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RESEARCH ARTICLE

Steroid Compounds Isolation from *Carthamus tinctorius* Linn as Antimalarial

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ABSTRACT:

Carthamus tinctorius Linn, also known as safflower, is a plant with the potential of being used in the production of antimalarial drugs. The purpose of this study was to isolate and identify the steroid compounds in the safflower and determine its antimalarial activity *in vitro*. The isolation process was conducted through extraction and chromatography methods. Then, the characterization of the isolated compounds was conducted through spectroscopic techniques which include Fourier Transform Infrared Spectroscopy (FT-IR), NMR 1-D (¹H and ¹³C-NMR), and NMR 2-D (HMQC, HMBC, and H-H COZY) as well as comparing data with the existing literatures. In addition, the tests conducted were with variations of isolate concentrations (10, 1, 0.1, 0.01, and 0.001 µg/mL) against 3D7 strain of *Plasmodium falciparum*. Based on the FT-IR spectroscopic data, the steroid compounds isolated from safflowers might be stigmasterols. In addition, the isolates had -OH functional group in the region of 3431 cm⁻¹, C-O in the region of 1053 cm⁻¹, and Csp³-H in regions of 2960, 2934, and 2865 cm⁻¹. The NMR 1-D data showed presence of 29 carbon atoms, while the protons were 48 in number. Furthermore, the IC₅₀ value of the compound was 34.03 µg/mL with a percentage inhibition of 43.92% against the growth of *P. falciparum*. Therefore, it was classified as inactive agent in inhibiting the growth of malaria parasites, however, it could be used as a marker compound in *C. tinctorius* Linn extract.

KEYWORDS: *Carthamus tinctorius*, antimalarial, Stigmasterol, NMR, Isolation.

INTRODUCTION:

Malaria is caused by plasmodium parasites in human blood cells, transmitted by female Anopheles mosquitoes. It is one of the oldest infectious diseases with widespread in the tropical regions^{1,2}. The dominant plasmodium species found in Indonesia are *Plasmodium falciparum* and *Plasmodium vivax*. Although, *Plasmodium ovale* and *Plasmodium malariae* are also found in Eastern Indonesia^{3,4}.

Infection with malaria parasites could lead to clinical symptoms such as fever accompanied with chills, sweating, headaches, nausea, vomiting, diarrhea, and muscle aches. However, people with parasitemia in the endemic areas do not usually come up with these symptoms^{1,5}.

There is a rapid development in the research of natural materials with the discovery of spectroscopic separation techniques. Through the use of this method, several structures of bioactive compounds have been discovered; like the quinine isolated from the bark of Chincona (Rubiaceae), being the first drug developed for treating malaria. Other antimalarial drugs have been developed since then, such as chloroquine and

primaquine. Despite being a standard antimalarial drug, chloroquine has been reported to be inactive for the treatment of malaria caused by *P. falciparum* while the efficacy of quinine has decreased on its efficacy⁵. The drug currently used for treating malaria is artemisinin and its derivatives are isolated from the *Artemisia annua* plant. It has a faster action compared with other antimalarial drugs because it has a better complex mechanism of action. However, there are indications it is resistant against the plasmodium-causing malaria⁶. There were clinical reports of two artesunate-resistant *P. falciparum* cases in Cambodia. There were cases of *P. falciparum* resistance and reduced efficacy of artemisinin and its derivatives at the molecular genetic level, thereby resulting in the increasingly complex and dangerous status of malaria. This parasite resistance problems could not be resolved until recently, as a result of bioactive compounds developed by pharmaceutical companies from plants used as lead compounds for the discovery of new drug^{7,8,9}.

Carthamus tinctorius Linn. plant, commonly called safflower, belongs to the Asteraceae family from Asterales order with 22,750 genera and over 1,620 species¹⁰. Other studies that have been done related to the plants that are one family (Asteraceae) is a compound artemisinin is a sesquiterpene lactone that can be used as an antimalarial, where hemozoin malaria can be inhibited by artemisinin^{11,12}. This endemic plant from Sulawesi is empirically used to treat diseases such as *sarampa*, smallpox and measles¹³. It has also been used as a laxative, antipyretic, analgesic and antidote to poisoning in traditional medicine. In addition, it has been shown to have antioxidant, anti-inflammatory, and antidiabetic activities^{14,15}.

Additionally, the plant has the huge potential of being used as a new antimalarial drug. Examination of its antimalarial activity against *P. falciparum* conducted *in vitro* showed that the extract from its flower water had about 52.68% inhibitory action on the growth of 3D7 strain of the parasite¹³. Ethanol and methanol extracts have also been found to show growth inhibitory actions against the malaria parasites. However, ethanol extract showed a higher inhibitory value of about 95.97% and an IC₅₀ value of 1.06 µg/mL, but the inhibitory value of methanol extract was around 62.39% with an IC₅₀ value of 15.89 µg/mL *in vitro*¹⁶. The ethanol extract of *C. tinctorius* Linn. have been found to inhibit the growth of *P. berghei* ANKA in mice *in vivo* with an ED₅₀ value of 24.79 mg/KgBB¹⁷. Furthermore, results from the fractionation process in the same study involving the antimalarial activity of ethanol extract from *C. tinctorius* Linn flowers *in vitro* showed that ethyl acetate fraction inhibited parasitemia. The largest inhibition percentage was 94.48% at a concentration of 100 µg/mL¹⁸.

Therefore, there is need to search for active compounds responsible for the antimalarial activity of this plant. Based on this background, the purpose of this study was to isolate and identify the steroid compounds in the safflower and determine its antimalarial activity *in vitro*.

MATERIAL AND METHODS:

Chemicals and Reagents:

The solvents used were purely distilled technical grades. Aluminum sheets kieselgel 60 PF254 0.25 mm (Merck 1.05554), silica gel 60 HF254 5-40 µm (Merck 1.07747), and silica gel 60 HF254 5-40 µm containing gypsum (Merck 1.07749) were subjected to thin layer (TLC), and vacuum liquid chromatography (VLC). Other materials used include laboratory bottles (Schott Duran[®]), microscopes (Olympus[®]), slide glass (Sail Brand[®]), disposable syringe with needle (Sigma-Aldrich[®]), closed centrifuge tubes (Labcon[®] dan Falcon[®]), micropipettes (Socorex[®]), incubator (WTC Binder[®]), cold centrifuge (Hettich[®]), and 24-well microplate (Costar 3524[®]).

Herbal Material:

The herbal sample was collected from Safflower plantation in Bone Regency, South Sulawesi. It has been marked at LIPI Purwodadi, Pasuruan, Indonesia, kept safe in the herbarium with certificate number 1795/IPH.3.04/HM/XI/2019.

Extraction:

The sample was made into powder form of about 1 kg. This was then macerated with 80% Ethanol (Et-OH) (3 x 3 L of the solvent and was replaced every 24 hours) at room temperature and filtered. The solvent from the process was evaporated using a vacuum rotary evaporator (Buchi[®]) and yielded 108.1 g thick brownish-yellow Et-OH concentrated extract (10.81% w/w).

Isolation, Purification, and Characterization of the Isolates:

Separation of the extract was conducted through the vacuum column chromatography (VCC) and radial chromatography (RC). Then, the profile was observed with the thin layer chromatography (TLC) Si-Gel F254 (Merck[®]) under UV lamps of 254 and 366 nm, and visible light by staining with cerium sulfate (CeSO₄).

The identification of the pure compounds obtained was conducted using the TLC profile. Also, the Infrared (IR) analysis of the compounds was conducted using a Nicolet iS5 spectrophotometer with iD5 ATR (Thermo Scientific[®]). However, the ¹H, ¹³C, and 2-D NMR spectra were recorded using JEOL ECP 400 MHz (Tokyo, Japan) spectrometers. Then, the data were compared with those already in literatures. Different chromatographic and spectroscopic methodologies are currently available for essential oil fingerprinting for

characterization of complex mixtures of volatile compounds. For this technique such as FT-IR and NMR spectroscopy play important role^{19,20}.

In vitro Antimalarial Assay of the Isolate:

The malaria parasite used was the chloroquine-sensitive 3D7 strain of *P. falciparum*. This was obtained from the Malaria Laboratory, Institute for Tropical Diseases, Airlangga University, Surabaya. It was cultured according to the modified Trager and Jensen’s method^{21,22}. The isolates were then tested against the cultures for antimalarial activity by checking the percentage inhibition of parasitemia (IC₅₀) in the chloroquine-sensitive 3D7 strain of *P. falciparum*.

Preparation of Cell Suspension Parasite:

The parasitemia suspension used for antimalarial test *in vitro* was 1% concentration and 5% hematocrit. About 0.5 mL of the 1% parasitemia were placed in each well making the total volume 6 mL. Then, the parasitemia was diluted to concentrations of 10% to 1% with a volume of 10 mL. This was achieved by adding 0.9 mL of 50% RBC and 8.1 mL of complete medium to 1 mL of 10% parasitemia to obtain 10 mL of 1% parasitemia. Then, to a thin blood smear, represented as D0, which is the initial parasitemia level at 0 hours before adding the test substance, add 0.5 mL of the parasite cell suspension into each well, already with 0.5 mL of the test solution.

Preparation of Isolate and Control Solution Test:

About 1 mg of the isolate was dissolved in 100 µL of DMSO (as stock of 10,000 µg/mL) and the concentrations were then varied to 0.001; 0.01; 0.1; 1; and 10 µg/mL (duplo). Then, 10 µL of the stock solution was taken and added with complete media of about 500 µL (200 µg/mL). A complete medium of 1080 µL was added in wells A1, A2, A3, A4, and A5, while 1000 µL was added to well A6 as the negative control.

About 120 µL of the stock solution was pipetted into well A1; then another 120 µL of the components in well A1 was taken and inserted in well A2; which continued to well A5. The intended volume in each well was 1000 µL, hence, an excess of 80 µL was discarded from it. Therefore, the total concentration obtained for wells A1; A2; A3; A4; and A5 were 20; 2; 0.2; 0.02; and 0.002 µg/mL, respectively.

The samples were made in duplo by taking 500 µL from A1 into B1, 500 µL from A2 into B2, 500 µL from A3 into B3, 500 µL from A4 into B4, 500 µL from A5 into B5 and then 500 µL from A6 to B6. Next, 500 µL of 1% parasitemia suspension was added to each well making the final concentration of wells A1 and B1 10 µg/mL, 1 µg/mL for A2 and B2, 0.1 µg/mL for A3 and B3, 0.01 µg/mL for A4 and B4, 0.001 µg/mL for A5 and B5, while wells A6 and B6 were the negative controls.

Parasitaemia Observations:

The cultures were incubated at 37°C for 48 hours and then harvested. A thin blood smear was made with 20% Giemsa staining to aid the observation of the red blood cells infected with *P. falciparum*. Also, the percentage of parasitemia and percentage of inhibition of *P. falciparum* growth were calculated by counting the number of infected erythrocytes in every 5000 erythrocytes under the microscope.

The percentage of parasitemia was calculated with the formula:

$$\% \text{ Parasitemia} = \frac{\sum \text{infected erythrocytes}}{\text{the number of erythrocytes}} \times 100\%$$

The percentage of inhibition was calculated with the formula :

$$\% \text{ Inhibiton} = 100\% \left[\frac{\text{growth percentage of test solutions}}{\text{growth percentage in negative control}} \right] \times 100\%$$

Statistical Analysis:

The IC₅₀ value was calculated through the probit analysis (unit probability) with SPSS 21.0 version. Then, a correlation curve was created between the percentage growth probit and the concentration logarithm through the linear regression line equation.

RESULT AND DISCUSSION:

Isolation and Purification of Isolates

The separation process was initiated by optimizing the eluent profile used in the VVC process. This eluent was a ratio of n-hexane and ethyl acetate, used because n-hexane has more non-polar properties compared with ethyl acetate, thereby facilitating the separation of non-polar and polar compounds. A good separation process was achieved with ratio 8:2 of n-hexane and ethyl acetate, while ratio 6:4 was used to separate the more polar compounds. Also, this separation process was conducted using Vacuum Column Chromatography (VCC) with silica gel as the stationery phase and hexane-ethyl acetate with increased polarity, as the mobile phase. The working principle of VCC is the separation of compounds based on their level of polarity. The process resulted in 19 fractions, after which TLC profiling was conducted to show the staining profile.

The spots exhibiting similar profile were merged into 4 fractions. The resulting fractions merged are shown in

Table 1:

Table 1. Result of fraction merging

S. No.	Fraction	Weight (g)
1	A	1.77
2	B	2.88
3	C	2.75
4	D	1.2

The combination of fraction A was found at the top, thereby making it difficult to separate. Also, the fraction was very minimal as shown in **Table 1**. The compounds in fraction B showed good separation profile and the stain formed had similar R_f value with ethyl acetate fraction. In addition, fraction B had the highest weight and after being subjected to the VCC, it resulted in 15 sub-fractions. Then, the fifth sub-fraction had a single spot with very few impurities. Therefore, subjected to decantation process by dissolving it in n-hexane solvent so as to remove the impurities in the isolates. This was followed by TLC profiling, the single stain was seen as darkish red after spraying with cerium sulfate and heated. However, it was not seen under the UV light at wavelengths of 254 and 366 nm. This gives the possibility of classifying the compound as steroid.

Characterization of the Isolated Compounds

The isolated compound was about 12 mg white crystal form and identified using FT-IR, 1-D NMR (¹H and ¹³C-NMR), and 2-D NMR (HMQC, HMBC, and H-H COZY). The results of FT-IR spectroscopy are shown in Figure 1.

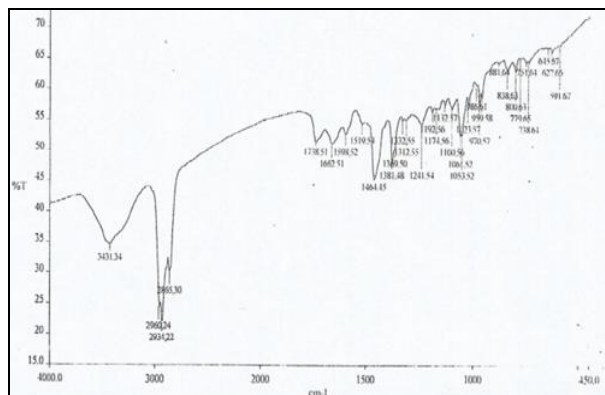


Figure 1. FT-IR spectrum of isolates

Figure 1 shows the –OH functional group produced by absorption in the region of 3431cm⁻¹, strengthened by the presence of C-O absorption at 1053 cm⁻¹. A sharp stretching shows the number of Csp³-H groups in the region of 2960, 2934, and 2865 cm⁻¹ supported by the bending of Csp³-H at 1464 and 1381 cm⁻¹ region. IR can show the main functional group in the degradation of a plant extract^{23,24}. The NMR spectrum data strengthen the existence of the functional groups above (Figure. 2).

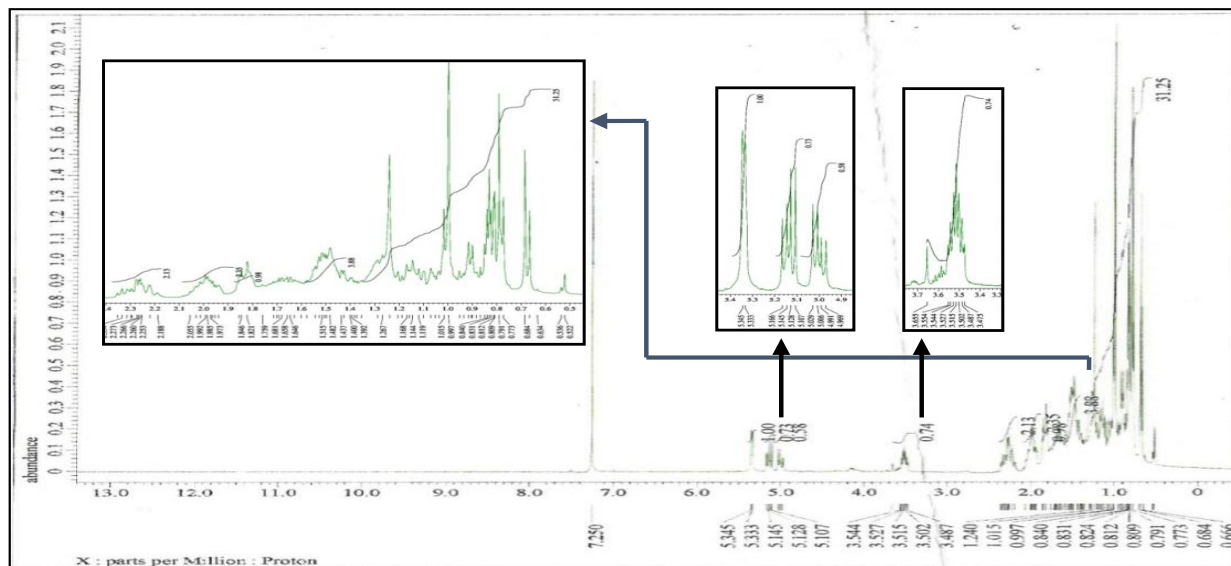


Figure 2. NMR-1D spectrum (¹³C-NMR)

The ¹³C-NMR spectrum shows 29 carbon signals of isolated compound structure. This was consistent with the data from IR spectrum, showing a sharp absorption in the region of 2935 and 2866 cm⁻¹ (Csp³ – H). In addition, there was δ_c of 140.8, 138.4 (C-23), 129.3, and 121.8 (C-4, C-7, C-8, C-10), indicating the presence of sp² carbon, although not visible in the IR spectrum. The absorption was invisible due to the large C-sp³ in the structure of the isolated compound. Also, carbon binding oxygen atoms were expected to appear in the region of 60-80 ppm and one signal expected to bind the oxygen atom at δ_c 71.9. Then, three carbons with methyl

substituents appeared at δ_c 21.2 (C-1, C-5, C-10), 21.1 (C-17), 19.5 (C-25), 19.0 (C-26), 12.4, and 12.1 (C-12, C-13).

The ¹H-NMR data in **Figure 3** shows the presence of 48 protons, of which 4-H had a significant chemical shift at δ_H 5.34 (H-6), 5.13 (H-22), 5.01 (H-23), and 3.51(H-3). This magnitude in as indication that the protons had a minimal electron density. Additionally, it was inevitable that the two protons were located at the transposition through the similarity of the coupled constant value (J=15 Hz).

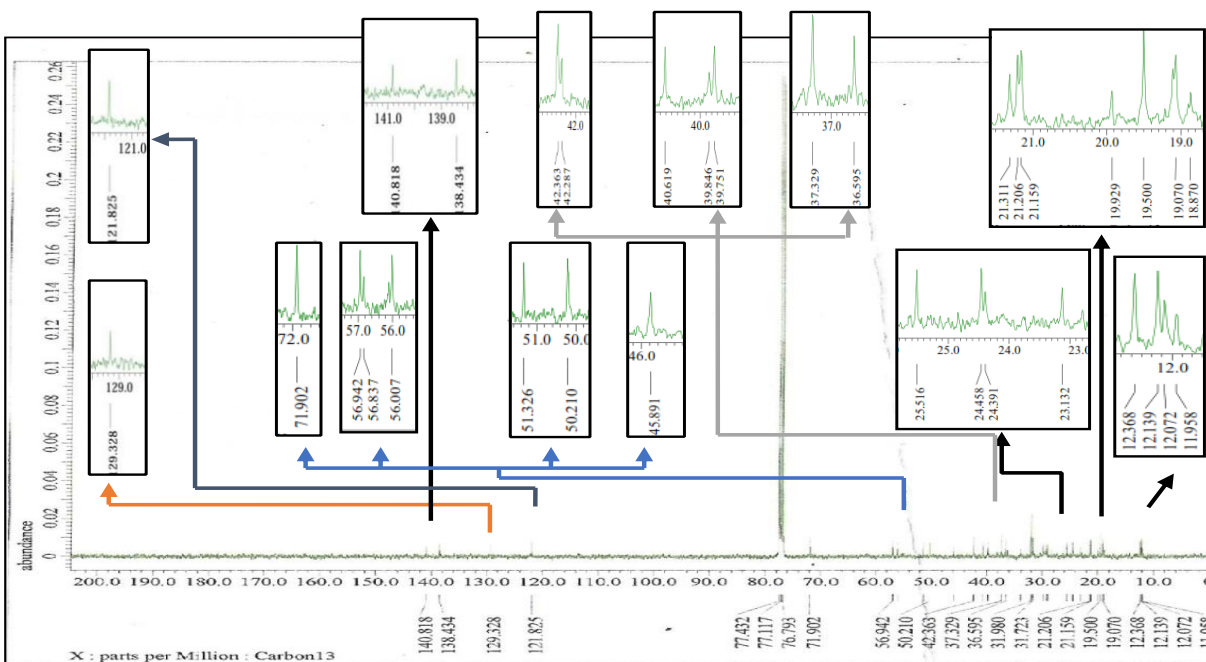


Figure 3. NMR-1D spectrum (¹H-NMR)

Table 2. Spectrum Data of HMQC Isolate

C atom	δ_c	H Position	δ_H (ΣH , mult, J dalam Hz)
1	37.3	1a	1.82 (1H, <i>m</i>)
		1b	1.15 (1H, <i>m</i>)
2	31.7	2a	1.95 (1H, <i>m</i>)
		2b	1.85 (1H, <i>m</i>)
3	71.9	3	3.51 (1H, <i>m</i>)
4	42.4	4a	2.27 (1H, <i>m</i>)
		4b	2.22 (1H, <i>m</i>)
5	140.8	-	-
6	121.8	6	5.34 (1H, <i>br d</i>)
7	32.0	7	1.93 (2H, <i>m</i>)
8	32.0	8	1.49 (1H, <i>m</i>)
9	50.2	9	0.91 (1H, <i>br d</i>)
10	36.6	-	-
11	21.3	11	1.47 (2H, <i>m</i>)
12	39.7	12	2.02 (1H, <i>m</i>)
13	42.3	-	-
14	56.9	14	0.97 (1H, <i>m</i>)
15	24.6	15	1.54 (2H, <i>m</i>)
16	29.0	16	1.27 (1H, <i>m</i>)
17	56.0	17	1.08 (1H, <i>m</i>)
18	12.1	18a	0.84 (1H, <i>br d</i>)
		18b	0.79 (1H, <i>br d</i>)
		18c	0.67 (1H, <i>br s</i>)
19	21.2	19	1.00 (3H, <i>br s</i>)
20	40.6	20	1.97 (1H, <i>m</i>)
21	21.1	21	1.00 (3H, <i>br s</i>)
22	138.4	22	5.13 (1H, <i>dd</i> , 15)
23	129.3	23	5.01 (1H, <i>dd</i> , 15)
24	51.3	24	0.91 (1H, <i>br d</i>)
25	31.1	25	1.66 (1H, <i>m</i>)
26	19.5	26a	1.00 (1H, <i>br s</i>)
		26b	0.81 (2H, <i>br d</i>)
27	19.0	27a	0.91 (1H, <i>br d</i>)
		27b	0.81 (1H, <i>br d</i>)
		27c	0.69 (1H, <i>br s</i>)
28	25.5	28	1.44 (2H, <i>m</i>)
29	12.4	29a	0.84 (1H, <i>br d</i>)
		29b	0.79 (1H, <i>br d</i>)
		29c	0.67 (1H, <i>br s</i>)

The ¹H-NMR spectrum showed proton buildup with numerous integration. Also, the proton multiplicity data (*m*, *br d*, and *br s*) showed the number of neighboring protons and were seen with very close chemical shifts. This makes the structure of the isolates to be similar to that of steroids. Furthermore, based on the data of ¹H and ¹³C-NMR, it was estimated that the molecular formula of the isolated compound was C₂₉H₄₈O with DBE (Double Bond Equivalence) of 6.2, derived from alkene groups formed by 4 C-sp³ atoms and 4 others derived from cyclic carbon.

This was followed by the NMR 2-D data analysis of HMQC, stating the correlation between carbon signals directly bonded to the proton in Table 2.

Based on the HMQC spectrum, there was a direct correlation between δ_H 5.34, 5.14 and 5.01 ppm protons with δ_C 121.8, 138.4 and 129.3 ppm carbons respectively. Also, there was a quaternary carbon with δ_C 141.8 ppm. Conversely, the protons with 3.34 ppm shift were directly bound to the carbon with a δ_C of 71.8 ppm, indicating that the carbon and proton have a low electron density close to the electron withdrawal group (-OH) as shown by the IR data.

The direct correlation between the protons and carbon was supported by HMBC data which interpreted the correlation to a maximum of 3 bonds. The spectrum of HMBC isolates compound was presented in Table 3.

Table 3. Spectrum Data of HMBC Isolate Compound

Atom C	δ_c	H position	δ_H [$\Sigma H, mult, J$ (Hz)]	HMBC
1	37.3	1a	1.82 (1H, <i>m</i>)	C-5
		1b	1.15 (1H, <i>m</i>)	-
2	31.7	2a	1.95 (1H, <i>m</i>)	-
		2b	1.85 (1H, <i>m</i>)	C-3
3	71.9	3	3.34 (1H, <i>m</i>)	-
4	42.4	4a	2.27 (1H, <i>m</i>)	C-3, C-5, C-6
		4b	2.22 (1H, <i>m</i>)	C-2, C-3, C-5, C-6, C-10
5	140.8	-	-	-
6	121.8	6	5.34 (1H, <i>br d</i>)	C-4, C-7, C-8, C-10
7	32.0	7	1.93 (2H, <i>m</i>)	-
8	32.0	8	1.49 (1H, <i>m</i>)	-
9	50.2	9	0.91 (1H, <i>br d</i>)	C-11, C-19
10	36.6	-	-	-
11	21.3	11	1.47 (2H, <i>m</i>)	C-8
12	39.7	12	2.02 (1H, <i>m</i>)	-
13	42.3	-	-	-
14	56.9	14	0.97 (1H, <i>m</i>)	C-7, C-8, C15, C-18
15	24.6	15	1.54 (2H, <i>m</i>)	-
16	29.0	16	1.27 (1H, <i>m</i>)	-
17	56.0	17	1.08 (1H, <i>m</i>)	C-18
18	12.1	18a	0.84 (1H, <i>br d</i>)	-
		18b	0.79 (1H, <i>br d</i>)	-
		18c	0.67 (1H, <i>br s</i>)	C-12, C-13
19	21.2	19	1.00 (3H, <i>br s</i>)	C-1, C-5, C-10
20	40.6	20	1.97 (1H, <i>m</i>)	-
21	21.1	21	1.00 (3H, <i>br s</i>)	C-17
22	138.4	22	5.14 (1H, <i>dd</i> , 15)	C-23
23	129.3	23	5.01 (1H, <i>dd</i> , 15)	-
24	51.3	24	0.91 (1H, <i>br d</i>)	C-26, C-27
25	31.1	25	1.66 (1H, <i>m</i>)	-
26	19.5	26a	1.00 (1H, <i>br s</i>)	-
		26b	0.81 (2H, <i>br d</i>)	C-25
		27a	0.91 (1H, <i>br d</i>)	C-26
27	19.0	27b	0.81 (1H, <i>br d</i>)	C-25
		27c	0.69 (1H, <i>br s</i>)	-
		28	1.44 (2H, <i>m</i>)	C-29
29	12.4	29a	0.84 (1H, <i>br d</i>)	C-24
		29b	0.79 (1H, <i>br d</i>)	-
		29c	0.67 (1H, <i>br s</i>)	-

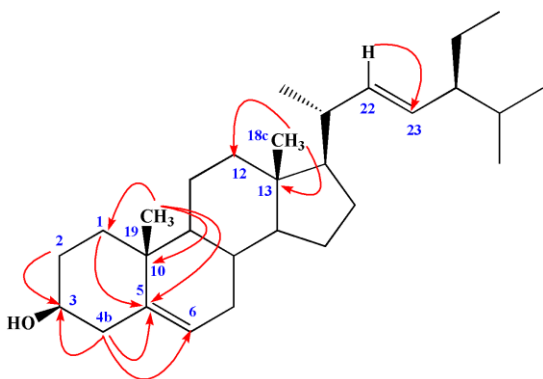


Figure 4. HMBC Isolate Compound

The data in **Table 3** and **Figure 4** show the correlation of protons at position 6 with C-4, C-10, C-7, and C-8. There was a correlation between H-19, C-5, and C-10, proving the accuracy of the proposed structure. Also, the correlation between H-18c, C-12, and H-13 contributed to the accuracy of the carbon location with chemical

shifts of 39.7 and 42.4 ppm. Therefore, the data confirmed the assumption that the isolated compound was an alcohol steroid group which generally have a basic framework with the -OH group usually located close to the sp^2 carbon quaterner. Then, further verification was performed using another NMR 2-D spectrophotometer known as H-H COSY. The result of H-H COZY the relative stereochemical correlation between the adjacent proton units. Also, the data showed no spatial correlation among the protons in methines which were close together, such as in H-8 and H-9, H-8 and H-14, H-17 and H-18, as well as in H-24 and H-25.

Furthermore, the spectrum data of IR, NMR 1-D (1H and ^{13}C -NMR with the DEPT technique) and 2-D NMR (HMQC, HMBC and H-H COZY) produced an assumption that the isolated compound was stigmasterol. The data was compared with past literatures showing similar profile^{25,26,27}, as shown in Table 4.

Table 4. Comparison of ¹H and ¹³C-NMR of Isolate Compound (ppm, measured under 100 MHz (¹³C) and 400 MHz (¹H) in CDCL₃, δ TMS = 0.

Atom C	δ C (ppm)	δ C _b (ppm) ²⁵	δ C _c (ppm) ²⁶	Atom H	δ H (ΣH, mult, J [Hz])	δ H ^d (ΣH, mult, J [Hz]) ²⁷
1	37.3 (CH ₂)	37.28	37.25	3	3.34 (1H, m)	3.35
2	31.7 (CH ₂)	31.91	31.64	6	5.34 (1H, br d)	5.36
3	71.9 (CH)	71.78	71.81	22	5.14 (1H, dd)	5.14
4	42.4 (CH ₂)	42.31	42.29	23	5.01 (1H, dd)	5.03
5	140.8 (Cq)	140.76 121.69	140.73			
6	121.8 (CH)	31.91	121.72			
7	32.0 (CH ₂)	31.91	31.89			
8	32.0 (CH)	50.18	31.89			
9	50.2 (CH)	36.52	50.12			
10	36.6 (Cq)	21.09	36.40			
11	21.3 (CH ₂)	39.70	21.08			
12	39.7 (CH ₂)	42.22	39.68			
13	42.3 (Cq)	56.88	42.29			
14	56.9 (CH)	24.38	56.87			
15	24.6 (CH ₂)	28.92	24.38			
16	29.0 (CH ₂)	55.97	28.24			
17	56.0 (CH)	12.02	55.93			
18	12.1 (CH ₃)	21.09	11.87			
19	21.2 (CH ₃)	40.20	19.38			
20	40.6 (CH)	21.23	40.05			
21	21.1 (CH ₃)	138.31	21.20			
22	138.4 (CH)	129.28	138.49			
23	129.3 (CH)	51.25	129.27			
24	51.3 (CH)	31.66	51.23			
25	31.1 (CH)	19.40	31.89			
26	19.5 (CH ₃)	19.00	19.00			
27	19.0 (CH ₃)	25.41	19.00			
28	25.5 (CH ₂)	12.25	25.45			
29	12.4 (CH ₃)		12.27			

The comparison of the data shows that the ¹H spectrum and ¹³C-NMR isolates were identical to the stigmaterol compounds in literatures. This indicates that the compound isolated might be stigmaterol (**Figure 5**). It was reported to be isolated from many plants such as *Ambroma augusta*, *Strychnos potatorum* and *Dalbergia volubilis* flowers²⁸.

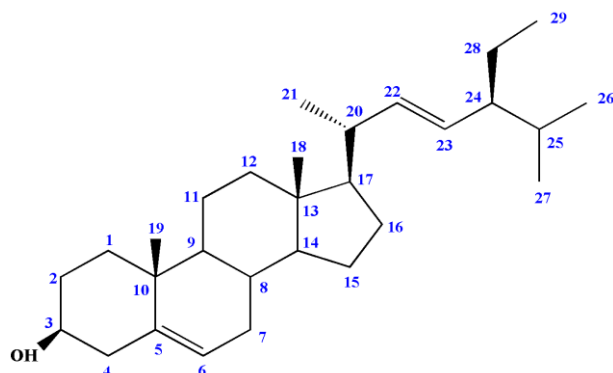


Figure 5. Stigmaterol Structure

In Vitro Antimalarial Activities:

The isolated compound was subjected to test to know its potency as antimalarial substance *in vitro*. *In vitro* antimalarial activities are experimental models for detecting antiplasmodial activity of plant extracts in the erythrocytic stage of malaria parasites²⁹. The life cycle of parasite in the mosquito is dependent on temperature

and other climatic factors. At very high temperature of around 45°C the cycle is interrupted because the parasite is unable to survive³⁰. The results showed that the concentration of 10 µg/ml exhibited the highest inhibitory ability at 43.92%. The probit log analysis conducted to determine the IC₅₀ value or the isolate concentration which inhibit the parasites' growth by 50% showed that the IC₅₀ of isolates and chloroquine phosphate were 34.03 and 0.006 µg/ml, respectively, as shown in **Table 5**. According to Lemma et al, antimalarial activity are classified into very strong, strong, moderate and inactive, if the IC₅₀ are < 0.1; 0.1-1; 1-5; and > 5 µg/ml, respectively³¹. A research by Hamsidi et al. (2015) tested the ethanol extract of *C. tinctorius* L against the 3D7 strain of *P. falciparum* and exhibited high inhibitory property at 95.97% and IC₅₀ of 1.06 µg/mL, therefore, classified as strong activity.

Table 5. IC₅₀ values of Isolates

Concentration (µg/ml)	% Inhibition	IC ₅₀ (µg/ml)
Control (-)	-	34.03
10	43.92	
1	35.01	
0.1	23.25	
0.01	17.30	
0.001	4.73	

Also, the antimalarial activity are classified as very active, active, less active, and inactive, if the IC₅₀ were < 5; 5-50; 50-100; and > 100 µg/mL, respectively³². Based

on the results, the isolated compound had a stronger antimalarial activity compared with stigmasterol isolates due to the many active compounds contained in the extract that work synergistically in inhibiting the parasitemia growth. This was supported by the research conducted by Indriani et al. (2020), which showed that three chemical compounds of the stigmastane steroid isolated from *Dryobalanops oblongifolia* stem bark were not promoted as antimalarial agents³³.

CONCLUSION:

The isolated compound from the *C. tinctorius* Linn was stigmasterol, with inhibition percentage of 43.92% at concentration of 10 µg/mL and IC₅₀ value of 34.03 µg/mL. Based on its IC₅₀ value, the compound was classified as inactive agent in inhibiting the growth of malaria parasites, however, it could be used as a marker compound in *C. tinctorius* Linn extract.

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CONFLICT OF INTEREST:

None declared.

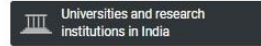
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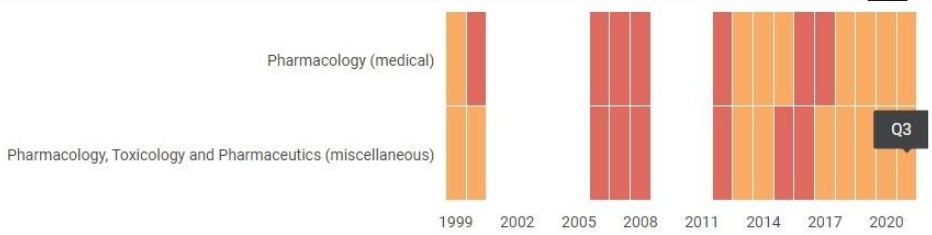
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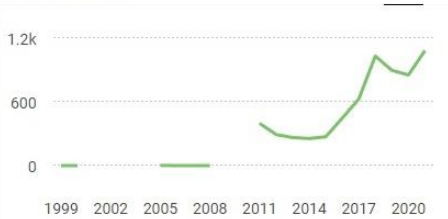
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CiteScore rank 2021

Category	Rank	Percentile
Pharmacology, Toxicology and Pharmaceutics	#13/30	58th
Pharmacology, Toxicology and Pharmaceutics (miscellaneous)		
Medicine	#180/255	29th
Pharmacology (medical)		