# CHAPTER II

# EXPERIMENTS AND RESULTS

## 2.1 Plant Materials

Roots of Harrisonia perforata Merr. were collected at Salang district, Chantaburi province, Thailand during the September 1990 and dried by sun light and milled to coarse powder.

## 2.2 Instruments and Equipment

### 2.2.1 Soxhlet Extraction Apparatus [36]

The Soxhlet extraction apparatus was used on Quickfit for continuous extraction.

### 2.2.2 Rotary Evaporator [37]

The Rotary Evaporator used was an Eyela Model N-1 for rapid removal of a large quantity of volatile solvent.

### 2.2.3 Infrared Spectrophotometer (IR)

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

2.2.4 Ultraviolet and Visible Spectrophotometer (UV and VIS)

The UV and VIS spectra were recorded on by a Shimadzu UV-VIS Spectrophotometer Model 240 (wavelength range : 190-900 nm. with double beam photometric system).

2.2.5 Mass Spectrometer (MS)

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

2.2.6 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 :

The $^1$H NMR Spectrometer : Jeol Fourier Transform NMR Spectrometer Model FX 902 and Bruker AC-F 200 operating at 200.13 MHz.

The $^{13}$C NMR Spectrometer : Jeol Fourier Transform NMR Spectrometer Model FX 902 and Bruker AC-F 200 operating at 50 MHz.

Tetramethylsilane (TMS ) was used as an internal standard. The Chemical shift ($\delta$) were given in ppm. down field from TMS signal.

2.2.7 Gas Chromatography (GC)

The GC analysis was carried out by using a Shimadzu Gas Chromatography Model GC-R1A.

### 2.2.8 Gas Chromatograph-Mass Spectrometry (GC-MS)

The GC-MS analysis was determined by either a Shimadzu GC-MS QP-1000 or a JMS-DX 300 GC-Mass Spectrometer, Jeol.

### 2.2.9 Elemental Analyzer

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

### 2.2.10 Amino acid analyzer (AAA)

The amino acid analysis results were obtained by a Hitachi Amino Acid Analyzer Model 835-50.

### 2.2.11 High Performance Liquid Chromatograph (HPLC)

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

### 2.2.12 Melting Point Apparatus (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, except solvents that were reagent grade.

## 2.4 Physical Separation Techniques

### 2.4.1 Thin-Layer Chromatography (TLC) [38]

Thin-layer chromatoplate was prepared in the following manners:

A mixture of silica gel (25.0 g.) and water (50 ml.) was stirred until homogeneous. It was then applied on a glass plates (20 cm. x 20 cm.) using a desaga spreader. After being dried at room temperature for half an hour, the plate was heated at 125 C for 30 min., cooled and stored in a dessicator until use.

### 2.4.2 Paper Chromatography (PC) [39]

Paper chromatography was performed similar to thin layer chromatography. Instead of an adsorbent-coated plate, a strip of paper was used. A thin film of water on the paper constitutes the adsorbent, therefore paper chromatography is a liquid-liquid partition technique, rather than a liquid-solid technique such as column chromatography and thin-layer chromatography. This method is most successful with very polar or polyfunctional compounds, for example, sugar, amino acid and other strongly polar compounds. The selection of solvent systems for paper chromatography is very important. The procedure

for preparing paper chromatography was explained briefly as follow :

Place a light pencil mark about 1.0 cm. from the bottom of the paper strip (Whatman No. 1 filter paper, 9.0 cm x 44.0 cm.). The sample was applied as a small spot with a capillary tube at the center of the line and allowed to dry. The paper was developed by descending method for developing in the tank 10.0 cm. x 48.0 cm. x 30.0 cm. This process might take up about 14-18 hours. After the developing, the paper was removed and allowed to dry. The separated spots of each compound were visualized by appropriate detector.

### 2.4.3 Quick Column Chromatography [40]

This method is especially useful for separating large quantitie of mixture compounds which have been obtained from natural resources into fractions. However, its speed and separating power depend on the used adsorbent to separate the desired components.



**Column Packing**

The column used was a glass column of 14.0 cm. diameter with sintered glass frit. Silica gel was added in the column and distributed evenly over the surface. 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 picked 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.

Separation of the extract on the column

The extract was dissolved in a small amount of suitable solvent and mixed with the adsorbent. It was then added directly on the column which was wetted evenly with the solvent 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 onto the top of the column. The polarities of the eluting solvent changed from n-hexane to a mixture of chloroform-hexane, chloroform and a mixture of chloroform-methanol, respectively.

### 2.4.4 Column Chromatography (CC) [41]

Column chromatography , also called elution chromatography, was used to separate the mixture. In this technique, a vertical glass column was packed with a polar adsorbent along with a solvent. The sample was added to the top of column then additional solvent was passed through the column to elute the components of the sample, one by one (ideally), down through the adsorbent to the outlet.

The sample on the column is subjected to two opposing forces: the solvent dissolving it and the adsorbent adsorbing it. The dissolving and the adsorption constitute an equilibrium process, with some sample molecule being adsorbed and others leaving the adsorbent as moving along with the solvent.

Because of the differences in the rates at which compounds move through the column of the adsorbent, a mixture of compounds was separated into bands, each compound forming its own band that moves through the column at its own rate.

**Column Packing**

The column used was a glass column of 4.0 cm. diameter. Pre-adjust the stopcock so that it was almost closed, but still allow the solvent to drip through. Allow approximately 0.5 cm. of the solvent to remain in the column when the slurry of the adsorbent was poured. After the bed was settled, a paper disk was inserted in the column. The sample was dissolved in the solvent as a 10% (w/v) solution and the solution was introduced onto the column 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 other solvent which it was soluble. The solution was next added to a minimun quantity of the 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.

## 2.5 Color Tests

### 2.5.1 Liebermann-Burchard's Test [42-43]

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. The developement of the purple or green color after a few minutes demonstrated that steroids or triterpenoids were presented in sample.

### 2.5.2 Molisch's Test [36]

This is a general method for testing carbohydrates. The sample (5 mg.) was placed in a test tube containing water (0.5 ml.) and mixed with 2 drops of 10% solution of $\alpha$-naphthol in alcohol and concentrated sulfuric acid (1 ml.) was dropped down along the side of the inclined tube until the acid formed a layer beneath the aqueous solution without mixing. If a carbohydrate was present in the sample, a red ring appeared at the common surface of the liquids. The color changed quickly when the mixture was shaken to result in a dark purple solution.

### 2.5.3 Shinoda's Reagent Test [44]

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 color developed within a few minutes could be observed. The shade of the color is suggestive of the class of flavonoid compounds.

### 2.5.4 Kraut's Reagent Test [44]

This is a test for alkaloid nucleus. The sample solution was added a few drops of Kraut's reagent. The positive test is an appearance of a brown precipitate.

### 2.5.5 Dragendroff's Reagent Test [44]

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

Other reagents for color tests used in this course of research, such as 2,4-DNP, 5% $FeCl_3$, $Br_2$ in $CCl_4$, $KMnO_4$ Benedict's, Selewanoff and etc. were carried out and observed by following the textbook of Practical Organic Chemistry [36] and the Systematic Identification of Organic Compounds. [45]

## 2.6 Extraction and Initial Fractionations

### 2.6.1 Extraction by soaking

The ground sun-dried roots (6 kg.) were extracted by soaking in methanol (25 liter) for several days at room temperature. The soaking was repeated until a clear solution was obtained. After methanol was removed by rotary evaporator under reduced pressure, the methanol extracts (Fraction Ia) was obtained as a dark-red crude residue 161.55 g. (2.69 % wt. by wt. of dried roots). Then crude Fraction Ia was re-extracted by hexane. The

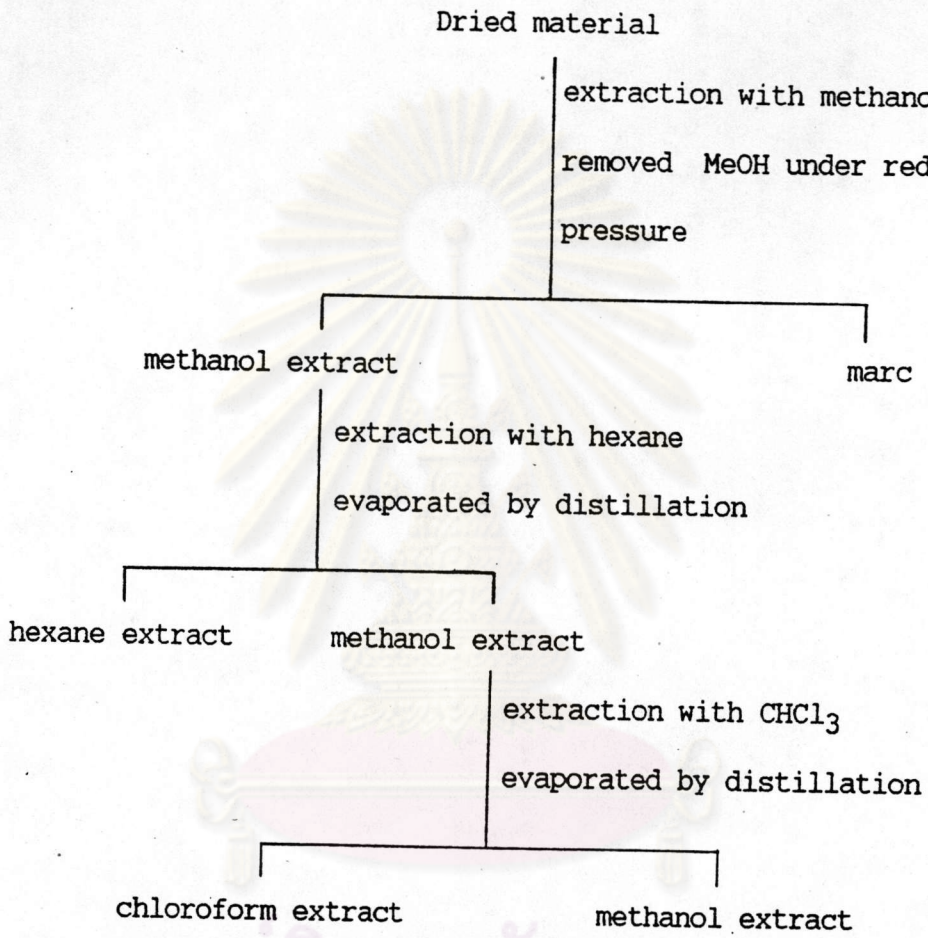
hexane extract was concentrated by simple distillation under mild condition. The hexane extract residue, fraction IIa was obtained as a dark-green crude residue 57.44 g. (0.96 % wt. by wt. of dried roots). The rest of Fraction Ia was further re-extracted by chloroform. After evaporation of chloroform, a dark-red crude residue (Fraction IIIa) 81.37 g., (1.36 % wt. by wt. of dried roots) was obtained.

### 2.6.2 Extraction by Soxhlet Extraction Apparatus

The sun-dried roots (14 kg.) were extracted with methanol (32 liters) by Soxhlet Extraction Apparatus. After extraction, the methanol extract solution was concentrated under reduced pressure, the methanol crude extract residue (Fraction Ib) was obtained 306.67 g.(2.19 % wt. by wt. of dried roots). Fraction Ib was then processed with hexane extraction and chloroform extraction in the same manner as Fraction Ia, to give hexane crude extract residue (Fraction IIb) 93.96 g. (0.67 % wt. by wt. of dried roots) and chloroform crude extract residue (Fraction IIIb) 142.38 g. (1.02 % wt. by wt. of dried roots).

The fractionation procedure by various solvents was shown in Scheme 2.1 and the results of each extraction were presented in Table 2.1.

Scheme 2.1 Extraction procedure for dry roots of *Harrisonia perforata* Merr.




Dried materials were extracted with methanol by :

1) Soaking with methanol at room temperature and standing for 5 days.

2) Soxhlet Extraction Apparatus.

Table 2.1 The results of extraction the sun-dried roots of *Harrisonia perforata* Merr.

| Procedure | Soaking | Soxhlet |
|---|---|---|
| Weight of dried roots (g.) | 6000 | 14000 |
| Weight of hexane extract (% wt. by wt.)* | 57.44 (0.96) | 93.96 (0.67) |
| Weight of chloroform extract (% wt. by wt.)* | 81.37 (1.36) | 142.38 (1.02) |
| Weight of methanol extract (% wt. by wt.)* | 161.55 (2.69) | 306.67 (2.19) |

* % wt. by wt. of crude extract by wt of raw material.

## 2.7 Separation of Fraction Ia and Fraction Ib

Thin-layer chromatography of Fraction Ia and Fraction Ib revealed that they were two major compounds in this crude (solvent : 50% methanol-chloroform ).

### 2.7.1 Separation of Fraction Ia

The crude extract of Fraction Ia (30 g.) was chromatographed on aluminium oxide 600 g. using quick column chromatographic technique to separate this fraction into small fractions by varying the polarities of eluents. First, the column was eluted with chloroform, and then solvents increasing polarity of solvent by increasing percentage of methanol. Finally, the column was stripped with methanol. The eluate solution was collected about 800 ml. for each fraction. Each fraction was monitered by using TLC technique and the equivalent fractions were combined. The results of separation were presented in Table 2.2.

The separation of this fraction (30 g.) was also performed on a silica gel (500 g.) quick column chromatography. The results were presented in Table 2.3.

Table 2.2 The results of Fraction Ia separated on aluminium oxide quick column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| $CHCl_3$ | 1-5 | - |
| | 6-8 | pale yellow oil |
| 5% MeOH-$CHCl_3$ | 9-11 | brownish tar |
| | 12-16 | yellow oil |
| | 17-21 | pale yellow oil |
| | 22-28 | very pale yellow oil |
| 10% MeOH-$CHCl_3$ | 29-34 | yellow amorphous solid in yellow oil (cpd. 3 ) |
| | 35-40 | pale yellow oil |
| 20% MeOH-$CHCl_3$ | 41-50 | brownish oil |
| | 51-61 | orange oil |
| | 62-70 | yellow oil |
| 30% MeOH-$CHCl_3$ | 71-76 | brownish oil |
| | 77-82 | yellow oil |
| 50% MeOH-$CHCl_3$ | 83-90 | deep yellow oil |
| | 91-93 | yellow oil |
| 70% MeOH-$CHCl_3$ | 94-99 | brownish oil |
| | 100-102 | brown solid |
| MeOH | 103-106 | dark brown solid |

Table 2.3 The results of fraction Ia separated on silica gel quick column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| $CHCl_3$ | 1-5 | - |
| | 6-8 | pale yellow oil |
| 5% MeOH-$CHCl_3$ | 9-11 | brownish tar |
| | 12-19 | yellow oil (cpd. 3) |
| | 20-28 | pale yellow oil |
| 10% MeOH-$CHCl_3$ | 29-34 | pale yellow solid |
| | 35-40 | pale yellow oil |
| 20% MeOH-$CHCl_3$ | 41-50 | brownish oil |
| | 51-58 | orange oil |
| | 59-70 | - |
| 30% MeOH-$CHCl_3$ | 71-76 | brownish oil |
| | 77-82 | orange oil |
| 50% MeOH-$CHCl_3$ | 83-90 | yellow oil |
| 70% MeOH-$CHCl_3$ | 91-97 | pale yellow oil |
| 90% MeOH-$CHCl_3$ | 98-101 | brownish oil |
| MeOH | 102-107 | thick brown solid |

2.7.2 Separation of Fraction Ib

The crude extract of Fraction Ib (30 g.) was chromatographed on aluminium oxide and silica gel quick columns. The procedure was treated in the same manner as that used for Fraction Ia. The results were presented in Table 2.4 and Table 2.5, respectively.

Table 2.4 The results of Fraction Ib separated on aluminium oxide quick column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| $CHCl_3$ | 1-3 | - |
| 5% MeOH-$CHCl_3$ | 4-8 | square solid in pale yellow oil (cpd.3 ) |
| | 5-11 | orange oil |
| | 12-18 | pale orange oil |
| 10% MeOH-$CHCl_3$ | 19-27 | yellow amorphous solid in deep orange oil (cpd.9) |
| 20% MeOH-$CHCl_3$ | 28-36 | orange oil |
| | 37-45 | yellow oil |
| 30% MeOH-$CHCl_3$ | 46-54 | brownish oil |
| | 55-59 | deep yellow oil |

(continued)

Table 2.4 (continued)

| Eluents | Fraction No. | Remarks |
|---|---|---|
| 50% MeOH-$CHCl_3$ | 60-65 | brownish oil |
| | 66-69 | yellow oil |
| 70% MeOH-$CHCl_3$ | 70-75 | brown solid |
| MeOH | 76-80 | brown solid |

Table 2.5 The results of Fraction Ib separated on silica gel quick column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| $CHCl_3$ | 1-3 | - |
| 10% MeOH-$CHCl_3$ | 4-20 | yellow ppt. in brownish tar |
| | 21-25 | orange oil |
| 20% MeOH-$CHCl_3$ | 26-34 | yellow oil |
| 30% MeOH-$CHCl_3$ | 35-42 | red-brown oil |
| | 43-51 | orange oil |
| 40% MeOH-$CHCl_3$ | 53-64 | brownish oil |
| 50% MeOH-$CHCl_3$ | 65-73 | brownish oil |
| | 74-80 | yellow oil |

(continued)

Table 2.5 (continued)

| Eluents | Fraction No. | Remarks |
|---|---|---|
| 60% MeOH-$CHCl_3$ | 81-88 | brownish oil |
| | 89-93 | pale yellow oil |
| 80% MeOH-$CHCl_3$ | 94-99 | brown solid |
| | 100-105 | yellow oil |
| MeOH | 106-111 | thick brown solid |

## 2.8 Separation of Fraction IIa and Fraction IIb

Thin-layer chromatography of Fraction IIa and Fraction IIb revealed that there were four major compounds in each crude (solvent 20% chloroform-hexane) using aluminium oxide as an adsorbent and revealed that their were three major compounds in this crude when silica gel was used as an adsorbent.

### 2.8.1 Separation of Fraction IIa

The crude extract of Fraction IIa (25 g.) was chromatographed on aluminium oxide (350 g.) by using column chromatographic technique to separate Fraction IIa into small fraction by varying the polarities of eluted solvent. First, the column was eluted with hexane, and then the polarity of the solvent was increased until the column was stripped with methanol.

The eluate was collected about 600 ml. for each fraction. Each fraction was monitored by TLC technique and the equivalent fraction were combined. The results were presented in Table 2.6.

Table 2.6 The results of Fraction IIa separated on aluminium oxide oxide column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| hexane | 1-3 | transparent oil |
| 10% $CHCl_3$-hexane | 4-7 | transparent oil |
| 20% $CHCl_3$-hexane | 8-12 | pale yellow oil |
| 30% $CHCl_3$-hexane | 13-18 | white needle solid in pale yellow oil (cpd.1) |
| | 19-24 | pale greenish oil |
| 40% $CHCl_3$-hexane | 25-27 | yellow needle solid in yellow oil (cpd.2) |
| | 28-35 | yellow ppt. in yellow oil (cpd.2) |
| 50% $CHCl_3$-hexane | 36-47 | orange oil |
| 70% $CHCl_3$-hexane | 48-55 | white needle solid in pale yellow oil (cpd.5) |
| $CHCL_3$ | 56-66 | yellow oil |
| 5% MeOH-$CHCl_3$ | 67-73 | pale yellow oil |

(continued)

Table 2.6 (continued)

| Eluents | Fraction No. | Remarks |
|---|---|---|
| 10% MeOH-$CHCl_3$ | 74-77 | pale yellow oil |
| | 78-82 | white ppt. (cpd. 4) |
| 30% MeOH-$CHCl_3$ | 83-87 | yellow oil |
| 50% MeOH-$CHCl_3$ | 88-94 | yellow oil |
| 70% MeOH-$CHCl_3$ | 95-98 | yellow oil |
| MeOH | 99-102 | deep orange oil |

Fraction IIa (25 g.) was also chromatographed on silica gel (450 g.) column. The results were presented in Table 2.7.

Table 2.7 The results of Fraction IIb separated on silica gel column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| hexane | 1-4 | - |
| 10% $CHCl_3$-hexane | 5-13 | pale yellow oil |
| 15% $CHCl_3$-hexane | 14-16 | white ppt. in pale yellow oil.(cpd.1) |
| 25% $CHCl_3$-hexane | 17-24 | yellow oil |
| 50% $CHCl_3$-hexane | 25-38 | yellow needle solid in pale yellow oil (cpd. 2) |
| 70% $CHCl_3$-hexane | 39-49 | brownish oil |
| $CHCl_3$ | 50-56 | orange oil |
| 10% MeOH-$CHCl_3$ | 57-60 | yellow oil |
| | 61-66 | brownish oil |
| 25% MeOH-$CHCl_3$ | 67-78 | white ppt. in yellow oil (cpd. 4) |
| 50% MeOH-$CHCl_3$ | 79-87 | brownish oil |
| 70% MeOH-$CHCl_3$ | 88-92 | yellow oil |
| 90% MeOH-$CHCl_3$ | 93-99 | pale yellow oil |
| MeOH | 100-105 | pale yellow oil |

2.8.2 Separation of Fraction IIb

The crude extract of Fraction IIb (25 g.) was chromatographed on aluminium oxide (350 g.) and silica gel (450 g.) columns. The separation of Fraction IIb was performed in the same manner as in Fraction IIa. The results of this fraction were presented in Table 2.8 and Table 2.9, respectively.

Table 2.8 The results of Fraction IIb separated on aluminium oxide column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| hexane | 1-7 | pale yellow oil |
| 10% $CHCl_3$-hexane | 8-16 | pale yellow oil |
| | 17-25 | orange oil |
| 20% $CHCl_3$-hexane | 26-32 | yellow oil |
| 30% $CHCl_3$-hexane | 33-40 | white needle solid in pale yellow oil (cpd. 1) |
| | 41-47 | pale yellow oil |
| 40% $CHCl_3$-hexane | 48-51 | yellow oil |
| 50% $CHCl_3$-hexane | 52-56 | yellow ppt. in yellow oil (cpd.2) |
| 60% $CHCl_3$-hexane | 57-63 | yellow ppt. in pale yellow oil (cpd. 2) |

(continued)

Table 2.8 (continued)

| Eluents | Fraction No. | Remarks |
|---|---|---|
| 80% $CHCl_3$-hexane | 64-68 | white ppt. in pale yellow oil (cpd.5) |
| $CHCl_3$ | 69-76 | yellow oil |
| 10% MeOH-$CHCl_3$ | 77-80 | white ppt.in pale yellow oil (cpd. 4) |
| 20% MeOH-$CHCl_3$ | 81-85 | pale yellow oil |
| 40% MeOH-$CHCl_3$ | 86-89 | yellow oil |
| 60% MeOH-$CHCl_3$ | 90-94 | pale yellow oil |

Table 2.9 The results of Fraction IIb separated on silica gel column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| hexane | 1-5 | - |
| 10% $CHCl_3$-hexane | 6-10 | yellow oil |
| 20% $CHCl_3$-hexane | 11-16 | white needle solid in pale yellow oil.(cpd. 1) |
| | 17-23 | very pale yellow oil |
| 35% $CHCl_3$-hexane | 24-31 | yellow oil |
| | 32-34 | very pale yellow oil |
| 50% $CHCl_3$-hexane | 35-41 | pale yellow needle solid in yellow oil (cpd.2) |
| 70% $CHCl_3$-hexane | 42-48 | yellow oil |
| | 49-55 | pale yellow oil |
| $CHCl_3$ | 56-64 | yellow oil |
| 10% MeOH-$CHCl_3$ | 65-72 | orange oil |
| 25% MeOH-$CHCl_3$ | 73-78 | white ppt. in pale yellow oil (cpd.4) |
| | 79-82 | - |
| 50% MeOH-$CHCl_3$ | 83-89 | pale yellow oil |
| 70% MeOH-$CHCl_3$ | 90-95 | pale yellow oil |
| 90% MeOH-$CHCl_3$ | 96-100 | yellow oil |
| MeOH | 101-108 | brownish oil |

## 2.9 Separation of Fraction IIIa and Fraction IIIb

Thin-layer chromatography of Fraction IIIa and Fraction IIIb revealed that they were four major compounds in this crude when using silica gel plate. (solvent : chloroform).

In the same ways, Fraction IIIa and Fraction IIIb were performed by silica gel column chromatography.

### 2.9.1 Separation of Fraction IIIa

The crude extract of Fraction IIIa (30 g.) was chromatographed on silica gel (450 g.) column chromatography and then separated this fraction into small fraction by varying the polarities of the eluent. First, the column was eluted with 50% chloroform-hexane and then solvents of increasing polarity. Finally, the column was stripped with 50% methanol-chloroform. The eluted solution was collected approximately 800 ml. for each fraction. Each fraction was monitored by TLC technique and equivalent fractions were combined. The results were presented in Table 2.10.

Table 2.10 The results of Fraction IIb separated on silica gel column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| 50% $CHCl_3$-hexane | 1-5 | - |
| 60% $CHCl_3$-hexane | 6-10 | yellow oil |
| 70% $CHCl_3$-hexane | 11-16 | orange oil |
| 80% $CHCl_3$-hexane | 17-24 | pale yellow oil |
| $CHCl_3$ | 25-30 | white ppt. in pale yellow oil (cpd 8) |
| 10% MeOH-$CHCl_3$ | 31-36 | brownish tar (4.88 g.) |
| 20% MeOH-$CHCl_3$ | 37-42 | orange oil |
| 30% MeOH-$CHCl_3$ | 43-50 | yellow oil |
| 40% MeOH-$CHCl_3$ | 51-56 | pale yellow oil |
| 50% MeOH-$CHCl_3$ | 57-63 | very pale yellow oil |

The crude extract of Fraction IIIa (30 g.) was also separated by aluminium oxide (300 g.) column chromatography and the separation was done in the same manner as above in Fraction IIIa when silica gel was used. The results were presented in Table 2.11.

Table 2.11 The results of Fraction IIIa separated on aluminium oxide column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| 50% $CHCl_3$-hexane | 1-4 | - |
| 70% $CHCl_3$-hexane | 5-12 | yellow oil |
| 90% $CHCl_3$-hexane | 13-18 | white ppt. in pale yellow oil (cpd.8) |
| $CHCl_3$ | 19-30 | white ppt. in yellow oil (cpd.8) |
| 10% MeOH-$CHCl_3$ | 31-38 | yellow oil |
| 20% MeOH-$CHCl_3$ | 39-46 | yellow ppt. in orange oil (cpd.3) |
| | 47-52 | pale yellow oil |
| 30% MeOH-$CHCl_3$ | 53-60 | pale yellow oil |
| 50% MeOH-$CHCl_3$ | 61-66 | yellow oil |
| 70% MeOH-$CHCl_3$ | 67-73 | yellow oil |
| 90% MeOH-$CHCl_3$ | 74-81 | pale yellow oil |
| MeOH | 82-90 | yellow oil |

The eluted fractions No. 31-36 (Table 2.10 , pp. 39 4.88 g.) were eluted from the silica gel column of Fraction IIIa with 10% methanol-chloroform. The aluminium oxide thin-layer chromatographic technique (5% methanol-chloroform) revealed that there

were two major compounds in this crude. However, this eluted crude could not be separated by thin-layer chromatography when silica gel was used.

The separation of eluted fractions No. 31-36 (Table 2.10 , pp. 39 , 4.88 g.) were repeated on aluminium oxide (60 g.) column. The eluted solution was collected approximately 50 ml. for each fraction. The results were presented in Table 2.12.

Table 2.12 The results of fractions 31-36 separated on aluminium oxide column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| $CHCl_3$ | 1-6 | - |
| 2% MeOH-$CHCl_3$ | 7-10 | pale yellow oil and white ppt. (luminescence)(cpd 6) |
| 5% MeOH-$CHCl_3$ | 11-16 | pale yellow ppt. in yellow oil (cpd.6) |
| 10% MeOH-$CHCl_3$ | 17-20 | pale yellow oil |
| 15% MeOH-$CHCl_3$ | 21-30 | yellow ppt.in yellow oil (cpd.7) |
| 30% MeOH-$CHCl_3$ | 31-37 | yellow oil |
| 50% MeOH-$CHCl_3$ | 38-46 | thick brown solid |
| 70% MeOH_$CHCl_3$ | 47-56 | thick brown solid |
| MeOH | 57-60 | thick brown solid |

2.9.2 Separation of Fraction IIIb

The crude extract of Fraction IIIb (30 g.) was chromatographed on silica gel (450 g.) and aluminium oxide columns. The separation was done in the same route as for Fraction IIIa (2.9.1). The results were presented in Table 2.13 and Table 2.14, respectively.

Table 2.13 The results of Fraction IIIb separated on silica gel column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| 50% $CHCl_3$-hexane | 1-3 | - |
| 70% $CHCl_3$-hexane | 4-8 | pale yellow oil |
| 90% $CHCl_3$-hexane | 9-16 | yellow oil |
| $CHCl_3$ | 17-23 | white ppt. in pale yellow oil (cpd.8) |
| 5% MeOH-$CHCl_3$ | 24-30 | very pale yellow oil |
| 10% MeOH-$CHCl_3$ | 31-42 | brownish tar (4.15 g.) |
| 20% MeOH-$CHCl_3$ | 43-50 | pale yellow oil |
| 30% MeOH-$CHCl_3$ | 51-57 | pale yellow oil |
| 40% MeOH-$CHCl_3$ | 58-65 | pale yellow oil |
| 50% MeOH-$CHCl_3$ | 66-72 | very pale yellow oil |

Table 2.14 The results of Fraction IIIb separated on aluminium oxide column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| 50% $CHCl_3$-hexane | 1-6 | - |
| 70% $CHCl_3$-hexane | 7-14 | pale yellow oil |
| 90% $CHCl_3$-hexane | 15-24 | white ppt. in pale yellow oil (cpd.8) |
| $CHCl_3$ | 25-32 | white ppt. in yellow oil (cpd. 8) |
| 10% MeOH-$CHCl_3$ | 33-40 | pale yellow oil |
| 20% MeOH-$CHCl_3$ | 41-46 | yellow oil |
| 30% MeOH-$CHCl_3$ | 47-52 | yellow ppt. in pale yellow oil (cpd. 3) |
| 40% MeOH-$CHCl_3$ | 53-60 | orange oil |
| 50% MeOH-$CHCl_3$ | 61-67 | yellow oil |
| 60% MeOH-$CHCl_3$ | 68-74 | pale yellow oil |
| 80% MeOH-$CHCl_3$ | 75-81 | pale yellow oil |
| MeOH | 82-90 | very pale yellow oil |

The eluted fractions No. 31-42 (Table 2.13 , pp. 42 4.15 g.) were eluted from the silica gel column of Fraction IIIb with 10% methanol-chloroform. The aluminium oxide thin-layer chromatography revealed that there were two major compounds in this crude.

However, this eluted crude could not be separated by thin-layer chromatography with silica gel.

The separation of eluted fractions No. 31-42 (Table 2.13 , pp. 42, 4.15 g.) were also repeated on aluminium oxide (60 g.) column. The results of separation were presented in Table 2.15.

Table 2.15 The results of fractions 31-42 separated on aluminium oxide column

| Eluents | Fraction No. | Remarks |
|---|---|---|
| $CHCl_3$ | 1-5 | - |
| 2% MeOH-$CHCl_3$ | 6-12 | white ppt. in pale yellow oil (luminescence) (cpd.6) |
| 5% MeOH-$CHCl_3$ | 13-20 | white ppt. in yellow oil (cpd.6) |
| 10% MeOH-$CHCl_3$ | 21-26 | pale yellow oil |
| 15% MeOH-$CHCl_3$ | 27-32 | yellow oil |
| 30% MeOH-$CHCl_3$ | 33-40 | yellow ppt.in yellow oil (cpd.7) |
| 50% MeOH-$CHCl_3$ | 41-50 | yellow oil |
| 70% MeOH-$CHCl_3$ | 51-56 | pale yellow oil |
| MeOH | 57-64 | pale yellow oil |

## 2.10 Isolation and Purification of Separated Compounds

### 2.10.1 Compound (1)

Compound (1) was obtained from the hexane part which was separated on the silica gel column with a mixture of *n*-hexane and chloroform as eluting solvent. The obtained compound was recrystallized from the mixture of *n*-hexane and chloroform for several times to give bright white needle crystals 80 mg. (0.16 % wt. by wt. of Fraction IIa ) and 60 mg. (0.12 % wt. by wt. of Fraction IIb), m.p. 128-130 C. The Rf value of this compound was 0.45 using 1:1 *n*-hexane-chloroform as a developing solvent.

Compound (1) was soluble in chloroform and slightly soluble in *n*-hexane. It gave a deep green color with Liebermann-Burchard's reagent and decolorized $Br_2$ in $CCl_4$ solution.

The IR spectrum (Fig. 1) showed the important absorption band at $\nu_{max}$ ($cm^{-1}$) : 3430 (O-H stretching) , 2960-2840 (C-H stretching of $R_1R_2=CHR_3$), 1670-1630 (C=C), and 1050 (C-O)

The $^1H$ NMR ($CDCl_3$) spectrum (Fig. 2) showed the significant signals at chemical shifts (ppm.) : 5.32 (2H, d, J = 4.39 Hz, olefinic protons), 5.08 (1H, t, J = 5.86 Hz, olefinic protons) 3.46 (m) and other signals around 2.30 to 0.70 ppm.

The $^{13}C$ NMR spectrum ($CDCl_3$) spectrum (Fig.3) illustrated the carbon signals at chemical shifts (ppm.): 140.76, 138.27, 129.28, 121.64 (olefinic carbon), 71.69 (carbon attached to oxygen atom) together with signals around 56.79 to 11.83.

The MS spectrum (Fig. 4) showed the important ion peaks at m/e (% rel int.) 414.0 (83.82), 412.0 (100.0) and 400(58.23) (calcd. for $C_{29}H_{50}O$, $C_{29}H_{48}O$ and $C_{28}H_{48}O$, respectively) and other vital peaks at m/e 396.0 (26.20), 382.0 (21.96), 329.0 (18.89), 303.0 (20.60), 273.0 (34.91), 255.0 (61.03) and 213.0 (31.22).

The GLC analysis (Fig. 5) indicated that there were three peaks on the chromatogram at retention times as shown in Table 2.16

Table 2.16 The retention times of various peaks from the gas chromatogram of Compound (1) compare with steroid standards

| Compound | Retention time (mins) | Peak area |
|---|---|---|
| chloresterol | 14.43 | |
| campesterol | 18.10 | |
| stigmasterol | 19.43 | |
| β-sitosterol | 21.95 | |
| Compound (1) | 18.37 | 86006 |
| | 19.32 | 651114 |
| | 21.98 | 628639 |

(column OV-1 2%, col. temp. 260 °C, inj. temp. 290 °C, detector temp. 290 °C, $N_2$ flow rate 50 ml/ min )

The comparison of the retention times of the gas chromatogram to those of steroids standards revealed that Compound (1) was a mixture of three steroids : campesterol, stigmasterol and β-sitosterol.

### 2.10.2 Compound (2)

Compound (2) was collected from the hexane parts using both silica gel and aluminium oxide columns. After the purification of this compound by recrystallization from *n*-hexane, bright pale yellow needles 3.60 g. (7.20 % wt. by wt. of Fraction IIa) and 2.92 g. ( 5.84 % wt. by wt. of Fraction IIb) were obtained. The Rf value of this compound was 0.68 using 1:19 *n*-hexane-chloroform as a developing solvent, m.p. 103.0-104.0 °C .

This compound was soluble in ethylacetate, chloroform acetone and slightly soluble in *n*-hexane. It gave positive test with 2,4-DNP reagent and decolorized $Br_2$ in $CCl_4$ solution.

The IR spectrum (Fig. 6) showed the important absorption bands at $\nu_{max}$ ($cm^{-1}$) : 3070 (C-H stretching of aromatic), 2980 (C-H stretching), 1660 (C=O conjugated with double bond), 1600, 1500 (C=C), 1270 and 1080 (C-O stretching) and 850, 830 (C-H bending of aromatic).

The $^1H$ NMR ($CDCl_3$) spectrum (Fig. 7) showed the significant signals at chemical shifts (ppm.) : 12.76 (hydroxyl group), 6.36 (s, 1H, CH-aryl), 5.99 (s, 1H, =CH-C=C), 5.14 (t, 1H, $-CH_2-CH=$), 3.88 (s, 3H, $-OCH_3$), 3.37 (d, J = 7.2 Hz., 2H, $-CH_2-$), 2.36 (s, 3H, $C-CH_3$), 1.78 (s, 3H-$CH_3$), and 1.67 (s, 3H, $-CH_3$).

The $^{13}C$ NMR ($CDCl_3$) spectrum (Fig. 8) illustrated the carbon signals at chemical shifts (ppm.) : 182.89 166.69, 162.69, 154.67, 108.64, 94.67 (aromatic carbon), 55.96 (s, 3H, $-OCH_3$).

The UV spectrum of this compound (Fig. 9) gave the $\lambda_{max}$ at 298 nm.($\log \varepsilon = 3.63$), and 330 nm. ($\log \varepsilon = 3.64$)

The MS spectrum (Fig. 10) showed the important fragmentation ion peaks at m/e (% rel int.) : 274 (66.02), 259 (100.0) 219 (18.23), 206 (52.48), 205 (10.79), 189 (3.52) and 177 (7.60).

### 2.10.3 Compound (3)

Compound (3) was obtained from the chloroform parts by aluminium oxide columns, using methanol as an eluent. After oil removal, it was recrystallized from a mixture of ethanol and chloroform to give a pale yellow amorphous compound, 0.96 g. (1.60 % wt. by wt. of Fraction IIIa) and 0.88 g. (1.47 % wt. by wt. of Fraction IIIb) m.p. 230 °C (dec.) and having Rf value 0.60 using 3:1 chloroform-methanol as a developing solvent.

The Compound (3) was soluble in methanol and slightly soluble in ethanol, insoluble in *n*-hexane, chloroform and ethylacetate. This compound decolorized $Br_2$ in $CCl_4$ solution, gave positive result with 2,4-DNP reagent and liberated $CO_2$ with 5% $NaHCO_3$ solution. Liebermann-Burchard's test gave negative result.

The IR spectrum (Fig. 11) showed the important absorption band at $\nu_{max}$ ($cm^{-1}$) : 3040 (C-H stretching), 2960 (C-H stretching), 1660 (C=O), 1620 (C=C), 1385, 1370 (geminal

dimethyl) and 820, 810 and 770 (C-H bending of aromatic).

The $^{1}H$ NMR (DMSO+$CDCl_3$) spectrum (Fig. 12) showed the significant signal at chemical shifts (ppm.) : 6.86 (d , J = 9.9 Hz., 1H, -CH=CH-), 5.67(d, J = 9.9 Hz., 1H, -CH=CH), 3.82 (s,3H,-$OCH_3$) and 1.44 (s, 6H, -$CH_3$).

The $^{13}C$ NMR (DMSO) spectrum (Fig. 13) illustrated the carbon signals at chemical shifts (ppm.) : 175.58 (carbonyl carbon), 161.46 (carboxylic carbon), 160.14, 157.85, 152.95, 150.33, 114.22, 108.81, 102.00, 97.03 (aromatic carbon) 128.19, 115.13 (C=C), 78.29 (C-O) , 56.30 (methoxy carbon) , 27.80 (geminal dimethyl).

The MS spectrum (Fig. 14 ) gave the important fragmentation ion peaks at m/e (% rel int.) : 301.0 (100.00) 287 (21.74), 258 (3.91), 243 (7.20), 217 (6.54) and 213 (10.10).

### 2.10.4 Compound (4)

Compound (4) was obtained from the hexane parts by both aluminium oxide and silica gel columns. After oil removal by chloroform, it was recrystallized from methanol for several times, white amorphous solid, 0.38 g.(0.76 % wt. by wt. of Fraction IIa) and 0.25 g. (0.50 % wt. by wt. of Fraction IIb) were obtained. It has m.p. 260 °C (dec.) and Rf value 0.28 using ethanol as a developing solvent.

The Compound (4) was slightly soluble in methanol and ethanol, but insoluble in *n*-hexane, chloroform, ether, ethylacetate. This compound gave deep green color with Liebermann-Burchard's

reagent decolorized $Br_2$ in $CCl_4$ reagent and showed positive test with Molisch's reagent.

The IR spectrum (Fig. 15 ) showed the important absorption band at $\nu_{max}$ ($cm^{-1}$) : 3600-3400 (b, -OH), 1640 (w, C=C), 1295, 1165 (m, C-O) and around 1030 (glycosidic linkage).

The $^1H$ NMR (DMSO+$CDCl_3$) spectrum (Fig. 16) showed the significant signals at chemical shifts (ppm.) : 5.36 (olefinic proton), 4.79-3.73 (proton on sugar moiety) and other signals around 2.43 to 0.68 typical to steroid compounds.

The $^{13}C$ NMR (DMSO+$CDCl_3$) spectrum (Fig. 17) illustrated the carbon signal at chemical shifts (ppm.) : 140.63 and 121.68 (olefinic carbon), 101.30 to 62.50 (6 carbon adjacent to oxygen) and other peaks around 56.77 to 9.99 ppm.

The MS spectrum (Fig. 18) showed the important ion peak at m/e (% rel int.) : 414 (50.04), 396 (100.00), 381.0 (28.66), 273.0 (19.58), 255.0 (38.56).



Acid hydrolysis of Compound (4)

Compound (4) (150 mg.) was hydrolyzed by refluxing with 10% hydrochloric acid in ethanol for 12 hours (The completion of the reaction was checked by TLC.). Solvent was removed under reduced pressure. The residue was diluted with water and extracted with diethylether and separared into two layers as aglycone in diethylether and sugar component in water.

Study of aglycone

Ethereal solution that described above was washed with water and dried over anhydrous sodium sulfate ($Na_2SO_4$). After the solvent was removed, the residue was recrystallized from a mixture of chloroform and *n*-hexane to afford white needle crystals of this compound which were assigned as compound (4a), 90 mg., m.p. 135.0-136.5 °C and Rf 0.48 (solvent : chloroform). Compound (4a) was soluble in chloroform, diethylether and slightly soluble in methanol and *n*-hexane. This compound decolorized $Br_2$ in $CCl_4$ and gave a deep green color with Liebermann-Burchard's reagent. This revealed that Compound (4a) was one type of steroids.

The IR spectrum (Fig. 19) showed the important absorption bands at $\nu_{max}$ ($cm^{-1}$) : 3400 (b, O-H), 1630 (w, C=C), 1050 and 1020 (m, C-O).

Compound (4a) was also analysed by gas liquid chromatography (col OV-1 2%, col. temp. 260 °C, injection temp. 290 °C, $N_2$ flow 50 ml./min, FID ) (Fig. 20b) by comparing its chromatogram to those of standard steroids : chloresterol, campesterol, stigmasterol and $\beta$-sitosterol (Fig. 20a.).

Table 2.17 The retention times of various peaks from gas chromatogram of ompound (4a) compared with steroid standards

| Compound | Retention time (mins) | Peak area |
|---|---|---|
| chloresterol | 14.43 | |
| campesterol | 18.23 | |
| stigmasterol | 19.63 | |
| β-sitosterol | 22.09 | |
| Compound (4a) | 14.33 | 5810 |
| | 19.56 | 18086 |
| | 21.96 | 62532 |

Study of the carbohydrate component of Compound (4)

After aglycone was removed from the hydrolysis reaction mixture of Compound (4), the aqueous phase was neutralized with silver carbonate. The unreacted silver carbonate was separated by filtration and the filtrate was concentrated under reduced pressure. The white powder (20 mg.) was obtained and designated as compound (4b).

The solution of Compound (4b) (1.5 mg. in 1.0 ml. of water) was identified by paper chromatography as described in Topic 2.4.2 with a mixture of various standard sugars (glucose, arabinose, rhamnose, galactose, xylose). The chromatogram was developed using 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 heated at 110-120 C for 15 mins. It showed only one spot which was identical to the authentic D-(+)-glucose .

TLC of the component in neutral aqueous phase of glycosidic hydrolysate was compared with a mixture of various standard sugars using n-butanol-acetic acid-diethyether-water 9:6:3:1 as a developing solvent. The sugars was detected with 25% sulfuric acid by spraying the acid solution onto the TLC plate and the plate was dried at 110-120 C to develope a brownish color spot at Rf 0.48 which was identical to the authentic D-(+)glucose.

Acetylation of Compound (4b)

Compound (4b) (15 mg.) was dissolved in a few drops of pyridine and the solution was refluxed on a steam bath with acetic anhydride (3.0 ml.) for 4 hours, The entire mixture was worked up in usual manner to give white precipitated solid. After recrystallization of this compound from a mixture of chloroform and methanol, white needle crystals, (13 mg.) m.p. 112.0 C was obtained.

The IR spectrum (Fig. 21) showed the important absorption bands at $\nu_{max}$ ($cm^{-1}$) : 1760-1740 (s,-COO-) and 1260-1240 (s,C-O of acetate).

2.10.5 Compound (5)

Compound (5) was obtained from the hexane parts which was separated on aluminium oxide column. After recrystallization from *n*-hexane for several times, Compound (5) was collected, 0.32 g. (0.64 % wt. by wt. of Fraction IIa) and 0.17 g. (0.34 % wt. by wt. of Fraction IIb). Although its TLC plate showed only one spot when various developing solvent systems were used, the melting point of Compound 5 was still of a wide range from 191.0 to 210.0 °C. This compound was readily soluble in chloroform and diethylether, but slightly soluble in *n*-hexane, methanol. It gave a purple color to Liebermann-Burchard's reagent and decolorized $Br_2$ in $CCl_4$ solution.

The IR spectrum (Fig. 22) of this compound revealed the major absorption peaks at $\nu_{max}$ ($cm^{-1}$) : 3350 (b, O-H) and 1640 (m, C=C).

The IR spectrum of this compound demonstrated that there was hydroxyl functional group in the molecule. The purification of this crystalline solid was attempted by forming acetyl derivative.

Acetylation of Compound (5)

Compound (5) (300 mg.) was dissolved in dry pyridine (5.0 ml.) and acetic anhydride 10.0 ml. was added into this solution. The solution was refluxed on a water bath for 5 hours. The entire reaction mixture was then poured into ice distilled water and the acetyl derivative was collected. After the recrystallization

from a mixture of chloroform and ethanol, bright white needle crystals (285 mg.) with Rf 0.70 (solvent : 50% chloroform-*n*-hexane) were obtained. This derivative melted at 216.5-218.5 °C

The IR spectrum of Compound (5) acetate (Fig. 23) showed the important absorption bands at $\nu_{max}$ ($cm^{-1}$) : 1740 (s, -C=O) 1240 (s, C-O) of acetate .

The $^{1}H$ NMR ($CDCl_3$) spectrum of Compound (5) acetate (Fig. 24) showed the significant signals at chemical shifts (ppm.) : 4.65, 4.52 (2H, J = 10.24 Hz., olefinic proton), 4.40 (1H, t, J = 9.04 Hz., proton on carbon attached to oxygen of acetate group), 2.02 (3H, s, methyl proton of acetoxy group) together with other signals around 2.25-0.75 ppm.

The $^{13}C$ NMR ($CDCl_3$) spectrum of Compound (5) acetate (Fig. 25) illustrated the carbon signals at chemical shifts (ppm.) 170.70 (carbon of ester), 150.79 and 109.35 (1C each, olefinic carbon), 80.90 (carbon attached to acetyl group) and other signals around 55.33 to 14.49 ppm.

The MS spectrum of Compound (5) acetate (Fig. 26 ) gives the important fragmentation ion peaks at m/e (%rel int.) 468.0 (44.55) (calcd. for $C_{32}H_{52}O_2$ : MW. 468.41) and other ion peaks at m/e 453.0 (11.70), 218.0 (40.17) and 189.0 (100.0) .

The GLC analysis (Fig. 27) (condition : OV-17 , SW 80/100 mesh, 3mm. x 2m., column temp. 300 °C , FID 320 °C, carrier gas $N_2$ 50.0 ml/min.) revealed that a merely peak at Rt 29.09 min. which was corresponded to the authentic lupeolacetate.

Hydrolysis of Compound (5) acetate

Compound (5) acetate 150 mg. was hydrolyzed by refluxing with 5% KOH in ethanol on a steam bath for 50 mins in usual manner to obtain a white solid which was recrystallized from a mixture of chloroform and ethanol for several times to yield bright white needle crystals (97 mg.). It had m.p. 213.5-215.0 °C, Rf 0.72 (solvent : 50% chloroform-hexane). The results of the color tests was identical to those of Compound (5).

The IR spectrum (Fig. 28) showed the important absorption bands at $\nu_{max}$ ($cm^{-1}$) : 3330 (b, O-H), 3080 (w, C-H of $C=CH_2$), 1640 (w, C=C) and 870 (m, C-H out of plane of $=CH_2$).

The $^1H$ NMR ($CDCl_3$) spectrum (Fig. 29) showed the significant signals at chemical shifts (ppm.) : 4.65, 4.54 (2H, J = 9.00 Hz., olefinic proton), 3.14 (1H, t, J = 7.83 Hz., proton on carbinol carbon) and other signals around 2.45 to 0.75 ppm.

The $^{13}C$ NMR ($CDCl_3$) spectrum (Fig. 30) illustrated the carbon signals at chemical shifts (ppm.) : 150.55 and 108.89 (1C each, olefinic carbon), 78.65 (carbon attached to oxygen atom) and other signals around 55.68 to 14.49 ppm..

The MS spectrum (Fig. 31) gave the important fragmentation ions peaks at m/e (rel int.) 426.0 (75.96) (calcd. for $C_{30}H_{50}O$ : MW. 426.39 and the other fragmentation ion peaks at m/e 218.0 (69.58), 207.0 (79.54), 189.0 (88.55) and 95.0 (100.0).

The GLC analysis (Fig. 33) (using the same condition as Compound (5) acetate) showed only one peak at retention time 21.76 min. which was corresponded to authentic lupeol.

2.10.6 Compound (6)

Compound (6) was obtained from the chloroform part by silica gel column. The collected fractions were eluted by mixture of chloroform and methanol (9:1), was repeated with aluminium oxide column. The compound eluted by mixture of chloroform and methanol (49:1) was recrystallized from mixture of *n*-hexane and chloroform to yield white needle crystals 120 mg. (0.20 % wt. by wt. of Fraction IIIa) and 42 mg. (0.07 % wt. by wt. of Fraction IIIb) was obtained. It has Rf value 0.67 (using 40% chloroform-*n*-hexane as developing solvent), m.p. 68.5-69.5 °C.

This compound was soluble in hot *n*-hexane, chloroform, ether, alcohol, and water. It gave negative results with Liebermann-Burchard's reagent, 5% $NaHCO_3$ solution and 5% $FeCl_3$ reagent. This results indicated that this compound was not a steroidal or triterpenoidal skeletons.

The IR spectrum (Fig. 34) displayed the important absorption bands at $\nu_{max}$ ($cm^{-1}$) : 3050 (s, C-H stretching) , 1700 (s, carbonyl ester), 1600-1400 (C=C of aromatic), 1120 (C-O stretching).

The $^1H$ NMR ($CDCl_3$) spectrum (Fig. 35) showed the signals at chemical shifts (ppm.) : 7.71 (d, 9.5 Hz, 1H), 7.53 , 7.45, 7.37, 7.35, 7.26 (aromatic proton) and 6.43 (d, 9.5 Hz., 1H).

The $^{13}C$ NMR ($CDCl_3$) spectrum (Fig. 36) exhibited the significant signals at chemical shifts (ppm.) : 160.66 (carbonyl carbon) 143.38 (∝-carbon of C=O) 131.73 , 127.80 , 124.34 118.74 , 116.74 and 116.55 (C=C of aromatic).

The MS spectrum (Fig. 37) gave the molecular ion peak at m/e (% rel int.) : 146 (100.0) and the others ion peaks at 118.0 (74.95), 90.0 (32.80) and 89.0 (25.67).

Reaction Test of Coumarin

Compound (6) (20 mg.) was treated with hot 6 M NaOH solution and the compound was hydrolysed slowly to give a yellow solution of the lactone ring-opened product that luminesced yellow light [46].

This characteristic behavior towards dilute alkali has been developed into a simple test for all kind of coumarins. This result exhibited that this compound was coumarin.

2.10.7 Compound (7)

Compound (7) was obtained from chloroform part by silica gel column chromatography. The collected fraction were eluting by mixture of chloroform and methanol (9:1) was repeated with aluminium oxide column chromatography. After purification by recrystallization from mixture of chloroform and methanol for several times. It gave pale yellow needle crystals 54 mg. (0.09 % wt. by wt. of fraction IIIa) and 38 mg. (0.06 % wt. by wt. of Fraction IIIb) with m.p. 183.5-184.0 °C and Rf value 0.18 (9:1 chloroform-*n*-hexane).

This compound was soluble in chloroform, ether and alcohol. It gave negative results with Liebermann-Burchard's reagent, 5% $FeCl_3$ reagent and 5% $NaHCO_3$ solution . These results indicated that it was not composed of steroidal or triterpenoidal

skeletons.

The IR spectrum (Fig. 38) gave the absorption bands at $\nu_{max}$ ($cm^{-1}$) : 3500-3100 (b, O-H stretch.), 1710 (C=O of lactone), 1620 to 1470 (C=C stretch.), 1260, 1120 (C-O-C) and 1200, 1150 (C-O).

The $^{1}H$ NMR ($CDCl_3$) spectrum (Fig. 39) showed the signals at chemical shifts (ppm.) : 3.89 (3H, s, $-OCH_3$), 3.92 (3H, s, $-OCH_3$), 6.21 (1H, d, J = 9.6 Hz.), 6.40 (3H, s), 6.44 (1H, s, OH) and 7.96 (1H, d, J = 9.5 Hz.).

The $^{13}C$ NMR ($CDCl_3$) spectrum (Fig. 40) displayed the important signals for 11 carbons at chemical shifts (ppm.) : 161.47 (C=O), 155.61 (C-6), 151.72 (C-7), 145.66 (C-5), 138.61 (C-4), 131.55 (C-6), 11.68 (C-3), 102.53 (C-10), 92.31 (C-8), 61.34 ($-OCH_3$) and 51.68 ($-OCH_3$).

The MS spectrum (Fig. 41) displayed the important peaks at m/e (%rel int.) : 222 (79.9,$M^+$), 179 (15.2) and 151 (29.11).

The test for coumarin by reacting with alkali gave a positive result which showed that this compound is coumarin.

**2.10.8 Compound (8)**

Compound (8) was eluted from silica gel column chromatography with the mixture of chloroform and *n*-hexane (3:7) of chloroform part. After recrystallization with *n*-hexane for several times, white amorphous solid 45 mg. (0.08 % wt. by wt. of Fraction IIIa) and 32 mg. (0.05 % wt. by wt.of Fraction IIIb), m.p. 84.5-85.5 °C were obtained. This compound revealed one spot on TLC plate at Rf value 0.57 (developing solvent : chloroform).

This compound was soluble in chloroform and diethylether but slightly soluble in *n*-hexane and insoluble in water. It gave negative results with all this reagents : Liebermann-Burchard's, $Br_2$ in $CCl_4$, $FeCl_3$, 2,4-DNP and $KMnO_4$ reagents.

The IR spectrum (Fig. 42) revealed the important peaks at $_{max}$ ($cm^{-1}$) : 3350 (b, O-H) and 1050 (m, C-O of 1 ROH).

The $^1H$ NMR ($CDCl_3$) spectrum (Fig. 43) gave the proton signal at $\delta$ (ppm.) 3.67 (2H,t) 1.56, 1.27 and 0.90 ppm.

The $^{13}C$ NMR ($CDCl_3$) spectrum (Fig. 44) revealed the carbon signals at chemical shifts (ppm.) : 63.10 (carbon attacted to oxygen atom) together with signals around 32.85-14.00 ppm..

The MS spectrum (Fig. 45) did not give the molecular ion peak, $M^+$, but it displayed mass fragment ion peaks at m/e (% rel int.) 476.0 (3.50, $M^+-H_2O$). The other fragment ions peak were at m/e 448.0 (12.85, $M^+-H_2O-(CH_2)_2$), 420.0 (14.25, $448-(CH_2)_2$) and other signals corresponded to loss of $-(CH_2)_2-$ (m/e 28) step by step.

The GLC analysis (Fig. 46) (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, $N_2$ flow 50 ml/min) reveals five peaks on gas chromatography with retention time 9.60, 11.76, 14.78, 18.29 and 22.96 min., respectively. The major component in this mixture is the peak with retention time 18.39.

Acetylation of Compound (8)

Acetic anhydride (1.0 ml.) was added dropwise to a mixture of Compound (8) (35 mg.) and a few drops of dry pyridine. The reaction mixture was kept overnight at room temperature. After the reaction took place,the mixture was poured into ice water with vigorous stirring to yield a white precipitate which was filtered off and followed by washing with water until no odour of pyridine could be noticed. The acetyl derivative was purified by recrystallization from acetone to give a white plate solid (30 mg.), m.p. 70.0-70.5 °C, Rf value 0.77 (solvent : 15% chloroform-hexane).

The IR spectrum of Compound 8 acetate (Fig. 48) displayed the major absorption bands at $\nu_{max}$ ($cm^{-1}$) : 1750 (s,-C=O) and 1245 (s, C-O) of acetate.

2.10.9 Compound (9)

Compound (9) was obtained from the methanol parts by aluminium oxide column. After oil removal, it was recrystallized from the mixture of chloroform and methanol which gave yellow amorphous compound, 24 mg.(0.16% wt. by wt. of fraction Ia) and 13 mg. (0.14% wt. by wt. of fraction Ib). This compound decomposed at 250 °C, having $R_f$ value 0.57 using 3:1 chloroform-methanol.

This compound was soluble in methanol and very slighty soluble in ethanol, insoluble in *n*-hexane. It decolorized $Br_2$ in $CCl_4$ and gave negative result with Liebermann-Burchard's reagent.

The IR spectrum (Fig.49) showed the important absorption bands at maximum ($cm^{-1}$) : 3600-3200 (b, O-H), 3040 (C-H strech.), 2980 (C-H strech), 1670 (C=O), 1620 (C=C), 1600-1400 (C=C aromatic), 1390,1380 (geminal dimethyl), 1060 (C-O strrech 1 ROH).

The $^{1}H$ NMR ($CDCl_3$) spectrum (Fig. 50) showed the significant signals at chemical shifts (ppm) : 6.44 (d, J = 10 Hz., 1H), 6.25 (s, 1H), 5.47 (d, J = 10 Hz., 1H), 4.45 (s, 2H), 3.85(s, 3H) 2.25 (s, 3H), 1.43 (s, 6H).

The $^{13}C$ NMR ($CDCl_3$) spectrum (Fig. 51) illustrated the carbon signals at chemical shifts (ppm) : 177.54 (carbonyl carbon) 164.86, 160.02, 157.10, 154.10, 127.20, 115.07, 114.06, 108.52, 102.17, 96.36, 77.92, 61.10 ($-CH_2OH$), 56.09 ($-OCH_3$), 30.06 ($-CH_3$), 27.76 (geminal dimethyl).

The mass spectrum (Fig.52) gave the important fraction ion peaks at m/e ( % rel intensity) : 302.00 (9.23), 301 (50.32), 287 (60.55), 284 (100.00), 273 (30.55), 258 (10.58), 243 (24.76), 217 (7.54) and 213 (5.81).

## 2.11 Saparation of Water Layer Extracts

The crude extract from water layer (25.0 g.) was analysed for inorganic elements, sugars and amino acids. The identification of this part was shown as follow :

### 2.11.1 The Inorganic Elemental Analysis

The water layer of the crude extract was analysed by the Energy Dispersive X-Ray Fluorescence Spectrometer. It displayed three major components of potassium (K) , chlorine (Cl) and sulfur (S) and the minor components were Aluminium (Al), Magnesium (Mg), Sodium (Na) , Phosphorus (P) , Calcium (Ca) , Iron (Fe) , Zinc (Zn) , Copper (Cu) , Manganese (Mn) , Nickel (Ni) and Bromine (Br). (Fig. 53)

### 2.11.2 The Carbohydrate Analysis

The crude extract from the water layer was decolorized by activated carbon and then analysed by High Performance Liquid Chromatography (HPLC). (LC-$NH_2$-Supelco column, mobile phase : 75% MeOH-$H_2O$, flow rate 2.0 ml/min, refractive index detector).

The HPLC analysis data (Fig. 54) indicated that this part contained a mixture of xylose, glucose, sucrose and maltose. The analysis data were presented in Table 2.18.

Table 2.18 The comparison data between standard sugars and the crude extract of the water layer

| Compound | Retention time (Min) |
|---|---|
| xylose | 2.89 |
| arabinose | 3.28 |
| fructose | 3.51 |
| glucose | 3.62 |
| sucrose | 4.83 |
| maltose | 5.66 |
| lactose | 6.24 |
| The water layer | 2.15 |
| | 2.57 |
| | 2.87 |
| | 3.65 |
| | 4.83 |
| | 5.72 |

2.11.3 **The Amino Acid Analysis**

The decolorized solution (From 2.11.2 ) was determined for amino acid by the Amino Acid Analyzer (Hitachi, Custom Ion Exchange Rasin column, Mobile phase : citrate buffer, flow rate 0.25 ml/min. injection temp. 40-70 °C ). The data revealed that this part consisted of aspartic acid, threonine, serine, proline, glycine, alanine, and valine (Fig. 55).