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Microbiology Section

In-vitro Antimicrobial Screening, Enzymatic Assays and Metabolome Profiling and In-silico Analysis for Anticancer Potency of *Talaromyces pinophilus* (AVK3), Isolated from the Rhizome of *Zingiber officinale*

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ABSTRACT

Introduction: Endophytic fungi are prolific sources of bioactive constituents with considerable therapeutic efficiency against various diseases, including cancer, by acting as anticancer and antimicrobial agents. The bioprospecting of novel endophytic secondary metabolites has gained momentum in the treatment of cancer with minimal side-effects. *Talaromyces pinophilus* (AVK3) is known to produce pinophilin and talaromycesins (A and B), which inhibit cancer cell growth.

Aim: To evaluate the isolation, antimicrobial screening, enzymatic assays, and metabolomic profiling (GC-MS analysis) of an endophytic fungus of ginger, *Talaromyces pinophilus* (AVK3), along with in-silico studies of its anticancer properties.

Materials and Methods: An in-vitro experimental study was conducted from June 2023 to May 2024 at Acharya Nagarjuna University, Guntur, Andhra Pradesh, India. Thirty fresh ginger rhizomes were collected from Mamillapalli village, Andhra Pradesh, following strict inclusion criteria (healthy, undamaged rhizomes harvested within 24 hours) and used for the isolation of endophytes. The endophyte AVK3 was isolated using surface sterilisation methods and identified as *Talaromyces pinophilus* through 18S ribosomal Ribonucleic Acid (rRNA) sequencing (GenBank accession: PP957929). Antimicrobial screening was performed against human pathogens using agar well diffusion and Minimum Inhibitory Concentration (MIC) methods. Enzymatic assays and metabolomic analysis were conducted to identify potential bioactive compounds. The anticancer potential of bioactive compounds was further evaluated through molecular docking studies against cancer target proteins.

Results: *Talaromyces pinophilus* exhibited a high zone of inhibition (30 mm in diameter) against *Salmonella* typhimurium, with a MIC value of 6.25 µg/mL and an Half maximal Inhibitory Concentration (IC^{50}) of 13.397 µg/mL, demonstrating efficient antibacterial activity. Metabolomic profiling through Gas Chromatography–Mass Spectrometry (GC-MS) analysis revealed bioactive compounds like 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one; 1,2,3-propanetriol; HMF; 3-acetoxy-3-hydroxy-propionic acid methyl ester; 2-piperidinone; furandimethanol; hexahydropyrrolo(1,2-a)pyrazine-1,4-dione; terrain; and 3,6-diisobutyl-2,5-piperazinedione. Enzymatic assays showed the presence of hydrolytic enzymes such as amylase, lipase catalse, gelatinase, and lipases.

Conclusion: In-vitro antimicrobial studies and in-silico analysis of anticancer potential demonstrated binding affinities of –19.5 kcal/mol against the lung cancer target protein (PDB ID: 7W8O) and –22.9 kcal/mol against the breast cancer target protein (PDB ID: 3HBS), confirming *Talaromyces pinophilus* as a promising source for anticancer treatment. The extensive array of bioactive compounds exemplifies the chemical diversity of *Talaromyces pinophilus*, particularly highlighting its potential in biomedical innovation.

Keywords: Bioactive metabolites, Endophyte-derived compounds, Metabolomic profiling, Molecular docking

INTRODUCTION

A member of the Zingiberaceae family, ginger (*Zingiber officinale*), has long been used as a spice and herbal remedy for various illnesses [1]. According to studies, gingerols, shogaols, and paradols are the primary phenolic compounds responsible for ginger's diverse bioactivities [2]. The presence of shogaols, particularly 6-shogaol, which is a dehydrated form of gingerols, is primarily responsible for the pungency of dried ginger, while gingerols are the main contributors to the pungency of fresh ginger [3]. Because of these health-promoting properties, ginger can be incorporated into food products as an active ingredient.

Plants produce secondary metabolites that exert a variety of therapeutic effects on both the plant and other living organisms. Endophytic fungi form symbiotic associations with plants, enhancing nutrient uptake, increasing resistance to pests and diseases, promoting growth and yield, and producing secondary metabolites similar to those of their host plants. At times, due to co-evolution and genetic transfer, they also establish colonies within plant tissues [4]. Fungal endophytes strengthen plant cell walls and produce antimicrobial compounds, phytohormones, and enzymes, thereby improving nutrient status, enhancing antioxidant activity, and enabling plants to respond more effectively to biotic and abiotic stresses. This contributes to evolutionary advantages and overall plant development. Additionally, endophytes can help plants tolerate abiotic stresses such as heavy metal toxicity and salinity by improving nutrient absorption and boosting antioxidant defense systems [5]. Importantly, endophytes can also produce bioactive compounds with pharmacological applications; in-silico studies, such as molecular docking, have confirmed their potential as anticancer and antibacterial agents [6].

GC-MS analysis is one of the most suitable analytical techniques for identifying and analysing the bioactive compounds of interest and for performing detailed metabolomic profiling. It provides valuable insights into metabolite composition, with the advantages of cost-

effectiveness, high resolution, reproducibility, and highly reproducible mass spectral fragmentation.

Although prior studies on *Talaromyces* species have highlighted their secondary metabolites—such as terrein and pyranones—as cytotoxic agents [7], their presence in *Zingiber officinale* rhizomes remains unexplored, highlighting a critical research gap. This study is the first report of *Talaromyces pinophilus* (AVK3) isolated from *Z. officinale*, identified as a strong antimicrobial agent (confirmed by in-vitro antimicrobial assays using agar well diffusion), and as an effective anticancer agent (confirmed by molecular docking studies).

Enzymatic assays in the present study were performed qualitatively to detect the presence of hydrolytic enzymes or primary metabolites, aiming to better understand the mechanisms by which *T. pinophilus* (AVK3) establishes and survives within its host system. The in-silico docking methodology was employed to evaluate the binding affinities of bioactive compounds such as terrein, furandimethanol, 2-piperidinone, and propanetriol, produced by *T. pinophilus* (AVK3), against selected cancer targets to validate its anticancer efficacy [8].

Although several *Talaromyces* species have been investigated for their secondary metabolites with antibacterial and anticancer potential, the existence and bioactivity of *T. pinophilus* specifically isolated from *Z. officinale* rhizomes have not yet been reported. Previous research, such as that by Lei LR et al., has primarily focused on *Talaromyces*-derived compounds like terrein and pyranones [7], but lacked systematic metabolomic profiling, enzymatic screening, and in-silico anticancer evaluations of ginger-associated isolates. Thus, this work represents the first comprehensive study to isolate and characterise *T. pinophilus* (AVK3) from *Z. officinale*, revealing its powerful bioactive profile, particularly its antibacterial and anticancer properties. This previously unexplored fungal-host relationship represents a novel and underutilised source for drug development and therapeutic innovation.

This study aims to isolate and characterise the endophytic fungus *Talaromyces pinophilus* (AVK3) from *Z. officinale* rhizomes, and to evaluate its antibacterial, enzymatic, antioxidant, and anticancer properties using both in-vitro and in-silico methods.

Primary objective: To isolate and identify morphologically and molecularly the endophytic fungus *T. pinophilus* (AVK3) from *Z. officinale* rhizomes, and to assess its antimicrobial efficacy against selected human pathogens.

Secondary objectives: To optimise culture conditions to enhance bioactive metabolite production by *T. pinophilus* (AVK3).

- To perform enzymatic and mycochemical screening for hydrolytic enzymes and secondary metabolites.
- To evaluate antioxidant properties of the fungal extract using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.
- To perform GC-MS analysis of the fungal extract to identify important bioactive compounds.
- To assess anticancer activity of selected metabolites using molecular docking studies against cancer-specific protein targets.

MATERIALS AND METHODS

This in-vitro experimental study was conducted over 12 months (June 2023 to May 2024) at Acharya Nagarjuna University, Guntur Andhra Pradesh, India, focusing on the exploration of the anticancer properties of ginger endophytes. Fresh ginger rhizomes (*Zingiber officinale*) were collected from Mamillapalli village, Ponnuru Mandal, Andhra Pradesh, India, following strict inclusion and exclusion criteria.

Inclusion criteria: Healthy, mature *Z. officinale* rhizomes collected from organic farms in the Guntur district; free of visible disease symptoms and degradation were included in the study.

Exclusion criteria: Rhizomes showing microbial infection, physical damage, or treated with chemicals/pesticides were excluded from the study.

The selected rhizomes were transported under sterile conditions to the Microbiology Laboratory, Acharya Nagarjuna University, Guntur, for isolation of the endophytic fungus *Talaromyces pinophilus* (AVK3) and further exploration of its bioactive constituents for potential cancer treatment. Chemical or pesticide-treated samples.

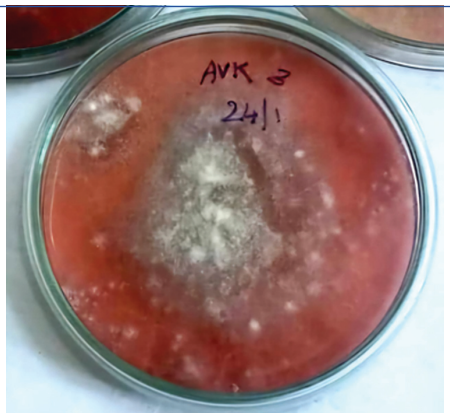
Calculation of sample size: The sample size was selected using conventional microbial endophyte isolation procedures commonly employed in phytochemical and bioactivity research. A minimum of three replicates was considered appropriate to ensure statistical significance and reproducibility of data (Eevers et al.,) [9].

Collection and processing of samples by surface sterilisation: Fresh ginger rhizomes were collected from Mamillapalli village, Ponnuru Mandal, Guntur district, Andhra Pradesh, and transported to the laboratory under sterile conditions. Surface sterilisation was carried out by rinsing the rhizomes thoroughly with running tap water, followed by sequential treatments with 20% H_2O_2, 95% ethanol, 4% NaOCl, 0.1% $HgCl_2$, and 20% formaldehyde. After sterilisation, the samples were rinsed with sterile distilled water to remove residual chemicals.

Isolation of endophytes: The final aliquot of the last rinse was used as a control to check the efficacy of surface sterilisation. The growth of fungal colonies or other microbes in this control indicated the presence of epiphytes, suggesting incomplete sterilisation.

For isolation, 0.1% NaCl solution was prepared in test tubes. Nine tubes (10^{-1} to 10^{-9} dilutions) were filled with 9 mL of 0.1% NaCl each. Sterilised rhizomes were crushed in a mortar with 1 mL of 0.1% NaCl to prepare the inoculum. Serial dilutions were performed, and 0.1 mL from each dilution (10^{-1} to 10^{-9}) was spread onto Sabouraud's Dextrose Agar (SDA) plates supplemented with streptomycin (10 µg/mL) to prevent bacterial contamination.

The inoculated plates were incubated for 21 days at 25°C, with daily monitoring to detect fungal growth [10]. Among the colonies obtained, three distinct isolates were identified, and one colony with unique morphology was selected for further studies and designated as AVK3 [Table/Fig-1]. AVK3 exhibited rapid growth (within 3 days of inoculation), strong antagonistic activity against contaminants, and tolerance to a wide range of culture conditions, including variations in pH, temperature, and salinity.



[Table/Fig-1]: Macroscopic view of *Talaromyces pinophilus* on Sabouraud's agar medium with extracellular pigmentation.

Molecular identification: Molecular identification of AVK3 was performed using Polymerase Chain Reaction (PCR) amplification and 18S rRNA sequencing by Macrogen, South Korea. Phylogenetic analysis confirmed the identity of the isolate as *Talaromyces pinophilus*. The sequence was deposited in the NCBI GenBank under accession number PP957929 (India).

Microscopic identification: The fungus was cultured using the agar block method and stained with lactophenol cotton blue to

study its morphology. Microscopic analysis revealed biverticillate conidiophores with dark blue, short stalk-like structures (metulae) and light blue, flask-shaped spore-producing cells (phialides). These features are characteristic of *Talaromyces* species [Table/Fig-2].

[Table/Fig-2]: Microscopic view of *Talaromyces pinophilus* under 100x magnification of a compound microscope and stained with Lactophenol blue.

Optimisation of fungal endophyte with different physicochemical parameters of culture conditions: To increase the production of bioactive compounds during the growth phase and enhance their potential activity, efforts were made to modify the composition of the culture media. Variations in the culture medium can influence metabolite levels and their biological roles. Optimising the culture medium [11] involved adjusting carbon and nitrogen sources, as well as pH, to determine the ideal conditions for the growth of the isolate and synthesis of secondary metabolites. Additionally, parameters such as temperature and pH were optimised to maximise metabolite production. The optimised conditions for AVK3 were: glucose as the carbon source, peptone as the nitrogen source, pH 6.0, temperature 35°C, and an incubation period of 21 days.

Preparation of crude extract from ginger rhizome: For fermentation and extraction of bioactive secondary metabolites, a 48-hour-old AVK3 mycelial culture was added to Sabouraud's broth in a 250-mL Erlenmeyer flask and incubated at 27°C on a rotary shaker at 100 rpm. Subsequently, 20 mL of this culture was transferred to each 500 mL Erlenmeyer flask containing 200 mL of Sabouraud's broth and cultured for 10 days at 27°C under stationary conditions. Bioactive compounds were extracted by centrifuging the culture at 5000 × g for 10 minutes to separate the mycelial mass. The supernatant was collected, mixed with an equal volume of methanol, and evaporated to obtain the crude extract of *Talaromyces pinophilus*.

Pathogenic strains selected for susceptibility assay: Antimicrobial activity is a key benchmark to evaluate the bioactive potential of endophytic microbes. Therefore, the most common opportunistic human pathogens were obtained from National Collection of Industrial Microorganisms (NCIM) for testing: *Staphylococcus aureus* (NCIM 2079), *Bacillus subtilis* (NCIM 2063), *Clostridium sporogenes* (NCIM 5113), *Pseudomonas aeruginosa* (NCIM 2200), *Salmonella* typhimurium (NCIM 2501), *Candida albicans* (NCIM 3471), and *Aspergillus brasiliensis* (NCIM 1196). Antimicrobial screening was performed to evaluate the efficacy of *T. pinophilus* in producing bioactive metabolites.

Antimicrobial assay by agar well diffusion method: The antibacterial activity of the AVK3 ethyl acetate extract was evaluated against opportunistic human pathogens, particularly those affecting immunocompromised individuals such as cancer patients [12]. In this study, antimicrobial activity was assessed against five pathogens: *S. aureus*, *C. sporogenes*, *P. aeruginosa*, *S.* typhimurium, and *C. albicans*. Plates were incubated for seven days at 37°C, and the zones of inhibition were measured in millimetres: 30 mm against *C. albicans* and *S.* typhimurium, 28 mm against *S. aureus*, 26 mm against *C. sporogenes*, and 25 mm against *P. aeruginosa*. Streptomycin (10 µg/mL) served as the positive control, while DMSO was used as the negative control. Results were expressed as mean±SD. The most potent endophytic fungi were selected for further optimisation of physicochemical parameters, including inoculum size, incubation period, pH, temperature, and carbon and nitrogen sources.

Minimum Inhibitory Concentration (MIC) of AVK3 extract: The MIC is defined as the lowest concentration of the extract that prevents visible growth of the test organism on an agar plate compared to the control. The MIC of the aqueous extract of ginger rhizomes was determined using standard procedures [13,14]. Mueller-Hinton agar was prepared and sterilised by autoclaving. Serial dilutions of the extract (100, 50, 25, 12.5, and 6.25 µg/mL) were added to respective wells, followed by inoculation with 2 µL of microbial suspension adjusted to the McFarland turbidity standard. Plates were incubated aerobically at 37°C for 18–24 hours, and the inhibition zones were measured in millimetres. The MIC of the ethyl acetate extract of *T. pinophilus* was 6.25 µg/mL, which was lower than the MIC of streptomycin (10 µg/mL), the standard antibiotic.

Antioxidant activity: A stock solution of the extract dissolved in methanol was prepared to create six different concentrations, with ascorbic acid used as a standard antioxidant, to evaluate antioxidant activity. The antioxidant potential of *Talaromyces pinophilus* (AVK3) was assessed using the DPPH assay [15] with slight modifications. A freshly prepared 1 mM DPPH radical solution in methanol was used. Two millilitres of the extract were mixed with 1 mL of DPPH solution, while 1 mL of methanol without DPPH served as a blank. The reaction mixtures were kept in the dark for 30 minutes, and absorbance was measured at 517 nm. The experiment was conducted in triplicate. The scavenging activity (AA%) of each extract was calculated using the following formula [16]:

%of DPPH Scavenging capacity=[(absorbance of control-absorbance of sample)/ absorbance of control]×100

Ascorbic acid was used as the standard antioxidant.

Enzymatic assays and mycochemical studies: Enzymatic assays were performed qualitatively to detect the production of primary metabolites, including cellulase, pectinase, gelatinase, catalase, asparaginase, amylase, protease, and lipase, confirming the hydrolytic enzyme activity of *T. pinophilus*. Secondary metabolites, which contribute to the defensive capabilities of microorganisms, were qualitatively evaluated using various mycochemical screening techniques [17,18]. Among these, phenolic compounds are particularly significant due to their clinical bioactivity, including anticancer effects such as apoptosis induction, cell cycle arrest, and angiogenesis inhibition. Therefore, total phenolic content in *T. pinophilus* was optimised to maximise the production of these bioactive compounds.

GC-MS profiling and molecular docking studies of bioactive metabolites: GC-MS analysis of ginger endophytes was performed to identify bioactive compounds with potential medicinal and industrial applications. Endophyte cultures were extracted using methanol, followed by separation through gas chromatography (GC) and detection using mass spectrometry (MS). The GC-MS settings included a DB-5 or HP-5 column, oven temperature range of 50–300°C, helium as the carrier gas, injection volume of 2 µL, and electron ionisation mode. Data were interpreted using the NIST library.

The major bioactive metabolites of *T. pinophilus* were docked into the active sites of target proteins using AutoDock Vina. SMILES IDs of the metabolites from PubChem were matched with the residues of target proteins obtained from PDB. Docking simulations were performed using stick-model representations of ligands, showing interacting residues and hydrogen bonds. A grid box centred on the active site was used to identify the best-docked conformation with the lowest free binding energy [19,20].

The compounds included volatile components (gingerol, shogaol, paradol, zingiberene) and non volatile substances such as flavonoids (quercetin, kaempferol), phenolic acids (ferulic acid, cinnamic acid), alkaloids (gingerenamines), and terpenoids (zingibereneol). These bioactive compounds exhibit antimicrobial, antioxidant, anti-inflammatory, anticancer, and immunomodulatory properties.

RESULTS

Isolation of AVK3 from rhizome of *Zingiber officinale:* The fungal endophyte *Talaromyces pinophilus* (AVK3) was successfully isolated from the rhizomes of *Zingiber officinale*, and a pure culture was maintained through repeated subculturing.

Molecular Identification of AVK3: The 18S rRNA gene of AVK3 was amplified by PCR. BLAST analysis showed 100% similarity with the partial 18S rRNA sequence of *Talaromyces* species. Phylogenetic analysis based on the partial 18S rRNA gene confirmed that AVK3 is closely related to *Talaromyces pinophilus*, as illustrated in the dendrogram in [Table/Fig-3]. The sequence has been deposited in NCBI under GenBank accession number PP957929.

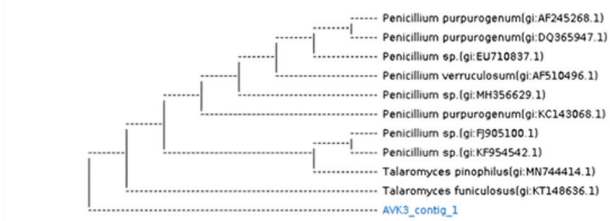
Macroscopic and microscopic identification: Based on macroscopic observation and microscopic examination, AVK3 was initially identified as a *Penicillium* species [Table/Fig-3]. Subsequent molecular identification and phylogenetic studies confirmed the strain as *Talaromyces pinophilus*.

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
NS1 5' (GTA GTC ATA TGC TTG TCT C) 3'	NS1 5' (GTA GTC ATA TGC TTG TCT C) 3'
NS24 5' (TCC GCA GGT TCA CCT ACG GA) 3'	NS24 5' (TCC GCA GGT TCA CCT ACG GA) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
MH356629.1	Penicillium sp.	1658	3	1656	99	3055	0.0	1654/1654	100.00

Kingdom	Family	Genus	Species
Eukaryota	Aspergillaceae	Penicillium	Penicillium sp.

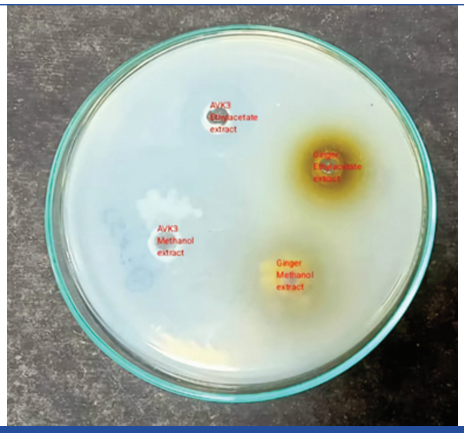


[Table/Fig-3]: Dendrogram of *Talaromyces pinophilus* (AVK3).

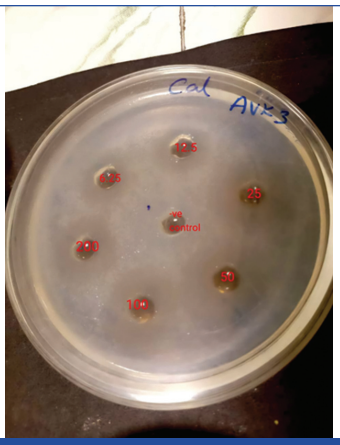
Antimicrobial activity of *T. pinophilus*: *Talaromyces pinophilus* AVK3 exhibited the highest growth inhibition against *Salmonella* typhimurium and *Candida albicans*, with a zone of inhibition of approximately 30 mm [Table/Fig-4]. The MIC values of *T. pinophilus* extract were determined using these two pathogens to establish the lowest concentration of extract required to produce a clear zone of inhibition. [Table/Fig-5] shows the MIC value of the *T. pinophilus* extract, which was 6.25 µg/mL. The IC^{50} values against *Salmonella* and *Candida* were 35.731 µL and 13.397 µL, respectively [Table/Fig-6].

The MIC of the *T. pinophilus* extract is very close to that of the standard stock solution of streptomycin (10 µg/mL), which was approximately evaluated as 3.15 µg/mL (≈4 µg/mL). These results demonstrate the high efficiency of *T. pinophilus*, as its zone of inhibition is comparable to that of the standard antibiotic.

Optimisation of fungal endophyte with different physicochemical parameters of culture conditions: Optimisation of *T. pinophilus* was performed to enhance its growth and antimicrobial potential by considering one physicochemical parameter at a time. Among bioactive metabolites, phenolic compounds enhance antiproliferative activity by inducing apoptosis or inhibiting the cell cycle. Therefore, total phenolic content of *T. pinophilus* was targeted for optimisation to maximise its anticancer potential.



[Table/Fig-4]: Antimicrobial activity of methanolic and ethyl acetate extracts of *T. pinophilus* in comparison to antimicrobial activity of ginger methanol and ethyl acetate extracts.



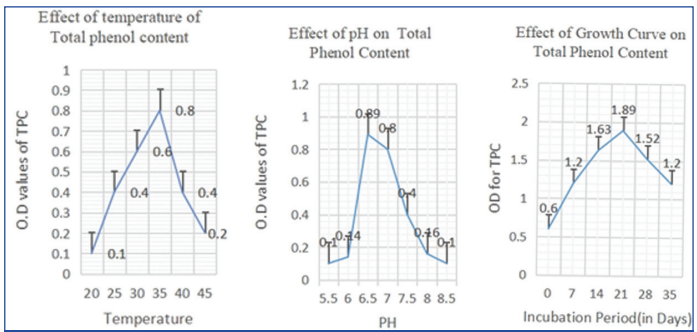
[Table/Fig-5]: The minimum inhibitory concentration of methanolic extract of *T. pinophilus*.

Isolate	Pathogen	IC50
AVK3	*Salmonella* Typhimurium	35.731
AVK3	*Candida albicans*	13.397

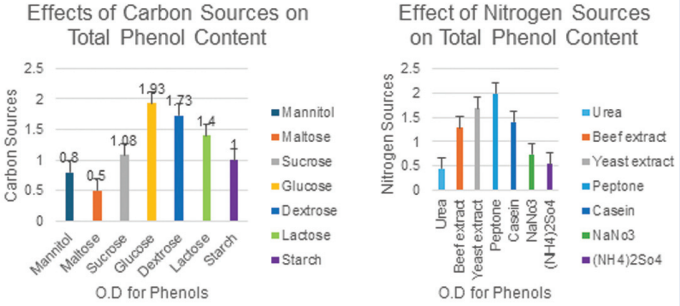
[Table/Fig-6]: IC^{50} values of the methanolic extracts of *T. pinophilus*.

Firstly, the minimum incubation period required by *T. pinophilus* was optimised, followed by adjustment of various culture media parameters. After optimisation, the ideal culture conditions for maximum growth and antimicrobial activity were determined as follows: temperature 35°C, pH 6, glucose as the carbon source, peptone as the nitrogen source, and an incubation period of 21 days [Table/Fig-7,8].

Enzymatic assays and mycochemical screening: Metabolomic profiling of *T. pinophilus* was performed using selective enzymatic assays and mycochemical screening following established protocols. *T. pinophilus* tested positive for primary metabolites, including amylase, asparaginase, gelatinase, and catalase. It also produced secondary metabolites like alkaloids, flavonoids, phenols, terpenes, phytosterols, and saponins [Table/Fig-9].



[Table/Fig-7]: Optimisation of physical parameters of culture conditions of *T. pinophilus*.



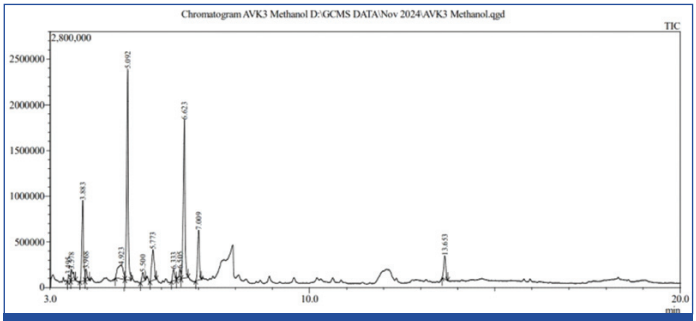
[Table/Fig-8]: Optimisation of chemical parameters of culture conditions of *T. pinophilus*.

	Metabolites	Result AVK3
Enzymatic assays	Cellulase	(-)
	Protease	(-)
	Lipase	(+)
	Asparaginase	(+)
	Amylase	(+)
	Pectinase	(-)
	L-glutaminase	(-)
	Gelatinase	(+)
	Catalase	(++)
Phytochemical screening assays	Alkaloids	(+)
	Flavonoids	(++)
	Tannins	(-)
	Phenols	(+++)
	Saponins	(+)
	Phytosterols	(+)
	Terpenes	(+++)

[Table/Fig-9]: Metabolomic profiling of *T. pinophilus*
+++ Highly produced / strongly present. ++ Moderately produced/presented. + Minimally produced / weakly present. – Not produced / absent

Antioxidant activity of *T. pinophilus* extract: The DPPH radical scavenging assay was performed using different concentrations of *T. pinophilus* extract. The percentage of antioxidant activity was determined based on absorbance and was found to be 59.7%. This indicates the extract's effectiveness against oxidative stress and other biological targets.

GC-MS report and molecular docking: GC-MS analysis of *T. pinophilus* revealed the presence of multiple bioactive compounds, as illustrated in the chromatogram [Table/Fig-10]. Major compounds included 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, 1,2,3-propanetriol, HMF, N-methyl-N-[2-(methylamino)ethyl] cyclohexanamine, 3-acetoxy-3-hydroxy-propionic acid methyl ester, 2-piperidinone, furandimethanol, hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, terrein, and 3,6-diisobutyl-2,5-piperazinedione [Table/Fig-11]. These compounds are associated with potent biological activities.



[Table/Fig-10]: GC-MS analysis report of methanolic extract of *T. pinophilus*.

In-silico docking studies revealed that identified phenolic and flavonoid compounds, including 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, 1,2,3-propanetriol, HMF, N-methyl-N-[2-(methylamino)ethyl]cyclohexanamine, 2-piperidinone, pyrrolo[1,2-a] pyrazine-1,4-dione, terrein, furandimethanol, 3,6-diisobutyl-2,5-piperazinedione, 3-hydroxy-2-methylbutyric acid, and 3-acetoxy-3-hydroxy-propionic acid demonstrated radical scavenging properties. The abundance of phenols and flavonoids likely contributes to the observed antioxidant activity of the ginger endophyte.

S. No.	Peak	Retention time	Area	Area%	IUPAC Name of identified bioactive compounds
1	6	5.092	6625096	26%	Pyrrolidin-2-one
2	11	6.623	5725623	22.47%	2-Furancarboxaldehyde, 5-(hydroxymethyl)- (aka Hydroxymethylfurfural)
3	3	3.883	2735953	10.74%	N-Methyl-N-[2-(methylamino) ethyl]cyclohexanamine
4	12	7.009	1821557	7.15%	Methyl 3-acetoxy-3-hydroxypropanoate
5	5	4.923	1577488	6.19%	Propane-1,2,3-triol (aka Glycerol)
6	8	5.773	1548218	6.08%	2-Piperidone
7	14	21.729	1123268	4.41%	Hexahydropyrrolo[1,2-a] pyrazine-1,4-dione
8	13	13.653	921884	3.62%	4,5-Dihydroxy-3-isopropylbenzaldehyde (Terrein)
9	16	30.097	678944	2.66%	3,6-Diisobutylpiperazine-2,5-dione
10	2	3.578	524156	2.06%	Ethyl 3-hydroxy-2-methylbutanoate
11	9	6.333	525970	2.06%	2,5-Bis(hydroxymethyl)furan (Furandimethanol)
12	4	3.968	392805	1.54%	Furan-2,5-dicarboxaldehyde
13	10	6.505	376195	1.48%	1,6:3,4-Dianhydro-D-mannitol (aka Dianhydromannitol)
14	7	5.5	374540	1.47%	(5S)-5-(Hydroxymethyl)-5H-furan-2-one
15	1	3.495	278845	1.09%	6-Methyl-3(2H)-pyridazinone
16	15	24.585	254125	1%	2,4-Diformyl-6-methoxyphenol

[Table/Fig-11]: Active metabolites of *T. pinophilus* (AVK3).

AutoDock Vina was used for molecular docking studies of pyran-4-one and furan carboxaldehyde to computationally assess their therapeutic potential as anticancer agents. The binding affinities of *T. pinophilus* bioactive compounds with target proteins are summarised in [Table/Fig-12].

DISCUSSION

The endophytic fungus *Talaromyces pinophilus* AVK3, isolated from *Zingiber officinale*, demonstrates noteworthy bioactive potential, aligning with previous studies that identified endophytes from Zingiberaceae family members as significant sources of antimicrobial and anticancer compounds (Arora DS et al., Krishnapura PR and Belur PD). The antimicrobial efficacy of AVK3, particularly against and *C. albicans*, aligns with prior work on *Talaromyces* species, which also reported effective inhibition of pathogenic bacteria and fungi [21,22]. This suggests that *Zingiber*-associated endophytes may possess conserved biosynthetic pathways for producing antimicrobial secondary metabolites.

The MIC value of *T. pinophilus* against *S.* typhimurium and *C. albicans* was 6.25 µg/mL, and the IC^{50} values were 35.731 µL and 13.397 µL, respectively, indicating higher potency than other known fungal extracts, such as those from turmeric endophytes, which showed IC^{50} values around 32.28 µg/mL [21]. Drugs with lower MIC values are considered more effective, as they require minimal concentrations to inhibit pathogen growth. In diagnostic laboratories, MIC values are essential for evaluating the efficacy of novel antimicrobial agents. Similarly, lower IC^{50} values

S. No.	Bioactive Compound/ Ligand	Simplified Molecular Input Line Entry System (SMILE) ID	Receptor/ target protein	Protein Data Bank (PDB) ID	Disease	Binding affinity	Residue involved in Hydrogen Interactions (Ligand Atom-Receptor)	Residue Involved in Hydrophobic Interactions (Ligand Atom-Receptor)
01	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	CC1=C(C(=O)C(CO1)O)O	disulfide-rich peptide that can bind MDM2	7W8O	All cancers	-22.9	O1 - Y18(B) O O4 - Y18(B) O O3 - W19(B) O O3 - E20(D) O O3 - W19(F) O O1 - Y18(H) O O3 - E20(I) O O3 - W19(J) O O3 - E20(K) OE2 O3 - W19(L) O O3 - W19(M) O O3 - W19(A) O O3 - E20(K) OE2	C1- W19(F) CZ3 C1 - W19(H) CZ2
02	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	CC1=C(C(=O)C(CO1)O)O	disulfide-rich peptide that can bind MDM2	7W8O	All cancers	-19.5	O3 - Y18(B) O O3 - W19(B) O O3 - W19(J) O O3 - W19(K) O3 - W19(L) O3 - W19(A) O	C4 - A21(B) CB C4 - A21(M) CB C4 - A21(N) CB C4 - A21(O) CB
03	N-Methyl-N-[2-(methylamino)ethyl] cyclohexanamine	CNCCN(C)C1CCCCC1	ABL kinase domain bound with a DFG-out inhibitor AP24534	3OXZ	Leukaemia	-5.2	N1 T315(A) OG1	C7 - L248(A) CD1 C7 - Y253(A) CE1 C10 - F317(A) CE1 C9 - L370(A) CD2 C6 - F382(A) CZ
04	3-acetoxy-3-hydroxy-propionic acid methyl ester	CC(=O)OC(CC(=O)OC)O	BCR-ABL Kinase domain	2VXT	Leukaemia	-4.7	O5 - L45(H) O O3 - Y96(L) O O1 - F98(L) O O5 - F98(L) O O1 - M4(L) N O3 - W47(H) N O4 - N60(H) ND2 O5 - F98(L) N	C4 - E46(H) CG C1 - T97(L) CG2
05	1,2,3-propanetriol	C(C(CO)O)O	KRAS Protein in Complex with GDP	1TRZ	Colorectal cancer	-3.4	O2 - F25(D) O O1- T27(D) O O3 - T27(D) O O2 - T27(D) OG1 O1 - T27(D) OG1 O3 - T27(D) N O1 - T27(D) OG1 O2 - T27(D) OG1	No Interaction
06	2-Piperidinone	C1CCNC(=O)C1	Human epidermal growth factor receptor extracellular domain	3L8V	Colorectal cancer	-4.6	O1 - T709(A) O N1 - T653(B) OG1 N1 - T653(B) OG1	C1 - R711(A) CG C1 - V720(A) CG1 C2 - N651(B) CB C2 - D676(B) CB
07	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	C1CC2C(=O)NCC(=O)N2C1	Anaplastic lymphoma kinase domain	4WKQ	Lung cancer	-5.4	N1 - D770(A) OD1 O1 - D770(A) OD1 O2 - A1013(A) O O2 - D1014(A) OD1 O1 - Q1020(A) NE2	C1 - L778(A) CD2 C1 - I1018(A) CD
08	Terrain	C/C=C/C1=CC(=O)[C@@H]([C@H]1O)O	Oestrogen receptor in complex with tamoxifen	3EBJ	Breast cancer	-4.7	O3 - K22(A) O O3 - E26(A) O O2 - N96(B) ND2	C3 - K22(A) CD C5 - D27(A) CB
09	3,6-diisobutyl-2,5-piperazinedione	CC(C)CC1C(=O)NC(C(=O)N1)CC(C)C	Human epidermal growth factor receptor 2	4XI3	Breast cancer	-5.7	O1 - E470(A) OE2 N1 - E470(A) OE2 N1 - Q441(B) OE1 O2 - E443(B) OE2	C11 - E470(A) CB C10 - E470(A) CG C1 - Q441(B) CB C1 - E443(B) CB C1 - E444(B) CG C2 - L489(B) CB
10	-hydroxy-2-methyl butyric acid, ethyl ester	CCOC(=O)C(C)C(C)O	Vascular endothelial growth factor A in complex with receptor	3RCD	Gastric cancer	-4.2	O3 - Y781(D) OH O2 - N824(D) ND2 O1 - Y781(D) OH O3 - K831(D) NZ	C7 - V725(B) CG2 C1 - Y781(D) CE1
11	Furandimethanol	C1=COC(=C1CO)CO	Phosphorylated RET tyrosine kinase domain with inhibitor	2X2K	Gastric cancer	-4.7	O2 - D892(A) OD1 O3 - D892(A) OD1 O3 - S891(A) OG O2 - K758(A) NZ O2 - D892(A) N	C1 - V738(A) CG1 C4 - V738(A) CG2 C1 - V804(A) CG2
12	2,5-Furandicarboxaldehyde	C1=C(OC(=C1)C=O)C=O	Human Angiotensin-Converting Enzyme 2 (ACE2) receptor.	6GGB	SARS-CoV-2	-4.6	O3 - S99(A) OG O2 - V197(B) O O2 - N200(B) O O1 - G199(B) N O3 - S99(A) OG O3 - Q100(A) N O2 - N200(B) N	No Interaction

[Table/Fig-12]: Molecular docking analysis of selected ligands produced from *T. pinophilus* using Autodock vina [31].

indicate strong activity of the test compound against the target pathogen.

In terms of enzymatic activity, *T. pinophilus* demonstrated the production of key enzymes, such as L-asparaginase and catalase. These enzymes are therapeutically significant: L-asparaginase is used in the treatment of acute lymphoblastic leukaemia due to its ability to deplete asparagine, an amino acid essential for tumour growth [23], while catalase neutralises hydrogen peroxide, protecting cells from oxidative damage and indirectly supporting anticancer activity [24,22]. This observation is consistent with earlier findings from *Chaetomium* species, which also exhibited strong enzymatic and anticancer properties [25,26].

The presence of secondary metabolites, such as flavonoids, alkaloids, saponins, and phenols in the AVK3 extract, as identified through mycochemical screening, further strengthens its therapeutic profile. These phytochemicals are well-documented for their multiple bioactivities, including antioxidant, anti-inflammatory, and antiproliferative effects [27,28]. For example, flavonoids can disrupt bacterial membranes, interfere with nucleic acid synthesis, and modulate signaling pathways involved in apoptosis and angiogenesis in cancer cells [29,30].

Previous studies by Zhai MM et al., demonstrated that *Talaromyces pinophilus* exhibits high bioactivity due to the production of various primary metabolites, including enzymes and secondary metabolites such as terpenoids, alkaloids, polyketides, esters, lactones, and furano-steroids, with potential applications in biotechnology, biocontrol, and biomedicine [31]. Specifically, Talaromycolides inhibit the growth of the human pathogen methicillin-resistant *Staphylococcus aureus*, squalene enhances antimicrobial activity against various *Candida* species, and pinophilin and *Talaromycesins* (A and B) exhibit strong antiproliferative activity by inhibiting cancer cell growth, inducing apoptosis, and preventing angiogenesis.

Medicinal plants rich in phenolic compounds are well-known for their antioxidant properties. Flavonoids, phenolic acids, tocopherols, and other phenolic compounds are the primary natural antioxidants in plants [32]. Flavonoids, which are hydroxylated phenolic compounds, are produced in response to microbial infections. They interact with soluble and extracellular proteins and the bacterial cell wall, exhibiting antibacterial properties that protect against various diseases. Additionally, flavonoids possess anticancer properties and act as potent antioxidants [33,34]. The extracts also contain saponins, which are known for their anti-inflammatory activity, hemolytic action, cholesterol-binding ability, foaming properties in aqueous solutions, and bitterness.

In terms of antioxidant potential, AVK3 showed notable free radical scavenging activity. This observation is in line with findings on other Zingiberaceae plants, like *Alpinia* and *Zingiber* species, which are known for their high phenolic content and potent antioxidant activity [35,36]. The ability of AVK3 to neutralise Reactive Oxygen Species (ROS) is significant, given the role of oxidative stress in chronic diseases such as cancer and cardiovascular disorders [37].

GC-MS analysis helps identify the potent bioactive constituents within a biological sample, whereas molecular docking studies aid in elucidating the precise mechanisms of these bioactive compounds against cancer cell lines, particularly in controlling proliferative pathways. Previous research on fungal metabolites has often identified compounds such as pyranones, furans, and flavonoids as bioactive agents [38]. In earlier studies, GC-MS profiling of AVK3 revealed compounds like 4H-pyran-4-one and 2-furan carboxaldehyde, both of which have been frequently reported in the literature for their antimicrobial, antioxidant, and anticancer potential [39-41]. The relatively high abundance of these compounds in AVK3 extract suggests that this strain is a rich source of pharmacologically relevant metabolites.

Furthermore, molecular docking studies using AutoDock Vina demonstrated that compounds such as 4H-pyran-4-one bind with high affinity to cancer-related proteins, including PDB ID: 3HBS (breast cancer) and 7W8O (lung cancer). These binding affinities are higher than those reported in studies of other fungal-derived compounds [42], suggesting that metabolites from AVK3 may serve as lead compounds for anticancer drug development. The high binding affinity may result from the controlled production of growth factors, inhibition of proliferation of new cells, or facilitation of apoptotic pathways.

Steroids, which interact with sex hormones, exhibit antibacterial properties and are considered significant bioactive substances. Alkaloids, used medicinally for millennia, have analgesic, antispasmodic, and antibacterial properties, while glycosides have been shown to reduce blood pressure. Plants are increasingly recognised as valuable reservoirs of bioactive compounds with therapeutic potential. Studies have found that AVK3 produces bioactive molecules with anticancer, anti-inflammatory, and antioxidant properties, highlighting its potential as a source of pharmacologically active phytochemicals.

AVK3 also demonstrates notable free radical scavenging activity, with a DPPH value of 59.70%.

%of antioxidant activity = [(absorbance of control - absorbance of sample)/ absorbance of control] × 100 [16].

$$\%\text{of antioxidant activity} = \frac{[(1.092-0.44)/1.092]\times 100}{= 59.70\%}$$

Several chronic disorders have been associated with the overproduction of ROS and other free radicals. In previous studies, in-vitro antioxidant activity was assessed using ferric-reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. According to the data, dried ginger exhibited the highest antioxidant activity, containing 5.2 times more phenolic compounds than fresh, stir-fried, and carbonised ginger. This activity was primarily attributed to its polyphenolic content. Overproduction of ROS leads to oxidative stress, which exacerbates various chronic illnesses, including cancer, cardiovascular disease, asthma, and neurological disorders [43].

Talaromyces pinophilus, a fungal endophyte isolated from ginger, produces flavonoids and phenolic compounds. The significance of compounds such as terpenoids, alkaloids, and polyketides identified in this study is supported by their historical use in medicine for antimicrobial and anticancer treatments [44]. Saponins, another metabolite class detected in AVK3, exhibit anti-inflammatory and cholesterol-binding properties, further supporting the pharmaceutical potential of this endophyte [45].

For the future application of *Talaromyces pinophilus* in cancer therapy with minimal side-effects, further research should include in vivo models, Cell toxicity studies, purification of significant pharmacokinetic compounds, and clinical validation. The present study highlights the potential of *Talaromyces pinophilus* in cancer therapy through in-vitro antimicrobial assays to evaluate bioactive metabolite production and molecular docking studies to computationally confirm anticancer efficacy by inhibiting growth factor activity in cancer cells via tight binding to target proteins.

The results of this study underscore the therapeutic promise of *Talaromyces pinophilus* AVK3 as a source of novel antimicrobial and anticancer compounds. Given the rising challenge of antimicrobial resistance and the demand for selective anticancer therapies, AVK3-derived metabolites offer a compelling alternative due to their potent biological activities and natural origin.

To translate these findings into clinical applications, several steps are essential:

- In vivo validation of antimicrobial and anticancer activity using appropriate animal models.
- Purification and structural characterisation of the most active compounds.
- Cytotoxicity and pharmacokinetic profiling to evaluate safety and bioavailability.
- Bioprocess optimisation using bioreactors for large-scale metabolite production.
- Genomic and transcriptomic analysis to identify and enhance biosynthetic gene clusters responsible for bioactive compound synthesis.

The integration of metabolomic, enzymatic, and molecular modelling approaches used in this study provides a strong foundation for the pharmaceutical exploitation of *T. pinophilus* AVK3. With further research, this endophyte may contribute to the development of next-generation antimicrobial and anticancer therapeutics with minimal side-effects.

Limitation(s)

While the present study provides significant insights into the antimicrobial and anticancer potential of *Talaromyces pinophilus* (AVK3) isolated from *Zingiber officinale*, several limitations should be acknowledged:

- The anticancer potential of the identified bioactive compounds was assessed solely through in-silico molecular docking; no in-vivo or in-vitro cytotoxicity assays were performed to validate their efficacy in biological systems.
- Although GC-MS analysis identified a range of bioactive compounds, the study did not include purification and structural elucidation of individual metabolites, which is necessary for precise pharmacological evaluation.
- Potential toxicity or side-effects of the fungal extract or its compounds were not assessed, which is essential for considering therapeutic applications.

CONCLUSION

This study demonstrates that GC-MS analysis of *Talaromyces pinophilus* AVK3, isolated from the ginger (*Zingiber officinale*) rhizome, revealed 4H-pyran-4-one with a peak area of 26% at a retention time of 5.500, and 2-furan carboxaldehyde with a peak area of 22.47%. AutoDock Vina software was used to assess the antibacterial and anticancer potential of these compounds. The findings revealed significant binding affinities of -22.9 kcal/mol against breast cancer (PDB ID 3HBS) and -19.5 kcal/mol against lung cancer (PDB ID 7W8O). Present research indicates that *T. pinophilus* is a promising source of new antibacterial and anticancer agents based on in-silico and in-vitro analyses.

This study aimed to isolate and characterise the endophytic fungus *Talaromyces pinophilus* (AVK3) from *Zingiber officinale* rhizomes, as well as to assess its antibacterial, enzymatic, antioxidant, and anticancer properties using in-vitro and in-silico methods. Following extensive experimental studies, the crude extract of *T. pinophilus* demonstrated antibacterial, antimicrobial, antioxidant, and anticancer activities. Thus, the fungal endophyte *T. pinophilus* from ginger rhizome shows considerable promise for pharmaceutical applications and may serve as a source of bioactive compounds for cancer therapy.

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