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ANTIPLASMODIAL ACTIVITY AND MALATE QUINONE OXIDOREDUCTASE INHIBITOR OF STEROID ISOLATED FROM *Fibraurea tinctoria*

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ABSTRACT

Malaria is an infectious disease that causes 400,000 deaths every year. Based on that fact, antimalarial exploration needs to be developed. This study aimed to explore another compound in *Fibraurea tinctoria* that is known to have antiplasmodial activity from the berberine compound. Lactate dehydrogenase (LDH) assay and malate quinone oxidoreductase (pfMQO) inhibitor assay against *Plasmodium falciparum* 3D7 was used to evaluate the activity. The result showed that methanol extract, n-hexane fraction, n-hexane subfraction, and steroid compounds isolated from n-hexane subfraction had the potential ability to inhibit the growth of *Plasmodium falciparum* 3D7 as well as the pfMQO enzyme. The results indicate that *Fibraurea tinctoria* may serve as a promising medicinal plant for future antimalarial drug development.

Keywords: *Fibraurea tinctoria*, LDH Assay, Malate quinone oxidoreductase *inhibitor, Plasmodium falciparum.*
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INTRODUCTION

Plasmodium parasites caused malaria-endemic disease[1]. World Health Organization (WHO) has reported 400,000 deaths every year, including babies and pregnant women every 2 minutes because of this disease.[2,3] Female mosquitoes are vectors that transmit the malaria parasite[4] to humans through mosquito bites. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae* are the four types of *Plasmodium* that can infect humans (in 2004, it was found that *Plasmodium knowlesi* also infect humans).[5,6] *P. falciparum* is the type that most causes death in children under five years.[7]

In the *P. falciparum* mitochondria, glycolysis, glutamate metabolism, electron transport, porphyrin, purine, and pyruvate metabolism occur. ATP formation plays an essential role in the process of electron transport. This process involves four enzyme complexes, consisting of complex I (NADH dehydrogenase), complex II (succinate dehydrigenase), complex III (coenzyme Q-cytochrome C reductase), complex IV (cytochrome oxidase), and complex V (ATP synthase). Electrons released from NADH will be bound to complex I and transported to complex II. This complex receives electrons from FADH2, which is produced by the Krebs cycle and then transported to complex III. The electrons from the coenzyme-Q are transported to cytochrome C and pumped from the matrix to the intermembrane space. The O_2 is reduced to H_2O when these electrons are transported from cytochrome C to O_2. This process is carried out by complex IV, in which two protons pump from the matrix into the space between the membranes. The electron transport reaction causes the formation of an electrochemical gradient (pH in the inter-membrane space is lower than

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in the mitochondrial matrix). The proton difference contains potential energy, so if the proton flows back through the complex V (ATP synthase), the energy is released and promotes the synthesis of ATP from ADP and inorganic phosphate.[8] Electron transport in *P. falciparum* can be seen in Fig.-1.

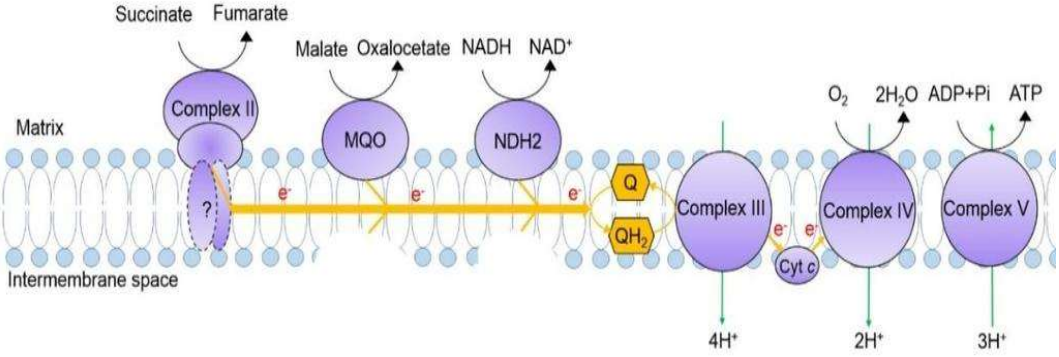

Fig.-1: The Process of Electron Transport in *Plasmodium falciparum*[6]

Malate Dehydrogenase (MDH) in *P. falciparum* is replaced by Malate Quinine Oxyreductase (MQO). In humans, the acceptor of MDH is NAD^+, whereas, in *P. falciparum,* the acceptor is ubiquinone. The MQO enzyme plays an essential role in the mitochondrial electron transport, tricarboxylic acid cycle, and fumarate cycle. Citrate, produced by the fumarate cycle is the precursor of coenzyme A in the pyruvate process, which is the main product of mitochondria. The MQO enzyme also plays a vital role in electron transport from complex II to complex III. Because of these functions, MDH and MQO are targeting the development of antimalarial drugs.[9]

Quinine and artemisinin are the two most effective drugs used in malaria treatment that are derived from nature. Many synthetic antimalarial drugs were derived from these natural compounds.[10] *Fibraurea tinctoria* is a medicinal plant used for the ethnomedical treatment of malaria, one of which is in Borneo Indonesia.[11] *F. tinctoria* is a large woody plant with yellow stems up to 40 meters long and 5 centimeters wide. The oval-shaped leaves resemble *Piper betel* leaves but without a distinctive aroma.[12] Methanol extract, methylene chloride, and methanol fraction of *F. tinctoria* stem are reported to have the ability to inhibit the growth of *P. falciparum* FcB1/ Colombia strain with IC_{50} values of 0.7 µg/ml; 0.5µg/ml, and 1.1 µg/ml respectively.[13] Methanol extract of *F. tinctoria* has antiplasmodial *berghei* anka at a dose of 10 mg/g of body weight.[14] Recent studies found that berberine-containing *F. tinctoria* inhibits the growth of *P. falciparum* with a mechanism that inhibits the *P. falciparum* telomeration activity.[15] Based on these facts, *F. tinctoria* was carried out for further research with the aim of identifying other compounds besides berberine that have the potential effect of antimalarial activity. The research began with extracting and isolating compounds beside berberines from *F. tinctoria,* continued with the antiplasmodium activity using the LDH assay in *P. falciparum* 3D7, and finally tested the inhibition assay of PfMQO enzyme.

EXPERIMENTAL

Plant Materials

The sample of *Fibraurea tinctoria* was collected from Wartono Kadri Samboja Forest in East Kalimantan. It was further analyzed at the Herbarium Center for the Conservation of Natural Resources, Samboja, East Kalimantan. The stems of the *F. tinctoria* were cleaned and chopped to make simplisia. It is then dried and milled.

Extraction and Isolation

The simplisia of *F. tinctoria* stem was extracted using methanol as a solvent by the reflux method. The solvent was then evaporated using a rotary evaporator. Isolation was begun with liquid-liquid extraction of methanol extract of *F. tinctoria*. It was dissolved in water-methanol in a ratio of 7:3 (the amount of water-methanol given was 20 fold of the extract used). It was then fractionated with organic solvents with stratified polarity starting from n-hexane, ethyl acetate, and water-methanol three times repeatedly. The extract and three types of fractions were tested using HPLC method to determine the berberine compound in them.[16]

The N-hexane fraction is selected and continues to be separated and purified. The separation process used column chromatography with the gradient of solvent starting from n-hexane: ethyl acetate of 10:0, 9:1, 8:2 until 0:10. The subfraction from this process was purified using thin-layer chromatography (TLC), which is characterized by obtaining a single spot. The monitoring process was continued by using The isolate was then characterized by identifying its melting point, NMR structural elucidation, and mass molecule using an LC-MS/MS instrument with a method described by Khalaf 2011.[17]

Lactate Dehydrogenase Plasmodium falciparum Assay
Parasite Culture
Plasmodium falciparum 3D7 was used as an infectious agent and was provided by the Biotechnology Center of BPPT Serpong. P. falciparum 3D7 strain was cultured in 3% hematocrit type A human RBC in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10µg/ml hipoxanthine, 25 mM sodium hydrogen bicarbonate, 0,5% (w/v) Albumax II (Gibco), and 40µg/ml gentamicin sulfate. The culture was maintained in a multi-gas incubator MG-70M (Taitec) at 5% O_2, 5% CO_2, and 90% N_2 at 37 ˚C. The medium was replaced daily, and parasitemia was maintained at below 5% for routine subculture.

***In-vitro* Assay**
10 ml of *P. falciparum* 3D7 culture in the complete medium of RPMI 1640 was synchronized with 6 ml of sorbitol 5% and homogenized for 6 minutes, followed by incubation for 15 minutes. The supernatant was then replaced by the new RPMI-1640 and the percent of parasitemia was calculated. The sample was prepared with a variety of concentrations using the microdilution method with DMSO as a solvent. DMSO was used as a negative control, and 250 µM of atovaquone was used as a positive control. The layout of the sample, negative control, and positive control in a 96-well-plate can be seen in Fig.-2.

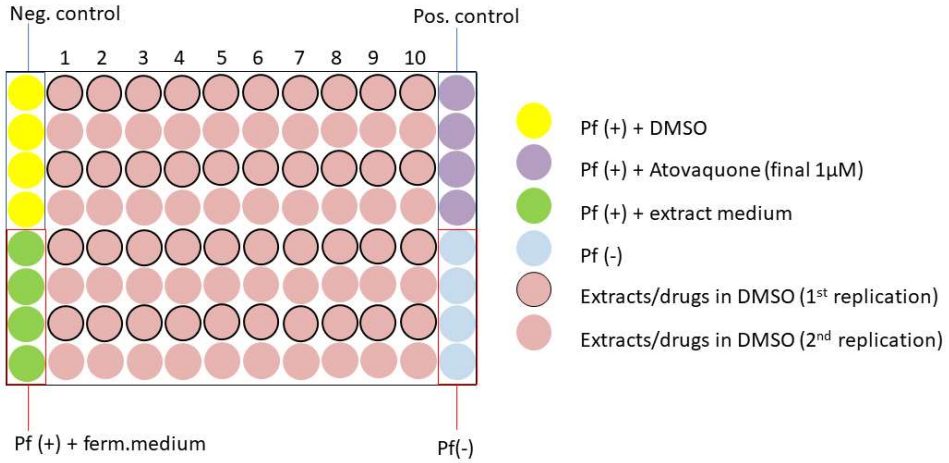

Fig.-2: The Layout of Samples, Negative Control, and Positive Control in a 96-well-plate for LDH Assay

The synchronized well-plate was diluted with RPMI complete medium until the parasitemia value was 0.3%, then 3% hematocrit was added. 0.4 µl serial dilution of extract, DMSO, complete medium with RBC, and atovaquone were added to the pink, yellow, blue, and purple columns respectively. *P. falciparum* (100 µl) was then added to each well, incubated at 37˚C, 5% O_2, and 90% N_2 for 72 hours. After 72 hours of incubation 200 µl of cold PBS was added and sealed with a plastic seal. It was spun for 5 minutes at 1300x g and removed the supernatant. The well-plate containing the pellets was sealed with a plastic seal, wrapped, and frozen at -30 ˚C to lyse erythrocytes.
The LDH buffer that had previously frozen at -30 ˚C was prepared. The LDH buffer was made by stirring slowly the mixture of 50 ml of Tris HCl 1M into 450 ml of Mili Q water, 2.8 g sodium L-lactate, and 1.25 ml of triton X 100 at room temperature. In 10 ml of LDH buffer, 2 mg of nitroblue tetrazolium (NBT) were dissolved in a dark container. 50 µl of acetylpyridine adenine dinucleotide (APAD)-3 in Mili Q water and 200 µl diaphorase was added to every 10 ml of the substrate. Plasmodium-containing pellets are suspended

and added to 90 µl of the substrate in each well. The well-plate was sealed and placed on the flat shaker at 650 rpm at room temperature. It was then incubated for 30 minutes and read using a microplate reader at 650 nm.[6] The percent of inhibition was calculated as follows (eqn.-1):

$$\% \text{ Inhibition } = \left(1\text{-} \frac{\text{A650 extracts or drug - A650 positive control}}{\text{A650 negative control - A650 positive control}}\right) \times 100\% \quad (1)$$

Notes: Positive control = Pf(+) + atovaquone; Negative control = Pf(+) + DMSO

IC_{50} was analyzed using GraphPad Prism v.7.0 from the variation of % inhibition in each concentration.

Plasmodium Falciparum Malate Quinon Oxidoreduktase Assay

10 mg/ml of extract was prepared in DMSO, and 2 µL was transferred into a 96-well-plate (flat clear bottom, Nunc cat no. 430341 bottom) using an electric multi-channel pipette. 20 ml of assay mix was prepared in a 50 ml tube (for one 96-well-plate) by mixing 20 ml of HEPES 50 mM pH = 7.5, 200 uL of DCIP 12 mM (final concentration = 120 uM), 8.3 uL of decyl ubiquinone 60 mM (final concentration = 25 uM), and 3.1 uL of recombinant enzyme PfMQO (final concentration 2.5 µg/ml). All the assay mix was transferred into a reagent reservoir, and 193 µL of assay mix was added into each well using a multi-channel pipette with the layout according to Fig.-3. The well-plate was placed on the plate mixer at 700 rpm for 10 sec, 900 rpm for 10 sec, and 1350 rpm for 10 sec. The absorbance was read in kinetic mode using a SpectraMax microplate reader at 600 nm, 37 ˚C for 1 minute. The data was saved as a background (refer to SOP for SpectraMax Microplate Reader). The well-plate was then transferred into a plate mixer and added 5 µL of 400 mM L malate into each well (except wells in row 12). The plate was placed on the plate mixer at 700 rpm for 10 sec, 900 rpm for 10 sec, and 1350 rpm for 10 sec. The absorbance was read in kinetic mode using a SpectraMax microplate reader at 600 nm, 37 ˚C for 8 minutes, and the data was calculated by the % inhibition **(2)**, which was saved as activity.[9]

$$\% \text{ Inhibition} = 100 - \left[\left(\frac{\text{sample-positive control}}{\text{negative control}}\right) \text{x } 100\ \%\right] \quad (2)$$

Notes: Sample = absorbance decreased in the sample after 20 minutes reading; Positive control = average of absorbance decreased of row no 12; Negative control = average of absorbance decreased of row no 1.

IC_{50} was analyzed using GraphPad Prism v.7.0 from the variation of % inhibition in each concentration

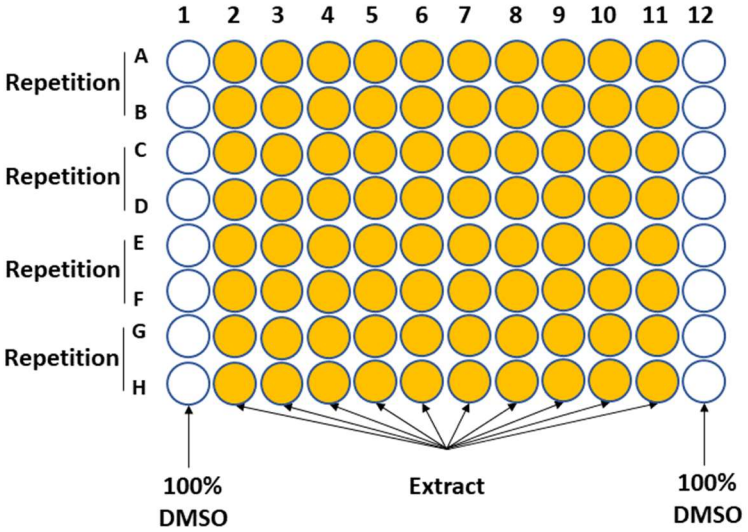

Fig.-3: The Layout of Samples in a 96-well-plate for PfMQO Assay

RESULTS AND DISCUSSION

The goal of this study was to find antimalarial candidates from *Fibraurea tinctoria.*The N-hexane fraction was carried out a series of tests, including the LDH assay and PfMQO enzyme inhibitor of *Plasmodium*

falciparum. Extraction was done by using the reflux method, and the fractionation was carried out by liquid-liquid column chromatography. Berberine was analyzed in the samples using HPLC with the Berberine chloride Merck® standard and the berberine standard calibration curve (Fig.-4). It was found in methanol extract, ethyl acetate, and water-methanol fraction, while the n-hexane fraction showed no berberine (Table-1).

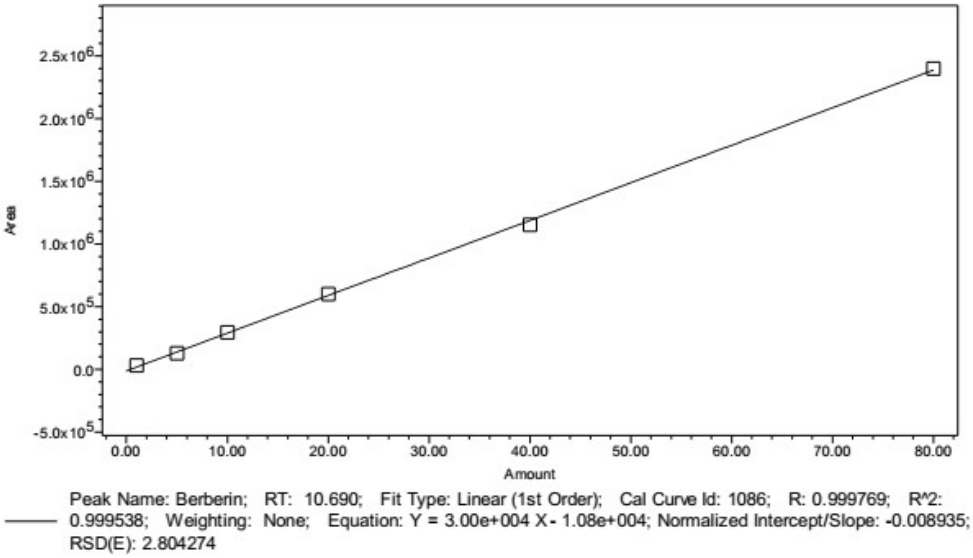

Fig.-4: Standard Calibration Curves of Berberine Chloride

Table-1: HPLC Results of n-hexane Fraction, Ethyl acetate Fraction, and Water-methanol Fraction ethyl acetate Fraction, and Water-methanol Fraction from methanol Extract of *Fibraurea tinctoria*

Sample	Berberine (%)
Methanol extract	9.67
n-hexane fraction	0
Water-methanol fraction	7.95
Ethyl acetate fraction	13.01

The selected n-hexane fraction was further separated by using column chromatography. Several compounds with similar polarity can be seen in the TLC profile in Fig.-5.

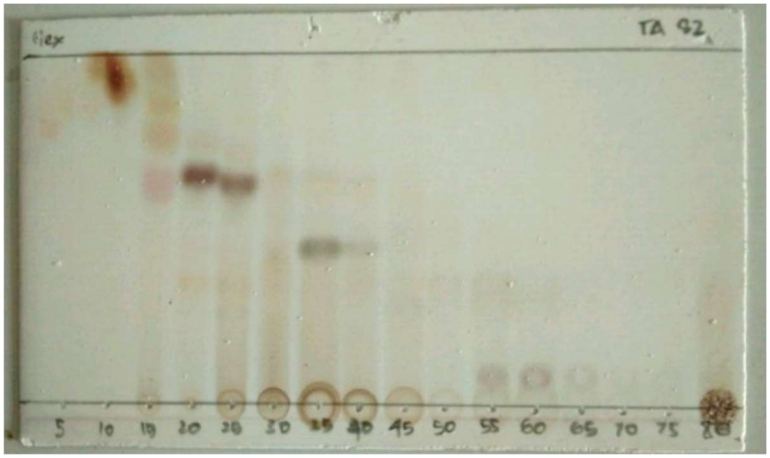

Fig.-5: TLC Profile of n-hexane Subfraction of *Fibraurea tinctoria* using Toluene: Acetone (8: 2)

Subfraction were grouped to 8 vials (from 80 vials) based on their compound similarity, vial A: 1 – 10 = 8%; vial B: 11 – 14 = 7%; vial C: 15 – 19 = 3%; vial D: 20 – 25 = 8%; vial E: 26 – 35 = 6%; vial F :36 –

44 = 3%; vial G : 45 – 59 = 3%; vial H : 60 – 80 = 4%. The compound from vial B showed good purity, so further analysis was carried out by separation and purification using decantation method. Crystals were rinsed with n-hexane-ethylacetate (9:1) until there appeared a single spot in TLC. The TLC profile of compound in vial B can be seen in Fig.-6.

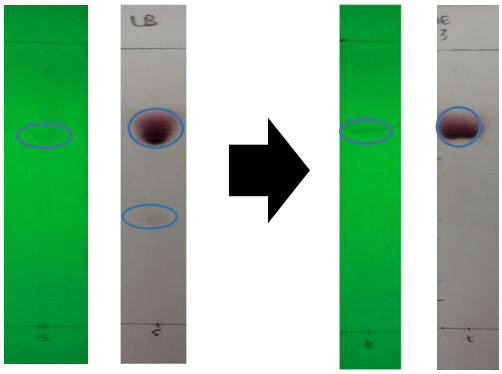

Fig.-6 : Crystal monitoring of HB Compounds

The melting point, nuclear magnetic resonance (NMR), and mass spectroscopy (MS) was used to identify and characterize the isolate. It has been found that the melting point of the isolate was 139.5ºC, which was shown to be a phytosterol coumpound.[18]

The ^{13}CNMR spectrum (Fig.-7) shows 59 carbon signals, indicating a mixture of compounds. ^{1}HNMR (Fig.-8) shows that there are alkane proton groups at shifts of 0.68 - 3.54 ppm and alkane proton shifts of 5.01 - 5.35 ppm. At the shifting proton of 0.68 and 0.69 ppm, there is a peak of the singlet, that coincides. This shows that other compound components have almost the same structure as the main component. The HSQC spectrum (Fig.-9) shows the relationship between protons and carbon, which has one bond, while the HMBC spectrum (Fig.-10) shows the correlation between protons and carbon.

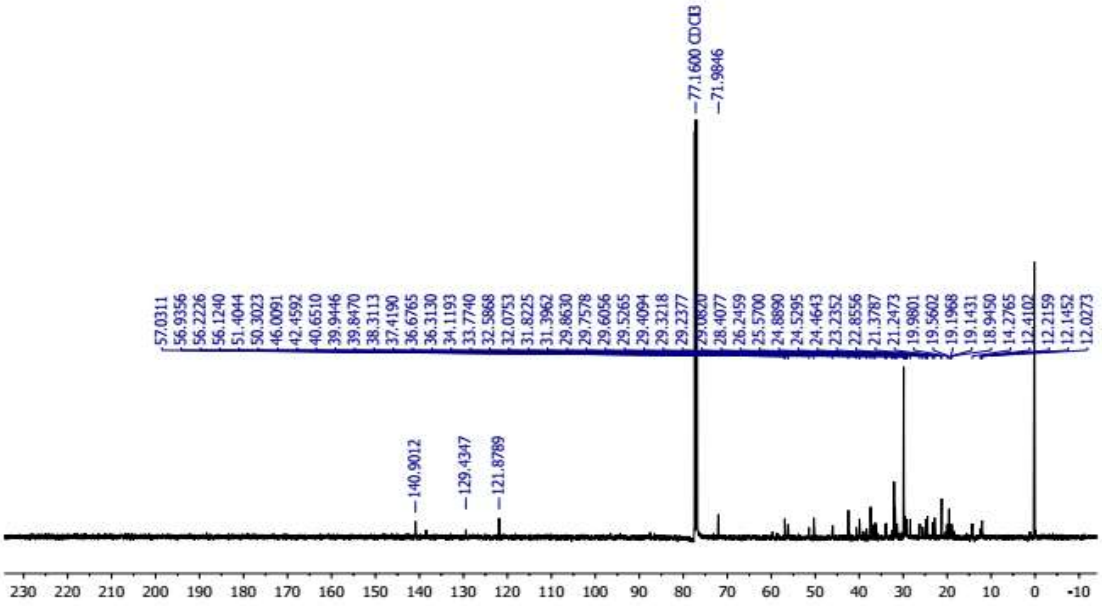

Fig.-7: ^{13}CNMR Spectra of HB Isolate in $CDCl_3$

From the interpretation of CNMR, HNMR, HSQC, and HMBC, the compound obtained from the HB isolate is a mixture of β-sitosterol and stigmasterol. Both of these compounds are steroid compounds derived from plants/phytosterols, with a fundamental difference seen in carbon atom double bonds numbers 23, 24. This bond shows from the spectrum of protons 5.16 and 5.03 ppm, which has a J-coupling value of 15.08 (trans relationship). The interpretation of NMR data can be seen in Table-2.

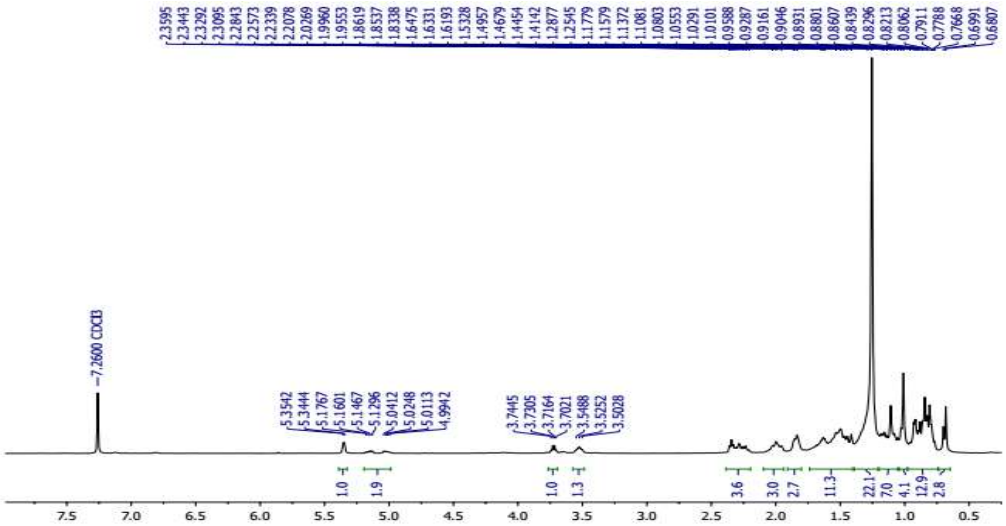

Fig.-8: ^{1}HNMR Spectra of HB Isolate in $CDCl_3$

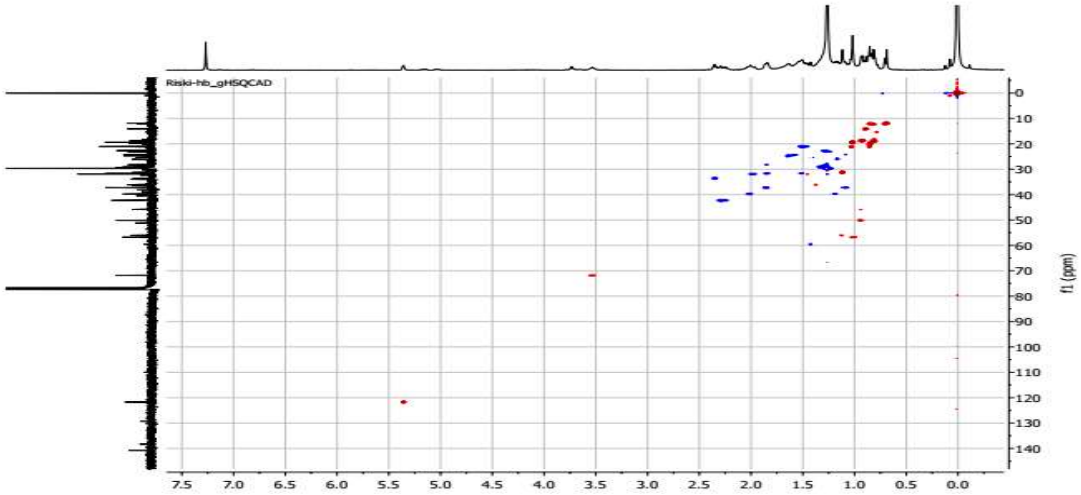

Fig.-9: HSQC Spectra of HB Isolate in $CDCl_3$

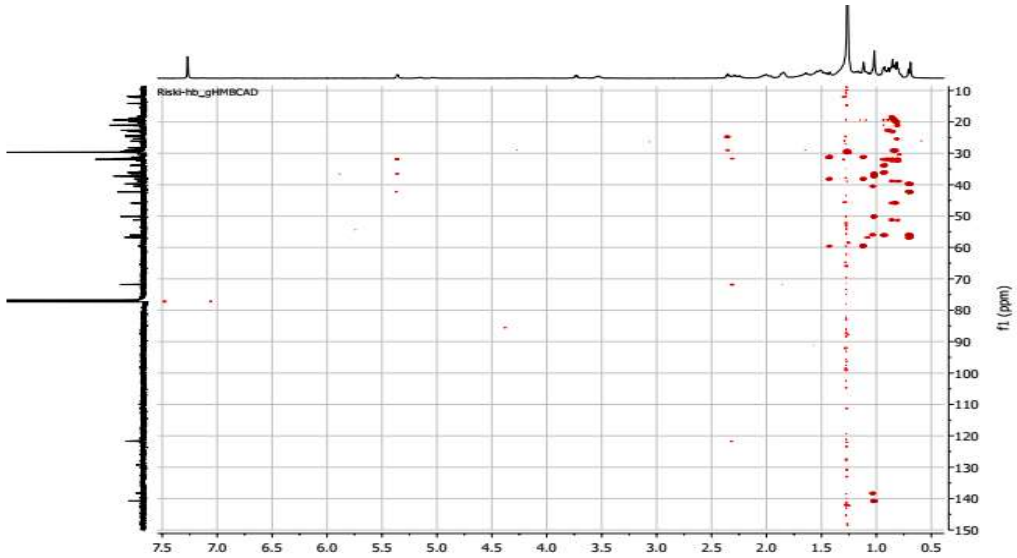

Fig.-10: HMBC Spectra of HB Isolate in $CDCl_3$

Table-2: [1]HNMR Spectrum and Correlation with HMBC Compound β-sitosterol and Stigmasterol from HB Isolate

Stigmasterol				β-sitosterol			
Shift (ppm)	H's	Type	HBMC Corelatin	Shift (ppm)	H's	Type	HBMC Corelatin
0.71	1	s	C18	0.68	3	s	C18
0.83	9	m	C29, C27, C30	0.77	9	m	C30, C29, C24
1.02	4	s	C4, C21, C2, C20	0.91	6	m	C27, C22, C2, C4
1.17	5	m	C6, C28, C7, C14	1.01	3	s	C25
1.47	5	m	C26, C3, C25	1.03	11	m	C21, C23, C8, C20, C19, C7,
1.71	+2	m	C8	1.42	7	m	C5, C3, C10, C9
1.86	1	m	C15	1.84	1	m	C28
2.02	2	m	C10, C19	1.81	3	m	C14, C15, C8
2.28	+2	m	C17	1.95	2	dd	C10
3.53	1	m	C15	2.21	1	m	C17
5.03	1	dd	C24, J-coupling 15.08	2.28	1	ddd	C17
5.16	1	dd	C23, J-coupling 15.08	3.50	1	m	C16
5.36	1	br.S	C11	5.35	1	m	C11

The result of the LC-MS/MS found that the HB compound's molecular weight was 397.372 with beta-sitosterol prediction and 395.3525 with stigmasterol prediction.

All the samples were subjected to the growth inhibition of *P. falciparum* 3D7 using the LDH assay. The result showed that all the samples could inhibit the growth of *P. falciparum* in a high category.[19] with IC_{50} <5 µg/ml. The LDH assay is used for the quantifying of lactate dehydrogenase (LDH) enzyme, which is a terminal enzyme in *P. falciparum* glycolysis pathway.[20] The LDH accumulation shows the survival of malaria and the effect of the test compound given in order to inhibit the growth of *P. falciparum*.[21] The principle of this assay is to convert the APAD and lactate to APDH and pyruvate by the pfLDH enzyme. APADH reduces the chromogenic NBT substrate by using the enzyme diaphorase. This step will develop Nitro Blue Formazan (NBF), a soluble, purple dye that can be quantified at 650 nm. The density of NBF will be directly proportional to the ability of the test compound to inhibit the growth of *P. falciparum*.

A further experiment was conducted to evaluate the ability of all the test compounds to inhibit the PfMQO enzyme. The principle of this assay is based on the electron exchange that involves malate, decilubiquinone (dUq), and dichlorophenolindophenol (DCIP). Under normal conditions, the malate in the assay will change to oxaloacetate.[22] This process will be followed by changing the decylubiquinone into decubiquinol, which releases electrons. The electrons are captured by DCIP and change the blue color to clear.[23] In the spectrophotometric assay, DCIP is a reagent used to measure reductor agents. Under resistance conditions, malate will not turn into oxaloacetate. It results in no change from decylubiquinone to dequbiquinol, and the result remains blue. The test conducted on HB isolate, n-hexane fraction, and methanol extract found an inhibition of the PfMQO enzyme of *P. falciparum*. The inhibition value can be seen in Table-3.

Tabel-3: Percentage of Yield, IC_{50} Antiplasmodial LDH Assay and IC_{50} PfMQO Inhibitor from *Fibraurea tinctoria*

Sample	Yield (% w/w)	IC_{50} of *P.falciparum* 3D7 (ug/ml)	IC_{50} of PfMQO (ug/ml)
Methanol extract	5.34	0.07	71953
N-hexane fraction	1.94	0.54	474.80
Isolate HA	8	1.150	N/A
Isolate HB	7	0.2056	724.7
Isolate HC	3	1.186	N/A
Isolate HD	7	1.402	N/A
Isolate HE	6	1.076	N/A
Isolate HF	3	1.186	N/A
Isolate HG	3	0.9860	N/A
Isolate HH	4	0.1517	N/A
Steroid compound*	5	0.2826	461.1

Notes: N/A : not performed, *β-sitosterol and stigmasterol from HB Isolate

With the inhibition of PfMQO, the electron transport chain will be inhibited. It shows *F. tinctoria* as a potential antimalarial.

CONCLUSION

The N-hexane fraction of methanol extract, all n-hexane subfractions, and steroid compounds from the stem of *Fibraurea tinctoria* could inhibit the growth of *Plasmodium falciparum*. Steroid compounds and n-hexane fractions also inhibit the PfMQO enzyme, which plays a vital role in the electron transport of *P. falciparum*.

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