CHAPTER II

EXPERIMENT AND RESULTS

PLANT MATERIALS

The dried leaves of Bridelia ovata were obtained from Vechapong Drug Store at Kanchanaburi province in June of 1993. The identity of these plant has been compared against a voucher specimen no. 050203 at the herbarium of the faculty of pharmaceutical sciences, chulalongkorn university.

INSTRUMENTS AND EQUIPMENT

- Rotatory Evaporator

The Eyela rotatory evaporator was used to evaporate the large amounts of polar solvent such as methanol. Solvents which were easily evaporated such as hexane, chloroform and dichloromethane will be evaporated by simple distillation.

- Melting Point(m.p.)

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

- Mass Spectra(MS)

The mass spectra were obtained on a Trio Fisons Instruments.

- Fourier Transform-Infrared Spectrophotometer(FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Model 1760 X Fourier Transform-Infrared Spectrophotometer. Solid samples which were examined by this instrument were incorporated into potassium bromide to form a pellet. Liquid samples were dropped on a sodium chloride cell.

- Gas Liquid Chromatography(GLC)

The GLC analysis results were obtained from Shimadzu Gas-Liquid Chromatography Model GC-7AG.

- ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectrometer

The ^{1}H-NMR and ^{13}C-NMR spectra were obtained from a Bruker Model ACF 200 Spectrometer which operated at 200.13 MHz. for ^{1}H and 50.32 MHz.for ^{13}C nuclei. The chemical shifts were assigned as ppm. and were compared to the reference signal of tetramethylsilane(TMS).

CHEMICAL SUBSTANCES

Solvents:

All solvents such as hexane, dichloromethane, methanol, ethylacetate and acetone were purified by distillation before use. Solvents of analytical grade were used in recrystallization.

Other substances:

- Merck' s silica gel 60 Art.7734 1000(70-230 mesh ASTM) was used as adsorbents for column chromatography.

- TLC aluminium sheets, silica gel 60 F254 precoated sheets, 20x20 cm^2, 0.2 mm. thick were obtained from Merck.

SEPARATION TECHNIQUES

Column Chromatography

Equipment :

The glass column with 4.5 cm. in diameter and 150 cm. in length was used. Merck' s silica gel 60 Art.7734 1000(70-230) mesh ASTM was used as the adsorbent with the ratio of approximately of 1:10 by weight.

Preparation :

The stopcock of the column was closed and then the glass rod was used to push the cotton wool to the bottom of column. The solvent was allowed to

remain about half of the column. When the slurry of the silica gel was poured in the glass rod was pulled out and the stopcock was let opened to release the solvent pouring down slowly until remained about 5 cm. above the surface of silica gel. The crude extracts to be identified were brought to incorporate with silica gel until friableness and then brought into the column. The dried silica gel was sprinkled on the top of the column and then inserted paper on top of silica gel in the column prior to the addition of the eluent.

Thin-Layer Chromatography(TLC)

In this research, Merck' s TLC aluminium sheets, silica gel 60 F254 precoated sheets, 20x20 cm^2, 0.2 mm. thick were used.

Two lines were drawn on each plate, the first one was 1.0 cm. from the lower edge, another line is 8.0 cm. above and parallel to the first line. The solution of substances to be identified was applied as small spots on the lower line of the plate. After the solvent had evaporated, the plate was placed in a closed glass utensil which had the eluting solvent to a depth of lower than 1.0 cm. The eluting solvent moved up the plate immediately. When the solvent reached the upper line, the plate was removed. Allow the solvent to evaporate, the plate was detected with UV and I_2

DISTILLATION

Solvents were purified by distillation before use. Distillation used in this work are of two types.

1. Simple distillation: for solvents of lower boiling points such as hexane, chloroform and dichloromethane.

2. Vacuum distillation: for solvents which are polar and have higher boiling points such as methanol, water, and etc. This distillation allows the solvents to distill at lower temperature than their normal boiling points and thus prevents decomposition of the substances. The instrument used in this distillation is a " Rotatory Vacuum Evaporator".

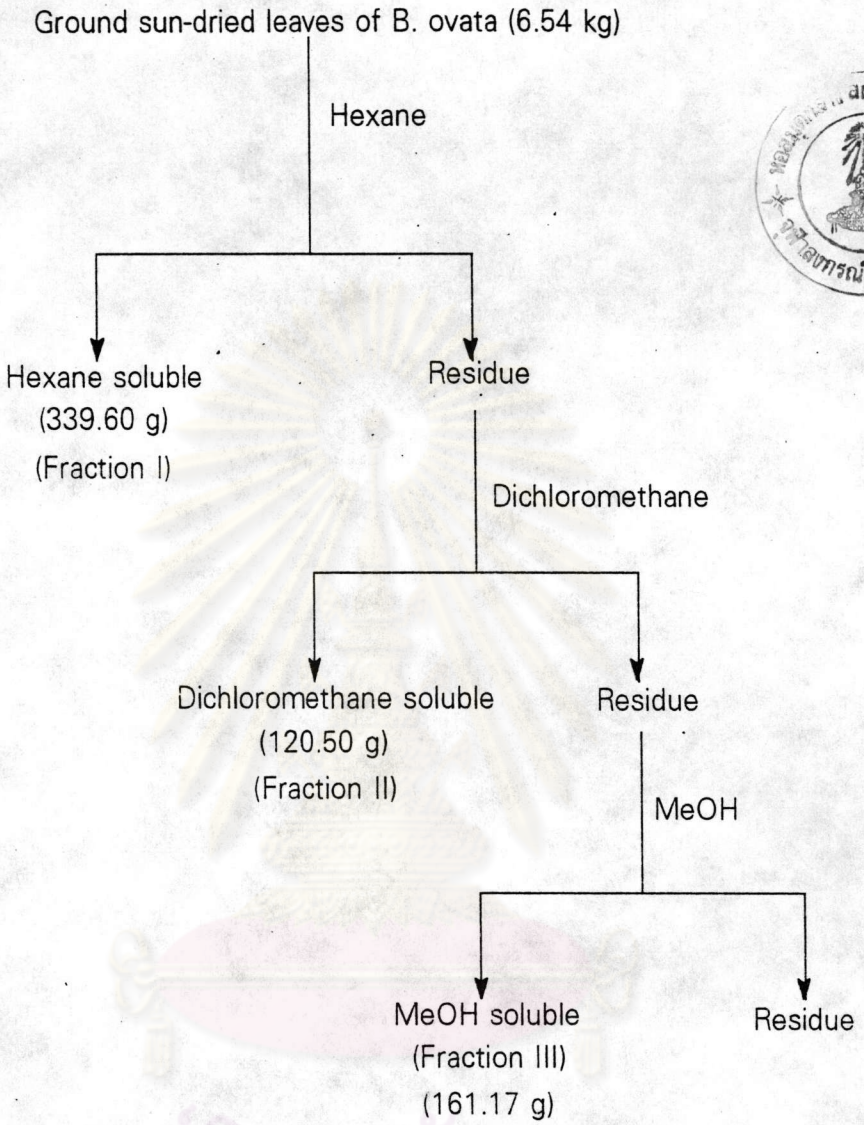
EXTRACTION

About 6,540 g of dried and ground Makaa leaves were extracted with hexane for 3 days. The solution was filtered and hexane was removed by simple distillation. This distilled hexane was used to extract the leaves again until the solution was colorless. The crude hexane extract of 339.60 g was obtained as a dark-green material(equivalent to 5.19% wt. by wt.of the leaves).

The residue left after hexane extraction was reextracted by dichloromethane until the solution was colorless and then the filtrated solution was evaporated. The crude dichloromethane extract of 120.50 g was obtained as a green oil(equivalent to 1.84% wt.by wt. of the leaves).

The residue after dichloromethane extraction was reextracted with methanol until the solution was colorless. The methanol extract was evaporated and a dark green oil of 161.17 g was obtained (equivalent to 2.46% wt.by wt. of the leaves).

The procedure of extraction is shown in **Scheme 1**



Scheme 1 The procedure of extraction

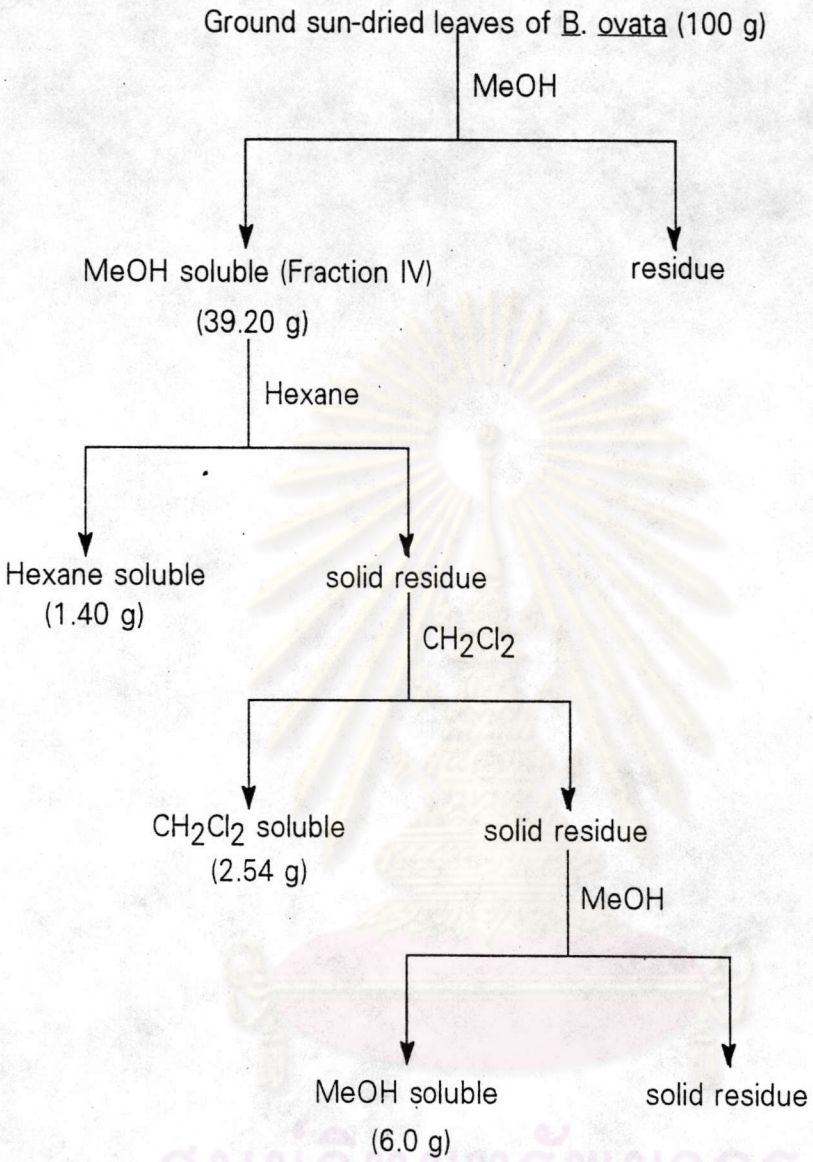
PRELIMINARY TESTS IN CHEMISTRY(20, 21)

About 100 g of the ground sun-dried leaves was extracted with methanol until the solution was colorless and the filtrated solution was evaporated to obtain a crude methanol extract of 39.20 g as a dark-green oil.

The crude methanol extract was reextracted by hexane until the solution was colorless. The hexane extract was vaporated and a dark-green oil of 1.40 g was obtained as a crude hexane extract. The crude methanol extract, after hexane extraction was reextracted by dichloromethane until the solution was colorless. The dichloromethane extract was evaporated to obtain a crude dichloromethane extract of 2.54 g as a green oil.

The crude extracts of hexane, dichloromethane and methanol including residue were brought for screening tests such as alkaloid, flavonoid, cardiac glycoside, saponin and cumarin tests.

The sample preparation for chemistry test is shown in **scheme 2**



Scheme 2 The sample preparation for chemistry tests

ALKALOID TEST

Preliminary alkaloid test: The crude methanol extract 20 cm^3 was evaporated by rotatory evaporator and then added 2 M. HCl about 15 cm^3. After the mixture was heated and stirred for 10 minutes, the residue was removed by filtering. The filtration was devided into six test tubes for testing with reagents namely Maeyer 's, Valser's, Wagner's, Dragendorff's, Kraut's and Marme's reagents, respectively. The color and quantity of precipitate were observed.

Confirmed alkaloid test: The residue solution from preliminary test was added NH_4OH and then was extracted with CH_2Cl_2 twice about 10 cm^3 each. Dichloromethane layer was evaporated and about 3 cm^3 of 2M HCl was added. The solution was boiled, stirred for 5 minutes and filtered. The filtrate was divided into six test tubes for six reagents as same as preliminary test. The water layer was brought to quaternary test.

Quaternary or Amine Oxide Base Test: Water layer from confirmed test was added 2M. HCl and the solution was divided into six test tubes for six reagents as same as preliminary test and confirmed test

In similar manner, the crude hexane, crude dichlromethane extract and the crude methanol residue were brought for preliminary, confirmed and quaternary test. The results of alkaloid test are shown in **Table 4**

Table 4 The results of alkaloid test

Reagent	Crude Hexane Extract		
	Preliminary Test	Confirmed Test	Quaternary Test
Mayer's	-	-	-
Valser's	+	-	-
Wagner's	+	-	-
Dragendorff's	+	-	-
Kraut's	-	-	-
Marme's	-	-	-

Reagent	Crude Dichloromethane Extract		
	Preliminary Test	Confirmed Test	Quaternary Test
Mayer's	+	-	+
Valser's	+ +	-	+
Wagner's	+ +	-	+
Dragendorff's	+ +	-	+
Kraut's	+	-	+
Marme's	+	-	+

Reagent	Crude Methanol Extract		
	Primary Test	Confirmed Test	Quaternary Test
Mayer's	+ +	+	+
Valser's	+ + +	+	+ +

Reagent	Crude Methanol Extract		
	Preliminary Test	Confirmed Test	Quaternary Test
Wagner's	+ + +	+	+ + +
Dragendorff's	+ + +	+	+ + +
Kraut's	+ +	+	+
Marme's	+ +	+	+

Reagent	Crude Methanol Residue		
	Preliminary Test	Confirmed Test	Quaternary Test
Mayer's	+	-	+
Valser's	+ +	+	+
Wagner's	+ +	+	+
Dragendorff's	+ +	+	+
Kraut's	+	-	+
Marme's	+	-	+

Note:

+ + + major precipitate

+ + minor precipitate

+ turbid

- no precipitate

CARDIAC GLYCOSIDE TEST

About 5 cm^3 of the crude methanol extract was added 10% of lead acetate solution 40 cm^3. After heating for 15 minutes, the solution was cooled and filtered. The filtrate was extracted with dichloromethane for three times, about 20 cm^3 each time. Dichloromethane layer was added anhydrous Na_2SO_4 in order to remove water and the solution was evaporated to 1/10 of the original volume. The solution was divided into three test tubes for three cardiac glycoside tests.

Liebermann- Burchard Reaction (steroid test): Dichloromethane solution in the first test tube was evaporated almost to dryness and 3 drops of acetic anhydride was added with shaking. One drop of conc. H_2SO_4 was poured along the side of the test tube. The color change was observed immediately from pink——> violet——> blue———>green.

Kedde's Reaction (α , β- unsaturated lactone test): Dichloromethane solution in the second test tube was evaporated almost to dryness and then about 10 cm^3 of Kedde's reagent and 2-3 drops of 1 M. NaOH were added. The positive test giving blue or violet / pink color was observed.

Keller - Kiliani Reaction (deoxy sugar test): Dichloromethane solution in the third test tube was added about 3 cm^3 of 5% $FeCl_3$. After shaking, conc. H_2SO_4 was poured along the side of the test tube. The positive test exhibiting brown ring at the junction between two layers and colour in the upper layer was observed.

All three reactions must be positive for Cardiac glycoside. The results of cardiac glycoside test are shown in **Table 5**

Table 5 The results of cardiac glycoside test of crude methanol extract

Reaction	Observation
Liebermann-Burchard reaction	red solution
Kedde's reaction	orange solution
Keller-Kiliani reaction	green solution in upper layer and brown ring at interlayer

FLAVONOID TEST

The crude methanol extract about 2.5 cm^3 was extracted by hexane for several times until solution was colorless. Solid residue(Scheme 2) was divided into 3 tubes. The first tube was blank control and the second, third tubes were brought for 2 reactions.

Cyanidin Test: The second tube was added conc. HCl about 0.5 cm^3 and magnesium about 1-2 pieces. Observe the color change. The positive test for flavone was orange-red color; flavonol was red—>dark red color; flavonone was dark red———>red-violet color but chalcone and aurone were of negative test. Observe the color of 1-octanol and water layer when 2 cm^3 of water and 1 cm^3 of 1-octanol were added and shaken violently. The positive test of free flavonoid is obtained in 1-octanol layer(upper layer) and the positive test of glycoside of flavonoid is obtained in water layer(lower layer).

Leucoanthocyanin Test: The third tube was added conc. HCl about 0.5 cm^3, warm the solution on water bath for 5 minutes. The positive test for flavonoid exhibits red-violet color. The results of flavonoid test were shown in **Table 6**

Table 6 The results of flavonoid test

Reaction	Observation
Cyanidin Test	violet color in 1-octanol layer
Leucoanthocyanin Test	red-violet solution

SAPONIN TEST

Foam Test: The ground dried-leaves of Makaa about 0.1 g. were brought into test tube and distilled water was added about 5 cm^3 . These was heat on water baht for 5 minutes and filtered. The filtrated was shaken violently for 1 minute and observe the foam for 20 minutes, dil. H_2SO_4 was added about 1 cm^3 and heated. Shake the mixture for 1 minute.

Color Test: The crude methanol extract about 5 cm^3 was added dil. H_2SO_4 10 cm^3 heated for 15 minutes, and then was extracted with dichloromethane about 15 cm^3. Dichloromethane layer was added anhydrous Na_2SO_4 and filtered. The filtrate was evaporated almost to dryness, acetic anhydride was added about 3 drops. About 1 drop of conc. H_2SO_4 was poured along the side of the test tube. The color change was observed within 1 hour. The positive test of steroid sapogenin was blue or blue-green color, the positive test of triterpenoid sapogenin was red, pink or violet color. The results of saponin test are shown in **Table 7**

Table 7 The results of saponin test

Reaction	Observation
Foam Test	no change
Color Test	red-violet color

COUMARIN TEST

The ground dried-leaves of Makaa were brought in small conical flask about 3 g. and small amout of distilled water was added. Filter paper which was wet by 1 M. NaOH was put to cover the mouth of flask. These was warm on the water baht for 20 minutes. The dried filter paper was brought under ultraviolet lamp for 10 minutes. Fluorescence on the filter paper was not shown. Therefore was negative.

ISOLATION TECHNIQUE

Separation of Fraction I

The crude hexane extract(Fraction I) was concentrated to a dark green liquid of 339.60 g. (equivalent to 5.19% wt. by wt.) (see Scheme 1). The technique used for separating 80 g. of the crude hexane extract into various fractions was column chromatography. Silica gel 60 G Art. 7734 was used as an adsorbent. Eluting solvent used for each fraction is about 800 cm^3. The solution in each fraction was evaporated to about 50 cm^3, they were transfered to small flask and concentrated on water bath to 10 cm^3 and checked the similarity by TLC plate. Those similar TLC patterns were combined together. The combined fractions of Fraction I are shown in **Table 8**.

Table 8 The results of separation of Fraction I by column chromatography

Eluent (% by volume)	Fraction No.	Remark	Weight (g)
100% Hexane	1-4	white plate	3.9431
100% Hexane	5-13	yellow oil	0.5705
100% Hexane - 5% CH_2Cl_2- Hexane	14-30	solid in yellow oil	5.4587
5-20% CH_2Cl_2- Hexane	31-53	solid in yellow oil	0.5150
20% CH_2Cl_2- Hexane	54-62	solid in orange oil	5.6315
20% CH_2Cl_2- Hexane	63-67	solid in yellow oil	1.5235
20-30% CH_2Cl_2- Hexane	68-87	yellow oil	0.6241
30% CH_2Cl_2- Hexane	88-93	solid in brown oil	5.3708
30-50% CH_2Cl_2- Hexane	94-101	solid in green-brown oil	5.7635
70% CH_2Cl_2- Hexane	102-117	brown-green oil	2.3874
85% CH_2Cl_2- Hexane	118-139	brown-green oil	4.9677
100% CH_2Cl_2 - 2% MeOH - CH_2Cl_2	140-156	solid in yellow-brown oil	3.5025
2-5% MeOH - CH_2Cl_2	157-173	brown oil	3.2584
10-30% MeOH - CH_2Cl_2	174-191	black brown oil	3.2729
50-70% MeOH - CH_2Cl_2	192-200	greenish oil	0.8127
70-100% MeOH	201-205	greenish oil	1.6376

Separation of fraction 54-62 (Fraction I)

When the solid in yellow oil from fraction 54-62 was checked by TLC technique, there were 3 spots (R_f = 0.82, 0.68 and 0.60; silica gel / dichloromethane). Most solid from fraction 54-62 dissolved in ethylacetate and hexane but the bright white solid was not dissolved. It was filtered and recrystallized by a mixture of dichloromethane and methanol. After recrystallization for several times, bright white amorphous as **Mixture I** was obtained about 0.57 g. (equivalent to 0.17% wt. by wt. of Fraction I). It gave 2 spots on TLC plate (R_f = 0.82 and 0.68; silica gel / dichloromethane). Thus **Mixture I** must be separated again using preparative TLC technique.

The solution which contained white solid dissolving in ethylacetate and hexane was evaporated and recrystallized by ethylacetate and hexane for several times to obtain 0.62 g of white amorphous solid as **BOV3**.

Separation of Fraction II

Crude dichloromethane extract 50 g.) was chromatographed on silica gel 60G Art.7734 (800 g.) using column chromatography technique. Hexane, a mixture of hexane and dichloromethane, dichloromethane, mixture of dichloromethane and methanol and methanol,respectively were used as eluents. The eluted solution was collected about 800 cm^3 each time and was evaporated to about 50 cm^3 by rotatory evaporator; then they were transferred to small flask and evaporated on water baht to 10 cm^3 and checked by TLC technique. The similar fractions were combined . The results of separation were shown in **Table 9**

Table 9 The results of separation of Fraction II by column chromatography

Eluent (% by volume)	Fraction No.	Remark	Weight (g)
100% Hexane	1-12	white plate	0.3051
100% Hexane	13-16	yellow oil	0.0186
100% Hexane - 20% CH_2Cl_2- Hexane	17-30	solid in yellow oil	3.5038
20% CH_2Cl_2- Hexane	31-35	solid in orange oil	1.3760
20% CH_2Cl_2- Hexane	36-40	solid in yellow oil	2.1880
30% CH_2Cl_2- Hexane	41-50	dark-greenish oil	8.5423
50% CH_2Cl_2- Hexane	51-55	greenish oil	2.3800
70-85% CH_2Cl_2- Hexane	56-80	solid in green oil	0.7087
100%CH_2Cl_2- 10%MeOH - CH_2Cl_2	81-111	solid in green oil	0.0632
30-50% MeOH - CH_2Cl_2	112-122	greenish oil	0.0201
70-100%MeOH - CH_2Cl_2	123-133	greenish oil	0.1967

Separation of fraction 31-35 (Fraction II)

As same as fraction 54-62(Fraction I), there are three substances in fraction 31-35. It was recrystallized by hexane and ethylacetate. The bright white solid which was not dissolved was filtered to obtain about 0.16 g (equivalent to 0.13% wt. by wt. of Fraction II). This bright white solid exhibited two spots by TLC as same as **Mixture I.** They were separated and recrystallized in the similar way to **Mixture I**. Substance which was dissolved in hexane and ethylacetate was **BOV3** about 0.20 g (equivalent to 0.17% wt. by wt. of Fraction II).

The bright white solid from this fraction were combined with **Mixture I** to obtain 0.73 g as **Mixture II** and must be separated by preparative TLC technique.

Separation of Mixture II by PTLC Technique

The bright white solid (**Mixture II**) about 0.75 g. was separated by preparative thin-layer chromatography (PTLC). The PTLC technique using silica gel 60G Art. 7729 as adsorbent on glass plate. Eluent for developing PTLC plate was the mixture of dichloromethane and hexane(1:1). After developing PTLC plate was detected by I_2, it appeared two bands. Each band of substances was extracted by dichloromethane and the solution was evaporated. Repeat PTLC technique until pure substance was obtained as

BOV 4 about 0.27 g., R_f = 0.82 and **BOV5** about 0.30 g., R_f = 0.68

Separation of Fraction III

Crude methanol residue (80 g) was chromatographed on silica gel 60G Art. 7734(600 g) using column chromatography technique. Eluents used in this column are a mixture of hexane-dichloromethane, dichloromethane, a mixture of dichloromethane-methanol and methanol respectively. The eluate collected was approximately 800 cm^3.,they were evaporated to about 50 cm^3 by rotatory evaporator. Transfer to small flask and evaporate to 10 cm^3 on water bath, combined the similar fractions by TLC plates. The combined fractions of Fraction III were shown in **Table 10**

Table 10 The results of the separation of Fraction III by column chromatography

Eluent (% by volume)	Fraction No.	Remark	Weight (g)
50% CH_2Cl_2- Hexane	1-2	brown oil	0.1150
50 - 60% CH_2Cl_2- Hexane	3-16	solid in yellow oil	0.2010
60 - 70% CH_2Cl_2- Hexane	17-23	needle participate in yellow oil	0.7040
70% CH_2Cl_2- Hexane	24-25	violet oil	0.0510
70 - 80% CH_2Cl_2- Hexane	26-31	jelly	0.1490
80%CH_2Cl_2-Hexane - 100%CH_2Cl_2	32-44	brown-green oil	0.1060
1 - 10% MeOH- CH_2Cl_2	45-70	black solid in dark greenish oil	0.6850
10 - 30% MeOH- CH_2Cl_2	71-86	concentrated black oil	0.8410
30 - 70% MeOH- CH_2Cl_2	87-109	brown oil	0.5120
70%MeOH-CH_2Cl_2-100% MeOH	110-123	brown oil	0.3745

Separation of fraction 45-70 (Fraction III)

The black solid in dark greenish oil from fraction 45-70 about 0.69 g. (equivalent to 0.43% wt. by wt. of Fraction III) appeared two spots on TLC, R_f =

0.88 and 0.90(silica gel/ methanol:dichloromethane = 1:10). The mixture was separated by two methods.

The first method; the mixture was separated by preparative HPLC Technique. Condition using in separation was flowrate 2.0 ml/min., sensitivity 0.01 min. The mobile phase was a mixture of dichloromethane-methanol (9:1). The chromatogram of separation is shown in Fig 1.

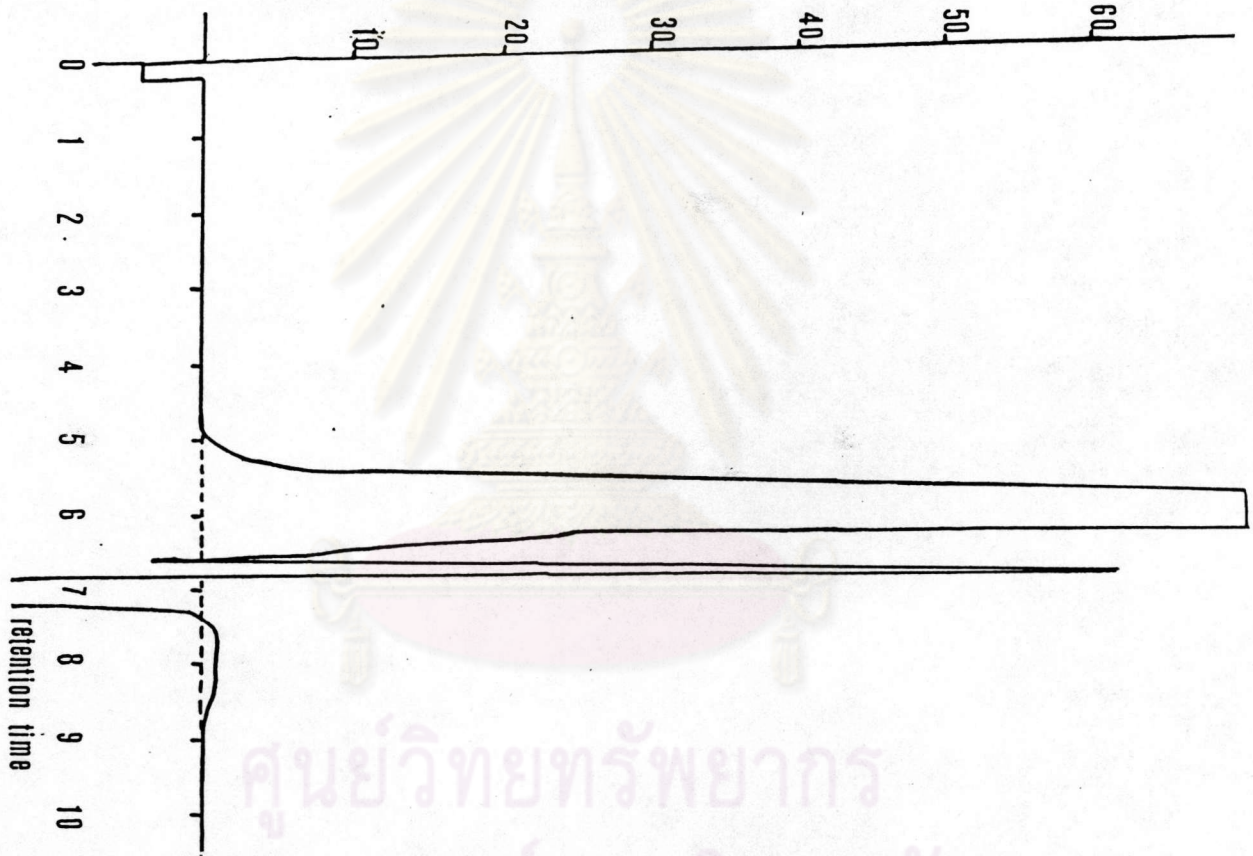


Fig. 1 HPLC Chromatogram of mixture in fraction 45-70

The second method; the mixture was separated by preparative TLC technique(PTLC), using silica gel 60G Art. 7729 as an adsorbent on glass plate. Eluent for developing PTLC plate was a mixture of dichloromethane-methanol (97 : 3). After developing, PTLC plate was detected by UV or I_2, there appeared two bands. Each band of substances was poured in small sintered glass connecting

with suction flask. Dichloromethane and methanol were used for extraction each band by suction(Fig. 2).

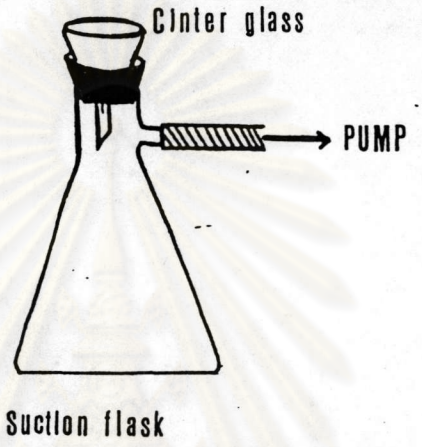


Fig. 2 The apparatus for extraction of the substances from PTLC plate

The solution obtained was evaporated and PTLC technique was repeated until obtaining pure substance.

From two methods, the mixture was separated to two substances as BOV7,

R_f = 0.88 about 0.020 g. and BOV8, R_f = 0.90 about 0.025 g.

Separation of Fraction IV

The crude methanol extract(Fraction IV) was concentrated to give a dark green viscous liquid of 39.20 g(equivalent to 39.20% wt. by wt. of the leaves, see Scheme 2). The technique used for separating 30 g of the crude methanol extract into fractions was column chromatography using about 400g of aluminium oxide 90

Art.1097 as adsorbent and hexane, a mixture of hexane-dichloromethane, dichloromethane, a mixture of dichloromethane-methanol and methanol respectively as eluents. About 500 cm^3 of the eluate was collected and evaporated to about 50 cm^3 by rotatory evaporator; then they were evaporated to 10 cm^3 on water baht and checked by TLC. The similar fractions were combined together. The results of combined fractions were shown in **Table 11.**

Table 11 The results of separation of Fraction IV by column chromatography

Eluent (% by volume)	Fraction No.	Remark	Weight (g)
100% Hexane	1-6	orange oil	2.0005
10 - 20% CH_2Cl_2- Hexane	7-20	needle precipitate in black oil	5.2277
20 - 70% CH_2Cl_2- Hexane	21-32	solid in yellow-brown oil	0.5912
70%CH_2Cl_2-Hexane - 100% CH_2Cl_2	33-40	solid in yellow oil	0.1946
100%CH_2Cl_2 - 5% MeOH- CH_2Cl_2	41-48	solid in yellow oil	2.4310
5 - 10% MeOH- CH_2Cl_2	49-53	black oil	0.5432
30 - 50% MeOH- CH_2Cl_2	54-59	green oil	0.2376
70%MeOH-CH_2Cl_2 - 100% MeOH	60-78	green oil	1.6440

PURIFICATION AND PROPERTIES OF SUBSTANCES FROM "MAKAA"

Purification and properties of BOV1

The solid in yellow oil was obtained from the combination of fraction 1-4(see **Table 8**, Fraction I) and the combination of fraction 1-12(see **Table 9**, Fraction II). It was purified by recrystallization from the mixture of hexane and ethylacetate for several times to obtain bright white plate as **BOV1**, 1.12 g (equivalent to 0.88g; 0.26% wt. by wt. of Fraction I and 0.24 g; 0.20% wt. by wt. of Fraction II), m.p. 61-63 °C. This substance dissolved in hexane; dissolved slightly in dichloromethane, did not dissolve in ethylacetate, methanol, ethanol and acetone. Result of TLC technique exhibited R_f 0.81 (silica gel/dichloromethane:hexane = 3:17)

FT-IR spectrum, ν_{max} (cm^{-1}) (Fig. 3): 2957, 2919, 2849(C-H stretch.), 1473, 1464, 1379(C-H bending) and 730, 729(C-H rock.)

GLC chromatogram (Fig. 5): (condition; column OV-1, column temp. 260 °C, injection temp. 290 °C, carrier gas N_2 50 ml/min. Chromatogram showed 8 peaks at retention time: 4.53, 5.62, 7.13, 8.81. 11.53, 14.36, 19.06 and 24.06 mins., respectively.

Purification and properties of BOV2

The solid in yellow oil was obtained from fraction 14-30(see **Table 8**, Fraction I) and fraction 17-30(see **Table 9**, Fraction II). After recrystallization from a mixture of hexane and ethylacetate for several times, white amorphous solid, 3.32 g.(equivalent to 1.90; 0.56% wt. by wt. of Fraction I and 1.42 g; 1.18% wt. by wt.

of Fraction II), m.p. 77-78 °C, was obtained as **BOV2**. This substance was soluble in dichloromethane, slightly soluble in hexane and ethylacetate and not soluble in methanol, acetone and ethanol.

FT-IR spectrum, ν_{max}(cm^{-1}) (Fig. 7): 2919,2850(C-H stretch.), 1737(-C=O stretch.), 1466(C-H bend.), 1173(C-O stretch.) and 723(C-H rock.)

^{1}H-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 8): 0.88-1.60, 2.29(t), 4.05(t)

Purification and properties of **BOV3**

The solid in yellow oil was obtained from fraction 63-67(see **Table 8**, Fraction I). It was purified with the mixture of ethylacetate and hexane for several times to obtain white amorphous about 0.16 g. as same as white amorphous which obtained from fraction 54-62 and fraction 31-35. After combining white amorphous solid was obtained, about 0.98 g.(equivalent to 0.78 g, 0.23% wt. by wt. of Fraction I; 0.2 g, 0.17% wt. by wt. of Fraction II). They were recrystallized again by hexane and ethylacetate. This substance was **BOV3**, m.p: 82-84 °C. This substance dissolved in chloroform, dichloromethane, dissolved slightly in hexane - ethylacetate and did not dissolve in methanol and ethanol. Result of TLC technique exhibited R_f 0.60(silica gel/ dichloromethane)

FT-IR spectrum, ν_{max}(cm^{-1}) (Fig. 9): 3672-3201(O-H stretch.), 2919, 2849(C-H stretch.), 1472, 1464(C-H bend.), 1123(C-O stretch.), 730, 720(C-H rock.)

GLC chromatogram (Fig. 11) (condition: column OV-1, column temp. 250 °C, injection temp. 290 °C, carrier gas N_2 50 ml/min.). Chromatogram showed 3 peaks at retention time : 5.36, 5.69 and 31.83 mins., respectively.

Purification and properties of **BOV4**

BOV4 was obtained from preparative TLC technique of **Mixture II**(from fraction 54-62 of Fraction I; fraction 31-35 of Fraction II) and recrystallized by methanol - dichloromethane for several times. The results from TLC technique exhibited R_f 0.82(silica gel/ dichloromethane). **BOV4** was white needle about 0.21 g (equivalent to 28% wt. by wt. of Mixture II), m.p. 257-260 °C. This substance was soluble in dichloromethane and chloroform, slightly soluble in ethylacetate and hexane and not soluble in methanol.

FT-IR spectrum, ν_{max}(cm^{-1})(Fig. 13): 2900, 2850(C-H stretch.), 1710(-C=Ostretch.), 1460(C-H bend.) and 1385(C-H sym. bend.)

Mass spectrum (% relative intensity) (Fig. 16): 426(M^+)(30), 411(7), 341(4), 302(20), 274(18), 273(35), 247(10), 218(29), 205(39), 191(21), 163(31)

^{1}H-NMR ($CDCl_3$) δ(ppm) (500 Mhz) (Fig. 14) : 0.72, 0.86, 0.87, 0.95, 1.01, 1.02, 1.05, 1.18(s, 24 protons), 0.93, 1.26-1.96(m, 23 protons),2.26-2.38(m, 3 protons)

^{13}C-NMR ($CDCl_3$) δ(ppm) (Mhz) (Fig. 15) : 6.83, 14.50, 18.00, 18.10, 18.50, 20.10, 22.10, 28.10, 30.00, 30.50, 31.80, 32.10, 32.39, 32.78, 35.00, 35.28, 35.58, 36.10, 37.40, 38.20, 39.20, 39.60, 41.10, 41.50, 42.10, 42.80, 53.00, 58.10, 59.50 and 207.50

Purification and properties of **BOV5**

BOV5 about 0.26 g (equivalent to 34.67 % wt. by wt. of **Mixture II**), m.p. 279-280 °C was obtained as the white plate from preparative TLC and HPLC of

Mixture II(from fraction 54-62 of Fraction I; fraction 31-35 of Fraction II) and recrystallized by mixture of methanol and dichloromethane for several times. The results of TLC technique exhibited R_f of 0.68 (silica gel/dichloromethane). This substance dissolved in dichloromethane and chloroform, dissolved slightly in ethylacetate, hexane and methanol.

FT-IR spectrum, $\nu_{max}(cm^{-1})$ (Fig. 23) : 3700-3400(O-H stretch.), 2900, 2850 (C-H stretch.), 1440(C-H bend.), 1380(C-H sym. bend.) and 1040(C-O stretch. and O-H bend.)

Mass spectrum (m/e) (% relative intensity) (Fig. 26): 428(M^+)(55), 413(56), 304(3), 275(25), 273(8), 249(10), 233(11), 218(32), 207(23), 205(29), 195(3), 193(3), 165(59)

^{1}H-NMR ($CDCl_3$) δ(ppm) (500 MHz) (Fig. 24): 0.86, 0.94, 0.95, 0.99, 1.00, 1.01, 1.51, 1.17(s, 24 protons), 0.89, 0.91, 0.97, 1.26-1.91(m, 27 protons) 3.74(s, 1 proton)

^{13}C-NMR ($CDCl_3$) δ(ppm) (500 MHz)(Fig. 25) : 11.60, 15.82, 16.40, 17.59, 18.26, 18.60, 20.10, 28.20, 30.02, 30.68, 31.80, 32.10, 32.40, 32.90, 35.01, 35.15, 35.20, 35.38, 35.60, 36.10, 37.90, 38.40, 39.30, 39.70, 41.80, 42.91, 49.25, 53.25, 61.40 and 72.20

Purification and properties of **BOV6**

The solid in brown oil obtained from fraction 88-93(see **Table 8**, Fraction I) and fraction 17-23(see **Table 10**, Fraction III) was recrystallized by a mixture of dichloromethane and methanol for several times to obtain white needle about 2.79 g.(equivalent to 1.89 g, 0.56% wt. by wt. of Fraction I; 0.65 g, 0.40 % wt. by wt.

of Fraction III; 0.25 g, (0.64 % wt. by wt. of Fraction IV), m.p. 160-162 °C as **BOV6**. The result from TLC technique exhibited R_f 0.46 (silica gel/dichloromethane). It was soluble in dichloromethane and chloroform, slightly soluble in hexane, ethylacetate and methanol.

FT-IR spectrum, $\nu_{max}(cm^{-1})$ (Fig. 33): 3712-3146(O-H stretch.), 2937, 2866(C-H stretch.), 1641(C=C stretch.), 1460(C-H bend.), 1381, 1370(C-H sym. bend.), 1157(C-O stretch and O-H bend.), 970(C-H wagging), 800(C-H wagging)

GLC chromatogram (Fig. 35) (condition): column OV-1, column temp. 260 °C, injection temp. 290 °C, carrier gas N_2 45 ml/min.), range 10^2, chromatogram showed 1 peak at retention time 19.42 min.

Mass spectrum (m/e) (% relative intensity) (Fig. 36) : 412(M^+)(10), 394(2), 369(2), 351(4), 300(9), 271(18), 255(19), 55(100)

^{1}H-NMR ($CDCl_3$) δ(ppm) (Fig. 37) : 0.69-2.28(m), 3.52(s), 5.09(t), 5.36(d)

^{13}C-NMR ($CDCl_3$) δ(ppm) (Fig. 38 : 12.04, 12.24, 18.98, 19.39, 21.08(two signals), 21.21, 24.36, 25.40, 28.91, 31.65, 31.89(three signals), 36.51, 37.25, 39.68, 40.49., 42.29(two signals), 50.15, 51.23, 55.95, 56.86, 71.80, 121.71, 129.27, 138.31, 140.74

DEPT-90 ^{13}C-NMR($CDCl_3$) δ(ppm) (Fig. 39) :

CH signals 11 peaks : 31.89(two signals), 40.49, 50.15, 51.23, 55.95, 56.86, 71.80, 121.71, 129.27 and 138.31

DEPT-135 ^{13}C-NMR($CDCl_3$) δ(ppm) (Fig. 40) :

CH_3, CH signals (up phase) 17 peaks : 12.04, 12.24, 18.98, 19.39, 21.08, 21.21, 31.89(two signals), 40.49, 50.15, 51.23, 55.95, 56.86, 71.80, 121.71, 129.27 and 138.31

CH_2 signals (down phase) 9 peaks : 21.08, 24.36, 25.40, 28.91, 31.65, 31.89, 37.25, 39.68 and 42.29

Purification and properties of **BOV7**

BOV7 was obtained from preparative TLC and HPLC technique of fraction 45-70 see **Table 10**, Fraction III). It was purified by recrystallization from the mixture of dichloromethane and hexane for several times to obtain the bright violet amorphous about 0.020 g(equivalent to 0.01% wt. by wt. of Fraction III), m.p. 205-207 °C. This substance dissolved in dichloromethane, ethylacetate and methanol, dissolved slightly in hexane. Result of TLC technique exhibited R_f = 0.88 (silica gel/methanol :dichloromethane = 1 : 9). This substance absorbed ultraviolet light 254 nm.

Mass spectrum (% relative intensity) (Fig. 41) : 490(1), 462(1), 450(1.5), 436 (1.5), 422(1.5), 341(1), 292(1), 278(2), 266(4), 252(5), 237(8), 233(2), 137(1), 122(42), 108(99), 94(67)

^{1}H-NMR ($CDCl_3$) δ(ppm) (Fig. 42): 1.21, 1.55-1.80, 3.10-3.90, 4.15, 5.29, 6.10-6.30, 7.70-8.08, 9.25, 9.40, 9.55

^{13}C-NMR($CDCl_3$) δ(ppm) (Fig. 43): 189.50 , 173.10, 172.05, 169.90, 161.10, 158.50, 155.99, 151.50, 149.90, 145.00, 143.10, 138.00, 136.50, 136.00, 135.90, 131.50, 129.00, 123.00, 122.00, 104.60, 97.90, 93.00, 92.90, 65.00, 53.80, 53.50, 52.10, 50.10, 31.00, 29.50, 23.50, 19.10, 18.50, 17.50, 13.10 and 11.00

DEPT-90 ^{13}C-NMR($CDCl_3$) δ(ppm) (Fig. 44):

CH signals 9 peaks : 129.00, 104.60, 97.90, 93.00, 92.90, 53.80, 53.50, 52.10 and 50.10

DEPT-135 ^{13}C-NMR($CDCl_3$) δ(ppm) (Fig. 45)

CH_3, CH signals (up phase) 13 peaks: 129.00, 104.60, 97.90, 93.00, 92.90, 53.80, 53.50, 52.10 ,50.10, 23.50, 17.50, 13.10 and 11.00

CH_2 signals (down phase) 7 peaks: 123.00, 122.00, 65.00, 31.00, 29.50, 19.10 and 18.50

Purification and properties of **BOV8**

BOV8 obtained from preparative TLC and HPLC technique of fraction 45-70 (see **Table 10**, Fraction III) was recrystallized by the mixture of dichloromethane and hexane for several times, the black amorphous solid was obtained about 0.025 g(equivalent to 0.02% wt. by wt. of Fraction III), m.p. 194-196 °C. This substance dissolved in dichloromethane, methanol and ethylacetate, dissolved slightly in hexane. The result of TLC technique exhibited R_f 0.90 (silica gel/methanol : dichloromethane = 1:9). This substance absorbed ultraviolet light.

FT-IR spectrum, ν_{max}(cm^{-1}) (Fig. 46): 3600-3080(O-H stretch.), 2990, 2950(C-H bend.), 1725, 1700(C=O stretch.), 1675, 1600(C=C stretch.), 1450(C-H bend.), 1390(O-H bend.), 1210(C-O stretch.)

Mass spectrum (% relative intensity) (Fig. 47): 292(2), 278(3), 263(4), 252(7), 237(8), 223(9), 135(11), 122(39), 108(100), 94(8), 57(27)

^{1}H-NMR($CDCl_3$) δ(ppm) (Fig. 48): 1.60-1.90, 2.10-2.70, 3.15, 3.35, 3.55-3.65, 3.85, 6.10-6.30, 7.70-8.00, 9.30, 9.45

^{13}C-NMR($CDCl_3$) δ(ppm) (Fig. 49): 189.50, 187.50, 173.10, 172.05, 169.90, 161.10, 158.50, 156.99, 151.50, 149.90,145.00, 143.10, 138.00, 136.50, 136.00, 135.90, 131.50, 129.00, 128.00. 123.00, 122.00, 106.50, 104.60, 101.50, 97.90,

93.00, 92.90, 65.00, 53.80, 53.50, 52.10, 50.10, 31.00, 29.50, 23.50, 19.10, 18.50, 17.50, 13.10 and 11.00

DEPT-90 ^{13}C-NMR($CDCl_3$) δ(ppm) (Fig. 50):

CH signals 14 peaks: 187.50, 129.00, 128.00, 106.50, 104.60, 101.50, 97.90, 93.90, 92.90, 65.00, 53.80, 53.50, 52.10 and 50.10

DEPT-135 ^{13}C-NMR($CDCl_3$) δ(ppm) (Fig. 51):

CH_3 and CH signals(up phase) 18 peaks: 187.50, 129.00, 128.00, 106.50, 104.60, 101.50, 97.90, 93.90, 92.90, 65.00, 53.80, 53.50, 52.10, 23.50, 17.50, 13.10 and 11.00

CH_2 signals(down phase) 6 peaks: 123.00, 122.00, 31.00, 29.50, 19.10 and 18.50