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Antibacterial activity of an active endophytic *Alternaria* sp. CATD-09 fraction In vitro and in silico evaluation

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ABSTRACT: Endophytic fungi have emerged as promising sources of bioactive secondary metabolites for pharmaceutical discovery due to their ability to produce metabolites similar to those of their host plants. This study investigated the antibacterial potential of strain CATD-09, an endophytic fungus isolated from *Cnidoscolus aconitifolius* (Mill.) I.M. Johnst., through antibacterial evaluation, chemical characterization, and molecular docking analysis. The antibacterial activity of fungal fractions was assessed against *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 using the minimum inhibitory concentration (MIC) method. The most active fraction was subsequently characterized by Gas Chromatography–Mass Spectrometry (GC–MS) to identify its major constituents. To predict the possible antibacterial mechanism, the identified compounds were subjected to molecular docking against penicillin-binding protein 2a (PBP2a), a validated molecular target associated with β-lactam resistance. Fraction 1 exhibited moderate antibacterial activity against *S. aureus* ATCC 6538 with an MIC value of 128 µg/mL and stronger activity against *E. coli* ATCC 8739 with an MIC value of 64 µg/mL. GC–MS analysis identified 9,12-octadecadienoic acid (Z,Z)- as the predominant constituent, accounting for 44.47% of the total composition. Molecular docking demonstrated that this compound exhibited a stronger predicted binding affinity toward PBP2a than the reference ligand, indicating its potential to inhibit bacterial cell wall biosynthesis. Overall, these findings suggest that *Alternaria* sp. CATD-09 is a promising source of antibacterial metabolites and provide a scientific basis for further investigation of its bioactive compounds as potential lead candidates for antibacterial drug discovery.

KEYWORDS: *Alternaria* sp CATD-09; antibacterial; endophytic fungi; in Silico; GC-MS.

▪ INTRODUCTION

Approximately 80% of the population in low-and middle-income countries depend on herbal medicine for their healthcare needs. The application of medicinal plants for disease treatment is referred to as phytotherapy. To date, numerous plant species have been extensively studied and shown to contain bioactive chemical compounds with diverse pharmacological properties [1]. The utilization of botanical sources for treatment has been passed down and is an essential part of the healthcare system. Herbal medications generated from plant extracts are increasingly employed in the treatment of a broad range of clinical disorders, but little is known about their mechanisms of action. According to research, regularly ingested medicinal herbs are high in polyphenols, saponins, flavonoids, and phenyl propanoids [2].

Cnidoscolus aconitifolius (Mill.) I.M. Johnston is a leafy green vegetable widely recognized for its rich content of essential nutrients, phytochemicals, and other functional bioactive compounds that may contribute to health promotion. The plant has been associated with several beneficial therapeutic effects, including glycemic regulation, anti-inflammatory, anti-anemic, anti-infective, and antioxidant activities [3]. Analysis of its aqueous extract has identified several phenolic constituents in different proportions, such as 1.86% phenols, 0.93% tannins, 0.30% flavonoids, 0.072% anthraquinones, and 0.065% phlobatannins [3]. Moreover, healthy plant tissues are naturally inhabited by endophytic microorganisms, which are broadly classified into fungal and bacterial endophytes. These endophytic microbes can produce secondary

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metabolites with structural resemblance to mirror those originating from the host plants in terms of structure and function, many of which exhibit significant biological activities [4].

This highlights their prospective role as a promising source of host-specific metabolites with broad applications in various fields, particularly in pharmaceuticals [4]. Endophytic organisms are recognized for producing a diverse array of bioactive compounds with pharmacological effects, including those with antibacterial, antitumor, anticancer, antifungal, antiparasitic, and antioxidant properties [5]. Numerous isolated compounds from endophytic fungi have been investigated for their biological and antimicrobial activities For example, kheiric acid, a newly identified polyhydroxy acid derived from *Curvularia papendorfii*, an endophyte derived from The compound isolated from the Sudanese medicinal plant the *Vernonia amygdalina* demonstrated antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA), with a minimum inhibitory concentration (MIC) value of 62.5 µg/mL [6]. Furthermore, phomoenamide, a newly identified an enamide dimer isolated from *Phomopsis* sp, an endophytic fungus obtained from the leaves of *Garcinia dulcis* (Roxb.) Kurz, demonstrated antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra, with a minimum inhibitory concentration (MIC) value of 6.25 µg/mL [7]. At present, The effectiveness of preventing and managing infectious diseases has become increasingly constrained. The declining effectiveness of existing antimicrobial agents highlights the urgent need for alternative therapeutic strategies. Endophytes represent a particularly promising option, as they possess a strong ability to produce novel bioactive metabolites with not only antibacterial properties but also a broad spectrum of biological activities [7].

MRSA represents a major public health issue due to its growing resistance to commonly used antibiotic, resulting in persistent infections, elevated healthcare expenditures, and increased mortality rates associated with infections caused by this pathogen [8]. This strain demonstrates substantial resistance to β-lactam antibiotics, which are widely utilized in the management of S. aureus infections. Resistance in MRSA is primarily associated with the mecA gene, which encodes Penicillin-Binding Protein 2a (PBP2a), a protein that remains unaffected by β-lactam antibiotics. PBP2a is essential for catalyzing the cross-linking of peptidoglycan during bacterial cell wall synthesis. Therefore, the discovery of novel therapeutic compounds capable of effectively targeting MRSA is considered highly important [8]. As a result, endophytes have piqued the interest of natural product researchers worldwide, resulting in a steady stream of publications revealing the separation of novel bioactive chemicals from endophytes [4].

Alternaria sp, is a plant-pathogenic fungal species that caused leaf spot disease and impacts a broad variety of crops and is widely recognized as a pathogen of wheat [9]. According to a prior study, an endophytic fungus of *A. alternata* ZHJG isolated from *Cercis chinensis* produced Isotalaroflavone and (±) -Alternaone demonstrated significant antimicrobial activity [10]. Endophytic fungi inhabiting the plant *C. aconitifolius* (Mill.) I.M. Johnston, including CATD-09, has garnered considerable attention due to its rich diversity of natural compounds with notable bioactive properties. Given the urgent demand for alternative treatments against resistant bacterial strains, these metabolites hold substantial promise for the development of novel antibacterial agents [11].

▪ MATERIALS AND METHODS

Materials

Endophytic fungus CATD-09 was isolated from *C. aconitifolius* plant collected from Pamulang, South Tanggerang City, Banten Province, Indonesia (S: 6°20' E: 106°43', 83 m.asl). The fungal isolate CATD-09 was molecularly identified by sequencing the internal transcribed spacer (ITS) region of the rDNA. The resulting sequence was compared with reference sequences in the NCBI GenBank database using BLAST. Identified as *Alternaria* sp The ITS sequence has been deposited in GenBank under accession number PZ039135 and were preserved at −80 °C. Chemicals used in this study include dimethyl sulfoxide/DMSO (Merck), Methanol P.A (Merck), quercetin (Sigma-Aldrich), Dragendroff (Merck), vanillin (Sigma-Aldrich), ethyl acetate, methanol, acetone, dichloromethanes were technical solvents and were distilled once with a purity of 96%–97%, chloramphenicol (merck), and Triphenyl tetrazolium chloride (TTC) (Sigma) and media such as Mueller Hinton Agar/MHA (Himedia), Mueller Hinton broth/MHB (Himedia), Potato Dextrose Broth/PDB (Difco™), Potato Dextrose Agar/PDA (Difco™).

Cultivation of endophytic fungus CATD-09

As per protocol [1], endophytic fungal organisms were cultured on a larger scale to obtain secondary metabolites. an axenic isolate of the fungal endophyte CATD-09 was cultured in 3 L of potato dextrose broth (PDB). Briefly, two agar plugs (0.5 × 0.5 cm²) from a PDA culture of CATD-09 were aseptically transferred into a 500 mL flask containing 200 mL of PDB. This medium is widely used for both screening and large-scale cultivation of endophytic fungi, as it supplies essential nutrients, including dextrose as a source of carbon and potato-derived components as a nitrogen source. Incubation was conducted under conditions adapted from previous studies, namely in a dark environment, at room temperature (approximately 25 °C), and without agitation (static conditions). These conditions are favorable for fungal development and the production of bioactive metabolites that may be influenced by light and temperature fluctuations. Furthermore, static cultivation offers economic advantages due to the absence of agitation equipment. The cultures were maintained under these conditions for three weeks.

Extraction and fractionation of endophytic fungal metabolites

After incubation, both the culture medium and fungal biomass were extracted three times using ethyl acetate. According to earlier studies, semipolar bioactive compounds can be effectively isolated using liquid–liquid extraction with ethyl acetate, which separates into two phases, allowing distinction between the aqueous medium and extracted components. This approach eliminates the need for freeze-drying, making it more efficient, straight forward, and cost-effective. Each maceration step lasted approximately 24 hours. The mixed extracts were concentrated with an evaporator and stored under refrigeration at 4°C for later analysis. This ethyl acetate extract (140 mg) was separated by gravity column chromatography (CC) method 3 cm x 80 cm with SephadexTM LH-20 (200 g) as stationary phase and eluted with methanol as solvent 2 mL/min. The separation results were collected using vials (10 mL) and obtained as many as 60 vials. The eluate was then analyzed using thin layer chromatography (TLC) with a mixed eluent of CH_2Cl_2-MeOH (10:1). TLC with similar chromatogram patterns were combined into one fraction. Based on the results of the chromatogram pattern obtained 4 fractions (F1-F4). These fractions were further tested for antimicrobial activity in vitro using againts *S. aureus* ATCC 6538 and *E. coli* ATCC 8739 and the compound content of the most potent fraction was identified using a Gas Chromatography-Mass Spectrometry (GC-MS) instrument [2].

Thin-layer chromatography (TLC) analysis of chemical compounds

Chemical profiling of the fractions was performed using thin-layer chromatography (TLC) plates coated with silica gel 60 F254 (Merck). The dried extract was prepared at a concentration of 10 mg/mL, and 10 µL of the solution was spotted onto the TLC plate prior to development using a CH_2Cl_2: MeOH (10:1) solvent system. After separation, the resolved compounds were visualized under ultraviolet (UV) light at wavelengths of 254 and 366 nm using a CAMAG UV detector. Additional visualization of the spots was achieved by spraying the plates with vanillin–sulfuric acid and cerium (IV) sulfate reagents [2].

Qualitative antibacterial activity by TLC bioautography assay

The antibacterial activity of the fraction was evaluated against *E. coli* ATCC 8739 as a Gram-negative and *S. aureus* ATCC 6538 as a Gram-positive bacterium. Aliquots (10 µg/µL) were applied onto silica gel GF254 TLC plates (Merck) and allowed to dry at room temperature. The plates were subsequently immersed in bacterial suspensions adjusted to 10^6 CFU/mL and incubated in humid chambers containing sterile moistened cotton at 37 °C for 18 h. Following incubation, the plates were treated with 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; Merck). Antibacterial activity was demonstrated by the formation of clear or whitish inhibition zones against a red-colored background [2].

Quantitative antibacterial assay for MIC determination

The antibacterial potential of the active extract was assessed by a microdilution assay using 96-well microplates based on the method described by Praptiwi et al [2]. Test concentrations ranged from 4 to 512 µg/mL, in which chloramphenicol functioned as the positive control substance. The MIC was defined defined as the lowest concentration that showed no variation in color was detected after the addition of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC, Merck).

GC–MS profiling of antibacterial compounds in active fractions

GC–MS was conducted with the use of an Agilent gas chromatograph (19091S-433UI:93.928) coupled to a mass spectrometer and fitted with a 5% phenyl methyl siloxane capillary column (30 m × 250 µm × 0.25 µm). Helium served with helium used as the carrier gas at a constant flow rate of 1.0 mL/min, and 1 µL of the sample was injected into the system. The oven temperature was initially maintained at 40 °C and subsequently raised at a rate of 10°C per minute until 300 °C was achieved, followed by a 4-minute holding period. Compound identification was carried out by matching the acquired mass spectra with the data contained in the NIST library. [3]

Molecular docking studies

Molegro Virtual Docker, licensed from 2013.6.0.0 to 2013.05-21 The software used included Chem Office was used for the molecular docking research. Structural protein information was sourced from the Research Collaboratory for Structural Bioinformatics Protein Data Bank [2]. An appropriate target protein was chosen from the Protein Data Bank (PDB), and the three-dimensional structure of PBP2a was retrieved from the publicly accessible database under PDB ID: 1MWT, available through RCSB Protein Data Bank https://rcsb.org. The receptor, a macromolecular entity, was separated from adjacent molecules such as water and the bound native ligand. Molecular docking and amino acid interaction analyses were performed using Molegro Virtual Docker (MVD). The docking poses were evaluated using the scoring functions implemented in MVD, including the rerank score, which was reported as a predicted docking score rather than an experimentally measured binding free energy. The docking protocol was subsequently validated by calculating the Root Mean Square Deviation (RMSD) between the redocked and crystallographic ligand poses. An RMSD value of ≤2.0 Å was considered acceptable for docking validation.

With all procedures visualized in three-dimensional (3D) representations. The docking process consisted of several steps: (a) the receptor was accessed via the Protein Data Bank (PDB), ensuring that the selected receptor structure contained a co-crystallized ligand; (b) hydrogen atoms were added to the receptor, as they are typically removed in deposited PDB files, and the receptor protein was repaired to correct any missing or erroneous amino acid residues. This step was generally performed automatically by the software; (c) the receptor binding site, where the ligand interacts, was identified. This site corresponds to cavities present within the receptor structure; (d) the 3D configuration of the compound was placed into the selected cavity. In Molegro Virtual Docker, ligand placement was carried out using an alignment procedure, in which three atoms of the tested compound were aligned with three corresponding atoms of the native ligand present in the receptor.

The selected atoms typically belonged to the pharmacophore group; and (e) the binding pose of the compound within the receptor cavity was visualized. Several visualization modes were used to examine the ligand environment, including hydrophobic surface mapping to assess hydrophobic interactions, electrostatic mapping to evaluate electronic interactions, and hydrogen bond visualization to identify hydrogen bonding between the ligand and the receptor [4].

In addition, in silico assessment of physicochemical and pharmacokinetic properties of compounds from the active fraction of *Alternaria* sp strain CATD-09 are also required. The assessment of physicochemical properties for potential drug candidates is commonly based on Lipinski's Rule of Five, which considers parameters such as molecular weight, Log P, the quantities of hydrogen bond donors and acceptors, together with the polar surface area. In addition, pharmacokinetic characteristics are evaluated through ADMET profiling, which encompasses absorption, distribution, metabolism, excretion, and toxicity. ADME and toxicity predictions are conducted to reduce the risk of compounds failing in clinical trials due to inadequate ADME properties. Therefore, the prediction of physicochemical and pharmacokinetic profiles is considered crucial in early-stage drug development, and parameter assessments were performed using the pkCSM online tools [5].

▪ RESULTS

Endophytic fungi cultivation

Endophytic fungus, *Alternaria* sp strain CATD-09 isolated from *Cnidoscolus Aconitifolius* (Mill.) I.M. Johnston collected from pamulang, South Tanggerang City, Banten Province, Indonesia (S: 6°20' E: 106°43', 83 m.asl). was culture in 3 l of PDB at 25 ºC for 21 days in the dark and static condition.

Extraction and fractionation

Following cultivation, the culture medium and fungal biomass was extracted with ethyl acetate (EtOAc), resulting in 140 mg of ethyl acetate extract. The EtOAc extract of *Alternaria* sp CATD-09 (140 mg) was fractionated by column chromatography using Sephadex™ LH-20 (GE Healthcare, 200 g) with methanol as the eluent, yielding four fractions (F1–F4) with respective weights of 50.6, 32.3, 35.9, and 10.3 mg.

Chemical compounds analysis thin layer chromatography (TLC)

In this study, flavonoids, terpenoids, and coumarins appeared as dark zones on the chromatogram when observed under UV light at 254 nm, as shown in fractions 1 and 2 Figure 1 (A) [6]. Meanwhile, several flavonoids, coumarins, anthracenes, and polyketides observed under UV light at 366 nm exhibited yellow, dark yellow, or brown fluorescence, as shown in fractions 2 and 3. Figure 1 (B) [6]. Spray reagents were applied to facilitate the characterization of the chemical composition of the fungal extracts. Cerium(IV) sulfate, a general staining reagent widely used for alkaloid detection, produced brown-colored spots as shown in Figure 1(C) [7]. Vanillin–sulfuric acid is another general spray reagent widely used for the detection of hydroxyl and carbonyl compounds, producing a variety of color reactions, i.e., green (terpenoids), Yellow, yellowish-brown, orange (Alkaloid), Brown, dark blue, black, Violet (Phenolic) as shown in Figure 1 (D) [7].

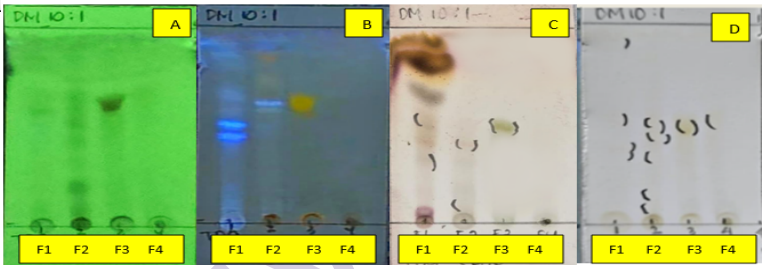

Figure 1. TLC chromatograms of *Alternaria* sp. extract fractions (F1–F4). (A) observed under visible light; (B) under UV light at 254 nm; (C) under UV light at 366 nm; (D) after spraying with cerium sulfate reagent; and (E) after spraying with vanillin–sulfuric acid reagent. The mobile phase consisted of CH_2Cl_2-MeOH (10:1), while the stationary phase used was silica gel 60 GF_{254}.

Antibacterial activity qualitative TLC-bioautography

The antibacterial potential of each fraction was subsequently examined employing the TLC-direct bioautography assay. TLC-bioautography demonstrated the presence of antibacterial constituents in several fractions, as indicated by the formation of clear inhibition zones on the chromatographic plate. Fraction F1 exhibited a greater number and wider distribution of clear inhibition zones compared to fractions F2 and F3, suggesting that F1 contained a higher abundance of antibacterial constituents and potentially possessed stronger antibacterial activity. In contrast, fractions F2 and F3 showed fewer inhibition zones, indicating relatively lower antibacterial potency, whereas fraction F4 showed no detectable antibacterial effect under the experimental conditions as shown in figure 2.

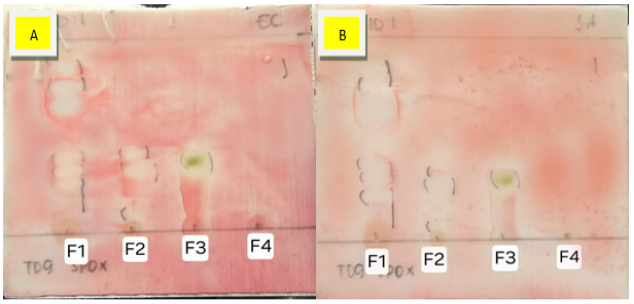

Figure 2. Bioautogram of extract fraction against *E.coli* (A) and *S.aureus* (B). Mobile phase: CH_2Cl_2 MeOH (10:1) Stasionary phase: Silica gel 60 GF254 (Merck).

Antibacterial activity fraction from fungal endophyte *Alternaria* sp Strain CATD-09

The MIC determination showed that F1 exhibited the strongest antibacterial activity among the tested fractions, with MIC values of 64 µg/mL against *Escherichia coli* ATCC 8739 and 128 µg/mL against *Staphylococcus aureus* ATCC 6538. In comparison, F3 showed weaker antibacterial activity, with MIC values of 256 µg/mL against *E. coli* and *S. aureus*. Fractions F2 and F4 showed no inhibitory activity within the tested concentration range figure 3. Therefore, F1 was selected for further GC-MS analysis and molecular docking based on its superior antibacterial activity, as indicated by its consistently lower MIC values against both tested bacterial strains figure 3 (A) (B). This selection criterion was based on the experimental antibacterial results.

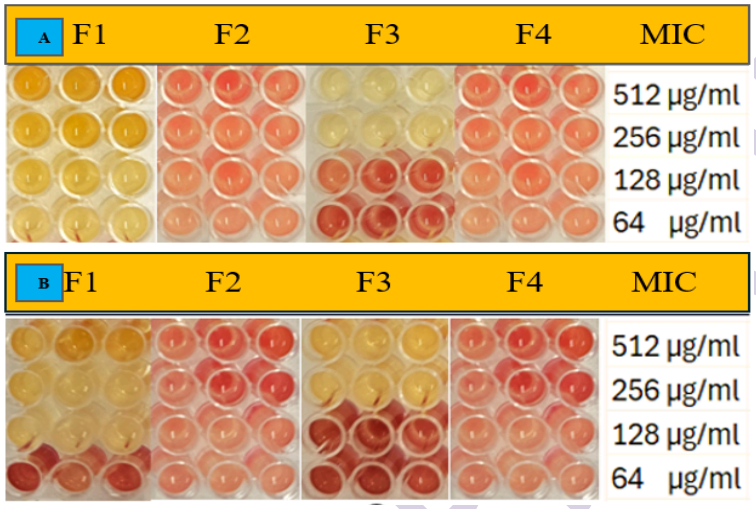

Figure 3. Assessment of antibacterial activity of *Alternasia* sp extracts Fraction No. 1, 2, 3 and 4 by microdilution assay against *E.Coli* (A) and *S.aureus* (B).

The composition of potential fraction *Alternasia* sp CATD-09 extract by GC-MS

GC–MS examination of the active fraction (fraction 1) of *Alternaria* sp strain CATD-09 extract showed several compounds, with palmitoleic acid, n-hexadecanoic acid, 9,12-octadecadienoic acid (Z,Z)-, cis-vaccenic acid, and octadecanoic acid being the most abundant compound (Figures 4 and 5).

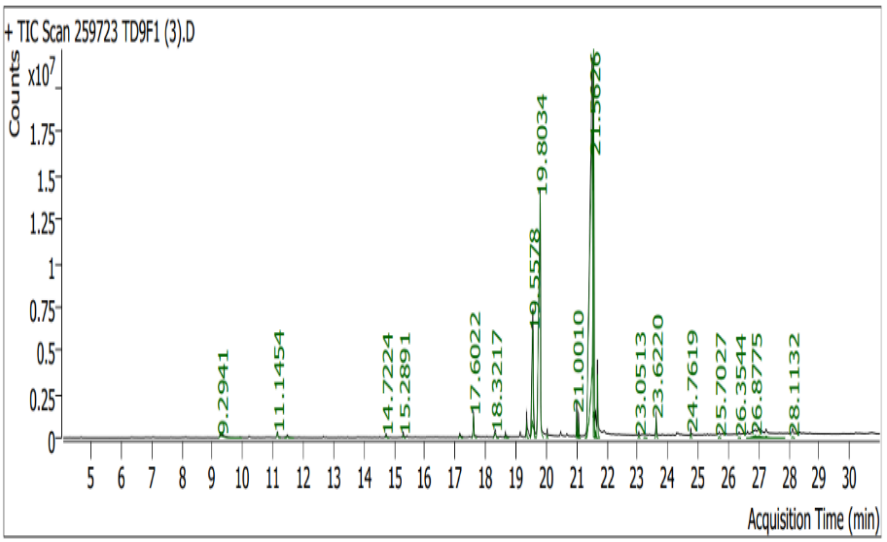

Figure 4. GC-MS chromatogram of Potential antibacterial fraction of *Alternasia* sp Strain CATD09.

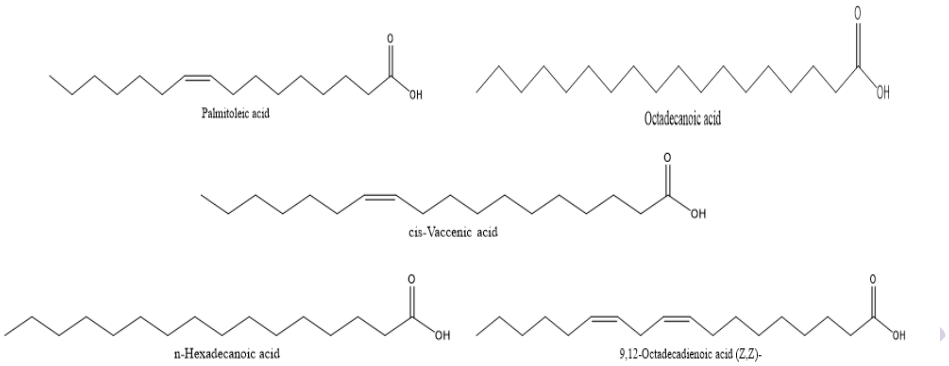

Figure 5. Major compound identified in fraction 1 by GC-MS instrument.

A potential contributor to the antimicrobial effect of the CATD-09 extract is its predominant constituent, 9,12-octadecadienoic acid (Z,Z)-, which accounts for approximately 44.47% of the relative composition (Table 1).

Table 1. Major compound identified in Potential antimicrobial fraction by GC-MS.

No	Name of the compound	Molecular formula	Retention time (Minute)	Peak area (%)
1	Palmitoleic acid	$C_{16}H_{30}O_2$	19.5578	8.26
2	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	19.8034	19.14
3	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	21.5120	44.47
4	cis-Vaccenic acid	$C_{18}H_{34}O_2$	21.5626	15.28
5	Octadecanoic acid	$C_{18}H_{36}O_2$	21.6900	2.55

Molecular docking studies

All docking procedures were validated using a redocking approach, in which the ligand was subsequently redocked into the receptor binding pocket and its predicted pose was compared with that of the original cocrystal structure. Root mean square deviation (RMSD) was used to evaluate the reliability of the docking procedure, with values below 2 Å indicating acceptable accuracy. Such results reflect a high level of precision and consistency of the simulation results, demonstrating that the docking methodology was able to successfully replicate the binding identified through experimental analysis conformations and confirming the validity of the molecular docking results.

The protocol of the docking was validated through the extraction of the native ligand (open-form penicillin G), followed by a redocking process. A simulation was considered valid when the resulting RMSD value was below 2.0. Validation using the Molegro Virtual Docker (MVD) software indicating that The receptor's binding location was positioned at the coordinates x = 28.14, y = 29.00, and z = 87.62, with a radius of 10 Å. Redocking of the native ligand produced a binding energy of −84.8287 kcal/mol and an RMSD value of 1.1149. The obtained pose was considered satisfactory as it closely aligned with the original binding conformation of the native ligand (Figure 6).

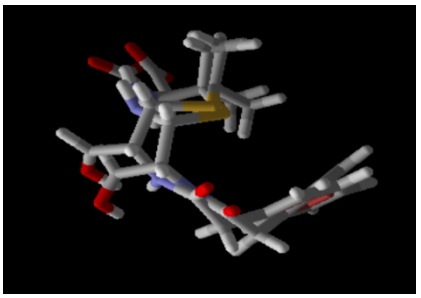

Figure 6. Comparison of native ligan with redocking result simulation.

Seven compounds, including the native ligand, were subjected to molecular docking against the PBP2a protein. The best binding conformation for each ligand was determined according to the rerank docking score. This rerank score was assumed to reflect the free binding energy between the inhibitory ligand and the target protein receptor. Lower or more negative rerank values indicate a more stable ligand–protein interaction, suggesting a greater potential to suppress the activity of the PBP2a receptor. The reranking docking analysis scores representing the binding affinities of each ligand toward the protein are presented in (Table 2).

Table 2. The result of molecular docking of each compound with 1MWT protein.

No.	Ligand Name	H Bond Interaction	Steric Interaction	Rerank Score (kcal/mol)
1	Open Form-Penicillin G	Asn 464, Ser 403, Thr 600, Ser 598	Thr 600, Ser 462, Ser 403, Ser 598, Asn 464, Gln 521	-85.3213
2	Palmitoleic acid	Ser 643, Ala 642, Thr 600	Ser 643, Ala 642, Thr 600, Ser 462	-84.3329
3	n-Hexadecanoic acid	Lys 430	Lys 430, Aan 464	-86.1729
4	9,12-Octadecadienoic acid (Z,Z)-	Ser 403, Thr 600	Gln 521, Ser 403, Thr 600, Ser 598	-88.2230
5	cis-Vaccenic acid	Ser 400, Thr 600	Lys 430	-84.6335
6	Octadecanoic acid	Lys 430, Thr 444	Lys 430, Thr 444, Gln 521, Thr 600, Aan 464	-83.0182
7	Chloramphenicol	Ser 403, Thr 600, Ser 462, Lys 406, Thr 444	Ser 598, Thr 600, Ser 462, Aan464, Ser 403, Lys 406, Gln 521	-84.0957

The determination of amino acids participating in drug–receptor binding, specifically concerning the pharmacophore group, can be determined by examining the compound's electronic and steric interactions with the receptor. Figure 7 illustrates that the ligands connect with protein residues through hydroxy (R-OH), ether (R-O-R), ester (RCOOR), and carbonyl (-C=O) functional groups. These connections occur as electrical interactions facilitated by hydrogen bonding within the active region of the receptor [5].

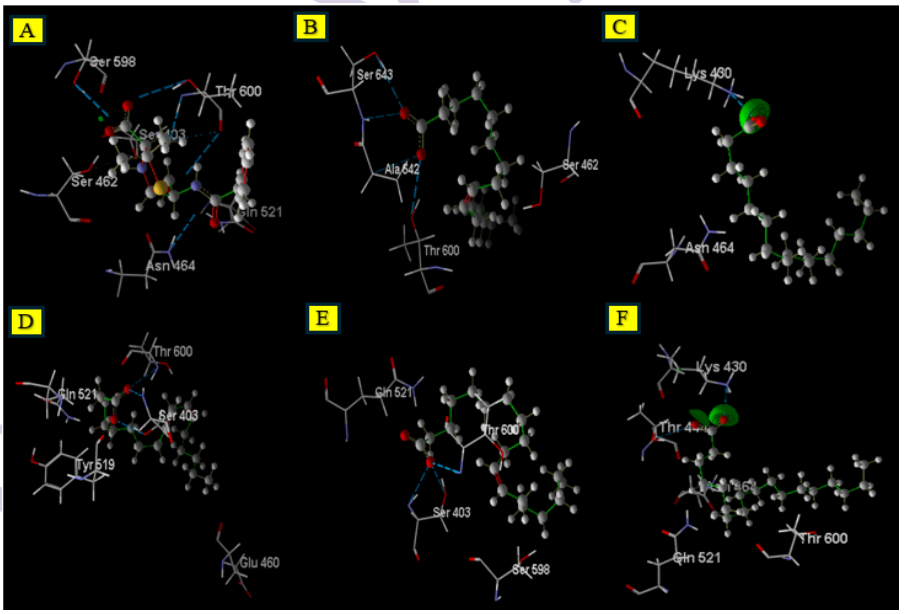

Figure 7. Visualisations of ligand interactions with protein PBP2a from the (PDB ID: 1MWT) (A) Open Form-Penicillin G (Native Ligand), (B) Palmitoleic acid, (C) n-Hexadecanoic acid, (D) 9,12-Octadecadienoic acid (Z,Z)-, (E) cis-Vaccenic acid, (F) Octadecanoic acid.

Prediction of the physicochemical and pharmacokinetic and toxicity profiles of active compounds from the potential fraction

Showed in Table 3 the Lipinski analysis of ligands with higher binding affinity than the natural ligand. These chosen ligands were next tested for their pharmacokinetic characteristics and toxicity, particularly hepatotoxicity. Table 3 shows the outcomes of these analyses.

Table 3. Result of Lipinski analysis for compound in potential fraction.

No.	Name	MW	Log P	HBD	HBA	PSA (Å)
1	Open form-penicillin G	336.41	0.81	3	5	138.58
2	Palmitoleic acid	254.41	5.33	1	1	112.48
3	n-Hexadecanoic acid	256.43	5.55	1	1	113.17
4	9,12-Octadecadienoic acid (Z,Z)-	280.45	5.88	1	1	124.52
5	cis-Vaccenic acid	282.47	6.11	1	1	125.21
6	Octadecanoic acid	284.48	6.33	1	1	125.89

Note : Molecular Weight (BM), Logarithm of Octanol (Log P), Hydrogen Bond Acceptors (HBA), Hydrogen Bond Donors (HBD), Polar Surface Activity (PSA).

Table 4. Pharmacokinetic and toxicity profiles of active compounds.

Name	Absorption		Distribution			Metabolism		Excretion	Toxicity
	Intestinal Absorption (%)	Skin Permeability (Log Kp)	Volume Distribution (Log L/kg)	Blood Brain Barrier / BBB (log BBB)	Fraction unbound (Fu)	CYP 2D6	CYP 3A4	Total clearance (log ml/min/kg)	Hepato toxicity
Open form-penicillin G	50.407	-3.024	-0.104	-0.824	0.523	No	No	0.533	Yes
Palmitoleic acid	92.058	-2.567	0.113	0.054	0.187	No	No	1.619	No
n-Hexadecanoic acid	92.004	-2.717	-0.543	-0.111	0.101	No	Yes	1.763	No
9,12-Octadecadienoic acid (Z,Z)-	92.329	-2.723	-0.587	-0.142	0.054	No	Yes	1.936	Yes
cis-Vaccenic acid	91.823	-2.725	-0.558	-0.168	0.052	No	Yes	1.883	No
Octadecanoic acid	91.317	-2.726	-0.051	-0.195	0.051	No	Yes	1.832	No

Note: (No) = Inactive, (Yes) = Active

The results of the pharmacokinetic and toxicity evaluation of active compounds present in Fraction 1 using absorption, distribution, metabolism, excretion, and toxicity (ADMET) parameters are presented in Table 4. The analysis in Table 4 shows that all active compounds in Fraction 1, as well as the native ligand, exhibited intestinal absorption values greater than 30%, indicating good absorption, as compounds are considered to have acceptable intestinal absorption when the value exceeds 30%. A compound is assumed to demonstrate favorable skin permeability if it has a LogKp value lower than −2.5. The results indicated that none of the compounds showed potential for skin absorption. Compounds are considered to have poor volume of distribution (VDss) when the value is below −0.15 and good distribution when the value exceeds 0.45. Table 6 shows the results demonstrate that all active substantial in Fraction 1 did not meet the criteria for good distribution. Active compounds are considered to possess the ability to readily crossing the blood–brain barrier (BBB) if the LogBB value is more than 0.3, whereas poor BBB distribution is indicated by LogBB values below −1. The BBB parameter is used as an indicator to assess a drug's ability to penetrate the brain barrier.

The results showed that none of the active compounds had LogBB values greater than 0.3. None of the active compounds exhibited a higher unbound fraction than the native ligand; a higher unbound value indicates increased binding to blood plasma proteins, which may reduce efficiency in diffusion or transmembrane transport. Four active compounds—n-hexadecanoic acid, 9,12-octadecadienoic acid (Z,Z)-, cis-vaccenic acid, and octadecanoic acid—were predicted to have the potential to inhibit the CYP3A4 enzyme, which plays a crucial role in drug metabolism in the body. Excretion parameters were evaluated based on total clearance values, and all active compounds in Fraction 1 exhibited higher total clearance values than the native ligand. A compound is considered hepatotoxic if it has the potential to cause pathological effects or impair liver function. The results indicated that one active compound in Fraction 1 which is 9,12-octadecadienoic acid (Z,Z)- exhibited hepatotoxic effects, whereas four compounds were predicted to be non-hepatotoxic.

DISCUSSION

The chemical compounds of endophytic fungal extracts were analyzed by thin-layer chromatography (TLC) due to their quick results, cost-effectiveness, and ease of use. TLC can also separate and identify the chemical compounds. The chemical compounds of the extracts were observed under long UV light (366 nm) and short UV light (254 nm) and visualized using a staining reagent to detect chemical compounds. After spraying with vanillin-sulfuric acid reagent, blue-colored spots were identified as saponin. Alkaloids are yellowish-brown, and flavonoids are orange after spraying with Cerium (IV)-sulfate. A prior study revealed that extracts obtained from endophytic fungi of *Alternaria* sp. exhibited diverse classes of metabolites, namely flavonoids, phenolics, terpenoids, and coumarins [8]. Two novel anthraquinones, namely anthrininones B and C, containing a 4,5-disubstituted butylaminolate moiety, were isolated from the marine fungus *Alternaria tenuissima* DFFSCS013 [9]. Isobenzofuranone A and indandione B, were isolated from liquid cultures of an endophytic fungus *Alternaria* sp [10].

The antibacterial activity of the endophytic fungal extracts was evaluated using the TLC-bioautography method. This technique is considered a rapid, sensitive, and practical approach for screening antibacterial compounds, requiring only a small quantity of sample and allowing straightforward visualization of bioactive spots. Antibacterial activity was characterized by the appearance of clear inhibition zones on the chromatographic plate against a purple-colored background [1]. The visualization of antibacterial activity on the TLC plate was enhanced using triphenyl tetrazolium chloride (TTC) as a colorimetric indicator. Viable microorganisms reduced TTC into colored formazan through microbial dehydrogenase activity, producing a dark-colored background, whereas antibacterial compounds appeared as clear inhibition zones due to suppression of bacterial growth. Similar applications of TTC in TLC-bioautography have been reported for the detection of antimicrobial metabolites from endophytic fungi and natural product extracts [11].

Minimum Inhibitory Concentration (MIC) value below 100 µg/mL against *Escherichia coli* is indicative of strong antibacterial potency. For comparison with standard antibiotics, the active compounds should be identified and benchmarked against chloramphenicol, which typically shows an MIC of ≥ 4 µg/mL [12]. Extensive reports describe differential antibiotic susceptibility between Gram-positive and Gram-negative bacteria, mainly as a consequence of differences in cell wall architecture and composition [1]. Previous studies have reported that several *Alternaria* species are capable of producing secondary metabolites that exhibit notable pharmacological activities, particularly antibacterial effects [8]. Several compounds have been successfully isolated from *Alternaria* fungi and have shown promising antibacterial activity such as, γ-pyranones isolated from *Alternaria* sp showed inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA), with MICs of 2.9, 3.2, and 2.0 µg/mL, respectively [13]. Two new natural compounds, namely 5-chloromoniliphenone and methyl8-dihydroxy-3-methyl-4-chloro-9-oxo-9H-xanthene-1-carboxylate were isolated from pearl barley culture of *A. sonchi* shows moderate antibacterial activity [14].

Compound such as n-Hexadecanoic acid may have bioactivities such as Anti-inflammatory, antioxidant, Anti-bacterial, anti-fungi, hypocholesterolemic, flavor, nematicide, pesticide, anti-androgenic [15]. Other molecules with bioactivities include octadecenoic acid, which has antibacterial effects against numerous microorganisms, antioxidant, and synthetic inhibitor of neurotoxins [16]. Octadecanoic acid has antimicrobial activities [17]. Previous studies have reported that *Alternaria alternata* KUDB15, isolated from soil collected in the interior of the Dandeli forest, produces linoleic acid [18]. The chemical 9,12-octadecadienoic acid (Z,Z) has been demonstrated to have strong broad-spectrum antibacterial action against the studied pathogens *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Salmonella typhi, Candida albicans, and Aspergillus niger* [19].

The virtual screening analysis identified two test ligands, *n*-hexadecanoic acid (−86.1729) and 9,12-octadecadienoic acid (Z,Z)- (−88.2230), which exhibited more favorable predicted docking scores than the native ligand (−85.3213). The native ligand also showed a more favorable predicted docking score than the reference drug, chloramphenicol. The rerank score (RS) is a computational scoring output generated by Molegro Virtual Docker (MVD) to evaluate ligand–receptor interactions. Therefore, the RS was interpreted as a predicted docking score rather than an experimentally determined binding free energy or receptor–ligand bond energy. A more negative RS indicates a more favorable predicted interaction according to the MVD scoring function. [20]. Molecular docking investigations were conducted to determine the compounds that may contribute to the antibacterial activity observed in this fraction. This method allows the prediction of

interactions between ligands and target proteins at specific binding sites. The pharmacological activity of extracts is associated with their bioactive constituents, which can interact with functional proteins, including receptors, leading to either activation or inhibition. Docking analysis was further employed to assess binding affinity, minimum binding energy, and hydrogen bond interactions within the protein–ligand complex. In this investigation, the docking simulation focused on PBP2a, a crucial protein involved in methicillin-resistant Staphylococcus aureus (MRSA) resistance. Since PBP2a enables bacterial cell wall production in the presence of most β-lactam antibiotics, targeting this protein represents a promising strategy for overcoming MRSA-related infections [21].

Lipinski's Rule of Five suggests that a viable drug candidate has a molecular weight of less than 500 Da, a LogP value of less than 5, fewer than five hydrogen bond donors, fewer than ten hydrogen bond acceptors, and a polar surface area (PSA) of no more than 140 Å². In this study, all tested ligands failed to satisfy the criteria due to their LogP values exceeding 5. Compounds with high positive LogP values generally exhibit poor water solubility and greater lipid solubility, which may reduce their bioavailability. In addition, such compounds tend to accumulate in adipose tissue, resulting in unstable plasma concentrations. Consequently, these properties may influence drug efficacy and excretion, potentially increasing toxicity despite strong in vitro activity [22].

Several possible chemicals found in this investigation have previously been shown to have antibacterial activity, including Palmitoleic acid compounds which have been reported to exhibited particularly high bactericidal activities against the *Staphylococci* strains.[23] n-Hexadecanoic acid also demonstrated modest antibacterial activity against A. *hydrophila, V. harveyi, B. subtilis, E. coli, and K. pneumoniae* [24]. 9,12-Octadecadienoic acid (Z,Z)- wich has the highest rerank score -88.233 in the previous studies also demonstrated a broad variety of antimicrobial activities against several species of microbes: *Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Salmonella typhi, Candida albican*, and *Aspergillus niger* [19].

The superior antibacterial activity of fraction 1 against *S. aureus* and *E. coli* is likely associated with the presence of nonpolar bioactive fatty acid compounds. Indeed, fatty acids and monoglycerides have been recognized as promising candidates for antibacterial agents. This study provides valuable insight into the isolation of bioactive antibacterial compounds from *Alternaria* sp strain CATD-09. Compounds exhibiting favorable docking affinities and ADMET profiles consistent with pharmacological standards may be considered promising candidates for antibacterial marker compounds. However, further bioassay-guided studies using isolated or purified constituents are essential to verify the in silico predictions.

▪ CONCLUSION

Fraction 1 of the ethyl acetate extract obtained from *Alternaria* sp. strain CATD-09 demonstrated in vitro antibacterial activity against *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538. GC–MS analysis identified five major compounds in the fraction. Molecular docking analysis further predicted favorable interactions of *n*-hexadecanoic acid and 9,12-octadecadienoic acid (Z,Z)- with SauPBP2a. These computational findings provide preliminary insights into the potential interaction of the identified compounds with PBP2a; however, PBP2a inhibition was not experimentally demonstrated in this study.

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