CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

Stem barks of *Moringa pterygosperma* Gaertn. were obtained from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand in October, 1994. The plant material was authenticated by comparison with the herbarium specimen (No. 110001) at the Department of Pharmacognosy, Chulalongkorn University, Bangkok, Thailand.

2. General Techniques

2.1 Analytical Thin-layer Chromatography (TLC)

Adsorbent	:	Silica gel 60 F254 (E. Merck) pre-coated plate.
Plate size	:	2.0 x 6.7 cm
Layer thickness	:	0.2 mm
Technique	:	One way, ascending
Distance	:	5.5 cm
Temperature	:	Laboratory temperature (28-34°C)
Detection	:	1. Ultraviolet light at the wavelength of 254 and 365 nm 2. Iodine vapor 3. Visual detection by spraying with 0.5% Anisaldehyde-sulfuric acid and glacial acetic acid reagent and heating at 105^0 for 5 minutes

2.2 Column Chromatography

Adsorbent : Silica gel 60 (Number 7734) particle size 0.063 - 0.200 mm (70-230 mesh ASTM)

Packing method

-Wet-packed : Adsorbent was mixed with solvent system to make slurry, then poured into the column. The solvent was sucked out, and the adsorbent allowed to settle tightly.

-Dry-packed : Adsorbent was poured into the column then tightly pressed.

Sample loading : Crude extract was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, dried under vacuum before being added gently on top of the adsorbent.

Examination of eluates :

Fractions were examined by TLC using visual detection under daylight, ultraviolet absorption, iodine vapour and anisaldehyde sulphuric acid spray reagent, respectively. The fractions were combined after TLC examination revealed their identical profiles, then evaporated in vacuo to dryness.

2.3 Gel Filtration Chromatography

Stationary phase : Sephadex® LH-20

Packing : The stationary phase was suspended in the eluant and left standing to swell prior to use for 24 hours., then poured into the column and allowed to settle tightly.

Sample loading : The extract was dissolved in a small volume of eluant and carefully loaded, to avoid disturbing the gel surface, on top of the column.

Examination of Eluates :

Same as that described in section 2.2.

2.4 High Performance Liquid Chromatography (HPLC)

Adsorbent : Pre-packed μ Bondapak™ C18 column

Column size : 3.9 x 300 mm

Solvent phase : Methanol - Water (1 : 1)

Addition of sample extract :

The sample was accurately weighed and dissolved with suitable amount of methanol to 5 mg/ml concentration and 50 μl was injected for each loading.

Solvent delivery system:

consta Metric® 4100

Sample injection system:

Rheodyne®

UV detector 254 nm :

Spectromonitor® 4100

Recorder :Linear® model 1202-0000

3. Spectroscopy

3.1 Ultraviolet (UV) visible absorption spectroscopy

Ultraviolet-visible absorption spectra were obtained on a Milton Roy Spectronic 3000 Array UV spectrometer (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University)

3.2 Infrared (IR) absorption spectra

The spectra were obtained on a Shimadzu IR-440 infrared spectrometer (The Scientific and Technology Research Equipment Center, Chulalongkorn University), using KBr disc or liquid cell (neat) to determine the spectra.

3.3 Mass spectroscopy (MS)

Mass spectra were recorded on a Kratos-Profile mass spectrometer for EIMS, operating at 70 eV. Inlet temperature 100-150 ^{0}C. (Department of Sciences Service, Ministry of Science, Technology and Environmental, Bangkok,)

3.4 Proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectroscopy

The NMR spectra were obtained on a Jeol JMN.-GSX spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) (The Scientific and Technological Research Equipment Center, Chulalongkorn University) using tetramethylsilane (TMS) as internal standard. The chemical shifts were reported in ppm.

4. Solvents

Throughout this work, all organic solvents used were commercial grade and had to be redistilled prior to use, excluding the solvents for HPLC which were analytical grade and filtered through membrane filter before use.

Extraction

The powdered stem bark of *Moringa pterygosperma* Gaertn. (7.5 kg) was macerated for 2 times in ethanol each, for 3 days, with 95% ethanol each 10 L.. The ethanol filtrate was concentrated under reduced pressure evaporator to give a crude extract (453 g). The extract was dissolved in chloroform and partitioned with distilled water.

The chloroform extract was concentrate to yield 185 g of dry extract. The extract was dissolved in 80% methanol, then. partitioned with hexane. The methanol fraction, evaporated to dryness under reduced pressure, to yielded 130 g. of extract.

The extraction scheme is summarized in Figure 4.

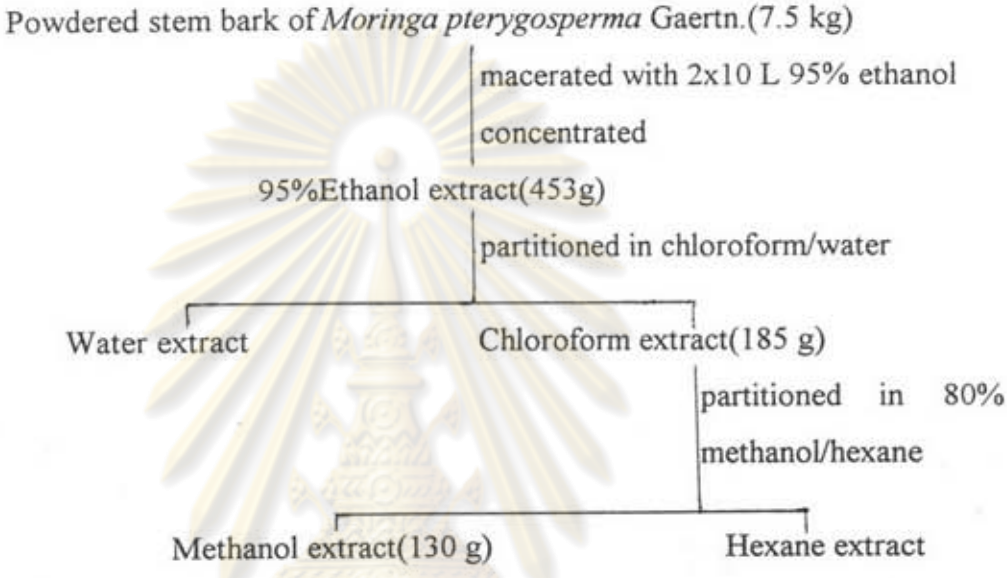

Figure 3 Extraction of *Moringa pterygosperma* Gaertn.

Isolation

The methanol extract (130g) was loaded onto a column of silica gel G (9.5 x 13 cm) and eluated with chloroform 500 ml and 300 ml of mixtures of chloroform-methanol was used to eluant, methanol each increased step 1:99, 2:98, 3:97,..., 21:78 methanol in chloform . Fractions of 100 ml were collected (65 fractions collected). Methanol was finally used for washing down the column until the eluate was clear.Each fraction was examined by thin layer chromatography (TLC) by used ethyl acetate-chloroform=5:2 as developing solvent. Fractions containing similar pattern were combined together. The fraction which eluated with methanol was combined.

The methanol eluant part showed a predominant spot in UV 254 nm.This collected fraction (46.537 g) was subject to further column chromatographywith a mixture of ethyl acetate-chloroform (1:1) 1000 ml and 500 ml of each ratio of mixture was ethyl acetate-chloroform =10:9, 10:8, 10:7,...10:4, and 100 ml of fraction was collected. The predominant spot was still in the last collected fraction, which weighed 18.876 g.

This fraction was further purified by gel filtration using Sephadex® LH-20, with chloroform-methanol (1:1) as solvent. Fractions based on colour bands (approximately 30 ml each) were collected. Fractions showing similar pattern on TLC (chloroform:ethyl acetate=2:5) were combined.

Fractions 8-10 yielded white precipitate which was filtered from mother liquor and rinsed with methanol. It was recrystallized in chloroform-methanol (1:1) to give white amorphous plate (65.60 mg), code-named PPT8, Thin layer chromatography of PPT8 using chloroform-methanol (1:1) and ethyl acetate-methanol (9:1) as developing systems showed only single spot on silica gel plate, with anisaldehyde in sulfuric acid as spraying reagent. The total yield of PPT8 was 8.76 x 10^{-4}% of dry weight of *Moringa pterygosperma* Gaertn. stem bark.

After fraction 18th until obtained clear and diluted eluants. This fraction gave sticky yellow compound after concentrated. It was further purified by column chromatography, gave eluated first with chloroform toget rid of non-polar components then with methanol. the methanol eluant was examined by TLC using chloroform - methanol(1:1), ethyl acetate-chloroform(1:1), ethyl acetate-methanol(99:1), and benzene-acetone(1:9) as developing solvent, it showed only single spot on silica 60 F 254 plate. They gave Rf value 0.42, 0.14, 0.52, and 0.50 respectively. It was named J1, which 12.7314 g.

J1 was separated by high performance liquid chromatography (HPLC) techniques by used μ Bondapak™ C-18 column, and chloroform-methanol (1:1) as eluant. It showed 3 peaks in chromatogram (Figure 20). All spectroscopic data confirm those 3 peaks were, niazirin(4-[(6-deoxy-α-L-manopyranosyl)oxy] Benzeneacetonitrile, niazinin A([[4-[(6-deoxy-α-L-manopyranosyl)oxy]phenyl]methyl] -*O*-methyl Carbamothioate(*E*) and Carbonimidothioate(*E*)), niazimicin ([[4-[(6-deoxy-α-L-manopyranosyl)oxy]phenyl]methyl]-*O*-ethyl Carbamothioate(*E*) and Carbonimidothioate(*E*))

Characterization of isolated compound

1. Characterization of PPT8

PPT8 was crystallized from the mixture of methanol-chloroform (1:1) as white flakes. It was soluble in methanol, chloroform and very soluble in pyridine.

EIMS : m/z (relative intensity); Figure 5.
577 (M+,2.0), 399 (3.0), 382 (3.8), 254 (3.3), 212 (3.4) 163 (3.9), 147 (6.2), 144 (3.8), 141 (6.5), 118 (4.3), 44 (100.0)

IR : υ cm^{-1}, KBr disc; Figure 6.
3400, 2900, 1700, 1500, 1050, 900

^{1}H NMR : δ ppm, 500 MHz in pyridine -d_5; Figure 7-9.
0.65(3H,s) 0.80-1.48(m) 1.54(2H,m) 1.72(2H,m) 1.90(2H,m) 2.12(1H, m) 2.45(1H,td,*J*=11.3,13.3) 2.72(1H,ddd,*J*=14.8,4.6,2.1) 3.97(1H,m) 4.04(1H,t,6.1) 4.26(1H,m) 4.40(1H,dd,*J*=11.9,2.4) 5.04(1H,d,7.94) 5.34(1H,m)

^{13}C NMR : δ ppm, 125 MHz in pyridine -d_5; Figure 10.
12.0, 12.2, 19.0, 19.2, 19.4, 20.0, 21.3, 23.4, 24.5, 26.4, 28.6, 29.5, 30.3, 32.1, 32.2, 24.2, 36.4, 37.0, 37.5, 39.4, 40.0, 42.5, 46. 1, 50.4, 56.3, 56.9, 62.9, 71.7, 75.3, 78.2, 78.5, 78.6, 102.6, 121.9, 141.0

2. Characterization of J1

J1 was obtained as sticky yellow liquid. miscible with chloroform, methanol, acetone and dimethylsulfoxide.

UV: λ_{max} (Absorbance), in $CHCl_3$/MeOH mixture(1:1); Figure 11.

246 (0.535), 210 (0.237), 228 (0.235), 221 (0.230)

IR : υ cm^{-1}, liquid cell; Figure 12.

3360, 2950, 1730, 1600, 1500, 1400, 1250, 1050, 925, 825, 680

^{1}H NMR : δ ppm, 500 MHz in DMSO-d_6; Figure 13-16.

-niazimicin

1.09 (3H,d, *J*=6.1), 1.19(3H,t, *J*=7.1), 1.23(3H,t, *J*=7.1), 3.29 (1H,dt, *J*=5.8, 9.2), 3.47(1H,dq,*J*=9.2,6.1) 3.64 (ddd, *J*=3.0, 6.1, 9.2), 3.82 (1H,m), 3.99 (2H,q, *J*=7.0), 4.23 (2H,d, *J*=6.4), 4.38 (2H,q, *J*=7.0), 4.39 (2H,q, *J*=7.0), 4.56 (2H,d, *J*=6.1), 4.71 (1H,d, *J*=6.1), 4.85 (1H,d, *J*=5.8), 5.02 (1H,d, *J*=4.6), 5.34 (1H,overlapping), 6.98 (2H,d, *J*=8.6), 7.16 (2H,d, *J*=8.6), 7.22 (2H,d, *J*=8.6), 9.52(1H,t, *J*=6.1), 9.54(1H,t, *J*=6.4)

-niazinin A

1.09 (3H,d, *J*=6.1), 3.29 (1H,dt, *J*=5.8, 9.2), 3.47(1H,dq,*J*=9.2,6.1), 3.53(3H,s) 3.64 (ddd, *J*=3.0, 6.1, 9.2), 3.82 (1H,m), 3.87 (3H,s), 3.89 (3H,s), 4.24(2H,d,*J*=6.4), 4.57(2H,d,*J*=6.1), 4.71 (1H,d, *J*=6.1), 4.85 (1H,d, *J*=5.8), 5.02 (1H,d, *J*=4.6), 5.34 (1H,overlapping), 6.98(2H,d, *J*=8.6), 7.16(2H,d,*J*=8.6), 7.21(2H,d,*J*=8.6), 9.56(1H,t,*J*=6.1), 9.60 (1H,t,*J*=6.4)

-niazirin

1.09(3H,overlapping), 3.29(1H, overlapping), 3.48(1H, overlapping), 3.82(1H,overlapping), 3.64(1H, overlapping), 3.93(2H,s), 4.73(1H overlapping), 4.86(1H, overlapping), 5.03(1H,d,*J*=4.3), 5.37(1H, *J*=1.8), 7.04(2H,d,*J*=8.8), 7.26(2H,d,*J*=8.6)

^{13}C NMR : δ ppm, 125 MHz in DMSO-d_6; Figure 18.

14.07, 14.26, 17.80, 17.88, 17.98, 21.67, 45.18, 47.23, 47.50, 47.63, 57.32, 56.56, 65.33, 66.20, 69.47, 70.25, 70.51, 71.84, 98.40, 98.54, 116.33, 116.92, 124.39, 128.25, 128.65, 128.65, 128.76, 129.34, 131.36, 131.46, 131.51, 131.63, 155.12, 155.25, 155.51, 188.0, 190.08, 190.93

EIMS : For J1 mixture compound. m/z (relative intensity), Figure 19.

279 (M+,1.2), 147 (20.4), 133 (38.7), 116 (8.7), 91 (92.8), 65 (46.2)

For niazinin pure compound. m/z (relative intensity), Figure 20.

343 (M+,0.6), 197 (20.2), 182 (9.7), 181 (3.0) 165 (5.8), 164 (2.5) 147 (11.2), 129 (11.8), 121 (8.2), 107 (51.2), 89 (6.3), 77 (25.6)

For niazimicin pure compound. m/z (relative intensity), Figure 21.

357 (0.1), 211 (9.8), 195 (0.3), 182 (9.9), 164 (0.8), 147 (9.8), 129 (8.3), 111 (3.9), 107 (79.3), 106 (18.3), 95 (11.7), 77 (29.2), 59 (20.9), 57 (42.6)