CHAPTER II

EXPERIMENTAL

Plant Material

Various parts of *Xylocarpus granatum*, namely fruits, branches, woods , leaves and seeds were collected at Tum Bon Bang-ta-boon, Chang Wad Phetchaburi during November 1994. The voucher specimens (BKF 54419, 71031, 07705) have been lodged with the Herbarium of the Bangkok Forestry Department. Each part was sun-dried and minced to coarse pieces.

General Procedure

Melting points were determined with a Fishers-Johns melting point apparratus and are uncorrected. Chromatotron equipment on Harrison Research Model 7924T was operated for certain separation.[28] Thin layer chromatography (TLC)[29] was performed on aluminium sheets precoated with silica gel (Merck's, Kieselgel 60 PF_{254}). Column chromatography[30] was performed on silica gel (Merck's, Kieselgel 60G) and flash chromatography[31] was proceeded on silica gel (40 μm average particle diameter).

The IR spectra were recorded on Perkin-Elmer Model TR 781, USA spectrophotometer and Shimadzu corporation , Japan. The ^{1}H and ^{13}C-NMR

spectra including 2D-NMR were performed in deuterated chloroform (unless specified otherwise) with tetramethylsilane (TMS) as an internal reference on Fourier Transformed Nuclear Magnetic Resonance Spectrometer of a Bruker, model AC-F200 and a Joel, model JNM-A500. Gas chromatography analysis was carried out on a Shimudzu Gas Chromatography GC-7AG instrument equipped with flame ionization detecter with N_2 as a carrier gas. The column used for chromatography was OV-1. Mass spectrometry (MS) analysis was conducted on Fisson Instrument Model Trio 2000. Ultraviolet-visible (UV-Vis) spectra were measured on Hewlett Packard Diode array Spectrophotometer. High performance liquid chromatography analysis was performed on a Water ^{TM} 996 , Photo Diode array detector and a Water ^{TM} 600 pump. The analytical column Noval-Pak C_{18}, 39 x 50 mm column was used. The semi-preparative high performance liquid chromatography was carried out on a Gilson Chromatography by UV/Vis detector and a pump model 803C. The column Lichrosorb RP 181q, 125 x 80 mm was used. The optical rotation was performed on a JASCO DIP-360 digital polarimeter, Japan.

Chemicals

All solvents used in this research were purified prior to use by standard methodology except for those which were reagent grade. Merck's silica gel 60 G Art 7734 (70-230 mesh) and silica gel 7749 60 PF_{254} containing gypsum

were used as adsorbents for column chromatography and chromatotron technique, respectively. The silica gel 40 μm average particle diameter from J.T. Baker was used for flash chromatography.

Chemical Tests

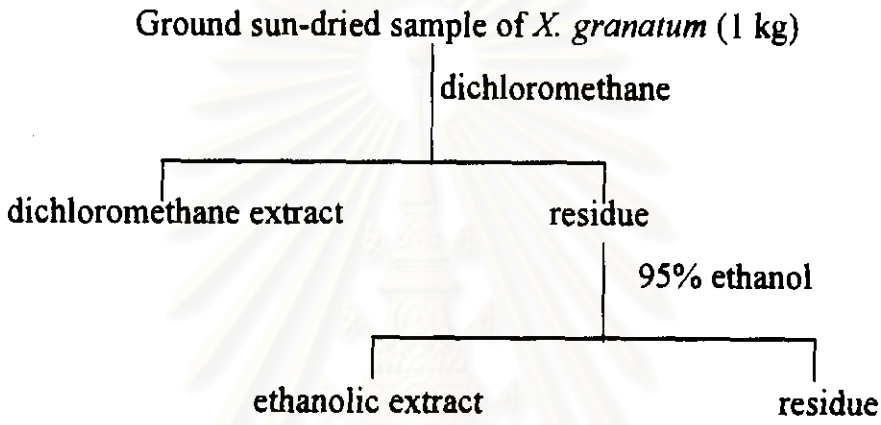
The colour tests : Liebermann Burchard's reagent[32], 2,4-DNP, 5% $FeCl_3$ and Br_2 in CCl_4 [33]

Extraction for Preliminary Screening Test

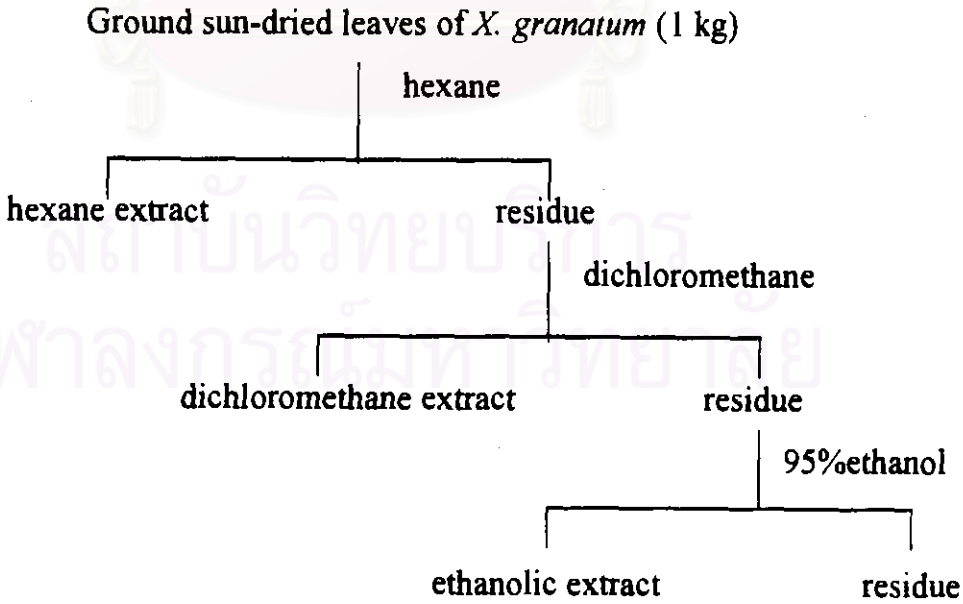
Each part (seeds, fruits, branches, heartwoods and leaves) of *X. granatum* was sun-dried and minced to coarse pieces. The former four samples, approximately 1 kg, was extracted by soaking in dichloromethane for five days at room temperature. The soaking was repeated for several times until the color of the extract was clear. The solution was filtered and the solvent was evaporated, yielding a dichloromethane crude extract. The plant residue was then extracted with 95% ethanol by using similar procedure to that of the extraction with dichloromethane. After evaporation of the solvent *in vaccuo*, the ethanolic extract of each part was obtained. In case of the leave samples, the initial extraction was performed by soaking with hexane prior to the extraction by dichloromethane. Other procedures were carreied out in the same

fashion as described above. The general schemes for extraction for preliminary screening tests are shown in Scheme 2.1 and 2.2.

Scheme 2.1 Extraction Procedure for the seeds, friuts, heartwoods and braches of *X. granatum*.



Sheme 2.2 Extraction Procedure for the leaves of *X. granatum*

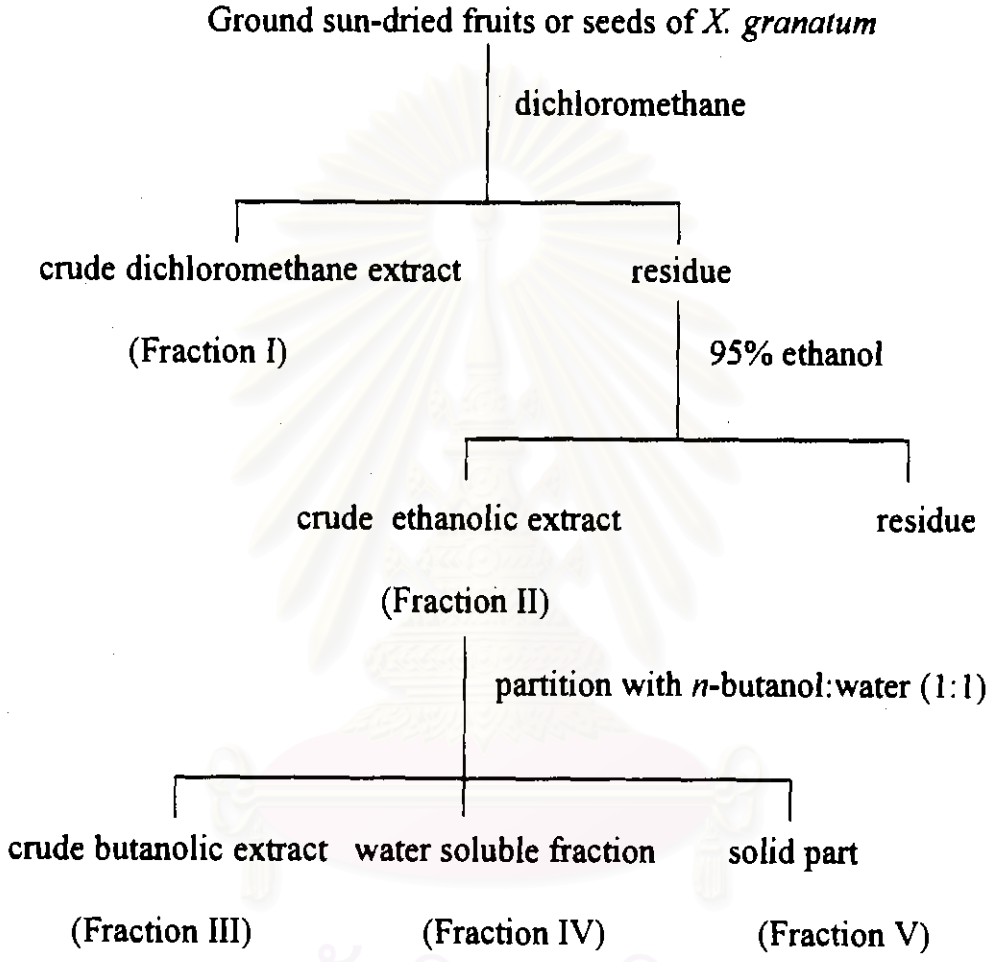


Extraction and Initial Fractionation

According to preliminary antifeedant activity against Greater Wax Moth (*Galleria mellonella*), the fruits and the seeds of *X. granatum* were selected for further investigation.

The ground sun-dried fruits and seeds of *X. granatum* were separately extracted with dichloromethane and ethanol using the same above-mentioned procedure, giving the dichloromethane and ethanolic crude extract, respectively. The ethanolic crude extract was further extracted by partition between *n*-butanol and water in ratio of 1:1 to gain a butanolic soluble fraction, water soluble fraction and a solid part. The general scheme for the extraction and initial fractionations of the seeds and the fruits of *X. granatum* is shown in Scheme 2.3

Scheme 2.3 Extraction and Initial Fractionations Procedure for the fruits and the seeds of *X. granatum*



Bioassay Experiments

As it was mentioned earlier, one of the major goal of this research is to search for an active principle from *X. granatum* which could possibly use for agricultural and/or medicinal proposes. The following bioassay experiments were therefore performed.

Insect Antifeedant Test [34]

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 a square bowl, folded aluminium foil of size 3x3 cm^2. The food bowls were weighed before use. The plant crude extract solutions of various concentrations (percentage wt. by wt. of food) were dripped into food bowl, labeled as "test". The control food was prepared by dripping the same solvent used to dissolve the crude extract or an appropriate solvent. The solvent was allowed to evaporate from each food bowl by air drying for 3 hours. After that each bowl was reweighed and then placed pair-wise (test + control) in a plastic box. Ten worms 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°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 of 100% represents total inhibition of feeding activity.

Insect Antifeedant Test (against *Anthrenocerous australis*) [35]

The extracts of the *Xylocarpus* species or pure compound(s) were supplied in a dry form by the Plant Extract Research unit, Otago University. Two millilitres of chloroform were added to each vial to give solutions of 4 (for pure compound) and 16 (for crude extract) mg/ml. Squares of woollen cloth (2.5 x 2.5 cm, standard abradant fabric, mean weight 164.4 mg) were treated with 100 microlitres of the appropriate solution, air dried overnight at ambient temperatures. This gave rates of 0.24 and 0.97 % w/w. In addition, four replicates were treated with 0.01 % w/w azadirachtin (Green Gold), so that activity could be compared with a chemical of similar structure. The sample were weighed and placed in individually labelled 150 ml plastic pots with well ventilated lids. Controls were cloth squares either left untreatd or treated with dichloromethane or methylated spirits (the diluent in Green Gold). Fifteen *A. austrralis* lavae were placed onto each of four replicates of the controls and

treated cloth, an the bioassay maintained in a controlled environment room at 25° C. After 14 days, larval condition and survival, and wool weight loss (*i.e.* amount consumed) were assessed. The wool weight loss was corrected for moisture content using weight changes observed in moisture content controls.