CHAPTER II
EXPERIMENTAL

2.1 Plant materials

The leaves of *Piper betle* were collected in Samutsongkhram province in July 1996. The specimen was compared with the Bangkok herbarium specimen number BK 20272 in the Botany-Weed Science Division Department of Agriculture, Ministry of Agriculture and Co-operatives.

2.2 Instruments and equipment

2.2.1 Melting Point (m.p.)

The melting points were obtained on a Fisher-John apparatus and are uncorrected.

2.2.2 Fourier Transformed- Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Perkin- Elmer Model 1760X Fourier Transformed-Infrared Spectrophotometer. Solid samples were examined by incorporating into a pellet of potassium bromide (KBr). For liquid samples a sodium chloride cell was used.

2.2.3 Gas Chromatography- Mass spectrometry (GC-MS)

GC-MS was performed on a Fisson Gas- Liquid Chromatography Model GC 8000- Fisson Mass Spectrometer Model Trio 2000.

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2.2.4 Gas Chromatography (GC)

Gas chromatography GC-7AG instrument was equipped with flame ionization detector and N_2 was used as a carrier gas. The column used for chromatography was OV-1.

2.2.5 ^{1}H- and ^{13}C-Nuclear Magnetic Resonance Spectrometer

The ^{1}H-NMR and ^{13}C-NMR spectra were obtained by using a Bruker Model ACE 200 Spectrometer and a Joel, Model JNM-A500 which operated at 200.13 MHz for ^{1}H and 50.32 MHz for ^{13}C-nuclei and 500.00 MHz for ^{1}H and 125.00 MHz for ^{13}C nuclei, respectively. The chemical shifts in δ (ppm) were assigned with reference to the signal from the residual proton in deuterated solvent. The signal due to residual proton in deuterated chloroform was assigned to be 7.24 ppm with the reference to TMS.

2.3 Chemical reagents

2.3.1 All solvents, such as hexane, chloroform, ethanol, ethyl acetate and methanol, were purified prior to use by distillation, except solvents that were reagent grade.

2.3.2 Merck silica gel 60 Art. 7734.1000 (70-230 mesh ASTM) was used as the adsorbent for column chromatography.

2.3.3 Merck silica gel Art.7749. 60 PF_{254} was used as the adsorbent for radial chromatography, using a Harrison Research Chromatotron model 7924T equipped with a solvent pump. The thickness of the adsorbent layer was 1 mm.

2.3.4 Merck TLC aluminum sheets, silica gel 60 F_{254} (E. Merck, 4271 Darmstadt, Germany) were used for identifying the fractions. Spots on the plate were observed under UV light and visualized by spraying with a 10 % H_2SO_4 in EtOH solution followed by heating.

2.4 Physical separation techniques

2.4.1 Column Chromatography (CC)

Column chromatography was performed as described in reference 161.

2.4.2 Thin- Layer Chromatography (TLC)

Thin-layer chromatography was performed as described in reference 162.

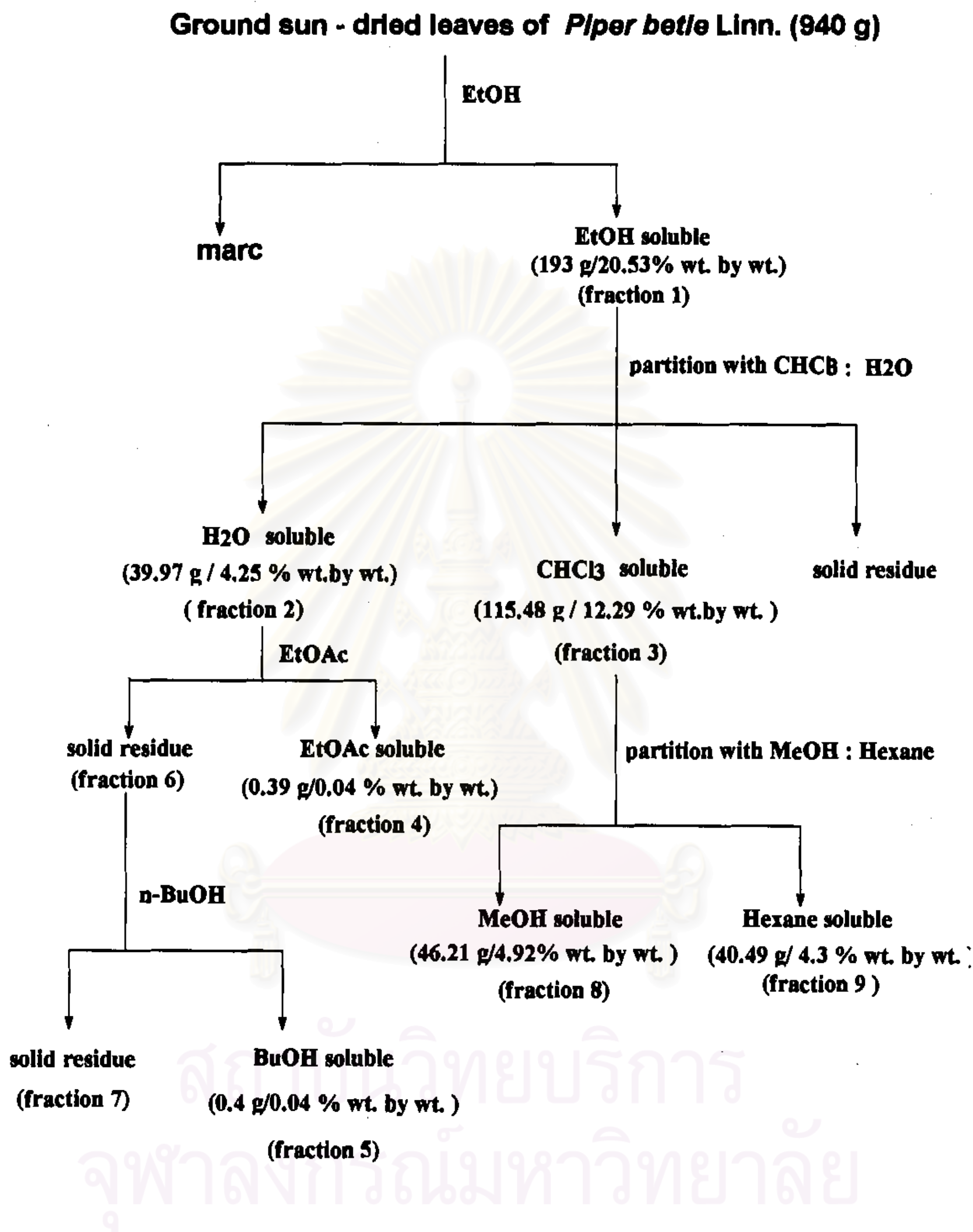
2.5 Extraction for preliminary screening test

The leaves of *Piper betle* Linn. were sun- dried for 7 days. Then they were minced to coarse pieces. The plant (940 g) was soaked in ethanol (4L) for 3-4 days at room temperature for 4 times (until the solvent became clear). It was then filtered and the solvents were evaporated. After evaporation of the solvent under reduced pressure, the crude extract (193 g) was obtained as a dark-green oil.
(20.53% wt. by wt. of dried leaves).

The ethanol crude extract was further extracted by partitioning between chloroform and water, using a liquid-liquid extractor until the chloroform solution was clear to give a chloroform soluble fraction. The chloroform and water fractions were evaporated to afford the chloroform crude extract (115.48 g) (12.29% wt. by wt. of dried leaves) as a dark- green oil, water crude extract (39.97 g) (4.25% wt. by wt. of dried leaves) as a dark- brown oil and the solid part as a black tar.

The chloroform extract was partitioned between methanol and hexane in a ratio of 1:1 until the solution was colorless to give the methanol crude extract (46.21 g / 4.92 % wt. by wt. of dried leaves) as a dark- green oil and the hexane crude extract (40.49 g / 4.30% wt. by wt. of dried leaves).

The water crude extract was reextracted with ethyl acetate until the solution was colorless. The ethyl acetate fraction was evaporated to afford the ethyl acetate crude extract (0.39 g / 0.04 % wt. by wt. of dried leaves) as a light brown oil. The residue left was reextracted with *n*-butanol until the solution was colorless. The *n*-butanol fraction was evaporated to afford the *n*-butanol crude extract (0.4 g / 0.04 % wt. by wt. of dried leaves) as a brown oil. The procedure of the extraction is shown in Scheme 1.



Scheme 1 Procedure prepared of crude extract for screening of inhibitory activity against the tumors cell *in vitro* and brine shrimp cytotoxic lethality test

2.6 Bioassay experiments

As mentioned earlier, one of the goals of this research was to search for an active principle from *Piper betle* Linn. which could possibly use for medicinal purposes, particularly as an anticancer agent. The following bioassay experiments were therefore performed.

The inhibition effect for tumor cell lines[163]

Preliminary screening test collaborators of the ethanol crude extract of the leaves of *P. betle* has been carried out by \at Beijing Medical University, Beijing, China. They reported the use of the MTT assay to study the inhibitory effect of the extract on tumor cell line. The 6 cell lines were Human *Leukemia Carcinoma* (HL-60), Human *Nasopharyngeal Carcinoma* (KB), Human *Gastric Carcinoma* (BGC-823), Human *Colon Carcinoma* (HCT-8), Human *Hepatocellular Carcinoma* (Bel-7402), and Proliferation of mouse (B) Lymphocyte.

Cell lines were cultured under conventional condition : 37 °C, 5% CO_2+95% Air, 100% relative humidity, in RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum, Penicillin 100 IU.ml^{-1} and Streptomycin 100 μg.ml^{-1}.

The tetrazolium dye (3,4,5- trimethylthiazol-2,5-diphenyltetrazolium bromide) assay is based on the reduction of MTT formazan by living cells. The reduced formazan can be measured with a microplate spectrophotometer.

MTT assay :

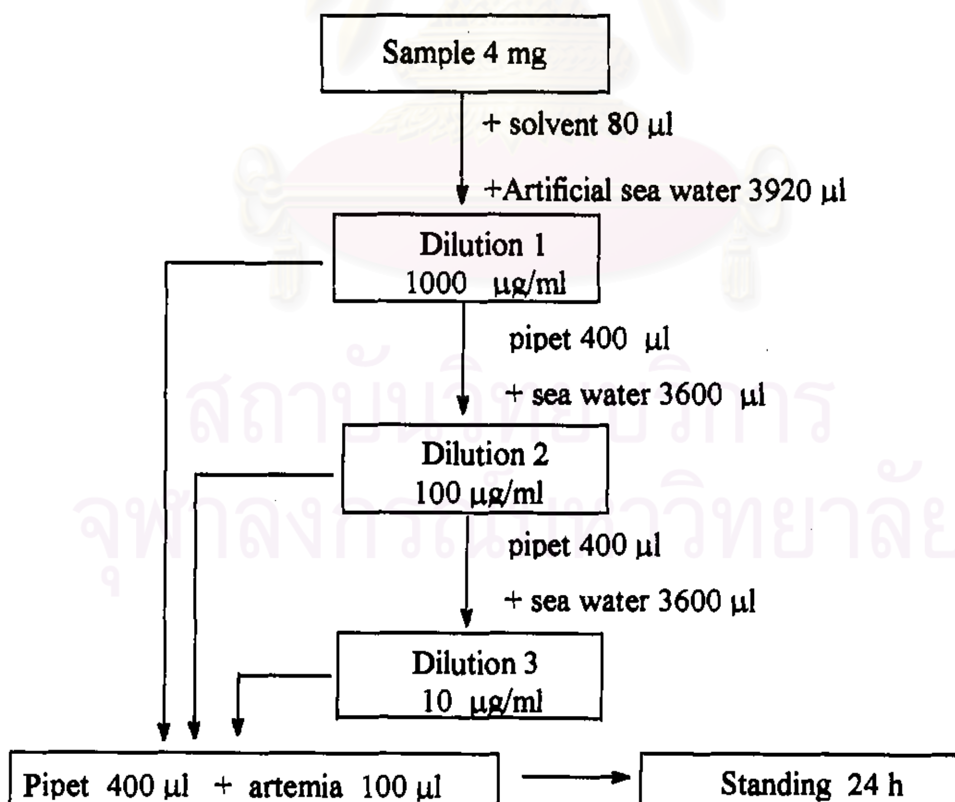
Cell lines were seeded in a 96 well microlitre plate in wells with 5 x 10^4 cells. A stock solution of the extract was added to each well, and 8 replicate wells without extract served as controls. The plates were incubated for 72 hr. After the incubation, 20 μl of PBS solution with MTT 5 mg.ml^{-1} was added to each well and the plates were reincubated for a further 4 hr. The plates were then inverted on blotting paper to remove the medium. The fomazan crystals formed were dissolved in 200 μl of acid - isopropanol. The plates were read on a Model 450 Microplate reader at 570 nm.

Brine Shrimp (*Artemia salina* Linnaeus) cytotoxic Lethality Test [164]

The assay begun 48 h after sowing of the cysts (with larvae that are 24 h old). Multiwelled culture plates can be used for the bioassay, although any clear glass container with flat bottoms (for example, small beakers or glass vials) will do. Five nauplii are collected, using a Pasteur pipette, from the hatching dish and are transferred to a well, using the sea water 100 μL. The concentrations of sample were 10, 100 and 1000 μg/ml. The tested solution are added, and the time is noted, thereby requiring 30 nauplii for each concentration of test sample. A parallel series of tests with the standard solution and blank control are always conducted. The procedure of brine shrimp cytotoxic lethality test is shown in Scheme 2.

Brine shrimp cytotoxic lethality test

By using microwell cytotoxicity assay using *Artemia salina*



Scheme 2 The procedure of brine shrimp cytotoxic lethality test