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Synthesis of (6-Methoxy-2,5-dinitro-quinolin-4-yl)-(5-vinyl-1-aza-bicyclo[2.2.2]oct-2-yl)-methanol) and *In Vitro* Assay Against *Plasmodium falciparum* 3 D7

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Abstract

Quinine, a naturally happening alkaloid initially utilized for the treatment of muscle cramps, is currently most usually utilized to treat malaria. Symptoms of poisonous quinine, called *Cinchonism*, include wooziness, tinnitus (ringing in the ears), blurred vision, nausea, vomiting, serious adverse reaction to excessive quinine use, vision impairment and deafness. This research aimed to obtain more polar quinine derivatives using reactions with sulfuric acid and nitric acid to reduce toxicity. The reactions were performed analogously to the procedures reported in the literature. The characterization of reaction products utilizing proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectroscopy showed that the reaction using reagents led to nitration of the quinoline ring with the yields of 7.09 %. The IC_{50} value of >10.000 µg/mL was obtained from the antimalarial test against *Plasmodium falciparum* 3D7. The IC_{50} values proved that the synthesis products (6-Methoxy-2,5-dinitro-quinoline-4-yl)-(5- vinyl-1-aza-bicyclo[2.2.2]oct-2-yl)-methanol) was not potential for malaria treatment.

Keywords: Antimalarial, nitration, *Plasmodium falciparum* 3 D7, quinine, quinine derivates.

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1. INTRODUCTION

Malaria is one of the most dangerous infectious diseases in the developing world and a major cause of public health problems in around 95 nations, primarily located in the tropical zone of the globe (notably South-East Asia, Africa, and the Eastern Mediterranean region (Aponte et al., 2015; Guillon et al., 2017). According to World Health Organization (2018), in the latest World Malaria Report, malaria is still a severe parasitic disease with 219 million infections and 435,000 deaths (Tajuddeen et al., 2019; WHO, 2019; Varo et al., 2020). Therefore, any efforts in antimalarial drug discovery and development are urgently required. Quinine has been used as a natural antimalarial drug since ancient times. The development of resistance to antimalarial drugs and higher the risk of adverse events stopped quinine use once. The drug has been staged again in recent years because of the resistance problem of the synthetic antimalarial drug. So far, new drug development is an urgent subject nowadays (Kumura et al., 2005; Stillwell et al., 1993).

Quinine has a strong track record believing that most antimalarial drugs encounter malaria drug resistance within a few years of introduction. Low-level parasite resistance to quinine could suggest a privileged natural structure that is difficult for the parasite to circumvent. While finding novel drug targets and new lead compounds for the treatment of malaria is essential, another is to

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use and further develop lead compounds from nature with proven attractive properties (Dinio et al., 2012).

Quinine resistance is still minimal despite over 400 years of usage, and quinine is now a recommended therapy for *P. falciparum* resistant to chloroquine and artemisinin. Quinine's low frequency of use is generally attributed to today's low parasite resistance. In Indonesia, *Plasmodium falciparum* (PF) is the most general cause of malaria (Elyazar et al 2011; Putra et al, 2017).

Artemisinin and its derivatives are also used in artemisinin-based combination therapies (ACTs), which are now recommended as the first line of treatment for uncomplicated *Plasmodium falciparum* malaria as the second part of treatment for three days of severe malaria in endemic areas (WHO, 2015; Guillon et al., 2017). Some P. falciparum strains or isolates that are chloroquine-resistant (CQR) show mild cross-resistance, although many do not. Further quinine modification could result in a well-tolerated treatment with better activity against CQR malaria.

Chloroquine, a quinine derivative, is a widely used and inexpensive malaria medication. Because of the resistance, Plasmodium strains have been demonstrated in this medicine. It has been shown that chloroquine is only about 50% effective. Quinine, a natural product derived from the cinchona tree and the first malaria therapy, will be used if chloroquine fails to work. Problems exist with the use of quinine, including severe adverse effects. Therefore, it is essential to find safe antimalarial drugs and new compounds (quinine) for antimalarial drug resistance. Synthesis of quinine derivate is one way to reduce the toxicity of quinine itself.

Quinine is the most effective antimalarial drug and is used as a preservative. However, the toxicity of quinine limits its use as an antimalarial drug. Lipophilicity and long half-life ($t_{1/2}$) of quinine which reaches 10-20 hours, are responsible for its toxicity. Symptoms of poisonous quinine, called Cinchonism, include wooziness, tinnitus (ringing in the ears), blurred vision, nausea, and vomiting, severe adverse reaction to excessive quinine use, vision impairment and deafness (Kartasasmita et al., 2015; Achan et al., 2011; Karlsson, 1990). This research aimed to obtain more polar quinine derivatives using reactions with sulfuric acid and nitric acid to reduce toxicity.

2. MATERIALS AND METHODS

Materials

The compound was prepared from quinine anhydrous (obtained from PT. SIL Indonesia) as the primary material; sulfuric acid and nitric acid were used as the catalyst. Other chemical reagents or solvents were gained from commercial sources such as methanol, hexane, ethyl acetate, dichloromethane, chloroform, silica plates, silica gel, sodium chloride, distilled water, and CD3OD. All solvents utilized in this research had been distilled prior to being used.

Media and Chemicals for *In vitro* Antimalarial Activity

Other reagents are used to *in vitro* test such as dimethylsulfoxide (DMSO), *Plasmodium falciparum* (strain: 3D7), human erythrocytes with type A (contain hematocrit 3%), RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Massachusetts, USA), lactic acid, nitroblue tetrazonium, and 3-acetylpyridine adenine dinucleotide (APAD; Sigma-Aldrich, Missouri, USA).

Tools

Some instruments are used in this research such as column chromatography, UV/Vis lamp, autoclave, laminar air flow, incubator, petri dish, centrifuge, microwell titer, rotary evaporator, analytical balance (KERN), spectrophotometer LC-MS HP 5972 series, ^{1}H and ^{13}C-NMR 500 MHz on JEOL (JNM-ECA).

Nitration

The appropriate quinine anhydrous (1 mmol) was added to 1 mL H_2SO_4 and 1 ml HNO_3. The reaction mixture was heated to 55 °C and stirred for 2 hours. After stirring for 2 hours at 55 °C, the mixture was alkalified with NaOH 1 N and extracted with ethyl acetate. The filtrate was purified and concentrated by silica gel column chromatography (n-hexane-ethyl acetate) to give compound 1.

Cytotoxicity Assay

Plasmodium falciparum strain 3D7 was treated in human type O-positive RBCs (red blood cells) in RPMI-1640 supplemented with 25 mM $NaHCO_3$, 10 ppm hypoxanthine, 40

ppm gentamicin sulfate, and 0.5% (w/v) Albumax II at 37 °C (Gibco, Thermo Fisher Scientific, Massachusetts, USA) under 5% oxygen and 5% carbon dioxide. The Japanese Red Cross Society provides Human RBCs. The experiments were carried out under the guidelines of The University of Tokyo's ethical committee (permission No. 10050). Parasite growth was monitored by the diaphorase-coupled P. falciparum lactate dehydrogenase (PfLDH) assay, as previously described (Hartuti et al., 2018; Wang et al., 2019). Briefly, sorbitol was used to synchronize cultures of P. Falciparum. The sorbitol-synchronized cultures at the ring stage were balanced to 2% hematocrit and 0.3% parasitemia and then placed in 96-well plates with an antimalarial control medication or the test compounds atovaquone at various concentrations. Tested compounds were dissolved in either Dimethylsulfoxide (DMSO) or Methanol (MeOH) (final DMSO concentration no more than 0.5%, which is non-toxic to P. falciparum parasites). In RPMI media, samples were put in 96 well plates, and serial dilutions were prepared in a final volume of 100/well. Plates were then incubated at 370C for 72 hours with a standard gas mixture of 5% O_2, 5% CO_2, and 90% N_2, for 72 h at 37°C. After spinning plates for five minutes at 1,300 x g with a low brake, 50 µL of supernatant was eliminated without disturbing the pellets. On the other hand, lyse erythrocytes were frozen at –30 °C. After that, Pre-thawing plates were added with 50 mM sodium-L-lactate, 100 µL of buffer containing 100 mM Tris-HCl pH 8.0, , 0.25 percent (v/v) Triton X-100, 0.7 mM APAD (3-acetylpyridine adenine dinucleotide), 1 unit/ml diaphorase, and 0.2 mg/ml nitro blue tetrazolium (Sigma-Aldrich, Missouri, USA). SpectraMax® Paradigm® (Molecular Devices, CA, USA) was measured absorbance after 30 minutes of incubation at a wavelength of 650 nm. Three independent experiments were used to measure inhibition values. Using GraphPad Prism, the IC_{50} values were estimated by least-squares curve fitting of the dose inhibition curves (GraphPad Software Inc., San Diego, USA) (Pramisandi et al., 2020).

3. RESULTS AND DISCUSSION

(6-Methoxy-2,5-dinitro-quinoline-4-yl)-(5-vinyl-1-aza-bicyclo[2.2.2]oct-2-yl)-methanol) was obtained as a smooth white crystal from a nitration reaction between sulfuric acid and nitric acid, as shown in Fig. 1. The purification and identification were conducted by SiO_2 column chromatography and obtained a 7.09 % final product yield. The yield is too low because the extraction process is not perfect using ethyl acetate in this research. Furthermore, H_2SO_4 and HNO_3 are strong acids, so that these reagents can destroy the synthesis products. The product was analyzed by LC-MS and gave the molecular ion at m/z 417 $[M+H]^+$, calculated for ($C_{20}H_{24}N_4O_6$), 416, which indicated the nitration product was formed. Quinine anhydrous was also analyzed by LC-MS and gave the molecular ion at m/z 325 [M+H], as shown in Fig. 2.

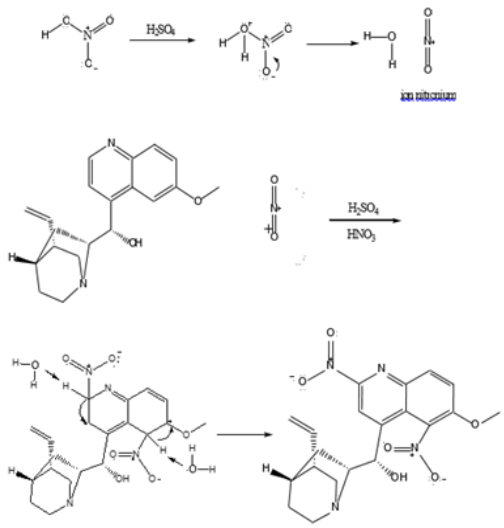


Figure 1. Reaction scheme proposed for the synthesis of (6-Methoxy-2,5-dinitro-quinolin-4-yl)-(5-vinyl-1-aza-bicyclo[2.2.2]oct-2-yl)-methanol

(6-Methoxy-2,5-dinitro-quinolin-4-yl)-(5-vinyl-1-aza-bicyclo[2.2.2]oct-2-yl)-methanol) was white solid (7.09%),[1] H NMR (500 MHz, $CDCl_3$) δ (ppm) 1,61 (m, 2H, *J* = 10), 2.29 (m, 1H, *J* = 7.5), 0.98 (m, 1H, *J* = 4), 1.29;0.93 (m, 2H, *J* = 8), 3.56 (t, 2H, *J* = 4.5), 1.30 (t, 2H, *J* = 7), 3.16 (m, 1H, *J* = 1.5), 5.01 (d, 1H, *J* = 15.5), 1.13 (m, 1H, *J* = 2), 0.90 (t, 3H, *J* = 7), 8,54 (s), 3.65 (s, 3H), 7.10 (d, 1H, *J* = 8.5), 6.81 (d, 1H, *J* = 8.5). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 55.55, 39.86, 24.31, 30.86, 45.48, 21.72, 62.25, 71.52, 23.83, 14.54, 168.54, 115.10, 148.76, 128.70, 56.56, 155.98, 124.92, 137.04, 122.50, 143.14.

The product was confirmed by ^{1}H and ^{13}C NMR spectroscopic data (500 MHz, CDCl3),

as shown in Fig. 3 & 4. The proton signal of the hydroxy group was not seen at proton NMR chemical shift, because sometimes the proton of hydroxyl group can or cannot appear in H-NMR spectra. The presence of quinolone ring was determined by the signal proton at δ_H 6.81-8.54, as shown in Table 1. The signals of quinolone ring was also determined by δ_C 115.10-168.54, and the signals ^1H and ^{13}C for OCH_3 were seen at δ_H and δ_C at 3.65; 56.56, respectively. These signals indicated the formation of an dinitro quinine.

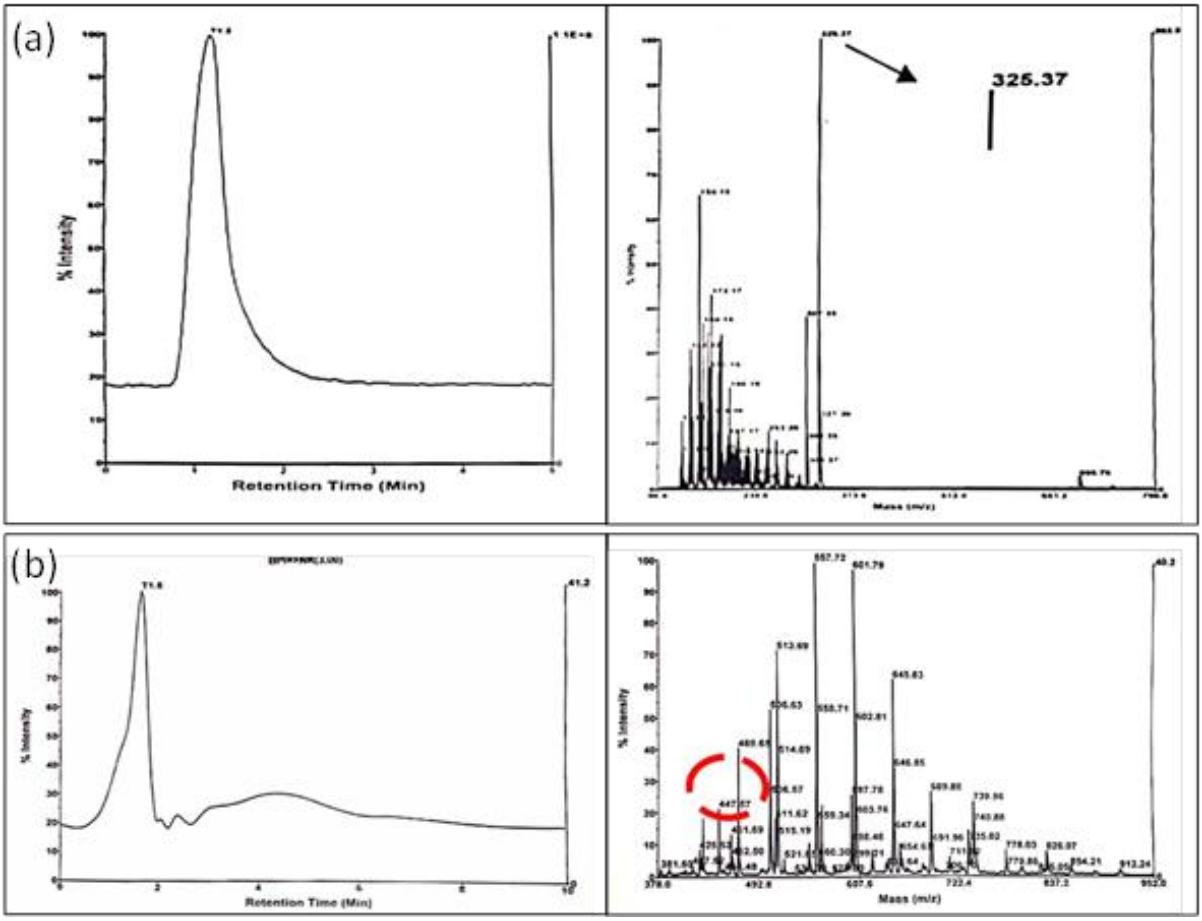
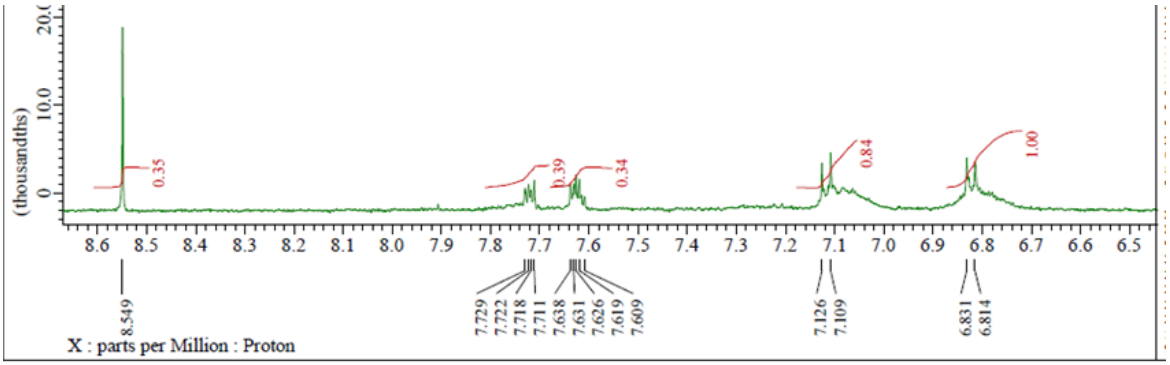
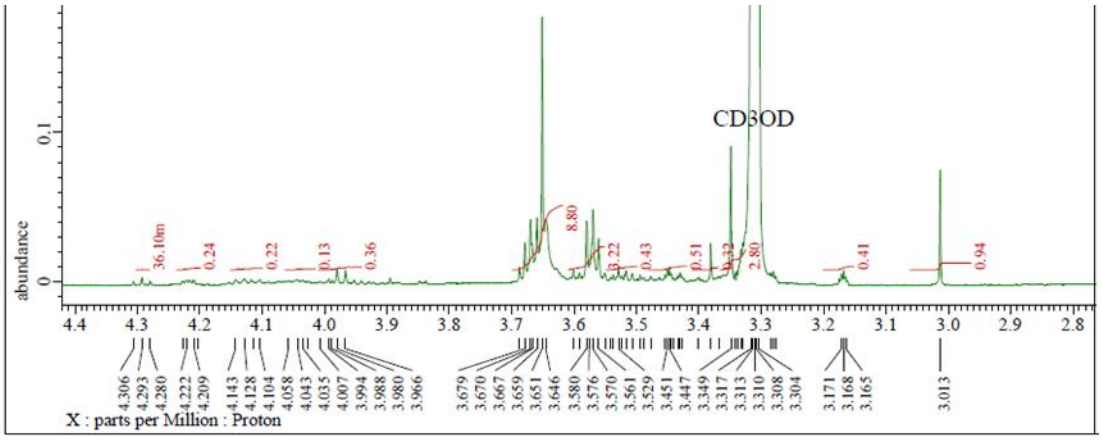
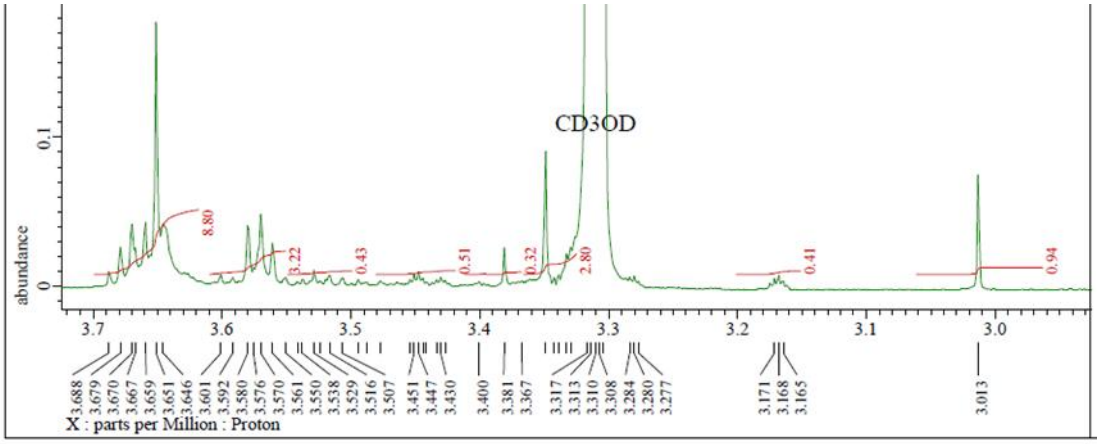


Figure 2. LC-MS Spectra (a) quinine anhydrous (b) nitration product




CD3OD
abundance
X : parts per Million : Proton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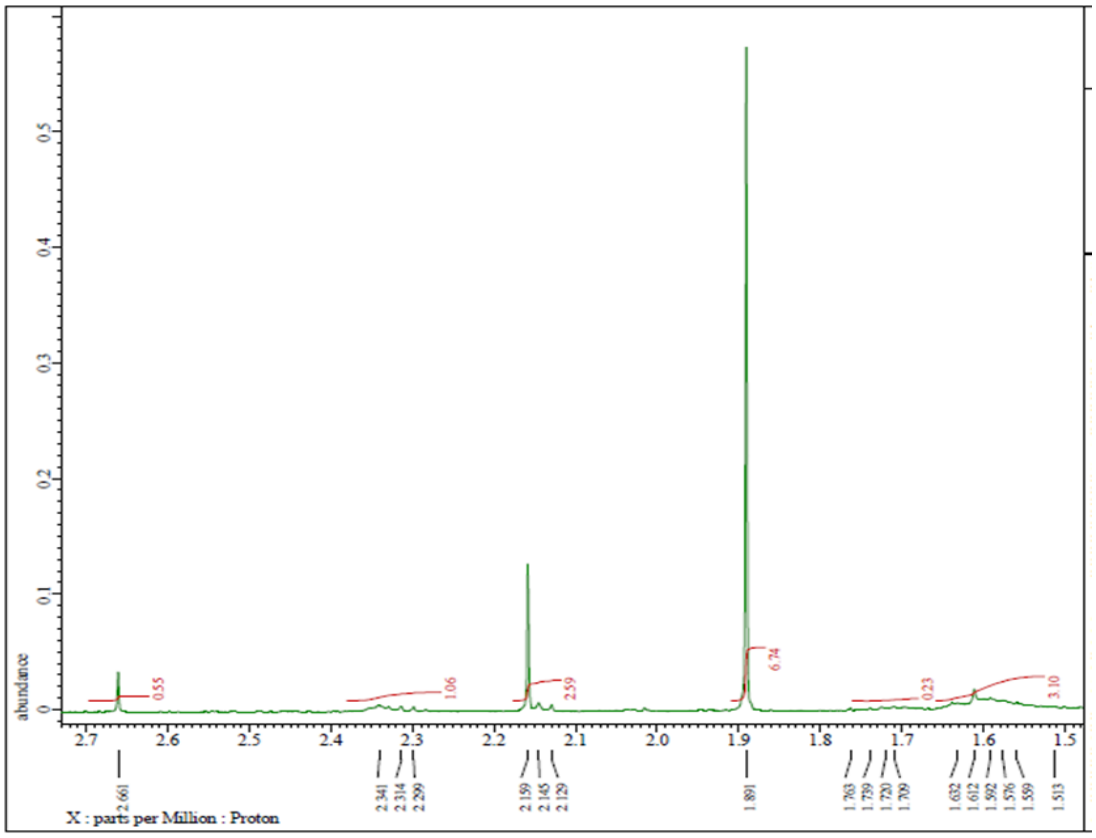
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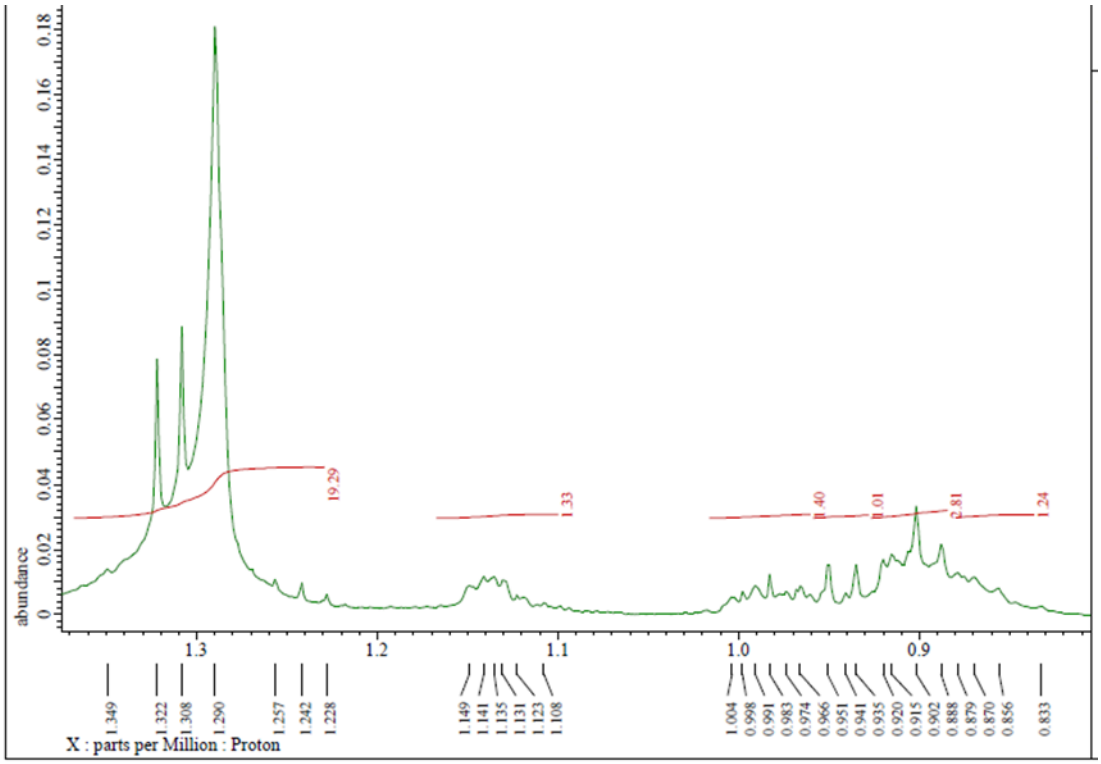
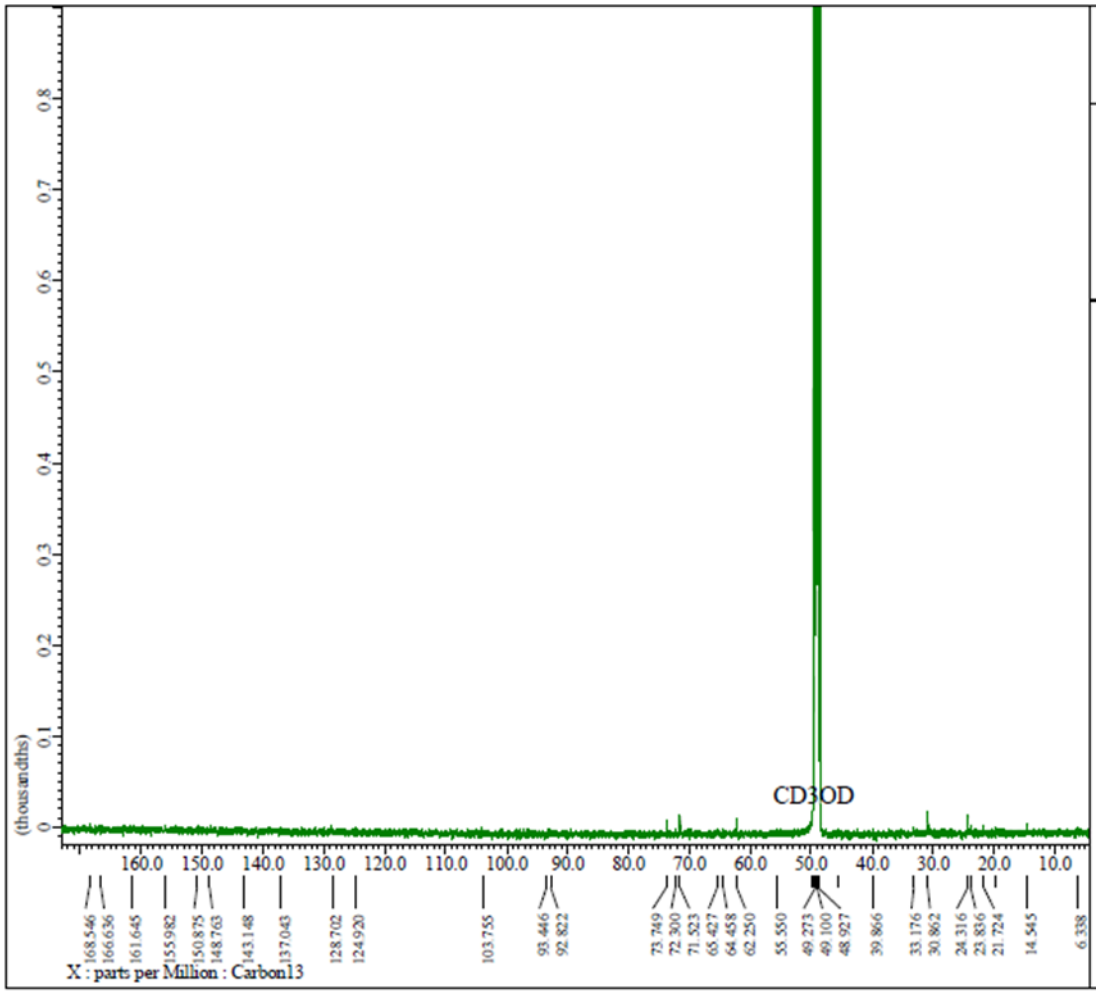


Figure 3. ^{1}H spectra (500MHz, CDCl3) of nitration product



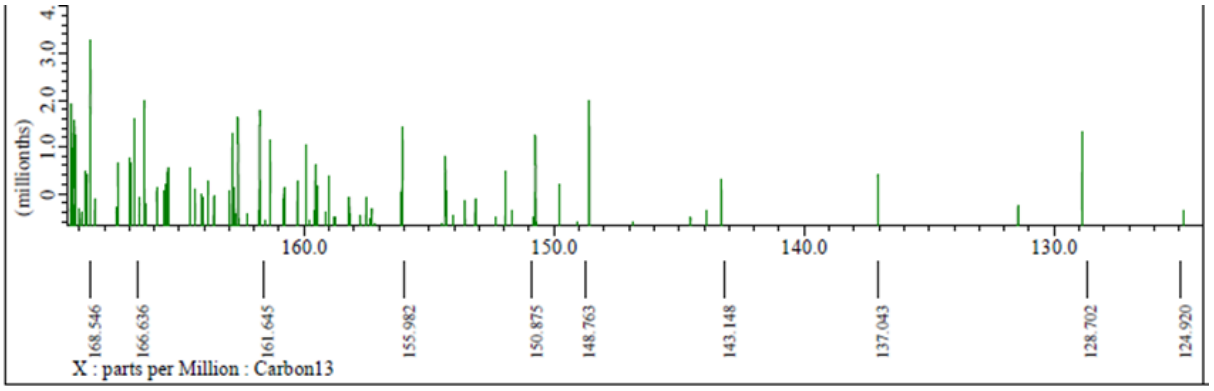


Figure 4. ^{13}C spectra (500 MHz, CDCl3) of nitration product

Table 1. Chemical shift data for (6-Methoxy-2,5-dinitro-quinolin-4-yl)-(5-vinyl-1-aza-bicyclo[2.2.2]oct-2-yl)-methanol

No	Chemical Shift (δ, J in Hz)	
	^{1}H-NMR	^{13}C-NMR
1	-	-
2	1,61 (m, 2H, J = 10)	55,55
3	2,29 (m,1H, J = 7,5)	39,86
4	0,98 (m, 1H, J = 4)	24,31
5	1,29;0,93 (m, 2H, J = 8)	30,86
6	3,56 (t, 2H, J = 4,5)	45,48
7	1,30 (t, 2H, J = 7)	21,72
8	3,16 (m, 1H, J = 1,5)	62,25
9	5,01 (d, 1H, J = 15,5)	71,52
(-OH)	-	-
10	1,13 (m, 1H, J = 2)	23,83
11	0,90 (t, 3H, J = 7)	14,54
1'	-	-
2'	-	168,54
3'	8,54 (s)	115,10
4'	-	148,76
5'	-	128,70
(-OCH3)	3,65 (s, 3H)	56.56
6'	-	155,98
7'	7,10 (d, 1H, J = 8,5)	124,92
8'	6,81 (d, 1H, J = 8,5)	137,04
9'	-	122,50
10'	-	143,14

Quinine was found to have good activity against *P. falciparum* in vitro. We hypothesized that dinitro quinine, which is more hydrophilic than quinine to reduce toxicity, would penetrate the membrane of P. falciparum and would have a higher anti-plasmodial activity than quinine or have the same activity as quinine. Based on the result, this hypothesis is wrong because the IC_{50} value showed that dinitro quinine has a lower anti-plasmodial activity than quinine. The hypothesis is wrong because two nitro groups ($-NO_2$) substituent or more polar quinine derivatives can reduce activity. One solution to

maintain activity we can change two nitro groups to other polar substituents (such as –OH, a halogen group).

Aromatics, amines, alcohol, glycol, and glycerine are frequently nitrated by an ion mechanism utilizing acid mixtures (containing strong acids such as sulfuric acid and nitric acid). The presence of nitronium ions in the acid mixture attacks these organic molecules, resulting in an unstable complex. The nitrated hydrocarbon is formed when a proton is released from this complex. When an aromatic is nitrated, a C-N bond is created, but when alcohol (or hydroxy group) is nitrated, a C-N bond is Formed. An N-N bond is created in the case of amine nitrations. When nitric acid is utilized, the strong acid in the acid mixtures works as a catalyst, promoting the creation of NO_2.

Nitrogen is utilized to a benzene ring through nitration, which can then be utilized in a substitution reaction. The nitro group deactivates the ring. The presence of nitrogen in a ring is advantageous since it can serve as both a directing and a masked amino group. In industrial chemistry, aromatic nitration products are particularly significant intermediates.

To examine the importance of sodium hydroxide in the modification of quinine into dinitroquinine, we reacted sulfuric acid solution of quinine with nitric acid for 2 hours, without acid-base a neutralization reaction using sodium hydroxide. After the reaction, we can not detect a spot of nitration product clearly in the Thin Layer Chromatography (TLC). Therefore, sodium hydroxide should be necessary to modify quinine into dinitroquinine. After the neutralization reaction, we extracted using organic solvent (ethyl acetate) to detect the spot.

The antimalarial activity of dinitro quinine was evaluated against Plasmodium falciparum 3D7. This compound has antimalaria activity against P. falciparum 3D7 with IC_{50} > 10.000 μg/mL, lower than quinine anhydrous ($IC_{50} \leq 10$ μg/mL), and epoxidation of vinyl substituents of the quinuclidine moiety in quinine ($IC_{50} \leq$ 1250-1500 μg/mL) (Salahuddin et al., 2021) based on our previous research studies. The Inhibition Concentration (IC_{50}) values showed that the synthesis products were not potential for malaria *Plasmodium falciparum* 3D7 treatment.

4. CONCLUSION

Synthesis of derivate quinine as an antimalaria compound has been done by a straightforward step of the reaction (nitration). In vitro test against Plasmodium falciparum, 3D7 showed that the IC_{50} of the synthesis compound was > 10.000 ppm. The IC_{50} values proved that the synthesis products did not have the potential for treating malaria disease.

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