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Research Article

Chemical Profile of The Ethyl Acetate Extract of *Aspergillus sydowi*, 22-PLP1-F1, as Antibacterial Agent Against Clinically Resistant Strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract
Mangrove endophytic fungi can produce bioactive substances with diverse biological functions. This study aims to evaluate the chemical profile of mangrove fungal endophytic extracts that inhibit clinical pathogenic bacteria resistant to various antibiotics. The fungi were collected from Petengoran mangrove forest, Lampung Province. Fungal isolates were grown on shrimp shell media using solid-state fermentation for 14 days. The fungal biomass was extracted using ethyl acetate, and the active components were evaluated using thin layer chromatography. The extract was partitioned with dichloromethane/water and its bioactivity was tested using TLC-bioautography and agar diffusion methods. The active fraction was identified using LC-MS/MS. The LC-MS/MS data was interpreted with SIRIUS 5.8.6, and the drug-likeness and toxicological characteristics were assessed using ADME/Tox and STopTox machine learning tools. Morphological analysis showed that isolate 22PLP1F1 was an *Aspergillus* sp., with spherical conidia at the hyphae tips. Through phylogenetic analysis it was confirmed that isolate 22PLP1F1 is *Aspergillus sydowii* with similarity 98.9%. Initial TLC examination indicated the production of alkaloids, polypeptides, and steroids. Antibacterial assays showed that the polar portion inhibited multi-drug resistance (MDR) *Staphylococcus aureus*, while the active fraction at 2 mg/mL inhibited MDR *Pseudomonas aeruginosa*. LC-MS/MS analysis revealed a major chromatogram peak at a retention time of 8.67; m/z 488.2196, suggesting a novel derivative of a compound at a retention time of 7.82; m/z 446.208. ADME/Tox analysis indicated that the compounds do not penetrate the BBB but remain in the GI absorption region. Further research is needed to elucidate the active compounds mechanism of action and conduct bioengineering studies.

Keywords: antibacterial agent, clinical bacterial pathogens, computer annotation, endophytic fungi, mangroves

1. INTRODUCTION

One major issue facing the 21st century is the rise in incidences of dangerous bacteria becoming resistant to different kinds of antibiotics. A new danger to global stability and human health is the rise of multidrug-resistant (MDR) and pathogenic microbes [1]. To combat the evolution of antimicrobial resistance and guarantee the safety and efficiency of existing medications, it is imperative to discover novel molecules with distinct modes of action sourced from natural sources [2]. Due to their growing resistance to recognized antibiotic classes, MDR bacteria pose one of the greatest dangers to human health worldwide [3]. Diseases caused by any of these MDR groups such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* have the potential to be fatal. There has been a lot of interest in the investigation of natural product molecules for the creation of treatments [4]. Since they are exposed to a range of extreme environmental stresses, which promote the evolution of distinctive bioactive compounds from their biosynthetic pathways, marine microorganisms in particular have the potential to serve as reservoirs of promising new antibacterial bioactive compounds [5]. The subject of marine pharmaceutical research has arisen as an autonomous scientific discipline, indicating the enormous potential of sea-derived substances, such as mangrove habitats [6].

Mangroves are a complex biosphere made up of mangrove plants and biologically active microorganisms that live in tropical and subtropical environments with high temperature, high salinity, intense light, tidal gradient, and low oxygen level [7]. Fungi are the most numerous and second biggest creatures in mangrove forests, living as endophytes. Endophytic fungi have emerged as

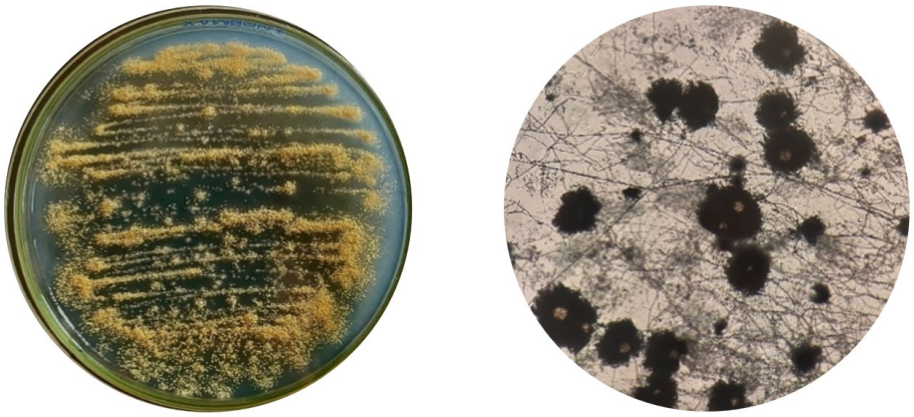

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Figure 1. Isolate 22PLP1F1 in (a) PDA media and (b) 100 magnification using a microscope.

interesting candidates for the identification of novel chemicals with potential anti-pathogen action. Endophytic fungi are tightly linked to plant tissues, producing unique ecological interactions that benefit both the fungus and the host plant [8]. Endophytic fungi can produce a wide range of secondary metabolites that can aid plant growth and development or give protection against diseases and severe conditions [9]. Jia et al. (2024) successfully isolated the chemical Penifuranone A, a novel alkaloid from the mangrove endophytic fungus *Penicillium crustosum* SCNU-F0006 [10]. In addition, Qin et al. (2024) discovered novel monoterpenoid compounds from the mangrove endophytic fungus *Aspergillus* sp. GXNU-Y85 [11]. The identification of this novel molecule serves as the foundation for further research into bioactive substances derived from mangrove endophytic fungus. Given Indonesia's oceanic region and 3 million hectares of mangrove habitats, there are several prospects for exploring the discovery of novel chemicals [12]. However, throughout the procedure, previously reported compound structures are frequently rediscovered. To circumvent this, quick bioactivity screening and culture procedures must be used, as cultivation medium have a significant impact on fungal compound synthesis. To assist close research and development gaps in novel antibacterial medications, particularly in compound re-discovery.

In this study, antimicrobial chemicals from mangrove fungus were examined utilizing the solid-state fermentation (SSF) method and metabolomics digitalization. Endophytic fungi have emerged as a source of bioactive chemicals, with promising possibilities for their discovery and use in the manufacture of useful medications [8]. In this investigation, endophytic fungi were revived on PDA medium to preserve viability, purity, and shelf life during the chemical extraction procedure [13]. The bioactive chemical extracts are then obtained by fungal culture. In present difficulties, culture in laboratory condition frequently yields previously found compounds (re-discovery), limiting the acquisition of novel compounds [14]. To avoid rediscovery of known molecules, it is critical to identify the acquired chemicals and select the possible strains for biosynthesis of novel compounds. The cultivation medium influences the variety of compound structures [15]. In this work, fungus was grown on selective media made from shrimp shell waste. Furthermore, to assess antibacterial activity, the bioactive fungal component extract was subjected to fast screening against pathogenic bacteria utilizing the thin layer chromatography (TLC)-bioautography technique. The structure of bioactive compound from endophytic fungus was then evaluated utilizing metabolomics methods, including a liquid chromatography-mass spectrometry (LC-MS/MS) methodology and digital dereplication.

The current research aims to assess the chemical profile of an extract from the mangrove endophytic fungus *Aspergillus* sp. 22PLP1F1 for its antibacterial properties against the clinical pathogens MDR *S. aureus* and MDR *P. aeruginosa*. Additionally, the ADME-related physicochemical properties of bioactive metabolites were predicted

using Swiss ADME web tools, and ProTox-II webserver was used to estimate *in silico* toxicity. This study paves the way for utilizing bioactive metabolites from mangrove endophytic fungi as novel sources for future in-depth research in bioengineering and natural product chemistry.

2. MATERIALS AND METHODS

2.1. Maintenance and Identification of Fungal Endophytic Mangrove

Fungal isolates were maintenance on potato dextrose agar (PDA) media at room temperature in an incubator for 14 days before being microscopically analyzed using a 45° coverslip [16]. Clean coverslips were planted at a 45° angle on agar plates and cultivated for 7 days. The cover slip was removed from the petri dish with tongs and put face up on a clean glass slide. The coverslip was seen using a Zeiss Axio Imager Microscope (100×). To detect endophytic fungi, a polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS), sequence area ITS1-5.8S-ITS4.

2.2. Cultivation, Extraction, and TLC Analysis

After being maintained on PDA media for 7 days, fungal isolates were inoculated into 20 mL Erlenmeyer flasks containing potato dextrose broth (PDB) media (26 ± 2 °C) for 3 days. Then, the fungal inoculum was cultivated in shrimp shell waste media (100 g of moist shrimp shell without the addition of artificial seawater) referring to the previous study [17]. Cultivation was carried out in 500 mL Erlenmeyer flasks and incubated statically for 14 days at a temperature of 26 ± 2 °C. After 14 days, the cultivation results were extracted using EtOAc and evaporated at 98 mbar at 40 °C. The ethyl acetate (EtOAc) extract was analyzed by TLC with a SiO_2 F_{254} as the stationary phase, and *n*-hexane:EtOAc (10:1) as the mobile phase. Further TLC observations used $Ce(SO_4)_2$ reagent and specific Dragendroff reagent to detect alkaloid components, Ninhydrin to detect polypeptides, vanillin sulphuric acid to detect steroid compounds. After the development of TLC spot, the partition was conducted using dichloromethane (DCM)/ water (H_2O) (1:3) to separate polar and nonpolar compound.

2.3. Antibacterial Assay using TLC-autobiography

Determination of antibacterial activity against resistant pathogens *S. aureus* and *P. aeruginosa* was carried out using the TLC bioautography test with an agar overlay. Preliminary bioactivity screening tests were carried out using the TLC-bioautography method [18]. TLC testing was carried out using a SiO_2 plate as the stationary phase, and *n*-hexane:EtOAc (10:1) as the mobile phase. The eluted chromatogram was visualized under ultraviolet (UV) light at two different wavelengths, namely $l_{254\ nm}$ and $l_{365\ nm}$. In the TLC bioautography testing process, the chromatogram is transferred to a sterile petri dish. Then 10 mL of Mueller–Hinton agar (MHA) was added with a mixture of 1 mL of 0.5 Mc Farland bacterial inoculum. The plate was incubated for 18 h at 37 ° C, after incubation 0.05% resazurin reagent was

Figure 2. Cultivation of isolate 22PLP1F1 on shrimp shell media.

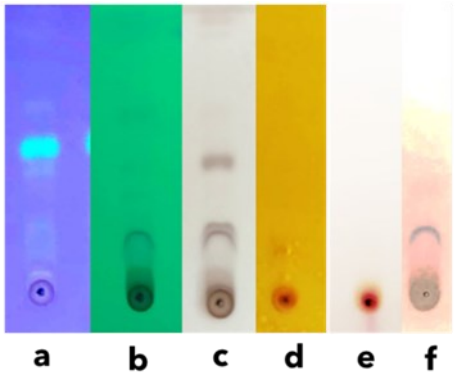

Figure 3. TLC analysis of crude extract of *Aspergillus sp*. 22PLP1F1 from shrimp shell waste media after 14 days.

added to visualize the inhibition zone on the thin layer chromatogram. The presence of a purple color in the retardation factor (Rf) component indicates an active compound.

2.4. Phylogenetic Analysis

The DNA of the fungal genome 22PLP1F1 was extracted following the method described by Landum et al., using the QIAamp DNA Minikit (Qiagen, Germany) [19]. The ITS region of ribosomal DNA from the fungal isolate was amplified with the forward primer ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3′) and the *reverse primer*, ITS4-R (5′-TCCTCCGCTTATTGATATGC -3′) [20]. The final reaction volume was 20.5 mL, which included 10 mL of the NEXpro TM PCR kit (PCR Biosystems, UK), 0.25 mL of each primer, 5 mL of ddH_2O, and 5 mL DNA template. For the negative control, distilled water was used instead of DNA to ensure no contamination. PCR was carried out using the Sensoquest Sensodirect Gradient Thermo block 96 (SensoQuest, Germany), with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C, 1 min at 72 °C, and a final extension at 72 °C for 5 min. The PCR products were separated on a 2% agarose gel in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0), stained with ethidium bromide (0.5 µg mL^{-1}), and documented using the QIAxcell Advanced (Qiagen, Germany). The PCR products were then sent for bidirectional sequencing using the ABIPRISM3730 × 1 Genetic Analyzer (Applied Biosystems, USA) at BASE First Laboratory Sdn. Bhd., Selangor, Malaysia.

2.5. Profiling Analysis

The active extract was analyzed using positive mode LC-MS/MS. The extract was dissolved in methanol and analyzed via LC-MS/MS analysis. The LC-MS/MS data files were converted to mzXML using Proteowizard (ver.3.0.21166) (part of ProteoWizard 3.0 package, Palo Alto, California, USA), then processed with Mzmine 2.53 software with data processing including mass detection by adjusting the noise level MS1= 3.0e3 and MS2 = 3.0e1, all data is centroided. Then the extension file is changed in mgf form for analysis in SIRIUS 5.8.6. Instrument profile: Q-Tof; Mass accuracy: 5 ppm for MS1 and 7 ppm for MS2; Molecular

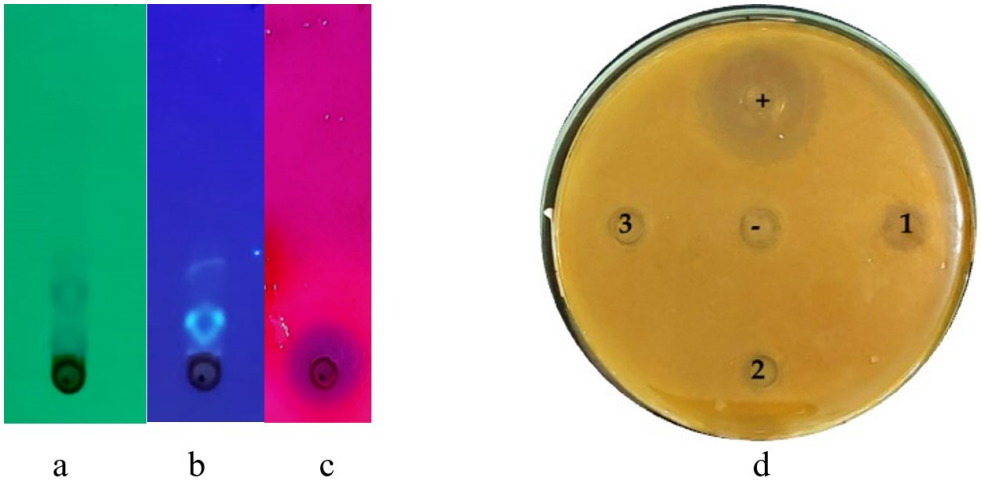

Figure 4. TLC bioautography assay at (a) UV $l_{254\ nm}$, (b) UV $l_{366\ nm}$ and (c) after incubation and addition of 0.02% resazurin, as well as (d) agar diffusion test against MDR *Pseudomonas aeruginosa*.

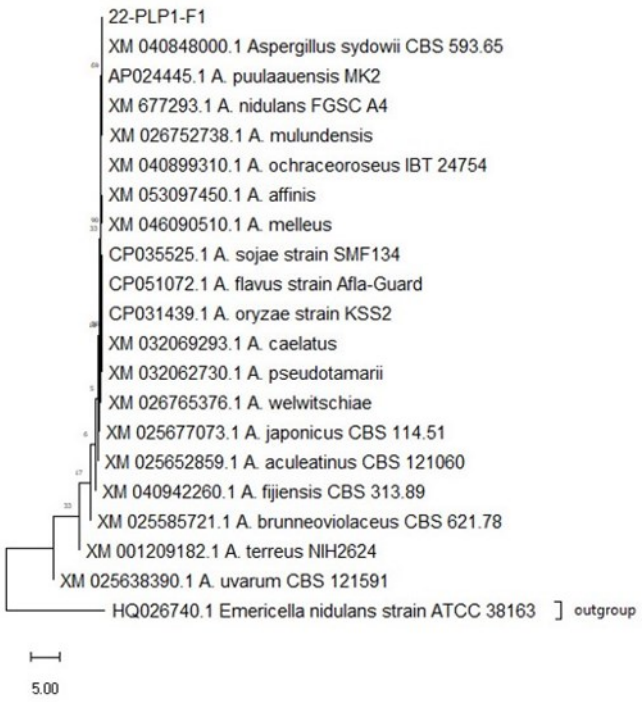

Figure 5. Phylogenetic analysis of *Aspergillus sydowi* 22-PLP1-F1.

formula and structure database: BIO; Maximum m/z to calculate: 700. ZODIAC was used to improve molecular formula predictions using a threshold filter of 0.99 [21]. Metabolite structure predictions were made with CSI: FingerID [22] and their significance was calculated with COSMIC [23]. Chemical class predictions were made with CANOPUS [24] using the NPClassifier chemical taxonomy [25].

2.6. Physiochemical and Pharmacokinetics Analysis

The physiochemical and pharmacokinetic properties including drug-likeness were calculated using the Swiss-ADME server (http://www.swissadme.ch/) with SMILES retrieved from PubChem. Based on Lipinski's rule of five frameworks and four parameters (Ro5) [26]. The first is molecular weight (MW < 500). The second criterion is the consensus log P value (log P < 5). The third parameter is the number of hydrogen bond donors (HBD ≤ 5). The fourth parameter is the number of hydrogen bond acceptors (HBA ≤ 10). Passing these criteria does not necessarily mean that the chemical is orally active. To further evaluate the active characteristics, other descriptors of drug similarity were explored, such as topological polar surface area (TPSA < 140 $Å^2$) and number of rotatable bonds (RB ≤ 10). Furthermore, OSIRIS software received from the organic chemistry portal is used to analyze the compound's irritating, mutagenic, or tumorigenic potential, as well as its reproductive efficacy in nature.

2.7. Toxicity Prediction

Bioactive compounds were tested for toxicity using the ProTox 3.0 (https://tox.charite.de/protox3/) and StopTox (https://stoptox.mml.unc.edu/) servers. The ProTox 3.0 server returns LD_{50} values in mg/kg body weight and toxicity classifications for the chemicals being analyzed. Next, the StopTox is utilized to examine acute toxicity produced by the exposed chemicals.

3. RESULTS AND DISCUSSIONS

3.1. Fungal Maintenance and Identification

Isolate 22PLP1F1 was obtained from a mangrove forest area in Petengoran, Pesawaran, Lampung (5°34'09"S 105°14'26"E) specifically from *Rhizophora apiculata*. Endophytic fungi were isolated from mangrove leaves and maintain on PDA media. Based on the previous research [27], the Petengoran mangrove forest has an area of

around 113 hectares and there are 4 types of mangroves that can be found, such as ringworm mangrove (*Rhizophora mucronata*), oil mangrove (*Rhizophora apiculata*), small mangrove (*Rhizophora stylosa*), and tengar (*Ceriops* sp.). Therefore, the Petengoran mangrove forest has unique diversity so it can be a sampling location. The macroscopic observation (Figure 1(a)) showed the typical characteristics of fungi with conidia in the form of yellow granules, and spread on the surface of the agar medium. Meanwhile, microscopic identification (Figure 1(b)) shows abundant globose and mycelium. The characteristic granule-shaped conidia indicate that the fungus comes from the genus *Aspergillus* sp.

Fungi found in mangroves exist in harsh environments and can produce a variety of chemical compounds. Several fungal metabolites play critical roles in the creation of novel anti-infective medicines. The search for novel chemical compounds of pharmacological significance has brought attention to mangrove environments, which provide unusual biodiversity [28].

3.2. Cultivation and Extraction

Production of the bioactive compound isolate 22PLP1F1 was carried out using SSF, the fungus was seen growing on the surface of the media with abundant yellowish conidia (Figure 2). This indicates successful cultivation. Shrimp shell media contains the main component chitin which can be degraded into its derivative glucosamine. Based on Ma et al., chitin media can induce genes that produce nitrogen-based compounds such as alkaloids and polypeptides [29].

TLC assay revealed the pattern of chemicals produced by *Aspergillus sp*. 22PLP1F1 grown on shrimp shell medium. A fluorosense yellow hue

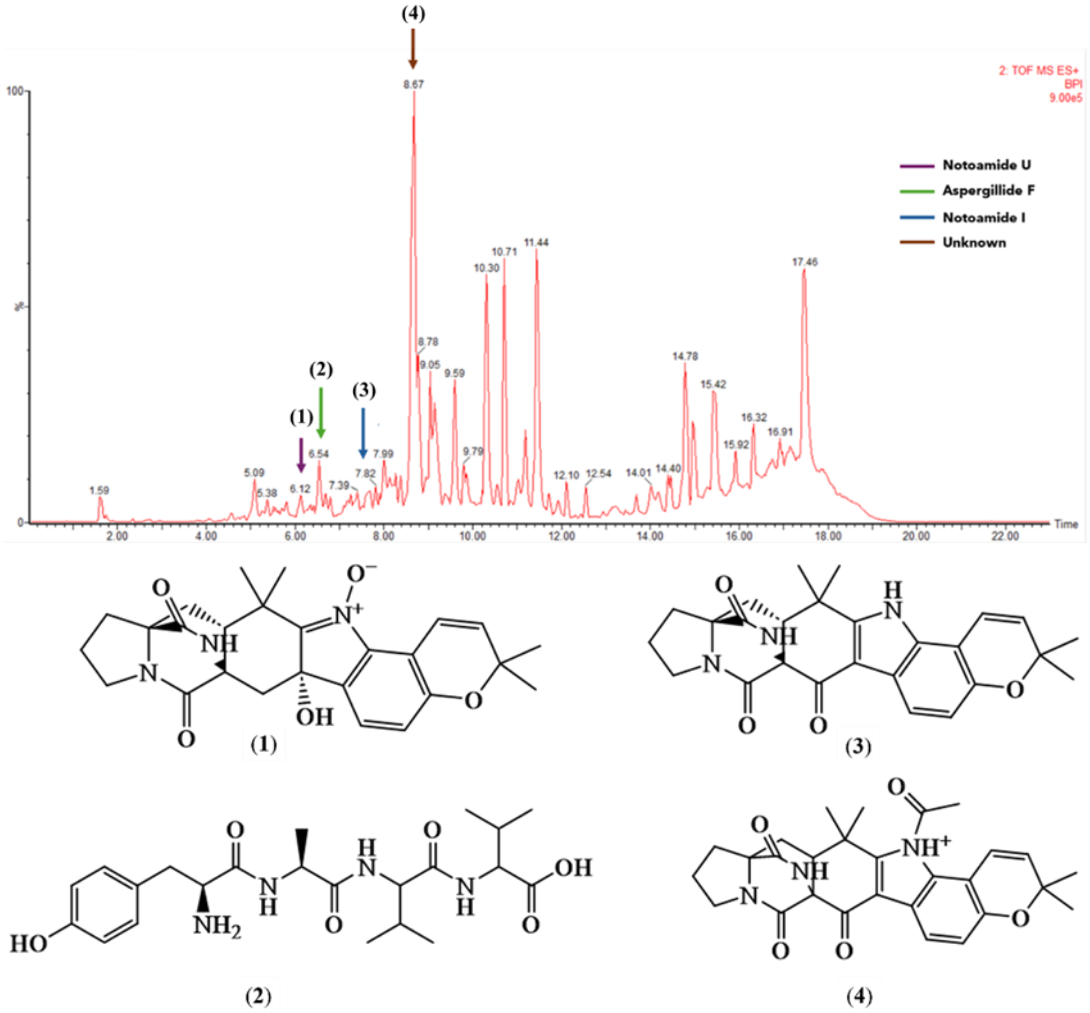

Figure 6. LC chromatogram of active fraction 22PLP1F1 from shrimp shell media and chemical structures of (1) notoamide U, (2) aspergillide F, (3) notoamide I, and (4) unknown compound.

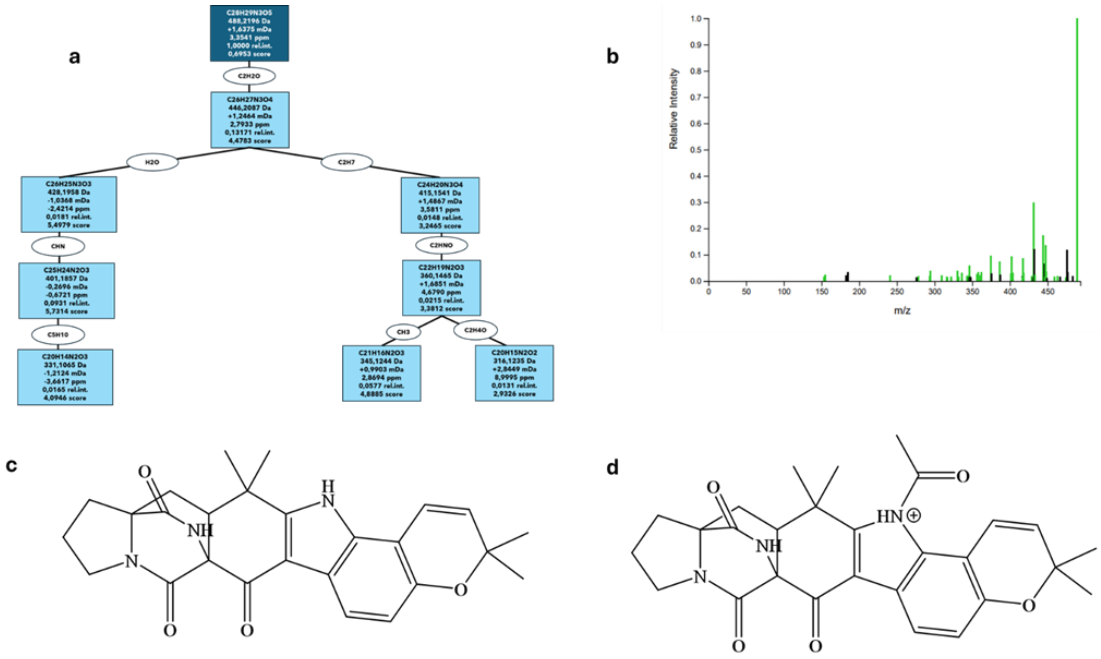

Figure 7. (a). Analysis CSI: fingerprint SIRIUS 5.6.2, (b) the m/z of MS2 detected is shown in green (c) notoamide I and (d) proposed unknown compound.

appears at 366 nm, showing that each medium creates distinct chemical compounds (Figure 3(a)). The UV measurements at $l_{254\ nm}$ (Figure 3(b)) revealed the existence of compounds with conjugated double bonds with varying patterns of component distribution. This study using UV light at 254 nm for detecting conjugated double bonds in compounds because it is non-destructive. UV detection allows the observation of compounds without altering the compound structure, which is crucial when further analysis is needed. Additionally, UV detection is specific to conjugated systems, making it a simple and efficient method compared to chemical reagents that require additional steps and may not be as selective for double bonds. TLC examination employing the universal reagent $Ce(SO_4)_2$ revealed the existence of numerous chemical components with varying polarity characteristics, with the polar component taking precedence (Figure 3(c)). Furthermore, the extract contain alkaloid compounds, as seen in orange spot (Figure 3(d)). Apart from alkaloids, TLC analysis utilizing Nynhidrin revealed positive peptide compounds with yellow and purple hues (Figure 3(e)). TLC examination with vanillin sulfate revealed terpenoid and steroid molecules characterized by brownish yellow and pink hues (Figure 3(f)). This is consistent with Mamangkey et al., who evaluate that *Aspergillus* sp. may produce anti-infective chemicals as well as antibacterials against resistant infections [30].

According to the TLC analysis result, DCM is ideal for separating diverse metabolites, was completely removed using nitrogen to ensure no residue interferes in bioactivity tests. Chosen over other toxic solvent such as chloroform for its stability and lower toxicity, DCM effectively aids compound purification without adding bioassay artifacts.

3.3. TLC Bioautography Assay

Based on the TLC investigation, the pattern of bioactive compound produced by fungi 22PLP1F1 grown in shrimp shell waste media is shown in Figure 4. Active UV l_{254nm} measurements (Figure 4 (a)) show the occurrence of compounds with conjugated double bonds that have varied patterns of component distribution. A fluorescence yellow hue appears at 366 nm, showing that each medium creates distinct chemical compounds (Figure 4(b)). The extract tested positive for antibacterial activity using TLC bioautography on the polar component spot, which was highlighted with a purple colour after adding the cell viability agent resazurin (Figure 4(c)). This TLC bioautography was performed using crude extract, without further

purification, to assess its antibacterial activity. Furthermore, MDR *P. aeruginosa* bacteria were tested using the agar diffusion technique. At a dosage of 2 mg/mL, *Aspergillus sydowii* 22PLP1F1 demonstrated 12 mm inhibitory zone activity (Figure 4(d)). This compound is included in the active category [31].

Bioautography TLC tests can detect the biological activity of substances in minute quantities, even in complicated combinations. This is because this approach depends on direct interactions between substances and microbes, allowing it to identify active compounds at low concentrations that other methods may miss. Another method that should be used during a screening run is to avoid rediscovering substances that are known to occur naturally in *Aspergillus* species. Based on bioactivity experiments, *Aspergillus* sp. 22PLP1F1 mangrove endophytic grown on shrimp shell media with SSF has the ability to produce antibacterial compounds against resistant clinical pathogens.

3.4. Phylogenetic Analysis

Phylogenetic analysis utilized Mega 11 software using the Neighbor-Joining method [32]. Evolutionary distance using the Tamura-Nei method [33]. The average variation of each sequence was analyzed using a gamma distribution model with a shape parameter of 0.41. *Emericella nidulans* strain ATCC 38163 was used as the outgroup [34]. The results of the phylogenetic analysis showed that isolate 22PLP1F1 was *Aspergillus sydowii*, in line with the results of the NCBI BLAST analysis which showed a similarity of 98.56% to *Aspergillus sydowii* CBS 593.65 (Acc. No. XM_040848000.1), as shown in Figure 5.

3.5. LC-MS/MS Profiling

The active fraction analysis revealed various molecular ion peak $[M+H]^+$ molecule with *m/z* 464.22 as notoamide U (**1**), m/z 451.26 as aspergilide F (**2**), m/z 446.21 as notoamide I (**3**), and the presence of a potential novel molecule of the type notoamide I (**4**) with m/z 488.22, as shown in Figure 6. Compounds **1** and **3** are an indole alkaloid substance found exclusively in marine *Aspergillus*. Their structures are unique and have a variety of interesting biological activities such as antibacterial activity [35]. Their core structures are formed by combination of tryptophan and a polyketide, followed by a series of enzymatic modifications that include the addition of various functional groups such as methyl, hydroxyl, and acetyl groups.

Figure 7(a) depicts a list of molecular formula candidates sorted by their matching spectrum scores, as well as the fragmentation tree of the selected molecular formula candidates. The stated peak was identified as a green peak in the spectra (Figure 7(b)). Furthermore, SIRIUS 5.6.0 analysis shows the presence of the notoamide I, compound with the molecular formula $C_{26}H_{27}N_3O_4$ with 15 degrees of unsaturation in accordance with Tsukamoto et al. [36] (Figure 7(c)), as well as an indication of a new structure at the major peak 8.67 min with the molecular formula $C_{28}H_{29}N_3O_5$ (m/z 488.2196) with 16 degrees of unsaturation. This

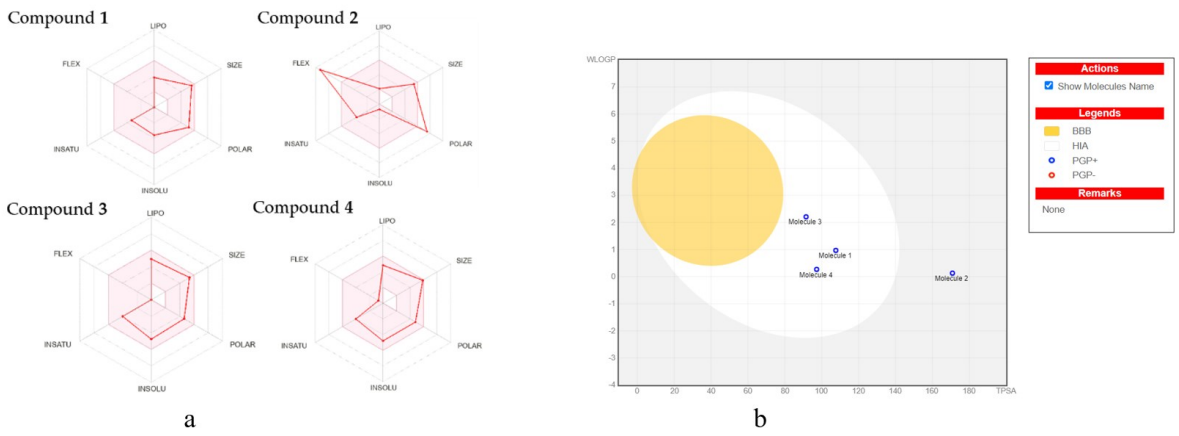

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Figure 8. ADME analysis: (a) the bioavailability radar of compounds 1–4 and (b) graph of the dependence of lipophilicity on the polarity of the studied molecules 1–11, determined by the BOILED-Egg method.

Table 1. Physicochemical analysis based on ADME.

Compound	Physochemical Properties				Lipophilicity		Drug-Likeness			Pharmacokinetics		
	ROTB (n) <10	HBA (n)<10	HBD (n)<15	ESOL (Log S)	TPSA (A_2)<140	ClogP (o/w)<5	Bio Score	Lipinski Filter	GIA	PGP Substrate	CYP3A4 Inhibitor	BBB Permeability
1	0	5	2	-3.61	107.62	1.75	0.55	Yes	High	Yes	Yes	No
2	14	7	6	-0.70	170.85	0.39	0.55	Yes	Low	Yes	No	No
3	0	4	2	-4.78	91.50	2.79	0.55	Yes	High	Yes	Yes	No
4	1	6	2	-4.79	97.20	1.65	0.55	Yes	High	Yes	No	No

Table 2. Toxicity prediction based on Protox-II.

Compound	Toxicity Values LD_{50} mg/kg	Probability				
		Toxicity Class	Hepatotoxicity	Cardiotoxicity	Carcinogenicity	Mutagenicity
1	1000	4	Inactive	Inactive	Inactive	Inactive
2	2287	5	Inactive	Active	Inactive	Inactive
3	340	4	Inactive	Inactive	Inactive	Inactive
4	150	3	Inactive	Inactive	Inactive	Inactive

Table 3. Acute toxicity prediction using StopTox.

Compound	Endpoints				
	Inhalation	Oral	Dermal	Irritation and Corrosion	Skin Sensinitazion
1	Non-Toxic	Toxic	Non-Toxic	Eyes (+) Skin (-)	Non-Sensinitizer
2	Non-Toxic	Non-Toxic	Non-Toxic	Eyes (-) Skin (-)	Non-Sensinitizer
3	Non-Toxic	Toxic	Non-Toxic	Eyes (+) Skin (-)	Non-Sensinitizer
4	Non-Toxic	Toxic	Non-Toxic	Eyes (+) Skin (-)	Non-Sensinitizer

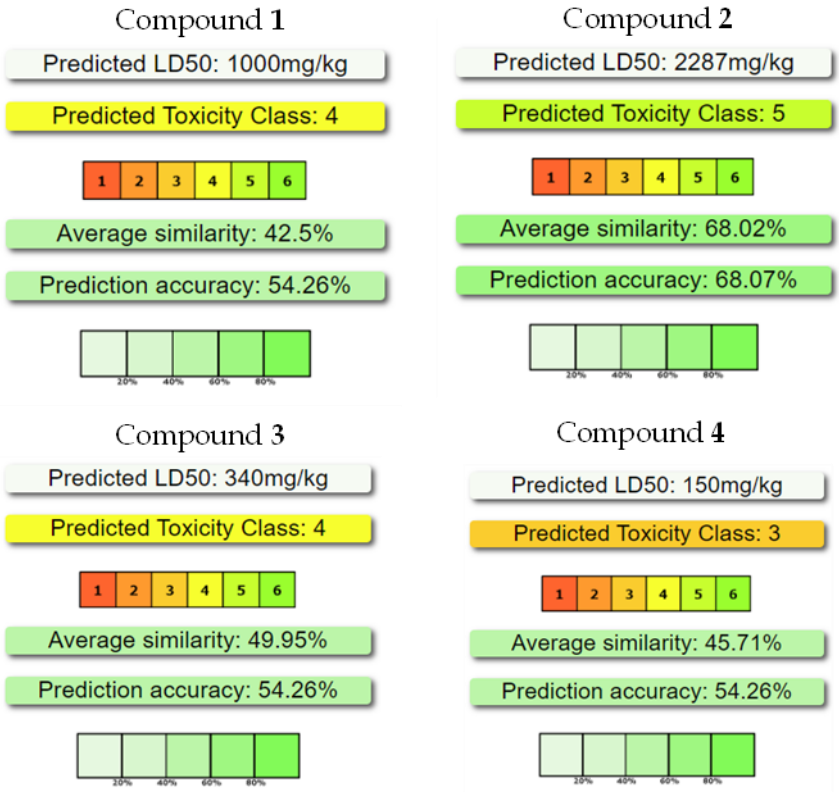

Figure 9. Toxicity report of compounds 1–4.

shows that the loss of 42 mass units might be attributed to acetyl group cleavage [37]. Compound 4 has the same fundamental structure as Notoamide I, which is an alkaloid from the prenylated indole alkaloid family, but it has an acetyl group to the tryptophan amine ring. It is also suggested that tryptophan and acetyl-CoA are essential starting ingredients in the production of notoamide. Tryptophan-derived intermediates, which are commonly generated by the NRPS process, interact with polyketide chains created by PKS. Then, hydroxylase inserts a hydroxyl group, which is acetylated by acetyltransferase [38].

3.6. Drug likeliness Properties

To evaluate the pharmacokinetic properties and potential toxicity of the compound. The active fraction 22PLP1F1 was evaluated *for drug-likeness* and vital pharmacokinetic properties related to bioavailability, Lipinski score, metabolism, and excretion properties (Table 1). Compounds **1** and **3** have the ability to inhibit cytochrome P450 3A4 and the 4 compounds are thought to be substrates for P-glycoprotein 1. P-glycoprotein 1 or multidrug resistance protein 1 is an efflux transporter which is responsible for pumping various organic compounds out of cells, including enterocytes, thereby regulating their bioaccumulation. Then, compounds **1**, **2**, **3**, and **4** have low bioavailability scores; has a bioavailability score of 0.55, which indicates that the compound passes the Lipinski Ro5 and thus shows a possible bioavailability of >10% or Caco-2 permeability. High molecular flexibility and polarity resulted in a (ROTB) of less than 10 and a TPSA of less than 140 $Å^2$ for all compounds except compound **3**, indicating high predicted oral bioavailability. TPSA has a unique role in the field of medicinal chemistry due to its study-based prediction of absorption in the gut and brain [39]. A TPSA value < 140 $Å^2$ indicates good intestinal absorption [40] while a TPSA value < 60 $Å^2$ indicates good blood brain barrier penetration. If the surface area of the polar topology is greater than 140 $Å^2$, HBA > 10, HBD > 5, MW > 500, or if the compound has a strong acid, the organic permeability of the cell is likely limited. In addition, for an organic compound to be normally active, the bioavailability score must be greater than 0.00, between 0.00 and 0.50 for moderate activity, and less than 0.50 for inactive [41]. Molecule weights

below 500 Da, increased lipophilicity (expressed as log P less than 5), and less than 10 HBA required, and less than 5 HBD and 40–130 is the best range for molar refraction [42].

The BOILED-Egg method was carried out to further confirm the GI absorption and blood brain barrier (BBB) permeant properties of the four selected compounds (Figure 8(b)) [43]. The yellow part represents egg yolk, containing compounds whose physicochemical properties make them less likely to penetrate through the BBB. The white part contains compounds that indicate a high probability of passive GI absorption, whereas the outer gray area contains molecules that have low absorption and limited brain penetration. As we can observe, there are four compounds are not found in the yolk (yellow part), so they can be developed further because they are not BBB permeant. In addition, compound **3** is close to the passive permeation area of the BBB and, thus, its use may cause potential harm. In contrast to compound **4**, it is safer because it has a position away from the BBB permeant. Compounds **1** and **4** show good physicochemical properties so that they can be absorbed passively in the GI tract. Only compound **2** has low absorption. Moreover, all of them represent blue dots, which means they are actively released by P-glycoprotein. P-glycoprotein pumps out substrate/drug, often the main cause of drug resistance.

3.7. ProTox

ProTox analysis categorizes ligands into 6 toxicity class categories based on the LD_{50} value. In this study, the active extract was evaluated for the risk of liver damage, heart damage, cancer-triggering properties, and the possibility of carrying mutations (Table 2). The ProTox-II program categorizes ligands into six toxicity classes based on the LD_{50} score, which is an estimate of the lethal dose at which 50% of test subjects die from oral exposure. Among the examined compounds, only compound **2** was found to be a member of class 5 ($2000 < LD_{50} \leq 5000$), indicating its possible non-toxic nature, and was generally found to be harmless if exposed orally. Then, the identified compounds **1** and **3** are class 4 candidates ($300 < LD_{50} \leq 2000$), which shows a low probability of being toxic when ingested orally. Meanwhile, compound **4** was found to be Class 3 ($50 < LD_{50} \leq 300$), indicating that both can be dangerous if consumed (Figure 9). Furthermore, these compounds are classified as safe and have no impact on liver damage, heart disease, cancer triggers, and mutation carrying agents, except compound **3** that have the potential to cause heart

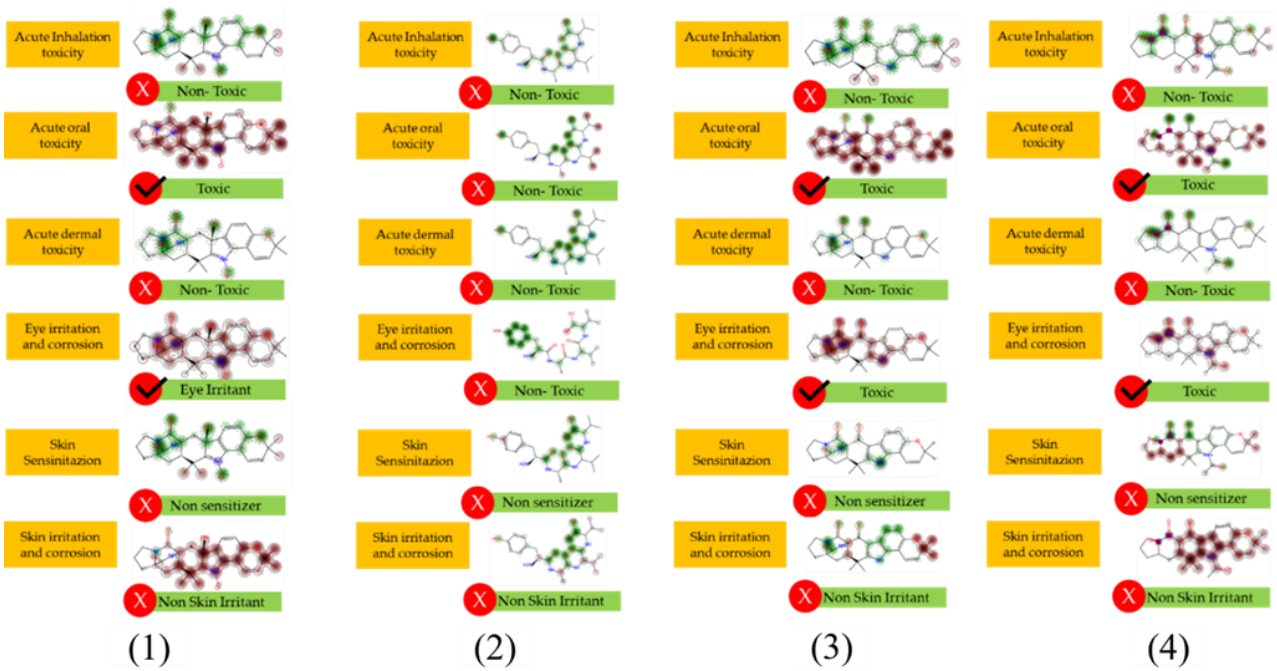

Figure 10. Stoptox prediction of compounds (1) – (4).

damage. The predicted results are examined in Figure 9.

3.8. StopTOX

Acute toxicity analysis with the machine learning-based web server, sTopTox (https://stoptox.mml.unc.edu/), which provides a six-pack of toxicity endpoints including (acute oral toxicity, acute skin toxicity, acute inhalation toxicity, irritation and skin corrosion, eye irritation and corrosion, and skin sensitization). The test results are shown in Table 3. Fragment contribution in addition to six-pack toxicity predictions for drug molecules. Compounds **1**–**4** do not show toxicity in inhalation and dermal, and do not have a skin sensitization effect. Furthermore, only compound **2** is not acutely oral toxic. Irritation and corrosion of eyes and skin, compounds **1**–**4** have no effect on the skin, however, compounds **1**–**3** have the potential to trigger acute irritation and corrosion to the eyes. Using this map (Figure 10), structural fragments predicted to increase toxicity are highlighted in red, and fragments predicted to decrease toxicity are highlighted in green [44].

4. CONCLUSIONS

The EtOAc extract of endophytic fungus *A. sydowii* 22PLP1F1, when cultivated in shrimp shell media, demonstrated an inhibitory effect on *S. aureus* and *P. aeruginosa* bacteria. Chemical analysis using LC-MS/MS identified natural compounds in the extract, specifically the polyketides alkaloids type of notoamide. *In silico* screening of dereplicated metabolites known as compounds **1**, **2**, **3**, and **4** revealed the potential to be antibacterial agent candidates. Also, pharmacokinetic and toxicity evaluation by ADME/ToX indicated that those compounds are non-toxic, supporting its potential as a therapeutic option for MDR bacterial infections. Additionally, *in silico* analysis of StopTox showed no sign of inhalation toxicity, dermal toxicity, or skin carcinogenicity. Further research is necessary to confirm the predicted target of components from the crude extracts.

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Conflicts of Interest

The authors declare no conflict of interest.

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