ANTIMALARIAL COMPOUNDS FROM ENDOPHYTIC FUNGI OF BROTOWALI (*Tinaspora crispa* L)

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ABSTRACT

*The term endophytic refers to a bacteria or a fungi microorganism that colonizes interior organs of plants, but does not have pathogenic effects on its host. In their symbiotic association, the host plant protects and feeds the endophytic, which "in return" produces bioactive metabolites to enhance the growth and compotitiveness of the host and to protect it from herbivores and plant pathogens. Plants with ethnobotanical history, for example brotowali (Tinaspora crispa L), are likely candidates to find bioactive compounds. Two alkaloids have been isolated from endophytic fungi of brotowali. The molecular structures of the isolated compounds were determined based on spectroscopic data, including UV, IR, NMR 1D and 2D spectrum. The compounds were determined as: 7- hydroxy-3,4,5-trimethyl-6-on-2,3,4,6-tetrahydroisoquinoline-8-carboxylic acid (**1**) and 2,5-dihydroxy-1-(hydroxymethyl)pyridin-4-on (**2**). The compound has antimalarial activity against Plasmodium falciparum 3D7, with IC_{50} values 0,129 µM and 0,127 µM.*

Keywords: *antimalarial, endophytic fungi, Tinaspora crispa L.*

INTRODUCTION

Malaria is disease which is caused by single cell obligate intracellular parasite from *Plasmodium*. *Plasmodium falciparum* is the most dangerous species for human because it can cause acute infection even death. This parasite is infected to human by female anoplheles mosquito [1]. Chloroquine is the most common drug for antimalarial because of easily obtaining, cheap and less side effect. Now chloroquine is first line drug for malaria treatment without any complication. However, to fight against malaria falsiparum of faces same serious barriers since the first discovery about *P falciparum* resistance to chloroquine in East Kalimantan (1974). Then, this tolerant widely spread and in 1996 cases of malaria that resistance to chloroquine has been discovered in all Indonesia provinces. Based on WHO guidance, if plasmodium resistance to chloroquine occurred more than 25% in one area, it suggested to stop using it as antimalarial drug except if combined with other antimalarial drug. The purpose of combined drug therapy is to increase antimalarial effect and sinergical activities and to inhibit resistance progressive of parasite for now drug. Therefore, to search antimalarial dug in Indonesia is very important to fight against of plasmodium resistance [2].

Isolation of bioactive compound from natural plants to serve active substance of drug, frequently has barrier which is rendemen is very low. To reach of this goal, high production of active substance is needed. Some efforts to obtain high production are by using culture method, search enzyme that play a role in synthesis of active substance, gene transplantation with bacteria, laboratory synthesis. Exploration of compound using above method has less relatively chance, high difficulty in produce and high cost [3].

Other methods to obtain bioactive compound is by using endophytic microbe that is available in every plant. This microbe lives together with host by mutualism symbiosis and generates certain secondary metabolites [4-5]. By isolation of endophytic microbe from host plant so this microbe cultivable in short time resulting secondary metabolites in enough amount is required. This method is necessary to develop because of some advantages in time and cost since it is plenty of plants in the world so it is needed one approach to make more simple in searching endophytic microbe that shows certain biological activity. One of them this plant which has ethnobotanical history linked to their several applications for medical or specific applications, thus possibility to obtain active substance is higher [6].

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Brotowali (*Tinaspora crispa* L) has been used traditionally to treatment for malaria [7-8]. Some researches have been carried out by researchers to prove that brotowali could be used as antimalarial drug. Two alkaloid compounds that potent as antimalarial has been isolated from plant endophytic mushroom sigend as 7- hydroxy-3,4,5-trimethyl-6-on-2,3,4,6-tetrahydroiso quinoline-8-carboxylic acid (**1**) dan 2,5-dihydroxy-1-(hydroxymethyl)pyridin-4-on (**2**). Structure determination of compound 2 has been published previously [9]. In this paper we elucidate structure of compound 1, furthermore antimalarial activity of compound **1** and **2** also described.

EXPERIMENTAL SECTION

Materials

Material for this research were brotowali plant (*Tinaspora crispa* L), medium of PCA, NA and PDA for isolation endophytic microbe, a series medium for physiologis assay or microbial identification, a series organics solvent, column chromatography was done silica gel 60 G (70-230 mesh), TLC analysis was carried out on precoated Si Gel plate (Kieselgel 60 F_{254}, 0.25 mm, 20x20 cm) and medium for antimalarial activity assay.

Instrumentation

The apparatus in the research were counter colony, autoclave, incubator, water bath, refrigerator, microscope, magnetic hotplate, UV lamp, column chromatography and generally apparatus in organic and microbiology laboratory.

Procedure

The preliminary research was isolation of fungi from brotowali plant and screening for fungi which have potential bioactive metabolites [10-11]. Potential fungi isolated were growth optimum condition to yield optimal culture [12]. Potential fungi isolated were cultivation on optimal condition to could give maximum bioactive metabolite and then harvested on the basis of growth phase obtained. Furthermore, isolation secondary metabolites from endophytic microorganism selected [5].

Potential isolate was cultured in PDB liquid medium culture (2L) and incubated on optimal condition to give bioactive compounds and then filtered to separate filtrate and biomass. Filtrate which containing bioactive metabolite extracted with n-hexane and EtOAc. The resulting extract was concentrated under reduced pressure to give crude extract of n-hexane and EtOAc. The fractions was preabsorbed on silica gel and chromatographed over a column of silica gel with gradient elution. Fractions which gave the same Rf on TLC were combined and rechromagraphed and recrystallized to give pure compound. The structures of pure compounds were determined on the basis of spectroscopic data UV, IR, ^{1}H, ^{13}C, HMQC, HMBC and COSY.

Antimalarial activity assay was done at parasitological laboratory of Airlangga University. In vitro antimalarial activity assay against *P falciparum* 3D7 [13].

RESULT AND DISCUSSION

From stem and leave of brotowali were isolated eight fungis, BB1-BB5 and BD4-BD6. Fungis isolated was cultivated in liquid medium PDB for four weeks and then filtered. TLC of ethylacetate extract showed fungi isolated BB3 and BB4 potential to give secondary metabolite.

BB4 recultivated in 2L PDB medium for four week, supernatant was extracted with n-hexane and EtOAc and then each fraction was evaporated to give 1.1 g n-hexane extract and 3.2 g EtOAc extract. TLC results of n-hexane extract showed that this extract was not potential while EtOAc extract showed there was one potential between three others. So EtOAc extract was sepatared further more.

BB4 extract (3.2 g) was preabsorbed and chromatographed of silica column with n-hexane-EtOAc of increasing polarity as eluent. Fraction which gave the same Rf on TLC were combined and gave four column fractions (F1-F4). F3 fraction was further rechromatographed with n-hexane-EtOAc (6:4) to afford 23 fractions which were combined to three fractions (F3.1-F3.3). F3.3 fraction purified by rechromatographed to yield compound **1** as yellow needles crystal (202 mg). Phytochemistry identification showed that compound 1 was alkaloid.

BB3 fungi was recultivated in 2L PDB medium for three weeks, supernatant was extracted with n-hexane and EtOAc and then evaporated to gave 0.6 g n-hexane extract and 3.1 g EtOAc extract. Based on TLC results n-hexane extract was not showed a potential metabolite while EtOAc extract showed there was a purple spot as major from six others. EtOAc extract was separated further more.

EtOAc extract (3.1 g) preabsorbted and column chromatographed eluted with n-hexane-EtOAc of increasing polarity as eluent to yield 80 fractions. Based on TLC results, these fractions were combined to five major fractions (F1-F5). F4 fraction showed a potential spot and recolumn chromatographed with n-hexane-EtOAc (5:5 to 1:9) of increasing polarity to afford 30 fractions which were combined to four fractions (F4.1-F.4.4). Purification of F4.3 fraction by column

Table 1. ^{13}C and ^{1}H NMR data of compound 1

No. C	δ_C ppm	δ_H ppm (ΣH. multiplicity. *J* in Hz)	HMBC	COSY
1	163.0	8.25 (1H; s)	81.8; 107.6; 139.3	
3	81.8	4.77 (1H; q; 6.7)	139.3; 163.0; 18.4	1.33
4	34.7	2.97 (1H; q; 6.8)	18.7; 107.6; 123.2; 139.3	1.21
4a	139.3			
5	123.2			
6	183.9			
7	174.4			
8	100.5			
8a	107.6			
3-CH_3	18.4	1.33 (3H; d; 6.7)	34.7; 81.8	4.77
4-CH_3	18.7	1.21 (3H; d; 6.8)	34.7; 81.8; 139.3	2.97
5-CH_3	9.6	2.01 (3H; s)	123.2; 139.3; 183.9	
9	177.6			
9-OH		15.10 (1H; s)	100.5; 177.6	

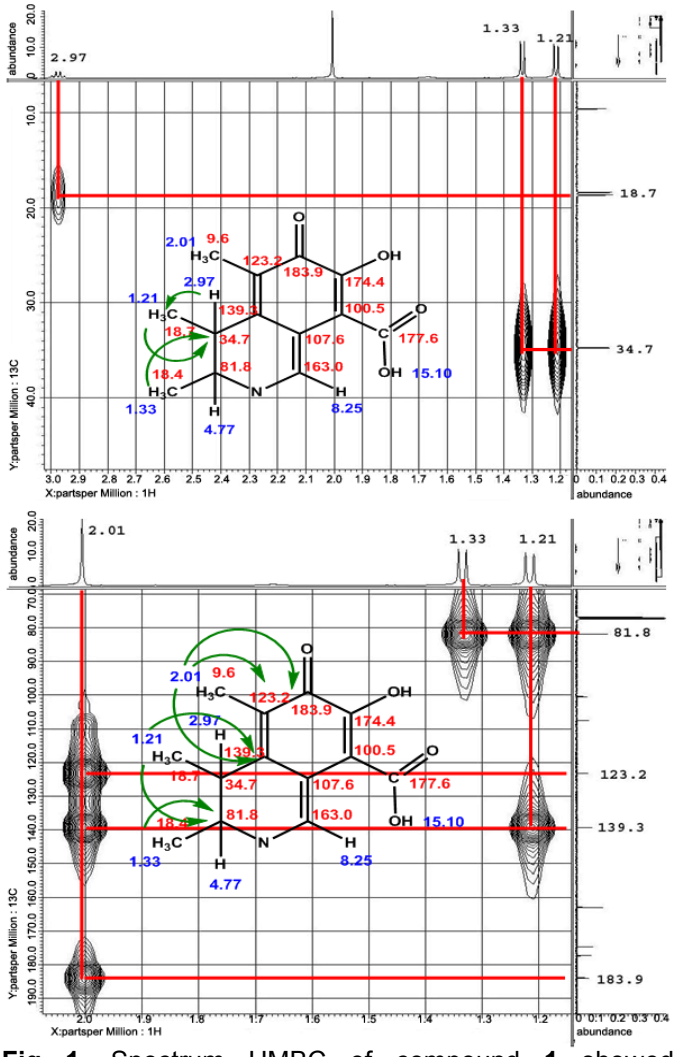

Fig 1. Spectrum HMBC of compound **1** showed correlations proton at δ_H 1.33 (3H; d; 6.7); 1.21 (3H; d; 6.8) and 2.01 ppm and methine proton at δ_H 2.97 (1H-500 MHz; ^{13}C-125 MHz, CDCl3)

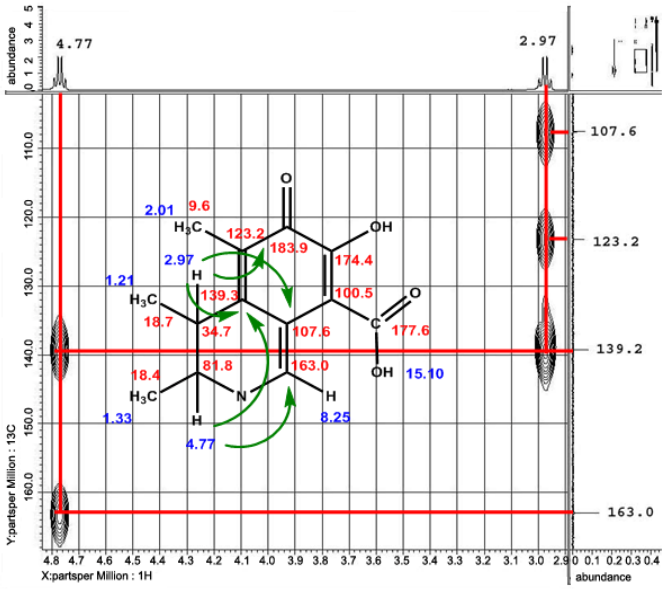

Fig 2. Spectrum HMBC of compound **1** showed correlation between proton at δ_H 2.97 and 4.77 (^{1}H-500MHz; ^{13}C-125 MHz; CDCl3)

chromatography gave compound 2 as white needles crystal (22 mg)

The UV spectrum of compound 1 (1 mg/10 mL MeOH) showed absorptions maxima at λ_{max} 213, 253 and 315 nm. These bands were not gave a batochromic shift with NaOH.

The IR spectrum exhibited absorptions at υ_{max} 3440.8 $cm^{-}1$ and 1018.3 cm^{-1} due to N-H group, (2979.8 – 2877.6 cm^{-1}) C-H stretch, (2555.5 cm^{-1}) typical of acid OH, (1627.8 cm^{-1}) for C=O stretch and 1577.7-1515.9 cm^{-1} typical of C=C conjugated.

The ^{1}H-NMR spectrum of compound 1 showed there were three signals for methyl groups δ_H 1.33 (3H; d; 6.7), 1.21 (3H; d; 6.8) and 2.01 ppm (3H; s), two signals for methines sp^3 at δ_H 2.97 (1H; q; 6.8) and 4.77

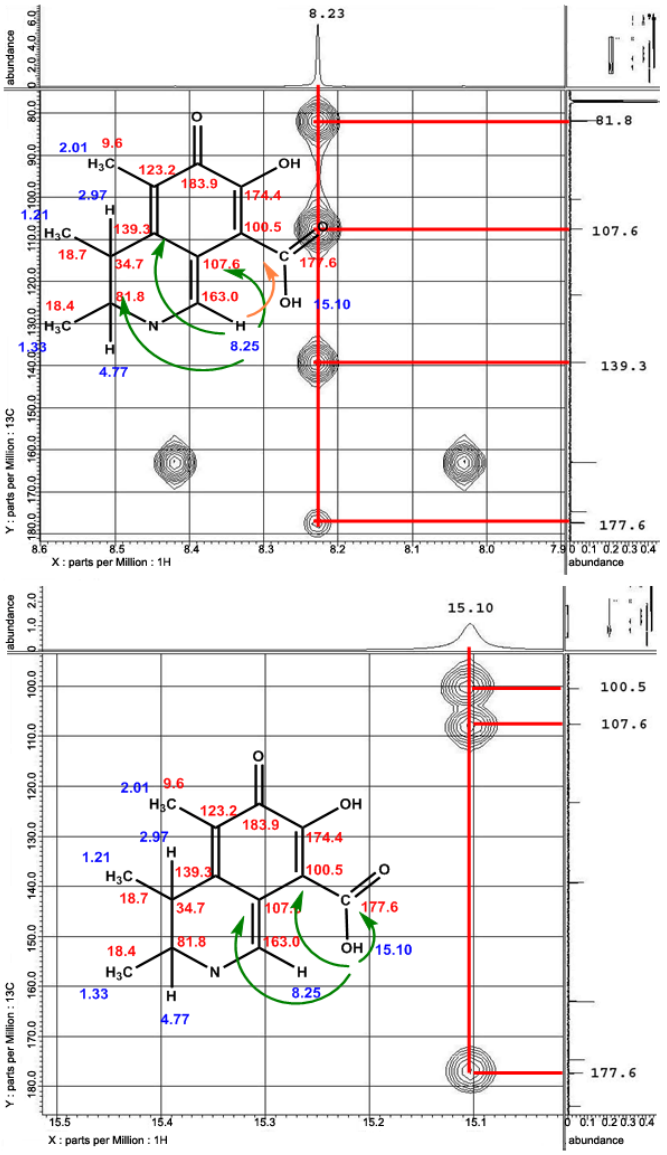

Fig 3. The HMBC spectrum of compound 1 showed correlations methine proton sp^2 at δ_H 8.23 with OH proton at δ_H 15.10 ppm (^{1}H-500 MHz; ^{13}C-125 MHz, $CDCl_3$)

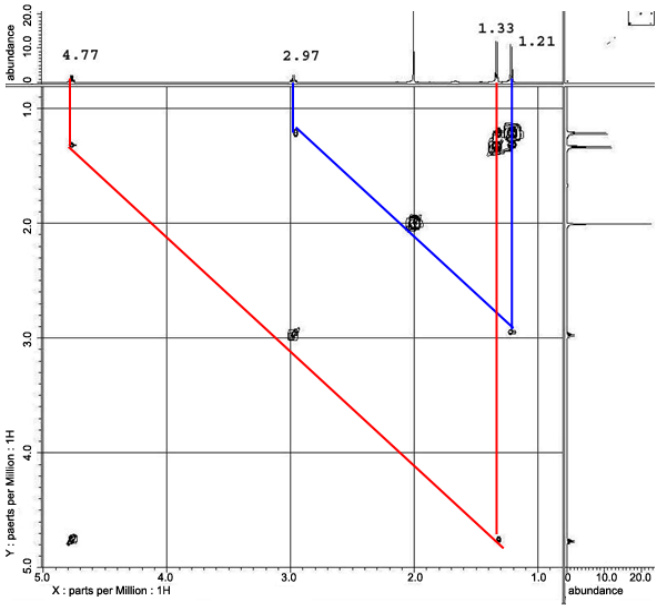

Fig 4. The COSY spectrum of compound 1 showed correlation between methine proton H-4 (δ_H 2.97) with methyl proton 4-CH_3 (δH 1.21) and methine proton at δ_H 4.77 with methyl proton 4-CH_3 (δ_H 1.33) (^{1}H-500 MHz; ^{13}C-125 MHz, $CDCl_3$)

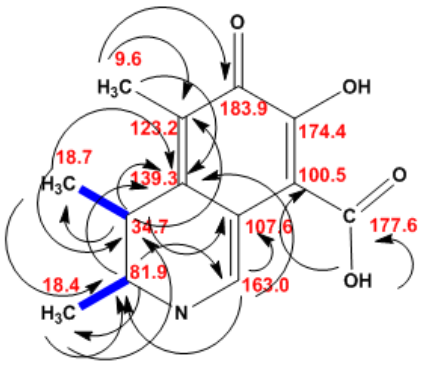

Fig 5. The HMBC and COSY correlations of 7-hydroxy-3,4,5-trimethyl-6-on-2,3,4,6-tetrahydroisoquinoline-8-carboxylic acid (**1**) (arrow bent for HMBC correlations and bold line for COSY correlations)

(1H; q; 6.7), and one for methine at δ_H 15.10 (1H; s). That signal indicated that compound 1 was having hydroxyl acid group.

The ^{13}C NMR data suggested that compound **1** contained thirteen carbons corresponded to three methyl at δ_C 9.6; 18.4; 18.7 ppm), two methine sp^3 at δ_C 34.7 and 81.8 ppm, and one methine sp^2 at δ_C 163.0 ppm, and there were six quarterner carbons at δ_C 100.5; 107.6; 123.2; 139.3; 174.4; and 183.9 ppm. Complete assignment of carbon and proton were made by analysis 2D NMR (Table 1).

In the HMBC spectrum (Fig. 1 and 2) of compound **1** methyl proton at δ_H 1.21 ppm (4-CH3) displayed correlations (^{2}J) with C-4 (δ_C 34.7) and long range correlations (^{3}J) couplings with C-3 (δ_C 81.8) and C-4a (δ_C 139.3). The methyl proton at δ_H 1.33 (3-CH3) showed correlation (^{2}J) with carbon C-3(δ_C 81.8) and long range correlations (^{3}J) with C-4 (δ_C 34.7). Furthermore, methyl proton at δ_H 2.01 (5-CH3) displayed correlation (^{2}J) coupling with carbon C-5 (δ_C 123.2) and ^{3}J couplings with C-4a (δ_C 139.3) and C-6 (δ_C 183.9).The HMBC spectrum also showed correlations (^{2}J) couplings between methine proton

Table 2. The Percentage of parasites growth and percentage of inhibitory of compound **1** against *P. falciparum.*

Concentration (µg/mL)	R	% Parasitemia 0 (h)	% Parasitemia 48 (h)	Growth (%)	Inhibitory (%)	mean Inhibitory (%)
Control (-)	1	1.15	5.70	4.55	-	
	2	1.15	4.07	2.92	-	
10	1	1.15	0.80	0.00	100.00	100.00
	2	1.15	0.82	0.00	100.00	
1	1	1.15	2.12	0.97	74.06	83.69
	2	1.15	1.40	0.25	93.32	
0.1	1	1.15	3.22	2.07	44.65	52.67
	2	1.15	2.62	1.47	60.70	
0.01	1	1.15	3.30	2.15	42.51	51.07
	2	1.15	2.66	1.51	59.63	
0.001	1	1.15	4.57	3.42	8.56	19.52
	2	1.15	3.75	2.60	30.48	

Table 3. The percentage of parasites growth and percentage of inhibitory of compound **2** against *P falciparum* 3D7

Concentrration (µg/mL)	R	% Parasitemia 0 (h)	% Parasitemia 48 (h)	Growth (%)	Inhibitory (%)	Mean Inhibitory (%)
Control (-)	1	1.15	6.22	5.07	-	
	2	1.15	6.12	4.97	-	
10	1	1.15	0.10	0.00	100.00	100.00
	2	1.15	0.20	0.00	100.00	
1	1	1.15	1.72	0.57	88.65	93.03
	2	1.15	1.28	0.13	97.41	
0.1	1	1.15	2.90	1.75	65.14	61.45
	2	1.15	3.27	2.12	57.77	
0.01	1	1.15	3.50	2.35	53.19	53.69
	2	1.15	3.45	2.30	54.18	
0.001	1	1.15	5.54	4.39	12.55	10.46
	2	1.15	5.75	4.60	8.37	

Table 4. The summary of percentage of inhibitory *P. falciparum* growth and results of probit by SPSS 11.5 programe.

Sample	Percentage of inhibitory at test dose (µg/mL)					IC_{50} (µg/mL)	µM
	10	1	0.1	0.01	0.001		
Compound **1**	100	83.69	52.67	51.07	19.52	0.03	0.129
Compound **2**	100	93.03	61.45	53.69	10.46	0.02	0.127

(δ_H 2.97) with carbon 4-CH3 (δ_C 18.7) and C-4a (δ_C 139.3) and long range (^{3}J) couplings with C-8a (δ_C 107.6) and C-5 (δ_C 123.2) while methine proton at δ_H 4.77 (H-3) showed correlation (^{2}J) couplings with 3-CH3 (δ_C 18.4) and (^{3}J) couplings with C-4a (δ_C 139.2) and C-1 (δc 163.0).

The HMBC spectrum (Fig. 3) showed correlation (^{2}J) coupling methine proton sp2 at δH 8.23 with C-8a (δ_C 107.6) and long range correlation (^{3}J) coupling with C-3 (δ_C 81.8 and C-4a (δ_C 139.3). Proton 9-OH wich appeared at downfield (δ_H 15.10) showed correlations (^{2}J) with C-9 (δ_C 177.6) and ^{3}J coupling with C-8 (δ_C 100.5).

Furthermore, COSY spectrum (Fig. 4) showed there was correlations proton with proton in compound **1**. Methine proton H-4 (δ_H 2.97) showed correlation with methyl proton 4-CH_3 (δ_H 1.21) and methane proton H-3 (δ_H 4.77) showed correlation with methyl proton 3-CH_3 (δ_H 1.33).

Based on UV, IR, ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC and COSY analysis we identified compound 1 as alkaloide 7- hydroxy-3,4,5-trimethyl-6-on-2,3,4,6-tetrahy droisoquinoline-8-carboxylic acid (**1**) with formula $C_{13}H_{15}O_3N$ (DBE = 7; BM = 233). Correlations proton with carbon in HMBC spectrum and proton with proton in COSY showed at Figure 5.

Result of antimalarial activity assay (Table 2-4) showed that compound 1 and 2 potential as antimalarial. In vitro antimalarial assay showed pure compounds has prospective as antimalarial based on IC_{50} < 1-5µM [14]. It has antimalarial activity if $IC_{50} \leq$ 7.71 µM [15], while extract with IC_{50} < 50 µg/mL and fraction with IC_{50} < 25 µg/mL affective as antimalarial [16]. Chloroquine has IC_{50} = 6.3 nmL^{-1} [13].

CONCLUSION

Endophytic microbe which grows in plant which has antimalarial activity could give a metabolite which has antimalarial activity too. From endophytic fungi of brotowali plant had been isolated alkaloids compound 7- hydroxy-3,4,5-trimethyl-6-on-2,3,4,6-tetrahydroisoquinoline-8-carboxylic acid (**1**) and 2,5-dihydroxy-1-(hydroxymethyl)pyridin-4-on (**2**). Both of them showed antimalarial activity against *Plasmodium falciparum* 3D7 in vitro with IC_{50} value 0.129 and 0.127 µM respectively.

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