CHAPTER III

MATERIALS AND METHODS

1. PLANT MATERIAL

Stembarks of *A. julibrissin* were collected from a genuine tree in Korea. They were investigated in collaboration with a taxonomist in Korea to prove that they were authentic and free of any contamination. They were carefully broken into small pieces and dried by shade-drying method to avoid any chemical changes.

2. CHEMICAL STANDARDS

α-spinasterol and α-spinasteryl-D-glucoside standards were previously obtained from *Phytolacca esculenta*. Their chemical structures had already been proved.[27]

Other sugar standards such as glucose, rhamnose, xylose, etc. were obtained commercially.

3. CHEMICAL REAGENTS AND EQUIPMENT

3.1 The solvents being used in this entire experiment were methanol (MeOH), n-hexane (n-C_6H_{14}), chloroform ($CHCl_3$), n-butanol (BuOH), ether (Et_2O) and ethyl acetate (EtOAc). They

were all commercial grades except the last two solvents which were reagent grades. The commercial grade solvents were distilled prior to use. Other chemicals mentioned in the experimental part were all reagent grades and obtained commercially.

Various types of silica gel from E. Merck, Darmstadt Co., Ltd. were used accordingly :

Art 7731 Kieselgel 60 G was used as silica gel for TLC.

Art 7729 Kieselgel 60 was used for column chromatography.

and Art 7736 Kieselgel H (type 60) was for PTLC.

3.2 Melting Point (m.p.)

The melting points were determined by using melting point apparatus Mitamura Riken Co., Ltd. and were uncorrected.

3.3 Ultraviolet (UV) Spectra.

The UV spectra were obtained from a multipurpose recording spectrophotometer MPS-50 L.

3.4 Infrared (IR) Spectra.

The IR spectra were recorded as KBr pellets on the Infrared Spectrophotometer Model IR-S Jasco Japan Spectroscopic Co., Ltd.

3.5 Proton Magnetic Resonance (^{1}H-NMR)

The ^{1}H-NMR spectra were run on a Varian model T-60 A

instrument operating at 60 MHz with a Nicolet, Model TT-7, Fourier Transform attachment. Tetramethylsilane was used as the internal reference and d_6-DMSO as the solvent.

3.6 ^{13}C-NMR Spectra

The ^{13}C-NMR spectra were taken on a varian model FT-80A ^{13}C-NMR instrument, Varian Company, U.S.A. Dimethyl sulfoxide was used as the solvent.

3.7 Mass spectra (MS)

The massspectra were determined using a Varian MAT 112S double focusing mass spectrometer, operating at 70 eV.

3.8 Gas Chromatography (GLC)

The GlC was carried out with a Pye-Unicam Pyes Series 104 Chromatography Philips.

4. PROCEDURES

4.1 PHYSICAL SEPARATION

4.1.1 Column Chromatography

A column chromatography was performed on a glass column using silica gel, Art 7729 Kieselgel 60, as an adsorbent. The size of the column used depended on the amount (weight) of the sample. Normally, a sample consisted of 1-4% (wt./wt.) of the adsorbent and the amount of adsorbent was sufficient to make

a maximum height to diameter ratio of 10:1

A slurry of the silica gel (25% wt./v.) in a suitable solvent was mixed and added to the chromatographic column. A sample was carefully added to the column and drawn onto the adsorbent. A small amount of the solvent was then added and passed through the column. The column was developed by the suitable solvents and the eluent was identified by thin layer chromatography.

4.1.2 Thin Layer Chromatography (TLC)

Thin layer chromatography for qualitative analysis was carried out by using the silica gel for TLC, Art 7731 Kieselgel 60 G., to coat the glass plates. The suspension for five plates (20×20 cm) was prepared by shaking 30 g of the silica gel and 60 cm^3 water for 30 seconds and applied uniformly to a thickness of 0.25 mm with an applicator. After 30 minutes at room temperature, the plates were heated in an oven at 100°C for 1 hour. The plates were used immediately after cooling.

The samples to be identified were dissolved in minimum amounts of methanol and the aliquots were applied on a plate by individual capillary tubes. The plate was developed by a suitable solvent system and the sample spots on the plate were detected by using UV, I_2, NH_3 vapour, and 50% H_2SO_4.

4.1.3 Preparative Thin Layer Chromatography (PTLC)

This was done in the same manner as it was in the case of TLC but using a specific silica gel, either for PTLC, Art 7736 Kieselgel H (type 60), or for column, to coat the plates and the thickness was 0.5 mm. A solvent system of either $CHCl_3$-Me_2CO-HOAc in the ratio of 16:4:1 or $CHCl_3$-MeOH-7% HOAc in the ratio of 25:8:5 v./v. was used to develop the plates. The desired band detected by UV light was extracted with MeOH and the eluted compound was crystallized from a suitable solvent.

4.2 COLOUR TESTS

4.2.1 Liebermann-Burchard Test

This is a test for a steroidal or triterpenoidal nucleus. To a solution of a tested sample (2-3 cm^3) in 0.5 cm^3 of chloroform a few drops of acetic anhydride were added followed by one drop of concentrated sulphuric acid. A change in colour occurred from blue to green within a few minutes suggested the presence of a steroidal or triterpenoidal moiety.

4.2.2 Molisch's Test

This is a general test for a carbohydrate. A sample (5 mg) was placed in a test-tube containing 0.5 cm^3 of water and was mixed with 2 drops of a 10% solution of β-naphthol in alcohol. 1 cm^3 of concentrated sulphuric acid was carefully dropped down

the side of the inclined tube so that the acid formed a layer beneath the aqueous solution. In the presence of a carbohydrate, a red ring appeared at the conjugation of the two liquids, the colour quickly changed on standing or shaking, resulting in a reddish violet solution.

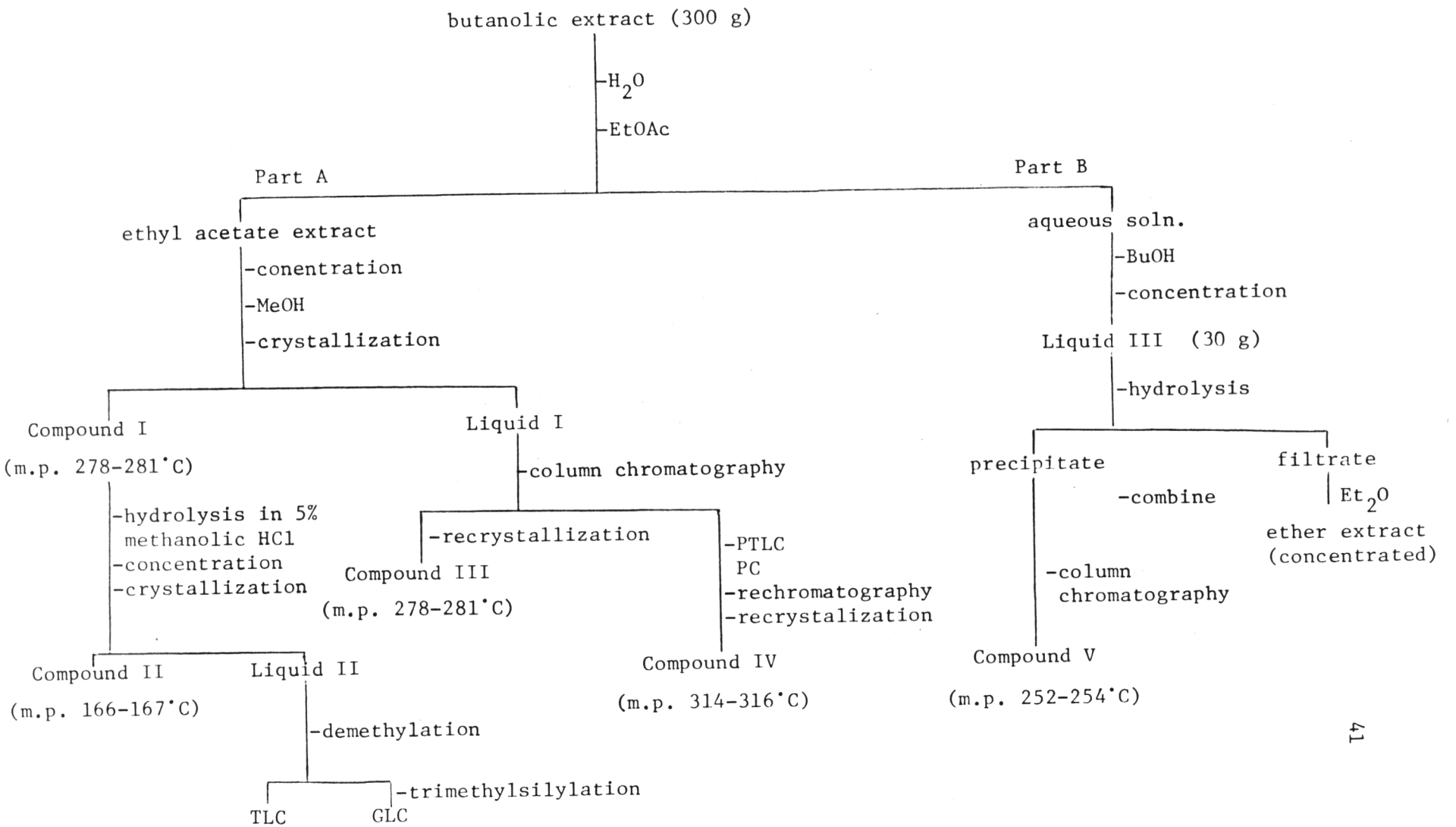
4.3 EXTRACTIONS

The dried plant material (5.4 kg) was cut into small pieces and extracted with hot methanol by refluxing on a water-bath for 3 hours. The methanolic extract was then concentrated by a rotary evaporator under reduced pressure and left at room temperature overnight. It was then filtered and the filtrate was mixed with distilled water in approximately 1:1 (v./v.) ratio. The aqueous mixture was fractionated in a separating funnel with n-hexane (upper layer), followed by chloroform (lower layer) and n-butanol (upper layer). The hexane, chloroform and butanol soluble fractions were concentrated separately under reduced pressure on the rotary evaporator. The fractionation procedure is shown in Scheme II.

4.4 SEPARATION AND PURIFICATION

Separation and purification procedure is presented in Scheme VI.

Scheme VI Separation and purification procedures.



Separation and Purification of the Butanolic Extract

300 g of the crude butanolic extract was dissolved in 300 cm^3 of distilled water and was partitioned with ethyl acetate in separating funnel several times until the color in the ethyl acetate layer (upper layer) disappeared. Two collected fractions, the ethyl acetate soluble and the aqueous soluble, were obtained. They were designated as Part A and Part B.

Separation and Purification of Part A (the Ethyl acetate Extract)

The ethyl acetate soluble extract was concentrated under reduced pressure to a nearly dry syrup. It was then dissolved in a small amount of methanol and kept at room temperature for few days to yield white needles which were recrystallized three times from methanol or ethyl acetate to give 54 mg of white crystals m.p. 278-281°C, designated as Compound I. The mother liquor was designated as Liquid I.

Hydrolysis of Compound I

30 mg of Compound I was dissolved in 10 cm^3 of 5% methanolic HCl. The reaction mixture was refluxed for 5 hours then was cooled by pouring onto ice. The precipitate was filtered and was recrystallized from methanol yielding 10 mg of white crystals, m.p. 166-167°C, designated as Compound II. The mother liquor was designated as Liquid II.

Identification of Liquid II

Demethylation of Liquid II

A sample of Liquid II was concentrated on high vacuum rotary evaporator to give a yellow syrup. The syrup was demethylated by refluxing with 5% aqueous HCl for 5 hours. The reaction mixture was neutralized with Ag_2CO_3 and the white precipitate was filtered off. The filtrate was then evaporated under reduced pressure to yield a concentrated syrup.

TLC of the Demethylated Liquid II

A comparison on TLC between the demethylated Liquid II and raffinose, methyl-β-D-xylose, rhamnose, fucose, arabinose, xylose, mannose, ribose and glucose in $CHCl_3$-MeOH-H_2O (52:25:8 v./v.) as a solvent system revealed that D-glucose was the sole sugar present in the demethylated liquid II.

Trimethylsilylation of Liquid II for GLC

10 mg of the demethylated Liquid II and 10 mg of glucose were trimethylsilylated separately by reacting with 1 cm^3 anhydrous pyridine, 0.2 cm^3 hexamethyldisilazane, and 0.1 cm^3 trimethylchlorosilane. The reaction mixture was shaken vigorously for about 30 seconds and allowed to stand for 5 minutes at room temperature. The reaction mixture was then evaporated to dryness under reduced pressure. Few drops of anhydrous heptane was added followed by filtration to get a clear

solution which was concentrated to about 0.1 cm^3 for injection into the gas chromatograph. Retention times of the two samples were recorded and compared.

Separation and Purification of Compound III and IV

20 g of Liquid I was fractionated by column chromatography using the silica gel for column, Art 7729 Kieselgel 60, as adsorbent. The column was developed with lower layer of the solvent system $CHCl_3$-MeOH-7% HOAc in the ratio of 25:8:5 v./v. The first crystalline Compound eluted from the column was recrystallized from methanol to yield 8 mg of crystals m.p. 278-281°C designated as Compound III.

Second eluted compound was further purified by PTLC, paper and column chromatographies over silica gel, Art 7729 Kieselgel 60, using the same solvent system. Finally, 81.4 mg of the yellow needles m.p. 314-316°C, designated as Compound IV, were obtained.

Separation and Purification of Part B (the Aqueous Soln.)

The total aqueous soluble fraction separated from the extraction with ethyl acetate (Part B) was partitioned between n-butanol and water until a colorless butanolic layer was obtained. The aqueous layer was discarded and the combined butanolic extracts concentrated under reduced pressure to a syrup

which was designated as Liquid III.

Hydrolysis of Liquid III

30 g of Liquid III was hydrolysed by refluxing with 500 cm^3 of 10% aqueous HCl for 3 hours. The precipitate was filtered off and the filtrate was extracted several times with ether until the disappearance of a color in the etheral layer. The combined ethereal extracts were evaporated under reduced pressure and the residue was combined with the previously obtained precipitate. The total precipitate was fractionated by column chromatography over silica gel using the lower layer of mixed solvent system $CHCl_3$-MeOH-7% HOAc in the ratio of 25:8:5 v./v. as an eluent to yield 13 mg of Compound V which was recrystallized from methanol to give white crystals, m.p. 252-254°C.