CHAPTER III

EXPERIMENTAL

3.1 Instruments and equipments

1. High Performance Liquid Chromatography (HPLC)

HPLC chromatogram were obtained with a Thermo Separation Products, model P-4000 SN spectrometer, Fortune Scientific Co., Ltd.

2. Liquid Chromatography-Mass Spectrometer (LC-MS)

Low resolution spectra were obtained liquid Chromatography-Electrospray Mass Spectrometry Model Quattro Micro, Water, Co., Ltd.

3. Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H and ^{13}C NMR spectra were recorded at 399.84 and 100.54 MHz, respectively, on a Varian, model Mercury 400 spectrometer in $CDCl_3$. Chemical shifts are given in part per million using residual protonated solvent as reference. HSQC, HMBC, COSY any NOESY experiments were performed.

4. Fourier Transform Infrared Spectrophotometer (FT-IR)

IR were recorded on a Nicolet Impact 410 spectrophotometer. Spectra of sample were recorded as thin films on NaCl cell.

5. UV Visible spectra

UV Visible spectra were recorded on Hitachi, model U2000 spectrrophotometer in acetonitrile.

6. Rotary vacuum evaporator

The Büchi, model R-200 rotary vacuum evaporator was used for the rapid removal of large of amounts of solvents.

7. Speed vacuum evaporator

The speed vacuum, model Maxi centrifuge was used for the rapid removal of litter of amounts of solvents.

3.2 Reagents and chemicals

Solvents used in this research such as acetonitrile (HPLC grade), ethyl acetate (AR grade), hexane 99 % (AR grade), dichloromethane (TG grade) were purchased from Labscan company, Bangkok, Thailand.

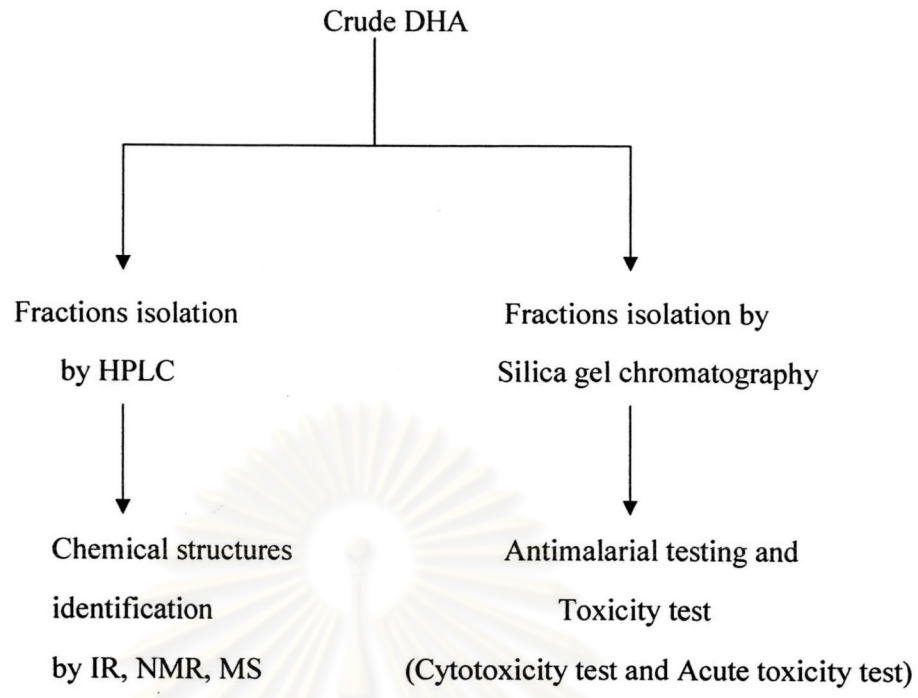
Merck's silica gel Art. 7734.1000 (70-230 mesh ASTM) were purchased from Merck company, Bangkok, Thailand.

3.3 DHA material

The sample, DHA were obtained the Government Pharmaceutical Organization, Bangkok, Thailand.

3.4 Thermal decomposition of DHA

The powdered, DHA was heated in reagent-bottom at 80 °C for 20 h. These products were named crude DHA. Then crude DHA was isolated and tested antimalarial activity and toxicity. All procedure are shown in Scheme 1.



Scheme 1 The procedures of degradation products study

3.5 Separation

3.5.1 Isolation of degradation products by HPLC

HPLC conditions [43]

Column : Phenomenex $C_{18,}$ model LUNA 5 µ, 250 mm. x 4.6 mm. (i.d.)

Precolumn : Guard cartridge column C18, 4.6 mm.

Mobile phase : Acetonitrile-Water (50:50 v/v)

Flow rate : 1.5 ml/min.

Detector : UV detector 205 nm.

Injection volume : 100 µl

Crude DHA was dissolved in 1:1 acetonitrile and water. Crude DHA solution was filtered through a nylon membrane with a pore size 0.45 µm then injected into HPLC. HPLC conditions are shown above. HPLC peaks were analyzed by LC-MS with MS scan mode and acetonitrile-water (50:50 v/v) as mobile phase. Major fractions was collected and similar peaks were pooled. Solvents were removed by

nitrogen blowing and these samples were repeated by LC-MS prior to investigate chemical structure using IR and NMR.

3.5.2 Isolation of degradation products by silica gel chromatography

3.5 Gram crude DHA was dissolved in 18 ml dichlorometane and 18 ml hexane added. These mixture were filtered through Whatman filter No 1 and then was evaporated by Rotary evaporator. The residue (2 g) was dissolved in 10 ml dichloromethane before loading into column chromatography. The degradation products were fractionated by Silica gel (100 g) column chromatography (bulb 100 ml, 3x60 cm.). Fractionated products were eluted with ethyl acetate-dichloromethane gradient in stepwise fashion[32] (eluted with 600 ml 10%, then 200 ml 15%, then 200 ml 20%, followed by 200 ml 30% ethyl acetate-dichloromethane). Each fraction was analyzed by LC-MS. Fractions with similar LC-MS were combined together, and solvents were removed under reduced pressure to obtain brown oil compound.

3.6 Toxicity test

3.6.1 Cytotixicity test[46]

Cytotoxicity test was carried out BIOTEC Central Research Unit, NSTDA, Thailand using the following protocol. Bioassay of cytotoxicity activity against mammalian cell culture *in vitro* was performed by MTT (3-(4, 5-dimethylthaiazol-2-yl)-2, 5-diphenyltetrazolumbromide) colorimetric method [47]. In principle, the viable cell number/well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

The mammalian cell line was harvested from exponential phase maintenance cultures, counted by trypan blue exclusion, and dispensed within replicate 96-well culture plate in 200 µl volumes using a repeating pipette. Following a 48 h incubation at 37 °C in an atmosphere of 2% CO_2 in air, 200 µl of culture medium, culture medium containing sample was dispensed within appropriate well. In addition, culture plate was incubated for 24 h. The test samples were removed from the cell cultures

and the cell were reincubated for a further 24 h in fresh medium and then tested with MTT assay. Briefly, 50 µl MTT in PBS at 5 mg/ml was added to the medium in each well and the cells were incubated for 4 h. Medium and MTT were then aspirated from the well, and formazan solubilized with 200 µl DMSO and 25 µl Sorensen's Glycine buffer, pH 10.5. The optical density was read with a plate reader at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC_{50} for each sample. Two controls were set up, one with medium, and the second with the 1% DMSO in medium; reagent or solvent control. There was no difference between two controls with no toxicity to tested cell lines.

A dose-response curve was derived from 8 concentrations in the test range of 0.78-100 µg/ml using 4 wells per concentration. Within each experiment 3-dose response curves were obtained. Results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells compound to controls and percentage of control absorbance (±1SD%) following subtraction of mean "background" absorbance.

Sample preparation

Three samples are DHA; fresh preparation and 20-month old stock at RT and its degradation products. These samples were dissolved in DMSO to give a stock concentration of 10 mg/ml. The stock solutions were further diluted in growth medium of cells.

Samples were tested for cytotoxic activity toward 6 cell lines, which contain L929 (Mouse, Connective tissue ECACC No. 85011425), BHK(21)C13 (baby hamster Syrian, Kidney ECACC No. 85011433), IEC-6 (Rat, small intestine epithelial cells ECACC No. 88701401), 3T3 (Mouse, embryonic fibroblast cells Riken Cell Bank, Japan), Vero (African green monkey, Kidney cells ATCC Cat. No. CCL-81), HepG2 (a human, Liver carcinoma)

3.6.2 Acute toxicity test[48]

Acute toxicity test was carried out Medicinal Plant Research Institute, Department of Medical Science, Nonthaburi. LD_{50} of sample administered orally in Mice ICR stain was investigated using the method of Weil [49].

The animals were fasted overnight for 18 h. Sample was given oral administration by gavage. The animals were then observed and recorded for lethality, any signs of toxic effects at ¼ , ½, 1, 3, 6, 24 h after oral administration until the end of 14-day. The does of sample caused death in 50% of the animals (LD_{50}) and 95% confidence interval (CI) were calculated by the method of Weil.

Sample preparations

Degradation products of DHA, brown oil, was diluted with corn oil at the ratio of 1:8, 1:4, 1: 2 and 1:1 (sample: corn oil).

3.7 Anitmalarial activity test[50]

Antimalrial activity test was carried out Armed Forces Research Institute of Medical Science (AFRIM). The bioassay[51,52] was based in part on a microdilution radioisotope method used for antimalarial drug susceptibility testing of Chloroquine-sensitive, Mefloquine-resistant;W2 and Chloroquine-resistant, Mefloquine-sensitive; D6 of *P.falciparum.*

Fifty microliters of each drug dilution were dispensed into the 96-well microtiter plate. A suspension of malaria parasite-infected erythrocytes (W2 or D6 clone; 0.5 % parasitemia at a 1.7 % hematocrit) was also dispensed (175 µl) into the same wells. Control wells (Without drug) were filled with either parasitized (positive controls) or normal (negative control) erythrocytes (1% suspension in completes medium without hypoxanthine). The microtiter plates were placed into a gas-tight Plexiglas chamber; flushed with a mixture of gas consisting of 90% CO_2, 5% O_2 and 5% N_2; and placed into an incubator (37 °C). Following 24 h of incubation, the microtiter plates were removed from the chamber and pulsed with [^{3}H]-Hypoxanthine (specific activity, 1 Ci/mmol) by addition of 25 µl (0.5 µCi) of isotope solution to each well. The microtiter plates were returned to the chamber and incubated for 18 to

20 h. The contents of the plates were then harvested with a Mach II harvester, in which the particulate material of the water-lyzed cell suspension was filtered though glass-fiber filter paper. The filter paper was dried in an oven and placed in a plastic bag, and 10 ml of scintillation fluid was added before the plastic bag was sealed. The level of incorporation of [^{3}H]-Hypoxanthine by the malaria parasites in each well was determined by counting in a liquid scintillation counter. The IC_{50} is defined as that concentration of drug producing a 50 % inhibition of uptake of [^{3}H]-Hypoxanthine by the intraerythrocytic parasites.

Sample preparation

Six samples are Mefloquine, Chloroquine, Quinine, fresh DHA, old DHA (20-month old stock at RT) and degradation products of DHA. Each drug to be tested were dissolved in 70% ethanol to final concentration of 1 mg/ml and diluted in the media, except for degradation products of DHA which one was dissolved in DMSO to final concentration of 10 mg/ml. The solutions were used as stock solutions. The stock solutions were further diluted in complete medium without hypoxanthine in order to keep the following serial dilutions.