Indonesian Journal of Pharmacy

VOL 36 (2) 2025: 263–271 | RESEARCH ARTICLE

Isolation, Structure Elucidation and Antiplasmodial Activity of Steroid Compound from Sponge *Hyrtios reticulatus*

Mahfur[1], Subagus Wahyuono[3], Indah Purwantini[3], Erna Prawita Setyowati[3*]

1. Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Pekalongan, Pekalongan, Indonesia
2. Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

Article Info

Submitted: 07-09-2023
Revised: 27-06-2024
Accepted: 03-07-2024

*Corresponding author
Erna Prawita Setyowati

Email:
erna_prawita@ugm.ac.id

ABSTRACT

Hyrtios reticulatus is a type of sponge that has antiplasmodial activity against *Plasmodium falciparum* 3D7 and FCR3 variants. However, the active compound that contributes to this activity is not yet known. The objective of this research was to isolate and structure elucidation of antiplasmodial compound from *Hyrtios reticulatus*. The chloroform fraction from ethanol extract was partitioned by column chromatography with a ratio of n-hexane to ethyl acetate of 5:5 v/v and was purified using preparative thin-layer chromatography. Then the result was identified and characterized using spectroscopic method. The antiplasmodial activity was studied in vitro against *Plasmodium falciparum* 3D7 and FCR3 using micro method. The purified compound was a white crystal with (λmaks) at 235 nm and was identified with UV-vis spectroscopy. The infrared spectrum showed that the isolate had functional hydroxyl (OH) groups, aliphatic C-H bond, C-O bond, vanillic C-H bond, and carbon-carbon double bonds (C=C). The result of the mass spectrometry analysis of the purified compound showed a molecular weight of 414 g/mol. The H-NMR, C-NMR, DEPT, COSY and HMBC spectrum and the spectral analysis revealed the presence of a compound of β-sitosterol, and it had antiplasmodial activity against *Plasmodium falciparum* 3D7 and FCR3 with IC_{50} 17.76 ± 2.86 µg/mL and 12.03 ± 1.60 µg/mL.

Keywords: β-sitosterol, Sponges, *Hyrtios reticulatus*, Antiplasmodial.

INTRODUCTION

Malaria is one of the deadliest infections in the world, and its resistance to drugs is rising. Malaria infected approximately 247 million people in 2021, killing over 625,000 people (World Health Organization, 2022). The major causative agent, *Plasmodium falciparum*, is a deadly parasite and is becoming resistant to current antimalarial drugs including chloroquine, quinine, sulfadoxine-pyrimethamine, and mefloquine (Gupta et al., 2013; Kumar, 2017; Simamora & Fitri, 2007). Consequently, new and more potent malaria medications are urgently needed (Alves et al., 2021; Hikmawan et al., 2020). Marine organisms are one source that can be investigated for antimalarial properties (Laport et al., 2009).

The marine ecosystem is widely acknowledged as a significant natural product source, offering a wide range of different chemical structures with potential medicinal applications (Islam et al., 2023; Wibowo et al., 2022). Over the past three decades, a total of 1,490 new compounds were discovered and extracted from marine biota in 2017 (Carroll et al., 2019). Many studies have successfully isolated compounds from marine organisms such as cyanobacteria, phytoplankton, sponges, cnidarians, bryozoans, mollusks, tunicates, echinoderms, and other iterdital plants. These compounds have been shown to have biological properties including antibacterial, anticancer, and antitumor properties, and the sponges are one source that can be developed as an antimalarial agent (Mahfur, Wahyuono, et al., 2022).

Hyrtios reticulatus is a type of sponge that has active compounds that have the potential as ingredients used for medicinal purposes (Mahfur, Setyowati, et al., 2022; Shady et al., 2017). Previous research found that the sponge has antimicrobial, anticancer, and antiplasmodial activities (Inman et

al., 2010). The extracts, fractions, and subfractions of *Hyrtios reticulatus* are shown to have good antiplasmodial activity against *Plasmodium falciparum* FCR3 and 3D7 (Mahfur et al., 2022b). Considering these findings, isolation was continued on subfractions to obtain pure compounds that also has antiplasmodial activity (Ozaki et al., 2019). Isolation of antiplasmodial compounds from the sponge *Hyrtios reticulatus* has not been reported, but other species, Hyrtios erectus, has been isolated. The sesquiterpenes isolated from its sponge are smenotronic acid, ilimaquinone, and pelorol, with IC_{50} 3.51 ± 0.63, 2.11 ± 0.23, 0.80 ± 0.19 (De Voogd, 2007; Ju et al., 2018; Shady et al., 2017).

The objective of this research is to isolate and structure elucidation of antiplasmodial compound from *Hyrtios reticulatus* with antiplasmodial activity test against *Plasmodium falciparum* 3D7 and FCR3. This research may also help identify the benefits of *Hyrtios reticulatus* for drug discovery and development.

MATERIALS AND METHODS

Extraction and isolation

Sponge *Hyrtios reticulatus* was obtained by scuba diving in about 12m depth in the Menjangan Island National Park West Bali, Indonesia. The identity of the material was authenticated at the University of Diponegoro, Indonesia. The extraction, fractionation, and column chromatography process of the *Hyrtios reticulatus* follows Mahfur et al., 2022b. The Preparative Thin Layer Chromatography (PTLC) was used to separate the target compounds from (5:5 v/v) column chromatography result. The PTLC system is used silica gel 60 GF_{254} Merck 1.07730.1000 for preparative thin-layer chromatography with 0.5 mm thickness as stationary phase, and n-hexane: ethyl acetate:toluene (3:1:0.5) (Merck) as mobile phase. The isolation outcomes were then filtered.

Characterization and Structure elucidation analysis

Characterization and Structure elucidation analysis were carried out using spectroscopic data UV (Shimadzu UH5300), melting point, Infrared spectroscopy with KBr pellet (Perkin Elmer FTIR 100), The spectra of ^{1}H-NMR (Jeol JNM-ECS 500 MHz), ^{13}C-NMR (Jeol JNM-ECS 100 MHz) with 1D and 2D, and MS fragmentation with GC (Shimadzu GCMSQP2010SE).

In vitro Antiplasmodial Activity Assay

The cultivation of *Plasmodium falciparum* was modified from the Trager and Jensen method. The samples antiplasmodial activity assays were carried out in triplicate. For every test, samples were incubated with parasite cultures for 48 hours in 5% CO_2 at 95% relative humidity. Compounds were dissolved in DMSO with 0.625 - 10 µg/mL concentration, samples were placed in 96-well flat-bottomed micro-plates consisting of negative control (DMSO (Merck)), samples and positive control (Chloroquine (Merck)). Parasitaemia was determined by observing the Giemsa-stained thin blood smears using a microscope. The culture of *Plasmodium* without sample was used as a negative control, which considered to be a 100% growth of *Plasmodium* (Trager & Jensen, 1997) and the % inhibition of parasitaemia was calculated.

Statistical analysis

The results of antiplasmodial activity of β-sitosterol and chloroquine were analyzed using the SPSS version 16. The IC_{50} was calculated by using statistical analysis regression probit with the logarithm between percent inhibition and sample concentration. The statistical analysis method was used an independent T-test with a 5% level of significance, i.e., $p \leq 0.05$.

RESULTS AND DISCUSSION

Extraction and isolation

The isolation outcomes obtained white crystal with a yield 23.8 % w/w from sub-fraction. The isolated compound was a white crystal with a melting point of 134–136 °C. Further identification of the sample was dissolved using chloroform.

Characterization of Isolated Compounds

UV-Vis and FTIR

The identification of the isolate using UV-vis spectroscopy indicated the peak of UV-vis spectra (λmax) was at 235 nm of wavelength and 0.101 of absorbance. The Fourier Transform Infrared (FTIR) spectrum (Supplemet 1) was used to show the functional groups of the isolated compounds. The hydroxyl group (-OH) owned by the isolate that can be seen from the absorbance stretching at 3430.86 cm^{-1}. The strong stretching at 2936.86 cm^{-1} indicated of aliphatic hydrocarbons (C-H).

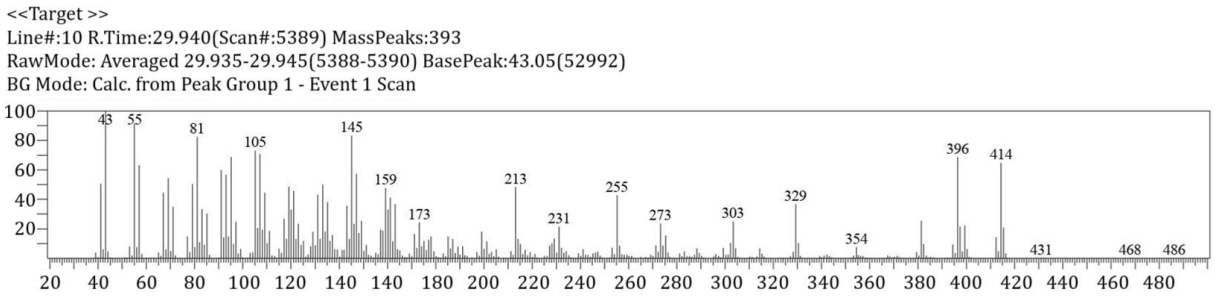

Figure 1. MS-Fragmentation of Isolate β-sitosterol from *Hyrtios reticulatus.*

Tabel I. NMR Data for β-sitosterol ($C_{29}H_{50}O$) 414 in $CDCl_3$

Position	δC sample	δC reference (Kamal et al., 2016)	δH (J in Hz) sample	δH reference (Permatasari et al., 2021)	COSY	HMBC
1	37.34	37.4	1.81	1.81		-
2	31.99	31.9	1.84	1.83	H-3	C3, C5, C10
3	71.90	71.8	3.51	3.52 (m)	H-4, H-2	-
4	42.40	42.3	2.28 (d, J = 15 Hz)	2.27	H-3	C2, C3, C5, C6, C10
5	140.85	140.3	-	-	-	-
6	121.82	121.7	5.34 (d, J = 5 Hz)	5.345 (dd)	H-7	C4, C8, C10
7	31.74	31.7	1.98	1.99	H-6	C9
8	32.18	32.0	1.45	1.45	-	C5, C6, C7, C9, C14
9	50.21	50.1	0.92	0.92	-	C1, C5, C10
10	36.59	36.5	-	-	-	-
11	21.17	21.1	1.47	1.47	H-12	-
12	39.86	39.6	1.97	1.99	H-11	-
13	42.38	42.3	-	-	-	-
14	56.85	56.7	1.08	1.08	-	-
15	24.38	24.3	1.55	1.54	H-16	C16
16	28.32	28.6	1.82	1.82	H-15	C15
17	56.10	55.9	1.06	1.06	-	C12, C13, C17, C18, C20
18	11.95	11.6	0.68	0.69 (s)	-	C12, C13 C17
19	19.49	19.4	1.07	1.07 (s)	H-1	C1, C5, C9
20	36.27	36.2	1.35	1.34	-	-
21	18.79	18.8	0.79	0.79	-	-
22	33.97	34.0	1.28	1.34	-	-
23	26.44	26.1	1.16	1.14	-	-
24	46.13	45.8	0.91	0.91	-	-
25	29.07	29.2	1.03	1.63	H-26	C23, C28
26	19.69	19.8	0.82	0.82	H-25	C24, C25
27	19.05	19.0	0.80	0.79	-	C26
28	23.08	23.1	1.24	1.24	-	C23, C24, C25, C29
29	12.13	12.0	0.83	0.83	-	C28

The weak absorbance at 1639.14 cm^{-1} showed have the C=C double bond. The infrared spectrum showed the C-H bending at 1376.18 cm^{-1} and the weak ribbon at 1051.86 cm^{-1} indicated stretching vibration of C-O bonds (Nandiyanto et al., 2019; Pavia et al., 2001; Silverstein et al., 2005). The weak absorbance at 1639.14 cm^{-1} showed the C=C double bond. The infrared spectrum showed the C-H bending at 1376.18 cm^{-1} and the weak ribbon at 1051.86 cm^{-1} indicated stretching vibration of C-O bonds (Nandiyanto et al., 2019; Pavia et al., 2001; Silverstein et al., 2005).

Interpretation of mass spectrum (MS)

The mass spectra (MS)-fragmentation (Figure 1) indicated that the molecular weight of the purified compound was 414 g/mol with

$C_{29}H_{50}O$ molecule formula and has five Double Bond Equivalent (DBE). The first fragmentation of the mass spectrum of the compound experienced the release of a water molecule, shown at m/z 396. The next fragmentation stage will be dealkylated to yield a peak at m/z 381. The peak at m/z 273 is for fragmentation of C17-C20 cleavage. The dehydration of m/z 273 fragment will yield m/z 255. The MS-Fragmentation of the sample is a characterization of β-sitosterol (Wahdaningsih et al., 2021).

^{1}H-NMR

The interpretation of ^{1}H-NMR spectra indicated characteristic signal of olefinic protons, proton at $^\delta$H 5.34 ppm chemical shift region showed H-6. The H-3 protons at $^\delta$H3.51 ppm showing –OH groups. Two methyl groups chemical shift at $^\delta$H 0.69 ppm and 1.07 ppm. The H-29 protons surfaced at $^\delta$H0.83 ppm, and protons of H-26 and H-27 surfaced at $^\delta$H 0.82ppm and $^\delta$H 0.80ppm respectively. The other protons signals of the isolated compound surfaced at chemical shift 0.6-2.0 ppm. The others chemical shift of protons (Table I) and compared to results of previous studies (Kamal et al., 2016).

^{13}C-NMR

The spectrum ^{13}C-NMR of the isolate showed the isolated compound has 29 carbon (Table I). The signal at $^\delta$c 71.9 ppm (C-3) showed bonding between carbon and hydroxyl group (C-OH). The signal at $^\delta$c140.85 ppm (C-5) and $^\delta$c121.82 ppm (C-6) indicated the double bond of carbon (C=C). These two carbons are part of the B ring of steroids, which has a more deshielded region chemical shift. The signals of the dimethyl carbons C-26 and C-27 appeared at $^\delta$c 19.69 ppm and $^\delta$c 19.05 ppm, respectively. The other chemical shifts in the 13C-NMR spectrum (Table I) were compared to the results of previous studies (Permatasari et al., 2021)

DEPT 135

The complexity of absorbance peak could be analyzed with Distortionless Enhacement by Polarization Transfer (DEPT) 135 (Supplement 2), so it was possible to differentiate the signals of methyl (–CH_3), methylene (-CH_2), and methine (-CH) of a compound; the signal of CH_3 and bCH was positive while the signal of CH_2 was negative (Luhata, 2015). The peaks of -CH were C3 at $^\delta$c 71.9 ppm, C6 at $^\delta$c 121.82 ppm, C8 at $^\delta$c 32.18 ppm, C9 at $^\delta$c 50.21 ppm, C14 at $^\delta$c 56.85 ppm, C17 at $^\delta$c 56.1 ppm, C20 at $^\delta$c 36.27 ppm, C24 at $^\delta$c 46.13 ppm and C25 at $^\delta$c 29.07ppm. The peaks of -CH_2 were C1 at $^\delta$c 37.34 ppm, C2 at $^\delta$c 31.99 ppm, C4 at $^\delta$c42.1 ppm, C7 at $^\delta$c 31.74 ppm, C12 at $^\delta$c 39.86 ppm, C15 at $^\delta$c24.38 ppm, C16 at $^\delta$c 28.32 ppm, C22 at $^\delta$c 33.97 ppm, C11 at $^\delta$c 21.17 ppm, C23 at $^\delta$c 26.44 ppm, and C28 at $^\delta$c 23.08 ppm. The peaks of -CH_3 were C18 at $^\delta$c 11.95 ppm, C19 at $^\delta$c 19.49 ppm, C21 at $^\delta$c 18.92 ppm, C26 at $^\delta$c 19.69 ppm, C27 at $^\delta$c 19.05 ppm and C29 at $^\delta$c 12.13 ppm. From the data showed that the isolate had 9 methine (-CH), 11 methylene (-CH_2), and 6 methyl (-CH_3).

COSY and HMBC

COSY (Correlated Spectroscopy) is cross-spectra between protons that are coupled. In the sample, the cross-peak signals indicated that the proton at C2 is coupled with the proton at C3 (Table I and Figure 2). The HMBC spectrum is the signal used to determine the relationship between protons and carbons. The correlation between the proton and carbon can be seen if they are separated by 2-4 bonds. Some of the HMBC signals that appear on the sample are proton in carbon no 2 have correlation with C3, C5, and C10 (Supplement 3 and data in Table I). Based on all available data characterization was determined that the isolate was a molecule β-sitosterol, with the chemical formula $C_{29}H_{50}O$ (Figure 3). β-sitosterol is a steroid compound often found in various plants. To date, notably in marine life, this compound has been successfully isolated from several sponges and tested for various activities. In sponges, the source of β-sitosterol is *Cinachyrella cf. cavernosa*, *Cliona sp*., *Callyspongia aff. implexa*, and *Didiscus sp*. (Abdelmohsen et al., 2015; Beesoo et al., 2017; Singh & Thakur, 2021; Taşdemir et al., 2003). This compound has antioxidant, antibacterial, anti-inflammatory, anticancer, hepatoprotective, and antidiabetic activities (Babu & Jayaraman, 2020; Rashed, 2020). One activity that has not been widely studied in this compound is its antiplasmodial activity.

Antiplasmodial Activity Assay

Antiplasmodial activities of β-sitosterol (Figure 4) were expressed by the inhibitory concentrations (IC_{50}) of the drug that induced 50% reduction in parasitemia compared to the control (100% parasitemia). The β-sitosterol with a concentration between 0.625-10 µg/mL was used to test the plasmodial activity. At the highest concentration the growth of parasitemia was 4.16±0.11% and inhibition was 41.11±1.59% (Table II). The β-sitosterol showed can inhibited of growth *Plasmodium falciparum*. β-sitosterol is a compound in the stigmasteroid group (Abdulqader et al., 2023).

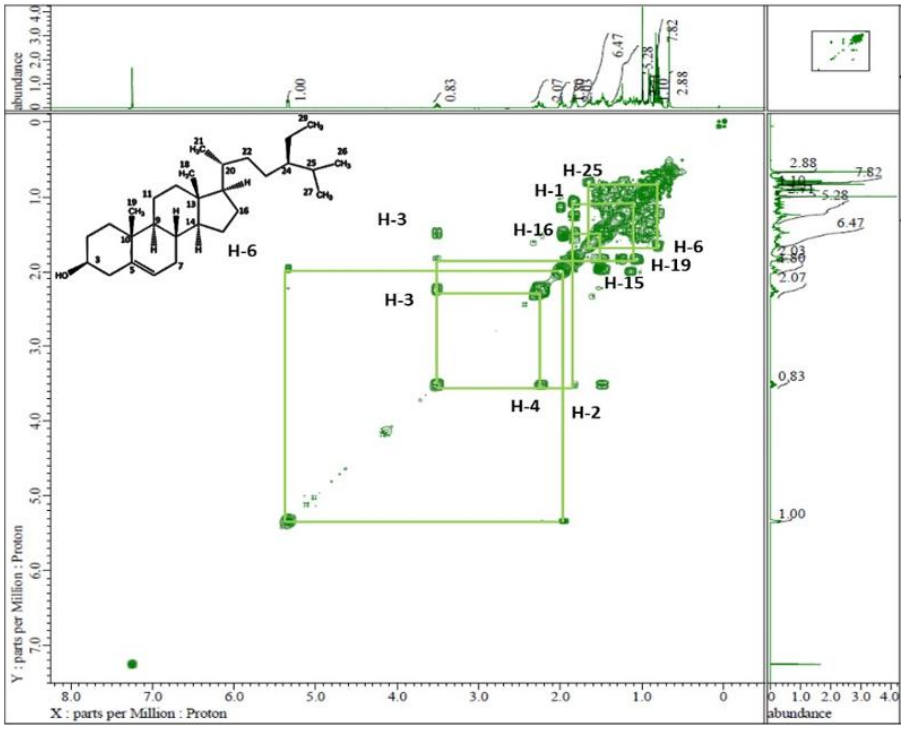

Figure 2. COSY Spectrum of β-sitosterol.

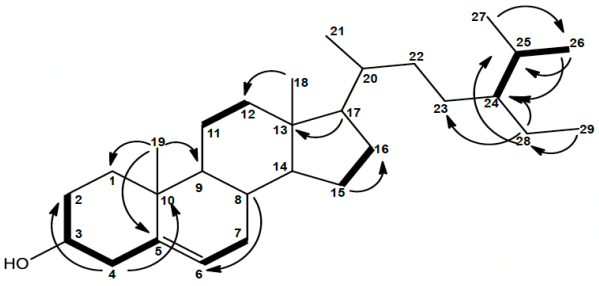

Figure 3. Structure of β-sitosterol (▬▬: COSY; ⟶ : HMBC)

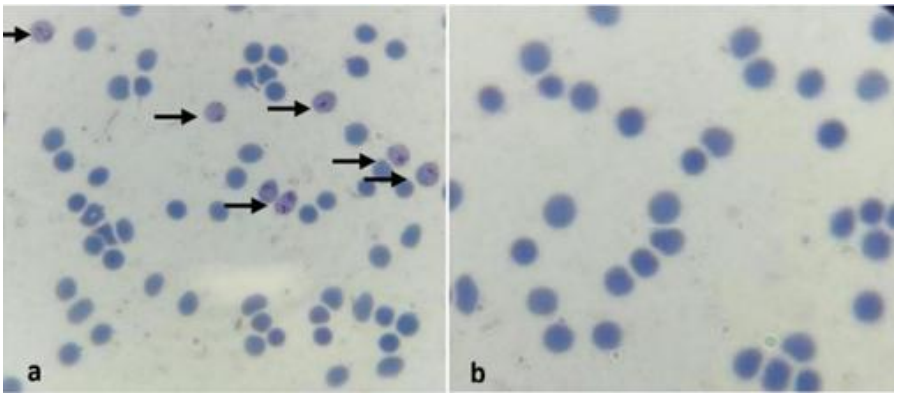

Figure 4. Morphology of *P. falciparum* after 24hour incubation. a) treatment with β-sitosterol; b) with Chloroquine (⟶: Parasitemia growth)

Table II. Activity of β-sitosterol against *P. falciparum* 3D7 and FCR3 variants

Sample	Consentration (µg/mL)	3d7		Fcr3	
		%parasetimia	%inhibition	%parasetimia	%inhibition
β- sitosterol	N.C	7.06 ± 0.21	0 ± 0	6.7 ± 0.19	0 ± 0
	10	4.16 ± 0.11	41.11 ± 1.59	3.57 ± 0.09	46.77 ± 1.37
	5	4.76 ± 0.16	32.61 ± 2.28	4.69 ± 0.05	29.95 ± 0.75
	2.5	5.44 ± 0.1	22.96 ± 1.38	5.24 ± 0.13	21.79 ± 1.89
	1.25	5.68 ± 0.12	19.53 ± 1.68	5.53 ± 0.28	17.47 ± 4.2
	0.625	7.06 ± 0.15	-0.04 ± 2.18	6.48 ± 0.17	3.21 ± 2.5

Data are expressed as mean ± SD, NC is negative control.

This compound is commonly found in plants such as *Alcea Kurdica*, *Baccaurea racemose*, and *Hylocereus Polyrhizus* (Abdulqader et al., 2023; Permatasari et al., 2021; Wahdaningsih et al., 2021). In addition to plants, a variety of sponges such as *Cinachyrella cf. cavernosa* (Singh & Thakur, 2021), *Cliona sp*. (Beesoo et al., 2017), *Callyspongia aff. Implexa* (Abdelmohsen et al., 2015), and *Didiscus sp.* (Taşdemir et al., 2003) are also a source of these compound.

The β-sitosterol showed antiplasmodial activity with an IC_{50} of 17.76 ± 2.86 µg/mL against *Plasmodium falciparum* 3D7 and 12.03 ± 1.60 µg/mL against *Plasmodium falciparum* FCR3. This activity is classified as moderate because it is >10 mg/mL. The classification of the antiplasmodials activity were divided into 5 groups: IC_{50} <1 µg/mL, potent; IC_{50} <10 µg/mL, good; IC_{50} 10–50 µg/mL, moderate; IC_{50} 50–100 µg/mL, low; and IC_{50} >100 µg/mL, inactive. The antiplasmodial activity of stigmasteroid-group compounds can be influenced by the presence or location of their hydroxyl groups. This ability may be due to the presence of hidroxyl group which could influence their antiplasmodial activity. The location of hydroxyl group in β-sitosterol is easier to interact with extracellular and intracellular fluids so that it can be easily carried to target molecule (Murtihapsari et al., 2019). Previous research reported that β-sitosterol has high activity against a chloroquine-sensitive (3D7) strain with an IC_{50} value of 5.51 µM or 13.30 µg/mL (Chaniad et al., 2022; Philip et al., 2019). In this study, the chloroquine had an IC_{50} of 0.018 ± 0.002 against 3D7 variant and 0.039 ± 0.004 µg/mL against the FCR3 variant and different significance with sample β-sitosterol ($p \leq 0.05$). The chloroquine as positive control had a mechanism of action against *Plasmodium* because of its ability to inhibit polymerization and heme detoxification and to bind and alter the nature of DNA (Inbaneson & Ravikumar, 2012).

Besides antiplasmodials activity, there have been several reports of the β-sitosterol compound's pharmacological activity. It is antibacterial activity, reportedly able to inhibit the growth of several bacteria (Sen et al., 2013). As an antidiabetic, reportedly able to reduce glucose and nitric oxide (NO) levels in mice induced by streptozocin (Gupta, 2015). As an antioxidant reported, it has an IC_{50} value of 74.33 µg/mL against DPPH free radicals (Erwin et al., 2020). As an anti-inflammatory, it is able to inhibit NFκB-induced phosphorylation by TNF-α (Loizou et al., 2010). As chemoprotective, reportedly able to inhibit the growth of colon and breast cancer (Novotny et al., 2017) and having immunomodulatory activity (Bouic et al., 1996).

CONCLUSION

The β-sitosterol compound isolated from *Hyrtios reticulatus* sponge has an IC_{50} of 17.767 ± 2.86 µg/mL against *Plasmodium falciparum* 3D7 and 12.03 ± 1.60 µg/mL against FCR3 and is classified as moderate activity. This research can be a guide to find other compounds from this sponge and β-sitosterol could be tested on other activities to find more potential ones.

ACKNOWLEDGMENTS

The authors would like to thank everyone who helped and supported us during development and implementation in this research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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