CHAPTER II

EXPERIMENT AND RESULTS

2.1 Plant Materials

Several parts of Rhizophora apiculata*, i.e., barks, branches, flowers, hypocotyls, leaves, heartwoods, overground and underground supporting roots, were collected at Klong Wat Mai distric, Samutsongkram province during October 1985. Each part was sun-dried and milled to coarse powder.

2.2 Instruments and Equipment

2.2.1 Infrared Spectra (IR)

The IR spectra were recorded on either a Shimadzu Spectrophotometer Model IR 440 or a Perkin-Elmer Model IR 718 and IR 1430 Infrared Spectrophotometer and calibrated with a polystyrene film. Solid samples were examined by incorporating the sample into a pellet of potassium bromide.

2.2.2 Ultraviolet and Visible Spectra (UV and VIS)

The UV and VIS spectra were determined by a Shimudzu UV-VIS Spectrophotometer Model 240 (wavelength range : 190-900 nm.

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* Identified as R. apiculata Bl. by Associate Professor Amorn Ubonchonlaket and Associate Professor Pipat Patanaponpaiboon (Department of Botany, Chulalongkorn University).

with double beam photometric system).

2.2.3 Mass Spectra (MS)

The mass spectra were obtained by a Jeol Mass Spectrometer Model JMS-DX-300/JMA 2000 at 70 eV.

2.2.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H NMR and ^{13}C NMR)

The ^{1}H and ^{13}C NMR spectra were performed using a Jeol Fourier Transform NMR Spectrometer Model FX 902. Tetramethylsilane (TMS) was used as an internal standard. The chemical shifts (δ) were given in ppm. down field from the TMS.

2.2.5 Elemental Analyses

The elemental analyses were made by using a Perkin Elmer CHNO Analyzer Model 240C.

2.2.6 Gas Liquid Chromatography (GLC)

The GLC analysis was carried out by using a Shimadzu Gas-liquid Chromatograph Model GC-7AG.

2.2.7 Flame Emission Spectrometer (FES)

The flame emission data was obtained by a Flame Emission Spectrophotometer Model Corning 400.

2.2.8 X-ray Fluoresence Spectrometer (XFS)

The X-ray fluoresence data was obtained by a Jeol X-ray Fluoresence Spectrometer Model JSX-60 PA.

2.2.9 Amino Acid Analyzer (AAA)

The amino acid analysis results were made by a

Hitachi Amino Acid Analyzer Model 835-50.

2.2.10 High Performance Liquid Chromatography (HPLC)

The HPLC analysis was carried out by using a Shimadzu High Performance Liquid Chromatography Model LC-3A.

2.2.11 Inductive Coupled Plasma Spectrometer (ICPS)

The ICP analysis results were obtained from a Shimadzu Ion Coupled Plasma Spectrometer Model ICPS-50.

2.2.12 Melting Point (m.p.)

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

2.3 Chemical Reagents

All solvents used in this research were purified prior to use by usual methods (55), except solvents that were reagent grade.

2.4 Physical Separation Techniques

2.4.1 Quick Column Chromatography (56)

This method is especially useful for separating large quantities of complex mixtures which were obtained from natural resources into fractions. Because of its speed and separating power, it can also be used for separating the reaction mixture.

Packing the column

The column was a glass column of 14.0 cm. diameter with sinter glass frit. Silica gel was added to the column and distributed evenly over the surface. The vacuum was then applied (water pump) and the silica gel was allowed to settle. Any cracks

which developed were pressed with a glass rod and more silica gel was added to give a packed bed of 6.0 cm. or less. When the bed was compressed, the application of vacuum was continued and the column was ready to be charged with the extract.

Separating the extract on the column

The extract was dissolved in small amount of chloroform and added directly on the column. It was performed to add a portion of solvent before adding the extract to ensure that the surface was wetted evenly to ensure smooth flowing of the solution in the column. When the column was about to go dry, add the extract quickly but gently in one portion to the top of the column, and 750 mL. fractions were collectd. Polarity of solvents was changed from n-hexane to a mixture of chloroform-hexane, chloroform, a mixture of chloroform-methanol and methanol, respectively.

2.4.2 Column Chromatography (CC) (57)

Merck's silica gel 700-325 mesh ASTM and aluminium oxide active, neutral for column chromatography, were used as adsorbents. The ratio of the mixture to be separated to the adsorbent used was approximately 1:30-40 by weight.

Packing the column

Pre-adjust the stopcock so that it was almost closed, but still allow the solvent to drip through. Allowed approximately 0.5 cm. of solvent to remain in the column when the slurry of the silica gel was poured. After the bed has settled, a paper disk, prepared from 3 mm. paper was inserted in the column. The sample was dissolved in the solvent as a 10% (w/v) solution and it was introduced when the solvent level was just above the paper disk in

the column. If the material was not soluble in the developing solvent, one could always dissolve the sample in which it was soluble. The solution was next added to a minimum quantity of adsorbent in a boiling flask and then removed the solvent in vacuum. The dried powder was sprinkled on the top of the column and the disk. Some adsorbent was usually added to the top of the column prior to the addition of the eluent.

2.4.3 Thin-layer Chromatography (TLC) (58)

The 0.25 mm. thin-layer chromatoplate was prepared in the following manner:

A mixture of silica gel (25.0 g.) and water (50.0 mL.) was stirred until it became slurry; it was then applied to glass plates (20x20 cm.) using a desaga spreader. After being dried at room temperature for an hour, the plates were heated at 125°C for 30 min., cooled and stored in a desiccator until use.

Two lines were drawn on each plate, one 2.0 cm. from one edge; this line will be referred to the base line. The other line is 14.0 cm. above and parallel to the first line called the upper line. The solution containing the compounds to be investigated was applied as small spots on the base line of the plate at 1.0 cm. interval, and after the solvent had evaporated, the plate was placed in a glass tank filled to a depth of 1.0 cm. with the eluting solvent and the tank was covered with a glass cover. The eluting solvent moved up the plate immediately. The rate of solvent moving became slower as the distance between the solvent front and the base line increased. The time taken for the solvent front to reach the upper line varied according to the solvents used; time between 20 to

45 min. was generally encountered. When the solvent front had reached the upper line, the plate was removed from the tank; the solvent was allowed to evaporate, and the plate was detected with a suitable detector such as UV, I_2 and 25% sulfuric acid to reveal the compounds.

2.4.4 Preparative Thin-layer Chromatography (PTLC) (58)

PTLC plates are prepared in the same ways as described for TLC plates, but PTLC plates are always coated thicker than the TLC ones.

The sample solution was applied as narrow band about 2.0 cm. from the edge of the plate. The plate after being dried by the air current, was developed by the ascending method. The components were detected by viewing under the UV lamp and appeared as purple bands against the white background of the adsorbent. These bands were cut out, dispersed in diethylether (or other appropriate solvents) contained in a flask. After shaking the diethylether mixture for a few minutes, it was filtered by suction. (The sohxlet apparatus can be used for continuous extraction).

2.2.5 Paper Chromatography (PC) (59)

Paper chromatography is somewhat similar to TLC except that high grade filter paper is used as the adsorbent as solid stationary phase. It is widely used preliminary when extremely polar or polyfunctional compounds, for example, sugars and amino acids, are to be separated. However, the selection of solvent systems for paper chromatography is very important. The brief procedure for preparing paper chromatography is shown below:

Place a light pencil mark about 1.0 cm. from the bottom of the strip cut from Whatman No.1 filter paper in size 9.0x44.0 cm. The sample was applied as a small spot with a micropipette at the center of the line and allowed to dry. The sample size should be about 10.0 g. Using the descending method for developing in the tank 10.0x48.0 cm., this process may take up to 14-18 hours. The paper was removed and allowed to dry and the compounds were visualized with an appropriate detector.

2.5 Colour Tests

2.5.1 Liebermann-Burchard's Test (60-61)

To a solution of the sample to be tested (2-3 mg.) in chloroform (0.5 mL.) was added a few drops of acetic anhydride, followed by one drop of concentrated sulfuric acid. Developement of the colour after a few minutes suggests the presence of steroids or triterpenoids.

2.5.2 Molisch's Test (55)

This is general test for carbohydrates. 5 mg. of the sample was placed in a test tube containing 0.5 mL. of water and it was mixed with 2 drops of a 10% solution of β-naphthol in alcohol. 1 mL. of concentrated sulfuric acid was dropped down the side of the inclined tube so that the acid formed a layer beneath the aqueous solution without mixing. If a carbohydrate was present, a red ring appeared at the common surfaces of the liquids, the colour quickly changed on standing or shaking, resulting in a dark purple solution.

2.5.3 Dragendroff's Reagent Test (62)

This is a test for alkaloid nucleus. Add a few drops of Dragendroff's reagent into the sample solution yielded orange precipitate. The positive test suggests the presence of alkaloid nucleus.

2.5.4 Kraut's Reagent Test (62)

This is also a test for alkaloid nucleus. The sample solution was added a few drops of Kraut's reagent yielded brown precipitate. This positive test exhibits the presence of alkaloid nucleus.

2.5.5 Shinoda's Reagent Test (62)

This is a test for flavonoid compounds. To an alcoholic solution (1.0 mL.) of the sample (2-3 mg.) was added a few pieces of magnesium and 1-3 drops of concentrated hydrochloric acid. Any colour developed within a few minutes was observed. The shade of colour is suggestive of the class of flavonoid substances.

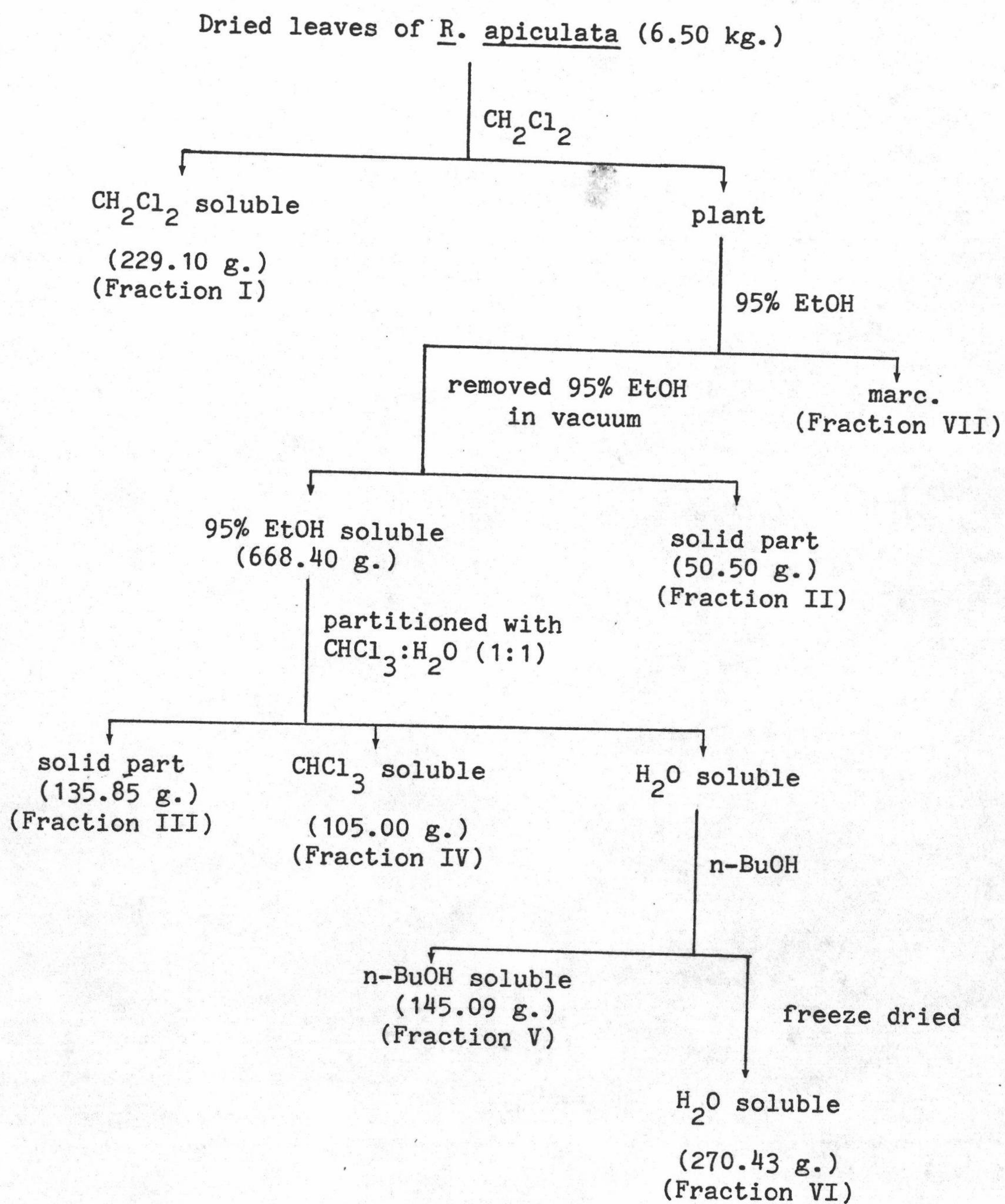
Other reagents for colour tests used in this course of research, such as 2,4-DNP, 5% $FeCl_3$, Br_2 in CCl_4, $KMnO_4$, Benedict's and etc. were carried out and observed by following the textbook of Practical Organic Chemistry (55) and the Systematic Identification of Organic Compounds. (63)

2.6 Extraction and Initial Fractionations

2.6.1 The ground sun-dried leaves (6.50 kg.) were extracted by soaking in dichloromethane for five days at room temperature. The soaking was repeated seven times. After evaporation the solvent, the

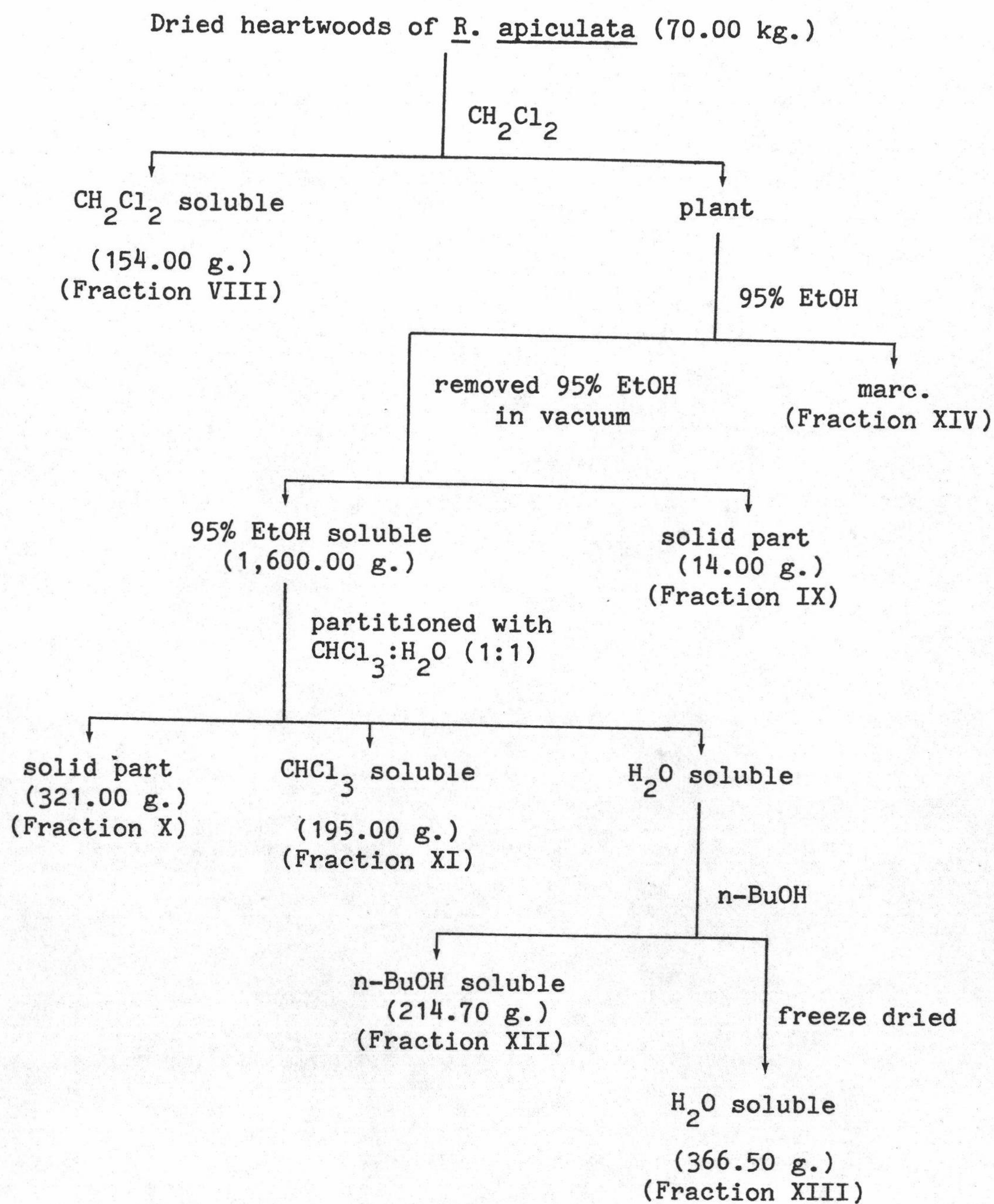
dichloromethane extracts, Fraction I, was obtained as a greenish crude 229.10 g. (4.60% wt. by wt.). The marc was then similarly extracted with 95% ethanol. The ethanolic extract was concentrated in vacuum to deposite nearly white solid, Fraction II, 50.50 g. (0.78% wt. by wt.). After filtration, the ethanol was completely removed from the extract to obtain a greenish crude 668.40 g. (10.28% wt. by wt.). The ethanolic crude was further extracted by partition between chloroform and water in ratio 1:1 by liquid-liquid extractor to gain chloroform soluble, water soluble and a solid part as red-brownish material 135.80 g. (2.09% wt. by wt.). The solid part which was insoluble in both chloroform and water was separated and labelled as Fraction III. The chloroform soluble part was dried over anhydrous sodium sulfate. After evaporation of chloroform, a dark greenish crude (Fraction IV) 105.00 g. (1.22% wt. by wt.) was obtained. The aqueous layer was extracted further with n-butanol to afford a reddish syrup of n-butanolic crude extract, Fraction V, 145.10 g. (2.23% wt. by wt.). The water soluble part after extraction with n-butanol was then freeze-dried to yield 270.40 g. of water soluble fraction, Fraction VI, (4.16% wt. by wt.). The 95% ethanol-exhausted marc was assigned as Frraction VII. The various extraction was processed as shown in Scheme 2.1.

Scheme 2.1 Extraction and initial fractionations procedure for the leaves of *R. apiculata*



2.6.2 The ground sun-dried heartwoods (70.00 kg.) were extracted in similar way to Topic 2.6.1 yielded dichloromethane extract as a yellow-brownish crude 154.00 g. (0.22% wt. by wt.) and deposited solid 14.00 g. (0.02% wt. by wt.) assigned as Fraction VIII and IX, respectively as well as ethanolic extract as a reddish crude 1,600.00 g. The ethanolic crude extract was then partitioned between chloroform and water in ratio 1:1 to gain a solid part as brown-reddish material which was insoluble in both chloroform and water, 321.00 g. (0.45% wt. by wt.), Fraction X, chloroform soluble as a brownish crude 195.00 g. (0.28% wt. by wt.), Fraction XI, and water soluble part as a reddish syrup, respectively. The aqueous layer was further extracted with n-butanol to afford n-butanolic soluble, Fraction XII, 214.70 g. (0.31% wt. by wt.). The water soluble part after extraction with n-butanol was then freeze-dried to yield a reddish crude 366.50 g. of water soluble fraction, Fraction XIII, 0.52% wt. by wt.. Fraction XIV was designated for the 95% ethanol-exhausted marc fraction. The various extraction and initial fractionations were processed as shown in Scheme 2.2.

Scheme 2.2 Extraction and initial fractionations procedure for the heartwoods of *R. apiculata*



2.6.3 The ground sun-dried barks, branches, flowers, hypocotyls and supporting roots were also extracted with dichloromethane and 95% ethanol, respectively for phytochemical screening tests as well as bioassay experiments. The procedure of extraction was shown in Scheme 2.3 and the results of extraction were presented in Table 2.1.

Scheme 2.3 Extraction for barks, branches, flowers, hypocotyls and supporting roots (overground and underground) of *R. apiculata*

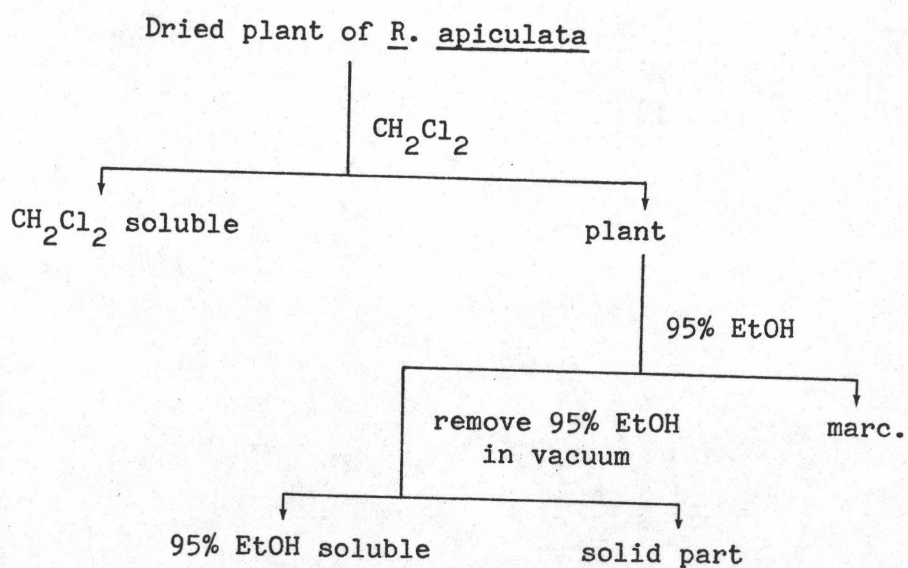


Table 2.1 The results of extraction from several parts of *R. apiculata*

Plant parts	Weight of plant (kg.)	Weight (g.) and percentage yield (% wt. by wt.)		
		dichloromethane crude extract	95% ethanolic crude extract	deposited solid
barks	1.00	7.50 (0.75)	168.00 (16.80)	2.20 (0.22)
branches	1.00	12.50 (1.25)	109.90 (10.99)	11.70 (1.17)
flowers	0.15	0.90 (0.60)	2.75 (1.83)	trace
hypocotyls	0.15	1.45 (0.97)	3.20 (2.13)	trace
leaves	6.50	229.10 (4.60)	668.40 (10.28)	50.50 (0.78)
heartwoods	70.00	154.00 (0.22)	1600.00 (2.28)	14.00 (0.02)
roots	1.00	7.00 (0.70)	57.80 (5.78)	0.80 (0.08)

2.7 Phytochemical Screening Tests

Every specimen of *R. apiculata* (barks, branches, flowers, hypocotyls, leaves, supporting roots (over and underground) and heartwoods) was extracted following the phytochemical screening techniques (62). The tests for alkaloids, cardiac glycosides, flavonoids, anthraquinone glycosides, tannins and polyphenols were conducted on 80% ethanolic extracts. For saponin tests, the additional test on the aqueous was performed and for the coumarin tests, the moist ground plants were used. The results of phytochemical screening tests were presented in Table 2.2.

Table 2.2 The results of phytochemical screening tests.

Plant parts	Alkaloid			Cardiac glycoside			A.G.	Flavoniods			Sap.	Cou.	Tan.
	Pri.	Con.	Qua.	L.B.	Ked.	K.K.		Oct.	Aq.	Leu.			
bark	+	+	-	+*	-	+	-	R	O	+	-	-	+**
branch	+	+	+	+*	-	+	-	O	Y	+	-	-	+**
flower	+	+	-	+*	-	+	-	O	Y	+	-	-	+**
hypocotyl	+	+	-	+*	-	+	-	O	O	+	-	-	+**
heartwood	++	+	+	+*	-	+	-	Y	Y	+	-	-	+**
leave	+	+	-	+*	-	+	-	O	O	+	-	-	+**
root	++	+	+	+*	-	+	-	O	Y	+	-	-	+**

Abbreviations:

A.G. anthraquinone glycoside
Sap. saponin
Cou. coumarin
Tan. tannin
Pri. preliminary test for alkaloid
Con. confirmed test for alkaloid
Qua. quarternary test for alkaloid

L.B. Liebermann-Burchard's test
Ked. Keddy reaction test
K.K. Keller and Kilani reaction test
Oct. octacosyyl layer
Aq. aqueous layer
Leu. leucoanthocyanin
R red
O orange
Y yellow

* positive test to both steroids and triterpenoids
** positive test to both hydrolysable and condensed tannin
\+ positive test
\- negative test

2.8 Bioassay Experiments*

2.8.1 Antifungal and Antibacterial Studies

This procedure involves the paper disc method. Commercial culture media (Potato-Dextrose agar) was prepared following to the directions on the container and then autocleaved at approximately 15 psi. for 20 mins.. The culture media will then be poured into petri dishes. Filter discs (6 mm. diameter) will be soaked in the test solution and then placed on cultural medium around the perimeter of the plate using sterile forceps. The plate is then inoculated with the fungus or the bacteria. As the fungus or the bacteria grow outward from inoculum or culture media, the black (with gray, fuzzy spots) mycelium approaches the treatment discs. The irregular zone of inhibition will be observed and noted. The fungi, Helminthosporium teres (H), Rhizoctonia solani (R) and Pythium ultimun (P) and the bacteria, Xanthramonas campestrous (XC) are used in this study.

$$\% \ T/C = \frac{\text{Inhibition zone radius in mm. caused by sample}}{\text{Inhibition zone radius in mm. caused by control}} \times 100$$

2.8.2 Boll Weevil Antifeedant Studies

The mixture of the dried cotton boll and the agar were made in the form of plugs by the procedure developed by P.A. Hedin in 1966 with a few modifications. 3.0 g. of freeze-dried

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* The bioassay experiments were carried out at Mississippi State University, USA.

cotton boll and 3.0 g. of agar were mixed in 100.0 mL. of distilled water and boiled to form the viscous solution. The solution was poured into 13 mm. diameter hollow glass rods and formed gellatin after cooling. These gellatinous rods were cut to individual 3.6 cm. plugs.

The plant extracts being tested were introduced onto 4 cm. squares of Whatman #1 chromatography paper by dipping the papers into it. After air drying, the papers were weighed. It was repeated until the sufficient amount was reached. Control papers were prepared by dipping the same size of paper into the solvent of the extract and let them dry. The papers were around the agar cotton plugs and fastened with staples. The ends of the plugs were placed staple-side down in the petri dishes so that the boll weevils could feed only by puncturing the papers.

Twenty newly emerged boll weevils were placed the petri dishes containing the test and control plugs. The bioassay was carried out in the dark at 80 °F for 4 hours. After the papers were removed, the punctures were counted.

Antifeedant activity was expressed as a % T/C value, where:

$$\% \text{ T/C} = \frac{\text{\# punctures on test paper}}{\text{\# punctures on control paper}} \times 100$$

% T/C values of zero represents total inhibition of feeding, while values greater than 100 represents feeding activity.

The bioassay results of preliminary antifungal, antibacterial and antifeedant activity were presented in Table 2.3.

The antifungal and antibacterial bioassay results of the crude extracts of various parts of R. apiculata were exhibited in Table 2.4.

The boll weevil antifeedant bioassay results of the crude extracts of the leaves of R. apiculata were shown in Scheme 2.4.

The boll weevil antifeedant bioassay results of the crude extracts of the heartwoods of R. apiculata were given in Scheme 2.5.

Table 2.3 The bioassay results of preliminary antifungal, antibacterial and antifeedant activity

Plant sample	Solvent extract			
	CH_2Cl_2		EtOH	
	Antifungal*	Boll weevil Antifeedant	Antifungal*	Boll weevil Antifeedant
R. apiculata (branches and leaves)	+	43 (% T/C)	++++	150 (% T/C)

* for antifungal activity against Helminthosporium teres

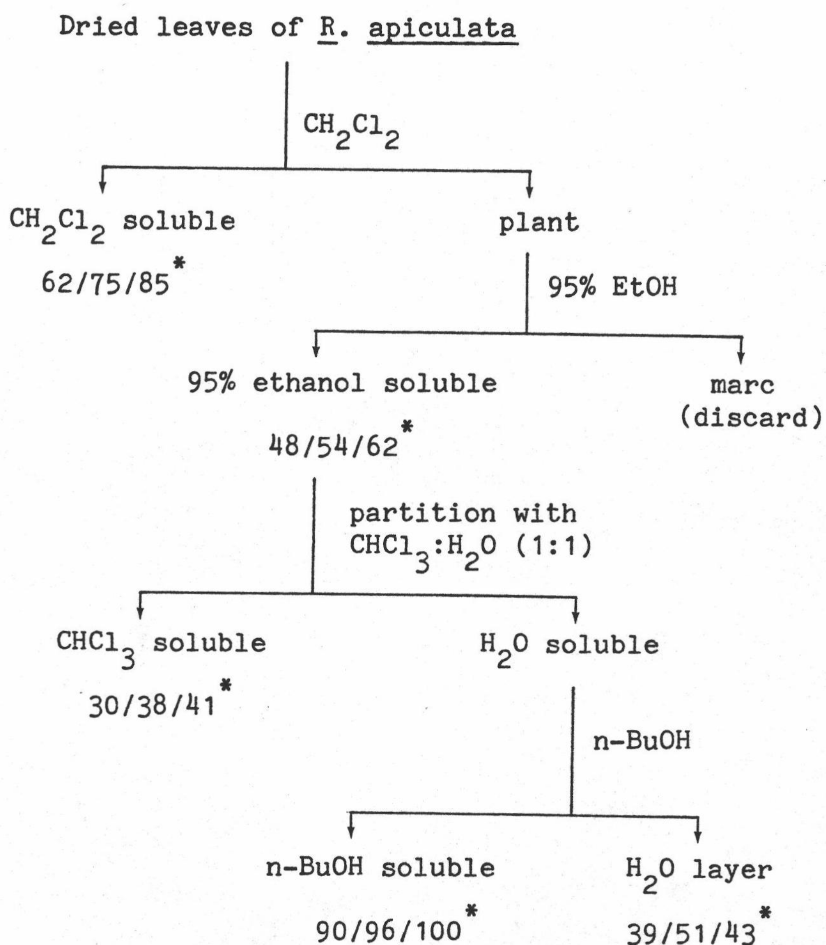
Note + weakly active ++ moderately active

+++ highly active ++++ extremely active

Table 2.4 The antifungal and antibacterial bioassay results of the crude extracts of various parts of *R. apiculata*

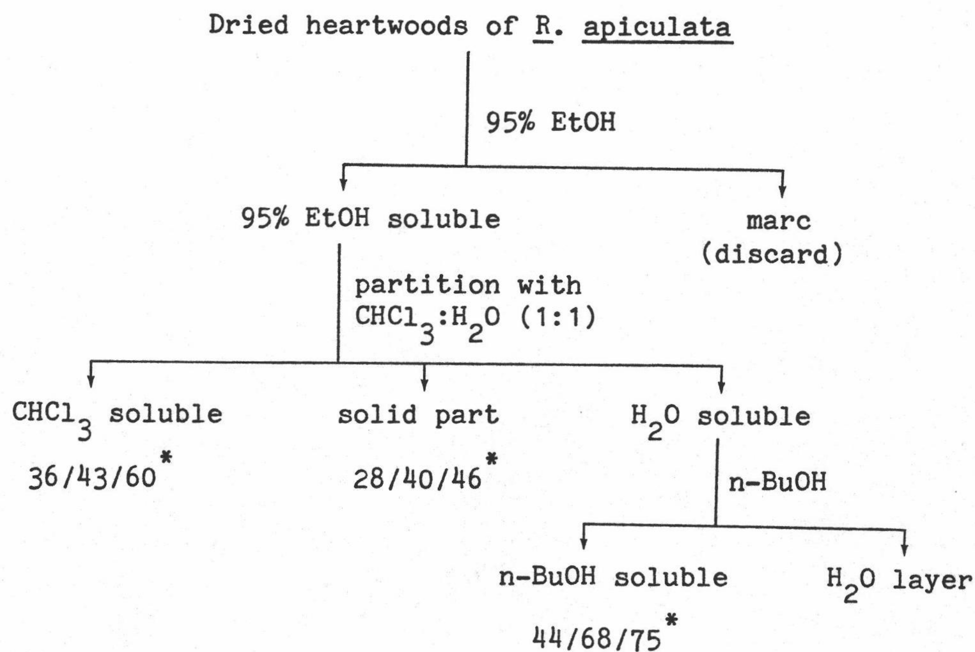
Plant parts	Solvent extract	% Inhibition			
		fungi			bacteria
		P	R	H	XC
leaves	CH_2Cl_2	-	-	-	25
	EtOH	-	-	-	-
	$CHCl_3$	-	-	-	12.5
	H_2O	-	-	-	25
barks	CH_2Cl_2	-	-	-	31.3
	EtOH	-	-	-	-
heartwoods	CH_2Cl_2	175	133	-	100
	EtOH	-	-	-	-
branches	CH_2Cl_2	-	-	-	12.5
	EtOH	-	-	-	-
supporting	CH_2Cl_2	-	-	-	38
roots	EtOH	-	-	-	-

<u>Scheme 2.4</u> The boll weevil antifeedant bioassay results of the crude extracts of the leaves of *R. apiculata*



* % inhibition of feeding against boll weevil indicated amount of samples use 10 mg./20 mg./30 mg., respectively.

<u>Scheme 2.5</u> The boll weevil antifeedant bioassay results of the crude extracts of the heartwoods of <u>R</u>. <u>apiculata</u>



* % inhibition of feeding against boll weevil indicated amount of samples use 10 mg./20 mg./30 mg., respectively.

Part I Chemical Constituents of the Leaves of *R. apiculata* Bl.

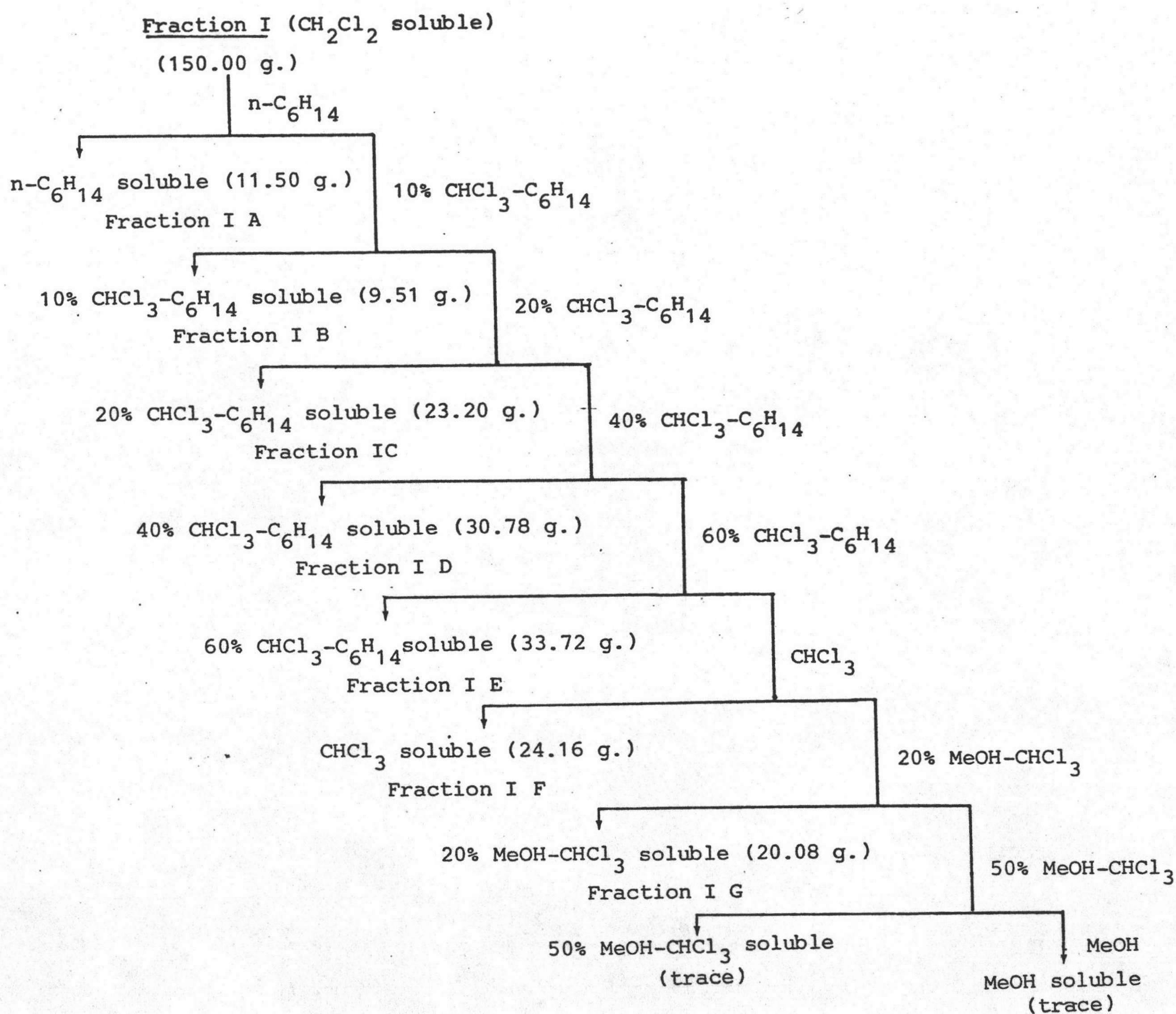
2.9 Separation of Fraction I

The dichloromethane extract, Fraction I, was concentrated to give a greenish sticky liquid of 229.10 g. (4.60% wt. by wt.) (see also Scheme 2.1). Thin-layer chromatography of concentrated extract showed that there were at least four components in this crude (solvent: chloroform). The portion of the concentrated 150.0 g. was chromatographed on silica gel using the quick column chromatography technique to separate Fraction I into small fractions due to their polarities. The column was initially eluted with n-hexane and changed to chloroform by gradual introduction of the latter. Finally, the column was stripped with methanol. The eluted solution was collected approximately 750.0 mL. for each fraction. Each one was monitored by using thin-layer chromatography technique and the equivalent fractions were combined. The results of separation Fraction I into small fractions were presented in Table 2.5 and the fractionation procedure by various solvents was shown in Scheme 2.6.

Table 2.5 The results of separation Fraction I by silica gel quick column

Eluents	No. of fraction. (750 mL.)	Remarks	Weights (g.)
n-hexane	10	golden oil + white ppt. (Fraction IA)	11.50
10% chloroform-hexane	10	yellow-greenish oil (Fraction IB)	9.51
20% chloroform-hexane	12	orange oil + needle ppt. (Fraction IC)	23.20
40% chloroform-hexane	11	dark greenish oil (Fraction ID)	30.78
60% chloroform-hexane	14	dark-greenish oil (Fraction IE)	33.72
chloroform	10	dark-greenish oil (Fraction IF)	24.16
20% methanol-chloroform	10	dark-greenish oil (Fraction IG)	20.08
50% methanol-chloroform	2	yellow oil	trace
methanol	2	yellow oil	trace

<u>Scheme 2.6</u> The fractionation procedures of Fraction I using quick column chromatography



2.9.1 Separation of Fraction IA

Thin-layer chromatography of Fraction IA revealed two major spots in its crude (solvent: 5% chloroform-hexane). The concentrated extracts of Fraction IA (11.50 g., 9.61% wt. by wt. of Fraction I) was then chromatographed on silica gel 300.0 g. using n-hexane and a mixture of n-hexane and chloroform as eluents. The eluted solution was collected about 250.0 mL. for each fraction. Each fraction was monitored by TLC technique and the equivalent fractions were combined. The results of separation this fraction were shown in Table 2.6.

Table 2.6 The results of separation Fraction IA by silica gel column (using column 2.5 x 120.0 cm.)

Eluents	Fraction No. (250 mL.)	Remarks	Weights (g.)
n-hexane	1-7	pale yellow oil + white ppt. (Fraction A)	0.79
	8-11	yellow oil	0.45
	12-30	yellow oil + white ppt. (Fraction B)	
5% chloroform-hexane	31-35	yellow oil	0.02
10% chloroform-hexane	36-40	yellow oil	0.02

2.9.1.1 Purification and Properties of Compound 1

The amorphous product with pale yellow oil, Fraction A, was obtained from the combination of Fraction 1-7 (see Table 2.6). It was purified by recrystallization from ethylacetate for several times to afford Compound 1 as bright white plate, 600 mg. (5.21% wt. by wt. of Fraction IA), m.p. 66.0-66.5 °C. (Rf 0.81, solvent: 3% dichloromethane-hexane). This compound was soluble in n-hexane, dichloromethane and was slightly soluble in ethylacetate and ethanol. It gave negative results to all these reagents: Liebermann-Burchard's, $FeCl_3$, 2,4-DNP, Br_2 in CCl_4 and $KMnO_4$ reagents.

The IR spectrum (Fig.7) showed the absorption peaks at ν_{max}^{KBr} (cm^{-1}): 2960,2920,2850 (C-H stretch.), 1475,1465,1375 (C-H bend.) and 730,720 (C-H rock.).

The mass spectrum (Fig.8) gave the molecular ion peak, M^+, at m/e (% rel int.) 464.0 (0.20) (Calcd. for $C_{33}H_{68}$: MW. 464.90) and other important fragmentation ion peaks at m/e 436.0 (6.10), 422.0 (1.40), 408.0 (5.30) together with other ion peaks corresponded to be lost $-CH_2-$ (m/e 14) step by step.

The GLC analysis (Fig.9) (condition: column 5% apiezon-L SW 60/100 mesh AW DMCS 1/8 inch x 7.55 fts., column temp. 300 °C, FID 300 °C, carrier gas N_2 50.0 mL./min.) revealed seven peaks on gas chromatogram at retention time 3.73, 4.76, 6.11, 7.83, 10.13, 12.93 and 16.60 mins., respectively.

Compound 1 did not show any absorption peaks in UV region.

2.9.1.2 Purification and Properties of Compound 2

Fraction B which was obtained from the concentration of the combination of Fraction No. 12-30 (see Table 2.6) was eluted from the silica gel column of Fraction IA with n-hexane. After washing yellow oil with methanol and recrystallization with a mixture of chloroform and acetone for several times, Compound 2 as white amorphous product, 4.98 g. (43.30% wt. by wt. of Fraction IA), m.p. 73.0-75.0 °C, was obtained. TLC exhibited only one spot at Rf 0.69 (solvent system: 20% chloroform-hexane). This compound was soluble in hot n-hexane and chloroform, but slightly soluble in acetone and methanol. Compound 2 gave a violet colour with Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.11) revealed the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 1735 (s,$-\overset{O}{\overset{\|}{C}}-O-$) and 1260, 1240, 1170, 1140 and 1100 (m,C-O).

The mass spectrum (Fig.12) gave the molecular ion peak, M^+, at m/e (% rel int.) 664.0 (0.6, calcd. for $C_{46}H_{80}O_2$: MW. 664.63) and other important peaks at m/e 649.0 (0.20, M^+-CH_3), 408.0 (32.70, $M^+-C_{16}H_{32}O_2$) and 256.0 (47.80, M^+-408).

The ^1H NMR ($CDCl_3$) spectrum (Fig.13) exhibited the proton signals at δ (ppm.): 5.18 (1H,t,J=3.51 Hz, olefinic proton), 4.52 (1H,t,J=7.90 Hz), 2.30 (2H,t,J=7.03 Hz), together with signals around 2.22 to 0.86 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.14) showed the carbon signals at δ(ppm.): 173.63 (carbon of ester), 145.19 and 21.62 (olefinic carbons) and other signals around 47.51 to 14.08 ppm..

The UV ($CHCl_3$) spectrum (Fig.15) gave the λ max at 203 nm. (log ε = 5.80) and 287 nm. (log ε = 3.99).

Hydrolysis of Compound 2

A solution of 10% ethanolic KOH (40.0 mL.) was added to Compound 2 (2.0 g.) and the mixture was heated under refluxing on a water bath for 4 hours (Check whether the reaction was completed or not by TLC). Evaporation of ethanol gave a solid which was further extracted with diethylether, 150.0 mL., three times. The combined diethylether was dried over anhydrous calcium chloride. Evaporation the solvent furnished a solid with pale yellow oil. After recrystallization this solid with a mixture of chloroform and methanol, bright white needle designated as Compound 2A (80 mg.), m.p. 196.0-198.0 °C, was obtained.

Acidification of the solid which was remained after extraction with diethylether afforded another impure solid. It was recrystallized for several times with a mixture of chloroform and methanol to yield Compound 2B (30 mg.), m.p. 62.5-64.0 °C.

Study on Compound 2A

Compound 2A as bright white feather (80 mg.), m.p. 196.0-198.0 °C, was obtained from basic hydrolysis of Compound 2. TLC of this compound showed a single spot at Rf 0.37 (solvent: chloroform). It was soluble in chloroform and dichloromethane, but slightly soluble in methanol. Compound 2A gave a violet colour with Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.16) showed the major absorption bands at ν_{max}^{KBr} (cm^{-1}): 3290 (b,O-H), 1645 (w,C=C) and 1040 (m,C-O).

The mass spectrum (Fig.17) displayed the molecular ion peak, M^+, at m/e (% rel int.) 426.0 (6.30) (calcd. for $C_{30}H_{50}O$: MW. 426.39) and other fragmentation ion peaks at m/e 411.0 (1.70, M^+-CH_3), 408.0 (32.70, M^+-H_2O) and 218.0 (100.00).

The ^1H NMR ($CDCl_3$) spectrum (Fig.18) revealed the proton signals at δ (ppm.): 5.19 (1H,t,J=3.50 Hz, olefinic proton), 3.23 (1H,m) and other signals around 1.89-0.71 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.19) exhibited the carbon signals at δ (ppm.): 145.19 and 121.73 (1C each, olefinic carbon), 79.07 (1C) and signals around 55.20 to 15.60 ppm..

Acetylation of Compound 2A (63)

Compound 2A (50 mg.) was mixed with acetic anhydride (1.0 mL.) and a few drops of dry pyridine. The mixture was kept at room temperature overnight with occasionally shaking. The reaction mixture was then poured, with vigorous stirring, into 5.0 mL. of iced water. Stirring was continued until the excess acetic anhydride was hydrolyzed. The crude precipitated acetate was filtered off, wash throughly with cold water until no more pyridine was remained and then purified by recrystallization from a mixture solvent of chloroform and ethanol to obtain colourless needle (50 mg., 91.02% yield), m.p. 237.0-238.5 ℃, Rf 0.58 (solvent: chloroform).

The IR spectrum of Compound 2A acetate (Fig. 20) revealed the important absorption peaks at ν_{max}^{KBr} (cm^{-1}): 1730 (s, $-\overset{O}{\overset{\|}{C}}-$) and 1245, 1020 (s,C-O) of acetate.

Study on Compound 2B

Compound 2B as white amorphous product, m.p. 62.5-64.0 °C, was designated for the acidic part derived from basic hydrolysis of Compound 2. This compound was easily soluble in many organic solvents such as dichloromethane, diethylether and chloroform. It did not show positive tests to Liebermann-Burchard's, Br_2 in CCl_4, 2,4-DNP and $FeCl_3$ reagents.

The IR spectrum (Fig.21) revealed the major absorption peaks at ν_{max}^{KBr} (cm^{-1}) : 3500-3000 (very b,O-H), 1700 (s, $-\overset{O}{\overset{\|}{C}}-OH$) and 940 (O-H bend.).

The GLC analysis (Fig.22) (condition: column OV-1, 2% CW 80/100 mesh, column temp. 220 °C, FID 280 °C, carrier gas N_2 45 mL./min.) exhibited the peak with retention time at Rt 3.44 min. which was corresponded to that of palmitic acid ($C_{15}H_{31}COOH$).

The HPLC analysis (Fig.23) (condition: column Zorbax-C18, 2.2% dioxane in methanol and water pH 2.8, column temp. 60 C, flow rate 1.0 mL./min., detector UV 205 nm.), revealed the peak with retention time at 6.48 min. which was also identical to that of palmitic acid ($C_{15}H_{31}COOH$).

Preparation amide of Compound 2B (59)

In a 10.0 mL. round-bottomed flask fitted with condenser and a calcium chloride tube, place 20 mg. of Compound 2B and add 1.0 mL. of redistilled thionyl chloride. Reflux gently

on water bath for 30 mins. Thereupon, arrange the equipment for distillation and distill off the excess thionyl chloride. The rest concentrated reaction mixture in the round bottom flask was cautiously poured into 5.0 mL. of ice-cold concentrated ammonia. The precipitated solid was collected by vacuum filtration and purified by recrystallization from aqueous ethanol to yield the amide of Compound 2B, 15 mg., m.p. 104.0-106.0 °C, Rf 0.53 (solvent: 2% methanol in chloroform).

The IR spectrum of Compound 2B amide (Fig. 24) revealed the important absorption bands at ν_{max}^{KBr} (cm^{-1}) : 3400-3200 (s,d., N-H) and 1640 (s,$-\overset{O}{\overset{\|}{C}}-N-$).

Preparation of β-amyrinpalmitate (55)

The palmitic acid 50 mg. was refluxed with redistilled thionyl chloride 5.0 mL. for 30 mins. Thereupon, the excess thionyl chloride was distilled off, the rest concentrated reaction mixture was carefully poured into 5.0 mL. of chloroform solution containing 40 mg. of β-amyrin. The entire solution was then evaporated the solvent in vacuum to gain white precipitated solid. After recrystallization from a mixture of acetone and chloroform for several times, white amorphous product, designated as synthetic Compound 2, 50 mg. (80.20% yield) was obtained. This compound showed the same Rf value as Compound 2 in various solvent systems.

The IR spectrum (Fig.25) revealed the vital absorption bands at ν_{max}^{KBr} (cm^{-1}): 1730 (s,$-\overset{O}{\overset{\|}{C}}-O-$) and 1260, 1240 and 1170 (m,C-O).

2.9.2 Separation of Fraction IB

Fraction IB, 9.51 g., 6.34% wt. by wt. of Fraction I, which was eluted by 10% chloroform-hexane from Fraction I was studied first by thin layer chromatography using 20% chloroform-hexane as a developing solvent. Thin layer chromatoplate showed two major spots, the upper one was 0.73 which was the same Rf value as Compound 2 and the other was at 0.70. Hence, Fraction IB 9.51 g. was separated by silica gel column chromatography (using adsorbent 150.0 g.). n-Hexane and a mixture of n-hexane and chloroform were used as eluents. Each fraction was collected about 500 mL.. Other procedures of separation were similar to Topic 2.9.1.. The results of separation this fraction were presented in Table 2.7.

Table 2.7 The results of separation Fraction IB by silica gel column (using column 2.5 x 120.0 cm.)

Eluents	Fraction No. (500 mL.)	Remarks	Weights (g.)
n-hexane	1-4	pale yellow oil	0.03
5% chloroform-hexane	5-8	yellow oil + white ppt. (one major spot)	1.02
	9-15	yellow oil + white ppt. (two major spots, Fraction C)	5.71
10% chloroform-hexane	16-25	yellow oil	0.27
20% chloroform-hexane	26-30	yellow oil	0.06

From Table 2.7, in Fraction No. 5-8 contained yellow oil and white precipitated solid 1.02 g. After the yellow oil was washed with cold acetone, the white remaining material was then recrystallized with a mixture of chloroform and acetone to gain white amorphous product 0.87 g. (9.15% wt. by wt. of Fraction IB), m.p. 73.0-75.0 °C. This compound gave the same Rf value as Compound 2 in various developing solvents. The mixed melting point of this compound and Compound 2 did not show different melting range from a single Compound 2. Moreover, the IR spectrum of this compound was also identical to that of Compound 2. Therefore, it is obvious to conclude that the compound in Fraction No. 5-8 was Compound 2.

2.9.2.1 Purification and Properties of Compound 3

Fraction C, which was obtained from the silica gel column chromatography of Fraction IB in Fraction No. 9-15, was composed of yellow oil and white precipitated solid. After the yellow oil was got rid of by washing with cold acetone, the remaining solid was recrystallized by a mixture of chloroform and acetone for several times to yield Compound 3 as white amorphous product, m.p. 70.0-71.5 °C, 4.93 g. (51.84% wt. by wt. of Fraction IB). The Rf value of this compound on TLC plate did not show merely spot, but it revealed two closely major spots at Rf value 0.73 and 0.70, respectively. Though the solvents for recrystallization were changed, the TLC results were still present two closely spots. Compound 3 showed physical properties like Compound 2. For instance, it could dissolve in chloroform, dichloromethane and hot n-hexane. This compound gave a violet colour with Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.26) showed the vital characteristic absorption peaks at ν_{max}^{KBr} (cm^{-1}): 1730 (s,$-\overset{O}{\overset{\|}{C}}-O$).

The mass spectrum (pattern 1) (Fig.27) displayed the mass fragmentation pattern idential to that of Compound <u>2</u> (Fig.12).

The mass spectrum (pattern 2) (Fig.28) exhibited the different fragmentation pattern to that of pattern 1, i.e., it did not give the molecular ion peak, but revealed other important peaks at m/e (% rel int.) 256.8 (0.59), 95.0 (7.69), 81.0 (6.38) and the base peak at m/e 43 (100.00).

The attempts to separate these two compounds in this mixture by both recolumn chromatography and PTLC were futile. Thus, Compound <u>3</u> was not investigated further.

2.9.3 <u>Separation of Fraction IC</u>

Thin layer chromatography of Fraction IC, 20% chloroform-hexane soluble fraction, revealed at least two components in this crude extract (solvent: 30% chloroform-hexane). One gave the same Rf as Compound <u>2</u> (Rf 0.85) and the other showed Rf value at 0.52. This fraction, 23.20 g., was separated by silica gel column using 450.0 g. of adsorbent and 10% chloroform-hexane as an initial eluent. Each portion was collected approximately 750.0 mL.. Other procedures of separation were similar to Topic 2.9.1. The results of examination Fraction IC were shown in Table 2.8.

Table 2.8 The results of separation of Fraction IC by silica gel column (using column 3.5 x 120.0 cm.)

Eluents	Fraction No. (750 mL.)	Remarks	Weights (g.)
10% chloroform-hexane	1-10	orange oil	0.51
	11-20	yellow oil + white ppt. (Fraction D)	2.73
20% chloroform-hexane	21-28	yellow oil + white ppt. (Fraction E)	1.68
	29-55	yellow oil + white ppt. (Fraction F)	10.23
	56-60	yellow oil	1.94
30% chloroform-hexane	61-70	yellow oil	0.55
40% chloroform-hexane	71-80	yellow oil	1.07
60% chloroform-hexane	81-90	yellow oil	0.48
	91-95	yellow oil	0.32

Fraction D, which was eluted from the silica gel column of Fraction IC in Fraction No. 11-20, consisted of yellow oil and white precipitated solid. The yellow oil was eliminated from this solid by washing with methanol. The remaining white material was then recrystallized by a mixture of chloroform and methanol. The crystallized solid was notified that there were two kinds in the crystalline product, i.e., one was the bright white plate and the other was white amorphous solid. Thus, the fractional recrystallization was used to separate these two solids.

2.9.3.1 Purification and Properties of Compound 4

Compound 4, which was obtained by the fractional recrystallization from the mixture solid in Fraction D, was white amorphous solid, 40 mg. (0.32% wt. by wt. of Fraction IC), m.p. 218.0-219.5 °C. It showed merely one spot on TLC plate at Rf value 0.68 using chloroform as a developing solvent. This compound was easily soluble in chloroform, dichloromethane and diethylether, but slightly soluble in cold n-hexane, methanol and ethanol. Compound 4 gave a purple colour to Liebermann-Burchard's and also decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.29) gave the vital characteristic peaks at ν^{KBr}_{max} (cm^{-1}): 3050 (w,C-H stretch. of $R_1R_2C{=}CR_3H$), 1730 (s,$-\overset{O}{\overset{\|}{C}}-O-$) and 1265 (m,C-O of ester).

The mass spectrum (Fig.30) gave the molecular ion peak at m/e (% rel int.) 678.0 (23.37) together with other fragmentation ion peaks at m/e 660.0 (78.23), 422.0 (23.0), 405.0 (38.11), 269.0 (100.00), 256.0 (19.68), 204.0 (63.70) and 181.0 (24.92).

The ^{1}H NMR ($CDCl_3$) spectrum (Fig.31) exhibited the proton signals at chemical shift δ (ppm.): 5.52 (1H,dd,J=2.50,7.51 Hz, olefinic proton), 4.53 (1H,d,J=13.40 Hz), 3.10 (1H,t,J=5.37 Hz), 2.29 (1H,t,J=7.08 Hz) and other signals around 1.81 to 0.82 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.32) revealed the significant carbon signals at chemical shift δ (ppm.): 173.52 (carbon of ester), 157.00 and 118.90 (1C each, olefinic carbon), 80.29 (carbon attached to oxygen atom) and other signals

around 58.13 to 14.14 ppm..

The UV (EtOH) spectrum (Fig.33) gave the λ max at 206 nm. (log ε = 5.48).

Hydrolysis of Compound 4 (55)

Compound 4, 30 mg., was refluxed with 10% KOH in ethanol 5.0 mL. for 4 hours (Check whether the reaction was completed or not by TLC). Evaporation of ethanol gave a solid which was extracted by diethylether 100.0 mL., two times. The etheral layer was dried over anhydrous magnesium sulfate and then evaporated the solvent to yield white solid with pale yellow oil. This fraction was purified by recolumn chromatography to gain a white crystalline solid, labelled as Compound 4A, 15 mg., m.p. 276.0-278.0 °C.

Acidification of the solid which was remained after extraction with diethylether furnished another impure solid. It was recrystallized by a mixture of chloroform and methanol for several times to afford Compound 4B, 8 mg., m.p. 61.0-63.0 °C.

Study on Compound 4A

Compound 4A, as white needle solid, 15 mg., m.p. 276.0-278.0 °C was derived from basic hydrolysis of Compound 4. This compound gave a single spot at Rf 0.47 (solvent: chloroform). It was soluble in chloroform, but slightly soluble in methanol, ethanol and hexane. Compound 4A showed a purple colour when being treated with Liebermann-Burchard's and also decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.34) revealed the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 3520 (s,O-H), 1630 (w,C=C), 1390,1370 (m,C-H bend. of gem-dimethyl) and 1050-1000 (m, C-O).

The mass spectrum (Fig.35) gave the parent ion peak, M^+, at m/e (% rel int.) 440.0 (68.22) (Calcd. for $C_{31}H_{52}O$: MW. 440.41) together with other fragmentation ion peaks at m/e 422.0 (21.20, M^+-H_2O), 259.0 (32.77), 220.0 (19.42), 204.0 (31.27), 189.0 (22.27), 161.0 (39.60), 133.0 (35.5) and 108.0 (100.00).

The ^1H NMR ($CDCl_3$) spectrum (Fig.36) exhibited the proton signals at δ (ppm.): 5.59 (1H,dd,J=3.53 Hz), 3.22 (1H,t,J=6.63 Hz), 3.11 (1H,t,J=4.64 Hz), 2.80 (1H,t,J=2.32 Hz) together with other signals around 2.16 to 0.82 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.37) displayed the carbon signals at chemical shift (ppm.): 157.00 and 118.86 (1C each, olefinic carbon), 78.99 (carbinol carbon) and other signals around 58.29 to 15.44 ppm..

Study on Compound 4B

Compound 4B as white amorphous product 8 mg. was labelled for the acidic part derived from basic hydrolysis of Compound 4. This compound was easily soluble in n-hexane, chloroform, dichloromethane and diethylether, but slightly soluble in methanol and ethanol. Compound 4B gave negative tests to all these reagents: Liebermann-Burchard's, Br_2 in CCl_4, 2,4-DNP and $FeCl_3$ reagents.

The IR spectrum (Fig.38) showed the strong absorption bands at ν_{max}^{KBr} (cm^{-1}): 3500-3000 (very b, O-H), 1700 (s,

$-\overset{O}{\overset{\|}{C}}-OH$) and 940 (O-H bend.).

The GLC analysis (Fig.39) (condition: column OV-1, 2% CW 80/100 mesh, column temp. 220 °C, FID 280 °C, carrier gas N_2 45.0 mL./min.) gave a solely peak at retention time 3.32 min. which was corresponded to palmitic acid ($C_{15}H_{31}COOH$).

2.9.3.2 Purification and Properties of Compound 5

Compound 5 was derived by the fractional recrystallization from the mixture solid in Frction D as bright white plate. After recrystallization with a mixture of chloroform and methanol for several times, bright white plate, 200 mg. (0.86% wt. by wt. of Fraction IC), m.p. 128.0-129.0 °C, Rf value 0.68 (using chloroform as a mobile phase), was obtained. Like Compound 4, this compound was easily soluble in chloroform, diethylether and dichloromethane, but slightly soluble in methanol and ethanol. Compound 5 gave a violet colour to Liebermann-Burchard's, decolourized Br_2 in CCl_4 and also gave a positive test to 2,4-DNP reagents.

The IR spectrum (Fig.40) revealed the important characteristic peaks at ν_{max}^{KBr} (cm^{-1}): 1730 (s,$-\overset{O}{\overset{\|}{C}}-O-$), 1650 (s,$-\overset{O}{\overset{\|}{C}}-$) and 1205 (m,C-O).

The mass spectrum (Fig.41) displayed the molecular ion peak, M^+, at m/e (% rel int.) 678.0 (8.75) (Calcd. for $C_{46}H_{78}O_3$: MW. 678.61) together with other important fragmentation peaks at m/e 660.0 (1.52), 422.0 (41.60), 407.0 (21.51), 273.0 (69.94), 256.0 (17.8), 232.0 (100.00) and 135.0 (53.84).

The ^1H NMR ($CDCl_3$) spectrum (Fig.42) exhibited the proton signals at chemical shift δ (ppm.): 5.59

(1H,s, olefinic proton), 4.55 (1H,t,J=7.56 Hz) and other signals around 2.36 to 0.88 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.43) expressed the significant signals at chemical shift δ (ppm.): 200.12 (carbon of ketone), 173.68 (carbon of ester), 170.60 and 128.07 (1C each, olefinic carbon), 80.29 (carbon attached to oxygen atom) together with other signals around 61.70 to 14.14 ppm..

The UV (EtOH) spectrum (Fig.44) gave the λmax at 248 nm. (log ε = 5.54).

Elemental analysis found: %C 81.74 %H 11.86

Calcd. for $C_{46}H_{78}O_3$, MW. 678.61: %C 81.42 %H 11.50

Hydrolysis of Compound 5

Compound 5, 50 mg., was basic hydrolysed by 10% KOH in ethanol 5.0 mL. using the same manner as described for Compound 4. The white solid, which was obtained after evaporation the ethanol, was then extracted with diethylether 100.0 mL., 2 times. The combined diethylether was dried over anhydrous magnesium sulfate and then distilled off to yield a white product with pale yellow oil. This portion was chromatographed on silica gel column chromatography using 20% chloroform-hexane as an eluent to gain white crystalline solid, marked as Compound 5A, 12 mg., m.p. 229.0-230.5 °C.

Acidification of the remaining solid after extraction with diethylether afforded another impure solid. It was purified by recrystallization by a mixture of chloroform and methanol for several times to yield Compound 5B 10 mg., m.p. 61.5-62.5 °C.

Study on Compound 5A

Compound 5A was obtained by recolumn chromatography of the alcoholic part from basic hydrolysis reaction of Compound 5. This compound gave a solely spot at Rf 0.40 (solvent: chloroform). It was easily soluble in chloroform, dichloromethane and diethylether, but slightly soluble in ethanol and methanol. It showed a purple colour to Liebermann-Burchard's, decolourized Br_2 in CCl_4 and gave a positive test to 2,4-DNP reagents.

The IR spectrum (Fig.45) revealed the major characteristic absorption bands at ν_{max}^{KBr} (cm^{-1}): 3400 (b,O-H), 1650 (s,$-\overset{O}{\overset{\|}{C}}-$), 1615 (w,C=C), 1385,1375 (m,C-H bend. of gem dimethyl) and 1030 (C-O).

Study on Compound 5B

Compound 5B, another product obtained from basic hydrolysis of Compound 5, was amorphous solid. It was soluble in many organic solvents such as chloroform, dichloromethane and n-hexane, but slightly soluble in methanol and ethanol. Compound 5B gave the same colour tests as Compound 4B.

The IR spectrum (Fig.46) displayed the major absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3500-3000 (very b,O-H), 1700 (s, $-\overset{O}{\overset{\|}{C}}-OH$) and 940 (b,O-H bend.).

The GLC analysis (Fig.47) using the same condition as that of Compound 4B revealed only one peak on gas chromatogram at Rt 3.26 min. which was corresponded to palmitic acid ($C_{15}H_{31}COOH$).

2.9.3.3 Purification and Properties of Compound 6

Fraction E was eluted from the column of Fraction IC with 20% chloroform-hexane in Fraction No. 21-28. After recrystallization with hot n-hexane for several times, white amorphous solid, m.p. 83.0-86.0 °C (105 mg., 0.45% wt. by wt. of Fraction IC) was obtained. This compound, assigned as Compound 6, revealed only one spot on TLC plate at Rf value 0.60 (solvent: chloroform). Compound 6 was soluble in chloroform and diethylether, but slightly soluble in n-hexane and insoluble in water. It gave negative results with all these reagents like Compound 1; Liebermann-Burchard's, Br_2 in CCl_4, $FeCl_3$, 2,4-DNP and $KMnO_4$ reagents.

The IR spectrum (Fig.48) revealed the important peaks at ν_{max}^{KBr} (cm^{-1}) : 3350 (b,O-H) and 1050 (m,C-O of 1° ROH).

The ^{1}H NMR ($CDCl_3$) spectrum (Fig.49) gave the proton signals at δ (ppm.): 3.66 (2H,t), 1.56, 1.26 and 0.89 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.50) exhibited the carbon signals at chemical shift δ (ppm.): 63.10 (carbon attached to oxygen atom) together with signals around 32.85-14.09 ppm..

The mass spectrum (Fig.51) did not give the molecular ion peak, M^+, but it displayed mass fragment ion peaks at m/e (% rel int.) 476.0 (6.62, M^+-H_2O). Other fragment ion peaks were at m/e 448.0 (21.91, $M^+-H_2O-(CH_2)_2$), 420.0 (16.94, $448-(CH_2)_2$) and other signals corresponded to lose $-(CH_2)_2-$ (m/e 28) step by step.

Compound 6 did not show any absorption peaks in UV region.

The GLC analysis (Fig.52) (condition: column 2% OV-1, CW 80/100 mesh AW DMCS 1/8 inch x 7.55 fts., column temp. 280 °C, FID 320 °C, carrier gas N_2 50.0 mL./min.) revealed seven peaks on gas chromatogram at retention time 7.71, 9.59, 11.75, 14.78, 18.28, 22.94 and 28.54 min., respectively. The peaks with Rt at 11.75 and 18.28 min. were the two major components in this mixture.

Acetylation of Compound 6

Compound 6 (30 mg.) was treated with acetic anhydride (1.0 mL.) and a few drops of dry pyridine in usual manner and kept at room temperature overnight. The reaction mixture was poured onto ice water with vigorous stirring to yield the precipitate which was filtered off, followed by washing with water until no left of odour of pyridine. The acetyl derivative of Compound 6 was purified from acetone to give white plate 27 mg., m.p. 70.0-71.0 °C, Rf value 0.83 (solvent: 20% chloroform-hexane).

The IR spectrum of Compound 6 acetate (Fig.54) revealed the major absorption bands at ν_{max}^{KBr} (cm^{-1}) : 1745 (s,$-\overset{O}{\overset{\|}{C}}-$) and 1245 (s,C-O) of actate.

Fraction F 10.23 g., which was obtained from the combination of Fraction No. 29-55 from the separation of Fraction IC, consisted of yellow oil and white precipitated solid. After yellow oil was removed by washing with cold n-hexane, the remaining white solid was recrystallized with a mixture of chloroform and n-hexane to gain white solid. This crystalline solid

did not give a unique shape, but they showed two shapes - a feather shape and a rhombic one. Moreover, this product melted in wide range from 185 to 280 °C. It was notified that the white precipitated solid of Fraction F was not composed of a single compound. Hence, this product was rechromatographed on silica gel column chromatography using 200.0 g. of an adsorbent and 5% chloroform in n-hexane as an initial eluent. The results of recolumn chromatography of Fraction F were presented in Table 2.9.

Table 2.9 The results of recolumn chromatography of Fraction F (using column 3.5 x 120.0 cm.)

Eluents	Fraction No. (500 mL.)	Remarks	Weights (g.)
5% chloroform-hexane	1-7	nothing	-
10% chloroform-hexane	8-14	white ppt. (one major spot at Rf 0.68) (Fraction F1)	0.25
	15-25	white ppt. (two major spots at Rf 0.68 and 0.60) (Fraction F2)	4.58
	26-45	white ppt. (one major spot at Rf 0.60) (Fraction F3)	4.92
	46-54	nothing	-
20% chloroform-hexane	55-60	nothing	-

2.9.3.4 Purification and Properties of Compound 7

Fraction F1 was obtained from the recolumn chromatography in Fraction No. 8-14 by 10% chloroform-hexane. It showed merely one spot on TLC plate at Rf value 0.46 using chloroform as a developing solvent. After recrystallization with a mixture of chloroform and methanol, Compound 7 was obtained as bright white feather, 150 mg. (0.65% wt. by wt. of Fraction IC), m.p. 196.0-198.0 °C. This compound was easily soluble in chloroform, diethylether and dichloromethane, but slightly soluble in n-hexane, methanol and ethanol. Compound 7 gave a violet colour with Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.55) revealed the major absorption peaks at ν^{KBr}_{max} (cm^{-1}): 3290 (b,O-H), 1645 (w,C=C) and 1040 (m,C-O).

The mass spectrum (Fig.56) gave the molecular ion peak at m/e (% rel int.) 426.0 (10.79) (Calcd. for $C_{30}H_{50}O$: MW. 426.39), together with other fragment ion peaks at m/e 411.0 (2.54, M^+-CH_3), 408.0 (0.73, M^+-H_2O), 218.0 (100.00, RDA) and 203.0 (27.43).

The ^1H NMR ($CDCl_3$) spectrum (Fig.57) displayed the vital signals at chemical shift δ (ppm.): 5.19 (1H,t,J=3.18 Hz, olefinic proton), 3.23 (1H,t,J=7.69 Hz) and other signals around 1.89 to 0.80 ppm..

The ^{13}C NMR spectrum (Fig.58) exhibited the carbon signals at chemical shift δ (ppm.): 145.13, 121.73 (1C each, olefinic carbon), 78.98 (carbon attached to oxygen atom) and other signals around 55.14 to 15.45 ppm..

The UV (EtOH) spectrum (Fig.59) gave the λ max at 203 nm. (log ε = 3.75).

Elemental analysis found: %C 84.07 %H 12.01

Calcd. for $C_{30}H_{50}O$, MW. 426.39: %C 84.51 %H 11.74

Acetylation of Compound 7

Compound 7 30 mg. in dry pyridine (2.0 mL.) and acetic anhydride (1.5 mL.) was allowed to stand at room temperature for 24 hours. Working up in usual way gave the acetate of Compound 7. After recrystallization with a mixture of chloroform and ethanol, bright white needle (30 mg., 91.02% yield), was obtained. This compound melted at 236.0-237.5 °C and gave Rf value 0.58 using chloroform as a mobile phase.

The IR spectrum of Compound 7 acetate (Fig.60) showed the significant absorption peaks at ν_{max}^{KBr} (cm^{-1}): 1730 (s, $-\overset{O}{\overset{\|}{C}}-$), 1245 and 1020 (C-O) of acetate.

The GLC analysis (Fig.61) (condition: column SE-30, column temp. 270 °C, FID 320 °C, carrier gas N_2 50.0 mL./min.) gave the peak with retention time at 27.97 min. which was corresponded to the authentic β-amyrinacetate*.

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* The authentic β-amyrinacetate was isolated and identified from the leaves of Streblus asper Lour. by Assist. Prof. Gaysorn Veerachato.

From Table 2.9, Fraction 15-25 (Fraction F2) which was eluted by recolumn chromatography revealed two closely major spots at Rf value 0.65 and 0.60 using chloroform as a developing solvent. This fraction was recrystallized by hot 95% ethanol to yield two shapes of crystalline products. One was a feather product and the other was a rhombic crystal. This result implied that this fraction was not composed of a single compound. Thus, this mixture was dissolved in n-hexane, only the feather product could dissolve, while a rhombic one was still in the solution. After filtration, the filtrate, labelled as Fraction F2A, was concentrated and kept to room temperature for 3 hours to yield a feather-like crystal, 2.64 g. (11.38% wt. by wt. of Fraction IC), m.p. 192.0-210.0 ˚C, Rf 0.65 (solvent: chloroform).

2.9.3.5 Purification and Properties of Compound 8

Compound 8, 2.64 g., was obtained from Fraction F2A by dissolving the precipitated solid in Fraction F2 with hot n-hexane. Although this compound revealed only one spot on TLC plate in various developing solvent systems, the melting point of Compound 8 was still shown in wide range from 192.0 to 208.0 ˚C. Compound 8 was easily soluble in chloroform, diethylether and dichloromethane, but slightly soluble in n-hexane, methanol and ethanol. It gave a purple colour to Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum of this compound (Fig.62) revealed the major absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3350 (b,O-H) and 1640 (m,C=C).

Since this compound revealed O-H as its functional group, the attempt to purify this crystalline solid was to convert this substance into its acetyl derivative.

Acetylation of Compound 8

Compound 8 300 mg. was dissolved in dry pyridine 5.0 mL. and acetic anhydride 10.0 mL. was added. The solution was shaken and refluxed on water bath for 5 hours. The entire reaction mixture was then poured onto ice distilled water and the precipitated acetate was collected. After recrystallization with a mixture of chloroform and ethanol, bright white needle crystal, 290 mg., Rf 0.69 (solvent: chloroform) was obtained. This acetate melted at 216.0-218.0 °C.

The IR spectrum of Compound 8 acetate (Fig.63) gave the strong absorption peaks at ν_{max}^{KBr} (cm^{-1}): 1740 (s, $-\overset{O}{\overset{\|}{C}}-$) and 1240 (s,C-O) of acetate.

The mass spectrum (Fig.64) displayed the molecular ion peak, M^+, at m/e (% rel int.) 468.0 (43.92) (Calcd. for $C_{32}H_{52}O_2$: MW. 468.41) and other fragmentation ion peaks at m/e 453.0 (11.16), 218.0 (33.87) and 189.0 (100.00).

The ^1H NMR ($CDCl_3$) spectrum (Fig.65) expressed the proton signals at chemical shift δ (ppm.): 4.67,4.55 (2H,J=10.26 Hz, olefinic proton), 4.42 (1H,t,J=9.04 Hz, proton on carbon attached to oxygen of acetate group), 2.03 (3H,s, methyl protons of acetoxy group) together with other signals around 2.23 to 0.78 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.66) exhibited the carbon signals at chemical shift δ (ppm.): 170.97

(carbon of ester), 150.88 and 109.38 (1C each, olefinic carbon), 80.94 (carbon attached to acetyl group) and other signals around 55.37 to 14.52 ppm..

The GLC analysis (Fig.67) (condition: column OV-17, SW 80/100 mesh, 3 mm. x 2 m., column temp. 300 °C, FID 320 °C, carrier gas N_2 40.0 mL./min.) revealed a merely peak at Rt 29.16 min. which was corresponded to the authentic lupeolacetate*.

Hydrolysis of Compound 8 acetate

Compound 8 acetate 100 mg. was hydrolyzed by refluxing with 5% KOH in ethanol on stream bath for 40 mins. in usual manner to obtain an alcoholic part as white solid. This solid was recrystallized by a mixture of chloroform and ethanol for several times to gain bright white needle, 80 mg., m.p. 213.0-215.0 °C, Rf 0.69 (solvent: 50% chloroform-hexane). This compound gave the same colour test results as those of Compound 8 acetate.

The IR spectrum (Fig.68) showed the important absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3340 (b,O-H), 3090 (w,C-H of $C=CH_2$), 1640 (w,C=C) and 875 (m,C-H out of plane of $=CH_2$).

The ^1H NMR ($CDCl_3$) spectrum (Fig.69) exhibited the significant signals at chemical shift δ (ppm.): 4.67, 4.57 (2H,J=9.00 Hz, olefinic proton), 3.16 (1H,t,J=7.82 Hz, proton on carbinol carbon) and other signals around 2.41 to 0.76 ppm..

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* The authentic lupeolacetate was separated and identified from the stembarks of Crataeva nurvala by Assist. Prof. Gaysorn Veerachato.

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.70) displayed the carbon signals at chemical shift δ (ppm.): 150.88 and 109.32 (1C each, olefinic carbon), 78.99 (carbon attached to oxygen atom) together with other signals around 55.37 to 14.57 ppm..

The UV (EtOH) spectrum (Fig.71) gave the λmax at 205 nm. (log ε = 3.64)

The mass spectrum (Fig.72) displayed the parent ion peak, M^+, at m/e (% rel int.) 426.0 (71.36) (Calcd. for $C_{30}H_{50}O$: MW. 426.39) together with other fragmentation ion peaks at m/e 218.0 (63.39), 207.0 (75.55), 189.0 (82.38) and 95.0 (100.00).

The GLC analysis (Fig.73) (using the same condition as Compound 8 acetate) showed only one peak on gas chromatogram at retention time 21.80 min. which was corresponded to the authentic lupeol.*

The rhombic crystal in Fraction F2, which was insoluble in n-hexane, was assigned as Fraction F2B. This fraction was further recrystallized by hot 95% ethanol to gain bright white crystalline product Rf 0.60 (solvent: chloroform), 1.05 g. (4.53% wt. by wt. of Fraction IC), m.p. 278.0-280.0 °C. The Rf value of this compound revealed the same position as the white crystallized product in Fraction 26-45 (Fraction F3). Moreover, the mixed m.p., Co. TLC and their IR spectra were found to be identical. Thus, the product in Fraction F2B and in Fraction F3 were combined.

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* The authentic lupeol was isolated and elucidated its structure from the stembarks of <u>Crataeva nurvala</u> by Assist. Prof Gaysorn Veerachato.

2.9.3.6 Purification and Properties of Compound 9

Fraction F3, 4.78 g. was obtained by the combination of Fraction No. 26 to No. 45 using 10% chloroform in n-hexane as an eluent from the recolumn chromatography of Fraction F. This fraction was found to be the same as Fraction F2B (1.05 g.). Hence, these two fractions were combined and recrystallized with a mixture of ethylacetate and n-hexane for several times to yield bright white rhombic product, 5.67 g. (24.44% wt. by wt. of Fraction IC), m.p. 278.0-280.0 °C, Rf 0.60 (solvent: chloroform). This compound, labelled as Compound 9, could also recrystallized by a mixture of chloroform and n-hexane to give bright white needle crystal. It was soluble in chloroform and dichloromethane, but slightly soluble in ethanol, methanol and n-hexane. Compound 9 gave a violet colour when it was treated with Liebermann-Burchard's and also decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.74) revealed the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 3490 (b,O-H), 3050 (w, C-H of $R_1R_2C=CR_3H$), 1645 (w,C=C) and 1040 (m,C-O).

The UV (EtOH) spectrum (Fig.75) showed the λmax at 204 nm. (log ε = 3.72).

The mass spectrum (Fig.76) displayed the molecular ion peak, M^+, at m/e (% rel int.) 426.0 (4.29) (Calcd. for $C_{30}H_{50}O$: MW. 426.39), 411.0 (4.68, M^+-CH_3), 408.0 (21.14, M^+-H_2O) and other fragmentation ion peaks at m/e 269.0 (57.53), 204.0 (100.00) and 189.0 (77.17).

The ^1H NMR ($CDCl_3$) spectrum (Fig.77) exhibited the proton signals at δ (ppm.): 5.53 (1H,dd,J=3.43,7.55

Hz, olefinic proton), 3.21 (1H,t,J=6.93 Hz) and other signals around 2.17-0.81 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.78) showed the signals at chemical shift δ (ppm.): 158.00 and 116.77 (1C each, olefinic carbon), 78.96 (carbon attached to oxgen atom) together with other signals around 55.45 to 15.20 ppm..

Elemental analysis found: %C 84.34 %H 12.04

Calcd. for $C_{30}H_{50}O$, MW. 426.39: %C 84.50 %H 11.74

Acetylation of Compound 9

Compound 9 (2.00 g.) was reacted with acetic anhydride in ususal manner to gain the acetyl derivative of Compound 9. The crude product was recrystallized from a mixture of chloroform and ethanol to give needle crystal 1.98 g. (90.40% yield), m.p. 296.0-298.0 °C, Rf 0.68 (solvent: chloroform).

The IR spectrum of the acetyl derivative of Compound 9 (Fig.79) showed the absorption peaks at ν_{max}^{KBr} (cm^{-1}): 1725 (s,$-\overset{O}{\overset{\|}{C}}-$) and 1245 (C-O) of acetate.

The ^1H NMR ($CDCl_3$) spectrum (Fig.80) displayed the signals at chemical shift δ (ppm.): 5.52 (1H,dd, J=3.42,7.96 Hz, olefinic proton), 4.45 (1H,t,J=7.80 Hz), 2.04 (3H,s, acetyl protons) and other signals around 1.94 to 0.82 ppm..

The mass spectrum (Fig.81) gave the molecular ion peak, M^+, at m/e (% rel int.) 468.0 (16.22) (Calcd. for $C_{32}H_{52}O_2$: MW. 468.41) and other fragmentation ion peaks at m/e 342.0 (34.31), 234.0 (100.00) and 189.0 (30.39).

Oxidation of Compound 9 (59)

Dissolve 100 mg. of chromic trioxide (CrO_3) in 2.0 mL. of concentrated sulfuric acid and carefully dilute with distilled water to 10.0 mL. This solution was added from an addition funnel to a stirred acetone solution of Compound 9 30 mg. maintained at 15 to 20 °C. After adding water 20 mL., the entire reaction mixture was extracted by dichloromethane. The dichloromethane extract was dried over anhydrous sodium sulfate and distilled off the solvent to yield pale greenish product. This product was purified by silica gel column chromatography using 10% chloroform in n-hexane as an eluent to gain a white solid. After recrystallized by a mixture of n-hexane and chloroform for two times, the bright white plate 10 mg., labelled as Compound 9A, m.p. 240.0-242.0 °C, Rf 0.65 (solvent: chloroform), was obtained.

The IR spectrum of this oxidative product (Fig.82) gave the strong absorption band at ν_{max}^{KBr} (cm^{-1}): 1700 (s, $-\overset{O}{\overset{\|}{C}}-$).

Conversion taraxerylacetate to β-amyrinacetate

To a suspension of taraxerylacetate (150 mg.) in glacial acetic acid (35.0 mL.) at 90 °C, a concentrated hydrochloric acid (1.0 mL.) was added. After the mixture had been heated on a stream bath for 10 mins., during which the suspended solid dissolved, the solvent was rapidly removed under reduced pressure. Recrystallization of the solid from a mixture of chloroform and methanol gave bright white needle (80 mg., 53.33% yield), m.p. 236.0-238.0 °C, Rf 0.37 using chloroform as a developing solvent, designated as Compound 9B.

The IR spectrum (Fig.83) exhibited the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 1730 (s,$-\overset{O}{\overset{\|}{C}}-$) and 1245, 1020 (s,C-O) of acetate.

The GLC analysis (Fig.84) (condition: OV-17 SW 80/100 mesh 3 mm. x 2 m., column temp. 300 °C, FID 320 °C, carrier gas N_2 40.0 mL./min.) revealed a sole component at Rt 18.90 min. and indentified as β-amyrinacetate by comparision with the authentic sample*.

The results from mixed m.p. and Co. TLC with the authentic β-amyrinacetate clearly confirmed that this compound was β-amyrinacetate.

2.9.4 Separation of Fraction ID

Fraction ID 30.78 g., 40% chloroform-hexane extract from Fraction I, was studied first by TLC using chloroform as a developing solvent. The result indicated that there were three major components in its crude. One was identical to Compound 9, the others showed spots on TLC plate at Rf value 0.19 and 0.14, respectively. Thus, the concentrated extract was subjected on silica gel 600.0 g. using 20% chloroform-hexane as an initial solvent and changing an eluent by gradual increasing of chloroform. Other procedures were carried out like Topic 2.9.1.. The results of separation this fraction were shown in Table 2.10.

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* The authentic β-amyrinacetate was derived and identified from the leaves of Streblus asper Lour. by Assist. Prof. Gaysorn Veerachato.

Table 2.10 The results of separation Fraction ID by silica gel column (using column 3.5 x 120.0 cm.)

Eluents	Fraction No. (500 mL.)	Remarks	Weights (g.)
20% chloroform-hexane	1-2	yellow oil	0.30
	3-26	yellow oil + Cpd. 9	4.32
	27-28	yellow oil	1.74
	29-37	yellow oil + white ppt. (Fraction G)	3.78
	38-42	greenish oil	2.61
30% chloroform-hexane	43-55	greenish oil + ppt. (Fraction H)	2.57
40% chloroform-hexane	56-70	greenish oil + ppt. (Fraction J)	2.98
60% chloroform-hexane	71-80	greenish oil	2.55
80% chloroform-hexane	81-90	greenish oil	3.10
chloroform	91-95	greenish oil	2.64

2.9.4.1 Purification and Properties of Compound 10

Fraction G was obtained from the combination of Fraction No. 29-37 which was eluted by 20% chloroform-hexane of Fraction ID. After washing a yellow oil with hot methanol, white needle material was obtained. It was purified by recrystallization with a mixture solvent of dichloromethane and n-hexane for four times to afford bright white needle, m.p. 143.0-145.0 °C, 550 mg. (1.79% wt. by wt. of Fraction ID). This compound, assigned as Compound 10, showed only one spot on TLC plate at Rf value 0.32 (solvent: chloroform). It was soluble in chloroform, acetone,

diethylether and dichloromethane, but slightly soluble in n-hexane, methanol and ethanol. This compound gave a deep green colour with Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.85) showed the significant characteristic absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3430 (b,O-H), 3030 (w,C-H stretch. of $R_1R_2C=CHR_3$), 1670-1630 (w,C=C) and 1050 (m,C-O).

The mass spectrum (Fig.86) gave the important fragmentation ion peaks at m/e (% rel int.) 414.0 (100.00), 412.0 (80.23) and 400.0 (40.83) (Calcd. for $C_{29}H_{50}O$, $C_{29}H_{48}O$ and $C_{28}H_{48}O$, respectively) together with other vital peaks at m/e 396.0 (41.14), 394.0 (10.55), 382.0(21.98), 381.0 (24.31) 329.0 (38.05), 303.0 (32.20), 273.0 (42.42), 255.0 (77.41) and 213.0 (52.45).

The ^1H NMR ($CDCl_3$) spectrum (Fig.87) revealed the significant sigals at chemical shift δ (ppm.): 5.32 (2H,d,J=4.39 Hz, olefinic protons), 5.09 (1H,t,J=5.86 Hz, olefinic proton), 3.52 (m) and other signals around 2.29 to 0.68 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.88) exhibited the vital carbon signals at position δ (ppm.): 140.80, 138.31, 129.31, 121.73 (olefinic carbons), 71.78 (carbon attached to oxygen atom) together with other signals around 56.77 to 11.86 ppm..

The UV (EtOH) spectrum (Fig.89) showed the maximum absorption peak at 203 nm. (log ε = 3.78).

The GLC analysis (Fig.90) (condition: column OV-1 2%, 3 mm. x 2 m., column temp. 260 °C, FID 320 °C, carrier gas N_2 48.0 mL./min.) gave three peaks on gas chromatogram at retention

time 18.40, 19.60 and 22.30 min., respectively.

Acetylation of Compound 10

Compound 10 50 mg. was mixed with acetic anhydride (1.0 mL.) and a few drops of dry pyridine in usual manner and kept at room temperature overnight. The reaction mixture was worked up in usual way to get white precipitated acetate 45 mg.. After recrystallization with a mixture of chloroform and ethanol, bright white plate 40 mg., m.p. 133.0-134.0 °C, Rf 0.70 (solvent: chloroform), was obtained.

The IR spectrum (Fig.91) revealed the major absorption peaks at ν_{max}^{KBr} (cm^{-1}): 1735 (s,$-\overset{O}{\overset{\|}{C}}-O-$), 1245 (s,C-O of acetate) and 1040 (m,C-O).

Fraction H 2.57 g., was obtained from the silica gel column chromatography of Fraction ID in Fraction No. 43-55 using 30% chloroform-hexane as an eluting solvent (see also Table 2.10). This fraction contained a mixture of greenish oil and precipitates. TLC of this fraction revealed two major spots at Rf value 0.19 and 0.14, respectively (solvent: chloroform). After removing greenish oil with n-hexane, the remaining material was then recrystallized by a mixture solvent of dichloromethane and n-hexane for six times to afford white crystalline product 0.30 g. (0.97% wt. by wt. of Fraction ID). Nevertheless, this compound revealed two spots at Rf value as mentioned above. Although the solvents for recrystallization were changed, this crystalline product still gave two spots on TLC plate. Hence, recolumn chromatography technique by using silica gel 20.0 g. as an adsorbent was used for separation this

crystalline product. The column was initially eluted by 60% chloroform-hexane and 20.0 mL. was collected for each portion. Each fraction was monitored by TLC and the equivalent ones were merged. The results of separation Fraction H by silica gel recolumn chromatography were presented in Table 2.11.

Table 2.11 The results of recolumn chromatography of crystalline product obtaining from Fraction H (using column 1.0 x 50.0 cm.)

Eluents	Fraction No. (20 mL.)	Remarks	Weights (g.)
60% chloroform-hexane	1-14	nothing	-
	15	white ppt. (Fraction H1)	0.02
	16-18	white ppt. (Fraction H2)	0.22
	19-30	nothing	-

Fraction H2 0.22 g. was still shown two major spots on TLC plate at Rf value 0.19 and 0.14 (solvent: chloroform). Thus, this fraction was then rechromatographed on silica gel 40.0 g. using 60% chloroform in n-hexane as an eluting solvent and collected about 10.0 mL. for each fraction. Other procedures were performed in the same ways as described for Fraction H. The results of separation Fraction H2 were presented in Table 2.12.

<u>Table 2.12</u> The results of recolumn chromatography of crystalline product obtaining from Fraction H2 (using column 1.0 x 50.0 cm.)

Eluents	Fraction No. (10 mL.)	Remarks	Weights (g.)
60% chloroform-hexane	1-20	nothing	-
	21-23	white ppt. (Cpd. 11)	0.03
	24-28	white ppt. (Fraction H3)	0.15
	29-35	white ppt. (Fraction H4)	0.01
	36-40	nothing	-

Fraction H3 still expressed two spots on TLC plate. Hence, PTLC was used for separation this fraction. (condition: TLC 0.50 mm. thick, 2% methanol in chloroform as a mobile phase and UV 254 nm. as a detector). After being separated and extracted completely with chloroform by soxhlet apparatus, the upper spot at Rf 0.50 was designated as Compound 11 40 mg. and the lower one at Rf 0.45 was labelled as Compound 12 90 mg., respectively.

2.9.4.2 Purification and Properties of Compound 11

Compound 11 was obtained by recrystallilza-tion the solid in Fraction H1, Fraction No. 21-23 from separation Fraction H2 and the upper part from PTLC plate (see also Table 2.11 2.12) with a mixture of chloroform and n-hexane for several times to furnish white amorphous product 50 mg. (0.16% wt. by wt. of Fraction ID). This compound, m.p. 275.0 °C (dec.), showed merely one spot on TLC plate at Rf value 0.19 (solvent: chloroform). Compound 11 was

soluble in chloroform and dichloromethane, but slightly soluble in n-hexane and methanol. This compound gave a violet colour when it was reacted with Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents. Besides, it gave a blue-green colour with 5% $FeCl_3$ reagent.

The IR spectrum (Fig.92) gave the important absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3600 (s,O-H), 3050 (w,C-H stretch. of alkene or aromatic), 1715 (s,$-\overset{O}{\overset{\|}{C}}-O-$), 1630, 1605, 1520 (s,C=C stretch. of alkene or aromatic), 1190, 1170 (s,C-O) and 845, 820 (m,C-H out-of-plane bend. of p-substituted benzene nucleus).

The mass spectrum (Fig.93) displayed the molecular ion peak at m/e (% rel int.) 572.0 (13.02) (Calcd. for $C_{39}H_{56}O_3$: MW. 572.44) and other fragmentation ion peaks at m/e 557.0 (3.36, M^+-CH_3), 408 (20.33, M^+-164), 204.0 (94.90) and 147.0 (100.00).

The UV (EtOH) spectrum (Fig.94) revealed the maxima absorption peaks at 312 nm. (log ε = 5.64), 208 nm. (log ε = 6.89) and a shoulder at 225 nm. (log ε = 4.14). The UV spectrum of this compound in ethanol containing 3 drops of 0.1 M. NaOH gave the maxima absorption peaks at 368 nm. (log ε = 4.48), 208 nm. (log ε = 4.48) and a shoulder at 225 nm. (log ε = 4.10).

The ^1H NMR ($CDCl_3$) spectrum (Fig.95) exhibited the signals at chemical shift δ (ppm.): 7.61 (1H,d,J=8.55 Hz), 6.83 (2H,d,J=11.69 Hz), 6.75 (1H,d,J=8.55 Hz), 5.82 (2H,d, J=12.69 Hz), 5.50 (1H,dd,J=2.56,7.05 Hz), 5.42 (1H,s, phenolic proton), 4.52 (1H,t,J=6.05 Hz) and other signals around 1.89 to 0.84 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.96) displayed the carbon signals at chemical shift δ (ppm.): 166.53 (carbon of ester), 158.19, 156.67, 142.91, 132.24, 118.10, 117.02, 115.07 (aromatic and olefinic carbons), 81.02 (carbon attached to oxygen atom) together with signals around 55.85 to 15.55 ppm..

2.9.4.3 Purification and Properties of Compound 12

Fraction H4, the lower spot on PTLC and Fraction J gave the same major spot on TLC plate at Rf 0.14 (solvent: chloroform). Fraction J contained greenish oil and crystalline solid. After washing greenish oil with n-hexane, the remaining solid was recrystallized with a mixture solvent of dichloromethane and n-hexane for several times to yield Compound 12 as white amorphous solid decomposed at 263 °C. Compound 12 gave only one spot on TLC plate at Rf 0.14 using chloroform as a mobile phase. It was soluble in chloroform and dichloromethane, but slightly soluble in n-hexane, methanol and ethanol. This compound gave a purple colour with Liebermann-Burchard's, decolourized Br_2 in CCl_4 and gave a deep blue-green colour with 5% $FeCl_3$, but it showed negative results to 2,4-DNP and 5% $KMnO_4$ reagents.

The IR spectrum (Fig. 97) revealed the major absorption peaks at ν^{KBr}_{max} (cm^{-1}): 3320 (b,O-H), 3050 (w,C-H stretch. of alkene or aromatic), 1720 (m,$-\overset{O}{\overset{\|}{C}}-O-$), 1670, 1640, 1615, 1580, 1520 (s,C=C stretch. of alkene or aromatic nucleus),1270-1250 (m,C-O), 1190, 1170 (s,C-O stretch. of phenol), 970 (m,C-H out-of-plane bend. of alkene in trans geometry) and 820 (m,C-H out-of-plane bend. of p-substituted benzene ring).

The mass spectrum (Fig.98) exhibited the molecular ion peak at m/e (% rel int.) 572.0 (8.36) (Calcd. for $C_{39}H_{56}O_3$: MW. 572.44) together with other fragmentation ion peaks at m/e 557.0 (2.54, M^+-CH_3), 408.0 (5.96, M^+-164), 204.0 (72.29) and 147.0 (100.00).

The ^1H NMR ($CDCl_3$) spectrum (Fig.99) displayed the proton signals at chemical shift δ (ppm.): 7.61 (1H,d,J=16.12 Hz), 7.43 (2H,d,J=8.30 Hz), 6.84 (2H,d,J=8.30 Hz), 6.29 (1H,d,J=16.12 Hz), 5.54 (1H,dd,J=3.22,8.87 Hz), 5.48 (1H,s, phenolic OH), 4.59 (1H,t,J=7.39 Hz) and other signals around 1.89 to 0.82 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.100) showed the significant carbon signals at chemical shift δ (ppm.): 167.29 (carbon of ester), 157.59, 153.06, 143.94, 129.91, 116.96, 116.42, 115.88 (aromatic and olefinic carbons), 81.04 (carbon attached to oxygen atom) together with other signals around 55.74 to 15.55 ppm..

The UV (EtOH) spectrum (Fig.101) gave the maxima absorption peaks at 306 nm. (log ε = 4.39), 208 nm. (log ε = 4.19) and a shoulder at 228 nm. (log ε = 4.09). The λ_{max} at 306 nm. was shifted to 388 nm. (log ε = 4.19) in alkalia medium (0.1 M. of NaOH, 3 drops).

Elemental analysis found:	%C 81.63	%H 10.06
Calcd. for $C_{39}H_{56}O_3$, MW. 572.44:	%C 81.73	%H 9.86

Hydrolysis of Compound 12 (63)

Compound 12 100 mg. was hydrolyzed by 10% KOH in 95% ethanol in usual manner for 10 hours. The alcoholic part was separated by extraction with diethylether 3 times to gain white needle product, 40 mg.. After recrystallization the latter using a mixture solvent of chloroform and n-hexane, the bright white needle crystal 35 mg., m.p. 278.0-280.0 °C, labelled as Compound 12A, was obtained.

Study on Compound 12A

Compound 12A, m.p. 278.0-280.0 °C, was soluble in dichloromethane and chloroform, but slightly soluble in n-hexane and methanol. This compound gave a violet colour with Liebermann-Burchard's and also decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.102) revealed the important absorption peaks at ν^{KBr}_{max} (cm^{-1}): 3480 (b,O-H), 3050 (w, C-H stretch. of $R_1R_2C{=}CR_3H$), 1640 (w,C=C) and 1025 (m,C-O).

The mass spectrum (Fig.103) gave the molecular ion peak at m/e (% rel int.) 426.0 (22.40) (Calcd. for $C_{30}H_{50}O$: MW. 426.39) and other fragmentation ion peaks at m/e 411.0 (12.35, M^+-CH_3), 408.0 (2.50, M^+-H_2O), 302.0 (44.10), 287.0 (30.05) and 204.0 (100.0).

The Co. TLC in several developing solvent systems and mixed m.p. techniques of Compound 12A with taraxerol resulted that these two compounds were coincided.

Acetylation Compound 12A (63)

Compound 12A 10 mg. was acetylated in usual way to obtain Compound 12A acetate as bright white plate 9 mg. (81.92% yield), m.p. 297.0-299.0 °C, Rf 0.68 (solvent: chloroform).

The IR spectrum of this acetate (Fig.104) was identical to that of taraxerylacetate.

The GLC analysis (Fig.105) (condition: column OV-17, SW 80/100 mesh 3 mm. x 2 m., column temp. 300 °C, FID 320 °C, carrier gas N_2 40 mL./min.) gave only one peak at Rt 25.73 min. which was corresponded to taraxerylacetate.

The acidic part of this reaction was acidified by 6N HCl to yield white cloudy solution which was not solidified. Hence, the acidic portion was extracted with ethylacetate and concentrated in vacuum to gain white impure amorphous product approximately 8 mg., m.p. 206.0-209.0 °C. This compound, designated as Compound 12B, gave a single spot at Rf 0.71 (solvent: 50% methanol-chloroform) which was corresponded to p-coumaric acid.

The HPLC analysis (Fig.106) (condition: column Lichrospher 100 RP-18 (5 µm) ODS, 4.6 mm. x 25 cm., mobile phase water (pH 2.5) : methanol by gradient elution 80/20→50/50 at 1% min. (GE), flow rate 1.2 mL./min., detector UV 320 nm.) revealed the solely peak at Rt 17.99 min. which was identical to the authentic trans-p-coumaric acid.

Acetylation of Compound 12

Compound 12 70 mg. was mixed with acetic

anhydride 6.0 mL. and 2.0 mL. of dry pyridine. The mixture was refluxed on a stream bath for seven hours and check whether the reaction was completed or not by TLC. The entire mixture was poured onto ice-water to yield white precipitated solid. After recrystallization this solid with a mixture of chloroform and n-hexane, white crystalline solid 53 mg., labelled as Compound 12 acetate, was obtained, m.p. 167.0-169.0 °C, Rf value 0.75 (solvent: chloroform).

The IR spectrum (Fig.107) revealed the significant absorption bands at ν_{max}^{KBr} (cm^{-1}): 1765 (-C(=O)-O-) and 1275 (C-O of acetate).

The mass spectrum (Fig.108) gave the molecular ion peak at m/e (% rel int.) 614.0 (1.50) (Calcd. for $C_{41}H_{58}O_4$: MW. 614.45) and other fagmentation ion peaks at 408.0 (9.00) and 204.0 (42.5).

2.9.5 Separation of Fraction IE

Thin layer chromatography of Fraction IE, 60% chloroform-hexane extract of Fraction I, using 5% methanol in chloroform as a developing solvent revealed that there were at least two major compounds in its crude. One was at Rf value 0.82 and the other was at 0.70. Like other fractions, the concentrated extract of Fraction IE 33.72 g. (22.48% wt. by wt. of Fraction I) was chromatographed on silica gel 650.0 g. using 20% chloroform-hexane as an initial eluent and changing a solvent system by gradual increasing of chloroform. The column was stripped off with methanol. Other steps were like Topic 2.9.1. The results of separation this fraction were presented in Table 2.13.

Table 2.13 The results of separation Fraction IE by silica gel column (using column 3.5 x 120.0 cm.)

Eluents	Fraction No. (750 mL.)	Remarks	Weights (g.)
20% chloroform-hexane	1-8	yellow oil	0.27
	9-15	yellow oil	3.15
40% chloroform-hexane	16-25	yellow oil + white ppt. (Cpd. 9)	4.61
	26-29	brown-yellowish oil	1.06
	30-41	pale green oil + white ppt. (Fraction K)	5.18
60% chloroform-hexane	42-54	greenish oil	2.03
	55-62	greenish oil	1.26
80% chloroform-hexane	63-75	greenish oil + ppt. (Fraction L)	2.10
chloroform	76-89	greenish oil + ppt. (Fraction M)	1.95
5% methanol-chloroform	90-100	greenish oil	6.26
10% methanol-chloroform	101-118	greenish oil	3.11
20% methanol-chloroform	119-130	greenish oil	1.05
40% methanol-chloroform	131-140	greenish oil	0.98
methanol	141-145	pale greenish oil	0.59

2.9.5.1 Purification and Properties of Compound 13

Fraction K, which was eluted from silica gel column chromatography of Fraction IE with 40% chloroform-hexane in Fraction No. 30-41, contained pale greenish oil and white crystalline solid. Removed pale greenish oil by washing with n-hexane and then purified the remaining white solid by recrystallization with a mixture solvent of chloroform and n-hexane to yield Compound 13 as bright white needle, 180 mg. (0.53% wt. by wt. of Fraction IE), m.p. 229.0-230.0 °C, Rf 0.31 (solvent: chloroform). It was soluble in chloroform and dichloromethane but slightly soluble in n-hexane and methanol. This compound gave a violet colour when it was treated with Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.109) revealed the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 3350 (b,O-H), 3090 (w, C-H stretch. of $R_1R_2C=CH_2$), 1645 (w,C=C), 1055, 1045, 1030 and 1010 (m,C-O) and 885 (s,C-H out-of-plane bend. of $R_1R_2C=CH_2$).

The mass spectrum (Fig.110) gave the molecular ion peak, M^+, at m/e (% rel int.) 442.0 (35.62) (Calcd. for $C_{30}H_{50}O_2$: MW. 442.39), and other fragmentation ion peaks at m/e 424.0 (18.75), 409.0 (10.70), 315.0 (17.69), 207.0 (100.00), 189.0 (86.67) and 135.0 (77.23).

The ^1H NMR ($CDCl_3$) spectrum (Fig.111) exhibited the proton signals at chemical shift δ (ppm.): 4.91 (2H,s, olefinic protons), 4.12 (2H,s, protons attached to electron withdrawing group), 3.19 (1H,t,J=12.80 Hz) together with other signals around 2.20 to 0.76 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.112) displayed the carbon signals at chemical shift δ (ppm.): 154.83, 106.89 (1C each, olefinic carbons), 79.91 (carbon attached to oxygen atom) and other signals around 65.06 to 14.57 ppm..

The UV (EtOH) spectrum (Fig.113) gave the λ max at 205 nm. (log ε = 3.64).

Elemental analysis found: %C 81.30 %H 11.56

Calcd. for $C_{30}H_{50}O_2$, MW. 442.39: %C 81.45 %H 11.31

Acetylation of Compound 13 (63)

To Compound 13 (30 mg.) in dry pyridine (1.0 mL.), acetic anhydride (4.0 mL.) was added and the reaction mixture was refluxed on a water bath for 4 hours. Then, the mixture was left at room temperature and poured onto ice water. The precipitate was filtered off, washed with water and recrystallized from a mixture of chloroform and ethanol to yield white needle 20 mg. (58% yield), m.p. 169.0-171.0 °C, Rf 0.71 (solvent: chloroform).

The IR spectrum (Fig.114) showed the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 3090 (w,C-H stretch. of $R_1R_2C{=}CH_2$), 1740-1730 (s,$-\overset{O}{\overset{\|}{C}}-O-$), 1660-1640 (w,C=C), 1240 (s,C-O of acetate) and 890 (w,C-H out-of-plane bend. of $R_1R_2C{=}CH_2$).

The mass spectrum (Fig.115) gave the molecular ion peak, M^+, at m/e (% rel int.) 526.0 (38.59) (Calcd. for $C_{34}H_{54}O_4$: MW. 526.42) together with other fragmentation ion peaks at m/e 466.0 (84.12) and 189.0 (100.00, base peak).

2.9.5.2 Purification and Properties of Compound A

Fraction L and M gave the same major spot at Rf 0.61 using 2% methanol in chloroform as a mobile phase. After the oil in these two portions was removed, the white product was recrystallized by a large amount of chloroform and mathanol to gain white amorphous product decomposed at 280-283 °C, Rf 0.61 (solvent: 2% methanol-chloroform). This compound, marked as Compound A, was hardly soluble in various organic solvents such as n-hexane, diethylether, dichloromethane, chloroform, benzene, acetone and methanol. Compound A gave a violet color with Liebermann-Burchard's, delourized Br_2 in CCl_4 and gave a positive test to Molisch's reagents.

The IR spectrum (Fig.116) gave the significant absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3430 (b,O-H), 3050 (very w, C-H stretch. of $R_1R_2C=CR_3H$), 1720 (s,$-\overset{O}{\overset{\|}{C}}-O-$), 1620 (w,C=C) and 1020 (s, C-O of glycosidic linkage).

The mass spectrum (Fig.117) did not give the molecular ion peak, M^+, but it displayed other important fragmentation ion peaks at m/e (% rel int.): 426.0 (0.34), 408.0 (20.00), 269.0 (48.00), 204.0 (100.00) and 189.0 (58.00).

2.9.6 Separation of Fraction IF

Fraction IF, chloroform soluble fraction, was obtained from silica gel quick column chromatography as a greenish crude, 24.16 g.. Thin layer chromatography of this extract using 10% methanol in chloroform as a developing solvent revealed one major spot at Rf value 0.54. Therefore, this fraction was subjected on silica gel column using an adsorbent 500.0 g. and 40% chloroform in

n-hexane as an initial eluent. Each portion was collected approximately 750.0 mL. Other procedures were similar to Topic 2.9.1. The results of separation this fraction were shown in Table 2.14.

Table 2.14 The results of separation Fraction IF by silica gel column (using column 3.5 x 120.0 cm.)

Eluents	Fraction No. (750 mL.)	Remarks	Weights (g.)
40% chloroform-hexane	1-10	yellow oil	0.51
60% chloroform-hexane	11-20	orange oil	1.27
80% chloroform-hexane	21-33	greenish oil	3.18
	34-40	greenish oil	5.63
chloroform	41-45	greenish oil	2.49
5% methanol-chloroform	46-60	greenish oil	1.30
10% methanol-chloroform	61-62	greenish oil	0.98
	63-74	greenish oil + ppt. (Fraction N)	3.26
20% methanol-chloroform	75-84	greenish oil	1.22
50% methanol-chloroform	85-90	pale greenish oil	0.87
methanol	91-93	brownish oil	0.08

2.9.6.2 Purification and Properties of Compound 14

Fraction N, which was eluted from silica gel column chromatography with 10% methanol in chloroform in Fraction No. 61 to 74, contained greenish oil and a crystalline solid. The greenish oil was removed by washing with ethylacetate and the remaining material was recrystallized by hot ethanol for several times to obtain white amorphous solid, 600 mg. (2.48% wt. by wt. of Fraction IF). This compound, labelled as Compound 14, decomposed at 260 °C and showed a single spot at Rf value 0.89 using 20% methanol-chloroform as a developing solvent. It was slightly soluble in many organic solvents such as chloroform, ethylacetate, methanol and ethanol. Compound 14 gave a green colour with Liebermann-Burchard's, showed positive tests to Molisch's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.118) showed the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 3420 (b,O-H), 1640 (w,C=C), 1295, 1165 (m,C-O) and 1075-1020 (s, glycosidic linkage).

The UV (EtOH) spectrum (Fig.119) gave the λ max at 203 nm. (log ε = 3.97).

The ^1H NMR (C_6D_5N) spectrum (Fig.120) exhibited the proton signals at δ (ppm.): 5.32 (olefinic proton), 4.79-3.73 (protons on sugar moiety) and other signals around 2.43 to 0.66 typical to steroid compounds.

The ^{13}C NMR (C_6H_5N) spectrum (Fig.121) displayed the carbon signals at δ (ppm.): 138.71 and 119.21 (olefinic carbons), 100.25, 78.36, 76.36, 75.81, 72.78 and 69.53 (6 carbons adjacent to oxygen) and other peaks around 56.77 to 10.00

ppm..

The mass spectrum (Fig.122) did not give the molecular ion peak, but it revealed important mass fragments at m/e (% rel int.) 414.0 (49.77), 396.0 (100.00, 414-H_2O), 381.1 (27.50, 414-H_2O-CH_3), 273.0 (18.07, 414-$C_{10}H_{21}$), 255.0 (39.74, 414-$C_{10}H_{21}$-H_2O) and 213.0 (32.22, 414-$C_{10}H_{21}$-42).

Acid hydrolysis of Compound 14

Compound 14 200 mg. was hydrolyzed by refluxing with 10% hydrochloric acid in ethanol for 10 hours (Check whether the reaction was completed or not by TLC). Solvent was removed under reduced presure. The residue was diluted with water and extracted with diethylether which was separated into two layers as aglycone in diethylether and a sugar component in water.

Study of aglycone

Etheral solution, that described above, was dried over anhydrous sodium sulfate and evaporated. The residue was recrystallized from a mixture of dichloromethane and n-hexane to afford white needle compound, assigned as Compound 14A, 120 mg., mp. 134.0-136.0 °C and Rf 0.32 (solvent: chloroform). Compound 14A was soluble in dichloromethane and chloroform, but slightly soluble in methanol and n-hexane. This compound decolourized Br_2 in CCl_4 and gave a deep green colour to Liebermann-Burchard's reagents.

The IR spectrum (Fig.123) exhibited the absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3420 (b,O-H), 1640 (w,C=C) and 1060 and 1020 (m,C-O).

The GLC analysis (Fig.124) (the same condition as Compound 10) revealed only one component at Rt 22.41 min. which was identical to the authentic β-sitosterol.

The mixed melting point of this aglycone with β-sitosterol did not show any depression. The Co-TLC technique of Compound 14A compared with the authentic β-sitosterol gave the same Rf value, though the solvent systems using as mobile phases were changed.

Acetylation of Compound 14A (63)

Compound 14A, aglycone part, 20 mg. was acetylated in usual manner to yield plate crystalline product, 16 mg., m.p. 132.0-134.0 °C. It showed only one spot at Rf value 0.70 using chloroform as a developing solvent.

The IR spectrum (Fig.125) expressed the vital absorption bands at ν_{max}^{KBr} (cm^{-1}): 1730 (s,$-\overset{O}{\overset{\|}{C}}-O-$), 1250 (s,C-O of acetate).

Study of carbohydrate component of Compound 14

After removing the aglycone from the hydrolysis mixture of Compound 14, the aqueous phase was neutrallized by silver carbonate, filtered off and concentrated in vacuum to gain white powder (35 mg.), designated as Compound 14B.

The solution of this carbohydrate (approximately 2 mg. in 1.0 mL. of water) was spoted on Whatmann No.1 chromatography paper as described in Topic 2.4.5 with a mixture of various standard sugars (arabinose, rhamnose, glucose, galactose and xylose). The chromatogram was developed by the descending

technique with n-butanol-benzene-pyridine-water (BBPW = 5:1:3:3) for 18 hours, dried at room temperature and detected with aniline hydrogen phthalate, further by heating at 110-120 C for 15 mins.. It gave one spot which was identical to the authentic D-(+)-glucose.

TLC of the component of neutral aqueous phase of glycosidic hydrolysate was compared with a mixture of various standard sugars using butanol:acetic acid:diethylether:water 9:6:3:1. The sugar was detected by spraying with 25% sulfuric acid and dried at 110-120 C to develope a brownish spot at Rf 0.45 which was identical to the standard D-(+)-glucose.

The HPLC analysis (Fig.126) (condition: column Li Chrosorb NH_2 5 µm, mobile phase: acetonitrile:water 75:25, flow rate 2.0 mL./min., pressure 140 kg./cm^2, detector: refractive index) revealed a single peak on chromatogram at Rt 5.99 min. which was corresponded to the authentic D-(+)-glucose.

Acetylation of Compound 14B

Dried Compound 14B, 15 mg., was refluxed on a stream bath with acetic anhydride 3.0 mL. and a few drops of pyridine for 4 hours. The entired mixture was worked up in usual manner to gain white precipitated solid. After recrystallization with a mixture of chloroform and methanol, bright white needle, m.p. 112.0-113.0 °C was obtained.

The IR spectrum (Fig.127) revealed the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 1760-1740 (s,$-\overset{O}{\overset{\|}{C}}-O-$) and 1260-1240 (s,C-O of acetate).

Peracetylation of Compound 14

Compound 14 (100 mg.) was acetylated in usual manner to yield bright colourless plates from a mixture of chloroform and ethanol, 95 mg., m.p. 169.0-171.0 °C, Rf 0.34 (solvent: 5% methanol-chloroform).

The IR spectrum (Fig.128) showed the important absorption bands at ν^{KBr}_{max} (cm^{-1}): 1759 (s,$-\overset{O}{\overset{\|}{C}}-O-$), 1660 (w,C=C) and 1250-1220 (s,C-O of acetate).

The ^{1}H NMR ($CDCl_3$) spectrum (Fig.129) displayed the proton signals at δ (ppm.): 5.39 (1H,d, olefinic proton), 5.15-4.20 (protons on sugar moiety), 2.02-2.08 (12H, methyl protons of acetyl groups) and other signals corresponded to those of steroidal compounds.

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.130) exhibited the carbon signals of 4 carbonyl groups at δ (ppm.): 173.41, 170.70, 170.40 and 169.40 ppm., two olefinic carbons at 140.37 and 122.16 ppm., together with other carbon signals around 99.68 to 11.86 ppm..

The mass spectrum (Fig.131) displayed mass fragments at m/e (% rel int.) 744.0 (0.02, detected with difficulty, Calcd. for $C_{43}H_{68}O_{10}$: MW. 744.53) together with other fragmentation ion peaks at m/e 684.0 (0.12), 624.0 (0.62), 564.0 (0.44) and 504.0 (0.21) corresponded to M^{+}-HOAc, M^{+}-2HOAc, M^{+}-3HOAc, M^{+}-4HOAc, respectively.

2.9.7 Separartion of Fraction IG

Fraction IG, 20% methanol in chloroform fraction, 20.08 g. (13.39% wt. by wt. of Fraction I), was obtained by silica

gel column chromatography. The TLC analysis using 20% methanol in chloroform as a developing solvent revealed one major spot at Rf 0.90. Therefore, this fraction was chromatographed on aluminium oxide column using 400.0 g. of the adsorbent and 50% chloroform in n-hexane as an initial eluent. Each 500.0 mL. portion was collected, monitored with TLC and combined the equivalent ones. Other procedure was carried out like Topic 2.9.1. The results of separation Fraction IG were shown in Table 2.15.

Table 2.15 The results of separation Fraction IG by silica gel column (using column 3.5 x 120.0 cm.)

Eluents	Fraction No. (500 mL.)	Remarks	Weights (g.)
50% chloroform-hexane	1-12	yellow oil	1.64
75% chloroform-hexane	13-25	greenish oil	2.10
chloroform	26-32	greenish oil	1.21
	33-40	greenish oil	1.58
5% methanol-chloroform	41-55	greenish oil	3.20
10% methanol-chloroform	56-58	greenish oil	0.85
	59-70	greenish oil + ppt. (Fraction O)	2.78
20% methanol-chloroform	71-80	greenish oil	2.51
50% methanol-chloroform	81-85	greenish oil	2.03
methanol	86-89	brownish tar	0.65

Fraction O contained greenish oil and white precipitated solid. After the greenish oil was removed by washing with ethylacetate, the remaining solid was recrystallized by hot ethanol for several times to yield white amorphous product 60 mg., m.p. 260-263 °C (dec.), Rf 0.89 (solvent: 20% methanol-chloroform). The mixed m.p. of this compound with Compound 14 did not show any depression, and the Co. TLC technique in various solvent systems of these two substances gave the same Rf values. Moreover, the IR spectra of both compounds were superimposed to each other. Hence, it was reasonable to conclude that the major product in Fraction IG was Compound 14.

2.10 Study on Fraction II

Fraction II, 50.50 g. (0.78% wt. by wt. of plant material), was white solid part which was deposited during evaporating the solvent from 95% ethanol crude extract. The deposited solid was filtered off and then washed with a small amount of hot ethanol to give white solid. This white solid was insoluble in chloroform, dichloromethane, hexane and diethylether, but it was able to dissolve in hot ethanol and water. After dissolving this solid with hot ethanol 250.0 mL. and then reconcentrating, white cubic crystalline product was obtained. Ignition test, chemical tests and its physical properties exhibited that this fraction should be an inorganic salt fraction. Quanlitative analysis of Fraction II found that there were Na^{+}, K^{+}, Mg^{2+} and Cl^{-} as major ions in this fraction. Flame emission spectroscopy technique was used for determination quantities of these cations. The quantitative

analysis results of Fraction II by FES were presented in Table 2.16.

Table 2.16 The quantitative analysis results of Fraction II by FES

Plant part	Percentage of cation		
	Na^+	K^+	Mg^{2+}
leaves (Fraction II)	59.60	40.10	0.30
heartwoods (Fraction IX)	99.98	trace	trace

2.11 Study on Fraction III

Fraction III, a brown-reddish solid 135.80 g. (2.09% wt. by wt. of plant material), was a solid part which was insoluble in both chloroform and water (see also Scheme 2.1). This fraction was soluble in alcohol, acetone and hot water, but insoluble in chloroform, diethylether and n-hexane.

2.11.1 Preliminary Study of Fraction III

Preliminary tests of Fraction III were carried out as follow:

a) Add NaOH 0.50 M. 1.0 mL. to Fraction III 0.1 g.

b) Add $NaHCO_3$ 0.50 M. 1.0 mL. to Fraction III 0.1 g.

c) Dissolve Fraction III 0.05 g. in 95% ethanol 1.0 mL. and then add 5% $FeCl_3$ 2-3 drops

The results of preliminary study of Fraction III were presented in Table 2.17.

Table 2.17 The results of preliminary study of Fraction III

Reagents	Remarks	Indication
0.50 M. NaOH	dissolve to brownish solution	may be carboxylic or phenolic group
0.50 M. $NaHCO_3$	does not dissolve and give gas	may be phenolic group not carboxylic group
5% $FeCl_3$	deep green colour	presence of phenolic group

2.11.2 Tannin and Polyphenol Tests on Fraction III

Fraction III, 3.0 g., was dissolved in 95% ethanol 50.0 mL., concentrated this solution to 20.0 mL. and then preliminary screened on tannin and polyphenol tests as Topic 2.2.7. The results of tannin and polyphenol tests on Fraction III were exhibited in Table 2.18.

Table 2.18 The results of tannin and polyphenol tests on Fraction III

Reagents	Remarks
gelatin solution	white precipitate
gelatin salt solution	white precipitate
1% ferric chloride	deep blue colour
bromine water	orange precipitate
formallin-HCl test	brownish precipitate
vanillin-HCl test	red solution and brownish precipitate
lime water	dark blue precipitate

2.11.3 Hydrolysis of Fraction III

Fraction III, 10.0 g., was dissolved in 20% HCl in ethanol 40.0 mL. and then refluxed on stream bath for ten hours. The entired reaction mixture was concentrated under reduced pressure, added water 30.0 mL. and then reextracted with ethylacetate 20.0, 10.0 and 10.0 mL., respectively. The combined ethylacetate extract was dried over anhydrous sodium sulfate and reconcentrated to gain a dark brownish crude. This crude gave a deep green colour to 5% $FeCl_3$ reagent.

The HPLC analysis (condition: column Lichrospher 100 RP-18 (5μm) ODS, 4.6 mm. x 25.0 cm., mobile phase: water (pH 2.5)/ methanol gradient elution 80/20 → 50/50 at 1% min. (GE), flow rate 1.2 mL./min., detector: UV 254 nm.) revealed one major peak on HPLC chromatogram at Rt 3.40 min. which was corresponded to the authentic

gallic acid.

2.12 Separation of Fraction IV

Fraction IV, chloroform soluble fraction, was obtained by equilibrating the ethanolic crude extract with chloroform and water (1:1). It was a dark greenish crude 105.00 g. (1.22% wt. by wt. of plant material). The concentrated extract 60.0 g. was chromatographed on aluminium oxide column (using adsorbent 1,000.0 g.). Firstly, the column was eluted by 50% chloroform-hexane and then the eluents were changed by gradual introduction of chloroform and methanol. The eluted solution was collected about 1000.0 mL. for each fraction and all fractions were monitored by thin layer chromatography. The equivalent ones were combined. The results of separation this fraction were shown in Table 2.19.

Table 2.19 The results of separation Fraction IV by aluminium oxide column (using column 5.0 x 150.0 cm.)

Eluents	Fraction No. (1000 mL.)	Remarks	Weights (g.)
50% chloroform-hexane	1-10	yellow oil	2.05
75% chloroform-hexane	11-25	yellow oil	1.85
	26-35	greenish oil	4.32
	36-40	greenish oil	2.39
chloroform	41-53	greenish oil	3.86
	54-60	greenish oil	2.74
5% methanol-chloroform	61-65	greenish oil	5.41
	66-70	greenish oil	2.78
10% methanol-chloroform	71-80	greenish oil	3.10
20% methanol-chloroform	81-85	greenish oil	1.23
	86-98	greenish oil + ppt. (Fraction P)	6.89
	99-100	greenish oil	2.14
50% methanol-chloroform	101-130	greenish oil	3.25
75% methanol-chloroform	131-140	brownish tar	2.10
methanol	141-145	brownish tar	0.95

Fraction P contained greenish oil and precipitated solid. After the greenish oil was removed by washing with ethylacetate, the remaining solid was purified by recrystallization with a mixture of chloroform and methanol 2 times and then with hot ethanol 2 times to yield white amorphous solid, 450 mg. (0.75% wt. by wt. of Fraction IV), m.p. 260.0-263.0 °C (dec.), Rf value 0.58 (solvent: 10% methanol-chloroform).

The mixed m.p. of this compound with Compound 14 did not reveal any depression and the Co. TLC between these two compounds in various mobile phases gave the same Rf value. Therefore, the only one crystalline solid obtained from Fraction IV was Compound 14.

2.13 Examination of Fraction V

Fraction V, n-butanolic extract, as a reddish syrup 145.10 g., was obtained by extraction the water soluble part with n-butanol. This fraction was studied by various ways as shown below:

2.13.1 Preliminary Tests

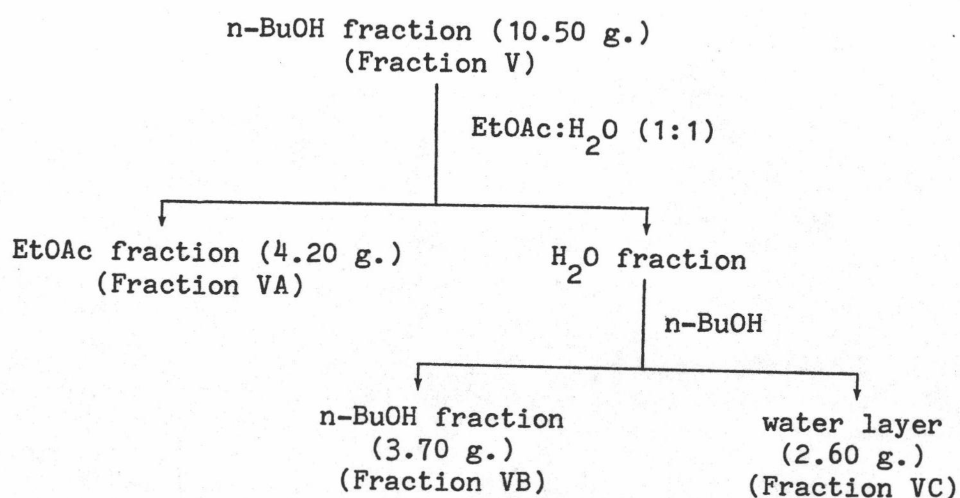
Preliminary tests on Fraction V were carried out by dissolving a concentrated reddish syrup 1.0 g. with 2.5 mL. methanol, divided into 5 portions and added Shinoda's, 5% $FeCl_3$, 2,4-DNP, Molisch's and Liebermann-Burchard's reagents, respectively. The results of preliminary study of Fraction V were shown in Table 2.20.

Table 2.20 The results of preliminary study of Fraction V

Reagents	Remarks	Indication
Shinoda's	red colour (like bird's blood)	may be presence of flavone compounds
5% $FeCl_3$	deep green colour	may be presence of phenolic compounds
2,4-DNP	-	the absence of carbonyl gr.
Molisch's	violet ring at junction	may be presence of carbohydrate moiety
Liebermann-Burchard's	-	the absence of steroid or triterpenoid compounds

2.13.2 Fraction V 10.50 g. was partitioned with water and ethylacetate in ratio 1:1 to yield ethylacetate soluble fraction (Fraction VA 4.20 g., 41.79% wt. by wt. of Fraction V). The water part after extraction with ethylacetate was then extracted with n-butanol to gain n-butanolic soluble fraction (Fraction VB, 3.70 g., 36.45% wt. by wt. of Fraction V), the rest water soluble fraction was assigned as Fraction VC 2.60 g. (25.62% wt. by wt. of Fraction V). The above extraction procedure was presented in Scheme 2.7.

Scheme 2.7 The extraction procedure of Fraction V



These three divided fractions were reexamined with Shinoda's, 5% $FeCl_3$ and Molisch's reagents. The results of reexamination were shown in Table 2.21.

Table 2.21 The colour test results of Fraction VA, VB and VC

Reagents	Fraction		
	VA	VB	VC
Shinoda's	-	+	-
5% $FeCl_3$	+	-	-
Molisch's	-	+	+

Note: + stands for the positive tests like the results in preliminary tests (see Table 2.20)

\- stands for the negative results

The information from Table 2.21 pointed out that Fraction VA should contain phenolic compounds, while Fraction VB ought to be present of flavone substances. The carbohydrate moiety may be present in both Fraction VB and VC.

Study on Fraction VA

Fraction VA was ethylacetate soluble fraction which was obtained from the extraction Fraction V with ethylacetate. This fraction revealed the possibility to contain phenolic compounds.

The HPLC analysis results for phenolic compounds (Fig.133) (condition: column Lichrospher 100 RP-18 (5 μm) ODS, 4.6 mm. x 25.0 cm., mobile phase water (pH 2.5):methanol by gradient elution 80/20→50/50 at 1% min. (GE), flow rate 10.2 mL./min., detector UV 240 nm.) revealed major peaks at 9.90, 24.07, 26.33, 28.97, 29.80 and 35.77 min., respectively.

The HPLC analysis results for gibberellins* (Fig.134) (condition: column Zobax C-8 (5 μm) ODS, 4.6 mm. x 25.0 cm., mobile phase: methanol: aqueous phosphoric acid (pH 3) (57:43), flow rate 8.0 mL./min., detector UV 208 nm.) displayed major peaks at 3.00, 3.40 and 4.27 min.. The peak at 3.40 min. might be corresponded to gibberellin A_3.

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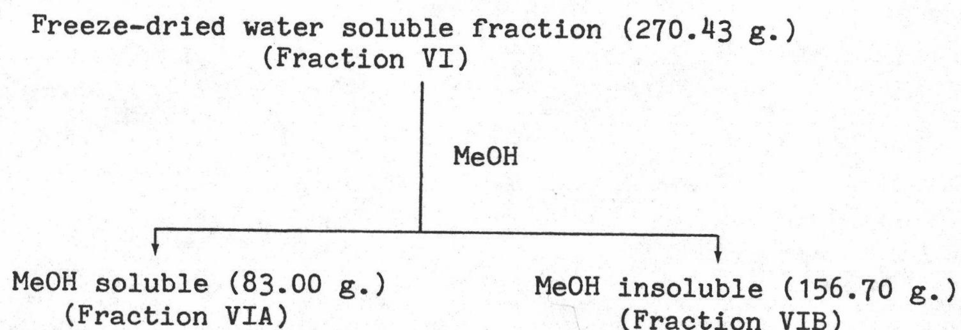
* The standard gibberellins A_3, A_4 and A_7 are obtained from Miss Wanrudee Nimcharoeunwongsa, Department of Biotechnology and Genetic Engineering, Chulalongkorn University.

2.14 Investigation of Fraction VI

Fraction VI, the freeze-dried water soluble, 270.43 g. (4.16% wt. by wt. of plant material) was then extracted with methanol at room temperature to yield two fractions of methanol soluble part (Fraction VIA), 83.00 g. (30.69% wt. by wt. of Fraction VI) and methanol insoluble part (Fraction VIB), 156.70 g. (57.94% wt. by wt. of Fraction VI). The extraction procedure of Fraction VI was presented in Scheme 2.8.

Scheme 2.8 The extraction procedure of Fraction VI



2.14.1 Preliminary Study on Fraction VIA

Fraction VIA, methanol soluble fraction, was obtained from methanolic extract of Fraction VI and concentrated to be a reddish syrup, 83.00 g.. Preliminary tests were conducted on some reagents as follow: Tollen's, Benedict's, Barford's, Molisch's and ninhydrin reagents by ususal method (55). The results of preliminary tests on Fraction VIA were presented in Table 2.22.

Table 2.22 The results of preliminary tests on Fraction VIA

Reagents	Remarks
Tollen's	silver mirror
Benedict's	orange precipitate
Barford's	orange precipitate
Molisch's	violet ring at junction
ninhydrin	pale purple colour

The preliminary information suggested that Fraction VIA ought to be composed of carbohydrate (reducing sugar) and amino acids.

Study on carbohydrate of Fraction VIA

Various methods were selected to study the carbohydrate component in Fraction VIA.

a) Paper chromatography of Fraction VIA

Fraction VIA 3.0 g. was dissolved in methanol 50.0 mL. and then decolourized with activated charcoal and filtered off till methanol soluble fraction became pale yellow solution. Thereupon, the solution was concentrated to 5.0 mL. and chromatographed on whatman No. 1 paper by descending method, as described in Topic 2.4.4 (59) using n-butanol:benzene:pyridine:water in ratio 5:1:3:3 as a developing solvent. Rhamnose, xylose, arabinose, glucose and galactose are used as standard sugars. The results of investigation Fraction VIA by paper chromatography were

presented in Fig.135.

b) The HPLC analysis of Fraction VIA

The aqueous solution which was prepared in Topic a) was analysed further by HPLC technique using condition as follow: Lichrosorb NH_2 5 μm, solvent acetonitrile:water = 75:25, flow rate 2.0 mL./min. pressure 140 kg/cm^2, detector: refractive index). The results were presented as shown in Fig.136.

c) The formation of osazone of Fraction VIA

The osazone formation of crude Fraction VIA 5.0 g. was performed as an usual method (55). An obtained yellow product was recrystallized by a mixture of ethanol and water to yield yellow needle crystal (40 mg.), m.p. 160.0-163.0 °C. Basing on the melting point of the repoted osazone derivatives, this osazone showed the possibility to be either arabinosazone or xylosazone. The comparision data of carbohydrate osazone derivatives was shown in Table 2.23. (55)

Table 2.23 The comparision data of carbohydrate osazones

carbohydrate osazones	m.p. (°C)
rhamnose	190
xylose	164
arabinose	166
glucose	205
galactose	201
Fraction VIA	160.0-163.0

d) The formation of acetate of Fraction VIA

The acetylation reaction was carried out for Fraction VIA in usual manner (63) to gain a precipitated crude. After recrystallization with a mixture of water and ethanol, the crystalline solid with m.p. 83.0-85.0 °C was obtained. The identification of this acetyl derivative was to compare its melting point with those of reported sugarpentaacetate. The comparision of the melting point of pentaacetate sugar was presented in Table 2.24 (55).

Table 2.24 The comparision data of carbohydrate pentaacetate

pentaacetate sugar components	m.p. (°C)
rhamnose	99
xylose	126
arabinose	86
glucose	112
galactose	142
Fraction VIA	83.0-84.0

Study on amino acids of Fraction VIA

The solution prepared in Topic a) was analyzed for amino acids by using Amino Acid Analyzer. The results of this examination were presented in Fig.137.

2.14.2 Study on Fraction VIB

Fraction VIB, 156.70 g. (57.94% wt. by wt. of Fraction VI) was methanol insoluble fraction. This fraction was purified by washing with methanol for several times, dissolved in water, decolourized with activated charcoal and filtered off. After evaporating the solvent, white solid 138.90 g., was obtained. Chemical tests showed that this fraction was inorganic salts. The preliminary anion qualitative test for this fraction displayed that the major component was Cl^-.

The information obtained from X-ray fluoresence spectrometer of this fraction (Fig.138) revealed that Fraction VIB was composed of Na, K, Mn and Cl.

The quantitative analysis of Fraction VIB by ICPS was carried out. The results were shown in Table 2.25.

Table 2.25 The quantitative analysis of Fraction VIB by ICPS

Plant parts	Fraction	Element concentration (ppm.)						
		Na	K	Mg	Ca	Mn	Fe	Cu
leaves	VIB	791.2	401.8	206.6	27.05	10.09	0.066	0.087
	VII	286.8	300.7	168.7	23.42	22.16	0.083	0.129
heartwoods	XIIIB	2747.0	503.3	451.3	74.04	15.02	0.117	0.037
	XIV	606.6	352.6	138.6	28.70	8.23	0.066	0.286

2.15 Examination of Fraction VII

Fraction VII was exhausted leaves after extraction with dichloromethane and 95% ethanol (see Scheme 2.1). This fraction 1.0 kg. was then extracted by 50% ethanol-water to gain a dark brownish crude 58.65 g. (5.86% wt. by wt. of Fraction VII). This fraction was preliminary tested on tannins and polyphenols following to the phytochemical screening method (62) and found that this fraction contained condensed and hydrolysable tannins. (It gave the same results as Fraction III, Table 2.18). Besides, this fraction 2.0 g. was dissolved in distilled water 25.0 mL. and decolourized with activated charcoal to gain clear pale yellow solution. Chemical tests on this solution showed the presence of Na, K, Mg and Cl.

The determination of the cation composition in this fraction by ICPS was performed. The results of this analysis were shown in Table 2.25.

Part II Chemical Constituents of the Heartwoods of *R. apiculata* Bl.

2.16 Separation Fraction VIII

The dichloromethane extract, Fraction VIII, was concentrated and gave a yellow-brownish crude 154.00 g. (0.22% wt. by wt. of dried plant material). Thin layer chromatography of concentrated crude revealed that there were at least three components in its crude (solvent: chloroform). This fraction 70.0 g. was chromatographed on silica gel column chromatography using 850.0 g. of adsorbent and n-hexane as an initial eluent. The eluents were changed from n-hexane to chloroform by gradual introduction of the latter. Finally, the column was stripped with methanol. The eluted solution was collected approximately 1,000.0 mL. for each fraction. Each eluted portion was monitored by thin layer chromatography and the equivalent ones were combined. The results of separation Fraction VIII using silica gel column chromatography were presented in Table 2.26.

Table 2.26 The results of separation Fraction VIII by silica gel column chromatography (using column 5.0 cm. x 150.0 cm.)

Eluents	Fraction No. (1000 mL.)	Remarks	Weights (g.)
n-hexane	1-5	yellow oil	0.92
	6-10	yellow oil	0.16
5% chloroform-hexane	11-33	golden oil + white ppt (Fraction AA)	3.56
10% chloroform-hexane	34-37	orrange oil	0.53
	38-44	yellow oil	0.44
20% chloroform-hexane	45-52	yellow oil + white ppt. (Fraction BB)	0.90
	53-65	pale yellow oil + white ppt. (Fraction CC)	3.52
	66-76	orange oil + needle ppt. (Fraction DD)	4.89
	77-85	orange oil	1.20
30% chloroform-hexane	86-89	brown oil	0.38
	90-106	brown oil	0.81
40% chloroform-hexane	107-116	yellow-brownish oil (Fraction EE)	0.56
	117-120	yellow-brownish oil	0.23
	121-132	yellow-brownish oil + yellow ppt. (Fraction FF)	2.54
50% chloroform-hexane	133-143	brownish oil	1.68
	144-149	yellow-brownish oil	0.54
60% chloroform-hexane	150-161	yellow-brownish oil	1.07
	162-165	yellow-brownish oil	0.12
80% chloroform-hexane	166-178	brownish oil + ppt. (Fraction GG)	3.86

Table 2.26 (Cont.)

Eluents	Fraction No. (1000 mL.)	Remarks	Weights (g.)
80% chloroform-hexane	179-187	brownish oil	2.05
chloroform	188-193	brownish oil	1.84
	194-199	brownish oil	0.32
5% methanol-chloroform	200-206	brownish oil + ppt.	0.26
	207-217	brownish oil + ppt. (Fraction HH)	1.67
10% methanol-chloroform	218-227	brownish oil + ppt. (Fraction JJ)	2.39
20% methanol-chloroform	228-245	brownish oil	4.61
50% methanol-chloroform	246-254	brownish oil	2.32
methanol	255-260	brownish oil	0.25

2.16.1 Purification and Properties of Compound 15

Fraction AA 3.56 g., which was eluted from silica gel column chromatography with 5% chloroform-hexane in Fraction No. 11 to 25, contained golden oil together with white precipitated solid. The golden oil was removed by n-hexane and then the white remaining material was recrystallized by a mixture solvent of chloroform and acetone for four times to yield white amorphous product 2.74 g. (3.91% wt. by wt. of Fraction VIII), m.p. 85.0-87.0 °C. This product showed 2 spots on TLC plate at Rf value 0.32 and 0.29 using 20% chloroform-hexane as a solvent system. Though the

solvents for recrystallization were changed, 2 spots on TLC plate were still shown. This compound was easily soluble in dichloromethane, chloroform and diethylether, but slightly soluble in acetone, methanol and 95% ethanol. It gave a deep green colour when it was treated with Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.139) revealed the major absorption peaks at ν_{max}^{KBr} (cm^{-1}): 1740 (s,$-\overset{O}{\overset{\|}{C}}-O-$), 1220, 1200 and 1180 (m,C-O).

The ^1H NMR ($CDCl_3$) spectrum (Fig.140) exhibited the proton signals at δ (ppm.): 5.38 (1H,d,J=3.98 Hz, olefinic proton), 4.05 (1H,t,J=6.52 Hz), 1.25 (high intensity, methylene protons) together with other signals around 2.27 to 0.88 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.141) displayed the important carbon signals at chemical shift δ (ppm.): 173.63, 173.3 (1C each, carbon of ester), 139.77, 122.60 (1C each, olefinic carbon) and other signals around 73.68 to 11.86 ppm..

The mass spectrum (Fig.142) exhibited a set of molecular ion peaks at m/e (% rel int.) 704.0 (0.14), 676.0 (0.23) and 648.0 (0.20) (Calcd. for $C_{48}H_{96}O_2$, $C_{46}H_{92}O_2$, $C_{44}H_{88}O_2$: MW. 704.76, 676.72 and 648.49, respectively) together with other fragmentation ion peaks at 396.0 (28.58), 381.0 (2.86), 255.0 (4.53) and 213.0 (3.43).

Basic Hydrolysis of Compound 15

Compound 15 260 mg. and 10.0 mL. of 10% KOH in ethanol were mixed and refluxed on stream bath for 5 hours (Check whether the reaction was completed or not by TLC). Evaporation of

ethanol gave a nearly white solid. This solid was extracted with diethylether, 150.0 mL., three times. The combined diethylether was dried over anhydrous calcium chloride. Evaporation the solvent furnished a solid with pale yellow oil 140 mg.. This solid gave two spots on TLC plate at Rf value 0.60 and 0.34 using chloroform as a mobile phase. This fraction, marked as Fraction 15A, was chromatographed on silica gel 7.0 g.. The results of separation Fraction 15A were presented in Table 2.27.

Table 2.27 The results of separation Fraction 15A by silica gel column (using column 1.0 x 50.0 cm.)

Eluents	Fraction No. (25 mL.)	Remarks	Weights (mg.)
n-hexane	1-7	yellow oil	8
	8-12	yellow oil + white ppt. (Fraction 15A1)	40
5% chloroform-hexane	13-15	yellow oil	10
	16-20	pale yellow oil + needle ppt. (Fraction 15A2)	65
	21-23	pale yellow oil	10
10% chloroform-hexane	24-27	nothing	-

Study on Fraction 15A1

Fraction 15A1, 40 mg., was gained from the recolumn chromatography of Fraction 15A in Fraction No. 8-12 by using n-

hexane as an eluent. After recrystallization with acetone for several times, Compound 15A was obtained as white amorphous solid, 29 mg., m.p. 74.0-76.0 °C, Rf 0.60 (solvent: chloroform). This compound was easily soluble in various organic solvents such as dichloromethane, chloroform and diethylether. It gave negative results to Liebermann-Burchard's, Br_2 in CCl_4, 5% $FeCl_3$ and 2,4-DNP reagents.

The IR spectrum (Fig.143) showed the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 3300 (b,O-H) and 1065 (m,C-O of 1° ROH).

The GLC analysis (Fig.144) (condition: 2% OV-1, CW 80/100 mesh AW DMCS 1/8 inch x 7.55 fts., column temp. 280 °C, FID 320 °C, carrier gas N_2 50.0 mL./min.) revealed six peaks on gas chromatogram at retention time 2.40, 3.29, 4.97, 7.60, 12.40 and 15.00 min., respectively. The peaks at Rt 12.40 and 15.00 min. were the major ones in this mixture.

Study on Fraction 15A2

Fraction 15A2 as a mixture of pale yellow oil and white needle 60 mg. was obtained from recolumn chromatography of Fraction 15A in Fraction No. 16-20. The pale yellow oil was removed by hot methanol and the remaining product was recrystallized by a mixture of dichloromethane and n-hexane to yield bright white needle crystal 40 mg., m.p. 134.0-136.0 °C, Rf 0.34 (solvent: chloroform). This compound, designated as Compound 15B, was easily soluble in diethylether, chloroform and dichloromethane, but slightly soluble in methanol and ethanol. Compound 15B revealed a deep green colour to Liebermann-Burchard's and also decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.146) gave the vital absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3420 (b,O-H), 1660-1640 (w,C=C) and 1060 (m,C-O).

The GLC analysis (Fig.147) (condition: column 2% OV-1, 80/100 mesh AW DMCS, 3 mm. x 2 m., column temp. 260 °C, FID 320 °C, carrier gas N_2 48.0 mL./min.) gave three peaks on gas chromatogram at retention time 18.32, 19.65 and 22.65 min., respectively. The peak at Rt 22.65 min. was the major one.

The other part from basic hydrolysis was a solid part which remained after extraction with diethylether. After acidification by dil. HCl, an impure solid was gained. It was recrystallized for two times with a mixture of chloroform and methanol to afford white amorphous solid 10 mg., labelled as Compound 15C. This compound was easily soluble in dichloromethane, chloroform and diethylether, but slightly soluble in methanol and ethanol. Compound 15C gave negative tests to all these reagents: Liebermann-Burchard's, Br_2 in CCl_4, 5% $FeCl_3$ and 2,4-DNP reagents.

The IR spectrum (Fig.148) gave the significant absorption bands at ν_{max}^{KBr} (cm^{-1}): 3600-3200 (b,O-H), 1700 (m,$-\overset{O}{\overset{\|}{C}}-OH$), 935 (b,O-H bend. of carboxylic acid).

The HPLC analysis (Fig.149) (condition: column Zorbax-C18, mobile phase: a mixture of 2.2% dioxane in methanol and water pH 2.8, column temp. 60 C, flow rate 1.0 mL./min., detector UV 205 nm.) revealed the peaks with retention time at 4.35, 6.47, 10.15 and 11.29 min., respectively. The peaks at Rt 6.47 and 11.29 min. were the major components in this mixture.

Fraction BB, obtained from the combination of the Fraction No. 45-52, was composed of yellow oil and white precipitated solid. This fraction was easily soluble in many solvents such as n-hexane, chloroform and dichloromethane. Fraction BB showed a major spot on TLC plate at Rf value 0.34 using chloroform as a developing solvent. Recolumn chromatography was used for purification this fraction (0.90 g.) using silica gel 20.0 g. as an adsorbent and 40% chloroform-hexane as an initial eluent. Each portion was collected about 20.0 mL. and each one was monitored on TLC plate. The equivalent portions were combined. The results of separation this fraction were presented in Table 2.28.

Table 2.28 The results of recolumn chromatography of Fraction BB by silica gel column (using column 1.0 x 50.0 cm.)

Eluents	Fraction No. (20 mL.)	Remarks	Weights (mg.)
40% chloroform-hexane	1-5	yellow oil	2
	6-7	yellow oil + white ppt. (Fraction BB1)	10
	8-12	pale yellow oil + white (Fraction BB2)	12
	13-17	pale yellow oil	7
	18-20	nothing	-

2.16.2 Purification and Properties of Compound 16

Fraction BB1 and Fraction BB2 gave the same major spot at Rf value 0.34 using chloroform as a solvent system. After removing yellow oil in Fraction BB1 and pale yellow oil in Fraction BB2, the white remaining products of these two fractions were combined and recrystallized with acetone for four times to yield Compound 16, 55 mg. (0.08% wt. by wt. of Fraction VIII), m.p. 78.0-79.0 °C. This compound was soluble in dichloromethane and chloroform, but slightly soluble in acetone and methanol. It gave negative results to all these reagents: Liebermann-Burchard's, Br_2 in CCl_4, 2,4-DNP and 5% $FeCl_3$ reagents.

The IR spectrum (Fig.151) revealed the major absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3300 (b,O-H) and 1065 (m,C-O of 1 ROH).

The ^1H NMR ($CDCl_3$) spectrum (Fig.152) exhibited the signals at chemical shift δ (ppm.): 3.67 (2H,m, proton on carbon attached to oxygen atom), 1.52, 1.26 (high intensity) and 0.87 ppm..

The mass spectrum (Fig.153) did not give the molecular ion peak, but it displayed the prominent peaks at m/e (% rel int.) 448.0 (0.67, M^+-H_2O), 420.0 (2.91, M^+-H_2O-$(CH_2)_2$), 392.0 (11.07, 420.0-$(CH_2)_2$) and other signals corresponding to lose -$(CH_2)_2$- (m/e 28) step by step.

The GLC analysis (Fig.154) (condition: column 2% OV-1, CW 80/100 mesh AW DMCS 1/8 inch x 7.55 fts., column temp. 280 C, FID 320 C, carrier gas N_2 50.0 mL./min.) revealed five peaks on gas chromatogram at retention time 4.89, 6.12, 7.49, 9.40 and 11.62 min., respectively. The peak at retention time 7.49 min. was the

major component in this mixture.

2.16.3 Purification and Properties of Compound 17

Fraction CC was obtained from the combination of Fraction No. 53 to 65 which was eluted by 20% chloroform in hexane. This fraction contained white precipitated solid and pale yellow oil. The oil was removed by n-hexane and then the remaining white solid was purified by recrystallization with acetone for five times to obtain white amorphous solid, labelled as Compound 17, 1200 mg. (1.71% wt. by wt. of Fraction VIII), m.p. 76.5-78.0 °C. It was soluble in dichloromethane and chloroform, but slightly soluble in acetone and methanol. Like Compound 16, Compound 17 gave negative results to all these reagents: Lieberman-Burchard's, Br_2 in CCl_4, 2,4-DNP and 5% $FeCl_3$ reagents.

The IR spectrum (Fig.156) revealed the major absorption bands at ν_{max}^{KBr} (cm^{-1}): 3300-2500 (very b.,O-H), 1710 (s, $-\overset{O}{\overset{\|}{C}}-OH$) and 940-930 (m, O-H out-of-plane bend.).

The ^{1}H NMR ($CDCl_3$) spectrum (Fig.157) displayed the important proton signals at δ (ppm.): 2.35 (t,J=10.52 Hz), 1.26 (high intensity) and 0.88 ppm..

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.158) exhibited the significant signals at δ (ppm.): 179.75 (carboxyl carbon of carboxylic acid) together with other carbon signals around 33.97 to 14.09 ppm..

The mass spectrum (Fig.159) gave the molecular ion peak, M^+, at m/e (% rel int.) 508.0 (0.54, M^+) (Calcd. for $C_{34}H_{68}O_2$: MW. 508.54) together with other fragmentation ion peaks which were corresponded to be lost $-CH_2-$ (m/e 14) step by step.

Methylation of Compound 17

Compound 17 30 mg. was dissolved in chloroform 2.0 mL. and treated with diazomethane* to yield the methyl ester of Compound 17 (Check whether the reaction was completed or not by TLC). This methyl ester gave Rf value 0.68 (solvent: 30% chloroform-hexane).

The GLC analysis (Fig.160) (condition: column 2% OV-1, CW 80/100 mesh AW DMCS 1/8 inch x 7.55 fts., column temp. 250 °C, FID 320 °C, carrier gas N_2 45.0 mL./min.) exhibited 11 peaks on gas chromatogram at retention time 3.25, 4.13, 5.26, 6.77, 8.78, 11.33, 14.70, 19.00, 24.63, 32.03 and 41.43 min., respectively. The peak at Rt 11.33 min. was a major one in this mixture.

2.16.4 Purification and Properties of Compound 18

Fraction DD which was eluted from the column with 20% chloroform-hexane in Fraction No. 66 to 76 was composed of orange oil and white needle crystals. The orange oil was eliminated by washing with cold n-hexane and the remaining product was recrystallized by a mixture of dichloromethane and n-hexane to yield a bright white needle crystal 870 mg. (1.24% wt. by wt. of Fraction VIII), m.p. 139.0-141.0 °C. This needle crystal, assigned as Compound 18, was soluble in hot n-hexane, dichloromethane and chloroform, but slightly soluble in methanol and ethanol. Compound 18 gave a deep green colour when it was treated with Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

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* The diazomethane was generated from methylnitrosourea.

The IR spectrum (Fig.162) revealed the important absorption peaks at ν_{max}^{KBr} (cm^{-1}): 3430 (b,O-H), 3030 (w,C-H stretch. of $R_1R_2C=CR_3H$), 1670-1630 (w,C=C) and 1050 (m,C-O).

The mass spectrum (Fig.163) displayed the vital fragmentation ion peaks at m/e (% rel int.) 414.0 (100.00), 412.0 (54.04), 400.0 (23.66) (Calcd. for $C_{29}H_{50}O$, $C_{29}H_{48}O$ and $C_{28}H_{48}O$: MW. 414.39, 412.38 and 400.38, respectively) together with other peaks at 396.2 (18.38), 382.0 (11.93), 381.0 (18.19), 329.0 (28.03), 303.0 (27.20), 273.0 (28.41), 255.0 (46.13) and 213.0 (32.06).

The GLC analysis (Fig.164) (condition: column 2% OV-1, 3 mm. x 2 m., column temp. 260 °C, FID 320 °C, carrier gas N_2 48.0 mL./min.) gave three peaks on gas chromatogram at retention time 18.28, 19.58 and 22.41 min., respectively. The peak with Rt 22.41 min. was the major component in this mixture.

Fraction EE was eluted from silica gel column in Fraction No. 107 to 116 with 40% chloroform-hexane as a mixture of white precipitated solid and yellow-brownish oil. Fraction EE 0.93 g. was dissolved in acetone approximately 25.0 mL. and heated till it gave a clear brownish solution. After it was cooled down to room temperature and kept it for two hours, a precipitated solid was separated. The solid was filtered off and found that there were two different kinds of solid. One was white amorphous product and the other was blue crystalline solid. The white and blue products were separated from each other by dissolving these solids with hot n-hexane and found that only white amorphous product could dissolve, while a blue one still remained in the solution.

2.16.5 Purification and Properties of Compound B

After dissolving Fraction EE with acetone on water bath and keeping it at room temperature for two hours, white amorphous solid together with a blue one were precipitated. Separation of these two compounds was carried out by dissolving in hot n-hexane. Only white amorphous solid could dissolve, while a blue one still remained in the solution. After filtration, the filtrate was concentrated to 10.0 mL. and found that white amorphous solid was formed. This compound, designated as Compound B, was recrystallized by hot n-hexane for four times to yield 5 mg. of Compound B (0.005% wt. by wt. of Fraction VIII), m.p. 73.0-74.0 °C and showed merely one spot on TLC plate of Rf value 0.55 (solvent: chloroform). Compound B was easily soluble in chloroform, dichloromethane and diethylether, but slightly soluble in methanol and n-hexane. Because of a limit of Compound B, this compound was not investigated further.

2.16.6 Purification and Properties of Compound C

Compound C was a blue crystalline solid which was separated from Compound B by dissolving in hot n-hexane. This compound was recrystallized with hot acetone for two times to yield blue crystalline solid, 2 mg.. It gave a single blue spot on TLC plate at Rf 0.30 (solvent: chloroform). Compound C was soluble in chloroform and dichloromethane, but slightly soluble in n-hexane and acetone. Due to unsufficient derived substance, this compound was not examined further.

2.16.7 Purification and Properties of Compound 19

From the separation Fraction VIII in Fraction No. 121-132 which was eluted with 40% chloroform-hexane yielded Fraction FF. Compound 19 was obtained by dissolving Fraction FF with 20% chloroform-hexane 40.0 mL. and then concentrated to 20.0 mL. The solution was kept at room temperature for two hours and found that there was a yellow precipitated solid in its solution. A yellow precipitated 510 mg. was filtered off and washed with n-hexane. The yellow solid was recrystallized from a mixture of dichloromethane and n-hexane for several times to gain bright yellow needle crystal, 380 mg. (0.54% wt. by wt. of Fraction VIII). This compound sublimated approximately at 230 °C and gave a single spot at Rf value 0.56 (solvent : 5% methanol-chloroform). Compound 19 was soluble in dichloromethane and chloroform, but slightly soluble in n-hexane and methanol. It gave negative tests to Liebermann-Burchard's and 5% $FeCl_3$ reagents, but it showed a positive result to 2,4-DNP and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.165) gave the major absorption bands at ν_{max}^{KBr} (cm^{-1}): 3060 (w,C-H stretch. of $R_1R_2C{=}CR_3H$), 1695, 1650 (s,$-\overset{O}{\overset{\|}{C}}-$ stretch. of unsaturated ketone) and 1595 (s,C=C).

The mass spectrum (Fig.166) gave the molecular ion peak, M^+, at m/e (% rel int.) 168.0 (100.00) (Calcd. for $C_8H_8O_4$: MW. 168.06) together with other fragment ion peaks at m/e 153.0 (4.02, M^+-CH_3), 138.0 (28.94, 153-CH_3) and 69.0 (85.35).

The ^1H NMR ($CDCl_3$) spectrum (Fig.167) displayed the proton signals at δ (ppm.): 5.86 (2H,s, olefinic protons) and 3.83 (6H,s, methoxy protons).

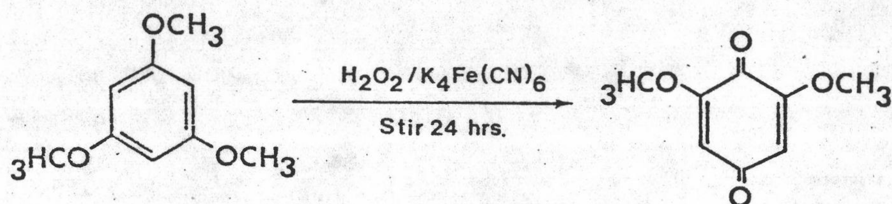
The ^{13}C NMR ($CDCl_3$) spectrum (Fig.168) exhibited the carbon signals at δ (ppm.): 186.85, 176.72 (1C each, carbonyl carbons), 157.32, 107.43 (2C each, olefinic carbons) and 56.50 (2C, methoxy carbons).

The UV (EtOH) spectrum (Fig.169) showed the λ max at 287 mm. (log ε = 4.34) and 376 nm. (log ε = 3.01).

Elemental analysis found:	%C 57.13,	%H 4.79
Calcd. for $C_8H_8O_4$, MW. 168.06:	%C 57.14,	%H 4.76

Synthesis 2,6-dimethoxy-p-benzoquinone (65)

To a solution of potassium hexacyanoferrate (50 mg.) in water (250.0 mL.) was added a solution of 1,3,5-trimethoxybenzene (420 mg.) in acetone (2.5 mL.) and hydrogen peroxide (30% aqueous solution), successively. After being stirred for 24 hours at room temperature, the solution was diluted with dichloromethane, washed with water and dried over anhydrous magnesium sulfate. The solution was concentrated and the residue was rinsed with methanol to separate yellow crystalline solid (250 mg., 59.52% yield).



The IR spectrum (Fig.170) revealed the important absorption bands at ν_{max}^{KBr} (cm^{-1}): 3060 (w,C-H stretch. of $R_1R_2C=CR_3H$), 1695, 1650 (s,$-\overset{O}{\overset{\|}{C}}-$ stretch. of unsaturated ketone), 1595 (s,C=C).

The mass spectrum (Fig.171) gave the molecular ion peak, M^+, at m/e (% rel int.) 168.0 (100.00) (Calcd. for $C_8H_8O_4$: MW. 168.06) and other fragment ion peaks at m/e 153.0 (4.02, M^+-CH_3), 138.0 (28.94, 153-CH_3) and 69.0 (85.35).

The ^1H NMR ($CDCl_3$) spectrum (Fig.172) expressed the chemical shift of protons at δ (ppm.): 5.86 (2H,s, olefinic protons) and 3.83 (6H,s, methoxy protons).

The mother liquor of Fraction FF after separation the yellow precipitated solid (Compound 19), designated as Fraction FF1, was studied further by TLC using a mixture of 5% methanol-chloroform as a developing solvent and found that there were two major spots at Rf value 0.56 and 0.48. The upper one gave the same Rf value as Compound 19. Hence, Fraction FF1 should be composed of at least two components; one of which was Compound 19. This fraction 70 mg., therefore, was rechromatographed on silica gel 7.0 g. as an adsorbent and 10% chloroform-hexane was used as an initial eluent. The results of separation Fraction FF1 were presented in Table 2.29.

Table 2.29 The results of recolumn chromatography of Fraction FF1 by silica gel column (using column 1.0 x 50.0 cm.)

Eluents	Fraction No. (20 mL.)	Remarks	Weights (g.)
10% chloroform-hexane	1-3	nothing	-
20% chloroform-hexane	4-10	yellow oil	0.10
	11-15	brownish oil + yellow ppt. (Cpd.19)	0.09
30% chloroform-hexane	16-17	brownish oil	0.03
	18-22	yellow oil + white ppt. (Fraction FF1A)	0.20
	23-25	yellow oil	0.18
50% chloroform-hexane	26-28	pale yellow oil	0.02

2.16.8 Purification and Properties of Compound 20

Fraction FF1A was collected from the combination of Fraction No. 18 to 22 by using 30% chloroform-hexane as an eluent. This fraction contained yellow oil and white precipitated solid. After removing the oil, white solid was recrystallized by hot n-hexane for five times to gain white needle crystal, 32 mg. (0.04% wt. by wt. of Fraction VIII)., m.p. 109.0-110.5 °C, Rf 0.48 (solvent: 5% methanol-chloroform). This compound, marked as Compound 20, was easily soluble in dichloromethane, chloroform and diethylether, but slightly soluble in methanol and ethanol. Compound 20 gave negative results to Liebermann-Burchard's and Br_2 in CCl_4 reagents, but showed positive tests to 5% $FeCl_3$ and 2,4-DNP reagents.

The IR spectrum (Fig.173) displayed the significant absorption bands at ν_{max}^{KBr} (cm^{-1}): 3290 (b,O-H), 3050 (w,C-H stretch. of substituted alkenes or aromatic), 1670 (s,$-\overset{O}{\overset{\|}{C}}-$), 1605, 1595, 1510 (m,C=C of aromatic skeleton) and 1330, 1250, 1205, 1135, 1100 (s, C-O).

The mass spectrum (Fig.174) exhibited the molecular ion peak, M^+, at m/e (% rel int.) 182.0 (100.00) (Calcd. for $C_9H_{10}O_4$: MW. 182.08) together with other fragment ion peaks at m/e 181.0 (52.60), 167.0 (15.76), 139.0 (13.92) and 111.0 (14.97).

The ^1H NMR ($CDCl_3$) spectrum (Fig.175) showed the proton signals at chemical shift δ (ppm.): 9.82 (1H,s, aldehyde proton), 7.15 (2H,s, aromatic protons), 6.10 (1H,b, phenolic proton) (This signal was absent when a sample was shaken with D_2O) and 3.97 (6H,s, methoxy protons).

The ^{13}C NMR ($CDCl_3$) spectrum (Fig.176) revealed the chemical shift of carbons at δ (ppm.): 190.75 (aldehyde carbon), 163.99, 147.30, 128.40 and 106.78 (aromatic carbons) and 56.45 (methoxy carbons).

The UV (EtOH) spectrum (Fig.177) displayed the λ max at 227 nm. (log ε = 4.60) and 309 nm. (log ε = 4.53).

Elemental analysis found:	%C 59.19,	%H 5.58
Calcd. for $C_9H_{10}O_4$, MW. 182.08:	%C 59.34,	%H 5.49

Fraction GG 4.60 g. contained brownish oil and precipitated solid. This fraction was studied first by TLC and found that there was one major spot at Rf value 0.75 (solvent: 20% methanol-chloroform). Therefore, silica gel column chromatography (90.0 g. of

adsorbent) was used for reseparation this fraction. The column was initially packed with 20% chloroform in n-hexane. The gradual introduction of chloroform in n-hexane was used as an eluent. The column was finally eluted with methanol. The eluted solution was collected approximately 30.0 mL. for each portion. The equivalent ones which were monitored by TLC were combined. The results of recolumn Fraction GG by silica gel column were shown in Table 2.30.

Table 2.30 The results of recolumn chromatography of Fraction GG by silica gel column (using column 2.5 x 100.0 cm.)

Eluents	Fraction No. (30 mL.)	Remarks	Weights (g.)
20% chloroform-hexane	1-10	pale yellow oil	0.02
40% chloroform-hexane	11-20	pale yellow oil	0.03
60% chloroform-hexane	21-25	yellow-brownish oil + ppt.	0.20
	26-40	yellow-brownish oil + ppt. (Fraction GG1)	0.96
80% chloroform-hexane	41-50	brownish oil	0.30
chloroform	51-60	brownish oil	0.52
20% methanol-chloroform	61-70	brownish oil	1.24
50% methanol-chloroform	71-75	brownish oil	0.54
methanol	81-83	pale brownish oil	0.30

2.16.9 Purification and Properties of Compound 21

Fraction GG1, which was eluted from the silica gel recolumn chromatography of Fraction GG by 60% chloroform in n-hexane in Fraction 26-40, contained brownish oil and precipitated solid. After removing the oil by washing with acetone, the remaining product was recrystallized by a mixture of acetone and methanol 2 times and then by hot n-hexane 2 times to yield white amorphous solid, 20 mg. (0.03% wt. by wt. of Fraction VIII), m.p. 80.5-81.5 °C, Rf 0.75 (solvent: 20% methanol-chloroform). This compound, labelled as Compound 21, was easily soluble in various organic solvents such as chloroform, dichloromethane and diethylether. Compound 21 gave negative results to all these reagents: Liebermann-Burchard's, Br_2 in CCl_4, 5% $FeCl_3$ and 2,4-DNP reagents.

The IR spectrum (Fig.178) exhibited the important characteristic peaks at ν^{KBr}_{max} (cm^{-1}): 3420 (b,O-H), 1740 (m,$-\overset{O}{\overset{\|}{C}}-O-$), 1170 and 1020 (m,C-O).

The mass spectrum (Fig.179) exhibited a set of molecular ion peaks at m/e (% rel int.) 846.0 (0.98), 818.0 (0.98), 790.0 (0.73) and 762.0 (0.19) (Calcd. for $C_{57}H_{114}O_3$, $C_{55}H_{110}O_3$, $C_{53}H_{106}O_3$ and $C_{51}H_{102}O_3$: MW. 846.90, 818.87, 790.83 and 762.80, respectively). Other fragmentation ion peaks were similar to those of saturated long chain aliphatic compounds.

The ^1H NMR ($CDCl_3$) spectrum (Fig.180) gave the proton signals at δ (ppm.): 4.15, 2.35, 1.26 (high intensity) and 0.88 ppm..

The ^{13}C NMR spectrum (Fig.181) displayed the carbon signals around 65.12 to 14.03 ppm.. The high intensity signal was

observed at 29.69 ppm..

2.16.10 Purification and Properties of Compound 22

Fraction HH, which was collected from the combination of Fraction No. 207-217, was composed of brownish oil and precipitated solid. After the oil was eliminated, the precipitated solid was recrystallized by a mixture of chloroform and methanol for four times to gain white amorphous solid, designated as Compound 22, 20 mg. (0.03% wt. by wt. of Fraction VIII), m.p. 145.0-147.0 °C, Rf 0.60 (solvent: 15% methanol-chloroform). This compound was soluble in chloroform, dichloromethane and slightly soluble in n-hexane, methanol and ethanol. Compound 22 gave negative results to all these reagents: Liebermann-Burchard's, Br_2 in CCl_4, 5% $FeCl_3$ and 2,4-DNP reagents.

The IR spectrum (Fig.182) showed the major absorption bands at ν_{max}^{KBr} (cm^{-1}): 3350,3200 (d,N-H) and 1620 (m, $-\overset{O}{\overset{\|}{C}}-N-$).

The ^1H NMR ($CDCl_3$-DMSO) spectrum (Fig.183) displayed the proton signals at δ (ppm.): 1.25 (high intensity) and 0.87 ppm..

The mass spectrum (Fig.184) gave a set of molecular ion peaks at m/e (% rel int.): 703.0 (0.41), 689.8 (1.26), 675.0 (6.83), 661.0 (10.41) and 647.0 (13.77) (Calcd. for $C_{48}H_{97}ON$, $C_{47}H_{95}ON$, $C_{46}H_{93}ON$, $C_{45}H_{93}ON$ and $C_{44}H_{89}ON$: MW. 703.77, 689.76, 675.74, 661.72 and 647.71, respectively). Other fragmentation ion peaks were resemble to those of saturated long chain aliphatic compounds.

2.16.11 Purification and Properties of Compound 23

Fraction JJ was obtained from the combination Fraction No. 218-227 which was eluted from silica gel column by 10% methanol in chloroform. This fraction was consisted of brownish oil and precipitated solid. Removed the brownish oil by washing with ethylacetate, the remaining product was recrystallized by hot ethanol for three times to yield white amorphous solid, 300 mg. (0.43% wt. by wt. of Fraction VIII), m.p. 260.0-263.0 °C (dec.), Rf 0.58 (solvent: 10% methanol-chloroform). This compound gave a deep green colour to Liebermann-Burchard's and decolourized Br_2 in CCl_4 reagents.

The IR spectrum (Fig.185) revealed the vital absorption bands at ν_{max}^{KBr} (cm^{-1}): 3420 (b,O-H), 1640 (w,C=C) and 1080-1020 (C-O of glycosidic linkage).

By direct comparision with Compound 14 which was obtained from the leaves of this plant, i.e, mixed m.p., Co. TLC and comparision their IR spectra, this compound was found to be identical to Compound 14 in all respects.

2.17 Study on Fraction IX

Fraction IX was a nearly white solid which was obtained during evaporating the solvent from the 95% ethanol soluble fraction. This fraction was purified by dissolving in distilled water and decolourized with activated charcoal. The clear solution was concentrated to gain white solid 14.40 g. (0.22% wt. by wt. of dried plant material). By means of chemical and ignition tests, this fraction was found to be an inorganic salt fraction, mainly

chloride salts of Na and K. The quantitative analysis of cation composition in Fraction IX by FES was performed. The results of this examination was presented in Table 2.16.

2.18 Study on Fraction X

Fraction X was assigned for the solid part which was insoluble in both chloroform and water, 321.00 g. (0.45% wt. by wt. of plant material). This fraction was preliminary studied following the phytochemical screening methods on tannins and polyphenols (62). The results of this examination were presented in Table 2.31.

Table 2.31 The results of tannin and polyphenol tests on Fraction X

Reagents	Remarks
gelatin solution	white precipitate
gelatin salt solution	white precipitate
1% ferric chloride	deep blue colour
bromine water	orange precipitate
formallin-HCl test	brownish precipitate
vanillin-HCl test	red solution and brownish precipitate
lime water	dark blue precipitate

2.19 Separation of Fraction XI

Fraction XI was designated for a chloroform soluble fraction, 195.00 g. (0.28% wt. by wt. of plant material). TLC of

the concentrated crude revealed one major spot at Rf 0.58 using 10% methanol in chloroform as a mobile phase. This fraction 60.0 g. was chromatographed on aluminium oxide column chromatography using adsorbent 1,000.0 g. and 50% chloroform in n-hexane as an initial eluent. Each fraction was collected approximately 1,000.0 mL. and monitored by TLC. The equivalent ones were combined. The results of separation Fraction XI were shown in Table 2.32.

<u>Table 2.32</u> The separation of Fraction XI by aluminium oxide column chromatography (using column 5.0 x 120.0 cm.)

Eluents	Fraction No. (1000 mL.)	Remarks	Weights (g.)
50% chloroform-hexane	1-10	yellow oil	1.05
75% chloroform-hexane	11-20	yellow oil	2.01
chloroform	21-30	brownish oil	1.68
	31-36	brownish oil	0.64
5% methanol-chloroform	37-46	brownish oil	6.89
	47-52	brownish oil	3.42
20% methanol-chloroform	53-62	brownish oil + ppt. (Fraction KK)	4.23
	63-68	brownish oil	1.58
50% methanol-chloroform	69-75	brownish oil	2.10
methanol	76-79	brownish tar	0.86

From Table 2.32, Fraction KK contained brownish oil and precipitated solid. After the brownish oil was eliminated by washing with ethylacetate and acetone, the remaining solid was then purified by recrystallization with hot ethanol three times to gain white amorphous solid 300 mg. (0.46% wt. by wt. of Fraction XI), m.p. 260.0-263.0 °C (dec.), Rf 0.89 (solvent: 20% methanol-chloroform). This compound was proved to be identical to Compound <u>23</u> by direct comparision methods, i.e., mixed m.p., Co. TLC in various solvent systems and comparision their IR spectra.

2.20 Examination on Fraction XII

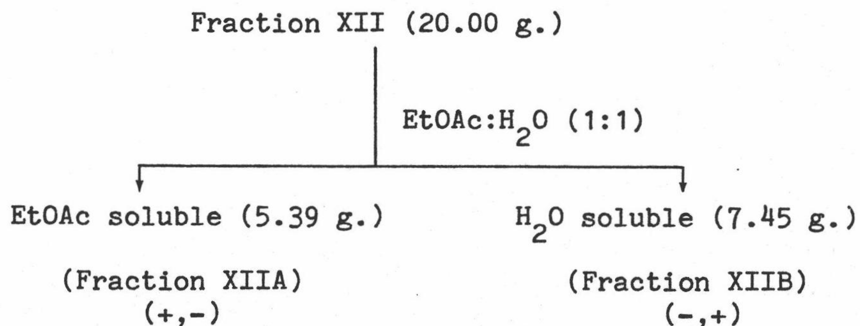
Fraction XII was the n-butanolic fraction obtained as a reddish syrup from the extraction of aqueous layer with n-butanol. This fraction was preliminary studied by dissolving Fraction XII 1.0 g. in 2.0 mL. of methanol and added these following colour test reagents: Shinoda's, 5% $FeCl_3$, 2,4-DNP, Molisch's and Liebermann-Burchard's reagents. The results of colour tests were shown in Table 2.33.

Table 2.33 The results of colour tests of Fraction XII

Reagents	Remarks	Indication
Shinoda's	-	the absence of flavonoid compounds
5% $FeCl_3$	deep blue colour	may be present of phenolic compounds
2,4-DNP	-	the absence of carbonyl gr.
Molisch's	violet ring at junction	may be present of carbohydrate moiety
Liebermann-Burchard's	-	the absence of streroid or triterpenoid compounds

This fraction 20.00 g. was further extracted by ethylacetate and water in ratio 1:1 to yield an ethylacetate soluble fraction, labelled as Fraction XIIA, 5.39 g. (26.95% wt. by wt. of Fraction XII) and a water soluble fraction, marked as Fraction XIIB, 7.45 g. (37.25% wt. by wt. of Fraction XII). These two fractions were reexamined with both 5% $FeCl_3$ and Molisch's reagents. The extraction procedure and the results of reexamination on colour tests were shown in Scheme 2.9.

Scheme 2.9 The extraction procedure and the results of reexamination on colour tests of Fraction XII



Note: (+,+) stands for positive tests to 5% $FeCl_3$ and Molisch's reagents, respectively

(-,-) stands for negative results

Study on Fraction XIIA

Fraction XIIA, an ethylacetate soluble fraction, was obtained from the extraction Fraction XII by ethylacetate. This fraction gave a deep blue colour to 5% $FeCl_3$ reagent.

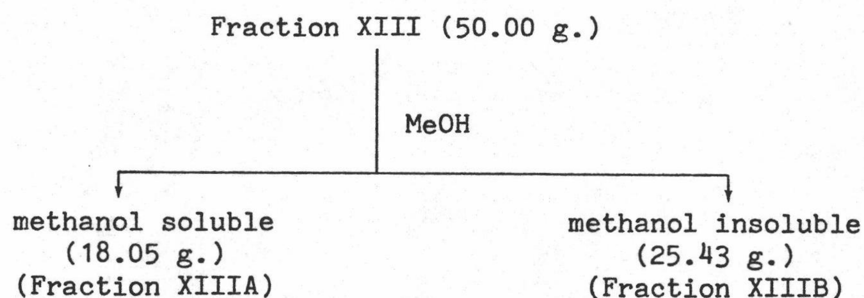
The HPLC analysis for phenolic compounds (Fig.186) (the same condition as investigation Fraction VA) revealed the major peaks on HPLC chromatogram at retention time (Rt) 3.36, 5.91, 6.72, 9.69, 11.37, 12.89, 20.26, 21.06, 22.72, 26.10 and 29.22 min..

The HPLC analysis for gibberellins (Fig.187) (the same condition as examination Fraction VA) exhibited three peaks on HPLC chromatogram at retention time (Rt) 2.89, 3.32 and 4.80 min.. The peak at Rt 3.32 min. might be corresponded to gibberellin A_3.

2.21 Investigation of Fraction XIII

Fraction XIII was a freeze dried water soluble fraction 366.50 g. (0.52% wt. by wt. of plant material). This fraction 50.00 g. was extracted further by methanol to yield a methanol soluble fraction 18.05 g. (36.10% wt. by wt. of Fraction XIII), designated as Fraction XIIIA, and a methanol insoluble fraction, assigned as Fraction XIIIB, 25.43 g. (50.86% wt. by wt. of Fraction XIII). The further extraction procedure was given in Scheme 2.10.

Scheme 2.10 The further extraction of Fraction XIII



2.21.1 Study on Fraction XIIIA

Preliminary tests on Fraction XIIIA were conducted on some reagents as follow: Tollen's, Benedict's, Barford's, Molisch's and ninhydrin reagents by usual methods (55). The results of this study were given in Table 2.34.

Table 2.34 The preliminary colour tests on Fraction XIIIA

Reagents	Remarks
Tollen's	silver mirror
Bendedict's	orange precipitate
Barford's	orange precipitate
Molisch's	violet ring at junction
ninhydrin	pale violet colour

The information obtained from Table 2.34 suggested that this fraction ought to contain carbohydrate (reducing sugar) and amino acids.

Study on carbohydrate of Fraction XIIIA

Fraction XIIIA was investigated on carbohydrate by the same methods as described for Fraction VA, i.e., paper chromatography, HPLC analysis and conversion this fraction into its osazone and acetyl derivatives. The results of examination Fraction XIIIA by paper chromatography and HPLC analysis were shown in Fig.188 and Fig.189, respectively. The osazone and acetyl derivatives derived from Fraction XIIIA were found to be identical to those compounds which were obtained from Fraction VA.

Study on amino acids of Fraction XIIIA

Fraction XIIIA was examined for amino acids by using Amino Acid Analyser. The results of this examination were shown in Fig.190.

2.21.2 Study on Fraction XIIIB

Fraction XIIIB was a methanol insoluble fraction. This fraction was purified by dissolving in distilled water and decolourized with activated charcoal and filtered off. The clear solution was concentrated to gain white solid. Chemical and ignition tests of this solid hinted that Fraction XIIIB was an inorganic fraction. The qualitative analysis of this fraction found that Fraction XIIIB was consisted of chloride salts of Na, K and Mg.

The X-ray fluoresence spectrum (Fig.191) showed the presence of Na, K, Mn, Cl and P.

The quantitative analysis of the cation composition in Fraction XIIIB was carried out by ICPS and the results were tabulated in Table 2.25.

2.22 Examination on Fraction XIV

Fraction XIV was labelled for the exhausted heartwoods which were obtained after the extraction with dichloromethane and 95% ethanol, successively. This fraction (1.00 kg.) was extracted further with 50% ethanol-water to gain a dark brown crude 67.52 g. (6.75 % wt. by wt. of plant material). The phytochemical screening tests on tannins and polyphenols were conducted on this fraction and found that Fraction XIV was composed of condensed and hydrolysable tannins. Besides, this fraction 2.0 g. was dissolved in distilled water and decolourized with activated charcoal to obtain clear pale yellow solution. Chemical tests on this solution revealed the presence of Na, K, Mg and Cl.

The determination of the cation composition in Fraction XIV by ICPS was carried out. The results of quantitative analysis were shown in Table 2.25.

2.23 Bioassay Results of Isolated Compounds

Several of the pure isolated compounds as well as mixtures from the leaves and the heartwoods were bioassayed against fungi, bacteria and the insect, boll weevil (see the experiment in Topic 2.8).

The antifungal and antibacterial activity results of the isolated compounds were presented in Table 2.35.

Table 2.35 The antifungal and antibacterial activity results of isolated compounds from *R. apiculata*

Plant parts	Compound	% inhibition*			
		Fungi			Bacteria
		P	R	H	XC
leaves	1	120	-	12	-
	2	-	-	-	-
	5	50	-	66	50
	6	-	-	-	-
	9	100	40	-	-
	10	-	-	-	-
	12	28	-	33	-
	13	81	73	100	-
	14	-	-	-	-
	Frac.III	20	11	-	36
heartwoods	15	-	-	-	-
	17	-	-	-	-
	18	-	-	-	-
	19	40	-	-	64
	20	30	-	80	25
	23	-	-	-	-
	Frac.X	20	11	-	33

* Concentration of the solution in which the paper discs were immersed (1 mg./mL.).

Inhibition zone radius of sample divided by that of the standard DITHANE.

Note: 1) The evaluation on the biological activity of some isolated compounds such as Cpd. 7 and 8 are in progress.

2) Some isolated compounds are obtained in unsufficient amount for evaluating their biological activity, such as Cpd. 4, 11, 21 and 22.

The antifeedant activity results against the insect boll weevil were presented in Table 2.36.

Table 2.36 The antifeedant activity results against the insect boll weevil of isolated compounds from *R. apiculata*

Plant parts	Compound	Dose level (mg)	% T/C	% Inhibition
leaves	1	2 5 6	30 0 0	70 100 100
	2	3.5 5 6.5	20 0 0	80 100 100
	6	3 5	145 214	0 0
	9	3 5 7	33 14 3	67 86 97
	10	2 4.5	36 21	64 79
	14	3 5	100 100	0 0
heartwoods	15	2 4	34 25	66 75
	17	3 5 7	36 14 0	64 86 100
	18	2 4.5	36 21	64 79
	19	3 6	55 11	45 89
	23	3 5	100 100	0 0