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Research Article

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Chemical and biological assessment of *Alternaria alternata* as an endophytic fungus isolated from *Thymus vulgaris*

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Abstract

Thymus vulgaris is a highly regarded herb known for its wide medicinal and culinary uses. Endophytic fungi, residing harmlessly within plant tissues, have attracted much attention as potential sources of novel bioactive compounds. This study aimed to evaluate the chemical and biological characteristics of *Alternaria alternata* as an endophytic fungus isolated from *Thymus vulgaris*. *Alternaria alternata* was isolated from *Thymus vulgaris* leaves and characterized using morphological and molecular identification techniques. Chemical characterization of dichloromethane fraction (DCM) of *Alternaria alternata* was conducted using Liquid Chromatography-Mass Spectrometry (LC-MS). The biological screenings revealed anticancer potential of the fungal secondary metabolites, while DCM extract showed strong antimicrobial activity against *Escherichia coli* and *Pseudomonas aeruginosa* compared to gentamycin. These findings imply that DCM extract has the potential as an alternative or complementary antibacterial agent; particularly in situations where antibiotic resistance is a concern. DCM extract exhibited moderate cytotoxicity with an IC_{50} of 70 µg/ ml compared to the more potent Doxorubicin with an IC_{50} of 10 µg/ ml. This suggests that *Alternaria alternata* extract may offer a less aggressive alternative for cancer therapy. Docking results revealed that key secondary metabolites such as dihydroaltersolanol showed strong binding activity across multiple bacterial targets; suggesting their broad-spectrum antibacterial potential, while

Bicycloalternarene F demonstrated specific high affinity for 3-hydroxy-3-methylglutaryl-coenzyme. Alternatone A, tenuazonic acid, and A reductase expressed strong multi-target antibacterial potential, while Bicycloalternarene F and Alternatone A displayed high binding affinity to several anticancer targets; particularly those proteins regulating apoptosis and cell survival. These findings highlighted the potential of these bioactive compounds as effective anticancer agents and warranting further experimental validation.

Keywords: Chemical and biological evaluation, *Alternaria alternata*, Endophytic fungus, Morphological and molecular identification techniques, Antibiotic resistance

1. Introduction

Thymus vulgaris (*T. vulgaris*), commonly known as thyme, is a widely recognized aromatic herb with various medicinal and culinary uses (Waheed *et al*., 2024). It is rich in several bioactive compounds such as essential oils, phenolic, flavonoids, and terpenes, and has been revered for centuries for its therapeutic properties. These compounds contribute to the plant's antimicrobial, antioxidant, and anti-inflammatory activities, making them valuable resources in traditional and modern medicine (Chaachouay and Zidane, 2024). Entophytic fungi, residing within the internal tissues of plants without causing any apparent harm, have gained significant attention as potential sources of novel bioactive compounds (Drożdżyński, *et al*., 2024). Among the numerous endophytic fungi, the *Alternaria* taxa has emerged as a prominent source of bioactive compounds with promising biological activities. *Alternaria* spp. produce various secondary metabolites, including alkaloids, terpenoids, quinones, and polyketides, demonstrating potent antimicrobial, antifungal, antitumor, and antioxidant potentials (Ragavendran *et al*., 2024). Furthermore, LC-MS analysis of *Alternaria* spp. metabolites provided a valuable chemical insight into their secondary metabolites profile and suggested them as rich sources of structurally diverse and potentially bioactive compounds, which merit further investigation for drug discovery and programs development (Fu *et al*., 2024). The significant findings obtained from this study should encourage more comprehensive studies to obtain wide scope of novel antimicrobial and anticancer agents.

Our study objective was to explore the untapped potential of *A. alternata* as an alternative source of bioactive compounds with diverse pharmacological activities. We employed a combination of isolation, identification, and fermentation techniques to isolate the endophytic fungal isolates from *T. vulgaris* and assess their chemical profiles (Nwobodo *et al*., 2024). Chemical characterization of the isolated *A. alternata* was conducted using various chromatographic and spectroscopic techniques, including LC-MS. Additionally, we performed extensive biological screenings to evaluate the anticancer activities of the isolated fungal secondary metabolites. The findings from this study could offer a wealth of valuable insights into the chemical diversity and biological potential of *A. alternata* endophytic fungus associated with *T. vulgaris*. Moreover, understanding the mutualistic relationship between *A. alternata* and *T. vulgaris* may illuminate the ecological significance of endophytic fungi in plant health and defense. This study contributes to the growing body of knowledge on the untapped potential of endophytic fungi and highlights their significances in the context of natural products discovery and drugs development. The insights gained from this study can pave the way for future investigations and applications in pharmaceutical sciences, agriculture, and biotechnology.

2. Material and methods

2.1. Plant material

Fresh and healthy leaves (200 g) of the *T. vulgaris* plant were collected in August 2022 from the Medicinal Plants Garden at Badr University, Cairo, Egypt, and transported immediately to the microbiology laboratory. The plant was authenticated by the Department of Pharmacognosy, Faculty of Pharmacy, Badr University, Cairo, Egypt.

2.2. Isolation of the endophytic fungi

Isolation of the endophytic fungi from *T. vulgaris* leaves followed a standard procedure previously established by Li *et al*., (2020) with minor modifications. The leaves were washed with sterilized dist. water, air dried, then successively surface sterilized by immersion in 95 % ethanol for 30 sec, 5 % sodium hypochlorite solution for 5 min., 95 % ethanol for 30 sec, and washed again with sterile dist. water for 3–5 sec. The leaves were cut into fragments, approximately 1 × 1 cm^2 each using a sterile scalpel. Four of these fragments were placed aseptically on the surface of potato dextrose agar medium (PDA) Petri plates supplemented with Gentamicin 340 µl (5 mg/ 10 ml) to suppress bacterial growth. The plates were sealed with parafilm, incubated at 27 ± 2 °C for 5 d, and observed daily for hyphal growth. After incubation, the developing hyphal tips were picked, and sub-cultured on PDA plates. Each fungal isolate was purified using a single spore technique, and the purified isolates were stored in PDA slants at 4 °C till further analysis.

2.3. Identification of the endophytic fungal isolates

Morphological characteristics of the isolated fungi (*i.e*., color, texture, and diameter of colonies) were identified at the Microbiology Department, Faculty of Pharmacy, Badr University, Cairo, Egypt, according to the procedure adopted by Wang *et al*., (2023). To confirm the identification, molecular characterization of the fungus was conducted according to Miller *et al*., (1999). Polymerase chain reaction (PCR) was used to amplify the Internal Transcribed Spacer (ITS) region of the genomic DNA of the fungal isolates using two universal ITS primers; *ITS5 5'* (TCCGTAGGTGAACCTGCG G) and *ITS4 5'* (TCC TCC GCT TAT TGA TAT GC) 3'. This technique involved the use of ITS genes and was conducted by the Macrogen Inc., Seoul, South Korea. The BLAST search tool in the National Center for Biotechnology Information (NCBI) was used for descriptive identification of the isolated fungal endophyte. The phylogenetic tree and bootstrap analysis were performed using MEGA10 software to infer the evolutionary history of the analyzed fungal taxa using the Neighbor-Joining method.

2.4. Preparation of the endophytic fungal extracts and their fractionation

Preparation of the endophytic fungal ethyl acetate (EtOAc) extracts was carried out according the procedure reported by Kumar and Kaushik, (2013). Mass production of the fungal secondary metabolites was performed in 10 flasks (1 l each) of rice grit solid medium. Rice medium was prepared as follows: 100 g of white rice was added to a 1 l conical flask containing 500 ml of dist. water. The flask was sterilized at 121°C for 20 min. at 15 psi. After cooling to room temperature, the rice medium was inoculated with the desired endophytic fungus growing on PDA that was aseptically cut into small sections (5–10 mm). The flasks were incubated at room temperature for 21 d in darkness. Afterward, 250 ml of ethyl acetate (EtOAc) were added to each 1 l culture flask and left overnight to extract the fungal secondary metabolites. The resulting extracts were concentrated under vacuum at 45 °C at a speed of 120 rpm and a vacuum pressure of 200 bar. The obtained crude extract was carefully removed and weighed, yielding approximately 50 g. The reported yield was based on experimental results and may vary slightly depending on the fungal strain. Liquid/ liquid fractionation of the crude extract was performed multiple times using a separating funnel, starting with n-hexane and 90 % aqueous methanol (MeOH), and then different gradients were used, involving the following

combinations: n-hexane: MeOH (90 %), chloroform ($CHCl_3$): MeOH (60 %), ethyl acetate (EtOAc): MeOH (60 %), dichloromethane (DCM), and aqueous methanol.

2.5. Isolation of the fungal secondary metabolites

2.5.1. Thin layer chromatography (TLC) of *Alternaria alternata* crude extract

The fungal extracts that showed the most promising results in biological activity assays were analyzed using thin layer chromatography (TLC) to identify the primary chemical classes in its active secondary metabolites (Azerang *et al*., 2019). TLC analysis of the fractions was carried out on silica sheets (Silica gel 60 F254, Merck, Germany). Samples of 25 µl each were loaded individually onto the silica plates and chromatographed in chloroform: Methanol (5:1) system. The plates were scanned under ultraviolet light at a wavelength of 254 nm. Fractions with the same retention factor (R_f) values were pooled, and then biological activity was checked to detect the most active fraction for further analysis.

2.5.2. Liquid Chromatography-Mass Spectrometry (LC\MS)

Chemical profiling of the fungal constituents was performed using liquid chromatography-mass spectrometry (LC\MS), where a Triple quadruple instrument (C-18 column 1.7 µm particle size - 2.1 × 50 mm) (XEVO TQD, Waters Corporation, Milford, USA) was used at the Center for Drug Discovery, Research and Development (CDDRD), Faculty of Pharmacy, Ain Shams University, in reference to Al-Saleem *et al*., (2022). Both positive and negative ion acquisition modes were carried out on a XEVO TQD triple quadrupole instrument (Waters Corporation, Milford, MA, USA).

The chromatographic separation was performed on an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm; Waters Corporation). The column was operated at a flow rate of 0.2 ml/ min. The mobile phase consisted of Solvent A: Water containing 0.1 % formic acid and Solvent B: Acetonitrile containing 0.1 % formic acid. A drying gas flow rate of 5 l/ min., drying gas temperature of 300 °C, ion acceleration voltage on the skimmer 35 V, fragmented 175 V, range MS 150-1000 m/z, target MS-MS 50-1000 m/z, collision energy - 30, 40, 50, 65, were used. Samples were injected onto a Zorbax SB C18 (3 µm, 150 × 0.5 mm) column (Agilent Technologies 1200) with a mobile phase composed of: A) 0.1 % formic acid and B) acetonitrile + 0.1 % formic acid. Elution on the Agilent Technologies 1260 Cap pump was performed at the rate of 15 µl/ min.: 5 min. on 60 %, 15-20 min. on 90 %, and 25 min. on 60 % of the mobile phase B.

2.6. Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration (MIC) assay was performed (Soheili *et al*., 2023) to assess the antibacterial activities of fungal DCM fractions against *Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 13316, *Klebsiella pneumoniae* ATCC 13883, *Streptococcus pneumoniae* ATCC 33400, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538, and *Candida albicans* ATCC 10231, which were obtained from VACSERA Co-operation, Giza, Egypt. The DCM fractions containing *A. alternata* metabolites were dissolved in Luria-Bertani (LB) broth medium and adjusted to concentrations ranging from 0.0625–8 mg/ ml using double dilution. In a 96-well plate, 100 µl of bacterial suspension in LB broth were mixed with varying volumes of the DCM fraction solutions to achieve the desired final concentrations (e.g., 40 µl of DCM solution for 8 mg/ ml, 20 µl for 4 mg/ ml, etc., with the remaining volume made up to 100 µl using bacterial suspension per well). Control wells received 100 µl of un-inoculated LB broth. After incubation at 37 °C for 24 h, 20 µl of 0.2 % 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) solution were added to each well and incubated for another 4 h. After incubation, the mixture color was observed and the fraction concentration at which the red precipitate was not observed in the well was considered as the MIC.

2.7. Evaluation of the fungal metabolites cytotoxic activity

The cytotoxicity of DCM fraction compounds was tested against the human breast cancer cell line (MDA-MB-231) with Doxorubicin (DOX) as a reference medication according to the previous procedure conducted by Ghanem *et al*., (2020). These compounds were applied at various concentrations ranging from 0.0625 to 8 µg/ ml typically following a double-dilution series to assess their cytotoxic effects and determine their IC_{50} values. 100 µl of these bioactive compounds were applied individually to each well of a 96-well plate with 4×10^4 cells/ ml in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum (FBS), and incubated for 24 h at 37 °C with 7 % CO_2. To achieve the best results, cells in the logarithmic growth phase were employed. Wells with 100 µl of cell culture media without the bioactive compounds served as the negative control. The blank control group consisted of wells filled with growth medium with no cancer cells. After 24 h of incubation, the plates were washed once with 200 µl of phosphate-buffered saline (PBS) for each well, followed by the addition of 100 µl per well of MTT solution (1 mg/ ml in DMEM with 10 % FBS). The plates were then re-incubated for 4 h. After removing the MTT solution, 150 µl of dimethyl sulphoxide (DMSO) were added to dissolve the formazan crystals generated during the incubation. After shaking for 10 min. on a shaker, the absorbance was measured at 570 nm. Each assay of a bioactive compound was conducted in three replicates. The cell proliferation ratio was computed using the following formula reported by Ghanem *et al*., (2020):

$$\text{Cell proliferation ratio} = \left(\frac{\text{mean OD of the experimental group} - \text{mean OD of blank}}{\text{mean OD of the control group} - \text{mean OD of blank}}\right) \times 100\ \%$$

2.8. Docking activity

2.8.1. Ligand preparation

The Schrödinger software suite's Maestro module was used according to Pandya *et al*., (2024) to prepare and draw the structures of the obtained six different bioactive compounds; mainly Alternatone A, 2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone, 2H-(2E)-tricycloalternarene 12a, Bicycloalternarene F, Dihydroaltersolanol, and Tenuazonic acid. LigPrep was used to optimize the geometry of the compounds, generating 3D conformations and ensuring the correct protonation states at a physiological pH of 7.0 ± 2.0.

2.8.2. Protein preparation

According to Thapa *et al*., (2023), the Protein Data Bank (PDB) was utilized to obtain the crystal structures of the bacterial target proteins, which included membrane-bound lytic murein transglycosylase, Rep C, DNA gyrase, dihydropteroate synthase, and 3-hydroxy-3-methylglutaryl-Coenzyme A Reductase. These proteins were prepared using Schrödinger's Protein Preparation Wizard, which included disulfide bonds formation, allocating bond orders, incorporating hydrogen atoms, and optimizing hydrogen-bond orientations. Water molecules were also removed from the active sites.

2.8.3. Receptor grid preparation

Receptor grids were generated around the active site of each target protein using the Glide module. The binding site was defined based on the co-crystallized ligands in the PDB structures or the known active site residues. This step ensured accurate docking by focusing the search space on the relevant region of the protein (Hassan *et al*., 2024).

2.9. Statistical analysis

The data were collected from three independent assays and analyzed using One-way analysis of variance (ANOVA) by Graph Pad Prism. Data are expressed as mean ± standard deviation (SD) of experiments performed in triplicates. Error bars on the different figures represent the SD.

3. Results

3.1. Phylogenetic analysis and molecular identification of the fungal endophyte

A single fungal colony morphotype was obtained and identified phenotypically as *A. alternata*. Characterization of the fungal endophyte was confirmed through molecular analysis using DNA barcoding techniques, specifically the Internal Transcribed Spacer (ITS) region of ribosomal DNA. BLAST analysis of the ITS sequence against the GenBank database revealed high sequence similarity with multiple *Alternaria* strains, clustering the strain closely with *A. alternata* strain Z3Y with 99.8 % similarity (Fig. 1).

Other closely related strains included *A. tenuissima* strain KUMCC-21-0684 (ON024740) showing 99.7 % sequence similarity. The highest sequence similarity (100 %) was observed with *A. alternata* strain NL-333-B (OQ561208), *A. alternata* strain-20 (OQ421569), and *A. tenuissima* strain GYTC61 (OQ612481). The ITS sequence of strain *A. alternata* Z3Y was deposited in GenBank under accession number OR717595. These results confirmed classification of the single endophytic fungal isolate within the *Alternaria* taxa. The close clustering between *A. alternata* strain Z3Y and *A. tenuissima* strains suggested strong genetic relationships, indicating that the fungal strain Z3Y may share similar phenotypic and pathogenic traits.

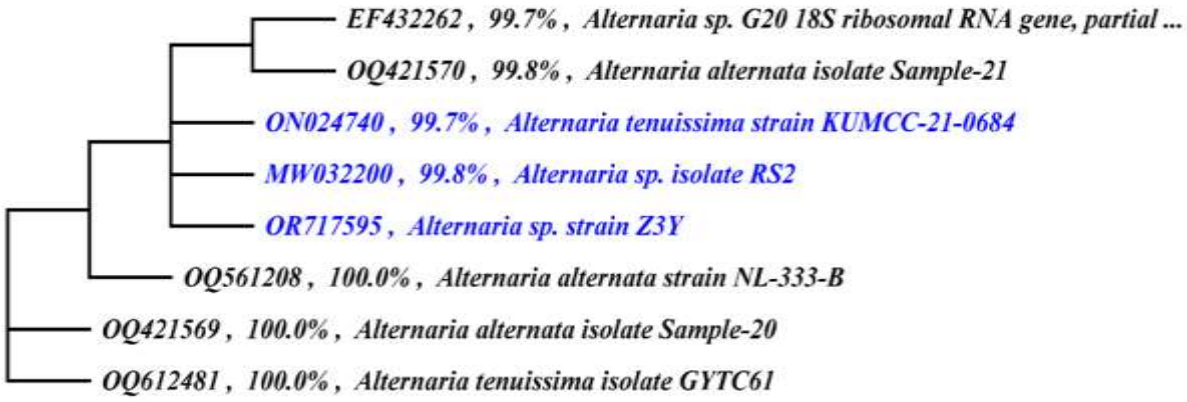
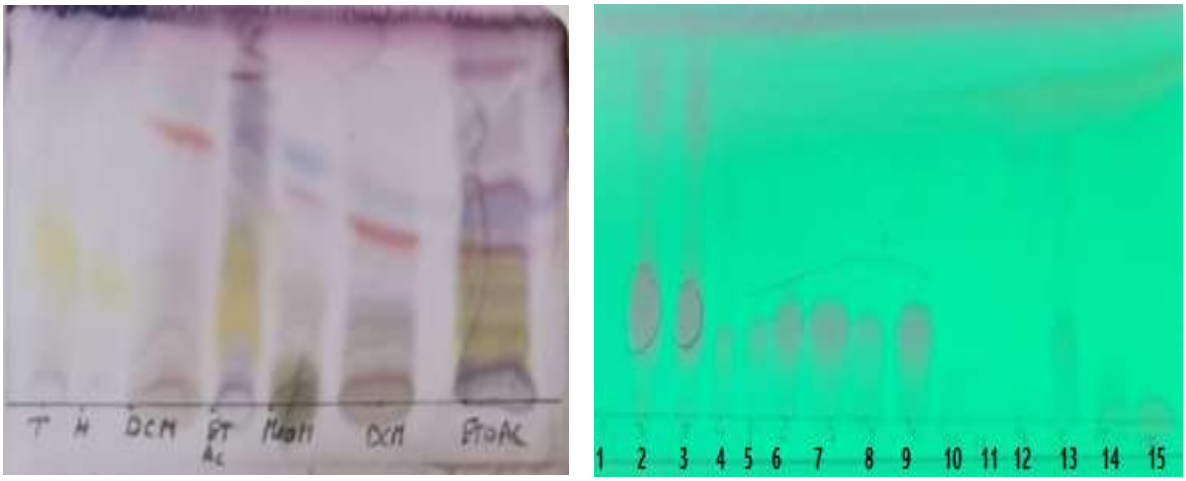


Fig. 1. Neighbor-joining (NJ) phylogenetic tree based on ITS-rDNA sequences of an endophytic fungus associated with the *Alternaria* spp. Digits above or below the branches indicate bootstrap values of NJ and MP analyses (> 50 %, right) from 1000 bootstrap replicates

3.2. Thin layer chromatography of *Alternaria alternata* strain Z3Y fractions

The TLC analysis revealed a diverse array of compounds within the *A. alternata* strain Z3Y extracts. The different bands observed under both normal and UV light showed the presence of several secondary metabolites. The complexity of the separation pattern suggested that *A. alternata* strain Z3Y produced a variety of bioactive compounds, which could be responsible for its observed biological activities (Fig. 2). This suggests that dichloromethane (DCM) solvent is effective in extracting a broad range of compounds from *A. alternata* strain Z3Y, including those with a significant bioactivity (Fig. 2a). Under UV light, there are two clear bands at lanes 2 and 3 in the DCM fraction, which suggests that they were pure compounds that typically appeared as sharp and well-defined spots on a TLC plate (Fig. 2b). Further isolation, characterization, and bioactivity studies are essential to unlock the full potential of these compounds for various applications. The promising results from DCM fractions highlighted its effectiveness and potential for future research and development in discovering novel bioactive compounds.



a **b**

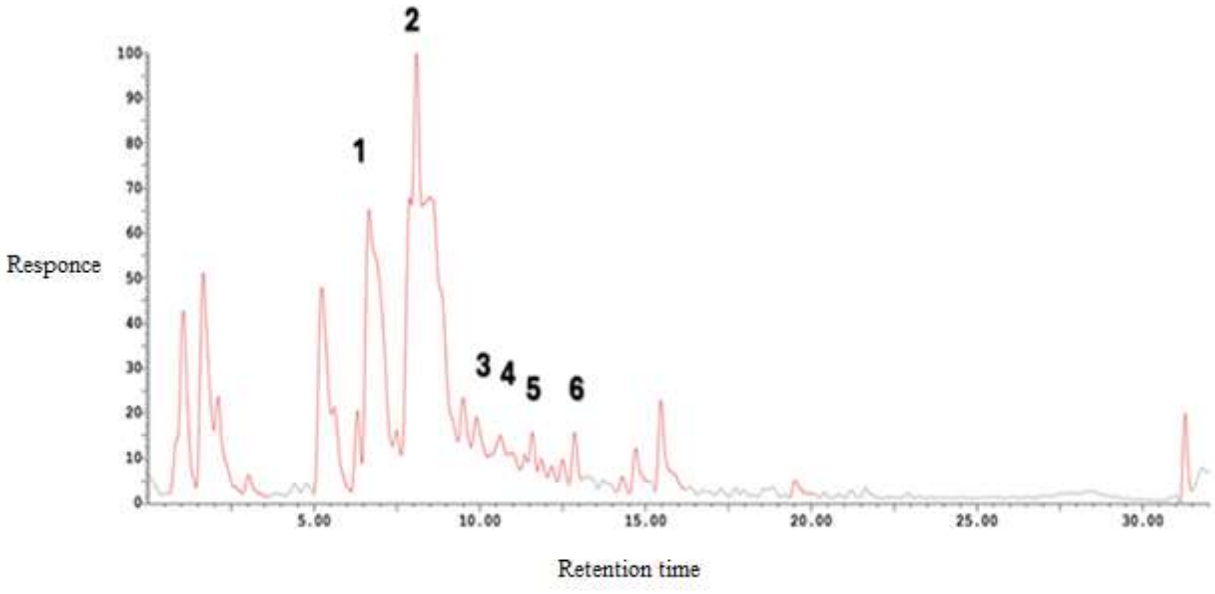
Fig. 2. Thin layer chromatography of *A. alternata* strain Z3Y fractions revealing a diverse array of compounds. The different fractions are visualized under visible light, where; (a) T indicate (Toluene), H (Hexane), DCM (Dichloromethane), EtOAc (Ethyl Acetate), MeOH (Methanol), and (b) the DCM fractions under UV light at 254 nm. The digits (1-15) below each lane indicate the different DCM fractions separated by chromatographic column made up of silica gel (kieselgel 60, mesh 70-230)

3.3. LC-MS analysis of *Alternaria alternata* strain Z3Y DCM fractions

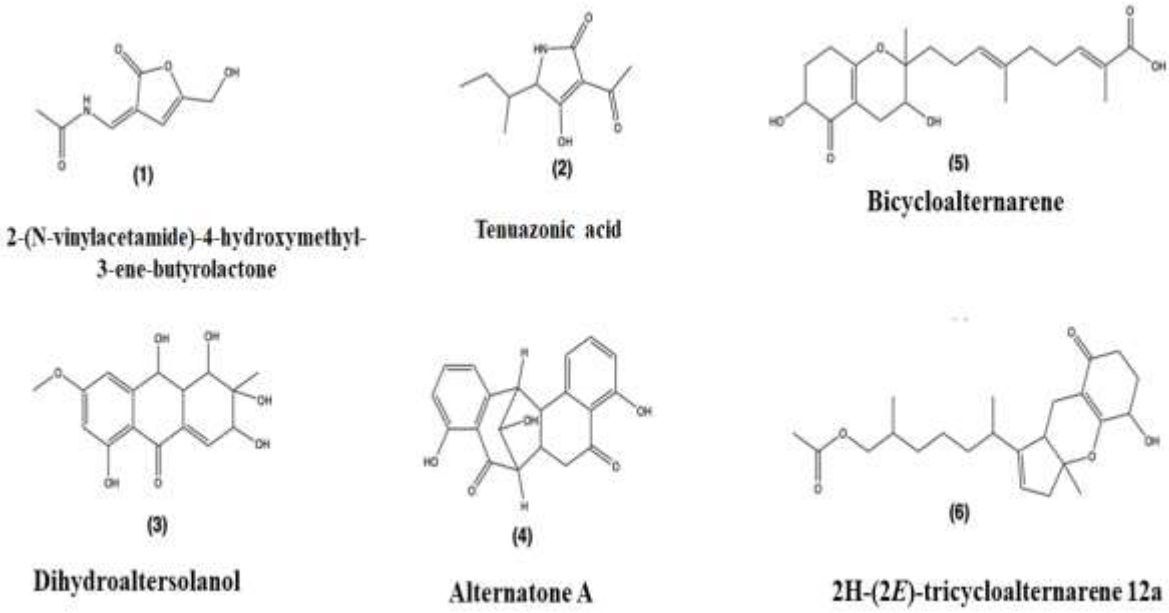
Liquid Chromatography-Mass Spectrometry (LC-MS) analysis was performed to identify the secondary metabolites constituents of *A. alternata* strain Z3Y DCM fractions. Six known compounds were obtained and identified as 2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone (1), Tenuazonic acid (2), dihydroaltersolanol A (3), alternatone A (4), Bicycloalternarene (5), and 2H-(2E)-tricycloalternarene 12a (6) (Table 1 and Fig. 3).

Table 1. LC-MS analysis of *Alternaria alternata* strain Z3Y DCM fractions

SN	Identified Compound	Rt	M+H	M.W.	M.F.	Reference
1	2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone	6.66	184.1681	183.05	$C_8H_9NO_4$	(Ding *et al*., 2017)
2	Tenuazonic acid	7.89	198.1246	197.23	$C_{10}H_{15}NO_3$	(You *et al*., 2023)
3	Dihydroaltersolanol A	10.61	323.2049	322.10	$C_{16}H_{18}O_7$	(Zheng *et al*., 2012)
4	Alternatone A	10.98	337.2461	336.10	$C_{20}H_{16}O_5$	(Zhao *et al*., 2019)
5	Bicycloalternarene	11.59	383.2301	382.24	$C_{21}H_{34}O_6$	(Zhang *et al*., 2013)
6	2H-(2*E*)-tricycloalternarene 12a	12.85	391.2815	390.24	$C_{23}H_{34}O_5$	(Shen *et al*., 2018)



(a)



(b)

Fig. 3. (a) LC-MS analysis of *Alternaria alternata* strain Z3Y DCM fractions, and (b) shows various compounds identified in the DCM fractions of *Alternaria alte*rnata strain Z3Y *via* LC-MS. The identified compounds included: 2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone, Tenuazonic acid, Dihydroaltersolanol A, Alternatone A, Bicycloalternarene, and 2H-(2E)-tricycloalternarene 12a

3.4. Antimicrobial activity

This study evaluated the MIC of two antimicrobial agents, the DCM extract of *A. alternata* strain Z3Y and gentamycin against six different microbial strains (Fig. 4). The average MIC values for the DCM extract and gentamycin were 10.5 µg/ ml and 14.0 µg/ ml; respectively, for *Bacillus subtilis* ATCC 6051, while for *Escherichia coli* ATCC 8739, the MIC values were 10.3 µg/ ml (DCM extract) and 16.0 µg/ ml (gentamycin). For *Klebsiella pneumoniae* NCTC 13465, the DCM extract showed an average MIC of 18.8 µg/ ml, compared to 24.1 µg/ ml for gentamycin. Against *Staphylococcus aureus* ATCC 25923, the MIC values were 13.5 µg/ ml for the DCM extract and 18.2 µg/ ml for gentamycin. In the case of *Pseudomonas aeruginosa* ATCC 27853, the DCM extract had a MIC of 16.4 µg/ ml, while gentamycin exhibited a slightly lower MIC of 15.3 µg/ ml. For *Candida albicans* ATCC 10231, the DCM extract and gentamycin had average MIC values of 15.4 µg/ ml and 16.3 µg/ ml, respectively. These results, supported by standard deviations from three replicates, highlighted the potential of the DCM extract of *A. alternata* strain Z3Y as an alternative or supplemental antimicrobial agent; particularly in cases of antibiotic resistance. Further optimization and studies on synergistic effects with other drugs are needed to enhance the DCM extract efficacy.

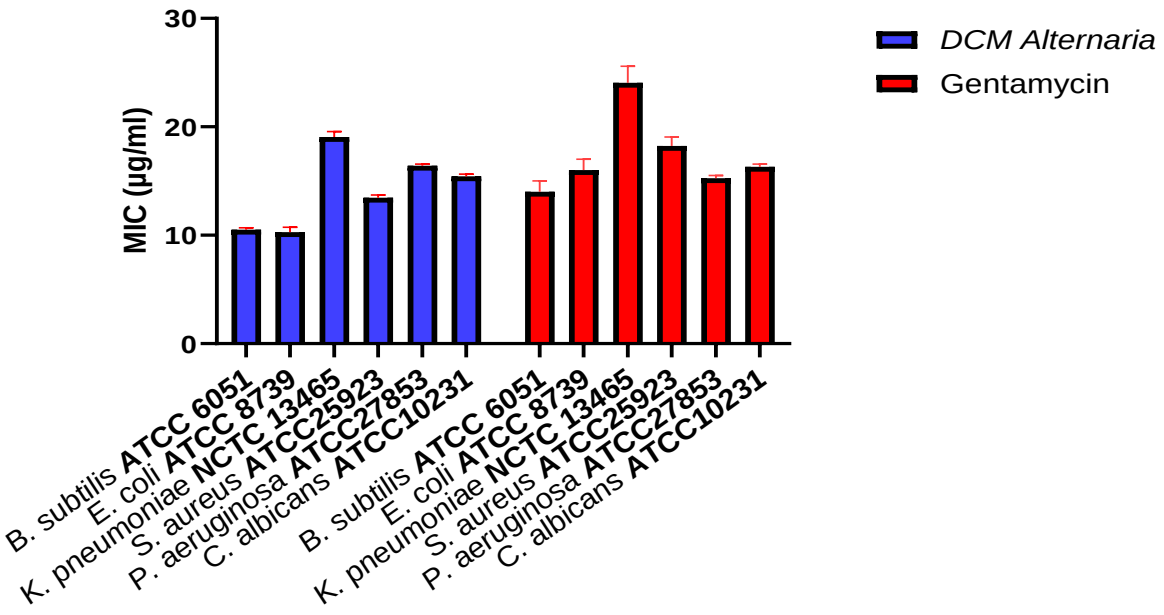
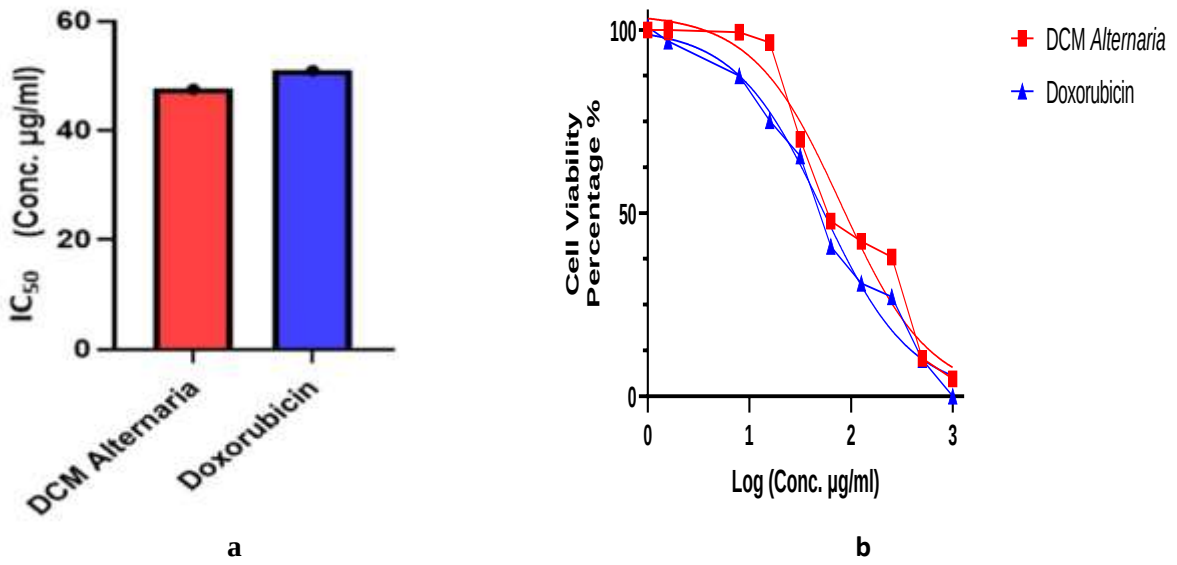


Fig. 4. The different bacterial and fungal strains MIC values for DCM extract of *Alternaria alternata* strain Z3Y and gentamycin antibiotic. The error bars represent the standard deviation. Results are averages of three replicates

3.5. Cytotoxic potency

In this study, the cytotoxic activity of *A. alternata* strain Z3Y DCM and Doxorubicin on breast cancer cell line (MDA-MB-231) was evaluated using the half-maximal inhibitory concentration (IC_{50}) metric. The IC_{50} value, which represents the concentration at which a substance achieves 50 % of its maximum inhibitory effect, was found to be 47.60 µM for DCM of *A. alternata* strain Z3Y and 50.96 µM for Doxorubicin, indicating that DCM *A. alternata* strain Z3Y was slightly more potent than Doxorubicin in this context (Fig. 5a). Moreover, the corresponding percentage of cell viability (%) and standard deviations were recorded for DCM of *A. alternata* strain Z3Y and Doxorubicin. At the lowest concentration (log 0), DCM of *A. alternata* strain Z3Y maintained high cell viability of 100 % with a standard deviation (SD) of 2.05. As the concentration increased to log 0.2, the cell viability remained high. A significant drop in viability was observed at log 1.5, where cell viability decreased to 70.12 %. The most notable decline occurred between concentrations of log 1.8 and log 3, where cell viability sharply decreased from 47.86 % to 4.79 %. Doxorubicin displayed a similar trend as DCM of *A. alternata* strain Z3Y at lower concentrations; however, it maintained high cell viability at log 0 and log 0.2 (100.73 % and 96.91 %, respectively). Meanwhile, the decline in viability began earlier, becoming evident at log 0.9 where cell viability reduced to 87.45 %. At log 1.5, viability decreased to 65.58 % and continued to decline sharply at higher concentrations. By log 3, Doxorubicin showed a complete loss of viability, reaching 0 % (Fig. 5b).

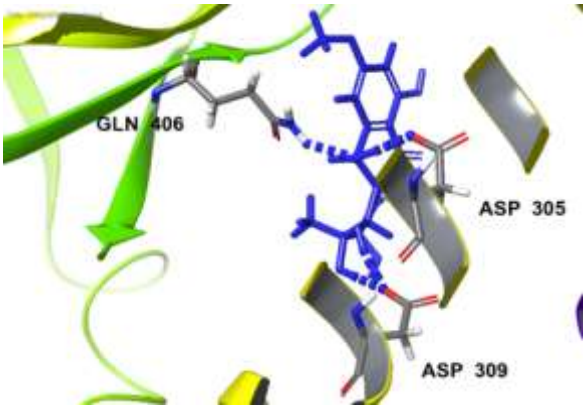


a **b**

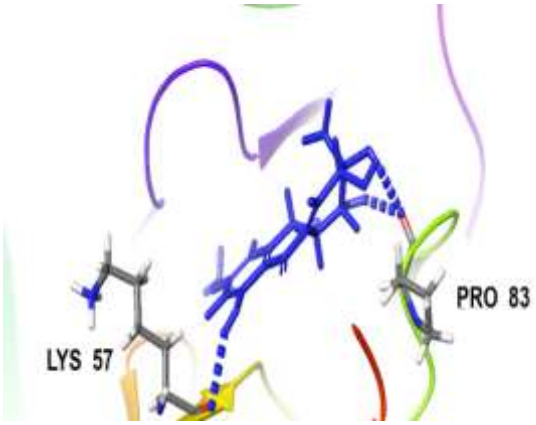
Fig. 5. The bar graph on the left (a) presents the IC_{50} values for the DCM extract of *Alternaria alternata* strain Z3Y and Doxorubicin. (b) presents dose-dependent effects of the DCM extract of *Alternaria alternata* strain Z3Y and Doxorubicin on cell viability percentage (%) across a range of concentrations (log scale). The smooth lines in the graph represent the regression line, which is a tool used in scatter plots to indicate the best-fit relationship between an independent and dependent variables

3.6. *In silico* docking studies

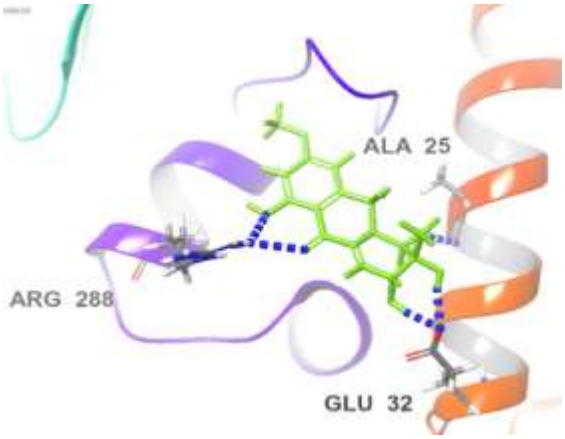
The current docking study provided insights into the binding affinities of six distinct compounds with various bacterial target proteins. Binding strength was assessed using docking scores measured in kcal/ mol, where more negative values indicated stronger affinities. Fig. (6) represents the distribution of docking scores for each compound across all target proteins; offering a visual representation of their binding affinities. The accompanying Table (2) details the docking scores of the various compounds with multiple targets and more negative scores, signifying stronger binding affinities. Alternatone A exhibited notable binding to Penicillin-binding protein (-4.287), Rep C (-5.339), DNA gyrase (-4.891), and membrane-bound lytic murein transglycosylase (-5.191). 2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone showed good binding affinities; particularly with Penicillin-binding protein (-4.587) and dihydropteroate synthase (-4.116). 2H-(2E)-tricycloalternarene 12a had its best binding with Penicillin-binding protein (-4.204) and membrane-bound lytic murein transglycosylase (-4.507). Bicycloalternarene F expressed exceptionally strong binding affinity with 3-hydroxy-3-methylglutaryl-coenzyme A reductase (-7.085), though positive values such as Rep C (0.118), indicating minimal or no binding affinity with certain targets. Dihydroaltersolanol showed the highest average binding scores, with significant targets, including Penicillin-binding protein (-5.186), Rep C (-5.879), 1-ddlB-D-alanine--D-alanine ligase (-5.303), and DNA Gyrase (-5.506). Tenuazonic acid also bounded effectively with multiple targets; notably membrane-bound lytic murein transglycosylase (-6.046) and Rep C (-5.754) (Table 2). In this study, the binding affinity of two natural compounds, Bicycloalternarene F and Dihydroaltersolanol, and the mycotoxin Tenuazonic acid to various enzymes with potential inhibitory effects was investigated.


GLN 406
ASP 305
ASP 309

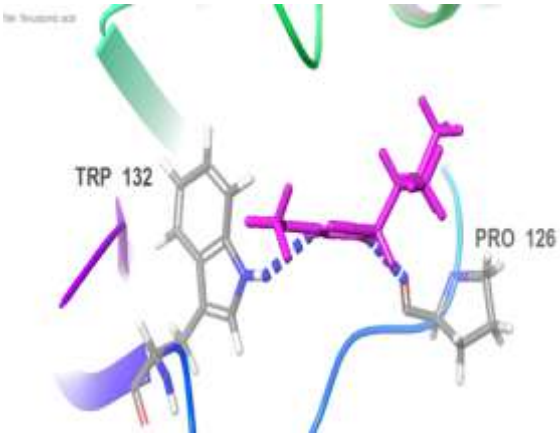
(a)


PRO 83
LYS 57

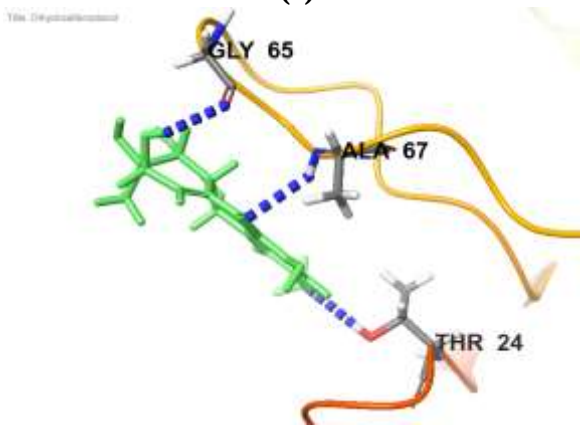
(b)


ALA 25
ARG 288
GLU 32

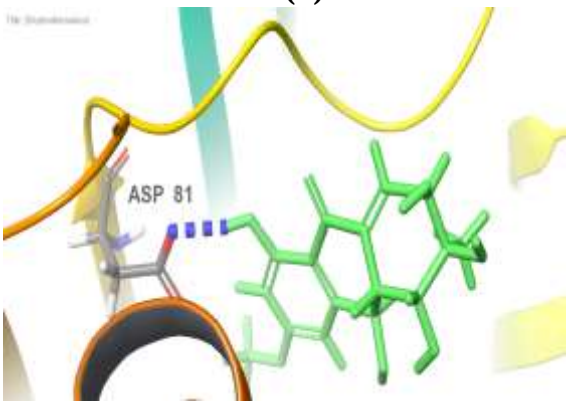
(c)


TRP 132
PRO 126

(d)


GLY 65
ALA 67
THR 24

(e)


ASP 81

(f)

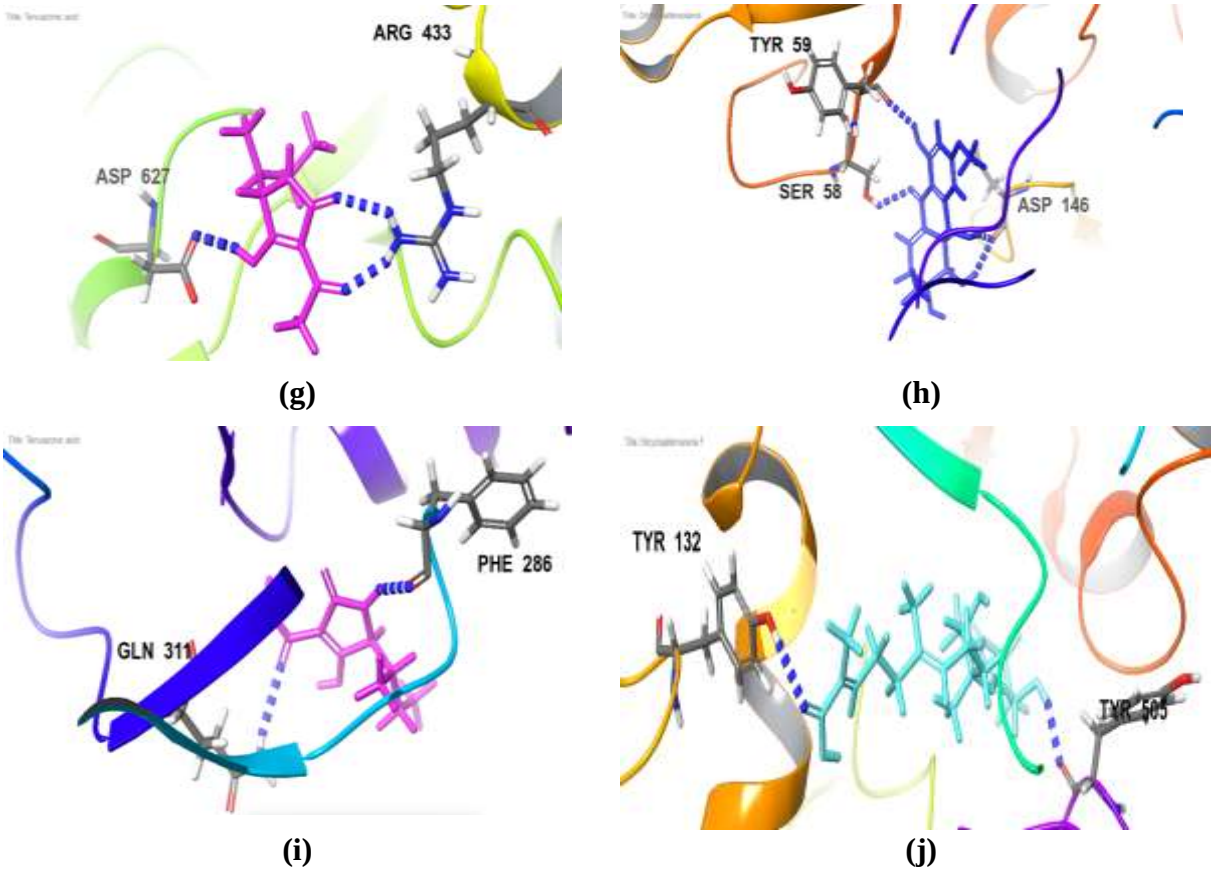


Fig. 6. Antimicrobial docking activity of DCM extract of *Altrernaria alternata* strain Z3Y on (a) Dihydroaltersolanol with Penicillin-binding protein, (b) Dihydroaltersolanol with Rep C, (c) Dihydroaltersolanol with 1- ddlB-D-alanine--D-alanine ligase, (d) Tenuazonic acid with Dihydrofolate Reductase, (e) Dihydroaltersolanol with Dihydropteroate synthase, (f) Dihydroaltersolanol with DNA Gyrase, (g) Tenuazonic acid with Isoleucyl-tRNA synthetase, (h) Dihydroaltersolanol with IV topoisomerase, (i) Tenuazonic acid with Membrane-bound lytic murein transglycosylase, and (j) 3hydroxy3methylglutaryl coenzyme A reductase with Bicycloalternarene F

Table 2. Docking scores of various compounds with selected targets

Compound	Penicillin-binding protein	Rep C	1- ddlB-D-alanine--D-alanine ligase	Dihydrofolate Reductase	Dihydropteroate synthase	DNA Gyrase	Isoleucyl-tRNA synthetase	IV topoisomerase	Membrane-bound lytic murein transglycosylase	3hydroxy3methylglutaryl_coenzyme_A_reductase
Alternatone A	-4.287	-5.339	-4.12	-3.634	-3.636	-4.891	-4.273	-3.61	-4.229	-5.191
2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone	-4.587	-4.676	-3.883	-3.472	-4.116	-4.757	-3.368	-3.745	-5.075	-4.549
2H-(2E)-tricycloalternarene 12a	-4.204	-4.829	-3.325	-3.064	-3.901	-3.914	-2.563	-3.031	-4.507	-4.518
Bicycloalternarene F	-4.34	0.118	-4.068	-4.189	0.567	0.453	0.453	0.384	0.432	-7.085
Dihydroaltersolanol	-5.186	-5.879	-5.303	-4.261	-4.911	-5.506	-4.325	-4.302	-4.463	-4.79
Tenuazonic acid	-3.963	-5.754	-4.659	-4.381	-4.679	-5.449	-4.455	-4.164	-6.046	-5.775

It was observed that Bicycloalternarene F specifically bonded to residues Tyr 132 and Tyr 505 of 3-hydroxy-3-methylglutaryl-Coenzyme A Reductase; a crucial enzyme in the mevalonate pathway for cholesterol biosynthesis. Dihydroaltersolanol demonstrated binding affinity at residues GLN 406, ASP 305, and ASP 309 of penicillin-binding proteins, indicating a potential inhibition of bacterial cell wall synthesis. Additionally, Dihydroaltersolanol was recorded to potentially inhibit Repc at residues Pro 83 and LYS 57, dihydrofolate reductase at residues ALA 25, ARG 288, and GLU 32, dihydropteroate synthase at residues GLY 65, ALA 67, and THR 24, isoleucyl-tRNA synthetase at residue ASP 81, and IV topoisomerase at residues Thr 59, Ser 58, and ASP 146. Meanwhile, Tenuazonic acid exhibited inhibitory potential for dihydrofolate reductase at residues PRO 126 and TRP 132, IV topoisomerase at residues ARG 433 and ASP 627, and 3-hydroxy-3-methylglutaryl-Coenzyme A Reductase at residues GLN 311 and PHE 286 (Fig. 6). Table (3) presents the in silico docking scores of various compounds with three key anticancer targets; mainly Caspase, Apoptosis regulator Bcl-2, and Protein Kinase B (AKT). The docking scores indicated the binding affinity between the compounds and the targets; with more negative scores suggesting stronger binding. For Caspase, the compound Bicycloalternarene F exhibited the strongest binding affinity with a docking score of -6.965, followed closely by Alternatone A (-6.933), and doxorubicin (-6.323). These negative values suggested that these compounds could potentially be very effective in interacting with Caspase; a key mediator of apoptosis. Regarding the Apoptosis regulator Bcl-2, Alternatone A demonstrated the highest binding affinity with a docking score of -4.740, indicating its potential to inhibit Bcl-2 effectively, which could lead to the induction of apoptosis in cancer cells. Other compounds such as Dihydroaltersolanol (-4.398) and Tenuazonic acid (-4.266) also displayed relatively strong binding affinities. For Protein Kinase B (AKT), Bicycloalternarene F again showed the highest binding affinity with a docking score of -5.970, suggesting its potential efficacy in inhibiting AKT; an important protein involved in the survival pathways of the cancer cells. Additionally, Tenuazonic acid (-5.266) and doxorubicin (-5.271) displayed comparable binding affinities. The docking studies revealed significant interactions between the compounds and the target enzymes, highlighting potential mechanisms for anticancer activity. Alternatone A demonstrated strong binding affinity with Caspase by forming hydrogen bonds with amino acid residues GLU 21 and ASP 60, suggesting that it may inhibit Caspase activity by stabilizing its inactive form and preventing substrate access. Bicycloalternarene F showed promising interactions with Apoptosis regulator Bcl-2, specifically binding to residues ARG 878, SER 904, and ASP 770 through hydrogen bonding and hydrophobic interactions, which could disrupt Bcl-2's anti-apoptotic function, thereby facilitating apoptosis in the cancer cells. Furthermore, Bicycloalternarene F exhibited strong binding affinity to the active site of Protein Kinase B (AKT), engaging residues VAL 228, GLU 226, and GLU 232, suggesting that it may effectively inhibit AKT activity. Inhibition of AKT may have triggered the apoptotic pathways, providing a potential therapeutic approach for cancer cells with hyperactive AKT signaling, making Bicycloalternarene F particularly promising for anticancer therapy (Fig. 7).

4. Discussion

Endophytic fungi, such as *A. alternata* offer a promising source of secondary metabolites, which frequently demonstrate a wide range of pharmacological activities, including antibacterial and anticancer (Wen *et al*., 2022). Thin Layer Chromatography (TLC) and LC-MS analyses of *A. alternata* strain Z3Y extracts revealed a wide diversity of secondary metabolites with potential bioactivity in agreement with Sonowal *et al*., (2024).

Table 3. *In silico* anticancer activity and docking scores of identified compounds with selected targets

Compounds	Caspase	Apoptosis regulator Bcl-2	Protein Kinase B (AKT)
2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone	-3.910	-3.237	-4.840
2H-(2E)-tricycloalternarene 12a	-5.810	-3.493	-3.590
Alternatone A	-6.933	-4.740	-5.070
Bicycloalternarene F	-3.965	-4.165	-5.970
Dihydroaltersolanol	-5.761	-4.398	-5.213
Doxorubicin	-6.323	-3.767	-5.271
Tenuazonic acid	-5.775	-4.266	-5.266

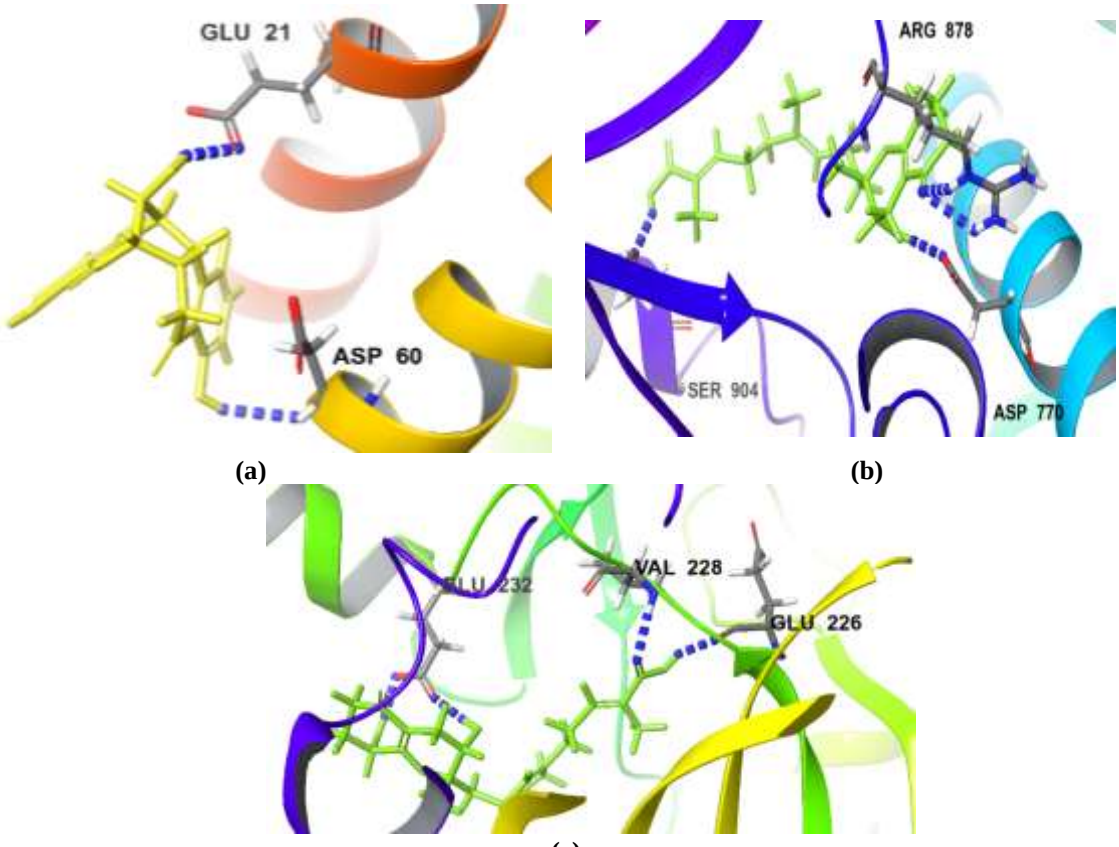


Fig. 7. *In silico* docking activity of (a) Alternatone A on Caspase, (b) Bicycloalternarene F on Apoptosis regulator Bcl-2, and (c) Bicycloalternarene F on Protein Kinase B (AKT)

In addition, the TLC analysis showed that the DCM fractions had more distinct bands under both normal and UV light compared to Hexane, EtOAc, and MeOH extracts, indicating that DCM's had a superior capacity for extracting the bioactive compounds. Six key secondary metabolites were identified by LC-MS of the DCM extract, including 2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone, Tenuazonic acid, Dihydroaltersolanol A, Alternatone A, Bicycloalternarene, and 2H-(2E)-tricycloalternarene. These compounds are known of their diverse biological activities, as supported by previous studies reported by Chen and Huang, (2023); Spina *et al*., (2023); Tian *et al*., (2023); Wang *et al*., (2023); Gao *et al*., (2024). Identification of these metabolites highlighted *A. alternata* strain Z3Y as a promising source of novel bioactive compounds for pharmaceutical and industrial applications (Khazaal *et al*., 2023). Further isolation, structural characterization, and functional studies are crucial to fully explore their potential and mechanisms of action. These findings underscored the importance of DCM as an extraction solvent in secondary metabolite discovery.

The present results indicated that the DCM fractions of *A. alternata* strain Z3Y possessed notable antimicrobial properties against both Gram-positive and Gram-negative bacteria. Evaluation of MIC values for the DCM extract of *A. alternata* strain Z3Y and gentamycin against six microbial strains revealed promising antimicrobial activity of the extract. For *Bacillus subtilis* ATCC 6051, the DCM extract outperformed gentamycin, and similarly, for *Escherichia coli* ATCC 8739, the DCM extract was more effective than gentamycin. Against *Klebsiella pneumoniae* NCTC 13465, the DCM extract also showed better efficacy compared to gentamycin. For *Staphylococcus aureus* ATCC 25923, the DCM extract was superior to gentamycin. However, for *Pseudomonas aeruginosa* ATCC 27853, gentamycin performed slightly better performance than the DCM extract. Both *A. alternata* strain Z3Y DCM and gentamycin showed similar efficacy against *Candida albicans* ATCC 10231, with the DCM extract nearly matching gentamycin. These results align with previous studies such as those reported by Khazaal *et al*., (2023); Kusmiati *et al*., (2024), where both studies demonstrated potent antimicrobial properties of extracts from other endophytic fungi like *Nigrospora oryzae* isolated from *Terminalia bellerica*, and with EtOAc extracts of *A. alternata* that showed antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, respectively. The presence of several bioactive compounds, as identified in the LC-MS analysis, supported the antimicrobial potential observed in the DCM fractions. Compounds such as tenuazonic acid and alternatone A are known for their antimicrobial activities, which likely contributed to the observed effects (Wang *et al*., 2022; Sotelo-Cerón *et al*., 2023). The *in silco* studies confirmed the activity of tenuazonic acid and alternatone A. Furthermore, Alternatone A and tenuazonic acid expressed robust multi-target affinities, while dihydroaltersolanol had strong binding affinity across multiple bacterial targets (Kusmiati *et al*., 2024). On the other hand, Bicycloalternarene F exhibited specific high affinity for 3-hydroxy-3-methylglutaryl-coenzyme A reductase, highlighting its targeted action and suggesting its promising potential for both antibacterial and anticancer applications. These findings suggested that further investigations on these compounds are needed for therapeutic development due to their potential as broad-spectrum antibacterial agents. Moreover, further studies, including purification and characterization of individual compounds and in vivo studies are essential to fully understand and harness the antimicrobial properties of these natural products (den Hollander *et al*., 2022; Sotelo-Cerón *et al*., 2023). This study underscored the potential for developing novel antimicrobial agents from fungal metabolites, which could be crucial in addressing the growing issue of antibiotic resistance. However, gentamycin has overall lower MIC values, suggesting that it may be more potent than DCM of *A.*

alternata strain Z3Y for most tested strains. DCM of *A. alternata* strain Z3Y displayed superior performance against *P. aeruginosa* indicating that it could be valuable for treating infections resistant to conventional antibiotics such as gentamycin; however, the different efficacy profiles of *A. alternata* strain Z3Y DCM and gentamycin suggested the potential for their synergistic combinations, which will enhance the overall antimicrobial activity and potentially reduce the risk of resistance development (Martelli and Giacomini, 2018; Freitas *et al*., 2022).

The anticancer activity of *A. alternata* strain Z3Y DCM extract was evaluated and compared to Doxorubicin, as a standard chemotherapeutic agent. The findings of this study provided valuable insights into the inhibitory effects of *A. alternata* strain Z3Y DCM and Doxorubicin on a specific cytotoxic function, as assessed by their half-maximal inhibitory concentration (IC_{50}). The IC_{50} values indicated that *A. alternata* strain Z3Y DCM was slightly more potent than Doxorubicin, with IC_{50} values of 47.60 µM and 50.96 µM, respectively. This slight difference in potency suggested that *A. alternata* strain Z3Y DCM could be more effective at lower concentrations, which is crucial for optimizing the therapeutic dosages. Both *A. alternata* strain Z3Y DCM and Doxorubicin demonstrated a clear concentration-dependent reduction in cancer cells viability, a common characteristic of cytotoxic agents. For the *A. alternata* strain Z3Y DCM extract, cell viability remained relatively stable at lower concentrations (log 0 to log 0.2). This stability suggested that *A. alternata* strain Z3Y DCM was well-tolerated up to a certain threshold, beyond which its inhibitory effects become more pronounced, in consistence with Khazaal *et al*., (2023). A significant reduction in cell viability was observed at log 1.5, where *A. alternata* strain Z3Y DCM efficacy began to sharply decline; with cell viability dropping to 70.12 %. This decrease indicated onset of the compound's potent inhibitory effect. As the concentration increased further (log 1.8 to log 3), cell viability continued to decrease rapidly, reaching as low as 4.79 %. This steep decline is typical for cytotoxic agents, where higher concentrations lead to greater inhibition of cell functions or viability. Doxorubicin showed a similar trend but exhibited earlier signs of reduced cell viability, starting at log 0.9. This earlier decline suggested that Doxorubicin begun exerting its inhibitory effects at lower concentrations compared to *A. alternata* strain Z3Y DCM. The rapid decrease in viability beyond log 1.5, culminating in a complete loss of cell viability at log 3, was aligned with Doxorubicin's known potency as a chemotherapeutic agent.

However, the observation that Doxorubicin exhibited a slightly higher IC_{50} than *A. al*ternata strain Z3Y DCM may indicate that while it was potent, its effect plateaued slightly higher, thus requiring marginally higher concentrations to achieve the same level of inhibition as *A. alternata* strain Z3Y DCM in accordance with Katoch *et al*., (2023). The cell viability (%) significantly dropped as the concentration of *A. alternata* strain Z3Y DCM increased, but the overall effectiveness remained inferior to Doxorubicin, which showed a steep decline in cell viability even at lower concentrations. These findings suggested that while *A. alternata* strain Z3Y DCM possessed notable anticancer activity, it was less potent than Doxorubicin, which agreed with several previous studies conducted by Barbero *et al*., (2018); Katoch *et al*., (2023). The higher IC_{50} value indicated that greater concentration of *A. alternata* strain Z3Y DCM was required to achieve similar inhibitory effects on cancer cell growth. Despite this, *A. alternata* strain Z3Y DCM cytotoxicity warrants further investigation; particularly in the context of its potential combinatorial therapies or modifications required to enhance its efficacy. Further studies should focus on elucidating the specific mechanisms of action of *A. alternata* strain Z3Y DCM and explore its potential synergistic effects with other anticancer agents. Additionally, optimizing the extraction and purification processes of *A. alternata* strain Z3Y fractions may improve the bioactivity and therapeutic potential of *A. alternata* strain Z3Y DCM in cancer treatment.

The docking results highlighted the varying affinities of different compounds toward bacterial targets. Dihydroaltersolanol was recognized due to its high affinity across multiple targets, indicating its broad-spectrum antibacterial properties (Fouillaud *et al*., 2016). Bicycloalternarene F presented a unique docking profile, while it showed weak binding to most targets; however, it exhibited an exceptional docking score with 3-hydroxy-3-methylglutaryl-coenzyme A reductase, suggesting a highly specific binding interaction. Alternatone A and Tenuazonic acid both displayed robust binding affinities, indicating their potential as multi-target antibacterial agents. These findings confirmed the antimicrobial potentials of these compounds and their mechanisms of action were computationally explained (Zhou *et al*., 2019; Chen *et al*., 2022). Conversely, 2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone and 2H-(2E)-tricycloalternarene 12a showed moderate to low affinities across most targets, which may limit their effectiveness unless they undergo specific structural modifications. The docking simulation results identified potential lead compounds for antibiotic development. Behera, (2024) reported that Dihydroaltersolanol emerged as a top candidate for further research due to its strong binding to multiple targets. In contrast, Bicycloalternarene F's showed mixed results with its strong binding to one specific target and minimal binding to others, underscored the need for target-specific investigations. These findings suggest that Alternatone A and 2-(N-vinylacetamide)-4-hydroxymethyl-3-ene-butyrolactone possessed promising attributes for developing new antibacterial agents. Their significant binding affinities with essential bacterial proteins call for further experimental validation to assess their effectiveness in inhibiting bacterial growth and survival (Gao *et al*., 2024). The analysis of binding affinities provided valuable insights into the potential inhibitory actions of Bicycloalternarene F, dihydroaltersolanol, and tenuazonic acid on various enzymes. Bicycloalternarene F showed specificity for residues on 3-hydroxy-3-methylglutaryl-Coenzyme A Reductase, which was indicative of its potential in managing cholesterol levels. Dihydroaltersolanol interacted with multiple proteins and formed penicillin-binding proteins to several key enzymes involved in folic acid and protein synthesis, pointing out its broad-spectrum antibacterial potential.

Anticancer activity studies on Tenuazonic acid, with its binding affinities to both dihydrofolate reductase and IV topoisomerase, suggested a dual-target mechanism that could enhance its efficacy as an antimicrobial agent (Kumar *et al*., 2024). The docking scores provided critical insights into the binding affinities of various compounds to specific anticancer targets and indicated their potential efficacy as anticancer agents. Bicycloalternarene F and Alternatone A emerged as promising compounds due to their strong binding affinities with multiple targets. These findings suggested that Bicycloalternarene F could be particularly effective in addition to its high binding scores across all three targets, indicating its broad potential as an anticancer agent. Additional experimental validation would be necessary to confirm these *in silico* predictions and evaluate the compounds' potential therapeutic efficacy in biological systems. The *in silico* docking studies revealed significant insights into the potential molecular actions of Alternatone A and Bicycloalternarene F, suggesting that these compounds were effective modulators of important proteins involved in apoptosis and cell survival. In terms of Caspase interaction, Alternatone A exhibited substantial binding affinity at the enzyme's active site, notably interacting with residues GLU 21 and ASP 60. This suggested that Alternatone A could inhibit Caspase activity, potentially halting apoptosis and providing a therapeutic pathway at conditions where controlling apoptosis was critical such as the case of neurodegenerative diseases. For Bicycloalternarene F, interactions with the anti-apoptotic protein Bcl-2; particularly with ARG 878, SER 904, and ASP 770, indicated that Bicycloalternarene F can effectively bind to and potentially inhibit Bcl-2. This inhibition could induce apoptosis in cancer cells, underscoring Bicycloalternarene F's potential as a promising

candidate for cancer therapy. Furthermore, Bicycloalternarene F demonstrated a strong inhibitory capacity against Protein Kinase B (AKT) by binding to its crucial residues VAL 228, GLU 226, and GLU 232. Protein Kinase B (AKT) played a vital role in cellular processes, including cell survival and proliferation. By inhibiting Protein Kinase B (AKT), Bicycloalternarene F triggered the apoptotic pathways and suppressed the cancer cells proliferation, positioning it as a potent anti-cancer agent. These findings underscored the therapeutic potential of Alternatone A and Bicycloalternarene F in targeting key apoptotic and survival pathways in cancer cells.

Conclusion

This study highlighted *A. alternata* strain Z3Y as a promising source of bioactive compounds with significant antimicrobial and anticancer potential. The DCM extract exhibited superior efficacy in its obtained active metabolites, outperforming gentamycin against several bacterial strains and showing comparable anticancer activity to Doxorubicin. Key compounds such as Tenuazonic acid, Alternatone A, and Bicycloalternarene F, demonstrated strong binding affinities to critical bacterial and cancer-related targets. These findings emphasize the importance of further isolation, mechanistic studies, and in vivo validation to optimize therapeutic applications of *A. alternata* strain Z3Y DCM extract. This study underscores the potential of fungal metabolites to combat antibiotic resistance and develop innovative cancer treatments.

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Conflict of interests

The authors declare no conflicts of interest.

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Ethical approval

None-applicable.

Authors' Contributions

A.G.: Conceptualization, Methodology, Formal analysis, software, Investigation, Resources, Writing-Original Draft. A.A.A: Conceptualization, Investigation, Resources, Supervision, Writing-Review and Editing. N.E.M: Conceptualization, Formal analysis, Investigation, Supervision, Writing-Review and Editing. M.E.: Conceptualization, Visualization, Investigation, Supervision, Writing-Review and Editing. A.M. R.: Conceptualization, Visualization, Investigation, Supervision, Writing-Review and Editing.

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