CHEPTER II
EXPERIMENTAL

Plant material

The roots of *Cladogynos orientalis* Zipp. ex Span. were purchased from Chao Krom Per. This plant was identified and compared with herbarium (voucher specimen, BKF 28024) by an expert botanist at Royal Forest Department, Bangkok, Thailand.

Instrumentation and Equipment

Spectroscopies Studies

Infrared Spectra were obtained using a Fouried Transform Infared Spectrophotometer, Perkin-Elmer Model 1760x. The sample of solid samples were examined by incorporating the sample into a potassium bromide (KBr) pellet. The liquid spectra were taken as films formed between two sodium chloride plates.

The ^{1}H-, ^{13}C- and 2D NMR spectra were obtained by using a Bruker Model ACF 200 and JEOL 500 MHz NMR spectrometer model JNM-A 500.Chloroform-d, 99.9%($CDCl_3$) and methanol-d_4, 99.5%(CD_3OD)were used as a solvent. Trimethylsilane(TMS) was used as an internal standard.

Mass spectral data were obtained using a Fison Mass Spectrometer model Trio 2000.

Gas chromatography was performed with a Shimadzu Gas Chromatography GC-7AG.

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

Chemical Reagents

In this research used commercial reagents such as hexane, dichloromethane, ethyl acetate, methanol. They were purified pior to use by distillation.

Color Tests

The Liebermann-Burchard test was used to test for steroids and triterpenoid. One milligram of the sample was dissolved in chloroform and a few drops of acetic anhydride. Then one drop of concentrated sulfuric acid was added. Development of color after a few minutes suggests the presence of steroid (violet→blue→green) or triterpenoid (violet→blue→pink).

Physical Separation

Column chromatography[22] was performed in glass column using silica gel (230-400 mesh, Merk) as the solid support.

Thin layer chromatography (TLC) was performed using E. Merk (Darmstadt) silica gel GF254 glass plates. [23] Chromatoplates were prepared at a thickness 1.0 nm for preparative TLC. The plates were activated at 110 ^{0}C for one hour. The analytical TLC plates[24] with precoated on aluminium sheet (Merck-Kieselgel 60F-254) was used. Ultraviolet light (254 nm and 366 nm), exposure to I_2 vapors, and 20% sulfuric acid were used as detecting agents.

Preparative TLC separations were performed using a Harrison Research Chromatotron model 7924T equipped with a solvent pump. Absorbent layers of 1 mm and 2 mm were used.

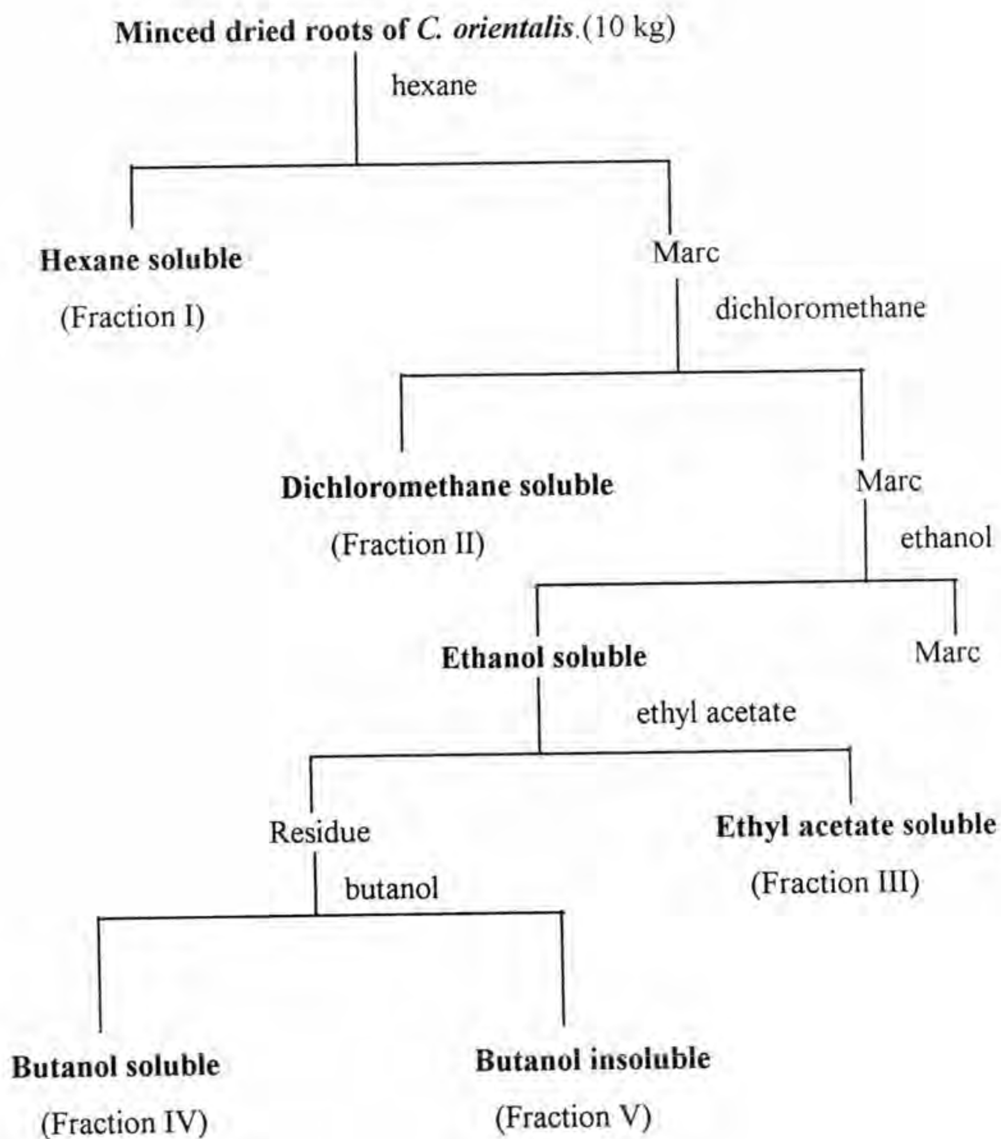
Extraction

The air-dried roots of *C. orientalis* (10 kg) were minced and extracted with solvents. The plant was soaked in each solvent for about 4-5 days several times until the solvents were evaporated.

The plant was initially extracted with hexane. The hexane crude extract (Fraction I) was a brown material (101.11g) the hexane insoluble part was extracted with dichloromethane in the same way and yieded the dichloromethane crude extract as a light brown material, 317.07 g (Fraction II).

The marc was soaked in methanol to obtained the methanol soluble part. This part was partitioned with ethyl acetate and butanol, respectively. It yielded the ethyl acetate crude extract, (Fraction III), a brown material, 46.47 g and butanol crude extract, (Fraction IV), a brown material, 93.50 g. The insoluble butanol was assigned as Fraction V, 117.60 g . The extraction procedure is shown in Scheme 1.

Scheme. 1 Extraction procedure of the roots of *C. orientalis*



Bioassay Experiments

In this research, one of the goals was to search for an active substances from the roots of *C. orientalis* that should possibility use for medicinal part such as anticancer agent. The following bioassay experiments were used.

Brine Shrimp (*Artemia salina* Linn.) Cytotoxicity Test [25]

A general bioassay, brine shrimp is proposed as simple bioassay for natural product research which can detect a broad spectrum of pharmacologic activities such as antimicrobial and cytotoxicity test. That can be employed by natural product chemists, in house at low cost, to guide phytochemical screening and fractionation.

The large petri dish is divided by a glass plate that did not quite reach to the bottom of the dish and is filled with the sterilized seawater. Cysts (about 100 to 200 mg) are placed in one compartment, and a 60 watt lamp is positioned to provide direct ligh and warmth (about 25 ^{0}C) thoughout the embriogenesis. Most of eggs should have hatched into free-swimming forms by 24 hr. After sowing of the cysts, the assay is begun 36 to 48 hr. Multiwelled culture plates can be used for the bioaasay, although any clear glass container with flat bottom (for example, glass vials) will do. Five nauplii are collected, using a Plasteur pipett from the hatching dish and are transferred to a well, using the 100 μl natural seawater (35 g of NaCl per 1 l of water). This is repeated for two additional wells, thereby requiring 30 nauplii for each concentration of test sample (10 μg/ml, 100 μg/ml, 1000μg/ml). A parallel series of test with the standard ethanol solution. Although evaluation is generally by lethal concentration for 50% mortality (LC_{50})* after 24 hr.

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Note : High activity (LC_{50} < 10μg/ml)

: Medium activity (LC_{50} < 100μg/ml)

:Low activity (LC_{50} < 1,000μg/ml)

Anticell Line Test

As a collaborative research between Natural Product Research Unit, Department of Chemistry, Chulalongkorn University and Beijing Medical University, Republic of China, this biological test was kindly conducted in China*. They reported the use of the MTT assay to study the inhibitory effect of the extract on tumor cell lines. The 4 cell lines were Human carcinoma of the Stomach, Human Leukemia (HL-60), Human Mamary Cancer, K562, Human Bladder Carcinoma and Human Castric Carcinoma (BGC 823). Cell lines were cultured under conventional conditions: 37 ^{0}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 (MTT) assay is based on a reduction of MTT formazan by living cells. The reduced formazan can be measured with a microplate spectrophotometer.

MTT assay :

Cell lines were seeded in 96 well microtritre plates in wells with $5X10^4$ cells. A stock solution of the extract was added to each well, and 8 replicate wells without extract servered as controls. The plates were incubated for 72 hr. After the incubation, 20 μl of PBS solution with MTT 5 $mgml^{-1}$ were added to each well and the plates were reincubated for a further 4 hr. The plates were then inverted on blotting paper to removed the medium. The formazan crystals formed were dissolved in 200 μl of acid-isopropanol. The plates were read on a Model 450 Microplate reader at 570 nm.

Antibacterial Bioassay [26]

This procedure involved the paper disc method, the bacteria, *Escherichia coli, Bacillus cereus, Staphylococus aureus* and *Staphylococus derby* are used in this study and used Steptomycin as standard. If some compounds show high inhibition, they will be further tested against more resistance bacterial. Stock solution was prepared by dissolving 10 mg of test sample in 1000 μl of proper solvent. Thirty milliliters of stock

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* These experiments were carried out in the collaboration with Beijing Medical University, Replubic of China.

solution were transferred by disposable pipette onto a disc. After 24 hr, diameter of clear zone was measured.

TLC Bioautography Assay

This bioassay was performed by direct bioautography method was applicable to microorganisms that can growed directly on the TLC plate and suitable precautions are required. Tests for biological activities have been carried out at the Instituted of Pharmacognosy and Phytochemistry, University of Lausanne, Switzerland*. The following bioautographic assays and subsequent analyses were performed.

Antifungal Activity [27]

1. Direct bioautography procedure have been described for spore producing fungi. Numerous antifungal compounds have been characterized using *Cladosporium cucumerinum* in a routine assay. Test sample were spotted on a TLC plate, migrated and sprayed with spores of the *C. cucumerinum*. Incubation of the plate in a tank for 3 days gave the color spots as positive test.

2. The agar-overlay technique was a hybrid of the above method and was applicable to a broad spectrum of microorganisms. This bioassay has been used for yeast such as *Candida albicans*. It produced well defined zones of inhibition and was not sensitive to contamination. Active compounds are transferred from the stationary phase to the agar layer (which contained the microorganism) by a diffusion process. After incubation, the plat is splayed with an aqueous solution (2.5 mg/ml) of thiazolyl blue (MTT) which is converted to a formazan dye by the microorganism. Inhibition zone are observed as clear spots against a purple background.

Antioxidative Activity [28]

1. The use of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as a TLC spray reagent proved also to be suited for the detection of antioxidants. The assay consisted of spraying TLC plates with a 0.2% DPPH solution in methanol. The plates

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* The TLC bioautography assay experiments were carried out at Lausanne University, Switzerland.

are examined 30 min. after spraying. Active compounds appeared as yellow spots against a purple background.

2. Bleaching of β-carotine

β-carotine was another reagent used for the TLC detection of antioxidants. TLC plates are sprayed with a β-carotine solution in chloroform (0.2 mg/ml) and then exposed for about 15 min. to λ=254 nm. UV light. β-carotine undergoed bleaching except in places where antioxidative substances prevent its degradation. Active compounds appeared as orange spots against a pale background.

Larvicidal Activity [(29)]

The testing procedure involved second instar larvae of *Aedes aegypti*. The eggs of *A. eagypti* were easy to handle and can be stored in a controlled atmosphere (26-28 ^{0}C, 70-80% rel. humidity) for up to six months. Larvae hatched readily when put into tap water (final DMSO concentration 1%) and incubated for 24 hr. The assay consisted of exposing approximately 20 larvae to various dilutions of the extracts (for a final test solution of 500 ppm) previously solubilized in DMSO. Mortallity is evaluated with the naked eye after 30 min and 24 hr. A sample is consisdered active when all larvae have been killed after 24 hr.

Insect Antifeedant test [(30)]

On literature review, various diterpenes was active on antifeedant. As it was mentioned earlier, one of the major goal of this research is to search for an active principle from the roots of *C. orientalis* which could possible use for agricultural and/or medicinal propose.

In general procedure, the larvae food consists of ceresol and bee pollen (approximately 3:1 ratio) and honey, mixed together (amount of honey about 10 ml in ceresol 3 g and bee pollen 1g). Two grams of food were put in square bowl, folded aluminium foil of size 3x3 cm^2. The food bowls were weighed before use. The pure compound solution of various concentrations (percentage wt by wt of the food) were

dripped into food bowl, labeled as " test". The control food was prepared by dripping the same solvent used to dissolve the pure compound or an appropriate solvent. The solvent was allowed to evaporate from each food bowl by air drying for 3 hr. After that each bowl was reweighted and then placed pair-wise (test + control) in a plastic box. Ten worms(*Galleria mellonella* Linn.) of the same size, 0.75 cm length, were chosen and put in the same box. It was kept in the incubator at temperature 35-36 ^{0}C. After 2 days, the worm were counted and both food bowls were weighed to determine the weight loss from tested food and controlled food. Antifeedant activity was expressed as a % T/C value, where:

%T/C = (1-weight loss of tested sample / weight loss of controlled sample) x 100

* T/C value >70 % represents high activity.