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Antimalarial Activity Screening from Endophytic Fungus of Red Ginger (*Zingiber officinale)*: *in vitro* and *in silico* Studies

(Penabiran Aktiviti Antimalaria daripada Kulat Endofit Halia Bara (*Zingiber officinale*): Kajian *in vitro* dan *in silico*)

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ABSTRACT

Diversity exploration of secondary metabolite compounds from plant endophytic fungi is expected to yield novel compounds that can efficiently overcome *Plasmodium resistance*. This study aimed to assess the antimalarial activity of the endophytic fungus derived from red ginger *Zingiber officinale* against *Plasmodium berghei*. Subsequently, the endophytic fungus was isolated from the sample using the dilution method, followed by evaporation. Nine isolates of endophytic fungi were successfully isolated that were assigned as JMR1, JMR2, JMR3, JMR5, JMB1, JMB2, JMB3, JMD1, and JMD2. The antimalarial efficacy showed that the JMR5 isolate exhibited significant activity in suppressing the proliferation of *P. berghei*. This activity was quantified by a per cent inhibition of 83.01% and an IC_{50} value of 5.81 µg/mL. The detected antimalarial activity of the JMR5 extract can be related to the presence of several phytochemicals, including alkaloids, flavonoids, and terpenoids. In addition, molecular identification was conducted using ITS primers on the JMR5 isolate, showing a complete genetic similarity of 100% with *Aspergillus flavus*. GNPS analysis was conducted using LCMS-MS data on ethyl acetate extract. Surafactin c, surafactin c14 and erucamide were probably secondary metabolites in the JMR5 extract. Furthermore, drug-likeness and molecular docking analysis were conducted. The result showed that erucamide is a potential antimalarial due to the fulfil of Lipinski's rule of five and also the binding affinity (- 4.2 kcal/mol) against Plasmepsin I. Based on the results obtained, the development of secondary metabolites from *Aspergillus flavus* JMR5 as potential antimalarial compounds is important to carry out.

Keywords: Antimalarial; endophytic fungal; molecular docking; *Zingiber officinale*

ABSTRAK

Penerokaan kepelbagaian sebatian metabolit sekunder daripada kulat endofit tumbuhan dijangka akan menghasilkan sebatian baharu yang boleh mengatasi rintangan *Plasmodium* dengan cekap. Penyelidikan ini bertujuan untuk menilai aktiviti antimalaria kulat endofit yang diperoleh daripada halia bara *Zingiber officinale* terhadap *Plasmodium berghei.* Selepas itu, kulat endofit telah diasingkan daripada sampel menggunakan kaedah pencairan, diikuti dengan penyejatan. Sembilan pencilan kulat endofit telah berjaya dipencilkan yang ditetapkan sebagai JMR1, JMR2, JMR3, JMR5, JMB1, JMB2, JMB3, JMD1 dan JMD2. Keberkesanan antimalaria menunjukkan bahawa pencilan JMR5 mempamerkan aktiviti penting dalam menyekat pembiakan *P. berghei.* Aktiviti ini dikira dengan perencatan peratus sebanyak 83.01% dan nilai IC_{50} sebanyak 5.81 µg/mL. Aktiviti antimalaria yang dikesan daripada ekstrak JMR5 boleh dikaitkan dengan kehadiran beberapa fitokimia, termasuk alkaloid, flavonoid dan terpenoid. Di samping itu, pengenalpastian molekul telah dijalankan menggunakan primer ITS pada pencilan JMR5, menunjukkan persamaan genetik lengkap 100% dengan *Aspergillus flavus*. Analisis GNPS dijalankan menggunakan data LCMS-MS pada ekstrak etil asetat. Surafactin c, surafactin c14 dan erucamide mungkin merupakan metabolit sekunder dalam ekstrak JMR5. Tambahan pula, analisis keserupaan dadah dan dok molekul telah dijalankan. Hasil kajian menunjukkan bahawa erucamide berpotensi sebagai antimalaria kerana memenuhi peraturan

lima Lipinski dan juga afiniti mengikat (- 4.2 kcal/mol) terhadap Plasmepsin I. Berdasarkan keputusan yang diperoleh, pembangunan metabolit sekunder daripada *Aspergillus flavus* JMR5 sebagai sebatian antimalaria yang berpotensi adalah penting untuk dijalankan.

Kata kunci: Antimalaria; dok molekul; kulat endofit; *Zingiber officinale*

INTRODUCTION

Research on new compounds that can suppress the activity of *Plasmodium* parasites has been encouraged by cases of malaria treatment resistance that have been reported to date (Belete 2020; Wicht, Mok & Fidock 2020). Malaria treatment is effective through Artemisinin combination therapy (ACT), however, certain drug combinations, such as the artesunate-pyronaridine (ASPY) combination, have been found to exhibit hepatotoxic consequences. New antimalarial agents with broad-spectrum efficacy against malaria and distinct molecular mechanisms of action are required for single-dose treatment to prevent the toxicity associated with these drug combinations (Belete 2020; Nsanzabana 2019). Hence, investigating natural products emerges as a significant approach in the quest for novel antimalarial compounds, represented by the alkaloid groups (Moyo et al. 2020; Tajuddeen & Van Heerden 2019).

Researchers are currently interested in investigating compounds derived from endophytic fungi. This interest originates from the fungi's ability to produce secondary metabolite compounds that exhibit bioactivity against various diseases, including the inhibition of *Plasmodium* growth. Several studies have explored this bioactivity, including those conducted by Calcul et al. (2013), Ferreira et al. (2019), Ibrahim et al. (2018), and Kumarihamy et al. (2019). Endophytic fungi are microorganisms that reside within plant tissues without causing any visible harm to the host plant's growth (Calcul et al. 2013). According to the findings of Ibrahim et al. (2018), the fusaripeptide compound derived from the endophytic fungus *Fusarium* sp. exhibits promising antimalarial properties against *Plasmodium falciparum*, with an IC_{50} value of 0.34 µM. In addition, the bioactive compound, oxalic acid, isolated from endophytic fungus *Lasiodiplodia theobromae* showed potential antimalarial against *P. falciparum* with an IC_{50} of 5.4 µg/mL (Nwobodo et al. 2024).

This research explored the endophytic fungi associated with *Zingiber officinale*, often known as red ginger. Limited information is available regarding the active compounds in *Zingiber officinale* that may be utilized as antimalarial candidates. However, a study by Faloye et al. (2024) showed that extracts from *Z. officinale* can inhibit 64% parasitemia in red blood cells infected with *P. falciparum*. Based on this information, research on the isolation of endophytic fungi from *Z. officinale* is important to conduct. The antimalarial activity of the ethyl acetate extract from each strain of the *Zingiber officinale* endophytic fungus was assessed *in vitro* using red blood cells infected with *P. berghei* and through molecular docking with Plasmepsin I for the *in silico* study. Plasmepsin is one kind of aspartic protease enzyme that appear in the Plasmodium. There are four types of Plasmepsin, Plasmepsin 1 until Plasmepsin IV. Inhibition of Plasmepsin will lead to the loss of haemoglobin degradation and inhibit the growth cycle of Plasmodium which ultimately prevents the severity of the disease in the infected people and triggers the recovery process (Bhaumik et al. 2011). Therefore, Plasmepsin I was chosen to be a protein target in molecular docking analysis. The potential of fungal isolate based on *in vitro* antimalarial test was analyzed by its compounds by LCMS-MS, then studied molecular docking analysis with plasmepsin I as a protein target to inhibit the proliferation of *Plasmodium*.

MATERIALS AND METHODS

SAMPLE PREPARATION

Red ginger *Z. officinale* is a medical plant collected from the Laboratory of Biota Sumatran, Indonesia. This plant was a host for exploring endophytic fungus to antimalaria agents. Stems, rhizomes, and leaves of this plant were separated then sterilized their surface using ethanol 70%. Samples were then brought to the laboratory to be collected on the isolation endophytic fungus using a diluted method (Artasasta et al. 2017). Once the pure isolate was obtained, it was cultivated into white rice (100 g) for four weeks at room temperature.

Extraction Metabolite Secondary

Extraction of secondary metabolite compounds from *Z. officinale* fungal isolates was carried out using ethyl acetate solvent with a ratio of (3:1). The ethyl acetate extract was then evaporated using a rotary evaporator to obtain a dry extract for further testing.

Antimalarial Assay

Antimalarial testing was conducted *in vitro* using *Plasmodium berghei* ANKA. The antimalarial testing method is based on the study by Orjuela-Sánchez et al. (2011). The *P. berghei* ANKA strain, obtained from the Parasitology Laboratory, Faculty of Medicine, Universitas Brawjaya, was injected into donor mice of the *Mus muculus* Balb/c species. Donor mice were allowed to experience *P. berghei* infection for 3-5 days until they reached a blood parasitemia percentage of 3-5%. Histopaque was used to isolate the red blood cells from the leukocyte- and monocyte-infected mice. Then, the infected red blood cells that were ready were cultured in 24 well plates using 20% FBS media 0.1% gelatin in RPMI for 2 × 24 h in a

CO_2 incubator at 32 °C and with a hematocrit of 2.5%. Testing can be performed if 4-6% of the red blood cells in the well are infected with *P. berghei.* A 24-well plate containing 200 µg/mL of the test extract was also used for the screening step. After testing, a blood smear was created using cover glass in each well to determine the per cent parasitemia result. In addition, the percentage of infected red blood cells for every 1000 cells was measured under a microscope. To find the IC_{50} value, extracts exhibiting a significant reduction in parasitemia percentage were subjected to a concentration series of 100, 10, and 1 µg/mL (link the statement with the equations herewith, should use the right multiply symbol, ×)

$$\text{Parasite percentage} = \frac{\Sigma \text{ infected red blood cells}}{1000 \text{ red blood cells}} \times 100\ \%$$

$$\text{Inhibition percentage} = \frac{\Sigma \text{ infected red blood cells (pretreatment)} - \Sigma \text{ infected red blood cells (after treatment)}}{\Sigma \text{ infected red blood cells (pretreatment)}} \times 100\ \%$$

PHYTOCHEMICAL DETERMINATION

The concentration of the crude extract tested was 2%. The appearance of a precipitate or discolouration serves as the test's indication (Shaikh & Patil 2020).

Triterpenoid (Steroid and Terpenoid)

Endophytic fungus extracts were solubilized and agitated in a ammonia-chloroform and 2N H_2SO_4 mixture. When two separate layers were generated, the underlying layer evaporated and subsequently mixed with anhydrous acetic acid and H_2SO_4 pro analyst. Steroids exhibited a chromatic transition towards a green-blue hue, whereas terpenoids manifested a distinct alteration in colouration towards a red colour.

Alkaloid

The alkaloid test uses three reagents: Dragendorff, Mayer, and Wagner. The top layer of the steroid test was added with each reagent in each test tube. The presence of brown, white, and orange precipitates indicates the presence of alkaloids.

Phenolic

A volume of 2 mL of endophytic fungal extract was combined with 1 mL of a solution containing 1% FeCl3. The indication of the existence of phenolic compounds is manifested through a visible alteration in colour, specifically to a pink hue.

Flavonoid

A volume of 2 mL of endophytic fungal extract was obtained and subsequently heated for approximately 5 min. Then, 0.1 grams of magnesium metal powder were added, followed by a strong hydrochloric acid solution. Flavonoid-induced positive outcomes are characterized by a noticeable change in colouration, typically shifting towards hues of red or orange.

IDENTIFICATION OF ENDOPHYTIC FUNGI POTENTIAL AS ANTIMALARIALS

Macroscopic and Microscopic Identification

Macroscopic and microscopic identification was performed on red ginger endophytic fungal isolates with potential antimalarial activity. Ni Putu, Ketut Srie and Ida Ayu (2018) employed macroscopic identification techniques, which involved analyzing the colour parameters of both the surface and bottom of the colony, as well as measuring the diameter of the colony on the seventh day of growth. The red ginger endophytic fungal isolates were subjected to macroscopic and microscopic identification techniques to assess their potential antimalarial activity. Barnet and Hunter (1998) employed the method for microscopic identification. Samples of potential isolates were obtained sterilely using a sterile inoculation needle and subsequently inoculated onto the surface of a glass plate. Subsequently, the cultural specimens were treated with lactophenol cotton blue solution, followed by the placement of a cover glass over them. The resulting preparations were then examined using a microscope, specifically at a magnification level of 400×. The observations included the identification of hyphae and reproductive structures.

Molecular Identification

Pure isolates of red ginger endophytic fungi with the most potential antimalarial activity were isolated from the DNA using the method of Saitoh et al. (2006). The DNA was amplified using ITS primer and then sequenced to First Base, Malaysia. Subsequently, the obtained DNA sequences were compared to fungal DNA sequences accessible on the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) algorithm (Handayani et al. 2019). The BioEdit application examined alterations in nucleotide bases inside DNA sequences. In addition, the DNA sequences of endophytic fungi were aligned with sequences from the database using Clustal W, resulting in generating a file in *FASTA* format. The MEGA-X application was utilized to input the alignment result file to identify the optimal substitution model for phylogenetic tree analysis (Engeset et al. 2003). Subsequently, a phylogenetic tree was constructed to illustrate the degree of similarity among sequences, using the neighbour-joining algorithm approach with *1000 Bootstrap*.

LCMS-MS Measurements and Molecular Networking

The potential of endophytic fungal isolate extract to antimalarial was determined its compounds by using LCMS-MS. The extract was dissolved in MeOH

to 1 mg/mL. The injection volume was 2 µL to LCMS-MS measurement. MicroOTOF-QII was used to detect the mass spectra utilizing an EC10/2 Nucleoshell C18 2.7 µM column. MS data was obtained in positive mode over a range from 100 to 1000 *m/z*. MS/MS fragmentation was performed with ions above a threshold of 100 and by increasing collision energy at 4Hz. MS data then was uploaded to https://gnps.ucsd.edu/) by using mzXML file format to compare reference spectra in GNPS (Global Natural Product Social) spectral libraries. The visualitation of network files was conducted by using Cystoscape 3.7.2 program (Riyanti et al. 2020).

Drug-likeness Analysis

The proper compounds in GNPS spectral libraries of potential antimalarial extract were evaluated for their drug-likeness properties based on Lipinski's rule of five. The SMILES codes of each compound are subjected to http://swissadme.ch to obtain drug-likeness data for each compound. Artemisinin was used as a positive control.

Molecular Docking Analysis

The molecular docking analysis was performed on a compound that conformed to Lipinski's rule with plasmepsin I (PDB ID: 3QRV). As receptor, Plasmepsin I (Plms I) was obtained from http://www.rcsb.org. The pdb format of the potential compound (ligand) is obtained by submitting the SMILE code to https://cactus.nci.nih.gov/translate/. AutoDock Vina was used to performing docking analysis with grid parameters of the receptor are X= 122; Y=30; Z=92 for grid box size (Å) and X=15.606; Y=7.511; Z= 19.33 for grid center coordinate (Å). The binding affinity value and amino acid residues of compounds tested to plasmepsin I are collected and comparing these values to the bond between artemisinin and plasmepsin I using Discovery Studio Visualizer (DSV).

RESULTS AND DISCUSSION

The emergence of drug-resistant malaria cases has led researchers to investigate the components of natural products to discover novel compounds with antimalarial properties (Pan et al. 2018). The investigation of secondary metabolite compounds derived from endophytic fungus residing in medicinal plants presents a viable approach for identifying prospective compounds that could address the issue of medication resistance in malaria cases (Alhadrami et al. 2021; Calcul et al. 2013; Kongsaeree et al. 2003; Tansuwan et al. 2007).

The present investigation involved the isolation of endophytic fungi from *Zingiber officinale*. A total of nine fungal isolates were isolated, each assigned a unique code: JMR1, JMR2, JMR3, JMR5, JMB1, JMB2, JMB3, JMD1, and JMD2. According to Table 1, it has been shown that various fungal isolate extracts exhibit distinct antimalarial activity, as determined by the percentage of red blood cell parasitemia evaluated *in vitro*.

After applying the test extracts at a concentration of 200 µg/mL each, the percent parasitemia value was determined. The result showed that extract isolate JMR5 was known to have the greatest reduction in percent parasitemia from 5.3% to 0.9% with a percent inhibition of 83.01%. Artemisinin used as a positive control, exhibited a significant reduction in the percentage of parasitemia, decreasing from 5% to 0.63%. This corresponds to a percent inhibition value of 88.11%. On the other hand, red blood cells that were infected with *P. berghei* but not treated showed a rise in the percentage of parasitemia from 4% to 8.63%. The antimalarial activity of the JMR5 isolate extract was further investigated to identify the IC_{50} value, which was 5.81 µg/mL (Table 2). Compared to artemisinin as a positive control, it has an IC_{50} value of 0.46 µg/mL.

The antimalarial activity of each isolate of endophytic fungal isolates from *Z. officinale* varies due to diverse secondary metabolites, which exhibit diverse bioactivities. Table 3 shows a qualitative description of the diversity of phytochemical content found in each extract of the fungal isolates. The antimalarial activity of the ethyl acetate extract of fungal isolate JMR 5 is attributed to its flavonoid, alkaloid, and terpenoid content. These chemical groups exhibit distinct ways of preventing the growth of *P. berghei*. The flavonoid and terpenoid drugs suppress the growth of *P. berghei* by preventing the formation of hemozoin through the inhibition of heme polymerization (Lehane & Saliba 2008). The alkaloid compounds function by stabilizing the erythrocyte membrane, which the invasion of *P. berghei* has disrupted. This stabilization prevents erythrocyte hemolysis and inhibits the conversion of heme into hemozoin, similar to flavonoids (Opajobi et al. 2022). Terpenoids can prevent the growth of *P. berghei* by inhibiting dolicol production during the trophozoite and schizont phases (Teka, Awgichew & Kassahun 2020).

Antimalarial activity testing is conducted when the percentage of red blood cells infected with *P. berghei* reaches 4-6% parasitemia. The percentage of parasitemia can be determined by examining the morphology of erythrocytes using a microscope (Figure 1). According to Lee, Cox-Singh and Singh (2009), normal erythrocytes exhibit a spherical morphology, possess a pale purple hue, and lack a cell nucleus. The infected erythrocytes display a spherical morphology and have a deep purple hue. Within these erythrocytes, the presence of *P. berghei* may be observed in the ring stage, trophozoite stage, and schizont phase.

The screening results indicate that the endophytic fungal isolate JMR5 has demonstrated promise in generating antimalarial drugs. Figure 2 displays the anterior and posterior perspectives of the JMR5 isolate, along with its microscopic examination. The JMR5 isolate cultivated on PDA media has a green colour on the front and a beige colour on the back. The growth diameter ranges from 25 to 30 mm and occurs throughout 4 to 7 days. Microscopic investigations showed that JMR5 has vesicles, spherical conidia, and robust conidiophores. JMR5 is classified within the genus Aspergillus based on its macroscopic and

TABLE 1. Percent red blood cell (RBC) parasitemia after administration of ethyl acetate extract of red ginger endophytic fungi isolates *Z. officinale* with a 200 µg/mL concentration

No	Sample	Percent parasitemia after testing	Percent inhibition
1	JMR1	2.7%	49.05%
2	JMR2	1.3%	75%
3	JMR5	0.9%	83,01%
4	JMB1	2%	62.26%
5	JMB2	2.9%	45.28%
6	JMB3	2.8%	47.16%
7	JMD1	1.8%	66.03%
8	JMD2	2%	62.26%
10	Negative control	8.63%	

TABLE 2. IC_{50} value of potential endophytic fungi JMR5

Concentration (µg/mL)	Percent inhibition (%)	IC_{50} (µg/mL)
200	83.01	5.81
100	65.62	
10	53.75	
1	37.50	

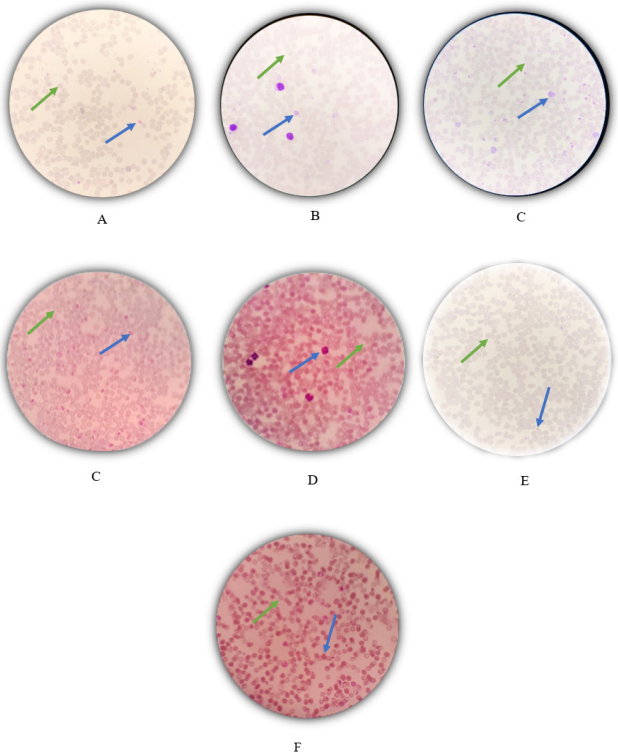


FIGURE 1. Observation of percent parasitemia (normal erythrocyte; infected erythrocyte-*P. berghei*); JMR5 extract assay for each concentration A: 200 µg/mL, B: 100 µg/mL, C: 10 µg/mL, D: 1 µg/mL; E: positive control (artemisinin); and F: negative control (without treatment)

TABLE 3. Phytochemical content of each ethyl acetate extract of *Z. officinale* endophytic fungal isolates

Sample	Phytochemical content			
	Alkaloid	Flavanoid	Terpenoid	Steroid
JMR1	+	-	+	-
JMR2	+	-	+	-
JMR5	+	+	+	-
JMB1	-	-	+	-
JMB2	+	-	-	-
JMB3	+	-	-	-
JMD1	+	-	+	-
JMD2	-	+	-	-

microscopic characteristics. Afzal et al. (2013) conducted research showing that the genus Aspergillus possesses circular conidia, vesicles, and conidiophores.

Furthermore, isolate JMR5 was identified molecularly using primers ITS to determine the isolate species. Figure 3 is a phylogenetic tree of DNA sequences of isolate JMR5 that illustrates its relationship with several other fungal DNA sequences. The isolate JMR5 has a 100% similarity in homology with the species *Aspergillus flavus*. Based on the Kimura 2- parameter model, JMR5 is identic with *Aspergillus flavus* LSPQ-NSM-001 with accession number KU761013.1 because there are no genetic differences (Table 4). Currently, there is less information regarding the antimalarial activity of secondary metabolites from *A. flavus*. However, research on the antimalarial activity of the *Aspergillus* genus has elucidated its potential as a source of antimalarial compounds. The astechcrome compound, an alkaloid group chemical derived from the fungus *A. terreus*, exhibits potential antimalarial activity against *P. falciparum* parasites, with an IC_{50} value of 0.94 µM (Bunbamrung et al. 2020). Furthermore, *A. aculeatus*, a different genus of *Aspergillus*, produces secalonic acid, variecolin, and ergosterol compounds (Yodsing et al. 2018). These three flavonoids have the potential to function as antimalarial agents against *P. falciparum*, with IC_{50} values of 1.03, 1.47, and 5.31 µg/mL, respectively (Yodsing et al. 2018).

The ethyl acetate extract of JMR5 was subjected to LCMS-MS analysis. Three compounds are dereplicated based on the GNPS library, namely, surfactin C, surfactin C14, and erucamide, with error per cent 13, 2 dan 2%, respectively (Table 5). Surfactin C and C14 are surfactant compounds that are often found in bacterial groups such as *Bacillus subtilis* and *Pseudomonas aeruginosa*. There is less information regarding the presence of surfactin C and C14 in fungal groups such as Aspergillus. However, Taofeeq Adekunle et al. (2015) reported a surfactant compound in A. *flavus*, namely glycosophrolipid sf2, which has an antibacterial activity. Otherwise, erucamide is an amide fatty acid compound often found in endophytic fungi (Tanvir, Javeed & Rehman 2018). As a study by Anisha and Radhakrishnan (2017), endophytic fungi in *Z. officinale* are often found to contain amide fatty acid derivative compounds which have roles as antibacterial, antifungal, antinematicidal and antimalarial.

Pharmacokinetics properties such as molecular weight, log P, number of hydrogen bond donors and acceptor, and polar surface area (PSA) of hit compounds of JMR5 extract in GNPS were carried out. These parameters show a compound's ability to be developed into a lead compound for a drug known as Lipinski's Rule of Five. The molecular weight of a compound is a parameter of its ability to pass through cell membranes, namely less than 500 Da; log P value of less than 5 indicates its ability to reduce toxicity when passing through the blood vessels; The number of hydrogen donors and acceptors less than 5 and 10, respectively, indicates potential biological activity; PSA value of less than 140 Å indicates its permeability (Kuo 2011; Lipinski 2004; Neidle 2012; Turner & Agatonovic-Kustrin 2006). Table 6 shows that only erucamide meets Lipinski's rule, which shows a good predicted ability to penetrate membranes, as shown by artemisinin as an antimalarial drug.

Furthermore, the bioactivity of erucamide as an antimalarial can be predicted *in silico* against plasmepsin I. This protein is a group of aspartic proteases in *P. falciparum* that plays a role in degrading hemoglobin for the invasion of red blood cells (Nasamu et al. 2020). Table 7 shows the binding affinity between erucimade and Plms I compared with positive control i.e., artemisinin and Plms I. The value of binding affinity between erucamide and artemisinin against Plms I were - 4.2 and - 7.2 kcal/mol, respectively. The interaction between the ligand and the receptor can be observed through the bond between the ligand and the active site (amino acid residue) of the receptor, shown in Figure 4 through a 2D visualization form. Erucamide-Plms I interaction showed two conventional H-bonds with two amino acids (Val237 and Phe232) and six pi-alkyl interactions with three amino acids (Lys239, Phe242, and Pro241). While artemisinin-Plms I interaction

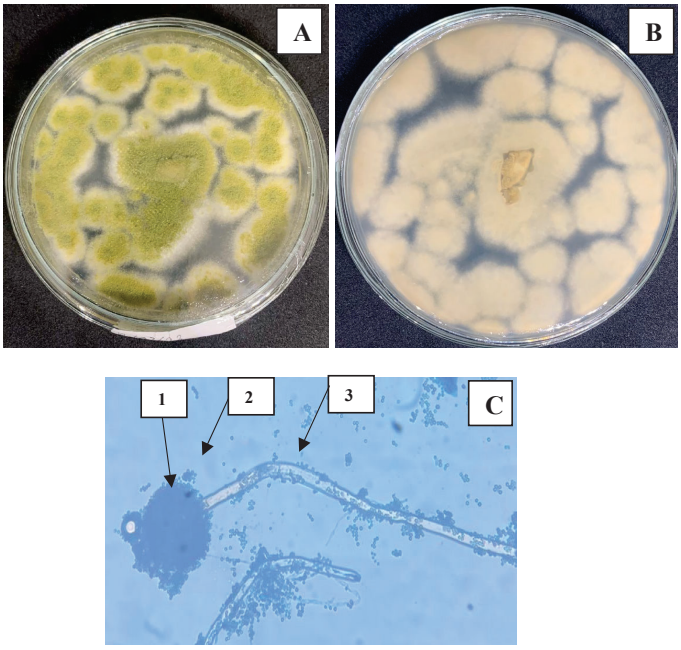


FIGURE 2. Macroscopic and microscopic observations of isolate JMR5; A: surface colony of JMR5, B: reverse colony of JMR5; C: Microscopic appearance of JMR 5, 1: vesicle, 2: conidia, 3: conidiophore

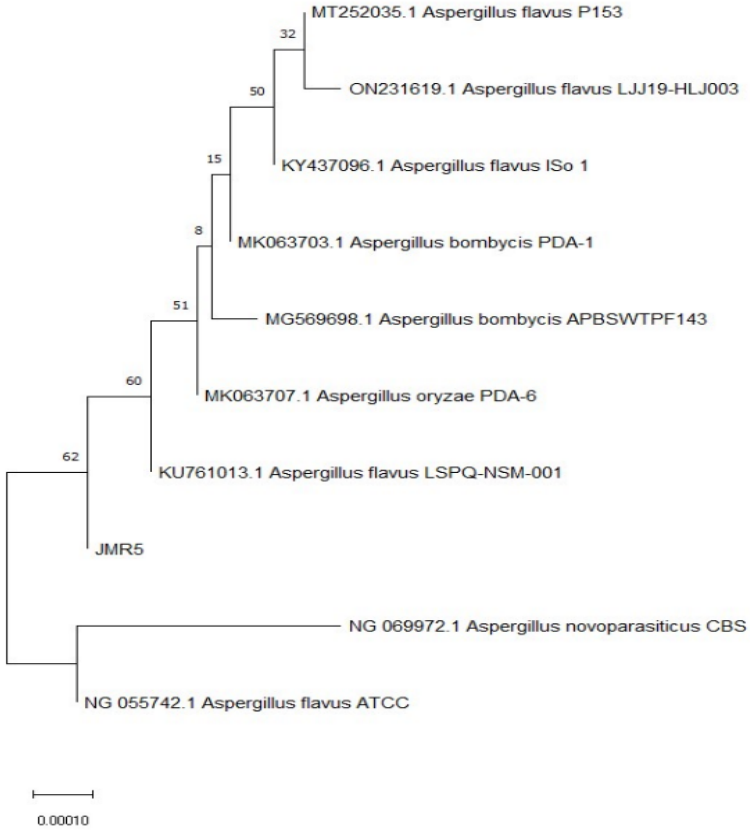


FIGURE 3. Phylogenetic tree showing the relationship of isolate JMR5 with *A. flavus* constructed by the neighbour-joining method through a bootstrap value of 1000

TABLE 4. The Kimura 2-parameter model between JMR 5 and other sequence strains

	Accession Number	1	2	3	4	5	6	7	8	9	10
1	JMR5										
2	KU761013.1	0.0000									
3	MK063703.1	0.0000	0.0000								
4	MK063707.1	0.0000	0.0000	0.0000							
5	MT252035.1	0.0000	0.0000	0.0000	0.0000						
6	NG069972.1	0.0000	0.0016	0.0000	0.0000	0.0015					
7	ON231619.1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0016				
8	NG055742.1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000			
9	KY437096.1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0016	0.0000	0.0000		
10	MG569698.1	0.0000	0.0008	0.0000	0.0000	0.0000	0.0016	0.0008	0.000	0.0000	

TABLE 5. Global Natural Product Social molecular networking **(**GNPS) data analysis of ethyl acetate extract of JMR5

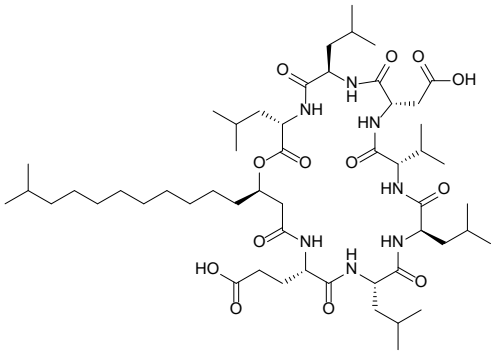
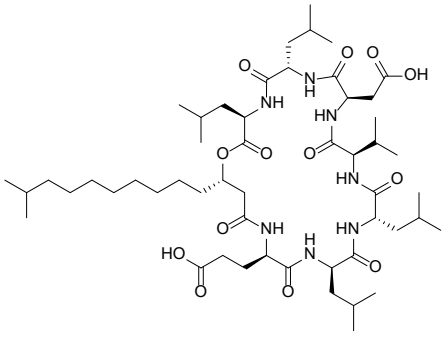
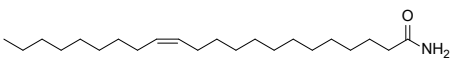
Parent mass	Compound Hit	Molecular weight (g/mol)	Percent Error (%)
1036.700/1036710	 Surfactin C	1036.3	13
1022.680	 Surfactin C14	1022.3	2
338.341	 Erucamide	337.6	2

TABLE 6. Compounds characteristics with the Lipinski's rule of five parameters

Ligand/Compound	Molecular weight (g/mol)	Log P	Number of hydrogen bond donors	Number of hydrogen bond acceptor	Polar surface area (Å)
Surfactin C	1036.3	3.90	9	13	304.60
Surfactin C14	1022.3	5.58	9	13	304.60
Erucamide	337.6	5.10	1	1	43.09
Artemisinin*	282.33	1.39	0	5	53.99

TABLE 7. The binding affinity value of antimalarial potential compound with Plms I. Why is both RMSD value is zero? Is it important to show in the table?

Ligand/Compound	Plms I (kcal/mol)	RMSD (Å)
Erucamide	-4.2	0.00
Artemisinin*	-7.6	0.00

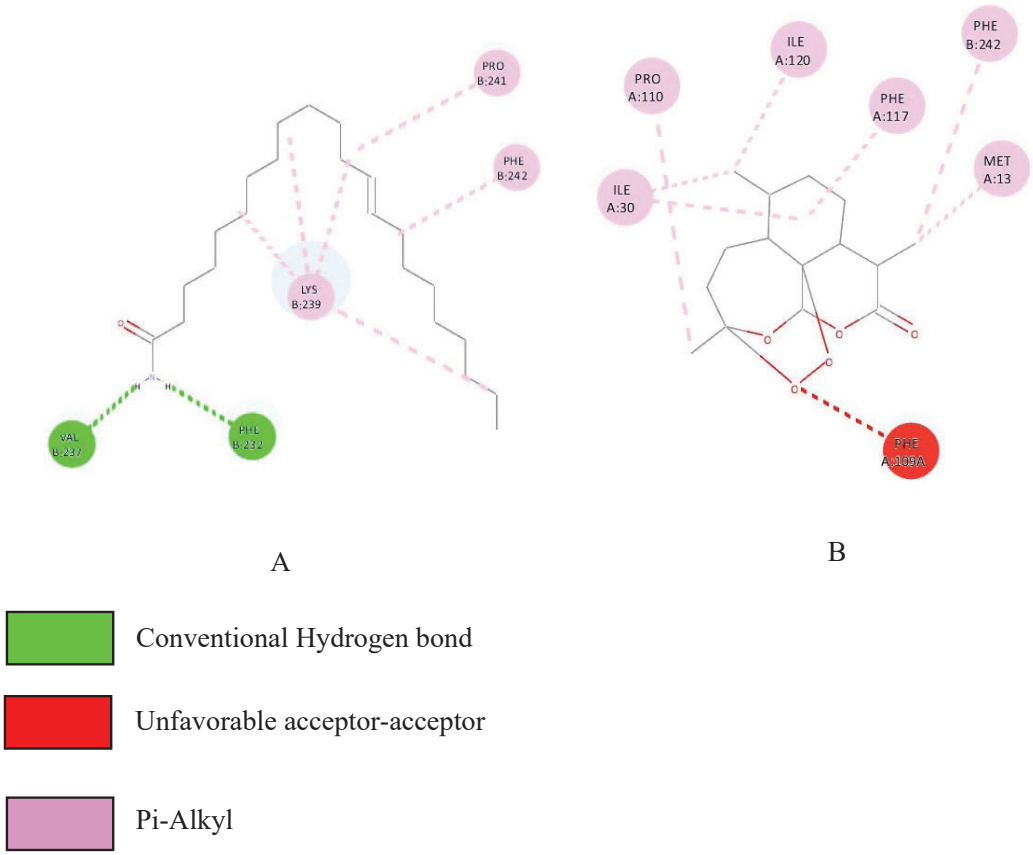


FIGURE 4. Ligand and receptor interaction; A. erucamide with Plms I; B. artemisinin with Plms I

showed one unfavourable acceptor-acceptor with one amino acid (Phe109) and seven pi-alkyl interactions with six amino acids (Ile30, Pro110, Ile120, Phe117, Phe242, and Met13).

Molecular docking is an *in silico* analysis that determines the bioactivity of a compound towards target proteins involved in molecular disease. The results of molecular docking can be seen from the binding affinity value; the smaller it is, the stronger the binding strength is. It concluded that the ligand has the potential to inhibit protein targets. In addition, there is an interaction between the ligand and the active site of the receptor (amino acid residues), which is shown in the form of 2D visualization (Astalakshmi et al. 2022; Pantsar & Poso 2018). Molecular docking results can be validated from the root mean square division (RMSD) value, which is valid if the value is below 2 Å (Bell & Zhang 2019). The results of the docking analysis stated that erucamide can influence the activity of Plms I in terms of its binding strength with a small binding affinity value. However, compared with the positive control, artemisinin, showed that it has a greater effect in inhibiting Plms I activity. Further information is needed regarding the abundance of secondary metabolites from JMR5 obtained through the isolation (chromatography) and elucidation process (spectroscopy). This should be done to find out the possible compounds that are responsible for inhibiting the growth of the *Plasmodium*.

CONCLUSION

Screening results from ethyl acetate extract of red ginger fungus isolate *Z. officinale,* which has potential antimalarial activity, is JMR5 has similarity with *A. flavus* species. The antimalarial activity of this isolate is thought to be due to the content of flavanoids, alkaloids and terpenoids. Moreover, GNPS analysis confirmed that erucamide is a secondary metabolite in JMR5 extract. The result of docking analysis against Plms I showed that erucamide is a potential antimalarial due to its ability in binding Plms I. Further analysis is needed to determine the abundance of secondary metabolites from JMR5 extract by spectroscopic means to find the compound most responsible for antimalarial activity.

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