CHAPTER II

EXPERIMENTAL

2.1 Plant materials

Alpinia galanga (Linn.) Swartz., *Amomum xanthoides* Wall., *Cyperus alternifolius* Roxb., *Cyperus rotundus* Linn., *Kaempferia galanga* Linn., *Zingiber cassumunar* Roxb., *Zingiber zerumbet* (Linn.) Smith. and *X. xylocarpa* were purchased from Vetchapong (Plant drug store), Bangkok, Thailand in 2001. The other plants used in this research were obtained from local medicinal plant store, Nongkhai, Thailand in 2001.

X. xylocarpa identified by comparison with voucher specimen BKF No. 124480 by Th. Wongprasert deposited in the Forest Herbarium, National Park, Wildlife and Plant Conservation Department, Bangkok, Thailand.

2.2 Instruments and equipment

The ^{1}H and ^{13}C-NMR spectra were preformed in deuterated chloroform or otherwise stated with tetramethylsilane (TMS) as an internal reference on Fourier Transform Nuclear Magnetic Resonance Spectrometer of Varian model Mercury+ 400. The Fourier Transform-Infrared spectrum (FT-IR) was recorded on Nicolet impact 410 spectrophotometer. The mass spectrum was obtained on Fisson Mass Spectrometer model Trio 2000 at 70 eV. Gas Chromatograph-Mass Spectrometry (GC-MS) was carried out on a Hewlette Packard model HP6890/5972 with helium as a carrier gas. The column used for chromatography was a fused-capillary column type of HP-5MS (30 m lengh, 0.25 mm i.d.) coated with immobilized poly(dimethylsilicone) of 25 μm thickness from J&W scientific company. Adsorbents used for isolation were silica gel 60 Merck, cat No. 7734 and 9385 for column chromatography and flash column chromatography. Merck's TLC aluminium sheet, silica gel 60 F_{254} precoated was used for qualitative analysis purpose. The spots on the plate were observed under UV light or visualized by spraying with 10% H_2SO_4 in

ethanol. Melting points were determined with a Fisher-John melting point apparatus and are uncorrected.

2.3 Chemical reaction

Reduction of compound 1[29]

Sodium borohydride 1.25 mmol (0.046 g) was added over a period of 5 min to a stirred solution of compound **1** 2.5 mmol (0.74 g) in 5 mL of ethanol. Stirring was continued for 30 min at RT. Water was added and the mixture was extracted three times with ether. The combined organic phases were washed with saturated NaCl solution and dried over anhydrous Na_2SO_4. The solvent was evaporated to gain 0.54 g of the desired product (75.0%).

Reduction of compound 9[29]

A solution of compound **9** 0.15 mmol (45 mg) in anhydrous ether was slowly added dropwise to a stirred suspension of lithium aluminum hydride 0.14 mmol (8 mg) in anhydrous ether. The mixture was then refluxed for 5 hours. After filtration, the solvent was evaporated *in vacuo* to obtain white needle 41.2 mg (95.4%).

2.4 Biological screening assay

Common cutworms (*S. litura*) obtained from department of agriculture, ministry of agriculture and cooperatives were used as an insect model in this research.

Insect feeding study, *choice leaf-disk bioassay*, was used as a main bioassay to follow bioactive compounds present.

Choice leaf-disk bioassay[30]

Leaf-disks, 1.9 cm diameter, were prepared with a cork borer from fresh sweet potato (*Ipomoea batatas*) leaves that had been cultivated without agrochemicals.

Two disks were treated with a specific amount of plant extracts or tested compounds dissolved in a proper solvent. Other two disks were prepared using the same solvent as the control. The four disks were set in alternating positions in the same petri dish. After complete removal of the solvent, 10 larvae (third instars) were released into the dish. The dishes were then kept in an insect rearing room at 25°C in the dark for 18 hours. Partially consumed leaf-disks were taped onto photocopier paper for monotone data conversion. The monotone data was photocopied, determined to contain no errors, and then converted to digital data files using a digital

scanner. Digital data analyses were performed on a PC computer using the public-domain Scion image program (developed by the U.S. National Institutes of Health and available from the Internet by anonymous FTP from www.scioncorp.com). For each experiment, the data file of an intact disk was measured and compared with that of treated disk. For evaluation of the antifeedant activity of the extracts and tested compounds, three citerias: antifeedant index (AFI), feeding inhibitory (FI) and control disk consumption (CDC) derived from the calculation according to the following formula were considered.

$$\text{Antifeedant Index} = \frac{\%\text{ treated disks consumed}}{(\%\text{ treated disks consumed} + \%\text{ control disks consumed})} \times 100$$

$$\text{Feeding Inhibitory} = 100\text{-}(\text{AFI} \times 2)$$

$$\text{Control Disk Comsumption} = \frac{\text{control disk (pixl) - residue control disk (pixl)}}{\text{control disk (pixl)}} \times 100$$

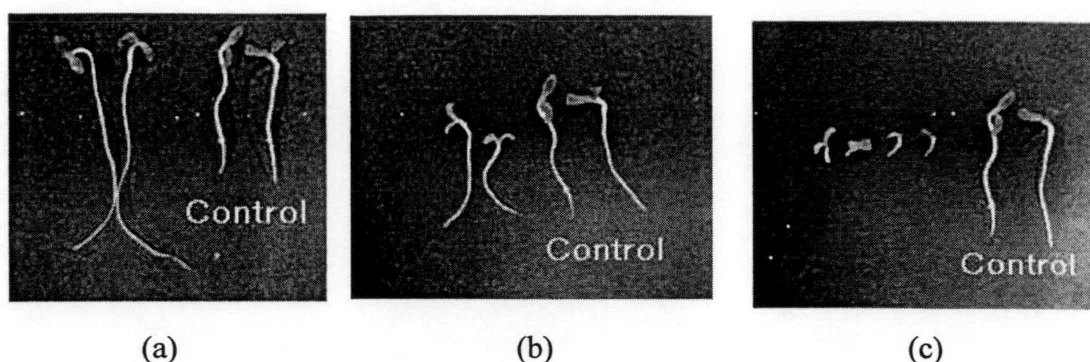
Phytotoxicity against lettuce seedlings[31]

Inhibition of the growth of lettuce (*Lactuca sativa* L.) seedlings (Great Lake 366) was assayed in petri dishes (43 mm x 8 mm) with filter paper (Toyo No. 5C) cut to a 40 mm diameter. An acetone solution of a test compound (2 mL) was poured into the petri dish, and the carrier solvent was completely evaporated under a hood. The paper was moistened with 2 mL of distilled water, and 10 germinated lettuce seeds were placed on the filter paper and incubated at 25 °C under the light condition. The symptom and growth inhibitory were evaluated comparing with the control by naked eyes at 6 days after treatment. The plant growth effects were decided into three types according to the following definitions (see also Figure 2.1):

Inactive: applied seedlings are almost the same growth toward control seedlings or more growth with radical elongation.

Slightly inhibition: applied seedlings are smaller growth than control set.

Strong inhibition: applied seedlings completely died.



(a) (b) (c)

Figure 2.1 (a) inactive with radical elongation, (b) slightly inhibition with normal growth and chlorosis and (c) complete inhibition and chlorosis

For evaluation of tested compounds, cotyledon, hypocotyl and radical length were recorded at 6 days after application. The plant growth effect was calculated with the formula:

$$\% \text{ Plant growth} = (T / C) \times 100$$

where 'T' is cotyledon, hypocotyl and radical length of treated seedlings and 'C' is also the length of controlled set.

Antifeedant test against termite (Reticulitermes speratus) [32]

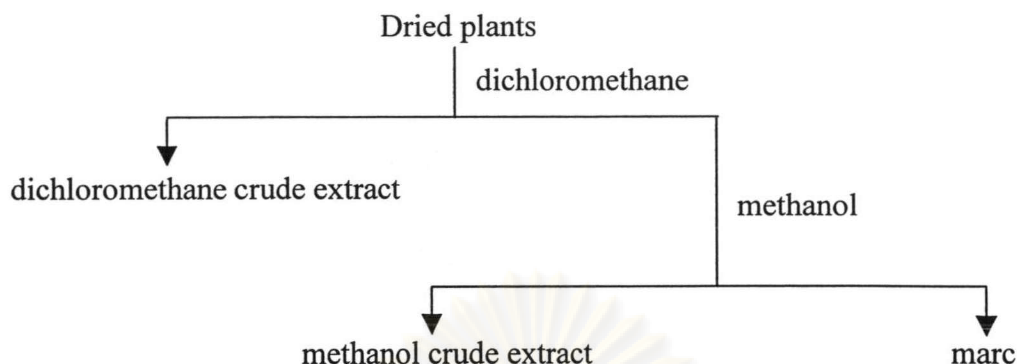
This test was conducted on the petri dishes (43 mm x 8 mm); each was filled with vermiculite moistened with distilled water. Filter papers (Toyo No. 1, 6 mm diameter) were treated with tested compounds dissolved in acetone and then air-dried. Both of 2 treated disks and 2 control disks were placed into the same dish with 20 workers of termite per disk. The petri dishes were placed on the insect raring room (26 °C controlled) for 2 weeks. The evaluation of the activity is similar to the leaf disk bioassay against *S. litura*.

2.5 Extraction procedure

2.5.1 For preliminary screening test

Seventeen Thai plants were extracted with dichloromethane by soaking at room temperature for three days. The solution was filtered and the solvent was evaporated under vacuum. This process was repeated for three times to obtain a dichloromethane crude extract. The marc was then extracted with methanol using the same procedure to receive a methanol crude extract. These two crude extracts were

further subjected to the bioassay activity tests. The scheme for the general extraction is shown in Scheme 2.1.

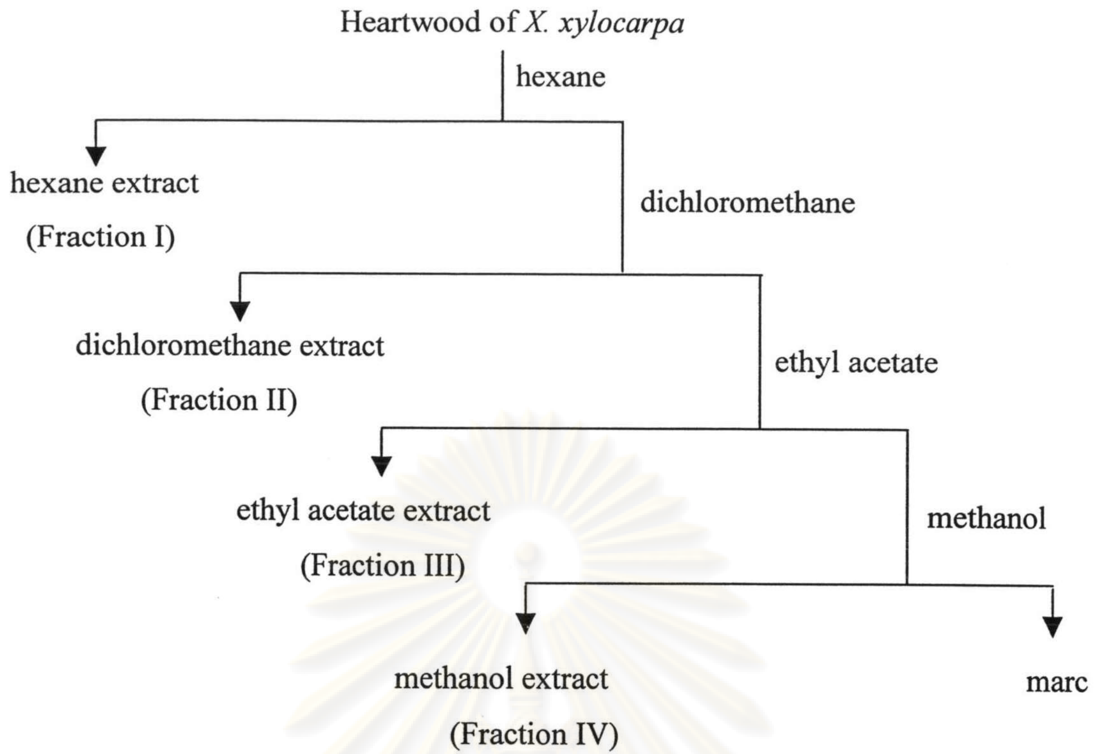


Scheme 2.1 Extraction procedure for preliminary screening test

2.5.2 Extraction of *X. xylocarpa*

The dried heartwood of *X. xylocarpa* was minced into small pieces. The ground heartwood (7 kg) was extracted with hexane, dichloromethane, ethyl acetate and methanol, respectively, by soaking employing the same procedure described above. After the solvent was removed, four crude extracts: hexane (Fraction I), dichloromethane (Fraction II), ethyl acetate (Fraction III) and methanol (Fraction IV) were obtained. The extraction procedure was summarized as shown in Scheme 2.2.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย



Scheme 2.2 The extraction procedure for *X. xylocarpa*

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย