

## The Synthesis of Cinchonine Tiglat Ester Compound and Cytotoxic Test Against MCF-7 Breast Cancer Cell

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### Abstract

Cinchonine is a type of cinchona alkaloid compound commonly found and/or isolated from *Cinchona sp.* plant. It is commonly used to treat malaria, and can potentially be used against cancer cells. In this particular study, cinchonine ester derivatives were extracted through esterification processes. Synthesized ester is aimed to gain higher lipophilicity of cinchonine in order to makes it easier to pass through cell membrane. Esterification was conducted using DCC activator as well as DMAP catalyst with tiglic acid which used to create cinchonine tiglat. Subsequent cinchonine tiglat was obtained in the form of oil with 25,28% yield. The compound obtained from the synthesis was then analyzed using LC-ESI-MS and <sup>1</sup>H-NMR spectroscopy instrumentation. Results show that the target compound has been successfully synthesized. Its cytotoxic ability against MCF-7 breast cancer cells was tested using the Alamar Blue method. Results concluded that cinchonine tiglat ester compound has a viable cytotoxic activity with IC<sub>50</sub> value of 1.22 ppm.

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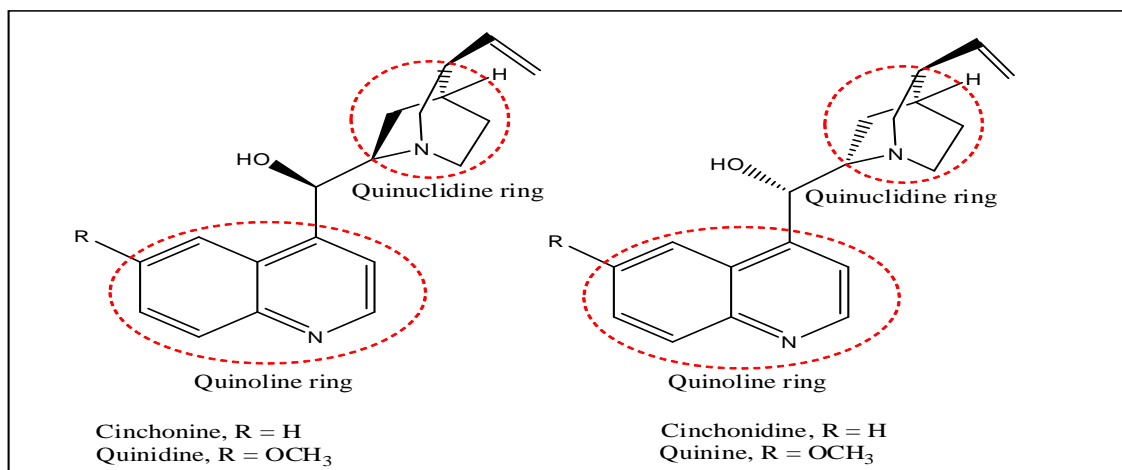
## 1. INTRODUCTION

Cancer is one of the main cause of death in developing Countries. GLOBOCAN estimated that by 2012, there were 14,1 million cases of newfound cancer and approximately 8,2 million deaths with relation to cancer in the whole world; with lung cancer and breast cancer being two of the most prominently diagnosed and suspected cause of death [1]. With 1,7 million cases and 521.900 deaths bay 2012, breast cancer is the most prevalent type of cancer which is found in women. Approximately 25% of all cancer cases and 15% of cancer induced deaths are attributed to breast cancer. Developed countries accounted 50% of breast cancer cases and 38% of death caused by breast cancer [2].

Quinine and its derivatives were found to contain potential anticancer agents. Quinine has been known for its benefits as antimalarial agent [3]; but more recent studies found that it contains chincona alkaloid derivatives with anticancerous properties against leukemia or

white blood cell cancer (K562/ADM), mouth cancer (KB and Hep-2), breast cancer (MCF-7), liver cancer (HepG2), lung cancer colon cancer, and neuroblastoma (SH-SY5Y) [4,5,6]. One of the quinine derivative has even been clinically tested for (phase-III) pancreatic cancer chemotherapy agent [3].

Chincona's alkaloid compound consist of two primary rings; the quinoline ring and the quinuclidine ring (Figure 1). The quinoline ring has anticancerous, anti malarial, and anti inflammatory properties [7,8]. Compounds with quinoline ring affect parasites in their *schizoints* phase and eject them from the blood stream [9]. Quinine and its derivatives have autophagic effect; the breaking down of intracellular components by lysosome [10]. This autophagic ability causes the compounds' cytotoxicity. Quinacrine's (a derivative of quinine) autophagic ability is able to cause death to cancer cell by increasing *p53* and *p21* [11].



**Fig. 1.** Cinchona alkaloid and their basic skeleton

Cinchonine is one of the cinchona alkaloids which has anticancer properties. An *in vitro* study conducted by Genne *et al* concluded that cinchonine goes against leukemic cell K562/ADM; with maximum effect being achieved at 5  $\mu$ M; better than quinine which is known to achieve its maximum potential at 10  $\mu$ M. When introduced to plasma, cinchonine's bond with protein is not as strong as that of quinine's. Cinchonine has 37-55% of free phase while quinine has 20-30%. This makes cinchonine more active than quinine [12]. When cinchonine is combined with Doxorubicine, it is very active against leukemic cell in rats P388/DOX [13]. Cinchonine shows activity against MES-SA uterine sarcoma [14]. Cinchonine is known to be more active compared to other cinchonian alkaloids against colon cancer's cells in rats (DHD/K12/Prob) and human leukemic cells (K562/ADM). Cinchonine also has much lower toxicity compared to other cinchonian alkaloids [15].

Cinchonine is a hematologic anti-multidrug resistance (MDR) agent for its tolerance and its ability to remain in serum. Cinchonine is also a more efficient *in vitro*, *ex vivo*, and *in vivo* anti-MDR agent when compared to quinine when administered with parenteral route. Cinchonine inhibits the function of P-glycoprotein as drugs' reflux pump on cancer cells [16]. Multiple drug resistances (MDR) is the prime cause of failure in cancer chemotherapies [17].

In this study, a cinchonine ester is synthesized, which will be mixed with tiglic acid through esterification. Ester is a prodrug that can easily be hydrolyzed to facilitate its distribution [18]. Cinchonine has only one hydroxyl group, hence why its esterification is relatively easy to achieve [19]. The combination of cinchonine and tiglic acid is expected to be an effective and efficient chemotherapy agent; by increasing its lipophilicity, its activity will increase [20].

## 2. EXPERIMENTAL SECTION

### 2.1. Materials and Instrument

Three neck Rounded Flask, Liebig Condenser, Magnetic stirrer (IKA-C MAG HS7), Chromatography Column, Spektrofotometer NMR (JEOL JNM ECA-500), Spektrofotometer LC-MS (Marinier Biospectrometry), incubator CO<sub>2</sub>, ELISA (Enzyme Linked Immunosorbent Assay) plate reader, hemocytometer, conical tube sterile, scapper, 96 well plate, micropipette and tissue culture flask.

Cinchonine obtained from Local supplier (PT. Sinkona Indonesia Lestari – Indonesia), tiglic acid p.a (Merck), Ethyl acetate p.a (Merck), Chloroform p.a (Merck), Dichloromethane p.a (Merck), ethanol p.a (Merck), n-heksan p.a (Merck), N, N-4-dicyclohexylcarbodiimide (DCC, Sigma D3128 p.a.), Dimethylaminopyridine (DMAP, Sigma D5640 p.a.), thin layer chromatography (KLT, Merck GF254 0,25 mm), Silica gel 60,

Medium culture DMEM (Dulbecco's Modified Eagle's Medium, Merck), Phospat Buffer Saline (PBS, Gibco), Trypsin EDTA 0,25% (Gibco, Invitrogen, Canada) dan Alamar Blue (Serotec Limited).

## 2.2. Methods

### 2.2.1. Synthesis of cinchonine tiglat

As much as 588,78 mg (2,0 mmol) of cinchonine was dissolved with dichloromethane as the solvent; then 240,05 mg (2,4 mmol) of tiglic acid and 495,19 mg (2,4 mmol) of N,N-dicyclohexylcarbodiimide (DCC) activator were added. The mixture was then stirred using magnetic stirrer for 1 hour. After an hour had passed, N,N-dimethylaminopyrine (DMAP 20%) was then added to the mixture as a catalyst. The mixture was then stirred with constant speed for 24 hours [21]. The resulting product was indentified using thin layer chromatography. The purification of cinchonincinnamate was done with column chromatography using 7:3 mixture of ethyl acetate and chloroform respectively as solvent.

### 2.2.2. Preparation of cancer cell lines

The cancer cell used in this study was from breast carcinoma Michigan Cancer Foundation (MCF-7). The cells were grown in Dulbecco's Modified Eagle Media (DMEM) culture media with 10% Fetal Bovine Serum (FBS). The cells were cultured inside a tissue culture dish and were incubated at a constant temperature and humidity of 37°C and 5% respectively for 3 days until culture growth of 60-70% was achieved. The media were then renewed with fresh plate and reincubated for another 24 hours. The media were then eliminated and the cultures were rinsed using Phosphate Buffer Saline (PBS) for 1-2 times. A 0,25% trypsin-EDTA solution was added to release the culture from the plate's wall. The cells' suspensions were then moved to sterile conical tubes containing new media.

### 2.2.3. In vitro anticancer activity

The testing was conducted using Alamar Blue method. The standard solution was created by dissolving 1 mg of sample into 1 mL of DMSO. It was then diluted serially to concentrations of 20 ppmp; 10 ppm; 5 ppm; 2,5 ppm; 1,25 ppm; 0,625 ppm; 0,3125 ppm; 0,1563 ppm; 0,0781 ppm, and 0,0391 ppm. Ten  $\mu$ L of sample solutions (with varying concentrations) were added to every 100  $\mu$ L of cancer cells; distributed into 96 well plates; and incubated for 24 hours at 37°C. The colouring process was done by adding Alamar Blue solution for 4 hours. The cell's spectral intensity was quantified using ELISA plate reader on 560 nm excitation wavelength and 590 nm of emission wavelength [22]. The viability percentage was calculated based on the following formula:

$$\frac{OD (cell+sample)-OD (negative control)}{OD (cells)-OD (negative controls)} \times 100$$

The IC<sub>50</sub> value was analyzed using linear regression between the percentage of living cells and the logarithm of sample's concentration.

## 3. RESULTS AND DISCUSSION

### 3.1. Synthesis of cinchonine tiglat

The cinchonine tiglat was successfully synthesized with the help of DCC activator as well as the DMAP catalyst in dichloromethane (Figure 2). The reaction took 24 hours to finish at room temperature and pressure inside a round-bottom flask while being constantly stirred. The combination of activator and catalyst's usage was expected to increase the product's yield and decrease the temperature and energy needed to fuel the reaction. The aforementioned reaction yielded 25,28% product; with the resulting product has an oily characteristic. The obtained yield was still relatively low; so optimizing the reaction by re-adjusting the temperature is recommended.

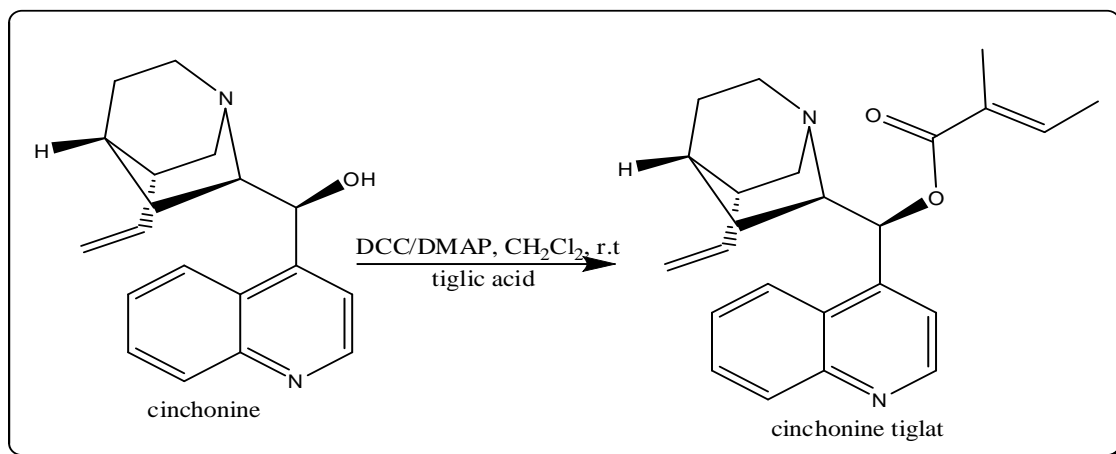


Fig. 2. Esterification of cinchonine tiglat

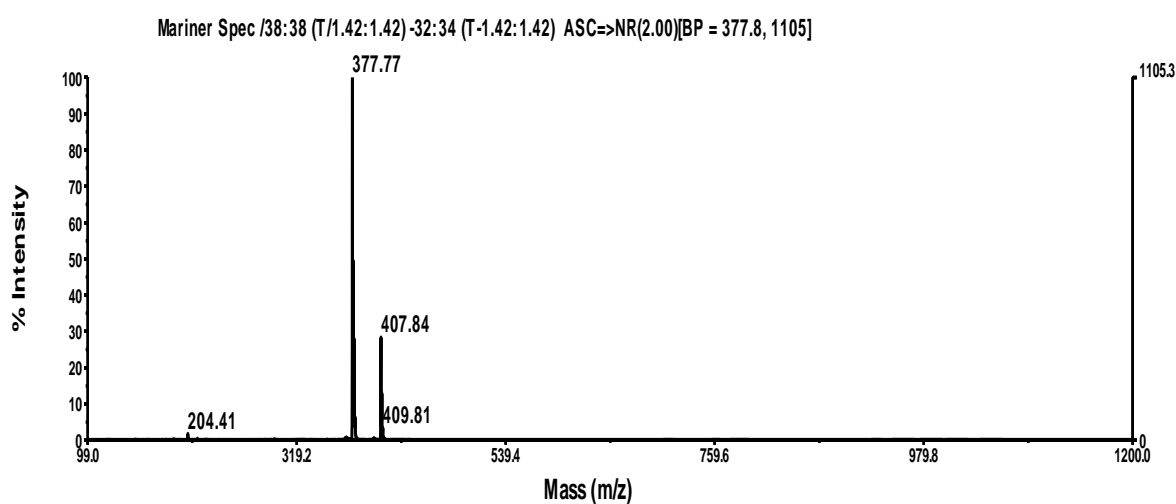


Fig. 3. The Spectrum of LC-ESI-MS

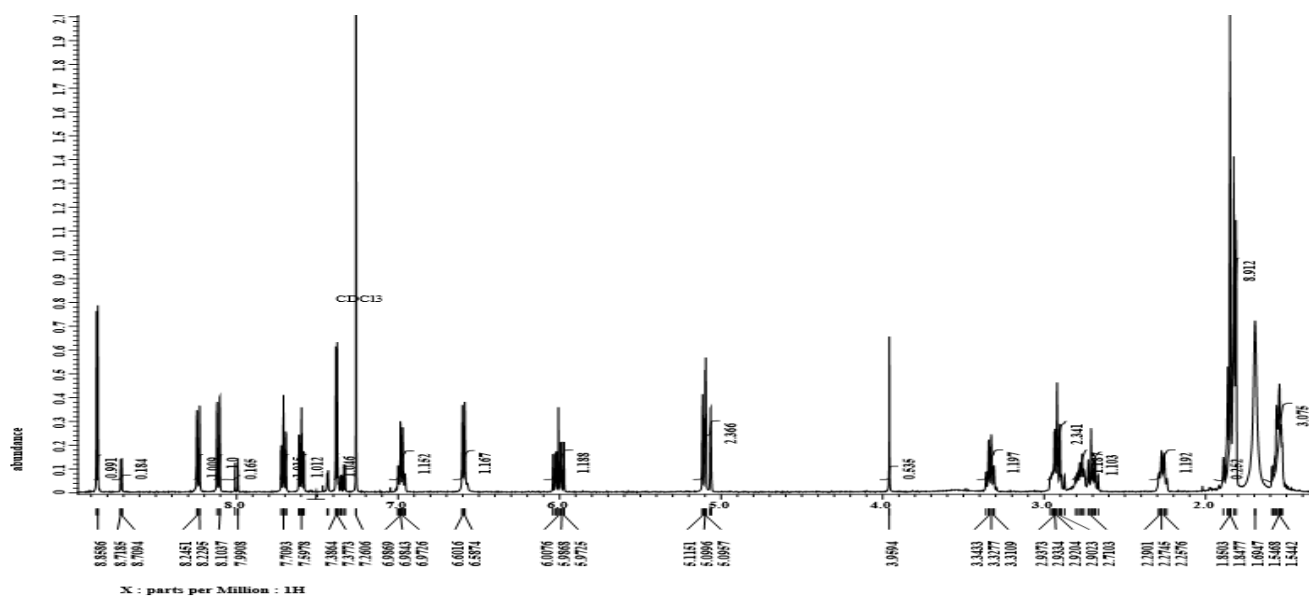


Fig. 4. <sup>1</sup>H-NMR spectra of cinchonine tiglat

### 3.2. Structural identification using LC-ESI-MS and <sup>1</sup>H-NMR

The cinchonine tiglat was indentified using LC-ESI-MS with MeOH as the solvent and type C-18 column (15 mm x 1 mm). The spectral figures in Figure 3. shows that the compound had a retention time (tR) of 1,42 minutes and the molecule's peak ion (m/z) was at 377,77. Tiglic cinchonine has the molecular formula of C<sub>24</sub>H<sub>28</sub>N<sub>2</sub>O<sub>2</sub> and a molar mass of 376,22; as stated by ChemDraw application. A delta of 1 molecule from the spectrum was caused by an additional proton [M+H<sup>+</sup>].

The <sup>1</sup>H-NMR spectrum of cinchonine tiglat can be divided into two separate areas. The first area was 5-8 ppm from the quinoline ring and the alkene bond. The second area was between 1-4 ppm for the quinuclidine ring. The <sup>1</sup>H-NMR spectrum from the second area proved to be difficult to observe due to its high density. A chemical shift δH 6,49 ppm indicated the peak of H-9 to have doublet multiplication. The H-9 peak was more downfield compared to protons from other quinuclidine ring due to its direct bond with O from the ester functional group (-COO-) at position α and with N in position β; with both exhibiting electronegativity, resulting in the peak's shift towards the right side [23]. A thorough spectrum list was stated in Figure 4. : <sup>1</sup>H-NMR (CDCl<sub>3</sub>) : 8.86 (1H, d, 4.55 Hz, H-2'), 8.23 (1H, d, 7.8 Hz, H-5'), 8.11 (1H, d, 7.8 Hz, H-8'), 7.71 (1H, dd, 7.1 Hz, H-7'), 7.61 (1H, dd, 7.1 Hz, H-6'), 7.37 (1H, d, 4.55 Hz, H-3'), 6.98 (1H, q, 5.85 Hz, H-4''), 6.60 (1H, d, 7.1 Hz, H-9), 6.00 (1H, m, H-10), 5.10 (2H, m, H-11), 3.34 (1H, q, 7.8 Hz, H-8), 2.95 (2H, m, H-6), 2.93 (1H, s, H-3''), 2.72 (2H, m, H-2), 1.16 (3H, d, 5.14 Hz, H-5''), 2.69 (1H, m, H-3), 1.86 (2H, m, H-5), 1.81 (1H, m, H-4), 1.54 (2H, m, H-7). Based on the results from LC-ESI-MS and <sup>1</sup>H-NMR, we concluded that the synthesis of cinchonine tiglat was successful.

### 3.3. In vitro anticancer activity of cinchonine tiglat

The cytotoxicity test was done in vitro to see if there were any anticancer potential against MCF-7 breast cancer cell's growth.

The first step of the test was filtering, followed by isolating the chemical compound which is responsible for the anticancer properties. This method does not explain the mechanism behind the anticancer properties. The test was employed to MCF-7 breast cancer cell using Alamar Blue method.

The Alamar Blue reagent contains resazurin; which exhibits bluish colour without fluorescence. This compound can be reduced into a fluorescent pink resorufin when meeting certain conditions. The change of colour from blue (resazurin) into pink (resorufin) indicated its reduction by cancerous cells [24]. The process of colouring cells with Alamar Blue needed 4 hours of incubation. The result of tigliccinchonine's test are as follows:

**Table 1.** Viability percentage of cinchonine tiglat

Concentration (ppm)	Viability (%)
0,0391	127,11
0,0781	111,61
0,1563	96,06
0,3125	80,54
0,625	65,00
1,25	49,47
2,5	33,94
5	18,40
10	2,87
20	-12,66

As shown by Table 1, the lower the concentration of cinchonine tiglat, the higher the rate of surviving cancerous cells (shown by the higher viability percentage). Dose dependency is a common trait among most anticancer compounds; the higher the concentration, the higher its ability to inhibit cancerous cells' growth.

The IC<sub>50</sub> value (the minimum concentration of sample to exhibit the ability to inhibit 50% of the cancerous cells' growth) of tiglic cinchonine was 1,22 ppm with correlation coefficient of 0,9000 from the regressive equation  $y = -51,60 \text{ Log } X + 54,47$ . The lower

the IC<sub>50</sub> value, the more efficient it is as anticancer agent.

As shown by its IC<sub>50</sub> value; cinchonine tiglat exhibits a powerful anticancerous activity. Tiglic cinchonine is a derivative compound from cinchonine and tiglic acid. By it self, cinchonine is already a great candidate as multidrug resistance (MDR) agent. Cinchonine's activity as an MDR agent is relatively more powerful than most other chincona alkaloids; hence why it is a great addition to combine with other chemotherapy drugs [16].

Cinchonine tiglat's structure follows Lipinski's rule. Lipinski's rule was made to determine compound's physicochemical properties as well as whether the stated compound has a hydrophobic or hydrophilic tendency to penetrate the cellular membrane through passive diffusion. Lipinski's rule stated that compounds with molecular mass of less than 500; will have a Log P value of less than 5, a number of hydrogen bond donor of less than 5, and a number of hydrogen bond acceptor of less than 10 [25]. Based on ChemDraw, cinchonine tiglat structure has a Log P value of 4,23; molecular mass of 376,22; 0 amount of hydrogen bond donor; and 4 amount of hydrogen bond acceptor.

MCF-7 cell is a strand of breast cancer cell that has a tendency to be more resistant against chemotherapy agents, especially when compared to T47D cell. This type of cell has been known to be resistant against Doxorubicin; a type of commonly used chemotherapy agent [26]. This is why finding a newer type of drug to combat the resistance is crucial.

The synthesization of cinchonine tiglat was done to increase cinchonine's lipophilicity to ultimately increase cinchonine's anticancerous ability [20]. Table 2 shows the IC<sub>50</sub> value of tiglic cinchonine is 1,22 ppm; lower than that of cinchonine at 1,32 ppm. This meant an increase of cinchonine tiglat's anticancerous activity compared cinchonine. Cinchonine tiglat's anticancerous ability is in line with the used positive control variable; antimycin, but cinchonine tiglat exhibits a better IC<sub>50</sub> value.

**Table 2.** Anticancer activity of cinchonine tiglat

Compounds	MCF-7
	(IC <sub>50</sub> in ppm)
Cinchonine	1.32
Cinchonine tiglat	1.22
Antimycin	1.24

#### 4. CONCLUSIONS

Structurally, cinchonine tiglat follows Lipinski's rule. Cinchonine's anticancerous property was increased after having its lipophilicity increased. Cinchonine tiglat exhibits a high number of cytotoxic activity against MCF-7 breast cancer cells with IC<sub>50</sub> value of 1,22 ppm.

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