CHAPTER III
EXPERIMENT

3.1 Source and authentication of the plant materials

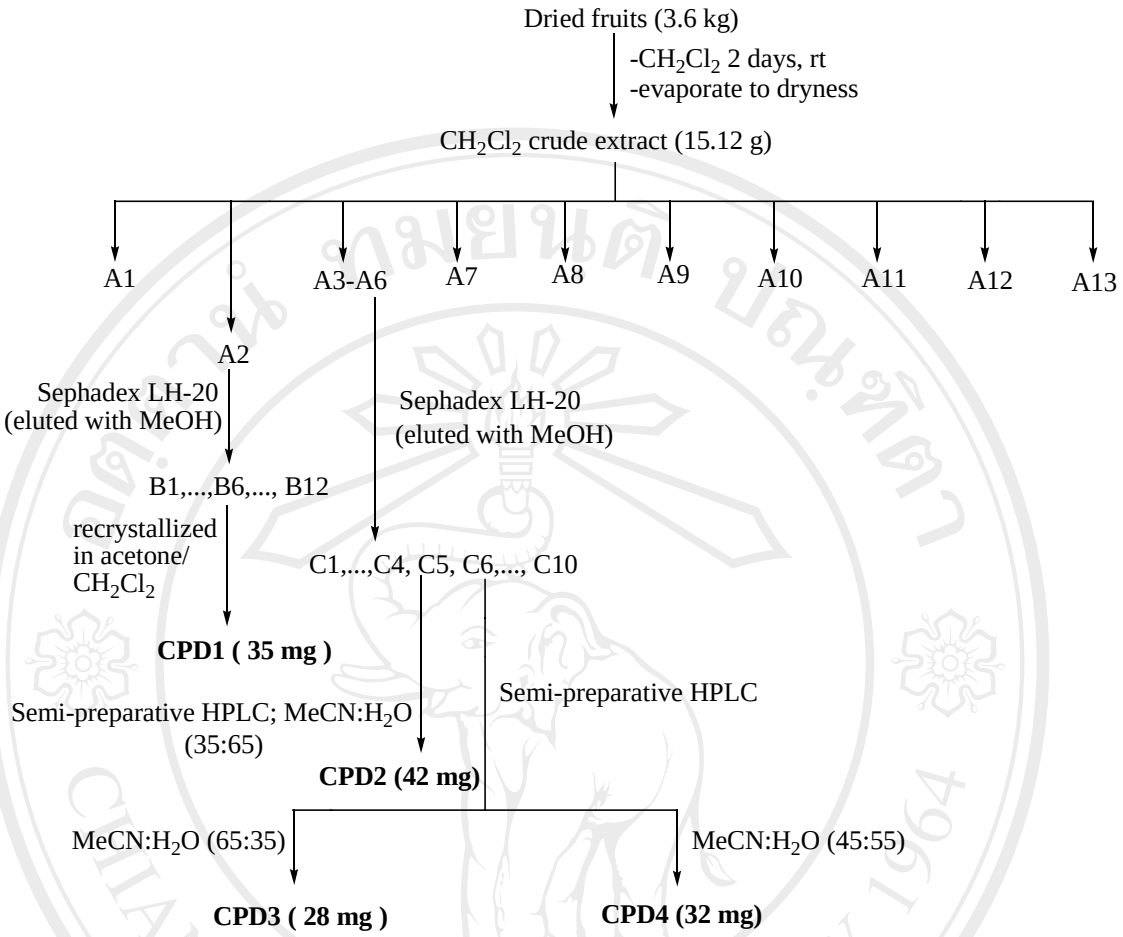
The fruits of *Diospyros ehretioides* were collected twice, the first batch collected was only the brown and dried fruits but the second batch collected was both the green and fresh, and brown and dried fruits from Kang Tana National Park, Ubon Ratchathani province. Moreover, the woods of *Diospyros rhodocalyx* were collected from Nakhon Sawan province and of *Diospyros glandulosa* were collected from Khao Yai National Park, Nakhon Ratchasima province. These plants were identified by P. Charoenchai, Kasetsart University. The *Diospyros ehretioides* voucher specimen (No. BRU 29) was deposited at the Bioresource Research Unit (BIOTEC) and the *Diospyros rhodocalyx* and *Diospyros glandulosa* voucher specimens (No. CRI 283 and 317) were deposited at Laboratory of Natural Products (Chulabhorn Research Institute), Thailand.

3.2 Extraction and Isolation

3.2.1 Fruits of *Diospyros ehretioides*

The first batch of *Diospyros ehretioides* fruits was collected in October 29, 2003. Dried fruits (3.6 kg) of *Diospyros ehretioides* were macerated with dichloromethane (CH_2Cl_2) for 2 days at room temperature. The filtrate, which was evaporated under reduced pressure, yielded a crude extract (15.12 g). The crude extract, which was chromatographed on Sephadex LH-20 (eluent: methanol; MeOH), yielded thirteen fractions (A1-A13). Further purification of fraction A2 on Sephadex

LH-20 column with MeOH afforded fractions B1-B12. Due to a promising crystal appearance in fraction B6, therefore, it was re-crystallized in acetone/CH_2Cl_2 to afford white crystals **[CPD1]** (35 mg). Fractions A3-A6 were also rechromatographed in the same manner as fraction A2 to provide ten fractions (C1-C10), from which fraction C5 was further purified by semi-preparative HPLC (reversed-phase, RP C_{18} Column), using acetonitrile (MeCN)/ water (H_2O) in the ratio of 35:65 as eluent to furnish light-brown solid **[CPD2]** (42 mg). Fraction C6 was also subjected to reversed phase HPLC, eluted with MeCN/ H_2O, in the ratio of 65:35 and later to the ratio of 45:55 to provide orange-brown solid **[CPD3] (**28 mg) and orange-red solid **[CPD4]** (32 mg), respectively (Scheme 1). All fractions obtained were identified by spectroscopic techniques such as UV, IR, ^{1}H NMR, ^{13}C NMR and HR-MS.



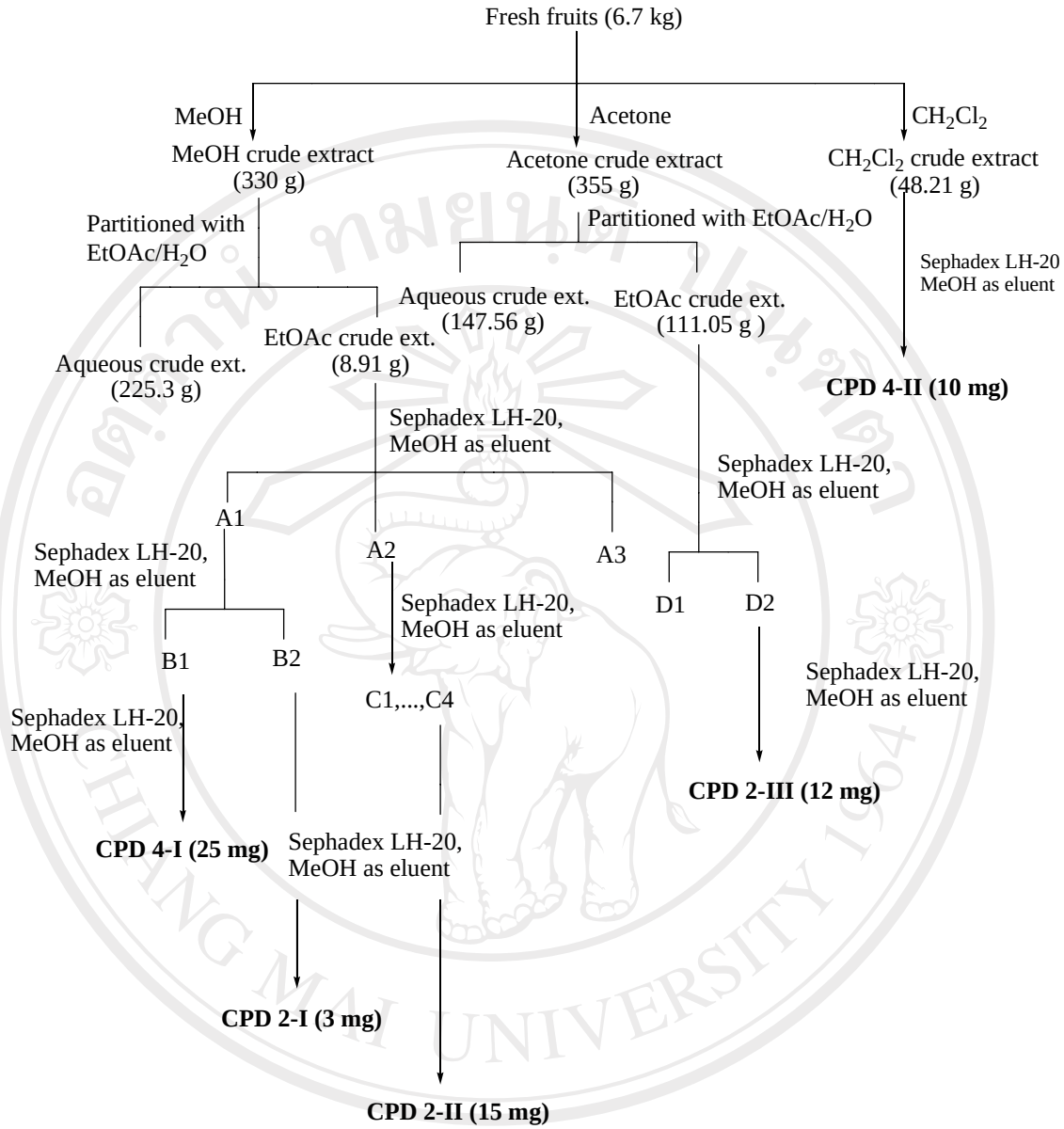
Scheme 1. The extraction and isolation of CH_2Cl_2 crude extract from dried fruits of *Diospyros ehretioides*

Fruits of *Diospyros ehretioides* were re-collected in September 22, 2004, as fresh and dried fruits. Fresh fruits (6.7 kg) were extracted sequentially with MeOH, acetone and followed by CH_2Cl_2 to yield 330, 355 and 48.21 g of dried crude extracts, respectively. The MeOH extract was added with H_2O (100 ml) and partitioned with ethyl acetate (EtOAc). The aqueous phase was concentrated under reduced pressure. The organic phase was evaporated until dryness and further purified on a Sephadex LH-20 column (eluent : MeOH) to obtain three fractions (A1-A3). Fraction A1 was re-chromatographed on Sephadex LH-20 column (eluent: MeOH) to give two

fractions (B1-B2) which afforded an orange-red **[CPD4-I]** and a light-brown solid **[CPD2-I]** after being loaded on a Sephadex column with the same eluent. Another fraction (A2) was re-chromatographed with MeOH to obtain four fractions (C1-C4). Fraction C4 was re-chromatographed on Sephadex LH-20 column (eluent:MeOH) to give light-brown solid **[CPD2-II]** (Scheme 2).

As with MeOH extract, acetone extract was added with H_2O (100 ml) and partitioned with EtOAc. The organic phase was evaporated and rechromatographed on Sephadex LH-20 column (eluent: MeOH) to give two fractions (D1-D2). Fraction D2 was rechromatographed with MeOH to give light-brown solid **[CPD2-III]**. The CH_2Cl_2 extract was evaporated and re-chromatographed on Sephadex LH-20 column (eluent: MeOH) to give orange-red solid **[CPD4-II]** (Scheme 2).

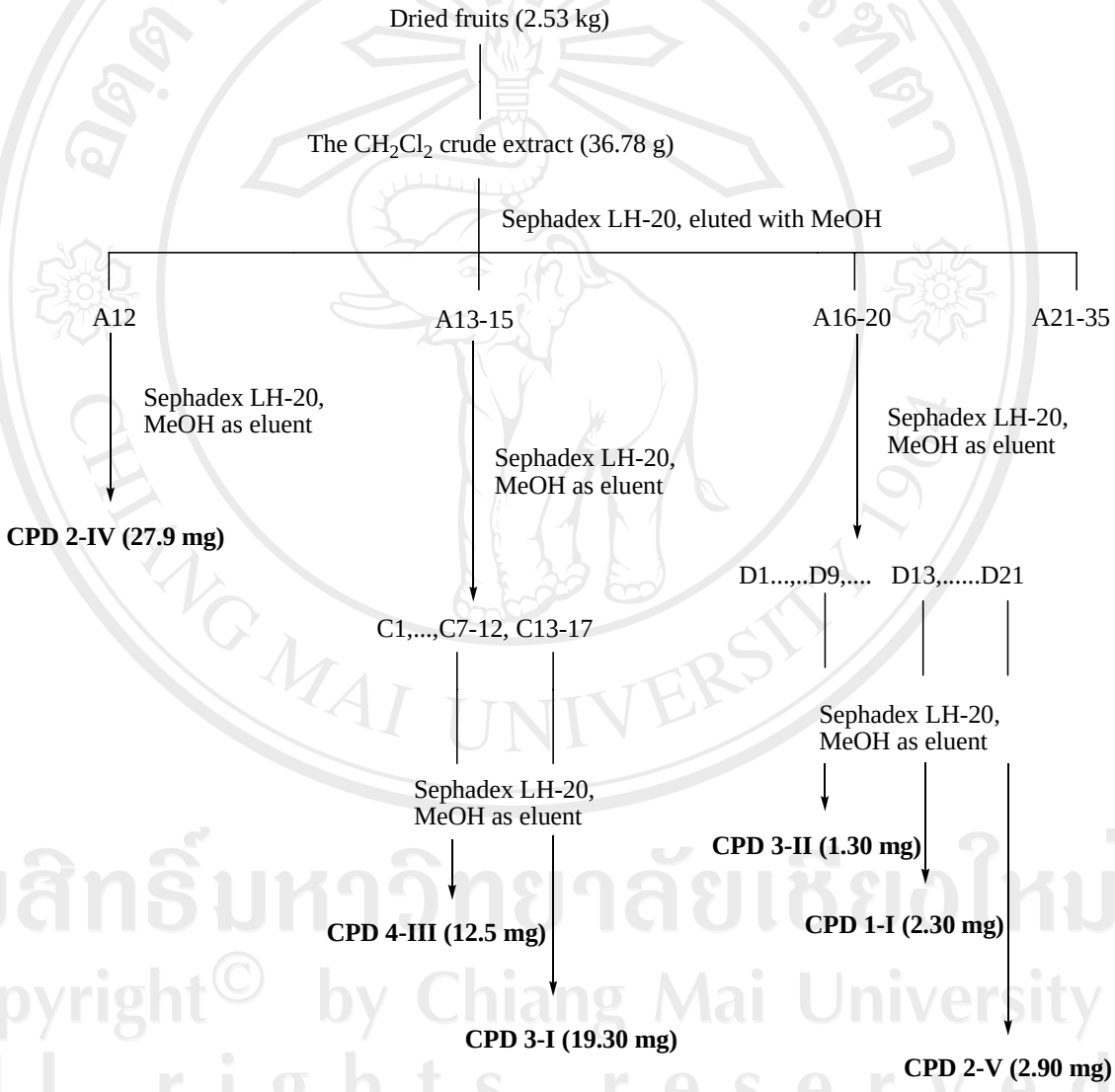
Using the ^{1}H-NMR analysis technique mentioned earlier, no trace of the white crystal **[CPD1]** and the orange-brown solid **[CPD3]** as found in the first batch was detected, while the orange-red **[CPD4-I, CPD4-II]** and light-brown solids **[CPD2-I, CPD2-II, CPD2-III]** were consistently found. Therefore, it was concluded that the orange-red **[CPD4-I, CPD4-II]** and the light-brown solids **[CPD2-I, CPD2-II, CPD2-III]** were present in both crude extract of green and fresh and brown and dried fruits which might be plant metabolite.



Scheme 2. The extraction and isolation of crude extract from fresh fruits

The dried fruits of *Diospyros ehretioides* collected in September 22, 2004 were macerated with CH_2Cl_2 and evaporated until dryness to give a CH_2Cl_2 crude extract (36.78 g) which was subjected to being re-chromatographed on Sephadex LH-20 column in the same manner as that of the first batch. NMR analysis of fractions

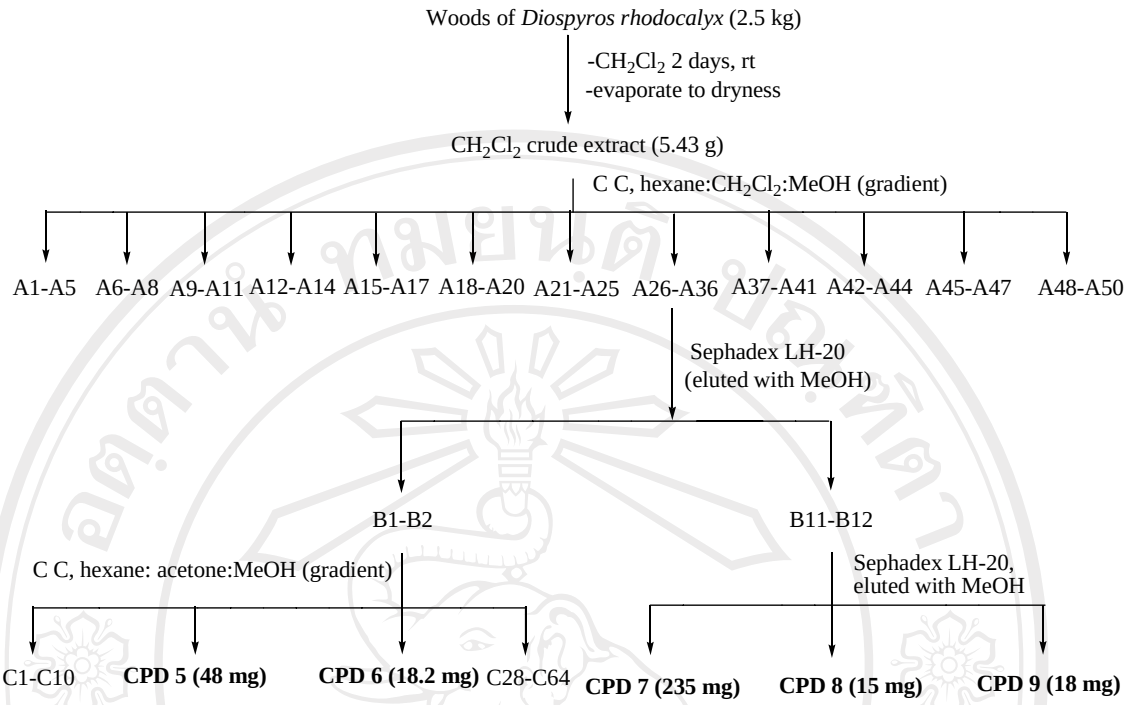
from Sephadex LH-20 column revealed the presence of white crystal **[CPD1-I]**, light-brown solids **[CPD2-IV, CPD2-V]**, orange-brown solids **[CPD3-I, CPD3-II]** and orange-red solid **[CPD4-III]** and further purification was carried out (as described earlier)(Scheme 3). All fractions obtained were identified using spectroscopic techniques.



Scheme 3. The extraction and isolation of crude extract from dried fruits

3.2.2 Woods of *Diospyros rhodocalyx*

The woods of *Diospyros rhodocalyx* were collected from Nakhon Sawan province in September 2006 and were ground and macerated with CH_2Cl_2 at room temperature for 3 days. After the organic solvent has been removed, a brown residue (17.15 g) was loaded on a silica gel column, using hexane as mobile phase, followed by gradual substitution of hexane with CH_2Cl_2 until neat. Then MeOH was introduced into the mobile phase step by step, and finally, the system was replaced with pure MeOH. Due to fractions A26-A36 presented interesting ^{1}H NMR spectrum, therefore, further purification was carried out by chromatography on Sephadex LH-20 column (eluted with MeOH) to yield twelve fractions (B1-B12). Fractions B1-B2 were further purified on a silica gel column (1.65 g), eluted with hexane, then gradually replaced with acetone, followed by increasing amount of methanol in acetone until neat. The solvents were evaporated to dryness to afford sixty four fractions (C1-C64). Due to a promising and interesting ^{1}H NMR spectrum appearance in fractions C11-C20 (100 mg), therefore, after being eluted with 4% acetone in hexane, a colorless solid **[CPD5]** (48 mg) was obtained. Same as fractions C11-C20 which presented interesting ^{1}H NMR spectrum, fractions C21-C27 (112 mg) were purified on silica gel column, eluted with 5% acetone in hexane and yielded an opaque white solid **[CPD6]** (18.2 mg). Other fractions B11-B12 were purified by Sephadex LH-20 column (eluted with MeOH) to afford orange-red solid **[CPD7]** (235 mg) together with a clear white solid **[CPD8]** (15 mg) and a white solid **[CPD9]** (18 mg), respectively (Scheme 4).

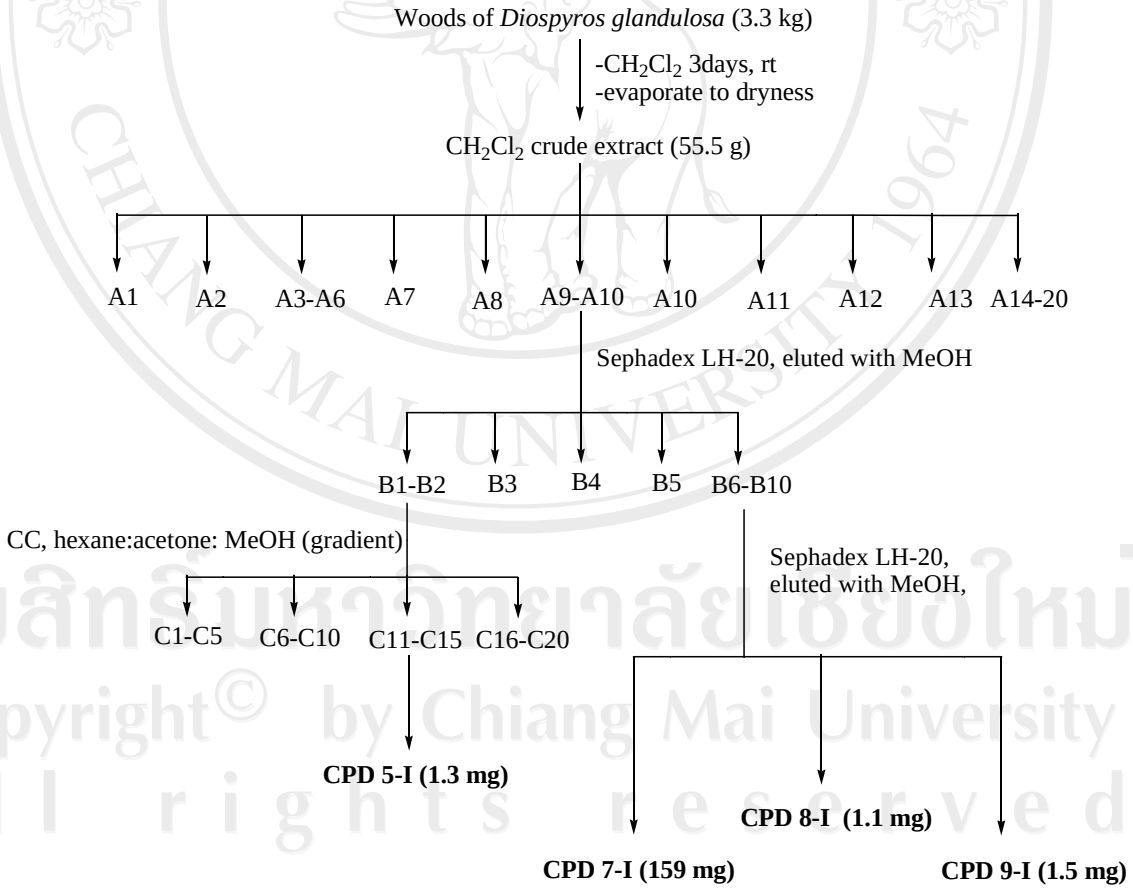


Scheme 4. The extraction and isolation of CH_2Cl_2 crude extract woods of *Diospyros rhodocalyx*

3.2.3 Woods of *Diospyros glandulosa*

The woods of *Diospyros glandulosa* (3.3 kg) were collected from Khao Yai National Park, Nakhon Ratchasima province in November 2005 and were ground and macerated with CH_2Cl_2 at room temperature for 3 days. After the organic solvent has been removed, a dark brown solid (55.5 g) was obtained which was subjected to column chromatography on a Sephadex LH-20 column (eluted with MeOH), yielded twenty fractions (A1-A20). Due to fractions A9-A10 presented interesting ^{1}H NMR spectrum, therefore, further purification by Sephadex LH-20 column (eluted with MeOH) was performed to yield ten fractions (B1-B10). Fractions B1-B2 were further purified on a silica gel column (1.5 g), eluted with hexane, then gradually replaced

with acetone, followed by increasing amount of methanol in acetone until neat. The solvents were evaporated to dryness to afford twenty fractions (C1-C20). Due to a promising and interesting ^{1}H NMR spectrum appearance in fractions C11-C15 (150 mg), therefore, after being eluted with 4% acetone in hexane, a colorless solid **[CPD 5-I]** (1.3 mg) was obtained. Moreover, fractions B6-B10 were further re-chromatographed on Sephadex LH-20 column (eluted with MeOH) to afford three different kinds of solid, orange-red solid **[CPD7-I]** (159 mg), clear white solid **[CPD 8-I]** (1.1 mg) and white solid **[CPD 9-I]** (1.5 mg) (Scheme 5). All fractions obtained were characterized, using ^{1}H NMR spectroscopic technique.



Scheme 5. The extraction and isolation of CH_2Cl_2 crude extract woods of *Diospyros glandulosa*

3.3 Bioactivity determination

3.3.1 Cytotoxic activity

The cytotoxicity test was performed according to the colorimetric method reported by Skehan *et al*.[20] The cell growth was monitored according to the cellular protein content. Cell lines used were small-cell lung cancer (NCI-H187), human epidermoid carcinoma of cavity, ATCC CCL-17 (KB), breast cancer cell line (BC) and kidney fibrobrast of an African green monkey (Vero). Ellipticine and DMSO (10% v/v) were chosen as a positive and negative control, respectively. Cells at logarithmic growth phase were harvested and diluted to 10^5 cells/ml with fresh medium, containing RPMI 1640 together with 10% fetal calf serum and gently mixed. Testing compounds were dissolved in 10% DMSO (conc at 20 mg/mL) to obtain a stock solution at 0.4 mg/ml. Both the cell suspension (190μL) and the stock solution (10μL) were subsequently transferred into microtiter plates before incubation at 37 ºC under 5% CO_2 atmosphere for 72 h. After the incubation period, the cells were then fixed with 50% trichloroacetic acid and re-incubated at 4 ºC for another 30 min before washing with tap water and air-drying. The cells were stained with 0.05 % (w/v) sulforhodamine B (SRB dissolved in 1% acetic acid) for 30 min. After the staining period, the remained solution of SRB was washed with 1% acetic acid. The plates were air-dried again to allow the color to develop.The dye was consequently solubilized in 10 mM Tris buffer pH 10 and left at room temperature for 5 min on a shaker before measuring its optical density (OD) at the wavelength of 510 nm, using microtiter plate reader. The absorbance at 510 nm was determined to indicate activity of living cells. The inhibitory concentration (IC_{50}) used in the study represents the concentration of the test sample which indicates 50% reduction in cell growth.

Calculate the percentage of cell-growth inhibition using the formulae below. For IC_{50} determination, plot a dose-response curve between the compound concentration and percent growth inhibition. IC_{50} values can be derived using curve-fitting methods with statistical analysis software or IC_{50} calculation software.

$$\% \text{ of control cell growth} = \frac{\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{day0}}}{\text{mean OD}_{\text{neg control}} - \text{mean OD}_{\text{day0}}} \times 100$$

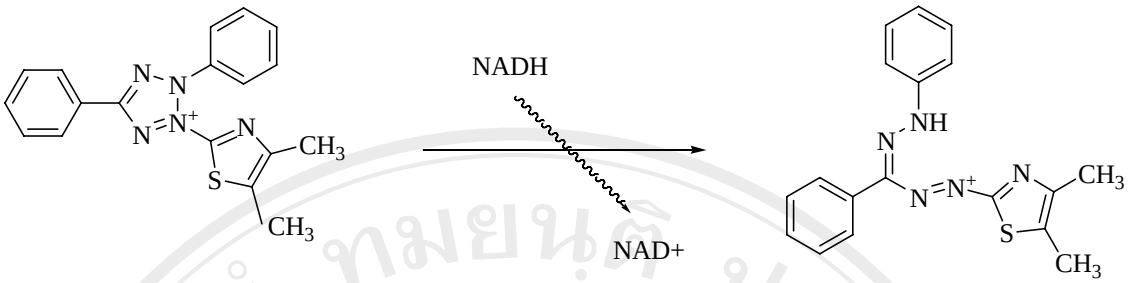
% growth inhibition = 100 – % of control cell growth

3.3.2 Antimalarial activity

The parasite *Plasmodium falciparum* (K1, multidrug-resistant strain) was cultured continuously according to the method of Trager and Jensen[21]. Quantitative assessment of *in vitro* antimalarial activity was determined by means of the microculture radioisotope technique, based upon the method described by Desjardins *et al.*[22]. Two hundred microliters of 1.5% erythrocytes with 1% parasitemia (at the early ring stage) was pre-exposed to 25 µL of the test sample (0.4 mg/ml in DMSO) for 24 h at 37 ºC under 5% CO_2 atmosphere. Subsequently, 25 µL of [^{3}H] hypoxanthine solution (10 µCi in culture medium) was added to each well, and the plates were incubated for an additional 24 h. Levels of incorporated radioactively-labeled hypoxanthine, indicating parasite growth, were determined using the Top Count microplate scintillation counter. Inhibitory concentration (IC_{50}) in this study represents the concentration of the test sample which indicates 50% reduction in parasite growth and dihydroartemisinin (DHA) and DMSO were used as a positive and negative control, respectively.

3.3.3 Antifungal activity

The antifungal activity was assessed according to the method described by Plumb *et al*.[23]. *Candida albicans* (ATCC 90028) was grown on a potato dextrose agar (PDA) plate at 30°C for 3 days. Three to five single colonies were then suspended in RPMI 1640 medium, a common medium for immune cell culture, and cultured in a shaking flask until cell density reached 2×10^6 CFU/mL. One hundred μL of the culture suspension was added to each well of a 96-well plate, containing 100μL of the test sample (0.4 mg/mL in DMSO) and then incubated at 37°C for 4 h. Fifty μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT, 5 mg/mL in culture media RPMI 1640) was added to each well and re-incubated at 37°C for an additional 4 h. The colorimetric MTT assay requires cellular metabolic activity to convert the colorless tetrazolium to the purple-colored formazan dye. Therefore, it detects only viable cells, whereas the sulforhodamine B(SRB) method does not distinguish between viable and dead cell (Figure 2). After the incubation period, the microplates were centrifuged at 200X *g* for 5 min. The MTT solution was removed from the wells whereas the formazan was developed. After 200 μL of DMSO and 25μL of Sorensen′s glycine buffer were added to the wells, they were determined for their absorbance at 570 nm, Using the multilabel counter *Victor* 3V. Amphotericin B and DMSO were used as a positive and negative control, respectively.



MTT tetrazolium (colorless) **MTT formazan (purple)**

Figure 2 Color changing of MTT tetrazolium reagent

3.3.4 Antitubercular activity

The antitubercular activity was assessed against *Mycobacterium tuberculosis* $H_{37}Ra$, using the Microplate Alamar Blue Assay (MABA)[24]. *M. tuberculosis* $H_{37}Ra$ was grown in 100 mL of Middlebrook 7H9 media containing 0.2 % v/v glycerol, 1.0 g/L casitone (7H9GC) and 0.005% Tween 80. The cell culture was incubated at 37°C in a 500 ml plastic flask and shaken continuously on a rotary shaker at 200 rpm until they reached an optical density of 0.4-0.5 at 550 nm. The bacteria were washed and resuspended in 20 mL phosphate-buffered saline and passed through an 8 μm-pore-size filter to eliminate clumps present in the culture media. The filtrates were aliquoted into Eppendorf tube and stored at -80°C for future use.

An antimicrobial susceptibility test was performed in a 96-well microplate. Outer perimeter wells were filled with sterile H_2O to minimize dehydration of the experiment wells. Test samples were initially dissolved (0.4 mg/mL in DMSO or H_2O). Frozen cell inocula were initially thawed and diluted to 1: 1000 with 7H9GC media. Consequently, 0.1 ml of the cell suspension was added into a 96-well

microplate to get a final bacteria titers of about 5×10^4 CFU/mL. One hundred microlitres of the test samples (0.4 mg/mL in DMSO or H_2O) were applied to the wells. The twofold diluted test samples (diluted in 7H9GC media) was done by adding 0.1 mL of 7H9GC media. Additional control wells were done by including *M. tuberculosis* (B), 7H9GC media (M) and test samples in the plates. Plates were incubated at 37 °C for 5 days. At day 6 of incubation, 20μL of Alamar Blue solution and 12.5 μL of 20% Tween 80 were added to B and M wells, and plates were re-incubated at 37°C. After 24 h, the B wells were examined for color changing from blue to pink. A blue color in the well was interpreted as no growth, and a pink color was scored as growth. If the color in the B well turned pink within 24 h, Alamar Blue solution was immediately added to the remaining wells. However, if color in B wells remained blue, the Alamar Blue solution would be added to another control well and the result would be read on the following day. The microplates were incubated for 24 h at 37°C. The B wells were further daily observed until the color of the B well changed. When the color of the B well changed, Alamar Blue solution was subsequently added to all remaining wells. Plates were then incubated at 37°C for another 24 h. The results were recorded using a fluorescence multi-well reader at the excitation and emission wavelengths of 530 and 590 nm, respectively. The control drugs used in this experiment were isoniazid and kanamycin sulfate as a positive control and DMSO as a negative control.