CHAPTER IV

RESULTS AND DISCUSSION

Four diterpene lactones isolated from the leaves of *Andrographis paniculata* in this research as the standard compound for the analysis.

Each compounds was identified on the basis of chromatographic method, spectrophotometric methods, physical constant, as compaired to the authentic samples.

1. Identification of Isolated Diterpene Lactones

1.1 C-2 Compound

C-2 compound appears as a colorless fine needle crystal which were recrystallized from methanol.

Melting Point : 202-203 ^{o}C (from methanol)
(Ref.17 : 203-204 ^{o}C)

Thin-Layer Chromatography : The C-2 compound was detected with TLC using solvent system no 1-6. The Rf value results of C-2 compound were shown in Table 3, and Figure 2.

UV-Spectrophotometry : Absorption spectra of C-2 compound in ethanol was shown in Figure 3. The compound showed a maximum absorption wavelength at about 248 nm and

210 nm.

Infrared Spectrophotometry : The IR spectra of C-2 compound in KBr were shown in Figure 4. The analysis of the absorption peaks of compounds were demonstrated below.

3,300 cm^{-1} O-H stretching of hydroxy group

1,758 cm^{-1} C=O stretching of α,β-unsaturated-γ--lactone

1,640 cm^{-1} C=C strrtching of exocyclic methylene

900 cm^{-1} C-H (out of plane) stretching of exocyclic methylene

Mass Spectrophotometry : Mass spectrophotometric identification of C-2 compound was done on electron impact mass spectrometer (EIMS). Mass spectrum was shown in Figure 5. The most mass/charge (m/e) was 332.

Acetylation : Acetylation of andrographolide has been reported to proceed with elimination to give the deoxydidehydroandrographolide diacetate with mp 134-135 ^{o}C (17).

Acetylation of C-2 (AC-1) yield the acetylated product with mp 134-135 ^{o}C. Both acetylated compound from C-2 (AC-1) and andrographolide (AC-2)prepared were detected in TLC (solvent system no 1) and gave identical Rf value (see Figure 6).

The IR spectrum of both compound gave the

identical peak at : ν_{max} = 2980, 2940, 1758, 1649, 910 cm^{-1} (see Figure 7).

All these information suggested that C-2 compound is dehydroandrographolide.

1.2 C-3 Compound

C-3 compound appears as a colorless plates crystal which were recrystallized from methanol.

Melting Point : 229-230 °C

(Ref.37 : 230-231 °C)

Thin-Layer Chromatography : The C-3 compound was detected with TLC using solvent system no 1-6. The Rf value results of C-3 compound were shown in Table 3 and Figure 2.

UV-Spectrophotometry : Absorption spectra of C-3 compound in ethanol was shown in Figure 8. The compound showed a maximum absorption wavelength at about 223 nm.

Infrared Spectrophotometry : The IR spectra of C-3 compound in KBr were shown in Figure 9. The analysis of the absorption peaks of compound were demonstrated below.

3,400 - 3,300 cm^{-1} O-H stretching of hydroxy group

1,735 cm^{-1} C=O stretching of α,β-unsaturated-γ-lactone

1,680 cm^{-1} C=C stretching of conjugated C=C

1,650 cm^{-1} C=C stretching of exocyclic methylene

906 cm^{-1} C-H (out of plane) stretching of exocyclic methylene

Mass Spectrophotometry : Mass spectrophotometric identification of C-3 compound was done on electron impact mass spectrometor (EIMS). Mass spectrum was shown in Figure 10. The most mass/charge (m/e) was 350.

All these information suggested that C-3 compound is andrographolide. The comparison of IR spectrum between C-3 compound and andrographolide were shown in Figure 9. They are identical in Rf value, melting point, form of crystal, IR spectrum, and also mass spectrum.

1.3 C-4 Compound

C-4 compound appears as a colorless needles crystal which were recrystallized from methanol.

Melting Point : 166-167 ^{o}C (from methanol)
(Ref.39 : 167-168 ^{o}C)

Thin-Layer Chromatography : The C-4 compound was detected with TLC using solvent system no 1-6. The Rf value results of C-4 compound were shown in Table 3 and

Figure 2.

UV-Spectrophotometry : Absorption spectra of C-4 compound in ethanol was shown in Figure 11. The compound showed a maximum absorption wavelength at about 205 nm.

Infrared Spectrophotometry : The IR spectra of C-4 compound in KBr were shown in Figure 12. The analysis of the absorption peaks of compound were demonstrated below.

3,400 cm^{-1} O-H stretching of four hydroxy group

1,748 cm^{-1} C=O stretching of α,β-unsaturated-γ-lactone

1,640 cm^{-1} C=C stretching of exocyclic methylene

909 cm^{-1} C-H (out of plane) stretching of exocyclic methylene.

All these information suggested that C-4 compound is neoandrographolide. The comparison of IR spectrum between C-4 compound and neoandrographolide are shown in Figure 12. They are identical in Rf value, melting point, form of crystal, and IR spectrum.

1.4 C-5 Compound

C-5 compound appears as a colorless fine needle crystals which were recrystallized frommethanol.

Melting Point : 188-190 ^{o}C (from methanol)

(Ref.40 : 187-188 ^{o}C)

Thin-Layer Chloromatography : The C-5 compound was detected with TLC using solvent system no 1-6. The Rf value results of C-5 compound were shown in Table 3 and Figure 2.

UV-Spectrophotometry : Absorption spectra of C-5 compound in ethanol was shown in Figuer 13. The compound showed a maximum absorption wavelength at about 205 nm.

Infrared Spectrophotometry : The IR spectra of C-5 compound in KBr were shown in Figure 14. The analysis of the absorption peaks of compounds were demonstrated below.

3,500, 3,400 and 3,362	cm^{-1}	O-H stretching of hydroxy group
1,755	cm^{-1}	C=O stretching of α,β-unsaturated-γ-lactone
1,640	cm^{-1}	C=C streching of exocyclic methylene
900	cm^{-1}	C-H (out of plane) stretching of exocyclic methylene

All these information suggested that C-5 compound is deoxyandrographolide-19β-D-glucoside. The comparison of IR spectrum between C-5 compound and deoxyandrographolide-19β-D-glucoside are show in Figure 14. They are identical in Rf value, melting point, form of crystal, and IR spectrum.

2 Determination of Adherence to Beer's Law

Chromatographic Procedure

For quantitative HPLC analysis, linearity of detectors is one of the instrumental requirements. Since the peak height and peak area are proportional to the amount of compound eluted, then the relationship between peak height or peak area and concentration of compound should be adherence to Beer's law.

A plot of peak height and peak area versus the amount of dehydroandrographolide (C-2) was linear over the concentration rang of 0.10 to 0.97 mg/ml with correlation coefficient 0.9990 and 0.9709, respectively. The curve extrapolated through the origin with a negligible intercept as shown in Table 4 and Figure 17.

A plot of peak height and peak area versus the amount of andrographolide (C-3) was linear over the concentration range of 0.10 to 0.42 mg/ml with correlation coefficient 0.9998 and 0.9925, respectively. The curve extrapolated through the origin with a negligible intercept as shown in Table 5 and Figure 18.

A plot of peak height and peak area versus the amount of neoandrographolide (C-4) was linear over the concentration range of 0.10 to 0.30 mg/ml with correlation coefficient 0.9998 and 0.9886, respectively. The curve

extrapolated through the origin with a negligible intercept as shown in Table 6 and Figure 19.

A plot of peak height and peak area versus the amount of deoxyandrographolide-19β-D-glucoside (C-5) was linear over the concentration rang of 0.02 to 0.20 mg/ml with correlation coefficient 0.9991 and 0.9917, respectivly. The curve extrapolated through the origin with a negligible intercept as shown in Table 7 and Figure 20.

3 Determination of the Reproducibility of Peak Height and Peak Area

With the external standard method, absolute amounts are calculated from peak heights or peak areas found in the sample analysis and the corresponding response factors previously obtained from the standard solution. Since absolute amounts are measured, high precision of injection repeatability is required.

Data in Table 8 for dehydroandrographolide (C-2) was generated by running 5 analyses. The precision from peak height and peak area measurements in term of relative standard deviation are 0.89% and 3.76%, respectively. Better precision can be obtained when peak height was used.

Data in Table 9 for andrographolide (C-3) was generated by running 5 analyses. The precision from peak

height and peak area measurements in term of relative standard deviation are 0.80% and 0.48% respectively. Since the precision of peak area measurement was better, but both the precision of peak height and peak area were accepted by USP XXI. Peak height was used in calculation of the amount of andrographolide (C-3) in this thesis.

Data in Table 10 for neoandrographolide (C-4) was generated by running 5 analyses. The precision from peak height and peak area measurements in term of relative standard deviation are 1.27% and 4.81% respectively. Better precision can be obtained when peak hight was used.

Data in Table 11 for deoxyandrographolide-19β-D--glucoside (C-5) was generated by running 5 analyses. The precision from peak height and peak area measurements in term of relative standard deviation are 1.67% and 2.62%, respectively. Better precision can be obtained when peak height was used.

4 Determination of Four Diterpene Lactone Contents in the Leaves of *Andrographis paniculata*, by HPLC

Reproducibility and linearity studies revealed that the HPLC system developed are applicable for four diterpene lactone determination.

For all sample collected, the analysis revealed that the four diterpene lactones dehydroandrographolide, andrographolide, neoandrographolide and

deoxyandrographolide-19β-D-glucoside are major compound detected (see Figure 16).

The analysis revealed that dehydroandrographolide are in high quantity in the period of January to August. The most highest 7.30% (w/w) was observed in April. And during September to December, the amounts of this compound were low. The most lowest quantity 0.61% (w/w) was observed in December (see Table 12 and Figure 21).

From January to March, the amounts of andrographolide were low. The most lowest quantity 0.82 % (w/w) was observed in Febuary. After April, the amounts of them were rised and the most highest quantity 6.02 % (w/w) was observed in November (see Table 13 and Figure 22).

Quantitative examination of neoandrographolide in the crude powder was found that, the most lowest quantity 0.61 % (w/w) was observed in April. And the most highest quantity 2.02 % (w/w) was observed in December (see Table 14 and Figure 23).

From January to March, the amounts of deoxyandrographolide-19β-D-glucoside were rised and the most highest quantity 3.81 % (w/w) was observed in March. After March, the amounts were fasly reduced. During October to November, the quantity of this compound in the crude powder were too low to be determine (see Table 15 and Figure 24).