillium herquei* ethyl acetate extract may contain bioactive compounds with anti-cancer properties. A concentration of 20 µg/mL emerges as a pivotal point, demonstrating marked inhibition and emphasizing the potential of the extract as a candidate for further investigation in cancer therapeutics. These results underscore the importance of exploring natural sources, such as fungal extracts, for potential cytotoxic compounds that could contribute to the development of novel anti-cancer agents. Additional research is needed to clarify the underlying mechanisms and to pinpoint the bioactive compounds responsible for the observed cytotoxic activity.

CONCLUSION

A comprehensive investigation of *Penicillium herquei* involved morphological and genotypic analyses, molecular identification, large-scale cultivation and the evaluation of its bioactive potential. Morphological characterization revealed distinctive features, such as fast-growing colonies with specific Metulae and Phialides characteristics. Genotypic analysis confirmed the identity of *Penicillium herquei*, exhibiting a 99% sequence similarity with the reference strain. The large-scale cultivation and extraction of bioactive secondary metabolites from *Penicillium herquei* demonstrated a robust approach for potential pharmaceutical applications. The ethyl acetate extract exhibited notable antibacterial activity, particularly against *E. coli* and *K. pneumoniae*, demonstrating its potential therapeutic significance. Comparison with the standard antibiotic, Cefixime-30, provided a valuable reference for the efficacy of the extract. Further exploration through Minimum Inhibitory Concentration (MIC) determination is crucial to understand its specific applications

and mechanisms of action against bacterial infections. Additionally, the antioxidant activity of *Penicillium herquei* ethyl acetate extract was evaluated using the DPPH method, showing its potential as a free radical scavenger. Cytotoxicity assessment of SK-MEL-3 cancer cells demonstrated an inhibition of cell growth, suggesting the potential of the extract in cancer therapeutics. Fluorescence microscopy provided visual insights into the effects of the ethyl acetate extract on SK-MEL-3 cells. The multifaceted approach, integrating morphological, genotypic and functional analyses, provides a comprehensive understanding of its potential applications in medicine and contributes to the ongoing exploration of microbial resources for therapeutic purposes. Future research studies must identify the activity mechanism and dose confirmation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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