CHAPTER II

EXPERIMENTALS

PLANT MATERIALS

The dried stems of Piper ribesoides Wall. were obtained from Kanchanaburi province in January of 1993 and identified by the herbarium of the faculty of botanical 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 and chloroform 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.

- Infrared Spectrophotometer(IR)

The IR spectra were recorded on a Perkin-Elmer Model 781 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.

- ^{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, chloroform, n-butanol, methanol, ethyl acetate and acetone were purified by distillation before use. Solvents of analytical grade were used in recrystallization.

Other substances:

- Merck' s silica gel 60 Art.7729 1000(70-230 mesh ASTM) was used as adsorbents for quick column chromatography.

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

SEPARATION TECHNIQUES

Quick Column Chromatography

This method is especially useful for separating large quantitie of mixture compounds which have been obtained form 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 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 solvents changed form n-hexane to a mixture of chloroform-hexane, chloroform and mixture of chloroform-methanol, respectively.

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 acetone.

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 20 kg of dried and ground Ta khaan phlu stem was extracted with methanol in soxhlet until the solution was colorless. The solution was filtered and methanol was removed by vacuum distillation. About 700 g. of crude methanol extract was obtained as a black-brown material(equivalent to 3.5% wt. by wt.of the stem).

The crude methanol extract was reextracted by hexane chloroform and n-butanol respectively until the solution was colorless and then the filtrated solution was evaporated. About 250 g. of crude hexane extract was obtained as a black-brown oil(equivalent to 1.25% wt.by wt. of the stem). About 300 g. of crude chloroform extract was obtained as a black-brown oil(equivalent to 1.5% wt. by wt. of the stem). About 100 g. of crude n-butanol extract was obtained as a black-brown oil(equivalent to 0.5% wt. by wt. of the stem).

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

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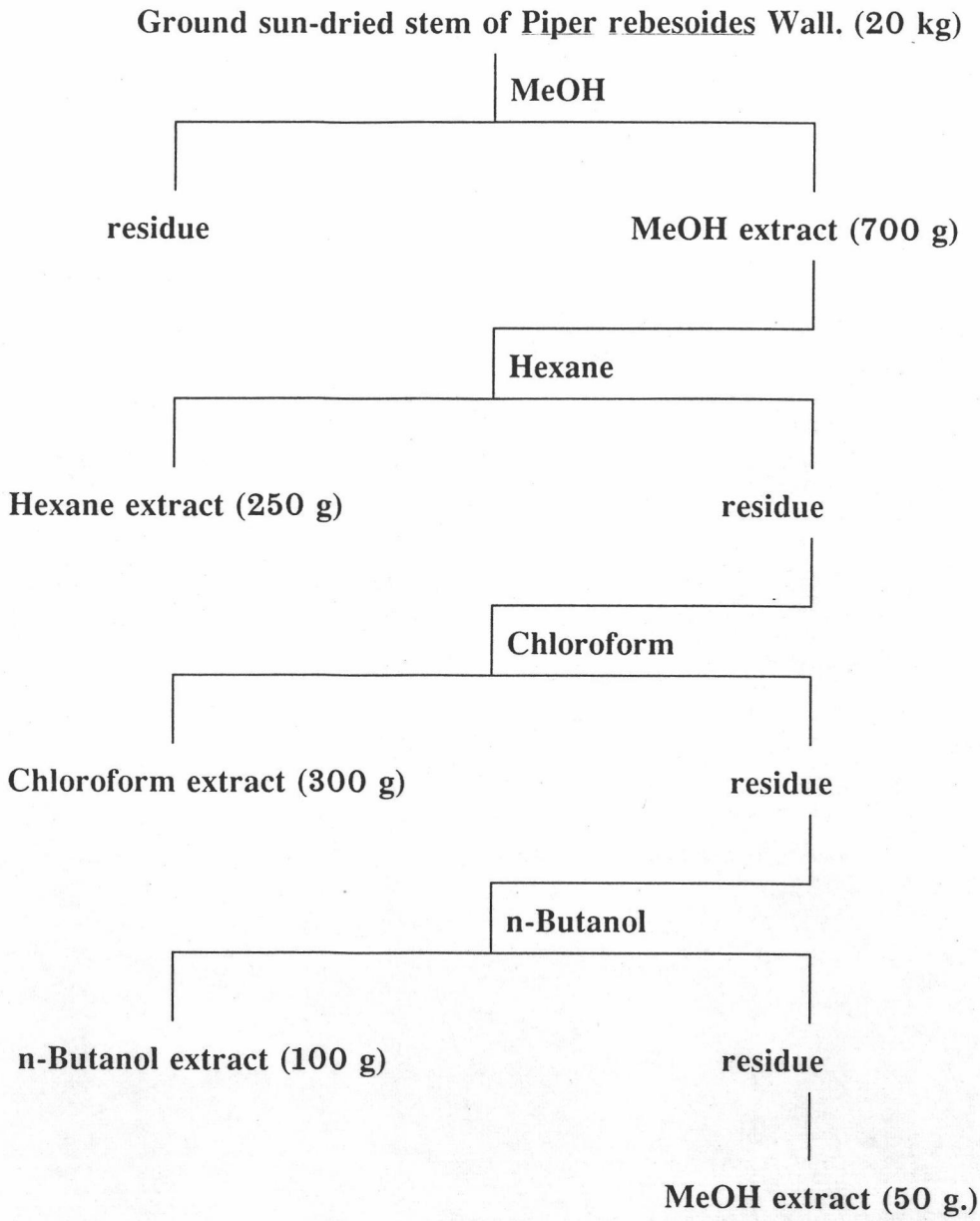
Scheme 1 The procedure of extraction



ISOLATION TECHNIQUE

Separation of crude hexane extract

The crude hexane extract was concentrated to a black-brown oil of 250 g. (equivalent to 1.25% wt. by wt.) (see Scheme 1). The technique used for separating

80 g. of the crude hexane extract into various fractions was quick column chromatography. Silica gel 60 G Art. 7729 was used as an adsorbent. Eluting solvent used for each fraction was about 100 cm^3. The solution in each fraction was evaporated to about 30 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 results of separation were shown in **Table 2**.

Table 2 The results of separation of crude hexane extract.

Eluent (% by volume)	Fraction No.	Remark
100% Hexane	1-15	orange oil
25% Chloroform - Hexane	16-26	white needle in orange oil
	27-35	orange oil
	36-45	white solid in orange oil
50% Chloroform - Hexane	46-54	brown oil
	55-65	yellow needle in brown oil
100% Chloroform	66-80	black-brown oil
5% MeOH - Chloroform	81-90	yellow needle in black-brown oil
10% MeOH - Chloroform	91-110	black-brown oil
25% MeOH - Chloroform	111-130	black-brown oil

Separation of crude chloroform extract

Crude chloroform extract (80 g) was chromatographed on silica gel 60G Art.7729 using quick column chromatography technique. Hexane, a mixture of hexane and chloroform, chloroform, mixture of chloroform and methanol and methanol, respectively were used as eluents. The eluted solution was collected about 100 cm^3 and evaporated to about 30 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 3**.

Table 3 The results of separation of crude chloroform extract

Eluent (% by volume)	Fraction No.	Remark
100% Hexane	1-20	yellow oil
25% Chloroform - Hexane	21-30	yellow oil
	31-40	white needle in yellow oil
	41-45	yellow oil
50% Chloroform - Hexane	46-50	yellow oil
	51-60	white solid in yellow oil
	61-69	yellow-brown oil
100% Chloroform	70-80	white solid in brown oil
	81-85	brown oil

Table 3 continue

5% MeOH - Chloroform	86-90	black-brown oil
	91-102	white solid in black-brown oil
	103-115	black-brown oil
	116-162	brown solid in black-brown oil
10% MeOH - Chloroform	163-172	black-brown oil
	173-185	black-brown oil
25% MeOH - Chloroform	186-197	black oil
	198-211	black oil

Separation of crude n-butanol extract

Crude n-butanol extract (80 g) was chromatographed on silica gel 60G Art. 7729 using q7uick column chromatography technique. Eluents used in this column are hexane, a mixture of hexane-chloroform, chloroform, a mixture of chloroform-methanol and methanol respectively. The eluate collected was approximately 100 cm^3.,they were evaporated to about 30 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 results of separation were shown in **Table 4**.

Table 4 The results of the separation of crude n-butanol extract

Eluent (% by volume)	Fraction No.	Remark
100% Hexane	1-30	yellow oil
25% Chloroform - Hexane	31-50	yellow oil
50% Chloroform - Hexane	51-70	yellow oil
100% Chloroform	71-90	brown oil
5% MeOH - Chloroform	91-99	brown oil
	100-106	black-brown oil
10% MeOH - Chloroform	107-210	black-brown oil
	211-222	black oil
25% MeOH - Chloroform	211-225	black oill

PURIFICATION AND PROPERTIES OF SUBSTANCES FROM *Piper ribesoides* Wall.

Purification and properties of **A**

The yellow needle in brown oil was obtained from the combination of fraction 55-65.(see **Table 2**, crude hexane extract) It was purified by recrystallization from the mixture of hexane and ethyl acetate for several times to obtain yellow needle as **A**, 0.030 g (equivalent to 0.037% wt. by wt. of crude hexane extract), m.p. 147-149 ^{o}C. This substance was soluble in hexane and chloroform and insoluble in ethyl acetate, methanol, ethanol and acetone. Result of TLC technique exhibited R_f 0.53 (silica gel/chloroform:hexane = 1:1)

UV absorption spectrum, λ_{max} nm (Fig. 1): 342

IR spectrum, ν_{max} (cm^{-1}) (Fig. 2): 3050, 2950, 1715, 1630, 1620, 1515, 1505, 1445, 1280, 1250

Mass spectrum, m/e (Fig. 3): 232(M^+)

^{1}H-NMR spectrum ($CDCL_3$) δ_{ppm} (Fig. 4): 3.5(3H, s), 5.75(1H, d), 5.8(2H, s), 6.5(1H, dd), 6.6(1H, d), 6.7(1H, dd), 6.8(1H, d), 6.6(1H, d)

^{13}C-NMR spectrum ($CDCL_3$) δ_{ppm} (Fig. 5) :50.5, 100.5, 105, 108, 119.5, 122.5, 124, 129.5, 139.5, 144, 147.5, 148, 166

^{13}C-NMR DEPT-135 ($CDCL_3$) δ_{ppm} (Fig. 6) :

CH, CH_3 signals(up phase) 8 peaks: 50.5, 105, 108, 119.5, 122.5, 124, 139.5, 144

CH signal(down phase): 100.5

^{13}C-NMR DEPT-90 ($CDCl_3$) δ_{ppm} (Fig. 7) :

CH signals 7 peaks: 105, 108, 119.5, 122.5, 124, 139.5, 144

Purification and properties of **B**

The white solid in orange oil was obtained from fraction 36-45(see **Table 2**, crude hexane extract). After recrystallization from a mixture of hexane and ethyl acetate for several times, white amorphous solid, 1.20 g.(equivalent to 4.5 g;1.50 % wt. by wt. of crude hexane extract) m.p. 102-105 ^{o}C, was obtained as **B**. This substance was soluble in hexane and chloroform, insoluble in ethyl acetate, methanol, acetone and ethanol. Result of TLC technique exhibited Rf 0.58 (silica gel / chloroform)

UV absorption spectrum , λ_{max} nm (Fig. 10): 268

IR spectrum, $\nu_{max(}cm^{-1}$) (Fig. 11): 3450, 3020, 2950, 2880, 1625, 1610, 1470 1460, 1300, 1280, 1240, 1225, 980

Mass spectrum, m/e (Fig. 12): 324(M^+)

^{1}H-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 13): 1.9(3H,d), 2.4(3H,s), 3.95(3H,s), 5.8(1H,s) 6.25(1H,qt), 6.5(1H,d), 6.8(1H,s), 6.95(1H,dd), 7.05(1H,s), 7.27(1H,d), 7.35(1H,dd)

^{13}C-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 14): 10.0, 19.0, 56, 56.2, 104.5 109, 109.5, 110.5, 114.0, 121, 123.5, 124, 131.5, 133, 133.5, 142.0, 144.5, 151.5, 146, 147

^{13}C-NMR DEPT-135 ($CDCl_3$) δ(ppm) (Fig. 15):

CH, CH_3 signals(up phase) 11peaks: 10, 19, 56, 56.2, 104.5, 114, 109, 109.5, 121, 124, 131.5

CH_2 signal(down phase): not show

^{13}C-NMR DEPT-90 ($CDCl_3$) δ(ppm) (Fig. 16):

CH signals 7 peaks : 104.5, 114, 109, 109.5, 121, 124, 131.5

Purification and properties of **C**

The white needle in orange oil was obtained from fraction 16-26(see **Table 2**, crude hexane extract). It was purified from the mixture of ethyl acetate and hexane for several times to obtain white needle about 1.20 g. (equivalent to 4.50 g, 1.50 % wt. by wt. of crude hexane extract). This substance was **C**, m.p. 146-148 ^{o}C. This substance was soluble in hexane and chloroform, insoluble in ethyl acetate, methanol and ethanol. Result of TLC technique exhibited R_f 0.60(silica gel/ chloroform)

UV absorption spectrum, λ_{max}nm (Fig. 19): 268

IR spectrum, ν_{max}(cm^{-1}) (Fig. 20): 3050, 2995-2900, 1620, 1515, 1490, 1460, 1270, 1240

Mass spectrum, m/e (Fig. 21): 322(M^+)

^{1}H-NMR spectrum ($CDCL_3$) δ(ppm) (Fig. 22): 1.9(3H,d), 2.4(3H,s), 4.0(3H,s), 5,95(2H,s), 6.2(2H,qd), 6.5(1H,dd), 6.8(1H,s), 6.85(1H,dd), 7.0(1H,s), 7.27(1H,dd,1H,d)

^{13}C-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 23): 19, 9.59, 56, 101.1, 105, 107, 108.0, 109.5, 110.5, 121, 124.5, 125.5, 131.5, 133, 133.5, 142, 145, 147.5, 148.0, 151.0

^{13}C-NMR DEPT-135 ($CDCl_3$) δ(ppm) (Fig. 24):

CH, CH_3 signals(up phase) 10 peaks: 19, 9.59, 56, 105, 107.0, 108.0, 109.5, 121, 124.5, 131.5

CH_2 signal(down phase): 101.0

^{13}C-NMR DEPT-90 ($CDCl_3$) δ(ppm) (Fig. 25):

CH signals 7 peaks: 105, 107, 108, 109.5, 121, 124.5, 131.5

Purification and properties of **D**

The white needle in yellow oil was obtained from fraction 31-40 of crude chloroform extract(see **Table 3**) and recrystallized from chloroform-hexane for several times to obtain white needle about 0.08g.(equivalent to 0.25 g, 1.00 % wt. by wt. of crude chloroform extract), m.p. 153-154 °C. This substance was soluble in chloroform and insoluble in hexane and methanol. Result of TLC technique exhibited Rf 0.09 (silica gel/ hexane:chloroform, 1: 3)

UV absorption spectrum, λ_{max}nm (Fig. 28): 292

IR spectrum, ν_{max}(cm^{-1}) (Fig. 29): 3500-3100, 3050, 2980, 2900, 2000-1700, 1680, 1640, 1620, 1600, 1525, 1390, 1380, 1230-1170, 840

Mass spectrum (% relative intensity) (Fig. 30): 300(M^+)

^{1}H-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 31): 0.87(3H,s), 0.90(3H,s), 1.06(3H,s), 1.20(2H,m), 1.80(5H,m), 4.75(1H,t), 6.27(1H,d), 6.85(2H,d), 7.41(2H,d), 7.55(1H,s), 7.61(1H,d)

^{13}C-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 32): 11.5, 19.99, 20.1, 27.0, 33.7, 39.0, 47.0, 45.0, 49.0, 81.5, 116.0, 116.0, 130.0, 130.0, 158.3, 126.8, 144.6, 115.6, 167.7

^{13}C-NMR DEPT-135 ($CDCL_3$) δ(ppm) (Fig. 33):

CH, CH_3 signals(up phase) 11 peaks: 11.5, 19.9, 20.1, 45, 81.5, 115.6, 116.0, 116.0, 130.0, 130.0, 144.6

CH_2 signals(down phase) 3 paeks: 27, 33.7, 39

^{13}C-NMR DEPT-90 ($CDCl_3$) δ(ppm) (Fig. 34):

CH signals 8 peaks: 45, 81.5, 115.6, 116.0, 116.0, 130.0, 130.0, 144.6,

Purification and properties of **E**

E about 0.08 g (equivalent to 0.25 g, 1.00 % wt. by wt. of crude hexane extract), m.p. 102-105 ^{o}C was obtained as a the yellow needle (from fraction 81-90, see **Table 2**) and recrystallized from mixture of chloroform and hexane for several times. The results of TLC technique exhibited R_f of 0.40 (silica gel/chloroform). This substance was soluble in chloroform and insoluble in hexane and methanol.

UV absorption spectrum, λ_{max}nm (Fig. 37): 260

IR spectrum, $\nu_{max}(cm^{-1})$ (Fig. 38): 3100, 2990, 2940, 2880, 1680, 1630, 1610, 1445, 1430, 1280, 1230, 1090, 980, 870, 750

Mass spectrum (m/e) (% relative intensity) (Fig. 39): 274(M^{+})

^{1}H-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 40): 2.35(3H,s), 3.3(2H,d), 3.85(3H,s), 5.05(1H,t), 5.90(1H,s), 6.25(1H,s), 12.6(1H,s)

^{13}C-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 41): 17.5, 20.5, 21.5, 25.5, 55.5, 94.5, 104.5, 107.5, 108.0, 122.0, 131.0, 154.5, 160.5, 162.5, 166.0, 182.5

^{13}C-NMR DEPT-135 ($CDCl_3$) δ(ppm) (Fig. 42):

CH, CH_3 signals(up phase) 8 peaks: 17.5, 20.5, 25.5, 55.5, 94.5, 122.0, 107.5, 108.0

CH_2 signals(down phase) 1 peak: 21.5

^{13}C-NMR DEPT-90 ($CDCl_3$) δ(ppm) (Fig. 43):

CH signals 4 peaks : 94.5, 107.5, 108.0, 122.0

Purification and properties of **F**

The white solid in yellow oil obtained from fraction 51-60(see **Table 3**, crude chloroform extract) was recrystallized from mixture of chloroform and hexane for several times to obtain white amorphous about 0.05 g.(equivalent to 0.16 g, 0.06 % wt. by wt. of crude chloroform extract), m.p. 149-150°C as **F**. The result from TLC technique exhibited R_f 0.85 (silica gel/chloroform:hexane, 9:1). It was soluble in chloroform and methanol and insoluble in hexane and ethyl acetate.

UV absorption spectrum, λ_{max}nm (Fig. 46): 240

IR spectrum, $\nu_{max}(cm^{-1})$ (Fig. 47): 3050, 2980, 1780, 1740, 1610, 1460, 1380, 1290, 1250, 1220, 1130, 1080, 1060, 1020, 730

Mass spectrum (m/e) (% relative intensity) (Fig. 48): not show

^{1}H-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 49): 1.93(3H, s) 2.05(3H, s) 3.09(1H, dd) 3.45(1H, dd) 3.67(1H, d) 4.25(1H, d) 4.50(1H, d) 5.01(1H, dd) 5.73(1H, d) 7.40(2H, ddd) 7.52(1H, tt) 7.95(2H, dd)

^{13}C-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 50): 20.0, 20.0, 48.0, 52.5, 53.5, 59.3, 62.0, 69.4, 70.2, 128.2, 128.2, 129.0, 129.8, 129.8, 133.0, 165.0, 169.3, 169.6

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

CH, CH_3 signals (up phase) 12 peaks: 20.0, 20.0, 48.0, 52.5, 53.5, 69.4, 70.2, 128.2, 128.2, 129.8, 129.8, 133.0

CH_2 signals (down phase): 62.0

^{13}C-NMR DEPT-90 ($CDCl_3$) δ(ppm) (Fig. 52):

CH signals 10 peaks: 48.0, 52.5, 53.5, 69.4, 70.2, 128.2, 128.2, 129.8, 129.8, 133.0

Purification and properties of **G**

The white solid in brown oil was obtained from fraction 70-80(see **Table 3**, crude chloroform extract). It was purified by recrystallization from the mixture of chloroform and hexane for several times to obtain the white needle about 0.09 g.(equivalent to 1.12 % wt. by wt. of crude chloroform extract), m.p. 116-117 °C. This substance was soluble in chloroform and methanol and insoluble in hexane. Result of TLC technique exhibited R_f = 0.60(silica gel/hexane:chloroform = 1 : 9).

UV absorption spectrum , λ_{max}nm (Fig. 55): 260

IR spectrum, $\nu_{max}(cm^{-1})$ (Fig. 56): 3320, 3050, 2940, 2880, 2000-1700, 1670, 1640, 1620, 1445, 1385-1350, 1320, 1260, 1270, 1170, 1050

Mass spectrum (m/e) (% relative intensity) (Fig. 57): 383(M^+)

^{1}H-NMR ($CDCl_3$) δ(ppm) (Fig. 58): 0.95(6H,d), 1.35(2H,s), 1.75(1H,m), 2.1(4H,m), 3.1(2H,t), 5.2(1H,s), 6.3-5.3(9H,d, 3H,m, 5.3(5H,m)), 1h,d), 5.85(2H,s), 6.7(2H,m), 6.85(1H,s), 7.15(1H,dd),

^{13}C-NMR spectrum ($CDCl_3$) δ(ppm) (Fig. 59): 20.5, 20.5, 28.5, 29.5, 29.5, 29.5, 29.5, 32.8, 32.8, 46.8, 100.8, 105.5, 108.2, 120.1, 122, 128.5, 124.5, 129.5, 132.5, 141.0, 142.5, 146.8, 147.8, 166.5

^{13}C-NMR DEPT-135 ($CDCl_3$) δ(ppm) (Fig. 60):

CH, CH_3 signals(up phase) 12 peaks: 20.5, 20.5, 28.5, 105.5, 108.2, 120.1, 122.0, 129.5, 129.5, 128.5, 141, 142.5

CH_2 signals(down phase) 7 peaks: 29.5, 29.5, 29.5, 32.8, 32.8, 46.7, 100.8

^{13}C-NMR DEPT-90 ($CDCl_3$) δ(ppm) (Fig. 61) :

CH signals 10 peaks : 28.5, 105.5, 108.2, 120.1, 122.0, 128.5, 129.5, 129.5, 141.0, 142.5