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Gymnascella thermotolerans-GTE-21, an Endophytic Fungus in *Euphorbia geniculata* as a Versatile Producer of Bioactive Metabolites

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Abstract

The thermophilic fungus *Gymnascella thermotolerans* GTE-21 produces a significant amount of both volatile and non-volatile compounds with potent antifungal and anticancer properties. Gas chromatography-mass spectrometry (GC-MS) was utilized to identify 14 volatile organic compounds (VOCs) released by *G. thermotolerans*. The most predominant compound identified was diisooctyl phthalate, constituting 71.99% of the total VOCs, followed by phenol, 2-methoxy-4-(2-propenyl) at 7.12%. The GTE-21's VOCs demonstrated significant antifungal growth inhibition activity of 42.5% against *Fusarium solani* and 39.03% against *Alternaria alternata*. Additionally, GTE-21's crude extract exhibited substantial antibacterial effects, evidenced by inhibition zones of 1.5 cm against *S. epidermidis*, 1.4 cm against *C. ciferrii*, and 1.3 cm against *S. aureus*. The CH_2Cl_2:MeOH (1:1) fraction was more effective against human pathogens, producing the largest zones of inhibition of 1.1 and 1.03 cm against *S. epidermidis* and *C. ciferrii*, respectively. The evaluation of GTE-21's antifungal activity using the Evans blue assay showed that fungal spores treated with GTE-21's crude extract exhibited 96.2% inhibition at a concentration of 25 mg/ml. Similarly, the fractions CH2Cl2:MeOH (1:1) and CH2Cl2:MeOH (9:1) demonstrated spore inhibition rates of 96.3% and 94.3%, respectively. The EtOAc crude extract demonstrated pronounced cytotoxic effects against the SKOV-3 and MDA-MB-231 cancer cell lines. For SKOV-3, cell viability decreased to 3.40% and 0.47% at concentrations of 50 and 100 µL, respectively. In the case of MDA-MB-231, cell viability was reduced to 0.19% at both 50 and 100 µL concentrations.

Keywords: Antimicrobial Activity, Anticancer, *Gymnascella thermotolerans*, Volatile Organic Compounds

1. Introduction

Euphorbia geniculata, a member of the *Euphorbiaceae* family, is characterized by its production of a white milky latex. This latex has traditionally been used as a purgative and lactogenic agent and for treating gonorrhea, migraines, and warts. Additionally, it is used in the manufacturing of fish poison, pesticides and toxins (Kumar *et al.*, 2010).

Endophytes, a beneficial class of microorganisms, are known for colonizing various plants while maintaining a symbiotic relationship that does not harm their hosts (Rodriguez *et al.*, 2009). Endophytes are considered invaluable in the realm of drug discovery due to their production of volatile and non-volatile bioactive metabolites with crucial biological functions (Priti *et al.*, 2009). These compounds are leveraged in biofuel production and exhibit a range of biological properties, such as cytotoxic, anticancer, antimicrobial, antidiabetic, antioxidant and antiproliferative activities (Fernandes *et al.*, 2015).

Endophytic fungi can generate complex combinations of volatile organic compounds (VOCs), which may protect their host plants from phytopathogens either directly or indirectly by enhancing plant resistance (Tahir *et al.*, 2017). These VOCs span various chemical classes, including esters, alkynes, alkenes, alkanes, alcohols, terpenoids, aldehydes, ketones, and sulfur-containing compounds (Mitchell et al., 2010). Notably, compounds such as 2-undecanone, nonanal, dimethyl trisulfide, n-decanal cyclohexanol, and benzothiazole have shown biocontrol activity (Xie *et al.*, 2018).

Currently, cancer ranks as the leading cause of death worldwide, with the World Health Organization (WHO) identifying it as a primary cause of morbidity and mortality. It has been determined that endophytic fungi

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provide a consistent and abundant source of anticancer agents. Early investigations have revealed that the bioactive compounds naturally produced by endophytes may offer a new foundation for the development of innovative anticancer medications (Xie and Zhou, 2017).

Thermophilic fungi are extremophiles that are able to reproduce and develop within a temperature range of 45 °C and 61 °C (De Oliveira *et al*., 2015). Therefore, temperature is considered as the key factor influencing the structural and functional properties of endophytic biomolecules, as well as maintaining the integrity of their cellular components. These unique biomolecules have garnered the interest of chemists and biotechnologists working in the field of natural products, and numerous applications of thermophiles-derived compounds have been seen in current biotechnology (Atalla, *et al*., 2019, 2020[a, b]; Elsehemy, *et al*., 2020).

The increasing problem of drug-resistant bacteria necessitates the urgent need for the development of more effective and powerful antimicrobial drugs. Recent theoretical developments have shown that numerous endophytic fungi have the potential to produce antimicrobial compounds, facilitating the creation of novel pharmaceutical medications. Fungi also produce a wide range of antioxidants, including flavonoids, alkaloids, steroids and phenols, and these compounds can scavenge the excess reactive oxygen species (ROS) that accumulate in our body (Hamed, *et al*., 2015, Gauchan *et al*., 2020).

In our study, we focused on thermotolerant endophytes that can produce bioactive secondary metabolites with cytotoxic, antibacterial and antioxidant effects. This study aims to demonstrate that endophytic fungi are valuable natural sources for the discovery of novel drugs, offering potential benefits to the food, pharmaceutical, and agricultural industries.

2. Materials and methods

2.1. Isolation and Identification of fungi

Euphorbia geniculata plant samples were collected from the field station at Aswan University, Egypt. The surface of the whole plant was sterilized using 70% ethanol for one minute followed by five minutes in a 5% sodium hypochlorite solution. Following sterilization, the plant was rinsed twice in sterile distilled water. The plant leaves, stems and roots were sliced longitudinally into small segments of 0.5-1.0 cm and placed on sterile Petri dishes containing Potato Dextrose Agar (PDA) medium (Sigma-aldrich, India). Three replicates of the Petri plates were incubated at a temperature of 45°C for two weeks. The emerging fungi were then transferred to fresh PDA plates for purification and stored on PDA agar slants at 4°C for further use. The morphological characteristics of the isolated fungus were utilized for its identification, primarily through an assessment of its colony visual appearance, mycelium color, structure and ascomata according to Zhou *et al.* (2016). Subsequently, we validated the identification through molecular means by sequencing the rDNA gene, using outsource service at Solgent Company located in Daejeon, South Korea. Fresh fungal cultures were incubated on PDA medium at 28° C for 7 days. Fungal DNA was extracted by CTAB method (Suarez *et al*., 2005, Gontia-Mishra *et al*., 2014). Prior to sequencing, amplification of the ribosomal rRNA gene (rDNA) was conducted by employing the polymerase chain reaction (PCR) technique. Two Universal primers, internal transcript scarcer 1 and 2 (ITS1 and ITS4, forward and reverse, respectively) were used in the reaction mixture. ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC - 33') primers were obtained from Sigma Korea. Finally, the specified PCR amplicon underwent verification using 1% electrophoresis agarose gel. To identify the sequence homology, the BLAST tool available on the NCBI website was utilized. Furthermore, the MEGA6 program was employed to construct the phylogenetic tree according to Tamura *et al*. (2013).

2.2. Extraction and fractionation

Fresh mycelium of *G. thermotolerans* grown on PDA medium for 6 days was inoculated into potato dextrose broth (PDB) medium and placed on a shaker at 180 rpm for 20 days at 45°C. The fungal culture was treated with 200 mL of ethyl acetate (EtOAc) (Sigma-aldrich, India) and allowed to stand overnight. Subsequently, the mixture underwent ultrasonication for 10 minutes and was filtered using a Büchner funnel under vacuum. Medium (water phase) and the filtrate containing EtOAc phase were collected for further work up, and the isolated mycelia (cell debris) were discarded. The water phase (medium) and EtOAc phase were then separated using a separator funnel. A rotary evaporator was then employed to concentrate and evaporate the EtOAc phase under vacuum conditions (Xu, 2010).

2.3. Flash Chromatography

Before loading the EtOAc fungal crude extract, the column was packed with dried Silica Gel and saturated with mobile solvents. The mobile phase was pumped through the column using air pressure. The solvents used in sequential order for the mobile phase were: Hexane, Methylene chloride (CH_2Cl_2), CH_2Cl_2: MeOH (1:1), CH_2Cl_2: MeOH (9:1) and MeOH. The fractions collected from each solvent system were then dried, following the method outlined by Gatto *et al*. (2013), with some modifications.

The GC-MS analyses were carried out by Nawah Scientific Inc., using a Thermo Scientific TRACE GC Ultra integrated with a Thermo Scientific ISQ quadrupole MS (Thermo Scientific, USA). The AMDIS software served as the de-convolution tool for the identification of compounds, by matching the obtained MS with those in the Wiley and NSIT library databases. Next, the relative abundance of each compound was determined by calculating its percentage of the total peak area in the chromatogram (Gomathi *et al*., 2015).

2.4. Screening of Bioactive properties of G. thermotolerans-GTE-21 metabolites

2.4.1. Antimicrobial assay of fungal endophyte-GTE-21

The antimicrobial activity of crude extracts derived from fungal endophyte GTE-21 and its fractions was assessed using multiple methods, including the disc diffusion method, antifungal activity of VOCs, the agar plate diffusion assay, and Evans blue method, as described by Balouiri *et al*. (2016).

2.4.2. Test organisms

Human pathogens, including *Escherichia coli* Ec31, *Klebsiella pneumoniae* Kp11, *Staphylococcus aureus* Sa9, *Staphylococcus epidermidis* Se 23, *Candida ciferrii* Cc 501 and *Candida albicans* Ca 20, along with plant pathogens such as *A. alternata*, *C. specifier* CSN-20 and *F. solani* FSN-20 were obtained from the Mycology Laboratory, Botany Department, Aswan University.

2.4.3. Disc diffusion method of crude extracts and fractions of GTE-21

The experiment involved inoculating Petri dishes filled with nutrient agar medium (Signa-Aldrich, India) with the test organisms, including human pathogens. Sterilized paper disks soaked in fungal crude extracts and fractions dissolved in a CH2Cl2 and MeOH (1:1) solution were then placed on a bacterial lawn spread on the agar medium surface.

The negative control consisted of a disk without any endophytic fungal extracts. We evaluated the presence or absence of an inhibition zone surrounding the discs by incubating the plate overnight at 37°C, as described by Schulz *et al*. (1995).

2.4.4. Antifungal activity of VOCs of GTE-21

Following the method established by Strobel *et al.* (2001), the antifungal activity of GTE-21's VOCs was assessed against plant pathogenic fungi *A. alternata*, *C. specifier*, and *F. solani*. For each assay, two 6.0 cm diameter Petri dishes were placed inside larger 12 cm diameter Petri dishes. In the first plate, GTE-21 was inoculated at the center of the PDA media and allowed to grow for four days at 28°C to enhance VOC production. Subsequently, the targeted fungal species was inoculated onto the second Petri dish. Both plates were securely sealed using Parafilm and subjected to an additional three-day incubation period. After incubation, the extent of fungal growth was measured, enabling the calculation of the inhibition percentage as shown in the following formula:

$$\% \text{ Inhibition} = \frac{Dc - Ds}{Dc} \times 100$$

Where: Dc = average diameter of colony growth in the control group and, Ds = average diameter of mycelial growth in treatment group (Singh and Tripathi, 1999).

2.4.5. Agar plate diffusion assay

Fungal EtOAc crude extracts and fractions, as previously mentioned, were added to PDA media at a concentration of 2.0 mg/mL and thoroughly mixed. The antifungal effect against microorganisms was evaluated using the poisoned food method. A mycelial disc of the pathogen (*A. alternata*, *C. specifier*, and *F. solani*) was placed at the center of a 6.0 cm plate (Balouiri *et al*., 2016). After 7 days of incubation at 28°C, the diameters of fungal growth on both control and treatment plates were measured. The percentage of growth inhibition was then calculated following the method described by Singh and Tripathi, (1999).

2.4.6. Evans blue method

Antifungal activity was also assessed by Evans blue staining method on *F. solani*, which exhibited clear results compared to *A. alternata* and *C. specifier*. Briefly, *F. solani* spores were cultured in Potato Dextrose Broth (PDB) at 28±2˚C for five days. Next, the mycelia were separated through filtration, and the conidial cells were isolated using centrifugation at 5000 rpm for 10 min. The EtOAc crude extracts and fractions at concentrations of 10 and 25 mg/mL were added to the tubes containing the spores. Additionally, a control set containing only the inoculum was prepared. All the tubes were incubated at 28±2˚C for intervals of 1h, 3h, and 24h. After each period, the tubes were centrifuged and 0.05% Evans blue was applied to 10 µl of the residual culture. Excess dye was then washed from the hyphae using sterile distilled water. The difference between dead and live cells was observed under a light microscope (Semighini and Harris, 2010).

2.5. 2.6. Cytotoxic activity

2.5.1. Cell culture

The breast and ovarian cancer cell lines were obtained from Nawah Scientific Inc, Egypt. Breast and ovarian cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute Medium (RPMI), respectively, both supplemented with 100 units/mL of penicillin, streptomycin and 10% heat-inactivated fetal bovine serum. The cancer cell lines were maintained at 37 °C in a humidified environment with 5% CO_2 (Allam *et al*., 2018).

2.5.2. In vitro Cytotoxicity assay

The cytotoxic effect of endophytic fungal extracts (GTE-21) was evaluated using Sulforhodamine B (SRB) assay against human breast and ovarian cell lines at different concentrations of 0.01, 0.1, 1, 10, 50 and 100 ug/mL. The surviving fraction and cell viability were determined (Allam *et al*., 2018).

2.6. Antioxidant activity assays:

To evaluate the antioxidant activity of endophytic fungal crude extracts and fractions, analytical assays were performed on all tested fractions using two different experimental techniques.

2.6.1. DPPH free radical scavenging activity method

To evaluate the antioxidant activity in the investigated samples, changes in the absorbance of DPPH radicals were determined. The endophytic fungal extracts and fractions at a final concentration of 1 mg/mL were prepared. A 0.5 mmol/L DPPH solution was prepared in 95% MeOH. Then, 0.1 mL of fungal extract/fraction was diluted with 95% MeOH, followed by the addition of 2 mL of the DPPH solution. The mixture was incubated at room temperature under dark conditions for 30 minutes. Following the incubation period, the absorbance was measured at 517 nm, as described by Brand-Williams *et al.* (1995).

2.6.2. Total antioxidant capacity method

The assay was conducted following the method outlined by Prieto *et al.* (1999). A 50 µl of each extract was combined with 1 mL of the reaction solution, consisting of 4 mM ammonium molybdate $(NH_4)_2MoO_4$, 0.6 M H_2So_4 and 28 mM Na_2PO_4. The capped tubes were then incubated in a thermal block at 95°C for 90 minutes. After cooling, the absorbance was measured at 695 nm for each sample, using a blank as a reference. The blank solution comprised 1 ml of the reagent solution and an

appropriate volume of the same solvent utilized for the sample, and it was incubated under the same conditions as the remaining samples. The antioxidant capacity was expressed in terms of ascorbic acid equivalent.

2.7. Statistical analysis

Data were collected and subjected to one-way analysis of variance (ANOVA). The significant difference at $P < 0.05$ between the treatment groups and the control was determined using Minitab's Student's t-test. Values represent mean ± standard errors (SEs) of four biological replicates. Correlation analysis was conducted using the Corrplot package in R v4.3.2.

3. Result

3.1. Morphological and Molecular identification

The colony of *G. thermotolerans* exhibits a white color on the obverse and a yellow color on the reverse, with a flat edge. Colonies are flat to low convex, with an irregular or regular and nearly transparent border. The hypha appears hyaline and light yellowish in color, with ascomata become visible at maturity after 15 days of cultivation. Subsequent molecular analysis confirmed that our *G. thermotolerans*-GTE-21 (Basionym: *Gymnoascus thermotolerans*), exhibited 99% similarity to the NCBI accession number MW590717 (Fig. 1).

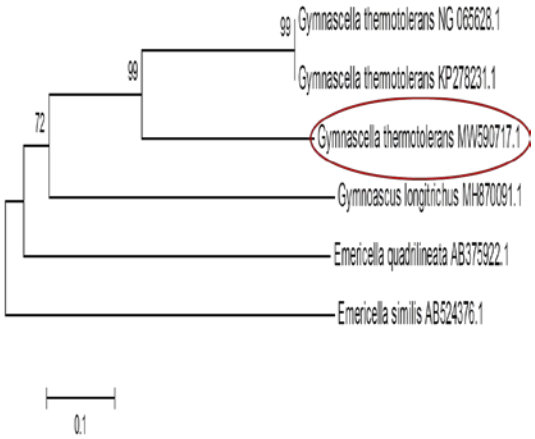


Figure 1. Phylogenetic analysis of the isolated *G. thermotolerans* endophyte and other closely related fungi obtained from the NCBI database. The phylogenetic relationships were determined based on the homology of their nuclear ribosomal internal transcribed spacer sequences. The scale bar represents the tree evolutionary distance.

3.2. GC-MS analysis of EtOAc crude

GC-MS analysis was conducted to determine the chemical compositions of the EtOAc extract of dried GTE-21 (Table 1). A total of 14 volatile organic compounds (VOCs) were identified, indicating the presence of various bioactive substances at different retention times (RT).The mass spectra were used to determine the peaks of each component (Fig. 2). These compounds were recognized by their chemical formulae, RT, molecular weight and percentage of peak area (Table 1). Among the identified VOCs, diisooctyl phthalate (71.99%), phenol, 2-methoxy-4-(2-propenyl) (7.12%), and 1,6-octadien-3-ol, 3,7-dimethyl emerged as the predominant compounds, based on their RT and peak area percentages.

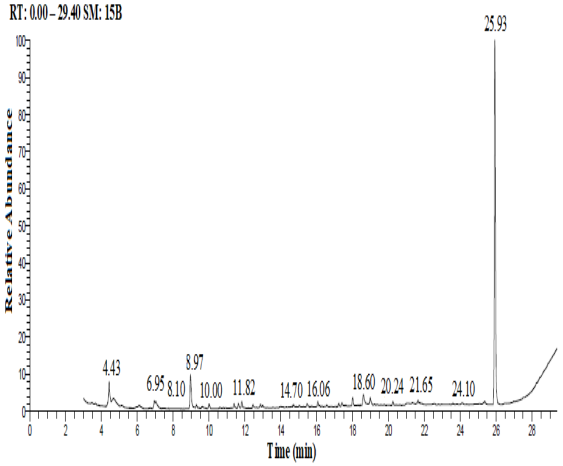
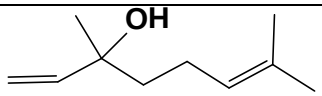
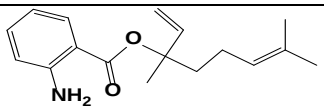
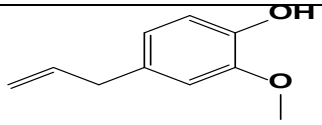
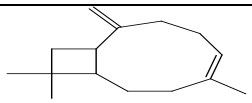
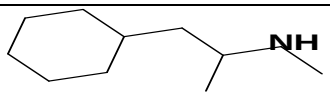
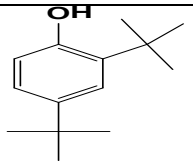
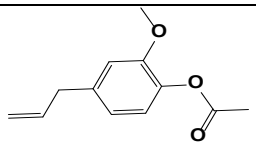
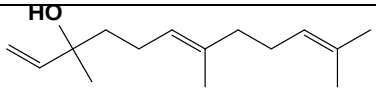
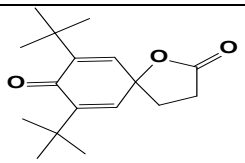
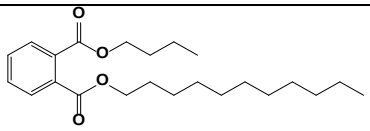
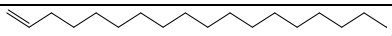
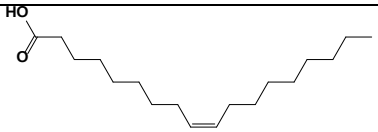
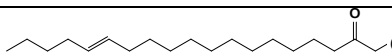
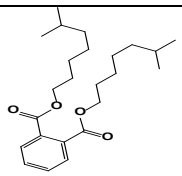


Figure 2: Chromatogram of GC-MS spectra of GTE-21 ethyl acetate crude extract.

Table (1): Volatile organic compounds of *G. thermotolerans* (GTE-21)

Chemical Compounds	Structure	Formula	RT (min)	M.W	Area %	Pharmacological actions
3,7-Dimethyl-1,6-Octadien-3-Ol (Linalool) (Monoterpenoid)		$C_{10}H_{18}O$	4.43	154	4.99	Anti-inflammatory, antioxidant, anticancer and antimicrobial Cherbal *et al.*, 2023
1,5-Dimethyl-1-vinyl-4-hexenyl-2- aminobenzoate (Linalyl aminobenzoate) or Linalyl ester		$C_{17}H_{23}NO_2$	7.02	273	2.09	Antioxidant and antibacterial Ouedrhiri *et al.*, 2015
Phenol,2- methoxy-4- (2-propenyl)- (Aromatic Compound)		$C_{10}H_{12}O_2$	8.97	164	7.12	Antioxidant and anticancer Satoh *et al.*, 1998
β-Caryophyllene (Bicyclic Sesquiterpene)		$C_{15}H_{24}$	9.99	204	1.01	Antioxidant, anticancer and antimicrobial (Dahham *et al*., 2015)
1-Cyclohexyl-N-methyl-propan-2-amine (Aliphatic Secondary Amine)		$C_{10}H_{21}N$	11.39	155	0.86	Heart block treatment (Day and Viar, 1951)
2,4-Di-tert-butylphenol (Aromatic Compound)		$C_{14}H_{22}O$	11.64	206	0.91	Antioxidant anti-Inflammatory cytotoxicity insecticidal and nematocidal antimicrobial and antiviral activity (Zhao *et al.*, 2020)
4-Allyl-2-methoxy phenylacetate (Aromatic Compound)		$C_{12}H_{14}O_3$	11.82	206	1.18	Antioxidant Hidalgo *et al*., 2009
3,7,11-Trimethyl-1,6,10-Dodecatrien-3-Ol (Sesquiterpene Alcohol)		$C_{15}H_{26}O$	12.45	222	0.67	Antimicrobial Madhumathi *et al*., 2014
7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione (Spiro compound)		$C_{17}H_{24}O_3$	18.00	276	1.60	Antioxidant Singh *et al*., 2018
Phthalic acid, butyl undecyl ester (Phthalic acid Ester)		$C_{23}H_{36}O_4$	18.59	376	2.45	Antioxidant and antimicrobial Nsofor et al., 2023
1-Nonadecene (Aliphatic Hydrocarbon)		$C_{19}H_{38}$	18.98	266	1.41	Antibacterial, antidiabetic and antitumor Amudha *et al*., 2018
Oleic Acid (Unsaturated Fatty Acid)		$C_{18}H_{34}O_2$	20.24	282	0.76	Anti-inflammatory, antioxidant, anticancer and antimicrobial Cherbal *et al.* 2023
Trans-13-Octadecenoic acid (Unsaturated Fatty Acid)		$C_{18}H_{34}O_2$	21.64	282	0.74	Anti-inflammatory Khan *et al*., 2022
Diisooctyl phthalate (Phthalic acid Ester)		$C_{24}H_{38}O_4$	25.93	390	71.99	Anti-inflammatory, antioxidant, anticancer, and antimicrobial Saeed *et al*., 2020

3.3. Disc diffusion method of crude and fractions of GTE-21

Antimicrobial compounds from fungi represent a highly promising source for the development of new antibiotics with novel mechanisms of action, aimed at overcoming bacterial drug resistance (Bhatnagar and Kim, 2010). Our results showed that the crude extract of GTE-21 exhibited the highest antimicrobial activity with inhibition diameter of 1.5, 1.4 and 1.3 cm against *S. epidermidis*, *C. ciferrii* and *S. aureus* respectively. The CH2Cl2:MeOH (1:1) fraction showed superior efficacy against human pathogens, producing the largest inhibition zones of 1.1 cm against *S. epidermidis* and 1.03 cm against *C. ciferrii*. In comparison, the MeOH fraction resulted in a 1.2 cm inhibition zone against *S. epidermidis*, but showed no activity against *C. ciferrii* and *S. aureus* (Fig. 3).

Our results indicate that the crude extract successfully inhibited *C. ciferrii*, producing an inhibition zone of 1.4 cm. Furthermore, the CH_2Cl_2:MeOH (1:1) fraction and hexane were more effective against *C. ciferrii* pathogen, yielding inhibition zones of 0.9 and 1.03 cm, respectively. In contrast, the CH_2Cl_2:MeOH (9:1) and MeOH fractions did not exhibit any inhibitory effect on *C. ciferrii*. The pathogen *S. aureus* was susceptible to the crude extract and the CH_2Cl_2 fraction, with inhibition zones of 1.3 cm and 0.6 cm, respectively, whereas the MeOH fraction showed no inhibitory activity. Additionally, the crude extract and the CH_2Cl_2:MeOH (1:1) and MeOH fractions inhibited *S. epidermidis*, resulting in inhibition zones of 1.5, 1.1 and 1.2 cm, respectively. In contrast, *C. albicans*, *E. coli* and *K. pneumonia* exhibited resistance against all tested fractions and the crude extract.

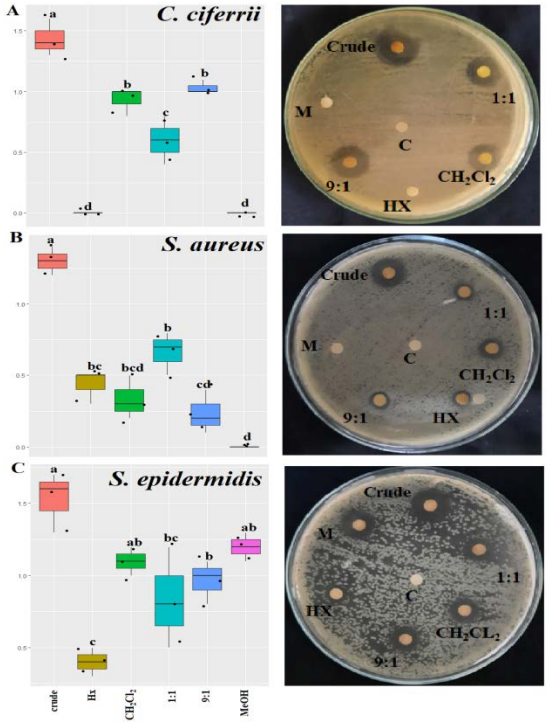


Figure 3: Effects of crude extracts and fractions on human pathogens, including *C. ciferrii* **(A)**, *S. aureus* **(B)** and *S. epidermidis* **(C)** using *in vitro* agar disc diffusion assay. Box plot diagram showing inhibition zones. Different alphabetical letters show significant differences at $P < 0.05$ using Tukey's HSD test.

3.4. Antifungal activity of VOCs of GTE-21

The antifungal activity of GTE-21's VOCs was evaluated against phytopathogenic fungi, including *A. alternata*, *C. specifier*, and *F. solani*. Among all tested pathogens, *F. solani* exhibited the highest inhibition at 42.5%, followed by *A. alternata* with 39.03%, while *C. specifier* showed no inhibition (Fig. 4).

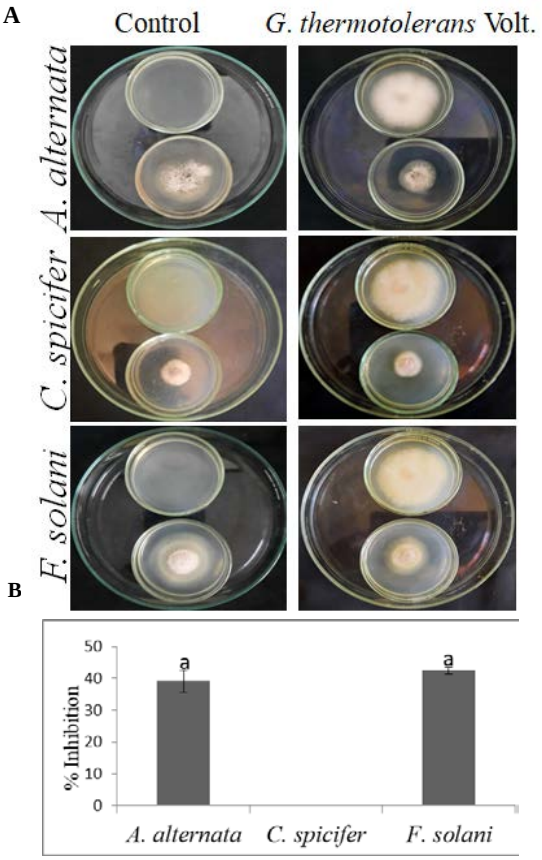


Figure 4. Antifungal efficacy of GTE-21's volatile organic compounds (VOCs) against different phytopathogens, illustrated through (A) Petri plate assay and (B) inhibition percentages. Different alphabetical letters indicate significant differences at $p < 0.05$ using Tukey's HSD test.

3.5. Agar diffusion method

The diameter of the inhibition zones can be used to calculate the percentage of growth inhibition of the chosen phytopathogenic fungus (*A. alternata*, *F. solani*, and *C. specifier*) at a concentration of 2.0 mg/ml of GTE-21 crude, and fractions were calculated (Balouiri *et al*., 2016).

Our research revealed that the crude extract of GTE-21, as well as the CH2Cl2:MeOH (1:1) and CH2Cl2:MeOH (9:1) fractions, demonstrated the highest inhibition rates against A. alternata, with percentages of 72.2%, 71.2%, and 70.3%, respectively, compared to the control. Conversely, the hexane, CH2Cl2, and MeOH fractions exhibited the lowest inhibition rates at 23.1%, 25.9%, and 12.03%, respectively.

"In addition, the crude extract inhibited fungal mycelial growth of C. specifier with a high inhibition percentage of 93.05%. The CH2Cl2:MeOH (1:1) fraction inhibited it by 68.05%, the CH2Cl2:MeOH (9:1) fraction reduced the growth by 58.3%, and both the CH2Cl2 and Hexane fractions reduced the pathogen growth by 30.5% and 25%

respectively." By contrast, the MeOH fraction inhibited the pathogen with low percentage 5.5%.

Also, the CH2Cl2: MeOH (9:1) and CH2Cl2: MeOH (1:1) fractions inhibited the growth of F. solani by 54.7%, 43.5%, and 38.4% respectively. CH2Cl2, Hexane, and MeOH inhibited by 33.3%, 28.2%, and 27.3%. (Fig. 5).

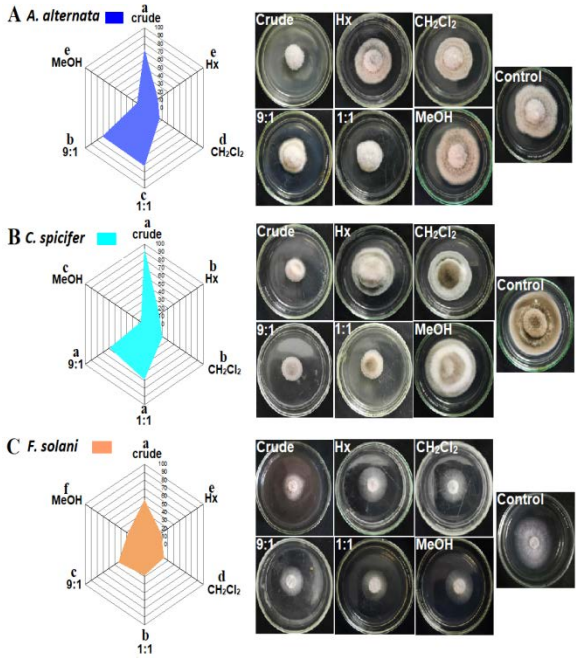


Figure 5. Effect of EtOAc crude extracts and fractions on phytopathogenic fungi using agar diffusion method. Radar plot indicate percentage of inhibition against **(A)** *A. alternata*, **(B)** *C. specifier* and **(C)** *F. solani*. Different alphabetical letters on rader plot indicate significant differences at $p < 0.05$ using Tukey's HSD test.

3.6. Evans blue method

Antifungal activity of the extract and crude was assessed by Evans Blue Staining method on *F. solani*. The fungal spores treated with crude, CH_2Cl_2: MeOH (1:1) and CH_2Cl_2 at concentration 10 mg/ml were more affected than Hexane, CH_2Cl_2: MeOH (9:1) and MeOH. Whereas the fungal spores treated with a concentration of 25 mg/ml were strongly affected by the crude, CH2Cl2: MeOH (1:1), CH2Cl2: MeOH (9:1), and MeOH, the spores showed little effect when treated with Hexane and MeOH. A relatively high suppression of spore germination (<80%) was detected at 25µg/ml. The untreated control conidia maintained their natural coloration. The crude extract showed 41.07% effect at a concentration of 10 mg/ml, while the CH2Cl2: MeOH (1:1) fraction showed 32.7% effect. Conidia treated with the crude extract showed 96.2% effect at a concentration of 25 mg/ml. The CH2Cl2: MeOH (1:1) and CH2Cl2: MeOH (9:1) fractions were affected by 96.3% and 94.3% respectively. But the spores of *Fusarium* were little affected by Evan's blue when treated with CH_2Cl_2 fraction at concentration 10 and 25 mg/ml (Fig. 6).

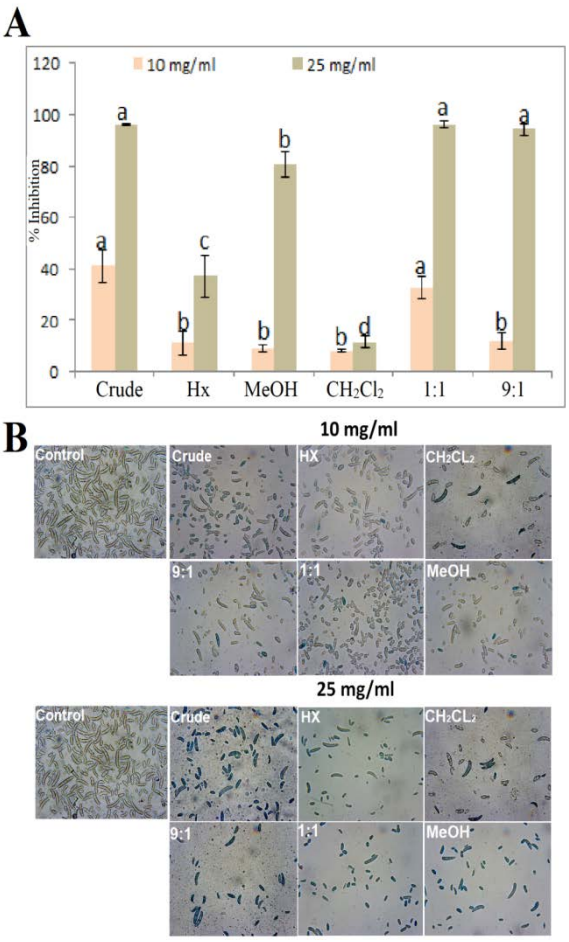


Figure 6. Effect of the crude and fractions on the viability of *F. solani* conidia by Evan blue method. Letters (a, b, c and d) indicate significant differences ($p < 0.05$) (ANOVA after Tukeys test analysis).

3.7. Screening of cytotoxic activity

Our findings on crude and fractions of GTE-21 showed highly cytotoxicity effects on the two cell lines (SKOV-3 and MDA-MB-231) with variable extent causing damaging (Figs. 7, 8, 9).

The EtOAc Crude displayed the highest significant cytotoxicity against SKOV-3 and MDA-MB-231 cell line with viability 3.4 and 0.47 % for concentration 50 and 100 µl respectively for SKOV-3 and 0.193 and 0.194% for concentration 50 and 100 ul respectively on MDA-MB-231.

The viability of SKOV-3 and MDA-MB-231 cells was strongly affected by the fraction CH2Cl2: MeOH (9:1) at 50 and 100 µl, resulting in viability values of 70.3% and 24.3% for SKOV-3 and 41.83% and 6.56% for MDA-MB-231, respectively (Fig. 8).

The concentration of 100 µl of the fractions MeOH, CH_2Cl_2: MeOH (1:1) and CH_2Cl_2 exhibited strong viability percentage 38.16, 37.02 and 26.75% respectively on breast cancer; on ovarian cancer they exhibited 44.56667, 49.45333 and 84.01667% respectively, yet, the Hexane fraction exhibited the least cytotoxicity on SKOV-3 and MDA-MB-231, with viabilities of 93.9933% and 68.9144% respectively (Fig. 9).

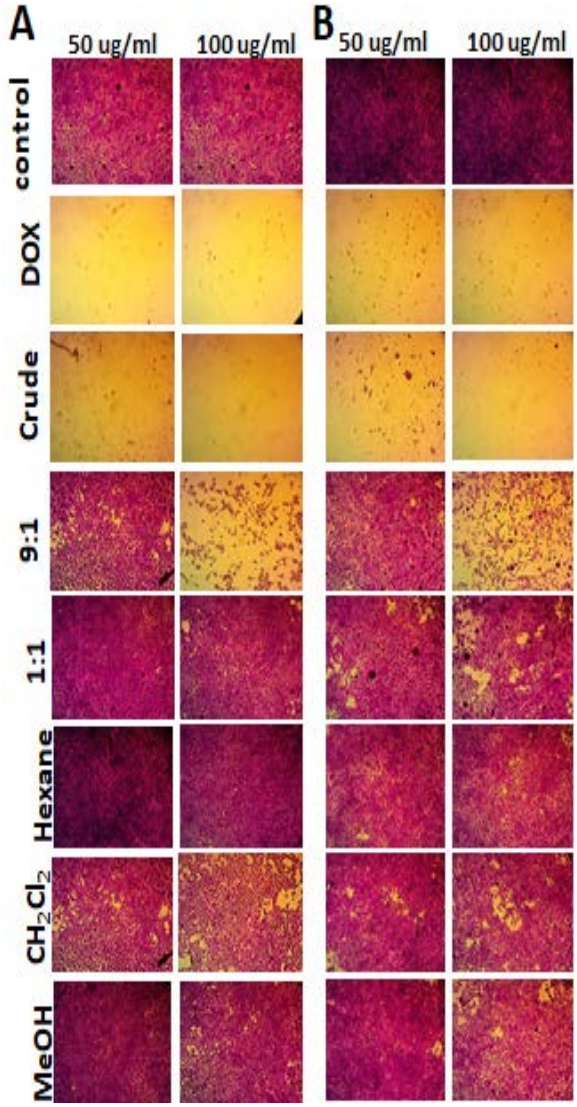


Figure 7. Crude and fractions effect on the cancer cells (A) MDA-MB-231 and (B) SKOV-3 at concentration 50 and 100 µg/ml.

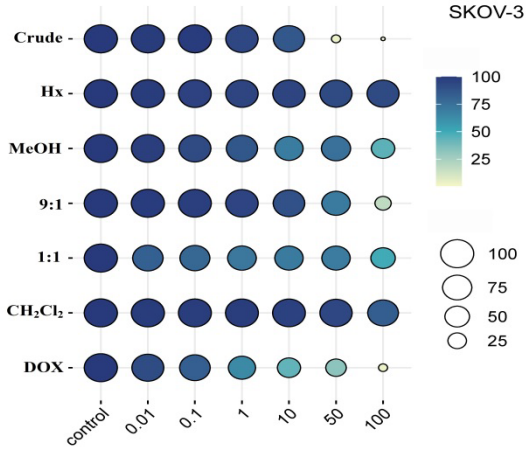


Figure 8. Dot plot illustrating viability percentage in SKOV-3 cells at various concentrations using a cytotoxicity Assay.

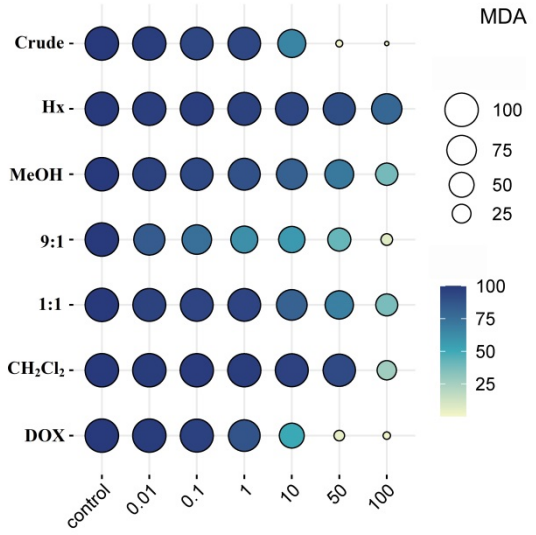


Figure 9. Dot plot illustrating viability percentage in MDA-MB-231 cells at various concentrations using a cytotoxicity Assay.

3.8. Antioxidant activity assays

3.8.1. DPPH free radical scavenging activity

The crude extract of EtOAc and the CH2Cl2: MeOH (1:1) fraction showed high scavenging activities of 63.1% and 58.06%, respectively. The methanolic fraction, CH2Cl2: MeOH (9:1), and the CH2Cl2 extract showed scavenging activities of 47.12%, 54.6%, and 52.56%, respectively. In contrast, the hexane fraction exhibited the least antioxidant activity at 11.4% (Fig. 10).

3.8.2. Determination of total antioxidant capacity (TAC)

Crude and CH_2Cl_2: MeOH (1:1) fraction showed high TAC (0.32±0.012 and 0.38±0.014) mg/ml followed by CH_2Cl_2: MeOH (9:1) and CH_2Cl_2 (0.29±0.01 and 0.24±0.03) mg/ml respectively. Hexane and MeOH fractions showed the lowest TAC (0.16±0.009 and 0.11±0.02 mg/ml respectively) (Fig. 10).

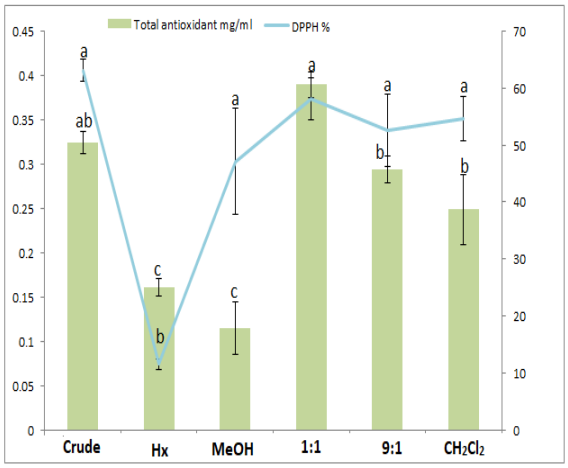


Figure 10. Antioxidant activity of the crude extracts and fractions of *G. thermotolerans*.

The correlation between all activities of *G. thermotolerans* crude and fractions extractions was clarified in figure 11. We did this correlation by metaboanalyst (https://genap.metaboanalyst.ca/ModuleView.xhtml)

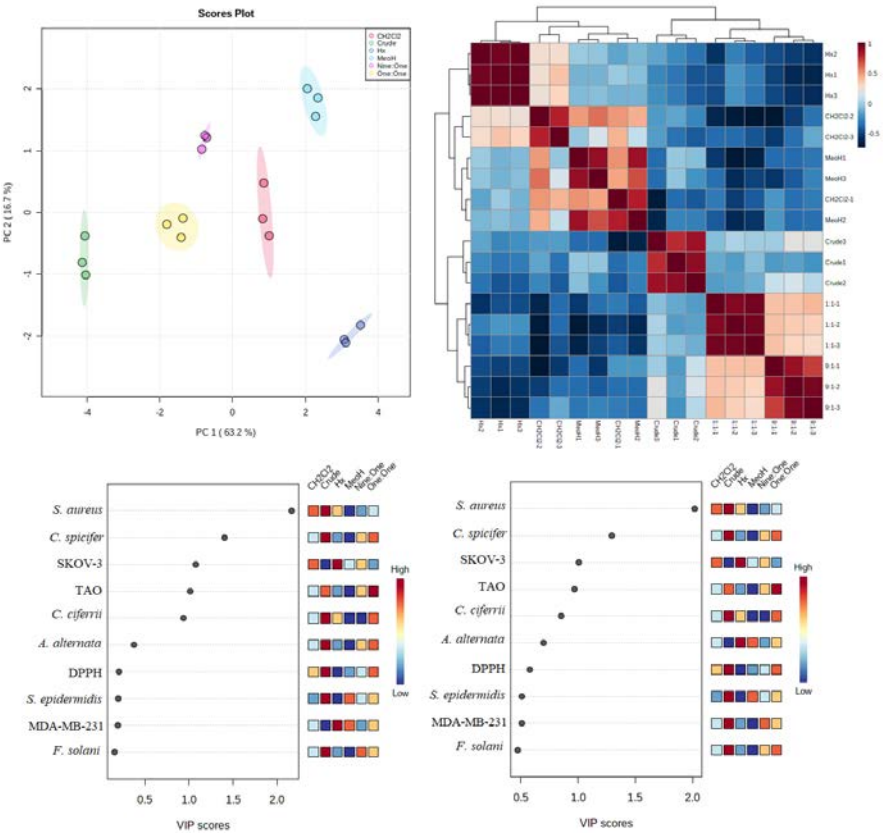


Figure 11. Principal component analysis (PCA), correlation heatmap, and variable importance in projection (VIP) scores for all examined parameters using crude extracts and fractions of *G. thermotoleran.*

4. Discussion

Several endophytic fungal strains have been discovered, and it has been claimed that they produce novel bioactive compounds in anticancer tests (Stierle and Stierle, 2015). Our results showed that the endophyte fungi *G. thermotolerans* produced VOCs and non-VOCs with antimicrobial, antioxidant and anticancer activities. Diisooctyl phthalate, Phthalic acid, 7, 9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione and 1-Nonadecene were the most active VOCs. These compounds are active due to their action in disrupting the membrane properties of pathogenic fungi and bacteria (Casillas *et al*., 2021). Moreover, as highlighted by Li *et al.*, (2021), certain active constituents such as fengycin induce alterations in the mycelial morphology of the cell membrane and cell wall in rice blast fungus. This process concurrently reduces the mitochondrial membrane potential, leading to the accumulation of reactive oxygen species (ROS) and consequent cytotoxic effects.

The anticancer medications derived from plants, such as taxol, etoposide, vincristine and irinotecan are currently used in clinical settings to treat a variety of human cancers (Balunas and Kinghorn, 2005). This work explores how some of these compounds were extracted from endophytes and reports on their cytotoxic effects on different cell lines. Endophytic fungi isolated from some medicinal plant like *Litsea cubeba* (Deka and Jha, 2018), *Pelargonium graveolens* (Yasser *et al.*, 2020) and *Solenostemma arghel* (Abdel-Motaal *et al.*, 2021) showed promising antimicrobial activity against selected pathogenic microbes.

Our result demonstrated that the Chemical composition of GTE-21 crude by GC-MS analyses was 14 VOCs compounds, and the predominant compounds were diisooctyl phthalate (71.99 %). This agrees with Weikl *et al*. (2016) who proved that fungi emitted more VOCs like *Alternaria* that produced VOCs like 3-octanone. Also, Plaszkó *et al*. (2020), reported *Macrophomina*, *Penicillium*, *Aspergillus* and *Fusarium* emitted numerous VOCs such as styrene and benzaldehyde.

In line with our observations, the crude extract of GTE-21 demonstrated the most significant antimicrobial activity, exhibiting inhibition diameters of 1.5 cm, 1.4 cm, and 1.3 cm against *S. epidermidis*, *C. ciferrii*, and *S. aureus*, respectively. This enhanced antimicrobial effect can be attributed to the presence of active compounds, including VOCs such as diisooctyl phthalate, oleic acid, butyl undecyl ester, and β-Caryophyllene, as well as non-VOCs like diisooctyl phthalate and dibutyl phthalate (unpublished data). Previous studies have indicated that these compounds possess antimicrobial properties (Stierle and Stierle, 2015). Despite the lack of a clear understanding regarding the mechanisms responsible for the antimicrobial activity of these VOCs and non-VOCs, several hypotheses propose that their action may involve disrupting membrane properties and inducing reactive oxygen species within the bacterial cells, potentially contributing to the observed biological effects activity (Casillas *et al*., 2021).

Handayani and Aminah, (2017) proved that the agar diffusion method was used to test the bioactivity of symbiotic fungi's EtOAc extract against four bacterial pathogens *Basillus subtilis* 1.4 cm, *Staphylococcus*

epidermidis 1.15 cm, *Salmonella typosa* 1.025 cm and *Escherichia coli* 1.47 cm.

The marine sponge-associated fungus *Acanthostrongylophora ingens* exhibits considerable promise as a prospective reservoir of bioactive compounds with potential applications in the pharmaceutical and medical domains, particularly in the context of generating antibacterial and anticancer agents (Handayani and Aminah, 2017). VOCs from GTE-21 were investigated for their antifungal properties against plant pathogenic fungus. The inhibition percentage was highly against *F.* solani at 42.5 %, and *A. alternata* at 39.03 %. This result is attributed to the presence of a phthalate compound with large percentage in EtOAc extract which, according to Habib and Karim (2009), possess antifungal and anticancer activities. Similarly, VOCs produced by *T. spirale* indicated numerous compounds with antifungal action, especially ethanol and phenyl ethyl alcohol. VOCs of *T. asperellum* increased resistance to leaf spot pathogens on lettuce (Baiyee *et al*., 2019, Wonglom *et al*., 2020).

The antifungal activity of GTE-21 was assessed using the Evans blue method. The results showed that spores treated with Crude were affected by 96.2% at a concentration of 25 mg/ml. Additionally, the fractions CH2Cl2: MeOH (1:1) and CH2Cl2: MeOH (9:1) were affected by 96.3% and 94.3% respectively. In a study by Dissanayake et al. (2015), it was reported that compounds from *Acorus calamus* had an effect on the *F. oxysporum* pathogen, resulting in dead cells that were stained by Evan blue stain.

The results of our study on crude and fractions of GTE-21 showed highly cytotoxic effects on two cell lines (SKOV-3 and MDA-MB-231). These findings are consistent with those of Uzma et al. (2018), who reported that 2,14-dihydrox-7-drimen-12,11-olide isolated from *A. glaucus* from *Ipomoea batatas* leaves has anticancer properties.

The production of novel chemicals by a number of endophytic fungal strains that are helpful in anticancer assays has been discovered (Stierle and Stierle, 2015). Mclaughlin *et al.* (1998) informed that the drug's cytotoxic effect has the potential to influence and disrupt the fundamental processes involved in cell division, growth, and differentiation. One of these pathways may be responsible for this extract's cytotoxic action.

5. Conclusion

The endophytic thermophilic fungus *G. thermotolerans* GTE-21 has been found to harbor bioactive secondary metabolites, comprising both volatile (such as Diisooctyl phthalate, Phthalic acid, 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, 1-Nonadecene, oleic acid, butyl undecyl ester, and β-Caryophyllene) and non-VOCs (specifically diisooctyl phthalate and dibutyl phthalate). These secondary metabolites have shown potential in inhibiting the growth of key plant pathogenic fungi and human pathogenic bacteria, alongside exhibiting anticancer and antioxidant properties. The VOCs from GTE-21 notably inhibited *F. solani* by 42.5% and *A. alternata* by 39.03%. On the other hand, non-VOCs within GTE-21's crude extract showed remarkable inhibitory action against *A. alternata* at 72.2% and were exceptionally effective against *C. specifier* with an inhibition rate of 93.05%. Thus, the thermophilic endophytic fungus *G. thermotolerans* GTE-21 is a promising source of bioactive compounds, with ongoing research in our laboratory aimed at isolating and identifying the most potent bioactive pure compounds.

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