Exploring the Bioactive Metabolites with Anti-Malarial Properties Derived from Endophytic Microbial Resources Indigenous to Indonesia

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Abstract: Malaria, caused by parasite infection, is still difficult to cure due to partial treatment resistance. This emphasizes the need for innovative molecules with different modes of action, particularly those derived from unconventional sources such as endophytic microbes. This study investigates endophytes from medicinal plants in order to find possible antimalarial drugs that target Plasmodium falciparum dihydroorotate dehydrogenase (PfDHODH). Endophytes were associated to Mitragyna speciosa, Zingiberaceae, Uncaria gambir, and others, yielding 586 species. Screening using a 96-well plate produced a z-factor of 0.92, indicating trustworthy findings. Hypomycetes (from Physalis angulata L.) and Fusarium sp. (from Hornstedtia scyphifera) showed considerable anti-malarial activity, suppressing PfDHODH by 51.53% and 66.37%, respectively. Metabolomic profiling using LC-HRMS demonstrated that Hypomycetes included bioactive substances such as uracil, kojic acid, and phloroglucinol, whereas Fusarium sp.

1 INTRODUCTION

Anopheles mosquitoes carrying the *Plasmodium* infection bite humans, transmitting the infectious disease malaria. The prevalence of malaria in the world in 2019 was 227 million, and it increased to 241 million people in 2020. Indonesia itself experienced an increase; in 2013, there were 0.4% of cases, and this increased in 2018 to 1.4% (Kemenkes 2018). There are five *Plasmodium* species that cause malaria in humans, but *Plasmodium falciparum* is the most potent and deadly.

The most effective treatment for malaria infection is artemisinin-based combination therapy, which has no side effects. However, over time, *Plasmodium* showed indications of partial resistance to artemisinin derivatives. Antimalarial drugs can inhibit key enzymes responsible for cell growth and parasite development. A group of genes known as *P. falciparum* lactate dehydrogenase (PfLDH), *P. falciparum* dihydroorotate dehydrogenase (PfDHODH), and *P. falciparum* dihydrofolate reductase (PfDHFR) play a big role in the growth of plasmodium parasites. PfDHODH is an important component of parasite metabolism and plays a key role in the de novo pathway of pyrimidine biosynthesis. This enzyme facilitates the conversion of dihydroorotate (DHO) into orotate. This compound

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is essential for generating the pyrimidine bases required for DNA and RNA synthesis (Akinnusi *et al.* 2023). However, it is essential to emphasize that targeted therapy must include safety and specificity, as these treatments must suppress the parasite while inflicting minimal harmful effects on the human body.

Developing antimalarial drugs targeting PfDHODH is a growing area of research to overcome malaria drug resistance and improve malaria treatment. The development of new malaria drugs begins with the exploration of bioactive plant metabolites and even plant microorganisms, one of which is endophyte. Endophytic fungi are microorganisms that grow in host tissue and produce bioactive compounds similar to those of their host without causing disease symptoms. Secondary metabolites produced by endophytic fungi have many activities, such as antioxidants and anti-bacterial. Research on endophytic fungi as a source of anti-malarial bioactive metabolites is still limited, whereas Indonesia is rich in germplasm sources.

Research related to the potential of endophyte bioactive metabolites as antimalarials was carried out by Ibrahim et al. (2017). The bioactive compound fusaripeptide found in *Fusarium* sp., which was isolated from the roots of the *Mentha longifolia* L. plant, potentially kill malaria parasites (Ibrahim et al., 2017). *Fusarium endophytes* from *Cinchona calisaya* trees could also stop *Plasmodium berghei* from growing by 62.18% to 78.41% (Hasbi, 2019).

The objective of this research was to: (1) Screening of extracts was carried out on 587 endophytic fungi that had PfDHODH inhibitory activity; (2) up-scaling cultivation and extraction of *Fusarium* sp. endophytes. which is associated with *Phyisalus angulata* and *Hornstedtia scyphifera var. fusiform*, which has a percentage of >50% inhibition of the PfDHODH enzyme; (3) identified the profile compounds of extract by using HRMS.

2 METHODS

2.1 Materials

The sample population consisted of 586 endophytes. Those endophytes was associated with the *Mitragyna speciosa* (36); *Soultalum album* L (15); *Phyllanthus urinaria* (4); *Myristica fragrans* (41); *Andrographis paniculata* (18); *Piper sarmentosum* (36); *Physalis angulata* L. (15) ; *Chilorantus officinalis* (5); *Staurogyne longata* (5); *Coleus amboinicus* (19); *Nigelia sativa* (22); *Ziziphus mauritiana* (50); *Artemisia annua* (8); *Artemisia vulgaris* (13); *Uncaria Gambier* (74); *Zingiberaceae* (95); *Piper nigrum* (21); *Kaempferia parviflora* (26); *Murraya koenigii* (4); medicinal plants from Mandalika Lombok (35); and medicinal plants from Bali (44).

Enzyme inhibition testing was: DMSO, HEPES, milli-Q water, KOH, NaCl, Triton-X 100, decyl ubiquinone (d-UQ), absolute ethanol, aluminium foil, parafilm, L-dihydro orotate (L-DHO), dichloroindophenol (DCIP), recombinant enzyme PfDHODH.

2.2 Endophytic Bioproduction and Extraction

The endophytic fungi were cultivated in potatoes dextrose broth (PDB) medium. Cultivation was carried out in large-scale media, 7–10 L, at room temperature for several weeks until bioactive metabolites were formed. The process of cultivating endophytic fungal isolates into PDB media was carried out sterilely in laminar air flow (LAF). The extraction was carried out by liquid-liquid extraction technique using ethyl acetate solvent. The extracted material was evaporated using a rotary evaporator, followed by a subsequent drying process with nitrogen gas. The dried extract was stored at a freezing temperature for further processing.

2.3 PfDHODH Inhibition Screening Assay

Measurement refers to the method developed by Pramisandi *et al.* (2021), with some modifications. Screening of extracts/isolates was carried out on 586 endophytic fungi that had PfDHODH inhibitory activity. The inhibitory activity of key target cells was assessed using the recombinant enzyme dihydroorotate dehydrogenase (PfDHODH) derived from *P. falciparum*. The inhibition experiments of PfDHODH were conducted by quantifying the decrease in the reduction of the electron acceptor 2,6-dichloroindophenol (DCIP). The 96-well plate was filled with 2 µL of extract and 190 µL of test solution. Centrifugation was used to mix the the solutions for 30 seconds at 500 rpm. L-dihydroorotate (L-DHO) 5 mM were added to each well to start the enzymatic reaction. Inhibitor activity was determined by spectrophotometer at 600 nm. Inhibitory activity was calculated based on equation (1).

$$\% \text{ Inhibition} = 100 - \left[\left(\frac{\text{Abs.sample} - \text{Abs.Positive control}}{\text{Abs.negative control}}\right) \text{x } 100\%\right] \quad (1)$$

The endophyte fungi that had an inhibition percentage above 50% were selected for bioproduction.

2.4 Compounds Characteristic Using LC-HRMS

Dried extract (1 mg) was soluble using 1 mL of MS grade MeOH. Sample analysis was performed according to Windarsih *et al.* (2022) with some modifications. The analysis was conducted using liquid chromatography with the Thermo Scientific™ Vanquish™ UHPLC Binary Pump, along with Orbitrap high-resolution mass spectrometry using the ThermoScientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ High Resolution Mass Spectrometer. Analytical column of Thermo Scientific™ Accucore™ Phenyl-Hexyl 100 mm × 2.1 mm ID × 2.6 µm was used for liquid chromatography. Using a gradient method and a flow rate of 0.3 mL/min, the mobile phases were MS-grade water containing 0.1% formic acid (A) and MS-grade methanol containing 0.1% formic acid (B). Initially, the mobile phase B was set at 5% and then gradually increased to 90% over a period of 16 minutes. After that, it remained at 90% for 4 minutes and then returned to the initial condition (5% B) until 25 minutes. The temperature of the column was adjusted to 40 ◦C, while the injection volume was set at 3 µL. An untargeted screening was conducted using full MS/dd-MS2 acquisition mode in either positive or negative ionization polarities/states. The spray voltage was set to 3.30 kV, while the capillary temperature and the auxiliary gas heater temperature were set at 320 ◦C and 30 ◦C, respectively. The scan range was conducted from 66.7 to 1000 m/z, with a resolution of 70,000 for full MS and 17,500 for dd-MS2, in both positive and negative ionisation modes.

3 RESULT AND DISCUSSION

3.1 Enzymatic Screening of Endophyte Extract as an Anti-Malarial

Endophytic fungi are a type of fungus that live in plant tissue without causing visible damage or disease in their hosts (Ababutain *et al.* 2021). The metabolites produced are generally similar as those of the host plant. Metabolites generated by endophytes exhibit numerous functions, including antimalarial activity. Malarial assay can be carried out enzymatically, parasitically and computationally. PfDHODH, *P. falciparum* Dihydroorotate Dehydrogenase, plays a vital role as an enzyme in the pyrimidine biosynthesis pathway of the malaria parasite *P. falciparum*.

Primary screening was conducted on 586 endophyte extracts derived from a variety of plant categories and parts, such as leaves, stems, roots, and seeds. Those extracts were plated on a 96-well plate for the PfDHODH assay, Fig.1. There are numerous critical stages that comprise its mechanism. The enzyme attaches to its substrate, dihydroorotate, at its active site and facilitates the chemical reaction that converts dihydroorotate into orotate. This procedure entails the extraction of two electrons and two protons from dihydroorotate. PfDHODH utilizes the coenzyme FMN (Flavin Mononucleotide) as an electron acceptor, facilitating the conversion of FMN from its oxidized state (blue form) to its reduced state (red color), Fig.2. FMN transports electrons to the electron transport chain located in the mitochondrial membrane of *P. falciparum*. This process produces vital energy for the parasite's survival and growth. The end product of the reaction, orotate, is liberated from the active site of the enzyme, so concluding the conversion process. PfDHODH plays a crucial role in the formation of pyrimidine, making it an important target for the development of antimalarial drugs. Inhibiting this enzyme can effectively hinder the growth and reproduction of *P. falciparum* (A. Phillips dan K. Rathod 2012). PfDHODH facilitates an enzymatic process that is linked to the reduction of DCIP. PfDHODH transfers electrons to DCIP, converting it from its oxidized blue form (DCIPox) to its reduced colorless form (DCIPred), as it converts dihydroorotate to orotate and reduces decylubiquinone to decylubiquinol, Fig. 2.

During this study, extracts that demonstrated inhibitory efficacy exceeding 50% were designated as primary findings. There were two extracts identified as the main hits (blue color), particulary endophytes associated with *Physalis angulata* L (51,53%), *Hornstedtia scyphifera* (66,37%). The two endophytes were fungi belonging to the *Hypomycetes* type, which was associated with *Physalis angulata* L, and the *Fusarium* sp., which was associated with *Hornstedtia scyphifera*. The batch codes for these fungi were BgPa1 and HSFP3, respectively.

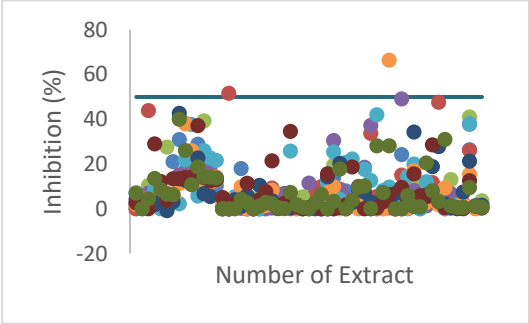


Figure 1: Inhibitory activity of 586 microbial extracts against *Pf*DHODH; purple-dashed line represents threshold line.

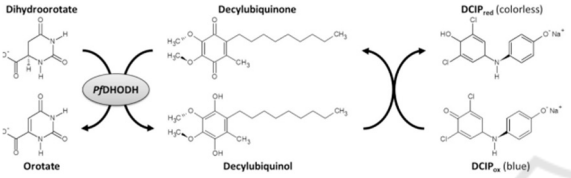


Figure 2: Endophytes extracts are subjected to enzyme-based screening assays to determine their inhibitory activities against PfDHODH (Waluyo *et al.* 2021)

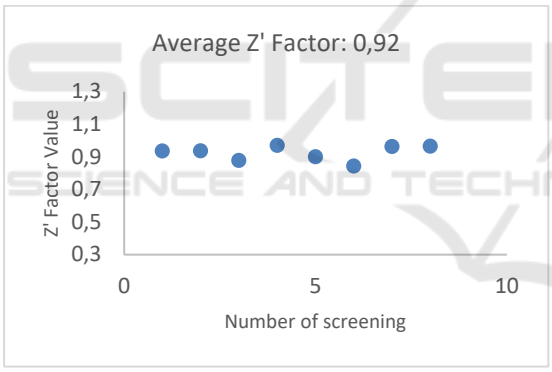


Figure 3: The z-factor value of each assay batch.

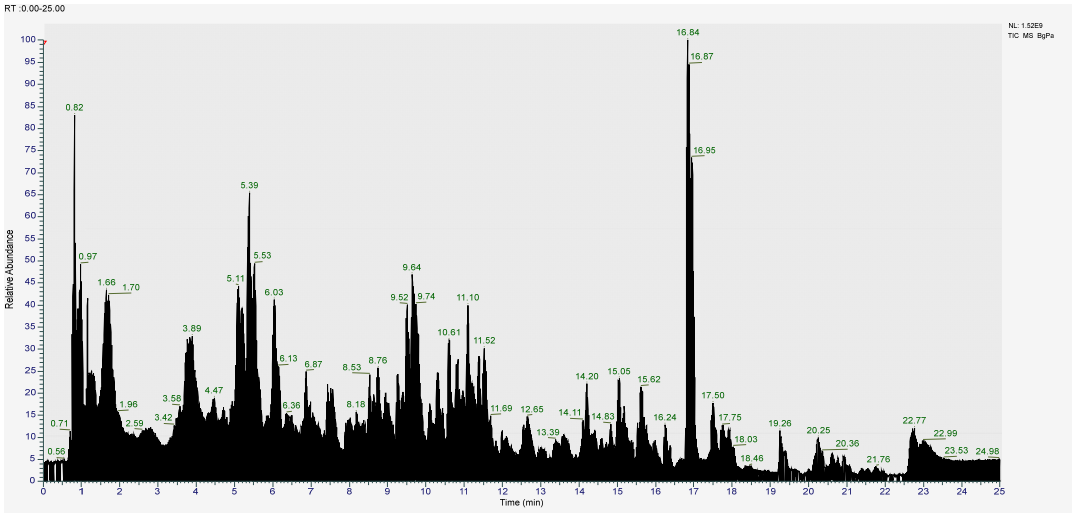
In this HTS study the z score value was also measured. The Z'-factor is a statistical metric that quantifies the reliability of data acquired from high-throughput screening (HTS) experiments. A high Z'-factor indicates that the assay is capable of accurately differentiating between active and inactive substances. The mean value of the z'factor in this research was 0.92 (Figure 3), indicating excellent results.

3.2 Untargeted Compounds Assay

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) was employed for untargeted compounds, allowing for a comprehensive screening of small compounds in the extract. The most often used approach for analysis is reversed-phase liquid chromatography (RPLC), specifically the C18-based method. LCHRMS enables the precise identification of a comprehensive metabolomic profile, encompassing both major and minor components in natural extracts. The novel information obtained from LC/HRMS analysis of the metabolomic profile of endophytes *Hypomycetes* (associated with *Physalis angulata*) and *Fusarium* sp (associated with *Hornstedtia scyphifera*) can be useful for advancing study. Results of the chromatogram analysis for each extract using LC/HRMS are displayed in Figure 1.

The LCHRMS results for the ethyl acetate extracts of endophytes associated with *Physalis angulate* and *Hornstedtia scyphifera* are displayed in Table 1. The three compounds with the largest areas for *Hyphomycetes* endophyte extracts were uracil, hexadecane, and 3-[(4-hydroxyphenyl)methyl]-octahydropyrrole[1,2-a]pyrazine-1,4-dione. The molecular structure of this chemical is complex, and it exhibits antifungal, anticancer, and anti-inflammatory properties, respectively (Zhang *et al.* 2013; Bao *et al.* 2023; Husnawati *et al.* 2023). A fungus belonging to the *Hyphomycetes* class was found in the flower of PA. This fungus showed both antioxidant and antibacterial properties (Palupi *et al.* 2021). Regarding *Fusarium* sp. fungus isolates related with the *Hornstedtia scyphifera* plant, the three predominant chemicals were amide compounds, specifically docosanamide, stearamide, and hexadecanamide. According to research conducted by (Gurning 2024) amide compounds exhibit potential as antidiabetic. Research related to the potential of endophyte bioactive metabolites as antimalarials was carried out by Ibrahim *et al.* (2018). The bioactive compound fusaripeptide found in *Fusarium* sp., which was isolated from the roots of the *Mentha longifolia* L, can kill malaria parasites (Ibrahim et al., 2017). *Fusarium* endophytes from *Cinchona calisaya* trees could also stop *Plasmodium berghei* from growing by 62.18% to 78.41% (Hasbi 2019) Considering that Indonesia is known as a source of germplasm with high biodiversity, it is certainly a great opportunity to explore endophytic bioactive metabolites as anti-malarials.

a



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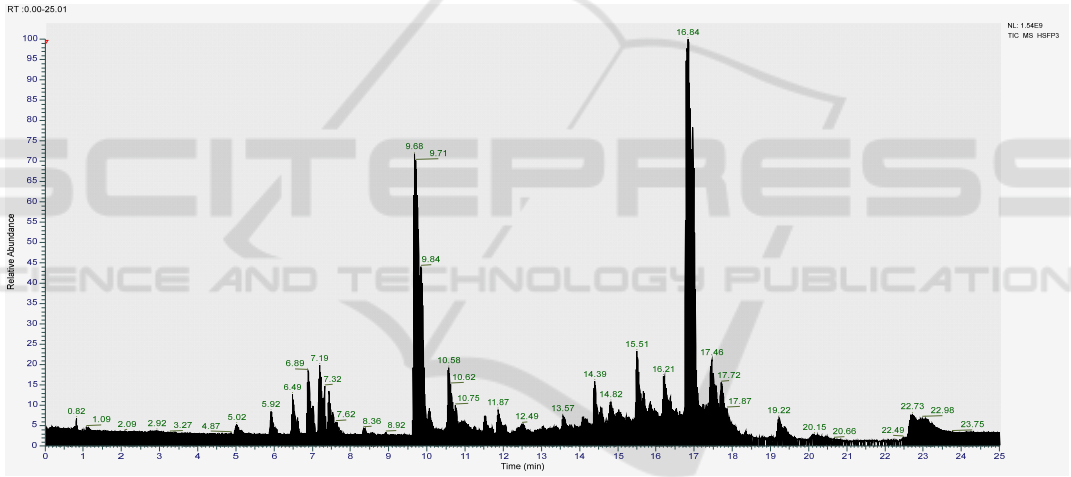

Figure 4: Profile LC/HRMS of endophyte extract: (a) Hypomycetes of endophytes associated with Physalis angulate (b) Fusarium sp. endophytes associated with Hornstedtia scyphifera.

4 CONCLUSIONS

There were two extracts had an inhibitory PfDHODH of more than 50%, from endophytes associated to *Physalis angulata* L (51.53%) and *Hornstedtia scyphifera* (66.37%). These endophytes were identified as *Hypomycetes* and *Fusarium* sp.

Analysis of untargeted compounds using LC-HRMS on ethyl acetate extracts from endophytes related to *Physalis angulata* and *Hornstedtia scyphifera* revealed various metabolites with potential biological activity. This research shows that endophytes have significant potential as a source of bioactive metabolites for the development of antimalarial drugs. Especially considering Indonesia's high biodiversity, further exploration of endophyte bioactive metabolites as antimalarials is very promising.

Table 1: Metabolomics components with the highest peak area in the etyl acetate extract.

Endophytes	Compounds	Formula	Calc. MW	% Area	Area (Max.)	Activity	Ref.
Hypomycetes of endophytes associated with *Physalis angulate*	Uracil	C4 H4 N2 O2	112,0274	12,40	335998122,8	Antifungal	(Zhang *et al.* 2013)
	Kojic acid	C6 H6 O4	142,0267	6,17	167278464,6	Antimicrobial	(Gurning 2024)
	Phloroglucinol	C6 H6 O3	126,0317	4,80	130028468,8	Anti breast cancer	(Kim *et al.* 2015)
	3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	C14 H16 N2 O3	260,1158	20,20	547431340,1	Anti breast cancer	(Husnawati *et al.* 2023)
	N-Acetyltyramine	C10 H13 N O2	179,0944	7,40	200609744,8	Antibacterial	(Driche *et al.* 2022)
	9-Oxo-10(E),12(E)-octadecadienoic acid	C18 H30 O3	294,2194	4,46	120787050	Anti ovarian cancer	(Zhao *et al.* 2015)
	1-Linoleoyl glycerol (1-monolinolein)	C21 H38 O4	354,2763	7,33	198644675,2	Antibacterial	(Jumina *et al.* 2019)
	Hexadecanamide	C16 H33 N O	255,2559	16,32	442141748,7	anti-inflammatory	(Bao *et al.* 2023)
	Monoolein	C21 H40 O4	356,2921	11,66	315992870,7	anti-inflammatory	(Ali *et al.* 2017)
	Stearamide	C18 H37 N O	283,2869	9,26	250852054,6	Antibacterial	(Hassan *et al.* 2012)
Fusarium sp. endophytes associated with *Hornstedtia scyphifera*	4-Methoxycinnamic acid	C10 H10 O3	178,06252	1,86	30813727,79	Antioxidant and antibacterial	(Nischitha dan Shivanna 2021)
	cis-12-Octadecenoic acid methyl ester	C19 H36 O2	296,2707	1,54	25454726	-	
	Citroflex A-4	C20 H34 O8	402,22435	1,97	32655666,27	-	
	Docosanamide	C22 H45 N O	339,34878	13,55	224460018,4	-	
	Hexadecanamide	C16 H33 N O	255,25543	54,91	909826078,2	anti-inflammatory	(Bao *et al.* 2023)
	Methyl palmitate	C17 H34 O2	270,25524	1,33	22054009,97	Antipoliperative	(do Nascimento *et al.* 2012)
	N,N-Dimethyldecylamine N-oxide	C12 H27 N O	201,20862	0,55	9170188,282	Acaricidal	(Zhu *et al.* 2023)
	Oleamide	C18 H35 N O	281,27114	0,90	14883383,66	-	
	Stearamide	C18 H37 N O	283,28636	23,14	383387130,3	Antibacterial	(Hassan *et al.* 2012)
	Triisopropanolamine	C9 H21 N O3	191,15152	0,25	4203345,083	-	

Note: % area based on displayed compounds

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