CHAPTER 2

EXPERIMENTAL

2.1 Chemicals and Instruments

IR spectra were recorded on a Jasco IR-810 Spectrophotometer (KBr) of Japan Spectroscopic Co., Ltd. and Perkin Elmer 1600 series FT-IR of Perkin Elmer Co., Ltd. ^{1}H and ^{13}C-Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on a FTNMR, Varian UNITY INOVA 500 MHz using either operating solvent or tetramethylsilane (TMS) as an internal standard. Spectra were recorded as chemical shift parameter (δ) value in ppm unit. A 90°-t_1-45° pulse sequence (COSY 45) was used for ^{1}H-^{1}H COSY experiments. Standard program for the library $^{n}J_{CH}$ = 8 Hz was used for the HMBC (heteronuclear multiple bond coherence) experiment. EI-MS data were recorded on a Hewlett-Packard HP 5890 Series II Plus GC-HP 5972 Mass Selective Detector (EI mode with mass range of 35-700 amu). Analysis of volatile oil was carried out by Gas chromatography/Mass spectrometry with a Hewlett-Packard HP 5890 Series II Plus GC-HP 5972 Mass Selective Detector. The operating conditions were as follows: inlet temperature 250 °C, initial temperature 70 °C, detector temperature 280 °C and final temperature 280 °C (hold for 5 min). It was used with column HP-5 length 30 m, film thickness 0.25 μm and ID 0.25 mm. Carrier gas was ultra high purity helium (UHP He). FAB-MS data were recorded by MAT 95 XL Mass Spectrometer which run high and low resolution techniques with solid probe (FAB probe). Ultraviolet spectra (UV) were measured (scanning mode) in the wave length 200-400 nm with Hewlett Packard 8452A Diode Array Spectrometer. The absorbance for free radical scavenging activity was measured at 520 nm with UV spectrometer of Milton Roy Company (Spectronic® Genesys). The absorbance (OD) of each well in cytotoxic activity assay was read on a Power Wave X plate reader (Bio-TEK Instruments Inc.) at 492 nm. Optical rotations were measured by POLAX-L Polarimeter. Silica gel 60 (Merck, 0.040-0.063 mm) was used for vacuum liquid chromatography (VLC) and column chromatography (CC). Sephadex® LH-20 of Amersham Biosciences AB was used for size-exclusion

chromatography. Preparative TLC was performed on silica gel 60 GF_{254} (Merck), 0.5 mm thick, activated at 105 °C for 60 min before use. The zones were detected using UV at 254 nm, scraped off, eluted with chloroform:methanol (3:1) and evaporated to dryness under reduced pressure. Analytical TLC was performed on precoated plates of silica gel $60F_{254}$ (Merck, 0.20 mm thick).

2.2 Plant Materials

Fresh rhizomes of *Alpinia galanga* (L.) Willd., *Boesenbergia pandurata* (Roxb.) Schltr., *Curcuma longa* L., *Kaempferia galanga* L. and *Zingiber officinale* Rosc. were purchased from a local market in Songkhla in April, 2001. They were identified by Assistant Professor Dr. Niwat Keawpradub. Authentication of plant materials were carried out at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, where the herbarium specimens have been kept.

2.3 Extraction and Isolation

Fresh rhizomes of *A. galanga, B. pandurata, C. longa, K. galanga* and *Z. officinale* were washed with water to remove the remaining sand and to reduce the microbial load. The cleaned rhizomes were cut into small pieces and then were divided into two portions.

2.3.1 Volatile oils

The first portion (1 kg of fresh rhizomes of each plant) was subjected to water distillation for 3 hr. After allowed the system cooling down overnight, the volatile oil of each plant was collected.

2.3.2 Water extracts

Marc and water from water distillation in 2.3.1 was filtered by filter paper and evaporated on a water bath (60 °C) to obtain water extract of each plant.

2.3.3 Methanol extracts

The second portion of fresh rhizomes of each plant was blended with methanol by electrical blender, soaked for 72 hr, filtered and the marc was then extracted with methanol repeatedly 2 times. The filtrates were combined and evaporated to dryness to yield methanol extract of each plant.

Volatile oils, water extracts and methanol extracts from the five fresh rhizomes were subjected to preliminary assays for free radical scavenging activity (section 2.5) and cytotoxic activity (section 2.6).

Results from the preliminary assays for free radical scavenging activity (section 3.1.1) and cytotoxic activity (section 3.1.2) of the methanol extracts, water extracts and volatile oils from the fresh rhizomes of *A. galanga*, *B. pandurata*, *C. longa*, *K. galanga* and *Z. officinale* gave evidences of the presence of active constituents in the volatile oils of the five fresh rhizomes, the methanol extracts of *A. galanga, C. longa*, and *Z. officinale*. Thus, separation of the active extracts were undertaken and the volatile oils were subjected to chemical analysis (GC/MS).

2.3.4 Isolation of chemical constituents from *Alpinia galanga*

An aliquot (50 g) of the methanol extract of fresh *A. galanga* rhizomes (AGM) was suspended in chloroform:methanol (9:1) overnight. Then it was sonicated 10 min and filtered by filter paper. It was repeatedly dissolved 3 times with 800 ml of chloroform:methanol (9:1). The combined filtrates were evaporated to dryness under reduced pressure to obtain 4 g of dark brown oily gum. Further separation was carried out on a silica gel column chromatograghy using gradient of solvents

chloroform:methanol (19:1, 9:1, 4:1) and finally being washed with methanol to afford thirty-nine fractions (50 ml each). Fractions with the similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure.

Fractions 9-12 were combined and obtained as green-yellow oil (0.666 g). Further separation by preparative TLC on silica gel plates using chloroform:methanol (19:1) as a mobile phase afforded three bands. Further separation of the middle band by preparative TLC on silica gel plates with chloroform:methanol (19:1) as a mobile phase gave **AGM1** (a pure component) as yellow oil (0.039 g).

2.3.5 Isolation of chemical constituents from *Curcuma longa*

An aliquot (30 g) of the methanol extract of fresh *C. longa* rhizomes (CLM) was chromatographed over a silica gel column chromatography using gradient of solvents chloroform:methanol (19:1, 9:1, 4:1) and finally being washed with methanol to obtain seventy-five fractions (50 ml each). Fractions with the similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure to obtain fractions 1-20, 21-23, 24-27, 28-53, 54-74 and 75.

Fractions 1-20 were obtained as pale yellow oil (2.137 g). Chromatogram characteristics on normal phase TLC with toluene:chloroform (2:1) indicated the presence of **CLM01** as a pure component. Fractions 21-23 obtained only few milligrams of residue, thus no further work have been carried out.

Fractions 24-27 were obtained as dark orange liquid (1.546 g) with orange crystals (0.267 g, mixture **CLM02+CLM03**). The total dark orange liquid was further separated by silica gel column chromatography using gradient of solvents chloroform:methanol (19:1, 9:1, 4:1) to obtain thirty-nine fractions (50 ml each). Fractions with the similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure to obtain fractions 13a-15a. Further separation of fractions 13a-15a by preparative TLC on silica gel plates with chloroform:methanol (19:1) as a mobile phase afforded four bands. The top band and

the lowest band yielded **CLM02** as orange crystals (0.161 g) and **CLM03** as reddish orange powder (0.167 g), respectively.

Fractions 28-53 was obtained as dark brown liquid with dark orange crystals. Further separation by preparative TLC on silica gel plates with chloroform:methanol (19:1) as a mobile phase afforded three bands. In the top band and the middle band yielded **CLM02** (0.025 g) and **CLM03** (0.025 g), respectively.

The lowest band was obtained as dark orange liquid. It was further separated by preparative TLC on silica gel plates with chloroform:methanol (19:1) as a mobile phase to afford two bands. The lower band was separated by preparative TLC on silica gel plates with ethyl acetate:chloroform:methanol (10:9.5:0.5) as a mobile phase to afford **CLM06** as reddish orange powder (0.002 g).

2.3.6 Isolation of chemical constituents from *Zingiber officinale*

An aliquot (50 g) of the methanol extract of fresh *Z. officinale* rhizomes (ZOM) was dissolved with methanol (10 ml) and mixed with silica gel to obtain dried sandy sample for vacuum liquid chromatography. Mobile phases used in vacuum liquid chromatography were chloroform:methanol (9:1, 4:1) and methanol, respectively. Three fractions were obtained and evaporated to dryness under reduced pressure.

Fraction 1 (chloroform:methanol; 9:1), upon chromatographic separation of fraction 1 using preparative TLC on silica gel plates and hexane:ethyl acetate (3:1) as a mobile phase, five bands were obtained (band1-5). Further separation of band 3 by sephadex column chromatography eluting with methanol, sixteen fractions were obtained. Fractions with the similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure to obtain fractions 8a-10a and fraction 13a.

Fractions 8a-10a were obtained as orange-yellow oil (0.131 g). Further separation by preparative TLC on silica gel plates with hexane:ethyl acetate (5:1) as a mobile phase gave **ZOM0** as yellow oil (0.042 g).

Fraction 13a, upon standing at room temperature, afforded yellow crystals (0.032 g) which was designated to be **ZOM1**. It gave violet colour by spraying with anisaldehyde reagent.

Band 4 of Fraction 1 was obtained as brown liquid. Upon separation by sephadex column chromatography eluting with methanol, fourteen fractions were afforded. Fractions with the similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure to afford fractions 7b-8b as pale yellow oil (0.154 g). Further separation was carried out by preparative TLC on silica gel plates with hexane:ethyl acetate (3:1) as a mobile phase to afford three bands. The lowest band was obtained as yellow oil (0.078 g) which was designated to be **ZOM3**.

Attempts had been made on purification of **Fraction 2** (chloroform:methanol; 4:1) and **Fraction 3** (methanol) of ZOM but none of pure compounds were obtained.

2.4 Physical and Spectral Properties of the Isolated Compounds

AGM1 (*p*-Coumaryl-9-methyl ether): $C_{10}H_{12}O_2$ (0.039 g); yellow oil; UV (MeOH) λ_{max} (log ε) 264 (4.01) nm; IR (KBr disc) ν_{max} 3350, 2920, 1610, 1520 cm^{-1}; HR-FABMS *m/z* 164.0840 (calc. for $C_{10}H_{12}O_2$ 164.0837); GC/MS (Electron Ionization) *m/z* (% relative intensity) 165 (M+H, 11), 164 (68), 163 (19), 137 (18), 131 (100), 121 (42), 115 (23), 103 (43), 91 (40), 77 (81). ^1H (500 MHz; $CDCl_3$) and ^{13}C NMR (125 MHz; $CDCl_3$) see Table 22; page 83

CLM01 (ar-Turmerone): $C_{15}H_{20}O$ (2.137 g); pale yellow oil; UV (MeOH) λ_{max} (log ε) 238 (3.94) nm; IR (KBr disc) ν_{max} 3000, 1700, 1600 cm^{-1}; GC/MS (Electron Ionization) *m/z* (% relative intensity) 217 (M+H, 4), 216 (23), 201 (14), 132 (17), 119

(59), 83 (100), 55 (25); $[\alpha]_D$ +64.3^o (c 0.7, $CHCl_3$); ^1H (500 MHz; $CDCl_3$) and ^{13}C NMR (125 MHz; $CDCl_3$) see Table 23; page 86

CLM02 (Curcumin): $C_{21}H_{20}O_6$ (0.186 g); orange crystals; UV (MeOH) λ_{max} (log ε) 236 (4.93), 424 (4.75) nm; IR (KBr disc) ν_{max} 3500, 1630, 1510, 1430 cm^{-1}; FAB-MS (low resolution) *m/z* (% relative intensity) 369 (M+H, 96), 350 (6), 285 (6), 219 (17), 177 (57), 137 (25), 133 (100); ^1H (500 MHz; $CDCl_3$) and ^{13}C NMR (125 MHz; $CDCl_3$) see Table 24; page 89

CLM03 (Demethoxycurcumin): $C_{20}H_{18}O_5$ (0.192 g); reddish orange powder; UV (MeOH) λ_{max} (log ε) 240 (4.31), 420 (4.77) nm; IR (KBr disc) ν_{max} 3450, 1650, 1600, 1500 cm^{-1}; FAB-MS (low resolution) *m/z* (% relative intensity) 339 (M+H, 25), 316 (5), 277 (13), 224 (21), 185 (100), 147 (10), 132 (21), 93 (85), 75 (18); ^1H (500 MHz; $CDCl_3$) and ^{13}C NMR (125 MHz; $CDCl_3$) see Table 25; page 92

CLM06 (Bisdemethoxycurcumin): $C_{19}H_{16}O_4$ (0.002 g); reddish orange powder; UV (MeOH) λ_{max} (log ε) 246 (4.06), 414 (4.57) nm; IR (KBr disc) ν_{max} 3500-3200 (broad) and 1600 cm^{-1}; FAB-MS (low resolution) *m/z* (% relative intensity) 309 (M+H, 63), 271 (8), 225 (16), 223 (61), 167 (86), 147 (100), 107(55); ^1H (500 MHz; DMSO-d_6) and ^{13}C NMR (125 MHz; DMSO-d_6) see Table 26; page 94

ZOM0 (6-Shogaol): $C_{17}H_{24}O_3$ (0.042 g); yellow oil; UV (MeOH) λ_{max} (log ε) 226 (4.28) nm; IR (KBr disc) ν_{max} 3400, 2960, 1700, 1500 cm^{-1}; FAB-MS (low resolution) *m/z* (% relative intensity) 277 (M+H, 29), 276 (35), 271 (3), 205 (5), 151 (7), 138 (10), 137(100); ^1H (500 MHz; $CDCl_3$) and ^{13}C NMR (125 MHz; $CDCl_3$) see Table 27; page 97

ZOM1 (6-Dehydrogingerdione also known as 1-Dehydrogingerdione): $C_{17}H_{22}O_4$ (0.032 g); yellow crystals; UV (MeOH) λ_{max} (log ε) 370 (4.29) nm; IR (KBr disc) ν_{max} 3350, 2960, 1600, 1500 cm^{-1}; FAB-MS (low resolution) *m/z* (% relative

intensity) 291 (M+H, 80), 289 (6), 276 (4), 219 (14), 191 (18), 177 (83), 137(100); ^{1}H (500 MHz; $CDCl_3$) and ^{13}C NMR (125 MHz; $CDCl_3$) see Table 28; page 100

ZOM3 (6-Gingerol): $C_{17}H_{26}O_4$ (0.078 g); yellow oil; UV (MeOH) λ_{max} (log ε) 282 (3.49) nm; IR (KBr disc) ν_{max} 3450, 2960, 1700, 1550 cm^{-1}; FAB-MS (low resolution) *m/z* (% relative intensity) 295 (M+H, 5), 294 (25), 277 (4), 179 (7), 151 (11), 137 (100); $[\alpha]_D$ +29.3° (c 0.478, $CHCl_3$); ^{1}H (500 MHz; $CDCl_3$) and ^{13}C NMR (125 MHz; $CDCl_3$) see Table 29; page 103

2.5 Assay for Free Radical Scavenging Activity

The antioxidative activity of these rhizomes was evaluated by DPPH radical scavenging assay which was originally described by Blois (1958).

DPPH (1,1-diphenyl-2-picrylhydrazyl) is considered as a stable radical because of the paramagnetism confered by its odd electron (delocalization of the spare electron over the molecule as a whole). The solution (in absolute ethanol) appears as a deep violet colour and shows a strong absorption band at 520 nm. DPPH radical can accept an electron or hydrogen radical to become a stable diamagnetic molecule and has pale violet. If substance for testing antioxidative activity is mixed with DPPH solution and gives rise to pale violet, it suggests that this substance has antioxidative effect by mechanism of free radical scavenging activity. The following assay procedure was modified from those described by Blois (1958) and Yamasaki, *et al*. (1994).

1. Dissolved samples for testing in absolute ethanol (for volatile oils), distilled water (for water extracts) and methanol (for methanol extracts).
2. Diluted each sample for at least 5 concentrations (two-fold dilutions). Each concentration was tested in triplicate.
3. Prepared $6x10^{-5}$ M of DPPH in absolute ethanol.
4. Pipeted 500 µl of sample solution into an eppendorf tube. Each concentration was tested in triplicate.

5. Pipeted 500 µl of DPPH solution to mix with sample solution.

6. Shaked and left at room temperature for 20 min.

7. Measured absorbance at 520 nm by comparing with blank solution of each concentration (sample solution 500 µl + absolute ethanol 500 µl).

8. Prepared standard solution and control in each experiment as follows:

-Control ethanol: 500 µl of absolute ethanol + 500 µl of $6x10^{-5}$ M of DPPH in absolute ethanol; **blank:** 1,000 µl of absolute ethanol.

-Control methanol: 500 µl of methanol + 500 µl of $6x10^{-5}$ M of DPPH in absolute ethanol; **blank:** 500 µl of methanol + 500 µl of absolute ethanol.

-Control water: 500 µl of distilled water + 500 µl of $6x10^{-5}$ M of DPPH in absolute ethanol; **blank:** 500 µl of distilled water + 500 µl of absolute ethanol.

9. Calculation of % inhibition.

$$\text{\% inhibition} = \frac{\text{OD control-OD sample}}{\text{OD control}} \times 100$$

10. Plotted dose-response curve between % inhibition and concentrations.

11. Linear regression analysis was carried out for calculating the effective concentration of sample required to scavenge DPPH radical by 50 % (EC_{50} value).

12. In each experiment BHT (butylated hydroxytoluene, a well known synthetic antioxidant) and caffeic acid were tested as positive standards.

2.6 Assay for Cytotoxic Activity

Volatile oils, methanol extracts, water extracts from the five fresh rhizomes and the isolated compounds were assessed for cytotoxic activity by the Sulphorhodamine B (SRB) assay (Skehan, *et al*., 1990). SRB is a pink aminoxanthene dye. It is an anionic protein stain containing two sulphonic groups that bind electrostatically to basic amino acid residues of cellular protein under mildly acidic conditions. The bound dye can be quantitatively extracted from cells and solubilized for spectrophotometry by weak bases (Skehan, *et al*., 1990). This colorimetric assay therefore can be used to estimate

cell number indirectly (for cell monolayer) by providing a sensitive index of total cellular protein content which is linear to cell density.

2.6.1 Human tumour cell lines

The human colon adenocarcinoma cell line LS174T was obtained from King's College London, University of London and the human breast adenocarcinoma cell line MCF7 was obtained from The National Cancer Institute, Bangkok, Thailand. The cells were cultured in Minimum Essestial Media (MEM) with Earle's salt, supplemented with 10 % heat-inactivated newborn calf serum, 2 mM L-glutamine, 50 IU/ml penicillin G sodium, 50 μg/ml streptomycin sulphate and 0.125 μg/ml amphotericin B. The cell were maintained at 37 °C in a 5 % CO_2 atmosphere with 95 % humidity.

2.6.2 Testing procedure

According to growth profile, the optimal plating density of the cell line MCF7 and LS174T were determined to be 2,000 and 1,000 cells/well, respectively to ensure the exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analysed by SRB assay (Skehan, *et al.,* 1990). Cells growing as monolayer in a 25 cm^3 flask were washed with phosphate buffered saline (PBS) pH 7.4 and trypsinized with 0.1 % trypsin-EDTA to make a single-cell suspension. The viable cells were counted by trypan blue exclusion in a haemocytometer (Freshney, 1994) and diluted with the medium to give a final concentration of $2x10^4$ cells/ml for cell line MCF7 (2,000 cells/well) and $1x10^4$ cells/ml for cell line LS174T (1,000 cells/well), respectively. 100 μl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated at 37 °C to allow for cell attachment. After 24 hr the cells were treated with the extracts or pure compounds. Each sample was initially dissolved in DMSO for the methanol extracts, or sterile distilled water for the water extracts and vinblastine sulphate, or absolute ethanol for the volatile oil and isolated compounds. They were further diluted in the culture medium to produce the required concentrations. Vinblastine sulphate (anticancer drug, Sigma, MW 909.1) and berberine were used as

positive controls. 100 µl per well of each concentration was added to the plates in 6 replicates. The final mixture used for treating the cells contained not more than 0.5 % of the solvent, the same as in solvent control wells. The 96-well microtiter plates were incubated for the exposure time of 72 hr. At the end of exposure time, the medium was removed. Then 200 µl of fresh medium was added to each well. The plates were further incubated for 72 hr. On the seventh day of culture period, cells were fixed by 100 µl of ice-cold 40 % trichloroacetic acid (TCA) per well, left in the refrigerator at 4 °C for 1 hr and washed 5 times with tap water. Non viable cells were washed and viable cells were fixed as monolayer in each well. 50 µl of SRB solution (0.4 % w/v in 1 % acetic acid) was added to each well and left in contact with the cells for 30 min. The plates were washed 5 times with 1 % acetic acid and dried overnight. On the day of reading plates, bound dye was dissloved with 100 µl of 10 mM Tris base (Tris [hydroxymethyl] aminomethane), shaked on a gyratory shaker 20 min. The absorbance (OD) of each well was read on a plate reader at 492 nm. The intensity of the colour formed in the wells is an indication of the viable cell number. Cell survival was measured as the percentage absorbance compared to the control (non-treated cells). The IC_{50} values (concentrations required to inhibit cell growth by 50 %) were calculated from the dose-response curves obtained by plotting the percentage of survival versus the concentrations. Based on probit analysis (Finney, 1971), computer program was used to determine the IC_{50} values.