Chapter 2
MATERIAL AND METHOD

2.1 Chemicals. See appendix

2.2 Preparation of lemon grass (Cymbopogon citratus Stapt)

Fresh lemon grass (Cymbopogon citratus Stapt) was purchased from local markets in Chiang Mai, Thailand, washed well with tap water, and minced with knife. Small pieces were freezed-dried by lyophillizer, ground with a mixer and kept in a refrigerator.

2.3 Preparation of lemon grass extract

There were two extracting conditions as the fallowing

2.3.1 Extraction at room temperature

Fifty grams of lemon grass powder was stirred with 500 ml of water at room temperature for 8 hours, the extract was centrifuged, supernatant was kept, the precipitate was continuously stirred with 500 ml of absolute methanol for 4 hours, and after centrifuge the precipitate was stirred again with 500 ml of hexane for 4 hours. The three extracts were evaporated to dryness in a vacuum rotatory evaporator at 50 ^{o}C.

The dry residues were weighed and redissolved in an appropriate solvent. The scheme of extraction method was shown in Figure 2.

2.3.2 Extraction by hot water

The same extraction and dissolution technic was used for another lemon grass powder, except the first step. The lemon grass powder was stirred with 500 ml of water at 100 ^{o}C for 8 hours, then following with the same method as described in section 2.3.1

The aqueous extract was freezed-dried while the organic solvents in extracts were fully evaporated to obtain residues. Each residue was dissolved in water (for aqueous extract) and dimethylsulfoxide (DMSO) (for methanol and hexane extract) and adjusted the concentration to 0.85, 1.50 and 3.00 mg/ml. The solutions were saved in screw-cap vial. All of the solutions were passed through Millipore filter membrane before the analysis for their mutagenicity and antimutagenicity .

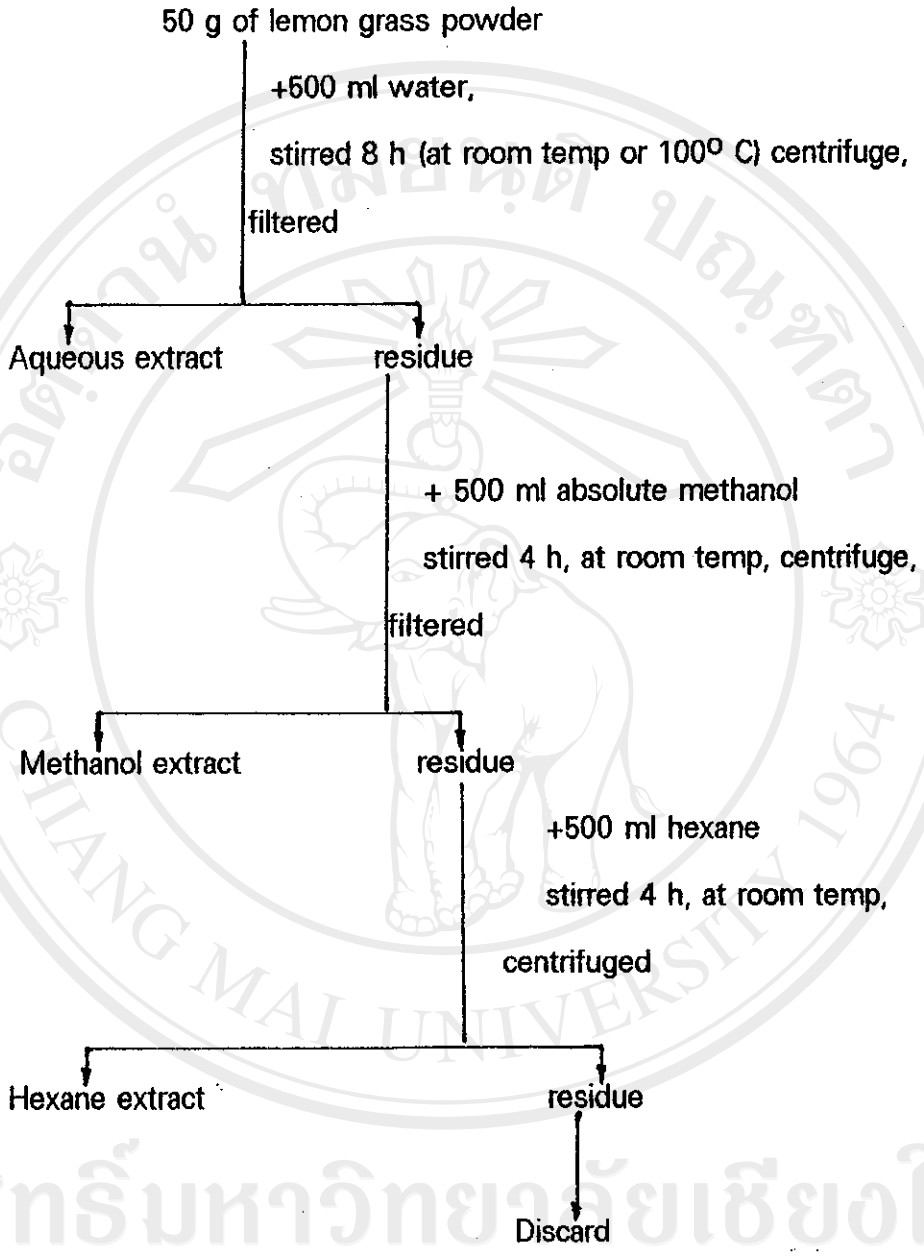

Figure. 2 Scheme of sample extraction

2.4 Mutagenicity and Antimutagenicity Assay

2.4.1 Mutagenicity Assay

The Salmonella mutation, with preincubation technique described by Matsushima and his colleagues (Matsushima, et al, 1980) was used in this study. The mutagenicity of lemon grass extracts was tested in Salmonella typhimurium strains TA 98 and TA 100, in the presence and absence of S9 mix. The following items were added in order to a capped culture tube, 50 ul of each extracts , 500 ul of S9 mix (or 500 ul of 0.2 M phosphate buffer, pH 7.4) and 100 ul of fresh overnight culture of tester bacteria strain.The mixture was preincubated at 30°C for 30 min in shaking water bath then 2 ml of top agar at 45 °C was added to the mixtures and was gently mixed, then poured onto a minimal glucose agar plates. The plates were incubated for 48 hours at 37° C, and histidine revertant colonies were counted. Positive (standard mutagen) and negative (H_2O or DMSO) control plates were included in this assay. The experiment was repeated twice, and the results were expressed as means of six plates for each concentration. The scheme of the mutagenicity assay is shown in Figure. 3.

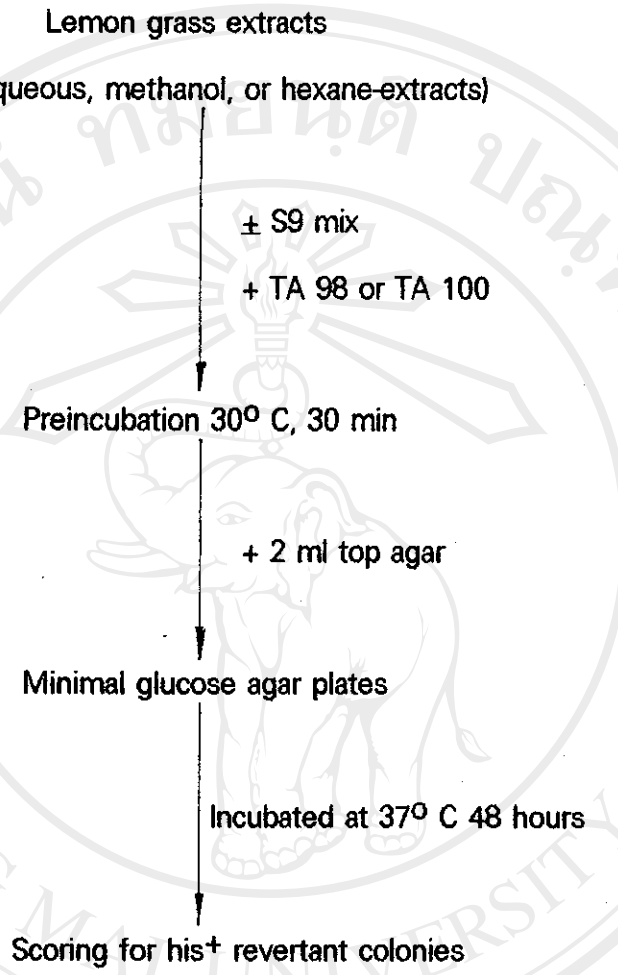

Figure 3 Scheme of mutagenicity assay

2.4.2 Antimutagenicity Assay

The effect of lemon grass extracts on the mutagenicity of various known mutagens, such as, aflatoxin B_1 (AFB_1), N-methyl-N'-nitro-N-nitroso guanidine (MNNG), furylfuramide (AF-2), sodium azide (NaN_3), 4-nitroquinoline oxide (4-NQO), dimethylbenzanthracene (DMBA), benzo pyrene [B (a) P] as well as typical amino acid pyrolysates, Trp-P-1, and IQ were investigated in the Ames test. The amount of each mutagens used in the investigation is shown in Table 1. The scheme of antimutagenic assay is shown in Figure. 4

Table 1. Concentration of various known mutagenes used in antimutagenic assay

Known mutagens	Amount (ug/plate) TA 98	TA 100
-S9 Mix		
AF-2	0.1	-
NaN_3	-	0.5
4-NQO	0.2	-
MNNG	-	0.5
+ S9Mix		
B(a) P	5	-
DMBA	20	-
IQ	-	0.1
Trp-P-1	0.02	-
AFB_1	0.05	-

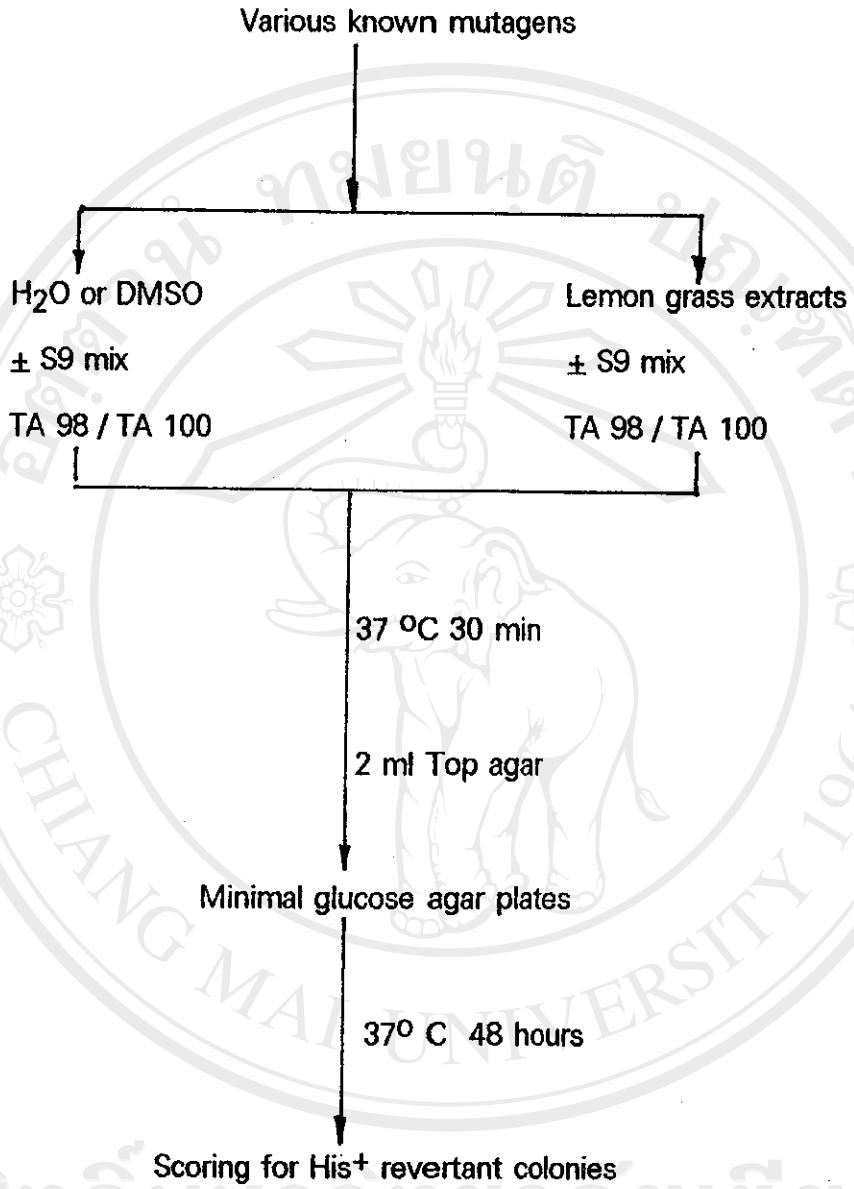

Figure 4. Scheme of antimutagenicity assay

2.5 Possible mechanism for inhibiting chemical mutagenesis

The procedure for possible mechanism for inhibiting of chemical mutagenesis are described as the following

2.5.1 Procedure 1, Antimutagenic effect on S9 mix

Lemon grass extracts were preincubated with S9 mix (AFB_1) or with phosphate buffer (MNNG) at 37 ^{o}C for 30 min. After incubation, bacterial culture TA 98 and AFB_1 or TA 100, and MNNG, and top agar were added to the mixture, then poured onto minimal glucose agar plates. The plate was incubated at 37 ^{o}C for 48 hour and the number of revertant colonies were counted afterward. All dose of methanol extract were carried out in triplicate and the experiment was repeated twice.

2.5.2 Procedure 2, Antimutagenic effect on mutagens

The methanol extract of lemon grass and known mutagens (AFB_1 or MNNG) were incubated at 37 ^{o}C for 30 min. After that bacteria culture of TA 98 (AFB_1) or TA 100 (MNNG) and S9 mix (AFB_1) or

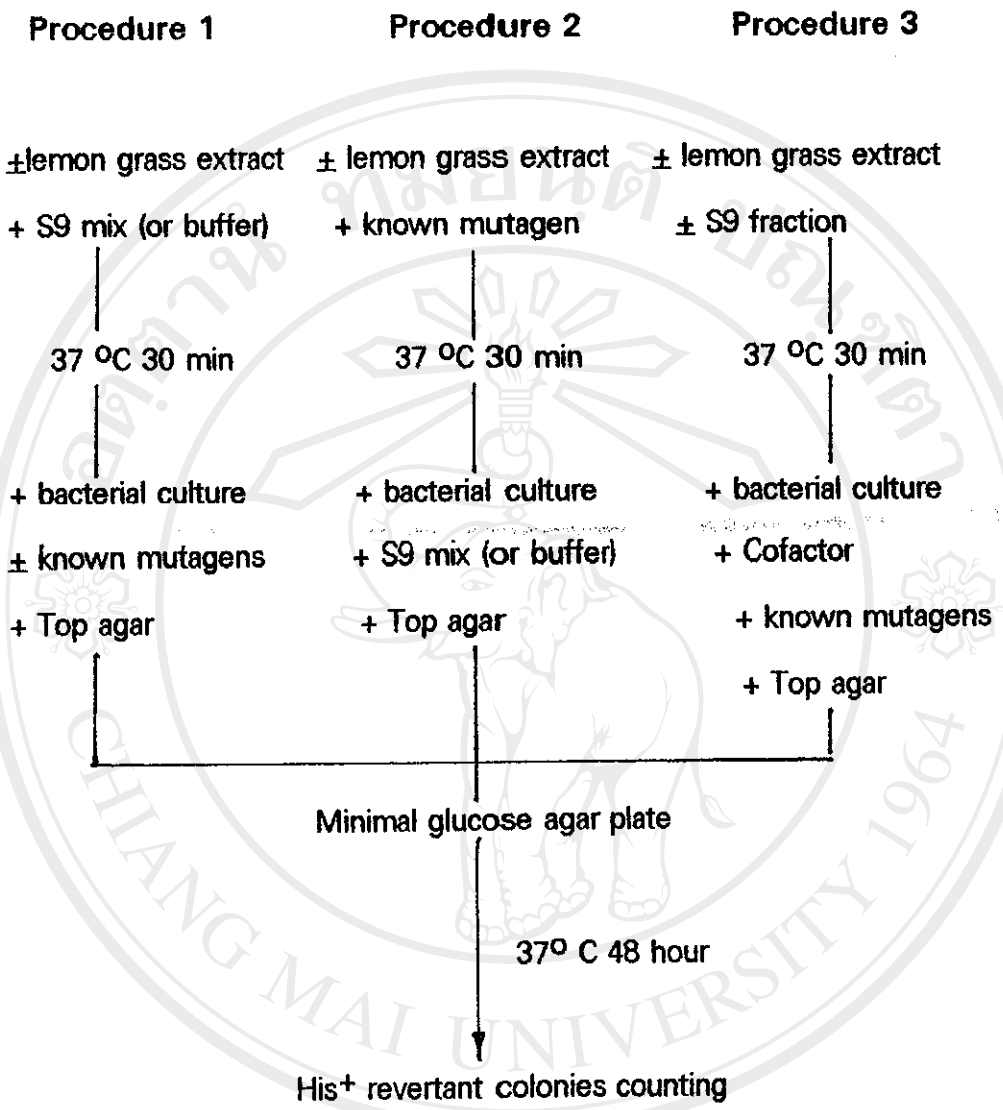

Figure. 5 Diagram showing desmutagenic activity assay

phosphate buffer (MNNG) and top agar were added to the mixture, poured onto a minimal glucose agar plates and incubated at 37 ^{o}C for 48 hours. Then the revertant colonies were counted. The experiment was repeated twice and each dose was done as triplicate plates.

2.5.3 Procedure 3, Antimutagenic effect on S9 fraction

The methanol extract of lemon grass was preincubated with S9 fraction (without cofactor) at 37 ^{o}C for 30 min. After incubation period bacterial culture, know mutagen, cofactor (NADPH, $NADP^{+}$) and top agar were added and poured onto minimal glucose agar plates. The plates were incubated at 37 ^{o}C for 48 hours then the number of revertant colonies were counted.

2.6 Bio-antimutagenic assay

Five ml of bacteria culture overnight was washed twice with 4 ml 1.5 M phosphate buffer pH 7.0 by centrifuge at 5,000 g for 20 min, then resuspended in 4 ml of phosphate buffer. Three ml of the bacteria suspension, 3 ml S9 mix and 2 ug of AFB_1 was incubated at 37 ^{o}C, 30 min in shaker bath. Then the reaction mixture was centrifuged and treated cells were washed twice with cold phosphate buffer and

the AFB_1 - treated cells were resuspended in 4 ml of phosphate buffer. A 0.1 ml of treated cells was mixed with various amount of the methanol extract of lemon grass (7.5 , 15, 30 75 and 150 ug/plate) and 2 ml top agar was added. Then the mixture was poured onto minimal glucose agar plate. The plates were incubated at 37 ^{o}C for 48 hours, the revertants were counted after that. The scheme of the assay is shown in Figure 6.

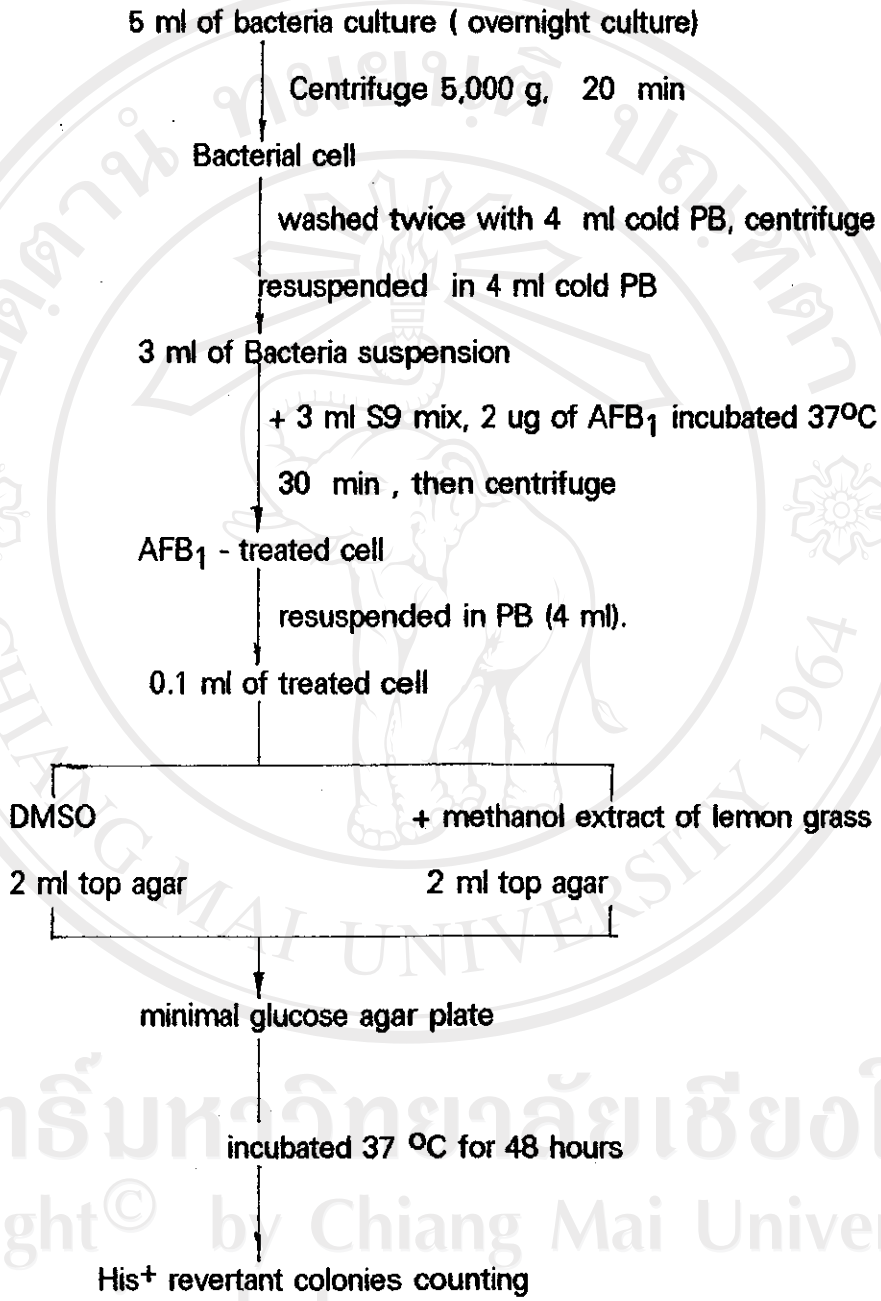

Figure. 6 Scheme for bio-antimutagenicity assay.

2.7 Antimutagenic fractionation of methanol extract of lemon grass.

The previeus result demonstrated that methanol extract of lemon grass showen highest capacity to inhibit various known mutagens. This investigation attempts to isolate and partial purify active antimutagenic substance from methanol extract of lemon grass.

The active antimutagenic substances were isolated and partial purified by Sephadex LH-20 column chromatography and high performance liquid chromatography with Bondclone 10 C_{18} column (Phenomenex)

2.7.1 Chromatographic fractionation step by Sephadex LH-20

The methanol solution of lemon grass extract at concentation of was applied to Sephadex LH 20 (40 x 1.5 cm.) and eluted with absolute methanol at flow rate 1 ml/min. The peaks with highest antimutagenic activity were subsequently purified by rechromatographed on the same column. The antimutagenic peak was collected and afterward evaporated to dryness. The residue after being dissolved in methanol (HPLC grade) was further purified by high performance liquid chromatography (HPLC).

2.7.2 Isolation of antimutagenic substances in peak2 by HPLC

The compounds in peak 2 were further separated by HPLC using Bondclone 10 C_{18} column (Phenomenex) (3.9 x 300 mm), operating conditions as following: the injection volume was 100 ul, 100 % methanol was used as elution solvent; flow rate was adjusted at 1 ml/min. The eluate was monitored by UV absorbance at 280 nm. The peaks were collected and analyzed for antimutagenic against AFB_1-induced mutation in Salmonella mutation assay.

2.8 Characterization of the partial purified antimutagenic substance from methanol extract of lemon grass.

The partial purified substance from HPLC was subjected to

2.8.1. Thin-layer chromatography with 2 different solvent systems :

systems 1 = acetonitrile : water 7: 3

system 2 = methanol : water 6:4

HPTLC silica gel 60F254 (precoated sheet) was purchased from EMerck, Darmstadt, Germany. The Rf values of the two partial purified peaks from HPLC were determined.

2.8.2 Chemical reactions

The peaks were checked for protein occurrence by colour reaction with Ninhydrin reagent and for carbohydrate presentation by colour reaction with Molisch's reagent and Benedict reagent.

2.8.3 Absorption spectra pattern from HPLC analysis

The ultraviolet absorption spectra of the isolated peaks were integrated by Spectromonitor 3200 during HPLC separation.

2.8.4 Gas-chromatographic-Mass spectrometry analysis.

THe partial purified peak were analysed for their molecular weight by GC-MS spectrum, at Department of Chemistry, Faculty of Science, Chaing Mai University.