CHAPTER III

EXPERIMENTAL

3.1 PLANT MATERIALS

The plant materials of *K. parviflora* Wall used in this study were obtained from Kanchanaburi Province, Thailand in November 2000. The whole plant and flowers were compared against the specimen no. BK 59342 at the Royal Forest Department, Bangkok, Thailand.

3.2 CHEMICAL REAGENTS

3.2.1 Solvents

All commercial grade solvents used in this research such as hexane, chloroform, ethyl acetate and methanol, were purified by distillation prior to use. The reagent grade solvents were used for recrystallization.

3.2.2 Other chemicals

1 Merck's silica gel 60 G Art. 7734 (70-230 mesh ASTM) and 9385 (230-400 mesh ASTM) were used as adsorbents for normal column chromatography and flash column chromatography.

2 Merck's TLC aluminum sheets, siliga gel $^{60}F_{254}$ precoated 25 sheets, 20x20 cm^2, layer thickness 0.2 mm were used for TLC analysis.

3 TLC spots were visualized with a UV lamp (254 and 365 nm) and with I_2.

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

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

6 Absolute EtOH (Merck)

7 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (Fluka).

3.3 INSTRUMENTS AND EQUIPMENTS

3.3.1 Melting point apparatus

The melting points were recorded on a Fisher - Johns melting point apparatus.

3.3.2 Rotary Evaporator

The Buchi rotary evaporator was used for the rapid removal of large amount of volatile solvents.

3.3.3 Optical Rotation

The optical rotation values were measured by a Perkin - Elmer 341 polarimeter.

3.3.4 Ultraviolet - visible Spectrophotometer (UV-VIS)

The UV - VIS spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer in chloroform and methanol.

3.3.5 Fourier Transform - Infrared Spectrophotometer (FT - IR)

The FT-IR spectra were recorded on a Nicolet Impact 410 spectrophotometer. Spectra of solid samples were recorded as KBr pellets.

3.3.6 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectra were recorded at 200.13 and 50.32 MHz, respectively, on a Bruker Model AC - F200 Spectrometer in deuterated chloroform ($CDCl_3$), and dimethylsulfoxide (DMSO).

3.3.7 Mass Spectrometer (MS)

The mass spectra were acquired using a Fisons Instruments Mass Spectrometer Model Trio 2000 in EI mode at 70 eV and the LC - MS spectra were obtained in Atmospheric pressure chemical ionization (ApcI) mode. The LC - MS solvent was MeOH: H_2O (1:1).

3.3.8 X - ray Diffractometer

The X - ray diffractometer were obtained on a BRUKER SMART CCD diffractometer at Department of Physics, Faculty of Science and Technology, Thammasart University.

3.3.9 Spectronic 21

Spectonic 21 (Milton roy company) is used to measure absorbance at 517 nm for antioxidant assay by DPPH method.

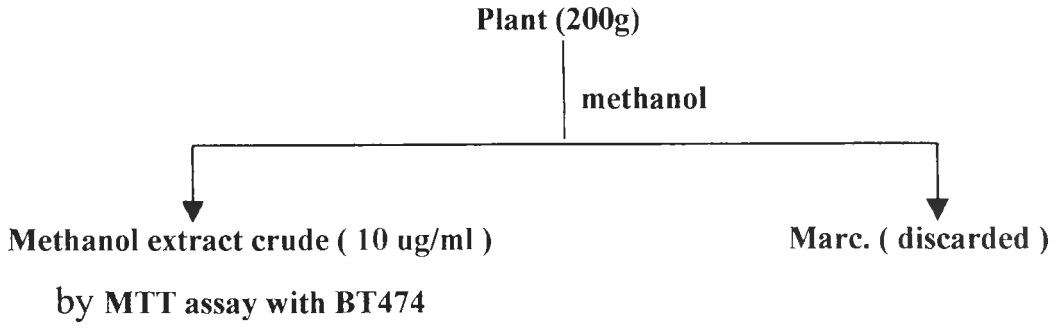
3.4 CYTOTOXIC SCREENING OF MEDICINAL PLANTS

Screening for cytotoxic activity against breast cancer cell lines of seventeen species of herbs were carried out. They were *Hydnophytum formicarum* (หัวร้อยรู), *Cuscuta chinensis* (ฝอยทอง), *Nelambo nucifera* (เกสรบัวหลวง), *Acanthus ebracteatus* (เหงือกปลาหมอ), *Kaempferia parviflora* (กระชายดำ), *Curcuma longa* (ขมิ้นชัน), *Orthosiphon aristatus* (หญ้าหนวดแมว), *Gelonium multiflorum* (ขันทองพยาบาท), *Salacia chinensis* (กำแพง 7 ชั้น), *Rhinacanthus nasutus* (ทองพันชั่ง), *Euphorbia lacei* (สลัดได), *Garcinia cowa* (ใบชมวง), *Rauvolfia seppentina* (ระย่อม), *Artemisia pallens* (โกฐจุฬาฯ), *Zingiber cassumunar* (ไพล), *Curcuma zedoaria* (ขมิ้นอ้อย), *Livisticum officinale* (โกฐเชียง). All 17 species were cut into small pieces, dried, and crushed in a blender. Fifty gram of each herb was soaked in ethanol at room temperature for 3 days. The methanol solution was filtered and evaporated under reduced pressure to dryness at 35°C, the crude methanolic extract of

each specie was tested for cytotoxic activity against breast cancer cell line using MTT method. Result of MTT assay revealed that *K. parviflora* have the highest activity for inhibition of breast cancer cell line. Therefore, *K. parviflora* was chosen for further study.

Table 3.1 The list of herbs in the primary screening this experiment.

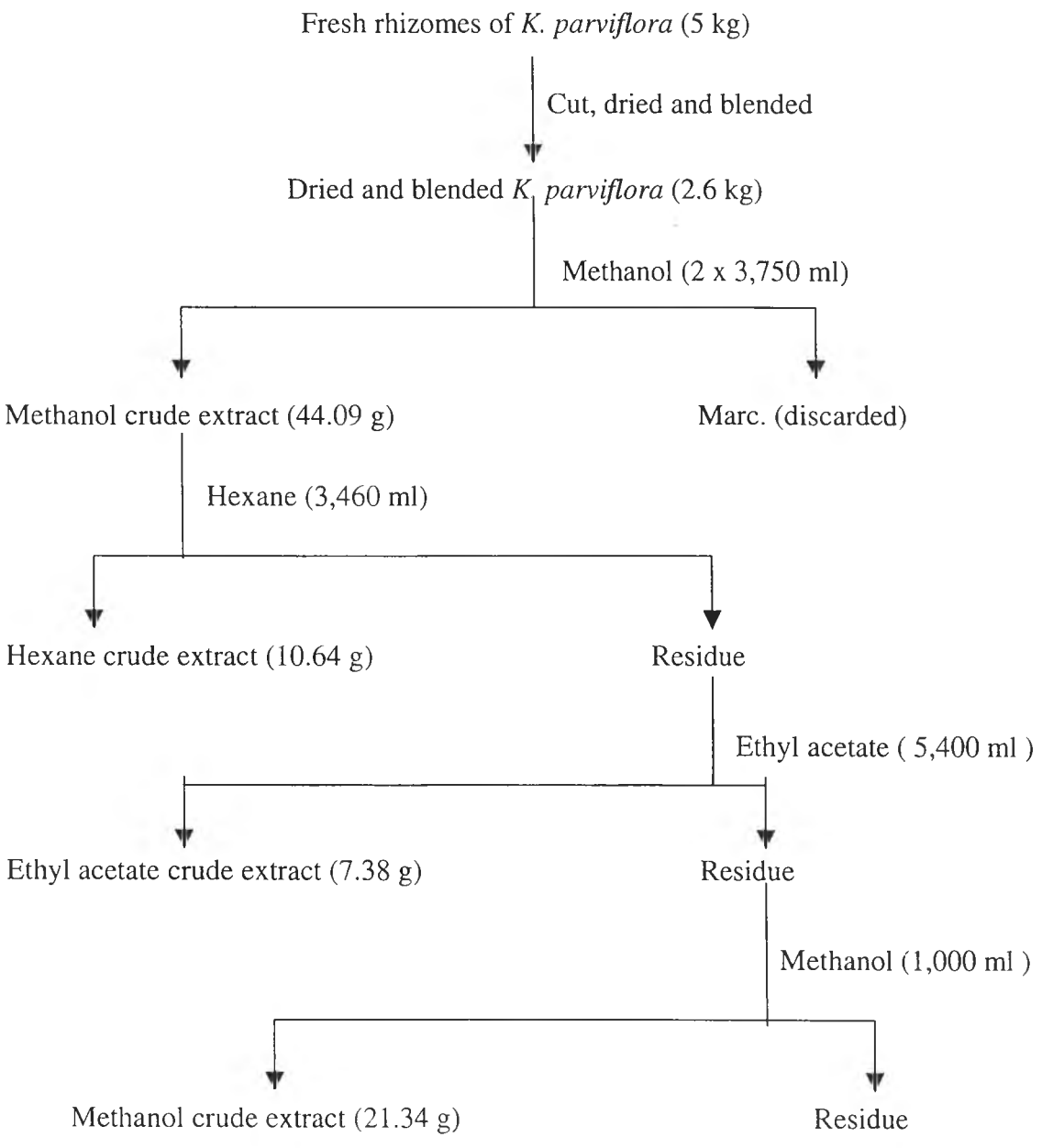
Herbs	Thai common name	Plant parts
Hydnophytum formicarum	หัวร้อยรู	Fresh rhizomes
Cuscuta chinensis	ฝอยทอง	Fresh stems
Nelambo nucifera	เกสรบัวหลวง	Dry pollens
Acanthus ebracteatus	เหงือกปลาหมอ	Dry leaves
Kaempferia parviflora	กระชายดำ	Fresh rhizomes
Curcuma longa	ขมิ้นชัน	Fresh rhizomes
Orthosiphon aristatus	หญ้าหนวดแมว	Fresh leaves
Gelonium multiflorum	ขันทองพยาบาท	Dry barks
Salacia chinensis	กำแพง 7 ชั้น	Dry barks
Rhinacanthus nasutus	ทองพันชั่ง	Fresh leaves
Euphorbia lacel	แก่นสลัดได	Dry barks
Garcinia cowa	ใบชมวง	Fresh leaves
Rauvolfia seppentina	รากระย่อม	Dry roots
Artemisia pallens	โกศจุฬา	Dry roots
Zingiber cassumunar	ไพล	Fresh rhizomes
Curcuma zedoaria	ขมิ้นอ้อย	Fresh rhizomes
Livisticum officnale	โกศเชียง	Dry roots



Scheme 3.1 The procedure for extraction of 17 Thai herbs.

3.5 EXTRACTION AND ISOLATION

Fresh black rhizomes of *K. parviflora* (5 kg) were cut, dried and crushed (2.6 kg). After extraction with methanol (3,750 ml, 2 times, each time soaked for 3 days) at room temperature, the filtrate was evaporated under reduced pressure to dryness at 55°C. The crude methanolic extract (44.09 g) was extracted with hexane (3,460 ml) until the solution was colorless. The filtered hexane solution was evaporated at 20°C to afford the hexane extract as a mixer of white-yellow solid and yellow oil (10.64 g). The residue of this step was extracted with ethyl acetate (5,400 ml) repeatedly until the solution was clear. The combined ethyl acetate solution was concentrated at 45°C on a rotary evaporator under reduced pressure to give the ethyl acetate extract as a mixer of white solid and yellow oil (7.38 g) and the final insoluble residue was evaporated at 55°C to obtain a brown - violet liquid (21.34 g). The crude extract of the black rhizomes of *K. parviflora* with various solvents are shown in **Table 4.2** and the procedure and results of the extraction are shown in **Scheme 3.2**.



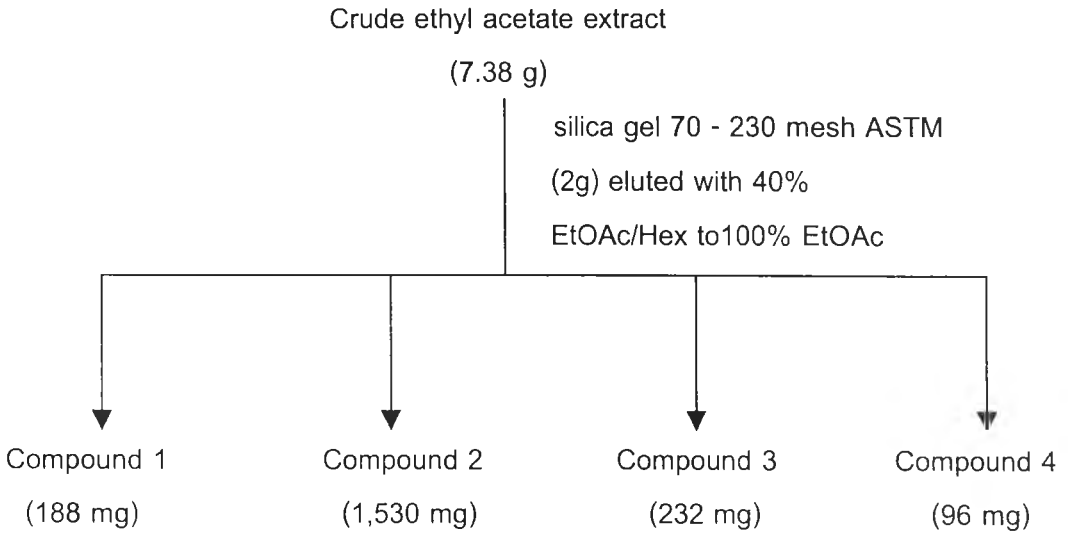
Scheme 3.2 The procedures and quantities of *K. parviflora* extraction.

3.6 ISOLATION OF CRUDE EXTRACT FROM *K. parviflora.*

The primary test for cytotoxic activity against breast cancer cell line revealed that the crude ethyl acetate and methanol extract were more active than the crude hexane as shown in **Table 4.3.** Therefore crude ethyl acetate extract was first separated by chromatography.

3.6.1 SEPARATION OF CRUDE ETHYL ACETATE EXTRACT.

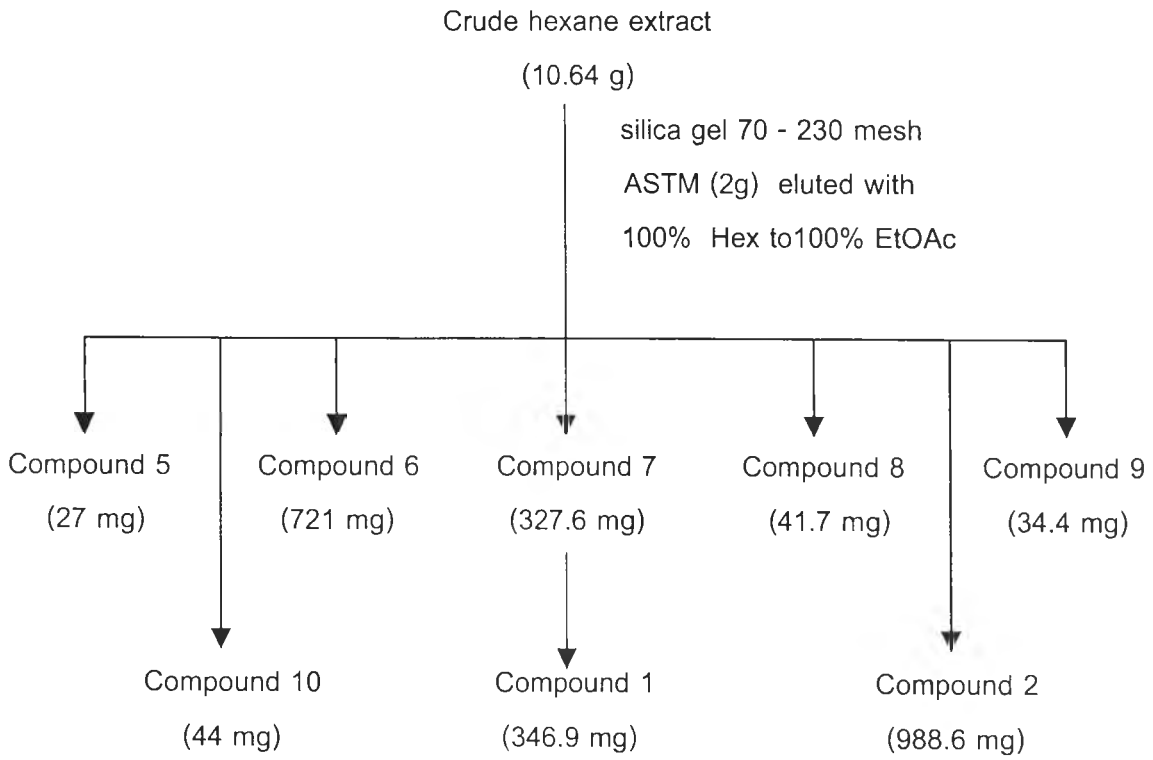
The crude ethyl acetate extract (7.38 g) was pre-adsorbed on silica gel 70 - 230 mesh ASTM (2g) prior to application on the top of the column. Firstly, the column was eluted with 40% hexane-ethyl acetate, concentration of ethyl acetate was slowly increased to 100% ethyl acetate. The similar fractions were combined and the solvent was removed by rotary evaporator to give compound 1, 2, 3 and 4, respectively. The isolation of compounds 1-4 from the crude ethyl acetate extract is briefly summarized in **Scheme 3.3**.



Scheme 3.3 Isolation procedures of the crude ethyl acetate extract of *K. parviflora.*

3.6.2 SEPARATION OF CRUDE HEXANE EXTRACT.

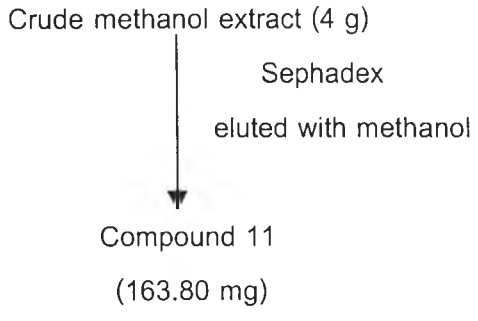
The crude hexane extract was obtained as a mixture of white - yellow solid and yellow oil (10.38g) after evaporation. The crude hexane extract (10.38g) was fractionated by open column chromatography using Merck's siliga gel 70-230 mesh ASTM (3g) as an adsorbent. The column was eluted with hexane-ethyl acetate gradient in a stepwise fashion. The similar fractions were combined and the solvent was removed by rotary evaporator to give compound 5, 6, 7, 8, 9, and 10. In addition, compound 1 and 2 which were obtained previously from crude ethyl acetate were found in crude hexane too. The isolation of compound 5-10 from the crude hexane extract is briefly summarized in **Scheme 3.4**.



Scheme 3.4 Isolation procedures of the crude hexane extract of *K. parviflora.*

3.6.3 SEPARATION OF CRUDE METHANOL EXTRACT.

The crude methanol extract was obtained as brown - violet vicous liquid (21.34 g). The crude methanol extract (4 g) was pre-adsorbed on a sephadex column. The column was eluted with methanol. The isolation of compound 11 from the methanol extract is briefly summarized in **Scheme 3.5**.



Scheme 3.5 Isolation procedures of the crude methanol extract of *K. parviflora.*

3.7 PURIFICATION AND PHYSICAL PROPERTIES OF ISOLATED COMPOUNDS.

3.7.1 PURIFICATION AND PROPERTIES OF COMPOUND 1.

Compound 1 from crude ethyl acetate extract (188 mg) and crude hexane (347 mg) is a colorless needle crystal (total 535 mg, 0.011% wt. by wt. of the fresh rhizomes). Compound 1 was obtained from the elution of silica gel column chromatography with 40% ethyl acetate in hexane and washed the crystals with 25% ethyl acetate in hexane. Compound 1 had m.p. 181-182 °C and showed a single spot at the R_f value of 0.41 on TLC plate using 70 % ethyl acetate in hexane as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and with I_2.

3.7.2 PURIFICATION AND PROPERTIES OF COMPOUND 2.

Compound 2 from crude ethyl acetate extract (1,558 mg) and crude hexane extract (989 mg) as white solid (total 2,547 mg, 0.051% wt. by wt. of the fresh rhizomes), was obtained by the elution of silica gel column chromatography with 60% ethyl acetate in hexane. It was re-crystallized with 50% ethyl acetate in hexane to obtain colorless needle crystals and was washed with 30% ethyl acetate in hexane. Compound 2 had m.p.134-135°C and showed a single spot at the R_f value of 0.21 on TLC plate using 70 % ethyl acetate in hexane as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and with I_2.

3.7.3 PURIFICATION AND PROPERTIES OF COMPOUND 3.

Compound 3 was obtained from crude ethyl acetate extract by the elution of silica gel column chromatography with 75% EtOAc / Hex, as a white powder (total 232 mg, 0.0046% wt. by wt. of the fresh rhizomes). Compound 3 had m.p.139-141°C and showed a single spot at the R_f value 0.23 on TLC plate using 80% EtOAc / Hex as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and with I_2.

3.7.4 PURIFICATION AND PROPERTIES OF COMPOUND 4.

Compound 4 was obtained from crude ethyl acetate extract as a white powder (total 96 mg, 0.002% wt. by wt. of the fresh rhizomes). Compound 4 was eluted from silica gel column chromatography with 80% EtOAc / Hex and washed with 40% EtOAc / Hex. Compound 4 had m.p. 252-254°C and showed a single spot at the R_f value 0.13 on TLC plate using 80% EtOAc / Hex as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and with I_2.

3.7.5 PURIFICATION AND PROPERTIES OF COMPOUND 5.

Compound 5 was obtained from crude hexane extract by the silica gel column chromatography with 5% EtOAc / Hex and washed with cool hexane.

Compound 5 is a greenish - yellow solid (27 mg, $5.4*10^{-4}$% wt. by wt. of the fresh rhizomes). Compound 5 had m.p. 121-122°C and showed a single spot at the R_f value 0.75 on TLC plate using 20% ethyl acetate in hexane as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and with I_2.

3.7.6 PURIFICATION AND PROPERTIES OF COMPOUND 6.

Compound 6 was found in crude hexane extract by the elution of silica gel column chromatography with 10% EtOAc/Hex. Compound 6 is yellow needle crystals (721 mg, 0.014% wt by wt of the fresh rhizomes). Compound 6 had m.p. 129-130 °C and showed a single spot at the R_f value of 0.58 on TLC plate using 20% ethyl acetate in hexane as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and with I_2.

3.7.7 PURIFICATION AND PROPERTIES OF COMPOUND 7.

Compound 7 is yellow plate crystals (508 mg, 0.01% wt. by wt. of the fresh rhizomes) in crude hexane extract. Compound 7 was obtained from the elution of silica gel column chromatography with 10% ethyl acetate in hexane. Compound 7 had m.p. 149-150°C and showed a single spot at the R_f value 0.45 on TLC plate using 20% ethyl acetate in hexane as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and with I_2.

3.7.8 PURIFICATION AND PROPERTIES OF COMPOUND 8.

Compound 8 was obtained from crude hexane extract as a yellow needles crystal (42 mg, $8.4*\ 10^{-4}$% wt. by wt. of the fresh rhizomes) by the elution of silica gel coloumn chromatography with 10% ethyl acetate in hexane. Compound 8 had m.p. 145-147°C and showed a single spot at the R_f value 0.35 on TLC plate using 20% ethyl acetate in hexane as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and I_2.

3.7.9 PURIFICATION AND PROPERTIES OF COMPOUND 9.

Compound 9 was obtained from crude hexane extract by the elution of silica gel column chromatography with 15 % ethyl acetate in hexane. Compound 9 was a greenish - yellow solid (34 mg, 6.8 * 10^{-4}% wt. by wt., of the fresh rhizomes) with m.p. 152-153 °C and showed a single spot at the R_f value 0.35 on TLC plate using 20% ethyl acetate in hexane as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and with I_2.

3.7.10 PURIFICATION AND PROPERTIES OF COMPOUND 10.

Compound 10 as colorless needle crystals (44 mg, 8.8 * 10^{-4} wt.by wt. of the fresh rhizomes). Compound 10 was obtained from crude hexane extract by the elution of silica gel column chromatography with 25% ethyl acetate in hexane. Compound 10 had m.p. 147-149 °C and showed a single spot at the R_f value 0.28 on TLC plate using 20% ethyl acetate in hexane as the mobile phase. TLC spots were visualized with UV lamp (254 and 365 nm) and with I_2 .

3.7.11 PURIFICATION AND PROPERTIES OF COMPOUND 11.

Compound 11 was obtained from the crude methanol extract by the elution of sephadex column chromatography with methanol as a brown - violet vicous liquid. (163.8 mg, 3.3*10^{-3} % wt. by wt. of the fresh rhizomes).

3.8 X-ray DIFFRACTION.

Crystal of compound 1, 2, 6, 7, 8 and 9 were identified by X-ray Diffraction analyses. All data were collected at room temperature using graphite monochromated MoK α Radiation (lamda = 0.71069 A°) on BRUKER SAMART CCD diffractrometer. The data were corrected for Lorentz and polarization effects. The crystal data of compound 1, 2, 6, 7, 8 and 9 are given in **Table A1, A6, A11, A16, A21 and A26** respectively.

The structures were solved by direct methods using SHELXLS - 97 and refined by full matrix least - squares on F^2 using SHELXLS - 97 with anisotropic thermal parameters for all non - hydrogen atoms. All hydrogen atoms were found from difference Fourier maps and were included in refinement. The fraction coordinates of non - hydrogen atom and selected bond distances and angles of compound 1, 2, 6, 7, 8 and 9 are listed in **Table A2-5, Table A7-10, Table A12-15, Table A17-20, Table A22-25 and Table A27-30,** respectively.

3.9 BIOLOGICAL ASSAY

3.9.1 Cytotoxicity assay

Bioassay of cytotoxicity activity against five cell lines, including Kato-III (gastric), BT 474 (breast), Chago (lung), SW 620 (colon) and Hep-G2 (hepatoma) cancer, *in vitro* was performed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] coloricmethod. In principle, the viable cell number per well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

Compounds were tested for cytotoxicity activity toward 5 cell lines as follow:

Kato-III : Gastric carcinoma, Human ATCC No. HTB-103

BT474 : Ductol carcinoma, breast, Human ATCC No. HTB 20

SW620 : Lymph node metastasis, colon adenocarcinoma, Human ATCCCCL227

HEP-G2 : Liver hepatoblastoma, Human ATCC No.HB 8065

Chago : Lung undifferentiated, Human J. Nat. Cancer Inst.

Cell lines, about $5*10^4$ cells / ml were cultured in RPMI 1640 culture medium, supplemented with 10%(V/V) fetal calf serum, penicillin and streptomycin. All cells were incubated in 5% CO_2 humidified incubator at 37°C. They were grown as monolayer and trypsin was added to disaggregate cells.

Cell lines at the exponential growth phase were harvested and centrifuged at 200* g for 5 min. counted under inverted microscope and resuspended in complete RPMI medium approxymately $2.5*10^4$ cells/ml and 200 µl of the cells suspension was added to each well of a flat bottom 96-well microtiter plate with a multichannel pipette. Number of cells per well is $5*10^3$. After 24 h incubation in a 5% CO_2 humidified incubator at 37°C, 2 µl of sample agents was added in appropriate wells to give various final concentration of samples (control group, N=6, each samples 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) "background" determinations. The concentration of DMSO used to dissolve the samples was adjusted to 0.5% and this concentration of solvent was used in control wells. After 72 hr incubation at 37°C, MTT stock solution was prepared as follow: 5mg MTT/ml PBS was filtered with 0.22 µm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in RPMI standard culture medium. MTT working solution (50 µl) 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 hr.

Incubation cell monolayers and formazan were then inspected microscopically. Culture plates containing suspension lines or any detached cells were centrifuged at low speed (200*g) for 5 min. All 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 pipette and mixed wells by plate mixer. Following through the formazan solubilization, the absorbance of each well was measured using a microtiter plate reader at 540 nm (single wavelength, calibration factor = 1.00)

Cell lines 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. In addition the IC_{50} was expressed as the sample concentration in µg/ml that caused a 50 % inhibition of growth compared with controls.

3.9.2 Antioxidant assay

The antioxidant assay of sample by DPPH method was used to study the potential of sample for reducing DPPH radicals (radical 2, 2-diphenylpicryhydrazyl). The sample that has high potential antioxidant activity can effectively reduce DPPH radicals. Vitamin E was used as a positive control.

The testing protocol

1) The absorbance of 4 ml DPPH solution was measured at 517 nm. (absolute ethanol was used as a blank adjustment for the spectrophotometer)
2) Samples and vitamin E were pipetted into each tube covered with foil. (each samples made duplicate)
3) Added 3.8 ml of DPPH solution into each tube (the final volume was 4 ml), throughly mixed and kept in the dark for 30 min.
4) After 30 min, the absorbance was measured at 517 nm. Compared the absorbance value of each sample with that of DPPH solution. The samples that had antioxidant activity would had absorbance value lower than that of DPPH solution.
5) The samples which had antioxidant activity were taken to examine for the IC_{50}value.

5.1) Each 5 mg / ml of sample which had antioxidant activity was diluted to 3.75, 2.5, 1.25 and 0.625 mg / ml. The ratio of dilution is shown in **Table 3.2**. Vitamin E was diluted to 0.375, 0.25, 0.125 and 0.0625 mg / ml and the ratio of dilution is shown in **Table 3.3**.

Table 3.2 Ratio of dilution of 5 mg / ml of samples with ethanol.

	5 mg / ml	3.75 mg / ml	2.50 mg / ml	1.25 mg / ml.	0.625 mg / ml
Vol. of sample (5 mg / ml)	200 ul	150 ul	100 ul	50 ul	25 ul
Vol. Of absolute ethanol	0 ul	50 ul	100 ul	150 ul	175 ul

Table 3.3 Ratio of dilution of 0.5 mg / ml of vitamin E with ethanol.

	0.5 mg / ml	0.375 mg / ml	0.25 mg / ml	0.125 mg / ml	0.0625 mg / ml
Vol. of sample (0.5 mg / ml)	200 ul	150 ul	100 ul	50 ul	25 ul
Vol. of absolute ethanol	0 ul	50 ul	100 ul	150 ul	175 ul

5.2) The samples in 5.1 were tested for antioxidation activity potential. Results were ploted as a correlation between absorbance value and concentration of samples.

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

Preparation for DPPH solution and samples for antioxidant assay.

1) DPPH solution.

DPPH solution containing 2, 2-diphenyl-1-picrylhydrazyl ($2*10^{-4}$ M) was prepared by weighing 20 mg DPPH (M.W. 394.3) and dissolved in 250 ml absolute ethanol. The DPPH solution had blueish-violet colour (ABS_{517} estimated 1.15 - 1.2).

- DPPH solution should be freshly prepared just before the testing and kept in dark to avoid the decomposition of DPPH.

2) Vitamin E for positive control.

Vitamin E 1.5 mg was dissolved in 1.5 ml of absolute ethanol. The concentration of vitamin E is 1 mg / ml. The two folds dilution resulted in 0.5 mg/ml of vitamin E.

3) The samples for antioxidant bioassay.

The samples were prepared to make 5 mg/ml final concentration by dissolving 7.5 mg of sample in 1,500 ml of absolute ethanol.