CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The bark of *Goniothalamus tenuifolius* was collected from Kaengkrachan, Phetchaburi province, Thailand. The plant was identified by comparison with herbarium specimens in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Co-operatives, Bangkok.

2. General Techniques

2.1 Analytical Thin Layer Chromatography (TLC)

Technique	: One way, ascending
Adsorbent	: Silica gel 60 F_{254} (E.Merck) precoated plate
Layer thickness	: 0.2 mm
Distance	: 6 cm
Temperature	: Laboratory temperature (30-35 °C)
Detection	: 1. Ultraviolet light at wavelengths of 254 and 365 nm
	: 2. 10% Sulfuric acid in ethanol then heated at 105 °C for 10 min.

2.2 Preparative Thin Layer Chromatography (TLC)

Technique	: One way, ascending
Adsorbent	: Silica gel 60 F_{254} (E.Merck) precoated plate
Layer thickness	: 0.2 mm
Distance	: 10 cm
Temperature	: Laboratory temperature (30-35 °C)
Detection	: Ultraviolet light at wavelengths of 254 and 365 nm

2.3 Column Chromatography

2.3.1 Quick Column Chromatography

Adsorbent : Silica gel 60 H (No.7736)

Packing : Dry packing

Sample loading : The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, dried and then placed gently on top of the column.

Detection : Fractions were examined by TLC observing under UV light at the wavelengths of 254 and 365 nm. The TLC plate was then sprayed with 10% sulfuric acid in ethanol and heated at 105 °C for 10 min. Fractions of similar pattern were combined.

2.3.2 Flash Column Chromatography

Adsorbent : Silica gel 60 (No.9385) particle size 0.400-0.063 mm (230-400 mesh ASTM) (E.Merck)

Packing : Dry packing

Sample loading : The sample was dissolved in a small amount of organic solvent, then applied gently on top of the column.

Detection : Fractions were examined in the same manner as described in Section 2.3.1

2.3.3 Gel Filtration Chromatography

Gel filter : Sephadex LH-20 (Pharmacia)

Packing : The gel filter was suspended in the eluent and left standing to swell prior to use (24 hours). It was then poured into the column and allowed to set tightly.

Sample loading : The sample was dissolved in a small volume of eluent and applied on top of the column.

2.4 Spectroscopy

2.4.1 Ultraviolet (UV) Absorption Spectra

UV (in methanol) spectra were obtained on a Milton Roy Spectronic 3000 Array spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4.2 Infrared (IR) absorption Spectra

IR (KBr disc) spectra were obtained from a Perkin Elmer FT-IR 1760X spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.4.3 Mass Spectra (MS)

Electron Impact Mass Spectra (EIMS) of GT-B, GT-C, GT-D and GT-F were determined on a JEOL JMS-AM20 spectrometer (Faculty of Pharmaceutical Sciences, Chiba University). EIMS of GT-A and GT-E were performed on a Finnigan MAT Incos 50 mass spectrometer (Department of Chemistry, Faculty of Sciences, Mahidol University).

2.4.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H and ^{13}C nmr) Spectra

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were obtained with a JEOL JMN-A 500 spectrometer (Faculty of Pharmaceutical Sciences, Chiba University or Scientific and Technological Research Equipment Center, Chulalongkorn University).

The NMR solvents used in this study were deuterated dimethylsulfoxide (DMSO-d_6) and deuterated chloroform (chloroform-d). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

2.5 Solvents

Throughout this work, all organic solvents were of commercial grade and were redistilled prior to use.

3. Extraction (Scheme 2)

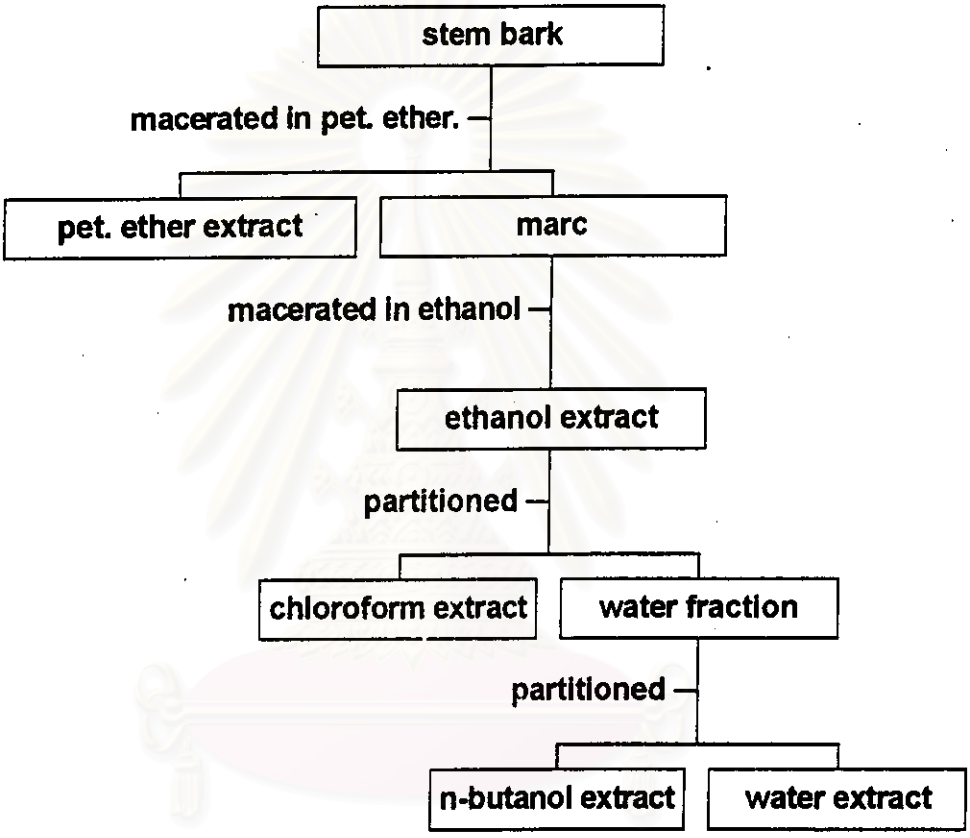
Two kilograms of the dried stem bark of *Goniothalamus tenuifolius* were chopped into small pieces. They were macerated in petroleum ether (8 liters, 1 day) and then filtered. The filtrate was evaporated under reduced pressure at temperature not exceeding 40 °C to yield a petroleum ether extract (syrupy mass 6.25 g, 0.31 % based on dried weight of stem bark).

The marc was repeatedly extracted five times with 95 % ethanol (8 litres, 5 days, each). The obtained extract was evaporated under reduced pressure to yield an ethanol extract (syrupy mass, 173.72 g, 8.96 % based on dried weight of stem bark).

The ethanol extract was partitioned between chloroform and water. The chloroform fraction was separated and evaporated under reduced pressure to give a chloroform extract (188.82 g, 9.44 % based on dried weight of stem bark).

The water fraction was partitioned with n-butanol. Then, the n-butanol fraction was separated and evaporated under reduced pressure to yield 13.20 g of brown solid mass (0.66 % based on dried weight of stem bark).

A water extract (76.80 g, 3.84 % based on dried weight of stem bark) was obtained from the water fraction after removal of water by lyophilization. Each extract was subject to antimalarial activity evaluation as described in Section 6.1.



Scheme 2 Extraction of *Goniothalamus tenuifolius* King

4. Isolation (Scheme 3)

The chloroform fraction, which was the most active fraction (see Section 6.1), was selected for further separation by quick column chromatography using a sintered glass filter column of silica gel 60 H (No. 7736) (50 g). The chloroform extract (20 g) was dissolved in a small amount of chloroform, triturated with silica gel 60 H (No. 7736) and dried under vacuum. The mixture was then applied on the top of the column. Elution was performed in a polarity gradient manner with hexane, ethylacetate and methanol as the solvents.

The eluents were examined by TLC using 10 % acetone in chloroform as the developing system. Fractions with similar chromatographic pattern were combined to yield 11 fractions: G1 (0.3 g), G2 (0.94 g), G3 (0.12 g), G4 (0.26 g), G5 (1.46 g), G6 (0.81 g), G7 (0.40 g), G8 (1.71 g), G9 (2.60 g), G10 (6.07 g) and G11 (14.48 g).

4.1. Isolation of GT-A

Fraction G4 (0.26 g) was dissolved in a small amount of chloroform and was then fractionated by silica gel column chromatography with polarity gradient elution, using mixture of Chloroform and ethyl acetate (10:0 to 9:1) as the solvents. All fractions were collected and similar fractions were combined after examining with TLC, using hexane:ethyl acetate (1:1) as the developing system. Fractions 15-19 were examined and further separated by preparative TLC on a precoated silica gel 60 F_{254} (0.2 mm, 10 x 20 mm) plate with triple development in hexane:ethyl acetate (85:15) to give GT-A (dark spot under UV light at 254 nm, 2 mg, $1x10^{-4}$ % based on the weight of dried bark). GT-A (1 mg, $5x10^{-5}$ % based on the weight of dried bark) was also obtained from fraction G3, using the same procedure.

4.2. Isolation of GT-B and GT-C

Fraction G5 (1.46 g) was equally divided into three portions. Each was fractionated on a silica gel column. Separation was carried out with gradient elution, using mixtures of chloroform and ethyl acetate (10:0 to 9:1). Fractions showing

similar pattern were combined. Fractions 20-39 were combined and further separated by a Sephadex LH-20 column, using methanol as the eluent to give GT-B (fluorescent blue color under UV light at 365 nm, 25 mg, 1.25×10^{-3} % based on the weight of dried bark) and GT-C (fluorescent green color under UV light at 365 nm, 3 mg, 1.5×10^{-4} % based on the weight of dried bark). These two compounds showed the same R_f value on a TLC chromatogram (silica gel, chloroform-acetone = 9:1).

Fraction G6 (0.81 g) was also separated in a similar manner to give GT-B (15 mg, 7.5×10^{-4} % based on the weight of dried bark) and GT-C (2 mg, 1×10^{-4} % based on the weight of dried bark).

4.3. Isolation of GT-D

Fraction G10 (6.07 g) was divided in to four portions. Each was fractionated by column chromatography using silica gel. Gradient elution was performed using mixtures of chloroform and acetone (10:1 to 9:1). Fractions of 300 ml were collected. Fractions with similar TLC pattern were combined.

Fractions 66-69 of the first portion, fractions 60-61 of the second portion, fractions 44-48 of the third portion and fractions 56-74 from the fourth portion were combined and further purified by recrytallizing from chloroform to give GT-D (75 mg, 3.75×10^{-3} % based on the weight of dried bark, fluorescent green color under UV light at 365 nm).

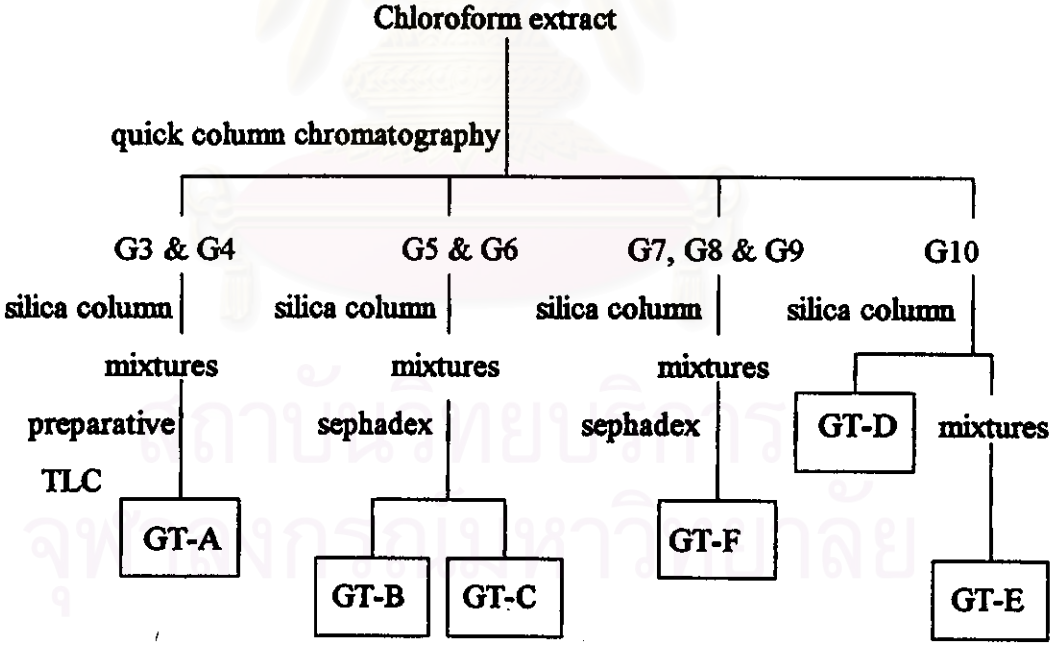
4.4. Isolation of GT-E

After the separation of GT-D from fraction G10, the remaining fractions of G10 were combined. Fractions showing fluorescent orange color under UV light at 365 nm. were further examined. They were combined and further purified by gel filtration chromatography using methanol as the eluent. The purification of GT-E by gel filtration chromatography was monitored by UV light at 365 nm.. All orange-fluorescent color portions were combined and dried to give compound GT-E (15 mg,

$7.5x10^{-4}$ % based on the weight of dried bark) as an orange solid with fluorescent orange color under UV light at 365 nm.

4.5 Isolation of GT-F

Fractions G7 (0.40 g), G8 (1.71 g) and G9 (2.60 g) showed similar TLC behavior. Each fraction was fractionated on a silica gel column with gradient elution, using mixtures of chloroform and acetone (2-50 % acetone in chloroform). Fractions 16-20 of G7 and G8 and fractions 11-17 of G-9 displayed a blue-fluorescent spot under UV light at 365 nm (GT-F). All these fractions were combined and then further purified by gel filtration chromatography (Sephadex LH 20) using methanol as the eluent to give GT-F (3 mg, $1.5x10^{-4}$ % based on the weight of dried bark).



Scheme 3 Isolation of pure compounds

5. Spectral Data of Isolated Compounds

5.1 Compound GT-A

EIMS : *m/z* (% relative intensity)

196 (M^+, 21), 150 (72), 122 (56), 94 (18), 77 (9), 69 (26), 53 (23), 39 (35), 28 (100)

UV : λ_{max} nm, in methanol

213, 240, 262, 285, 297

^{1}H nmr : δ ppm, 500 MHz, in chloroform-*d*

1.41 (3H, dd, *J* = 7.02, 7.02 Hz, H-9), 2.51 (3H, s, 6-CH_3), 4.39 (2H, ddd, *J* = 7.02, 7.02, 7.02 Hz, H-8), 6.23 (1H, d, *J* = 2.14 Hz, H-5), 6.28 (1H, d, 2.14 Hz, H-3), 11.80 (1H, s, 2-OH)

^{13}Cnmr : δ ppm, 125 MHz, in chloroform-*d*

14.23 (q, C-9), 24.36 (q, 6-CH_3), 61.26 (t, C-8), 101.27 (d, C-3), 105.76 (s, C-1), 111.25 (d, C-5), 144.00 (s, C-2), 160.14 (s, C-4), 165.38 (s, C-6), 171.67 (s, C-7)

5.2. Compound GT-B

EIMS : *m/z* (% relative intensity)

279 (M^+, 100), 264 (6), 236 (14), 221 (13), 209 (14), 193 (34), 181 (34), 164 (52), 150 (14), 138 (27), 97 (11), 83 (27)

UV : λ_{max} nm (log ε), in methanol

231 (2.85), 262 (2.75), 275 (2.79), 286 (2.78), 315 (2.23), 382 (2.15)

IR : ν cm^{-1}, KBr disc

3316-3104, 1715, 1379, 741

^{1}H nmr : δ ppm, 500 MHz, in DMSO-d_6

4.03 (3H, s, 4-OCH_3), 4.04 (3H, s, 3-OCH_3), 7.13 (1H, s, H-9), 7.55 (1H, dd, *J* = 7.81, 7.81 Hz, H-6), 7.58 (1H, dd, *J* = 7.81, 7.81 Hz, H-7), 7.85 (1H, s, H-2), 7.94 (1H, d, *J* = 7.81 Hz, H-8), 9.11 (1H, d, *J* = 7.81 Hz, H-5), 10.84 (1H, s, NH)

^{13}Cnmr : δ ppm, 125 MHz, in DMSO-d_6

56.93 (q, 3-OCH_3), 59.91 (q, 4-OCH_3), 104.61 (d, C-9), 109.91 (d, C-2), 119.91 (s, C-4a), 121.55 (s, C-1), 123.33 (s, C-10a), 125.47 (d, C-6), 125.92 (s, C-4b), 126.84 (d, C-5), 127.46 (d, C-7), 129.03 (d, C-8), 134.82 (s, C-8a), 135.12 (s, C-10), 150.40 (s, C-4), 154.24 (s, C-3), 168.39 (s, C=O)

5.3. Compound GT-C

EIMS : *m/z* (% relative intensity)

309 (M^+, 100), 294 (30), 279 (6), 251 (18), 236 (5)

UV : λ_{max} nm (log ε), in methanol

210 (3.39), 242 (2.85), 254 (2.85), 297 (2.47), 400 (2.17)

IR : ν cm^{-1}, KBr disc

3700-3500, 3400-3300, 1652

^{1}H nmr : δ ppm, 500 MHz, in DMSO-d_6

3.98 (3H, s, 8-OCH_3), 4.01 (3H, s, 4-OCH_3), 4.04 (3H, s, 3-OCH_3), 7.21 (1H, br d, J = 7.81 Hz, H-7), 7.42 (1H, s, H-9), 7.51 (1 H, dd, J = 7.81, 7.81 Hz, H-6), 7.87 (1H, s, H-2), 8.75 (H, br d, J = 7.81 Hz, H-5), 10.79 (1H, s, NH)

^{13}Cnmr : δ ppm, 125 MHz, in DMSO-d_6

55.89 (q, 8-OCH_3), 56.98 (q, 3-OCH_3), 59.94 (q, 4-OCH_3), 97.89 (d, C-9), 108.25 (d, C-7), 110.31 (d, C-2), 119.25 (d, C-5), 119.92 (s, C-4a), 121.58 (s, C-1), 123.07 (s, C-10a), 124.80 (s, C-8a), 125.69 (d, C-6), 126.70 (s, C-4b), 134.60 (s, C-10), 150.60 (s, C-4), 154.28 (s, C-3), 155.29 (s, C-8), 168.29 (s, C=O)

5.4. Compound GT-D

EIMS : *m/z* (% relative intensity)

295 (M^+, 100), 252 (9), 237 (5), 225 (3), 209 (11), 197 (12), 180 (7), 153 (13), 140 (11), 126 (30), 91 (22), 69 (19), 55 (45)

UV : λ_{max} nm (log ε), in methanol

245 (2.48), 293 (2.03), 406 (1.82)

IR : ν cm^{-1}, KBr disc

3531-3000, 1672, 1283

^{1}H nmr : δ ppm, 500 MHz, in DMSO-d_6

3.99 (3H, s, 4-OCH_3), 4.03 (3H, s, 3-OCH_3), 7.06 (1H, d, J = 8.30 Hz, H-7), 7.36 (1H, dd, J = 8.30, 8.30 Hz, H-6), 7.40 (1H, s, H-9), 7.84 (1H, s, H-2), 8.61 (1H, d, J = 8.30 Hz, H- 5), 10.11 (1H, s, 8-OH), 10.78 (1H, s, NH)

^{13}Cnmr : δ ppm, 125 MHz, in DMSO-d_6

56.94 (q, 3-OCH_3), 59.87 (q, 4-OCH_3), 98.71 (d, C-9), 109.92 (d, C-2), 112.13 (d, C-7), 117.97 (d, C-5), 120.19 (s, C-4a), 121.61 (s, C-1), 123.31 (s, C-10a), 123.96 (s, C-8a), 125.78 (d, C-6), 127.08 (s, C-4b), 133.89 (s, C-10), 150.54 (s, C-4), 153.74 (s, C-8), 154.21 (s, C-3), 168.35 (s, C=O)

5.5. Compound GT-E

EIMS : *m/z* (% relative intensity)

307 (M^+, 54), 279 (26), 264 (12), 236 (15), 221 (15), 209 (11), 193 (23), 181 (15), 164 (29), 138 (12), 97 (9), 83 (20)

UV : λ_{max} nm (log ε), in methanol

209 (3.61), 235 (3.54), 301 (3.16), 315 (3.19), 440 (3.06)

IR : ν cm^{-1}, KBr disc

3600-3300, 3200, 2780, 1700, 1394

^{1}H nmr : δ ppm, 500 MHz, in DMSO-d_6

4.05 (3H, s, 2-OCH_3), 4.09 (3H, s, 1-OCH_3), 7.52 (1H, s, H-7), 7.60-7.68 (2H, m, H-10 , H-9), 7.93 (1H, d, J = 7.57 Hz, H-8), 8.16 (1H, s, H-3), 9.43 (1H, d, J = 7.57 Hz, H-11), 12.05 (1H, s, NH)

^{13}Cnmr : δ ppm, 125 MHz, in DMSO-d_6

56.49 (q, 2-OCH_3), 60.15 (q, 1-OCH_3), 112.63 (d, C-3), 112.79 (d, C-7), 118.24 (s, C-11c), 123.62 (s, C-11b), 124.67 (s, C-3a), 125.99

(s, C-11a), 126.93 (d, C-9), 127.23 (d, C-11), 128.02 (d, C-10), 128.50 (d, C-8), 130.18 (s, C-6a), 132.42 (s, C-7a), 152.73 (s, C-2), 153.95 (s, C-1), 155.53 (s, 5-C=O), 176.87 (s, 4-C=O)

5.6. Compound GT-F

EIMS : *m/z* (% relative intensity)

265 (M^+, 100), 250 (45), 222 (16), 166 (77), 150 (16), 139 (66)

UV : λ_{max} nm (log ε), in methanol

233 (2.90), 275 (3.86), 285 (3.86), 389 (2.26)

IR : ν cm^{-1}, KBr disc

3600-3048, 2921, 1706, 1293

^{1}H nmr : δ ppm, 500 MHz, in DMSO-d_6

4.01 (3H, s, 4-OCH_3), 7.08 (1H, s, H-9), 7.54 (1H, ddd, *J* = 7.81, 7.81, 1.47 Hz, H-6), 7.56 (1H, ddd, *J* = 7.81 , 7.81, 1.47 Hz, H-7), 7.61 (1H, s, H-2), 7.93 (1H, dd, *J* = 7.81, 1.47 Hz, H-8), 9.09 (1H, dd, *J* = 7.81, 1.47 Hz, H-5) , 10.78 (1H, s, NH)

^{13}Cnmr : δ ppm, 125 MHz, in DMSO-d_6

59.46 (q, 4-OCH_3), 103.88 (d, C-9), 113.40 (d, C-2), 120.35 (s, C-4a), 121.80 (d, C-10), 122.28 (s, C-10a), 125.29 (d, C-6), 126.00 (s, C-4b) , 126.79 (d, C-5), 127.28 (d, C-7), 128.97 (d, C-8), 134.85 (s, C-8a), 135.32 (s, C-1), 148.83 (s, C-4), 152.27 (s, C-3), 168.49 (s, C=O)

6 Antimalarial Activity Evaluation

6.1 Preparation of Material for Malarial Culture

All containers and pipettes should be sterile, and aseptic technique was used throughout. Serum was prepared from the clotted blood and separated by centrifugation at 700x g at 4 °C for 15 min.

Stock RPMI-1640 culture medium was prepared by adding 10.4 g of the medium and 5.94 g HEPES buffer and then 1 mg of gentamycin solution (40 μg/ml) into 960 ml of distilled water. The medium was sterilized by filtration through 0.45 micron Millipore filter. It was dispensed aseptically as 200 ml portions into sterilized bottles, and stored at 4 °C for up to 4 weeks.

Complete culture media (cRPMI) was prepared by adding 8.4 ml of 5 % sodium bicarbonate solution to 200 ml of RPMI culture-1640 medium and 20 ml of serum. The cRPMI was stored at 4 °C for up to 1 week.

6.2 Preparation of Sample Solutions

Dimethylsulfoxide (DMSO) was used to initially solubilize the compounds and then diluted with cRPMI to give the final concentration of 0.1 % DMSO. Appropriate concentrations of the tested compounds were diluted with cRPMI.

6.3 Preparation of Parasites

The parasite used is the $T_{9/94}$ line of *Plasmodium falciparum*. The cultivation was carried out in a candle jar at 37 °C (Trager and Jensen, 1976).

Red blood cells (from human group O donor, with citrate-phosphate-dextrose (CPD) as anticoagulant) were prepared by washing red blood cell (10 ml) twice with incomplete medium (5 % sodium bicarbonate 8.4 ml + RPMI-1640 200 ml),

and the buffy coated layer was discarded. The red blood cells were resuspended in cRPMI to give cell suspension of 50 %.

6.4 Test Procedure and Data Analysis

6.4.1 Extract

The technique used for determining antimalarial activity of crude extract was microscopic technique or morphology end-point method (Rieckmann *et al.*, 1978).

By using 24-well microtiter plate (flat-bottom), each well was added with 1 ml of 5 % cell suspension and 0.3-0.5 % parasitemia. After incubation for 2 h, the medium was aspirated and 1 ml of the initial sample solution was then added into the appropriate wells. Each known concentration of crude extracts was duplicated. The sample solution was changed every 24 h for 4 days, using aseptic technique.

The percentage of parasitemia was determined from Giemsa's stain thin blood films on day 4. The thin blood films were fixed in acetone-free absolut methanol for 1 min and then air dried. The slides were then stained with 3 % Giemsa's stain in 0.06 M phosphate buffer pH 7 for 30 min, washed with tap water and air-dried.

EC_{50} (effective concentration at 50 % inhibition of parasite growth) of samples were obtained from the plot between log concentration and % inhibition.

Using microscopic technique as described, petroleum ether extract and ethanol extract were prepared in three concentrations (50 μg/ml, 10 μg/ml and 1 μg/ml), chloroform extract and water extract were prepared in three concentrations (100 μg/ml, 10 μg/ml and 1 μg/ml) and n-butanol extract was prepared in three concentrations (25 μg/ml, 10 μg/ml and 5 μg/ml). The EC_{50} values of extracts from *Goniothalamus tenuifolius* are shown in the Table 2.

Table 2. EC_{50} values of extracts from *Goniothalamus tenuifolius*

Crude extract	EC_{50} (μg/ml)
Petroleum ether extract	25
Ethanol extract	3
Chloroform extract	5
n-Butanol extract	8.5
Water extract	60

6.4.2 Pure Compound

To determine antimalarial activity of pure compounds, the radioisotope microdilution technique was used (Desjardins *et al.*, 1979).

Twenty five microliters of initial solution of pure compound was added to appropiate wells in columns 11 and 12 of 96-well microtiter plate (8x12 matrix) (Figure 1), each compound solution was duplicated. Two-fold serial dilution starting from columns 11 to 2 were performed, while column 1 served as control.

Two hundred microliters of 1.6 % parasitized red blood cell suspension in complete medium, was added to each well of the microtiter plate. The plate was then incubated in a candle jar at 37 °C for 24 h. In the column 1, Rows A-D served as control of parasitizied red blood cell in complete medium and rows E-H served as control of parasitized red blood cell in complete medium containing 0.1 % DMSO.

[^{3}H]-hypoxanthine solution was prepared by adding 75 μl (1 mCi/ml) of [^{3}H]-hypoxanthine to 3 ml of complete medium for each plate. Proper handling and disposal of radioactive materials were undertaken. Twenty five microliters of [^{3}H]-hypoxanthine solution was added to each well. The microtiter plate was then placed in the candle jar and incubated for 18 h at 37 °C.

The red blood cells were harvested by using cell harvester with distilled water for rinsing. The filter strips were dried, and the filter disks were cut out and placed into scintillation vials. One ml of scintillation fluid was added in each vial.

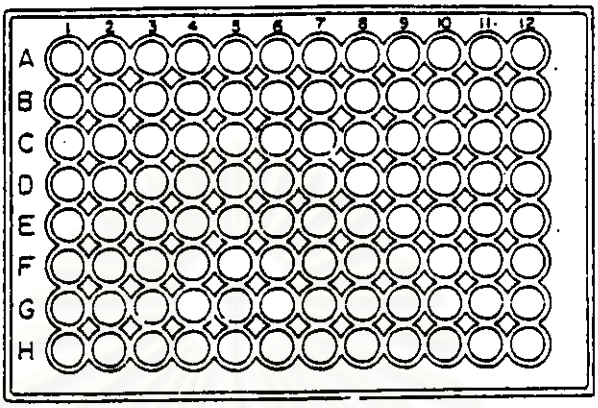


Figure 1 96-well microtiter plate

Counting activity of each vial was done in a liquid scintillation counter. The EC_{50} was calculated from the plot between concentration and cpm (count per min).

Using radioisotope microdilution technique, all pure compounds were prepared at 12.5 μg/ml except GT-A which was prepared at 25 μg/ml. Two-fold serial dilutions were made, and then the procedure as described in section 6.4.2 was followed. The EC_{50} values of pure compounds from *Goniothalamus tenuifolius* are shown in Table 3.

Table 3 EC_{50} values of pure compounds from *Goniothalamus tenuifolius*

Pure compound	EC_{50} (μg/ml)
GT-A	33
GT-B	11
GT-C	10.5
GT-D	7.5
GT-E	4.5
GT-F	9.5