CHAPTER III

EXPERIMENTAL

3.1 General experimental procedures.

All solvents were distilled prior used. UV-VIS spectra were recorded using a Hewlett Packard 8452A diode array spectrophotometer in $CHCl_3$. IR spectra were obtained using a Nicolet Impact 410 Fourier Transform Infrared Spectrophotometer. Spectra of solid samples were recorded as KBr pellets and liquid samples were recorded as thin films (KBr cells). Low resolution mass spectra were obtained using a Fisons Instruments Mass Spectrometer model Trio 2000 at 70 ev. ^{1}H and ^{13}C NMR spectra were recorded at 200.13 and 50.32 MHz, respectively using a Bruker Model AC-F200 Spectrometer in $CDCl_3$ and DMSO. Chemical shifts are given in parts per million using residual protonated solvent as reference. Optical rotation values were measured on a Perkin Elmer instruments model 341 polarimeter, using $CHCl_3$ and MeOH as a solvent. Silica gel (Merck Kieselgel 60 and silica TLC plates (Si gel 60 F_{254}) were purchased from Merck Company. Spectrophotrometric measurement for antioxidant test were carried out on Spectronic 20.

3.2 Plant materials for cytotoxicity preliminary screening test.

Table 4 Selected plants used as anticancer agents in Thai traditional medicine.

Species (Family)	Common name	Plants part
Croton oblongifolius Roxb.	เปล้าใหญ่	B
Hydnophytum formicar Jack.	หัวร้อยรู	S
Cuscuta chinensis	ฝอยทอง	A
Curcuma caesia *Curcuma Zedoaria* Rose.	ขมิ้นอ้อย	S

Table 4 continued

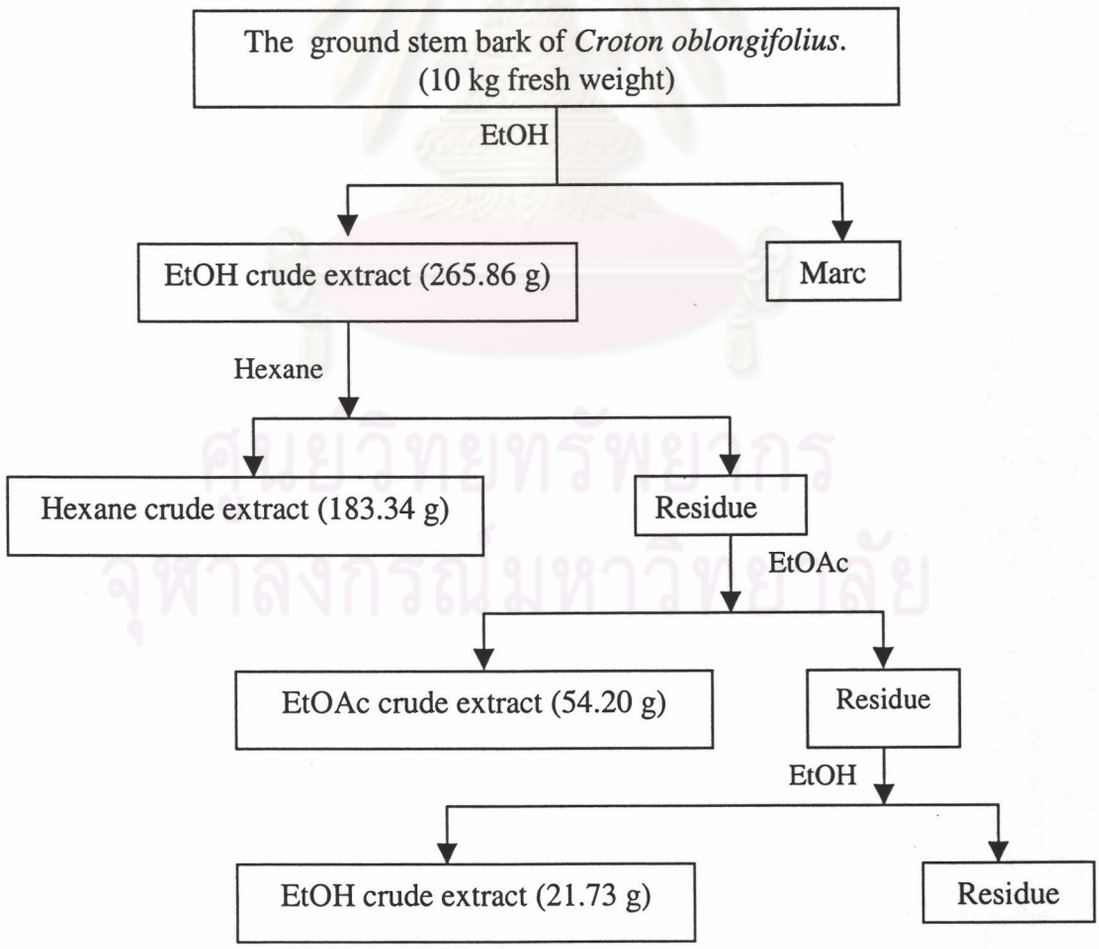
Species (Family)	Common name	Plants part
Nelambo nucifera	บัวหลวง	P
Livisticum officnale	โกฐเชียง	R
Acanthus ebracteatus Vahl.	เหงือกปลาหมอ	L
Mucuna collettii	กวาวดำ	S
Kaempferia parviflora	กระชายดำ	S
Curcuma spp.	เอ็นเหลือง	S
Zingiber cassumunar	ไพล	S
Zingiber ottensii Val.	ไพลดำ	S
Curcuma domestica Val. ; *Curcuma longa*	ขมิ้นชัน	S
Orthosiphon aristatus (Blume) Mig.	หญ้าหนวดแมว	A
Gelonium multiflorum	ขันทองพยาบาท	W,B
Salacia chinensis	กำแพงเจ็ดชั้น	W, B
Rhinacanthus nasutus Kurz. (*Rhinacanthus communis* Nees.)	ทองพันชั่ง	A
Garcinia cowa Roxb.	ชมวง	L
Zingiber rubens Roxb.	ชิงแห้ง	R
Euphorbia lacei	สลัดได	W
Rauvolfia serpentina (L.) Benth. ex Kurz	ระย่อม	R
Curcuma spp.	ม้าเหลือง	S
Artemisia pallens	โกฐจุฬาลำพา	S
Phyllanthus emblica Linn.	มะขามป้อม	F
Murdannia loriformis (Hassk.) Rolla Rao et Kammathy	หญ้าปักกิ่ง	A

References for use:(1)A: all parts, B: bark, L: leaf, F: fruit, P: pollen and pollen stick, S: storage root, R: root, W: wood. (เต็ม สมิตินันท์, 2523; เสงี่ยม พงษ์บุญรอด, 2520)

The plant material of *Croton oblongifolius* used in this study was collected from Amphur Vicheinburi Petchaboon Province, Thailand in March 2001. The plant specimen was compared against voucher specimen no. BKF 084729 deposited in the herbarium of the Royal Forest Department of Thailand.

3.3 Extraction.

The ground stem bark of *Croton oblongifolius*. (10 kg fresh weight) was repeatedly extracted with ethanol. The ethanol extract was filtered and evaporated under reduced pressure to obtain a dark-red gummy residue that was repeatedly extracted with hexane, ethyl acetate and ethanol, respectively. The extraction procedure is shown in scheme 1. The result of extraction were summarized in Table 5.



Scheme 1 Extraction procedure of the stem bark of *Croton oblongifolius* Roxb.

3.4 Isolation of crude extract of *Croton oblongifolius* Roxb.

3.4.1 Separation of hexane crude extract.

The hexane crude extract was obtained as a yellowish green oil (183.34 g, 1.83 %wt by fresh wt) after evaporation. The crude hexane extract (50.0 g) was fractionated by Silica gel column chromatography using Merck's silica gel Art.7734.1000 (70-230 mesh ASTM) as adsorbent. The column was eluted with hexane-ethyl acetate gradient in a stepwise fashion. The each eluted fractions of about 50 ml were collected and then each fractions were checked using thin layer chromatography (TLC) to combine the fractions which had the same components and the solvent was removed by rotary evaporation to about 20 ml. The separation result of hexane crude extract gave compounds 1-5 as shown in Table 9.

3.4.2 Separation of ethyl acetate crude extract.

The ethyl acetate crude extract was obtained as a dark-red semisolid residue (54.20 g, 0.54 %wt by fresh wt) after evaporation. The ethyl acetate crude extract (15.0 g) was separated on Silica gel 70-230 mesh ASTM using column chromatographic technique. The column was eluted with hexane, hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, respectively. The eluted fractions of about 50 ml were collected. Similar fractions were combined and evaporated to about 20 ml. The result of the separation of ethyl acetate crude extract gave compound 6 as shown in Table 9.

3.4.3 Separation of methanol crude extract.

The ethanol crude extract (21.73 g, 0.22 % wt by fresh wt) was gummy residues and insoluble in all solvents except ethanol and methanol. Therefore, the ethanol crude extract was not separated by column chromatographic technique.

Table 5 The weight of the crude extract

Extract	Weight (g)	% wt by fresh wt
Hexane	183.34	1.83
Ethyl acetate	54.20	0.54
Ethanol	21.73	0.22

3.5 Biological evaluation

3.5.1 Chemical for biological activity test

1. For cytotoxicity assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (Fluka)

2. For antioxidant assay

(1) DPPH (2,2 - Diphenyl - 1 - picrylhydrazyl), $C_{18}H_{12}N_5O_6$, M.W. 394.33 (Fluka)

(2) Vitamin E, $C_{29}H_{50}O_2$, M.W. 430.72 (Fluka)

(3) Absolute EtOH (Merck)

3.5.2 Biological assay procedure

Cytotoxicity test

Bioassay of cytotoxic activity against human tumor cell *in vitro* was performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method. In principle, the viable cell number / well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

This test was carried out at the Institute of Biotechnology and Genetic Engineering using the following protocol. The human tumor cell line was harvested from exponential-phase maintenance cultures (T-75 cm^2 flask), counted by trypan

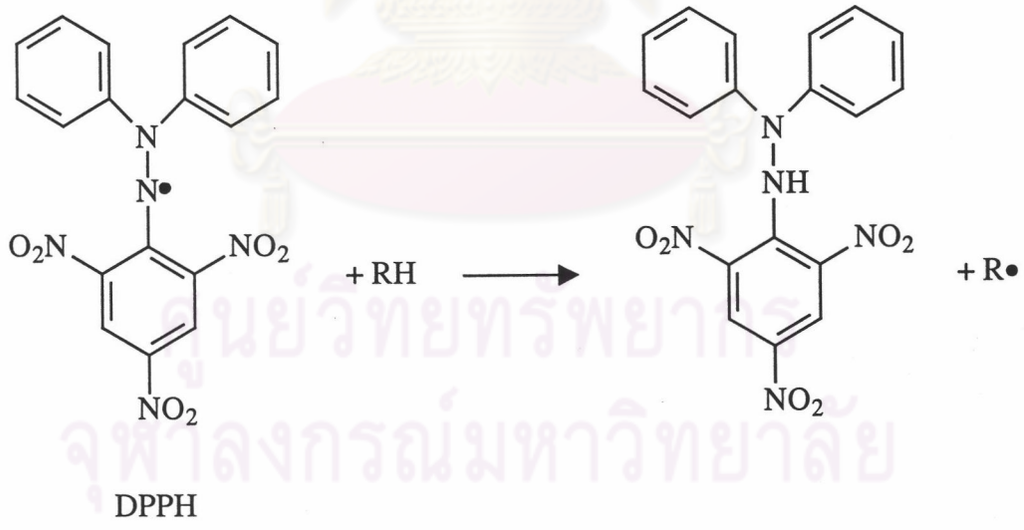
blue exclusion, and dispensed within replicate 96-well culture plates in 100-µl using a repeating pipette. Following a 24-hour incubation at 37°C, 5% CO_2, 100% relative humidity, 100 µl of culture medium, culture medium containing sample was dispensed within appropriate wells (control group, N=6; each sample treatment group, N=3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N=2) and medium / tetrazolium reagent blank (N=6) "backgroud" determinations. Culture plates were incubated for 4 days prior to the additions of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ml PBS was sterile and filtered with 0.45-µm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. MTT working solution (50µl) was added to each culture well resulting in 50 µg MTT / 250 µl total medium volume) and cultures were incubated at 37°C for 4 to 24 hours depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically. Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 µl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 µl of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00).

Cell line growth and growth inhibition were expressed in terms of mean (±1 SD) absorbance units and / or percentage of control absorbance (±1 SD%) following subtraction of mean "background" absorbance.

Samples were also tested for cytotoxic activity towards 6 cell lines, which contain fibroblast (HS27), gastric carcinoma (KATO-3), breast carcinoma (BT474), lung carcinoma (CHAGO), colon carcinoma (SW 620) and hepato carcinoma (HEP-G2) following the experiment method of bioassay of cytotoxic activity.

Antioxidant assay

The antioxidant assay of samples by DPPH method was used to investigate the potential of samples to reduce DPPH radicals (radical 2,2-diphenylpicryhydrazyl). This method is based on the reduction of DPPH, a stable free radical. The structure of DPPH and its reduction product by an antioxidant is shown in scheme 2. Because of its odd electron, DPPH gives a strong absorption maximum at 517 nm by visible spectroscopy (purple color). As the odd electron of the radical the radical becomes paried off in the presence of a hydrogen donor, that is, a free radical-scavening antioxidant, the absorption strength is decreased, and the resulting decolorization is stoichiometric with respect to the number of electrons captured (Bolis, M.S., 1958). This reaction has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate the antioxidantive activity of foods and plant extracts (Yen, G.C. and Duh, P.D., 1994; Cotelle, N. et al., 1996; Gadow, A.V., Joubert, E., and Hansmann, C.F., 1997; Soares, J.R., et al., 1997).



DPPH

Scheme 2 Scavenging the DPPH radical by an antioxidant.

The method of Yamaguchi et al. (1998) was adapted for assessment of the radical-scavenging activity. The sample which has high potential antioxidant activity can effectively reduce DPPH radicals. The absorbance of the sample was measured by

spectrophotometer. All tests were performed in duplicate and the results were averaged. For this experiment, vitamin E was used as a positive control.

The testing protocol

1) Prepared DPPH solution at 2 x 10^{-4} M in absolute ethanol and kept in dark until use.
2) The absorbance of 4 ml DPPH solution was measured at 517 nm (absolute ethanol was used as a blank adjustment for the spectrophotometer)
3) Samples and vitamin E (positive control) were pipetted into each tube covered with aluminum foil. The appropriate concentration of sample in absolute ethanol were added in vary concentration such as12.5, 25, 50, 100, 150, 200 and 400 μg/200 μl. Ratio of dilution is shown in Table 6.
4) Added 3.8 ml of DPPH solution (freshly prepare) into each tube. Thus its has about 4 ml final volume.
5) The mixture was shaken vigorously and left in the dark for 30 min.
6) After the solution left at room temperature for 30 min, the absorbance at 517 nm was measured. Compared the absorbance value of each sample with that of DPPH solution. The samples that had antioxidant activity would have absorbance value lower than the original absorbance value of DPPH solution.
7) The samples which had antioxidant activity were subjected to examine the IC_{50} value.

Table 6 Ratio of dilution of samples from 1 mg / ml with absolute ethanol.

	200 μg	150 μg	100 μg	50 μg	25 μg	12.5 μg
Vol. of sample (1 mg/ml)	200 μl	150 μl	100 μl	50 μl	25 μl	12.5 μl
Vol. of absolute ethanol	0 μl	50 μl	100 μl	150 μl	175 μl	187.5 μl

8) Results were plotted as correlation between the aborbance value and concentration of samples.
9) All tests and analyses were run in duplicate and averaged.

IC_{50} = Concentraion of samples at 1/2 of absorbance value of DPPH solution.

3.6 Single crystal x-ray diffraction experiment

A colorless monoclinic crystals of compound 1 which recrystallized from ethyl acetate and a colorless triclinic crystals of compound 2 which crystallized from 20% ethyl acetate in hexane and recrystallized in ethyl acetate respectively was determined for their structures by single crystal x-ray diffraction experiment. The x-ray diffractometer were obtained on a BRUKER SMART CCD diffractometer at the Department of Physics, Faculty of Science and Technology, Thammasart University.