CHAPTER III

EXPERIMENTAL

1. Source of Plant Materials

The dried heartwoods of *X. xylocarpa* var. *kerrii* were collected from Loei province, in May 2006 (Figure 2) and from Mae Hong Son province (Figure 3) in January 2008, Thailand. The plant materials were authenticated by comparison with voucher specimen No. BKF 124480, deposited in the herbarium of the Royal Forest Department, Ministry of Agriculture and Co-operatives of Thailand. The dried heartwoods were dried at the temperature of about 50°C and ground for extraction.

2. General Technique

2.1 Analytical Thin-layer Chromatography (TLC)

Technique	One dimension, ascending
Adsorbent	Silica gel 60 F_{254} (E.Merck)
Layer thickness	0.2 mm
Distance	5 cm
Temperature	Laboratory temperature (30-35 °C)
Detection	1. Ultraviolet light at the wavelengths 254 nm and 365 nm 2. Visual detection in iodine vapor 3. Anisaldehyde-H_2SO_4 reagent and heat at about 100 °C for a few minutes

2.2 Column Chromatography

2.2.1 Conventional Column Chromatography

Adsorbent	Silica gel (No.7734 E. Merck) particle size 0.063-0.200 nm (70-230 mesh ASTM)
Sampling loading	The sample was dissolved in small amount of eluent and then applied gently on top of the column.
Packing method	Wet packing

Detection — Fractions were examined using TLC technique. The TLC plate was observed under UV light at the wavelengths 254 nm and 365 nm and then exposed to iodine vapor and anisaldehyde-H_2SO_4 reagent, respectively. Fractions of similar chromatographic pattern were combined.

2.2.2 Flash Column Chromatography

Adsorbent — Silica gel (No.7734 E. Merck)
particle size 0.063-0.200 nm (70-230 mesh ASTM)

Sampling loading — The sample was dissolved in small amount of eluent and then applied gently on top of the column.

Packing method — The adsorbent was wet-packed after being suspended in eluent. The slurry of adsorbent was poured into the column, tapped and pressed down under air pump, and then allowed to settle.

Detection — Fractions were examined using TLC technique. The TLC plate was observed under UV light at the wavelengths 254 nm and then exposed to iodine vapor and anisaldehyde-H_2SO_4 reagent, respectively. Fractions of similar chromatographic pattern were combined.

2.3 Spectroscopy

2.3.1 Ultraviolet (UV) Absorption Spectra

The spectra were obtained on a Shimadzu UV-160A UV/VIS spectrophotometer at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.3.2 Infrared (IR) Absorption Spectra

IR spectra (KBr disc) were recorded on a Perkin-Elmer FT-IR 1760x spectrometer at the Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.3.3 Mass Spectra (MS)

The spectra were obtained on an ES TOFMS Mass Spectrometer at the National Center for Genetic Engineering and Biotechnology, Thailand.

2.3.4 Proton and Carbon-13 nuclear Magnetic Resonance (^{1}H and ^{13}C-NMR) Spectra)

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained with a Bruker Avance DPX-300 FT-NMR spectrometer at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were obtained with a JEOL JMN-A 500 spectrometer at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

Solvent for NMR spectra was deuterated chloroform ($CDCl_3$) Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

2.4 Physical properties

2.4.1 Melting Points

Melting points were obtained on a Gallenkamp Melting Points Apparatus at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University

2.4.2 Optical Rotations

Optical rotations were measure on a Perkin-Elmer Polarimeter model 341 using a sodium lamp operation at 589 nm at the Pharmaceutical Research instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.5 Solvent

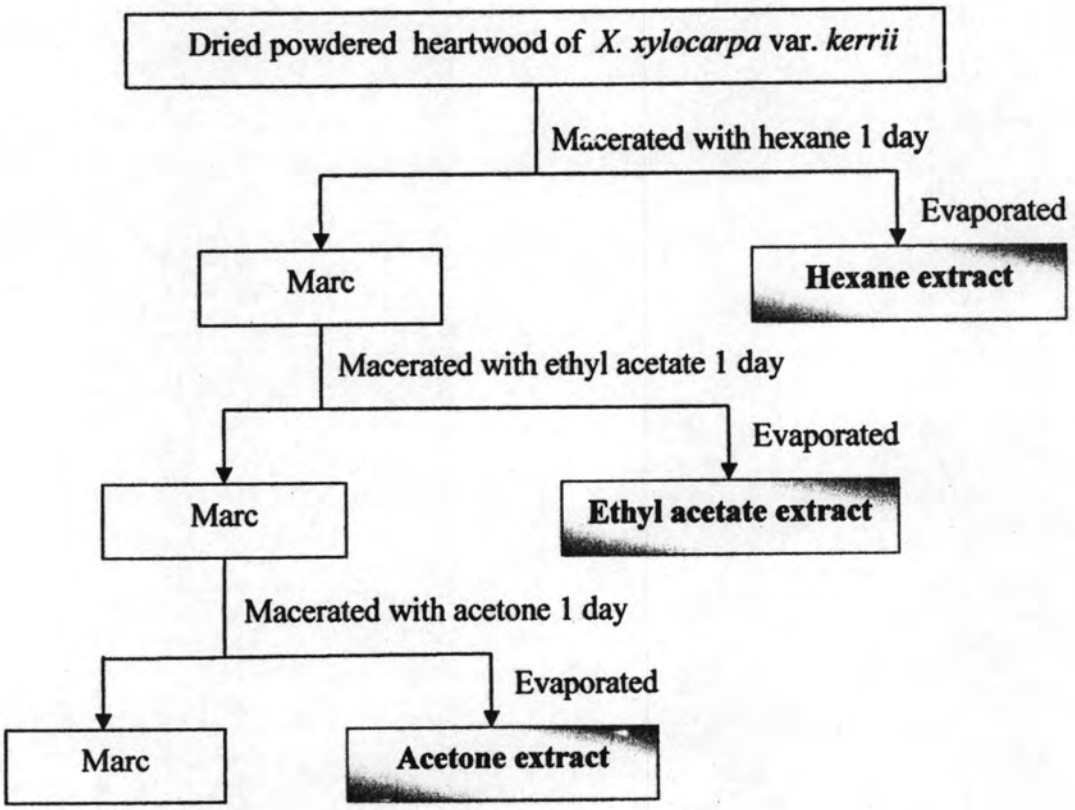
Throughout this work, all organic solvents were of commercial grade and were redistilled prior to use.

3. Extraction and Isolation

Part I : Heartwood of *X. xylocarpa* var. *kerrii* from Loei province.

1. Extraction

The dried heartwood of *X. xylocarpa* var. *kerrii* (1 Kg) was extracted with hexane, ethyl acetate and acetone (each 3000 ml for 1 day), respectively. The filtrate of each extraction was concentrated to remove solvent under reduced pressure at temperature not over 50 °C to yield hexane extract 21.34 g (2.13 % w/w), ethyl acetate extract 16.78 g (1.68 %w/w and acetone extract 35.55 g (3.56 % w/w). (Scheme 3)



Scheme 3 Extraction of the heartwood of *X. xylocarpa* var. *kerrii*

2. The Separation and Isolation

2.1 The separation of the crude hexane extract

The crude hexane extract (15 g) was dissolved in a small volume of hexane and packed onto the top of a wet silica gel column (silica gel 60, 150 g). It was fractionated by conventional column chromatography. The column was eluted with eluents (ethyl acetate-hexane mixtures of increasing polarity) each 200 ml in the order and collected 50 ml for each fraction. Fractions were combined according to their TLC patterns (using 25% ethyl acetate in hexane as developing solvent) to give a total of eight fractions (H-I-H-VIII), as shown below. (Table 1)

Table 1 Combination of fractions from crude hexane extract by conventional column chromatography

Fractions code	Number of fraction	Weight (g)
H-I	1-44	13.2621
H-II	45-48	0.3375
H-III	47-56	0.2079
H-IV	57-60	0.0939
H-V	61-72	0.1944
H-VI	73-84	0.1916
H-VII	85-92	0.3824*
H-VIII	93-100	0.1669

Remark : * = **Compound A-1**

Fraction H-VII was crystallized as white needles and recrystallized from hexane to give compound A-1 (0.3824 g)

Fraction H-I (13.2621 g) was further separated by column chromatography on silica gel (silica gel 60) and eluted with ethyl acetate-hexane mixtures of increasing polarity (2-50 % ethyl acetate in hexane). Fractions (approximately 50 ml each) were collected and combined according to their TLC patterns (using 25% ethyl acetate in hexane as developing solvent) to give a total of eight fractions (HI-1-HI-8). (Table 2)

Table 2 Combination of fractions from fraction H-I (13.2621 g) by column chromatography

Fractions code	Number of fraction	Weight (g)
HI-1	1-6	9.4142
HI-2	7-15	1.1918
HI-3	16-23	0.6422
HI-4	24-30	0.2731
HI-5	31-38	0.5048
HI-6	39-41	0.1619
HI-7	42-45	0.1057
HI-8	46-48	0.0544

Fraction HI-1 (9.4142 g) was further separated by flash column chromatography on silica gel (silica gel 60) and eluted with ethyl acetate-hexane mixtures of increasing polarity (5-50 % ethyl acetate in hexane). Fractions (approximately 50 ml each) were collected and combined according to their TLC patterns (using 25% ethyl acetate in hexane as developing solvent) to give a total of eleven fractions (I-1-I-11). (Table 3)

Table 3 Combination of fractions from fraction HI-1 by flash column chromatography

Fractions code	Number of fraction	Weight (g)
I-1	1-4	0.0417
I-2	5-6	0.2543
I-3	7-21	1.7930
I-4	22-32	5.4884
I-5	33-38	0.3503
I-6	39-40	0.0833
I-7	41-43	0.0494
I-8	44	0.0179
I-9	45-49	0.1087
I-10	50-56	0.1415
I-11	57-64	0.0666

Fraction I-3 (1.7930 g) was further separated by flash column chromatography on a silica gel (silica gel 60 and eluted with dichloromethane-hexane mixtures of increasing polarity (5-70 % dichloromethane in hexane). Fractions (approximately 50 ml each) were collected and combined according to their TLC patterns (using 40% hexane in dichloromethane as developing solvent) to give a total of five fractions (I3-1-I3-5). (Table 4)

Table 4 Combination of fractions from fraction I-3 (1.7930 g) by flash column chromatography

Fractions code	Number of fraction	Weight (g)
I3-1	1-15	0.0718
I3-2	16-31	0.3911
I3-3	32-34	0.0213
I3-4	35-42	0.1314*
I3-5	43-50	0.0441

Remark : * = Compound A-2

The white powder from fraction I3-4 were further purified by recrystallization from MeOH to yield compound A-2 (0.1314 g).

Fraction HI-2 (1.1918 g) was further separated by flash column chromatography on silica gel (silica gel 60) and eluted with ethyl acetate-hexane mixtures of increasing polarity (2-10 % ethyl acetate in hexane). The column was eluted with eluents each 200 ml in the order and collected 50 ml of each fraction. Fractions were combined according to their TLC patterns (using 25% ethyl acetate in hexane as developing solvent) to give a total of six fractions (I2-1-I2-6), as Table 5.

Table 5 Combination of fractions from fraction HI-2 (1.1918 g) by flash column chromatography

Fractions code	Number of fraction	Weight (g)
I2-1	1-9	0.0041
I2-2	10-13	0.0319
I2-3	14-16	0.0146
I2-4	17-18	0.0240
I2-5	19-44	0.4881*
I2-6	43-52	0.1061

Remark : * = Compound A-3

The white solid from fraction I2-5 (0.4881 g) was further purified by recrystallization from MeOH to yield compound A-3 (0.0350 g)

2.2 The separation of the crude ethyl acetate extract

The crude ethyl acetate extract (12 g) was dissolved in a small volume of ethyl acetate and packed onto the top of wet silica gel column (silica gel 60). It was fractionated by conventional column chromatography and eluted with ethyl acetate-hexane mixtures of increasing polarity (5-100 % ethyl acetate in hexane). The column was eluted with eluents each 200 ml in the order and collected 50 ml of each fraction. (Table 7) Fractions were combined according to their TLC patterns (using 25% ethyl acetate in hexane as developing solvent) to give a total of seven fractions (E-I-E-VII) as shown below. (Table 6)

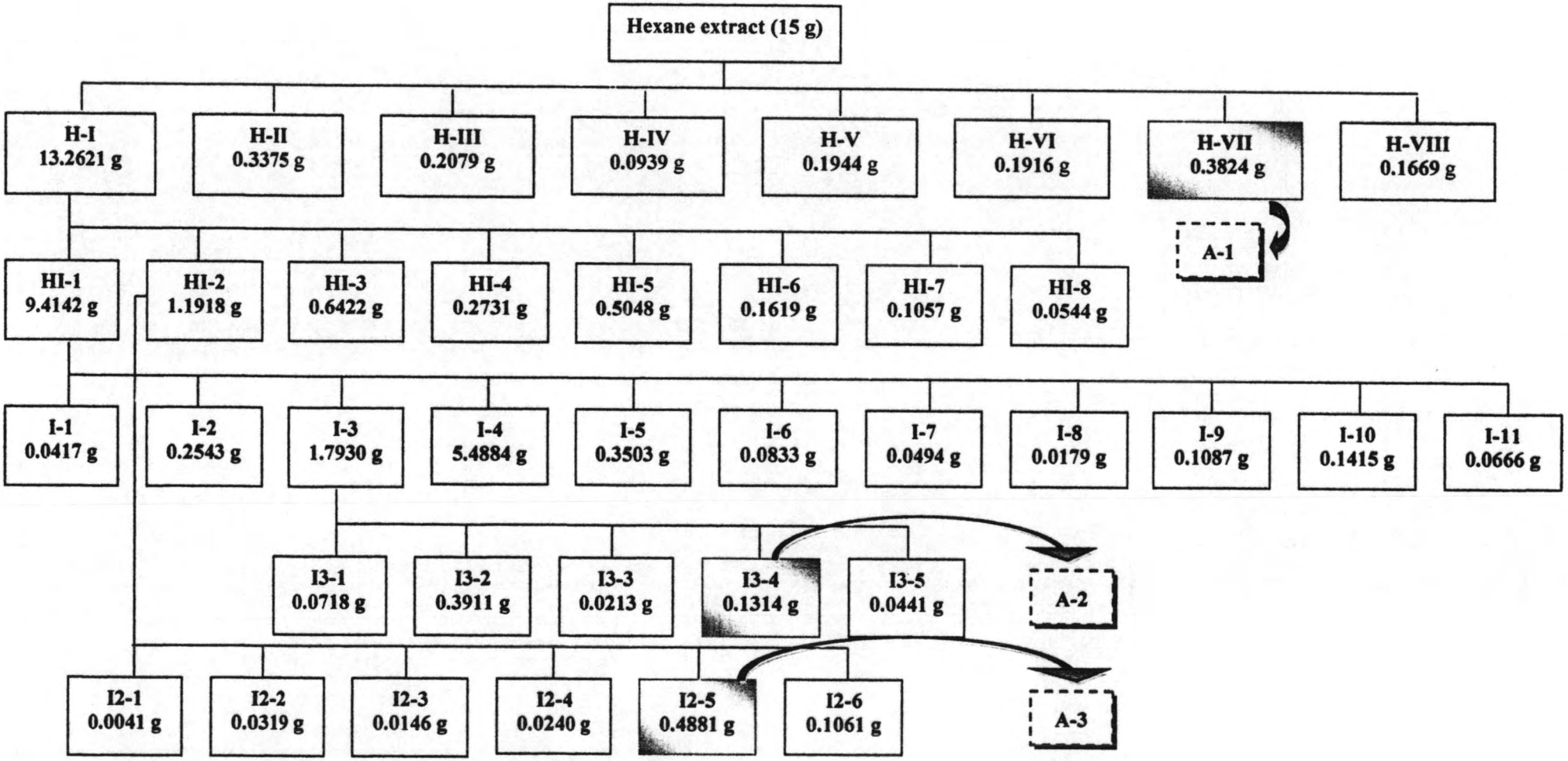
Table 6 Combination of fractions from crude ethyl acetate extract (12 g) by conventional column chromatography

Fractions code	Number of fraction	Weight (g)
E-I	1-20	0.4804
E-II	21-36	4.0393
E-III	37-40	0.1189*
E-IV	41-56	0.4098
E-V	57-60	0.4325
E-VI	61-72	0.3373
E-VII	73-88	3.9531

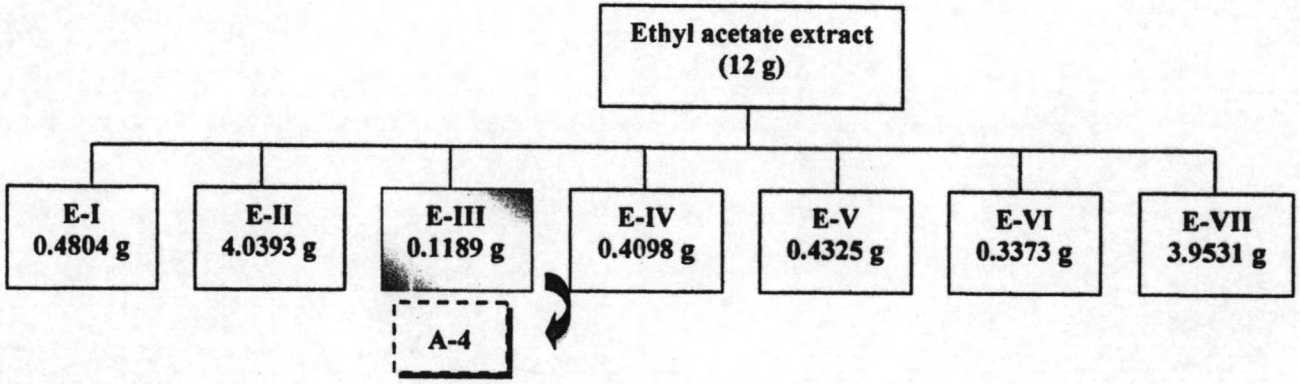
Remark: * = Compound A-4

The crystals from fraction E-III were further purified by recrystallization from hexane to yield compound A-4 (0.1189 g).

Separation and isolation of these compounds from hexane extract and ethyl acetate extract are summarized in Scheme 4 and Scheme 5, respectively, and the yields (%w/w) are shown in Table 7.



Scheme 4 Isolation from the hexane extract of *X. xylocarpa* var. *kerrii* from Loei province



Scheme 5 Isolation from the Ethyl acetate extract of *X. xylocarpa* var. *kerrii* from Loei province

Table 7 Yield of the hexane extract and ethyl acetate extract of *X. xylocarpa* var. *kerrii* from Loei province

Compound	Hexane extract (g)	Ethyl acetate extract (g)	%Yield of extract (%w/w)	%Yield (%w/w)
Crude extract	15	12	100	1.5 (hexane extract) 1.2 (ethyl acetate extract)
A-1	0.3423	-	2.28	0.0342
A-2	0.1211	-	0.81	0.0121
A-3	0.0350	-	0.23	0.0035
A-4	-	0.0809	0.67	0.0081

Part II : Heartwood of *X. xylocarpa* var. *kerrii* from Mae Hong Son province

1. Extraction

The dried heartwood of *X. xylocarpa* var. *kerrii* (400 g) was extracted with solvent in a similar extraction to that from Loei province (each 800 ml for 1 day). The hexane extract 7.57 g (1.89 % w/w), ethyl acetate extract 2.41 g (0.60 % w/w) and acetone extract 4.74 g (1.19 % w/w) were obtained.

2. The Separation and Isolation

The crude hexane extract (6.7 g) was dissolved in a small volume of hexane and packed onto the top of a wet silica gel column (silica gel 60, 150 g). It was fractionated by conventional column chromatography and eluted with ethyl acetate-hexane mixtures of increasing polarity (2-100 % ethyl acetate in hexane). The column was eluted with eluents (400 ml each) in the order and collected 100 ml of each fraction. Fractions were combined according to their TLC patterns (using 25% ethyl acetate in hexane as developing solvent) to give a total of fourteen fractions (MH-1-MH-14), as shown below. (Table 8)

Table 8 Combination of fractions from crude hexane extract (6.7 g) by conventional column chromatography

Fractions code	Number of fraction	Weight (g)
MH-I	1-19	0.2396
MH-II	20-24	3.5017
MH-III	25-32	1.1917
MH-IV	33-37	1.0224 *
MH-V	38-40	0.126
MH-VI	41-42	0.0302 **
MH-VII	43-44	0.0363
MH-VIII	45-50	0.0680 ***
MH-IX	51-57	0.0785
MH-X	58-65	0.0887
MH-XI	66-67	0.0042 ****

Fractions code	Number of fraction	Weight (g)
MH-XII	68-69	0.0145
MH-XIII	70-74	0.1185 *****
MH-IVX	75-76	0.0127

Remark: * = Compound B-1

** = Compound B-2

*** = Compound B-3

**** = Compound B-4

***** = Compound B-5

The white solid from fraction MH-IV (1.0224 g) was further purified by recrystallization from MeOH to yield compound B-1

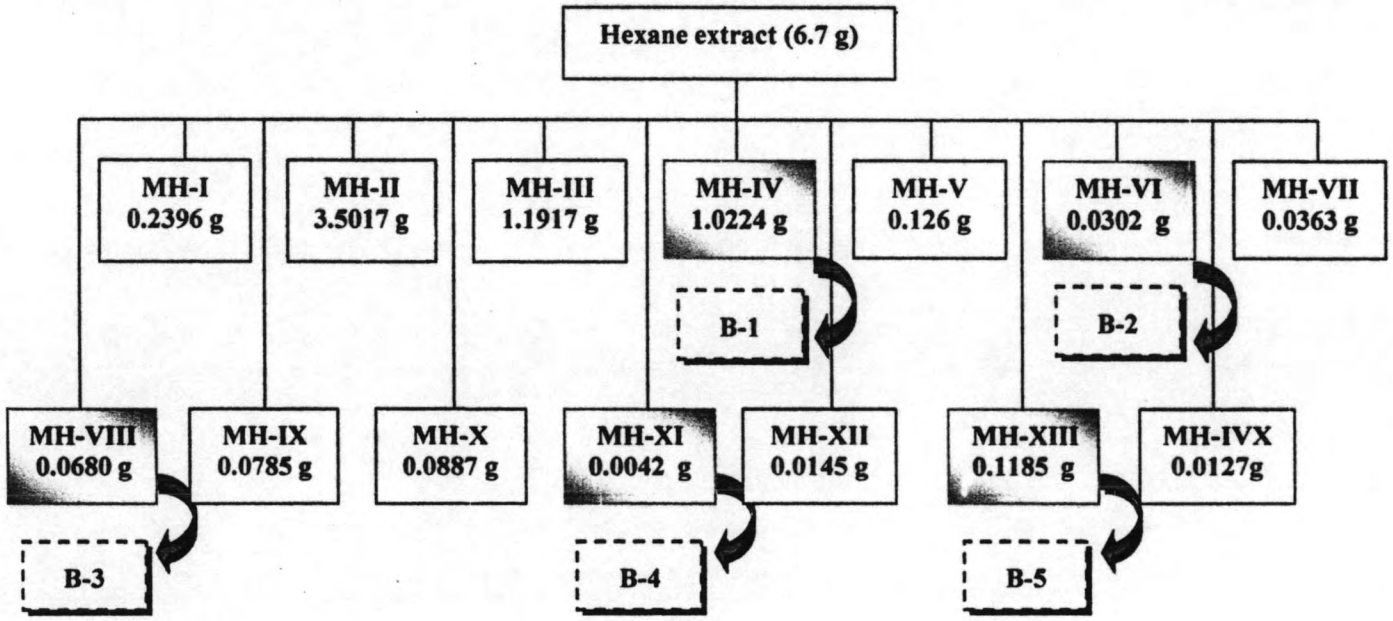
The white solid from fraction MH-VI (0.0302 g) was further purified by recrystallization from MeOH to yield compound B-2

The white solid from fraction MH-VIII (0.0680 g) was further purified by recrystallization from MeOH to yield compound B-3

The white solid from fraction MH-XI (0.0042 g) was further purified by recrystallization from hexane to yield compound B-4

The white solid from fraction MH-XIII (0.1185 g) was further purified by recrystallization from hexane to yield compound B-5

The separation and isolation of these compounds from hexane extract are summarized in Scheme 6 and the yield (%w/w) are shown in Table 9.



Scheme 6 Isolation from the hexane extract of *X. xylocarpa* var. *kerrii* from Mae Hong Son province

Compound	Hexane extract (g)	%Yield of extract (%w/w)	%Yield (%w/w)
Crude extract	6.7	100	1.6800
B-1	1.0040	14.99	0.2510
B-2	0.0228	0.34	0.0057
B-3	0.0595	0.88	0.0149
B-4	0.0031	0.05	0.0008
B-5	0.1065	1.59	0.0266

Table 9 Yield of the hexane extract and ethyl acetate extract of *X. xylocarpa* var. *kerrii* from Mae Hong Son province

4. Physical and spectral data of the isolated compounds

1. Compound A-1

Compound C-1 was obtained as white solid

$[\alpha]^{20}_{D}$: -20.7° (c, 0.34 in MeOH)

UV : λ max nm, (log ε) in MeOH; Figure 4

220.0 (3.34)

ES TOFMS : *m/z*; Figure 5

327.22 $[M+Na]^+$

IR : υ cm-1, KBr disc; Figure 6

3412, 2948-2830, 1448-1361, 1060, 1090-996, 908

^{1}H NMR : δ ppm, 300 MHz, in chloroform-d; Figure 7

0.82 (s), 0.91 (s), 1.01 (s) 3.40 (d, J = 10.5 Hz), 3.67 (m), 4.87 (dd, J=1.5, 10.5 Hz), 4.89 (dd, J = 1.5, 17.7 Hz), 5.21 (s), 5.74 (dd, J = 10.5, 17.7 Hz)

^{13}C NMR : δ ppm, 75 MHz, in chloroform-d; Figure 8

11.6, 15.6, 18.9, 22.6, 26.1, 27.3, 34.6, 35.7, 37.0, 37.5, 38.1, 42.3, 48.7, 50.4, 72.1, 77.1, 110.1, 128.9, 136.1, 148.7

2. Compound A-2

Compound C-2 was obtained as white solid

$[\alpha]^{20}_{D}$: -30.9° (c, 0.10 in MeOH)

UV : λ max nm, (log ε) in MeOH; Figure 9

221.0 (3.35)

ES TOF MS : *m/z*; Figure 10

308.96 $[M+Na]^+$

IR : υ cm-1, KBr disc; Figure 11

2988-2826, 1701, 1461-1427, 1385, 1369, 1001, 912

^{1}H NMR : δ ppm, 300 MHz, in chloroform-d; Figure 12

0.98 (s), 1.05 (s), 1.07 (s), 1.43 (m), 1.72 (d, J = 7.4 Hz), 1.99 (m), 2.30 (m), 2.63 (ddd, J = 5.8, 14.6, 14.6 Hz), 5.27 (s), 4.89 (m) 5.75 (dd, J = 10.7, 17.3 Hz)

^{13}C NMR : δ ppm, 75 MHz, in chloroform-d; Figure 13

14.7, 18.9, 22.3, 23.3, 25.8, 26.1, 34.4, 34.8, 35.6, 37.5, 37.7, 38.0, 47.8, 49.5, 55.4, 110.4, 129.6, 135.8, 148.6, 216.8

3. Compound A-3

Compound A-3 was obtained as white solid

$[\alpha]^{20}_{D}$: -32.5° (c, 0.02 in MeOH)

UV : λ max nm, (log ε) in MeOH; Figure 14

221.0 (3.35)

ES TOF MS : *m/z*; Figure 15

288 $[M]^+$

IR : υ cm-1, KBr disc; Figure 16

3388, 2941-2869, 1706-1639, 1458,1382, 1090, 1032, 997, 911

^{1}H NMR : δ ppm, 300 MHz, in chloroform-d; Figure 17

0.78 (s), 0.80 (s), 0.99 (s),1.02 (s), 1.45 (m), 1.98 (m), 2.25 (d, J = 12.4 Hz), 3.25 (dd, J = 4.2, 11.4Hz), 4.88 (m), 5.21 (br s), 5.75 (dd, J = 10.5,17.7 Hz)

^{13}C NMR : δ ppm, 75 MHz, in chloroform-d; Figure 18

15.0, 15.7, 18.8, 22.3, 26.0, 27.6, 28.5, 34.5, 35.9, 37.3, 37.4, 38.9, 39.0, 50.4, 54.2, 79.2, 110.1, 128.9, 136.7, 149.0

4. Compound A-4

Compound A-4 was obtained as white solid

$[\alpha]^{20}_{D}$: +77.2° (c, 0.03 in MeOH)

UV : λ max nm, (log ε) in MeOH; Figure 19

244.0 (3.21)

ES TOF MS : *m/z*; Figure 20

325.21 $[M+Na]^+$

IR : υ cm-1, KBr disc; Figure 21

3483, 2969, 2936, 1692, 1013, 909

^{1}H NMR : δ ppm, 500 MHz, in chloroform-d; Figure 22-23

1.07 (s), 1.10 (s), 1.11 (s), 1.44 (s), 1.58 (m), 1.67 (m), 1.96 (m), 1.99 (dd, J = 3.5, 13.3 Hz), 2.44 (ddd, J = 3.7, 7.3, 15.9, Hz), 2.56 (m), 2.60 (m), 3.48 (br s), 4.95 (dd, J = 1.2, 17.7, Hz), 4.98 (dd, J =1.2, 11.1 Hz), 5.71 (dd, J = 11.1, 17.7, Hz)

^{13}C NMR : δ ppm, 125 MHz, in chloroform-d; Figure 24

19.2, 19.9, 21.2, 21.4, 23.1, 26.5, 29.0, 29.7, 34.3, 34.8, 37.2, 39.5, 47.3, 51.1, 74.4, 112.7, 128.7, 139.3, 143.7, 217.5

5. Compound B-1

Compound B-1 was obtained as white platelet and was identified as compound A-3 by NMR spectra

^{1}H NMR : δ ppm, 300 MHz, in chloroform-d; Figure 32

0.78 (s), 0.80 (s), 0.99 (s), 1.02 (s), 1.45 (m), 1.98 (m), 2.25 (d, J = 12.4 Hz), 3.25 (dd, J = 4.2, 11.4 Hz), 4.88 (m), 5.21 (br s), 5.75 (dd, J = 10.5, 17.7 Hz)

^{13}C NMR : δ ppm, 75 MHz, in chloroform-d; Figure 33

15.0, 15.7, 18.8, 22.2, 26.0, 27.7, 28.5, 34.5, 35.9, 37.3, 37.5, 38.9, 39.0, 50.4, 54.2, 79.2, 110.1, 128.9, 136.7, 150.0

6. Compound B-2

Compound B-2 was obtained as white solid

$[\alpha]^{20}_{D}$: -50.7° (c, 0.04 in MeOH)

^{1}H NMR : δ ppm, 300 MHz, in chloroform-d; Figure 34

0.66-2.29 (overlapping), 3.49 (m), 4.98 (dd, J = 8.4, 15.2 Hz), 5.13 (dd, J = 8.4, 15.2 Hz), 5.33 (br. d)

^{13}C NMR : δ ppm, 75 MHz, in chloroform-d; Figure 35

11.9, 12.0, 12.0, 12.2, 18.8, 19.0, 19.0, 19.4, 19.8, 21.1, 21.2, 23.1, 24.3, 24.4, 25.4, 26.1, 28.2, 28.9, 29.2, 30.3, 31.7, 31.9, 31.9, 34.0, 36.2, 36.5, 37.3, 39.7, 39.8, 40.5, 42.3, 45.9, 50.2, 51.2, 56.0, 56.1, 56.8, 56.9, 71.8, 121.7, 129.3, 138.3, 140.8

7. Compound B-3

Compound B-3 was obtained as white solid

$[\alpha]^{20}_{D}$: -30.3° (c, 0.10 in MeOH)

UV : λ max nm, (log ε) in MeOH; Figure 36
221.0 (3.21)

ES TOF MS : *m/z*; Figure 37
324.93 $[M+Na]^+$

IR : υ cm-1, KBr disc; Figure 38
3429, 3050-2832, 1694, 1276

^{1}H NMR : δ ppm, 300 MHz, in chloroform-d; Figure 39
0.82 (s), 1.02 (s), 1.19 (s), 1.51 (m), 1.76 (m), 1.90 (dd, *J* = 12.3, 2.3 Hz), 2.14 (m), 4.85 (d, *J* = 10.1 Hz), 4.90 (d, *J* = 17.6 Hz), 5.20 (br. s), 5.75 (dd, *J* = 10.6, 17.6 Hz)

^{13}C NMR : δ ppm, 75 MHz, in chloroform-d; Figure 40
15.2, 16.8, 18.2, 18.6, 24.9, 26.1, 34.5, 35.5, 37.1, 37.4, 37.8, 38.3, 47.3, 48.9, 50.6, 110.2, 129.2, 136.6, 148.9, 184.4

8. Compound B-4

Compound B-4 was obtained as white platelet

$[\alpha]^{20}_{D}$: -52.3 ° (c, 0.02 in MeOH)

UV : λ max nm, (log ε) in MeOH; Figure 41
220.0 (3.37)

ES TOF MS : *m/z* (relative intensity); Figure 42
326.94 $[M+Na]^+$

IR : υ cm-1, KBr disc; Figure 43
3395, 2965, 2942, 2870, 2852, 1456, 1093, 1057, 995

^{1}H NMR : δ ppm, 500 MHz, in chloroform-d; Figure 44-45
0.84 (s), 0.85 (s), 1.02 (s), 1.12 (dd, *J* = 2.4, 12.2, Hz),1.36 (m), 1.44 (ddd, *J* =4.0, 4.0, 12.5, Hz), 1.52 (dd, *J* =3.2, 6.4 Hz), 1.62 (dd, *J* = 1.83, 13.7 Hz), 1.73 (m), 2.03 (dd, *J* = 4.3, 12.2 Hz), 3.64 (m) 4.89 (dd, *J* = 1.5, 17.4 Hz) 4.86 (dd, *J* = 1.5, 10.5 Hz), 5.25 (br s), 5.70 (dd, *J* = 10.5, 17.4 Hz)

^{13}C NMR : δ ppm, 75 MHz, in chloroform-d; Figure 46

15.9, 16.9, 18.9, 22.1, 26.0, 27.0, 34.4, 35.7, 37.5, 39.0, 39.3, 45.2, 50.4, 54.1, 68.8, 83.8, 110.2, 129.5, 136.0, 148.8

9. Compound B-5

Compound B-5 was obtained as white platelet and was identified as A-1

5. Cytotoxicity test

The human cancer cell line was harvested from exponential-phase maintenance culture (T-25 cm^3 flask and dispensed within replicate 96-well culture in 200 µL volume using a repeating pipette. Following a 24 hours incubation at 37°C, with 5% CO_2, 100% relative humidity and 200 µl of culture medium. Culture medium containing sample 10 µg/ml was dispensed within appropriate wells (control group, N=3: each sample treatment group, N=3) Peripheral wells of each plate (lacking cells) were utilized for sample blank (N=3). Culture plates were then incubated for 3 days prior to the addition of tetrazolium reagent

MTT stock solution was prepared as follows: 5 mg MTT/ml NSS was steriled and filtered with 0.45 µm filtered units. MTT working solution (10 µL) was added to each culture well resulting in 50 µg MTT/200 µL total medium volume and culture were incubated at 37°C for 4 hrs. Following incubation, cell monolayer and formazan were inspected microscopically; culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 minutes. All of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 µL of DMSO using pipette. Following the formazan with solubilization was measured using a microculture plate reader and spectrophotometer at 540 nm (single wavelength, calibration factor = 1.00)

Samples were tested for cytotoxic activity towards 5 human cancer cell lines, including KATO-3 (human gastric carcinoma), SW620 (human colon adenocarcinoma), BT474 (human breast ductal carcinoma), HEP-G2 (human liver hepatoblastoma) and CHAGO (human undifferentiated lung carcinoma). Following the experimental method for bioassay of cytotoxic activity.