CHAPTER IV

RESULTS AND DISCUSSION

4.1. Collection and storage of plant sample

CA plant samples were collected during the second week of each month of the year 2003 (except in April) from two gardens (garden A and garden B) in Nakornpathom province, as shown in table 4.1.

For each 10 kg of fresh plant sample, obtained 1 kg of ground dried plant.

4.2. Extraction and Isolation of triterpene glycoside

A 300 g of ground dried herb was extracted and isolated of the triterpenoid glycoside as mention in the method as scheme 3.1 yielded the precipitate 4.936 g (1.65%).

4.3. Preparation of AS, AA, MS and MA as working standard

4.3.1 Preparation of AS and MS

Each 100 mg of powder A was isolated by column chromatography yielded 22 mg of AS (22%) and 25 mg of MS (25%).

4.3.2 Preparation of AA and MA

Preliminary investigation revealed that source of CA contain very small amount of the aglycone compounds (MA and AA), in addition, to analyze the content of interested aglycone compound, there must be enough amount of working standard of each interested aglycones. Therefore, the aglycones (MA and AA) were prepared by the transformation from the abundant glycosides (MS and AS) *via* a alkaline hydrolysis.

Each 2.5 g of powder A, after the alkaline hydrolysis, yield 1.4 g (56%), powder B.

The powder B, after isolation and purification from column chromatography, gave 20 mg (20%) AA and 39 mg (39%) MA.

4.3.3 Identification of the isolated triterpene glycoside and its aglycone

The isolated triterpene glycoside and its aglycone can be identified by both chromatographic techniques and the spectrophometric techniques.

Two chromatographic methods (TLC and HPLC method) were selected by comparing the R_f value and the retention time of the isolated compounds to the references standards.

4.3.3.1 TLC method

The R_f values of isolated MS (0.37), AS (0.48), MA (0.77) and AA (0.92) were corresponded to theirs standards, as shown in Figure 4.1.

4.3.3.2 HPLC method

The retention times of isolated MS (3.4 min), AS (5.4 min), MA (6.3 min) and AA (18.2 min) were corresponded to theirs standards, as shown in Figure 4.2.

4.3.3.3 Spectrophotometric methods

Two spectrophotometric methods (infrared spectrometry, and nuclear magnetic resonance spectrometry) were selected for identification of the isolated compounds.

4.3.3.3.1 Infrared spectrometry (IR)

Both glycosides, MS and AS, showed the characteristic absorption band in the IR spectrum as shown in figure 4.3 and 4.4; broad band at 3411 cm^{-1} for OH stretching, 1735 cm^{-i} for

ester carbonyl stretching, 1647 cm^{-1} for C=C stretching , 1064 cm^{-1} for C-O stretching.

Both aglycones, MA and AA, showed the characteristic absorption band in the IR spectrum as shown in figure 4.5 and 4.6; broad band at 3429 cm^{-1} for OH stretching, 1692 cm^{-1} for carboxylic carbonyl stretching, 1035 cm^{-1} for C-O stretching.

4.3.3.3.2 Nuclear magnetic resonance spectrometry (NMR)

^{1}H-NMR spectrum of MS and AS showed in figure 4.7 and 4.8 and ^{13}C-NMR spectrum of MS and AS showed in figure 4.11-4.12.

For ^{1}H-NMR spectrum (500 MHz) of the isolated AS, showed the signal in range 0.5-1.2 (four singlets and two doublets of six methyl group), 5.14 (triplet-like, 1H-12) and 2.10 (doublet, J = 10.98 Hz, 1H-18) indicating the presence of a Δ^{12}-ursene skeleton. The sugar part of the ^{1}H-NMR spectrum showed the three doublets of the anomeric protons (δ5.18, J = 5.8 Hz; 5.16, J = 8.24 Hz; 4.84, J = 4.88 Hz).

For ^{1}H-NMR spectrum (500 MHz) of the isolated MS, showed the signal in range 0.7-1.2 (four singlets and two doublets of six methyl group), 5.14 (triplet-like, 1H) and 2.10 (doublet, J = 10.98 Hz, 1H) indicating the presence of a Δ^{12}-

ursene skeleton. The sugar part of the ^{1}H-NMR spectrum showed the three doublets of the anomeric protons (δ5.15, J = 5.8 Hz; 4.25, J = 7.93 Hz; 4.85, J = 4.87 Hz). Furthermore, ^{1}H-NMR spectrum showed additional signal at 4.04 of –C<u>H</u>OH-(doublet of doublet, 1H).

For ^{13}C-NMR spectrum of the isolated AS and MS, showed the signal around 175 (-COOR), 137 and 124 (-C=C), 60- 100 (17 C of sugar and C-2 and C-23 of ursene skeleton)

^{1}H-NMR spectrum of MA and AA were shown in figure 4.9 and 4.10 and ^{13}C-NMR spectrum of MA and AA were shown in figure 4.13 and 4.14.

For ^{1}H-NMR spectrum (300 MHz) of the isolated AA, showed the signal in range 0.7-1.2 (four singlets and two doublets of six methyl group), 5.18 (triplet-like, 1H) and 2.12 (doublet, J = 10.98 Hz, 1H) indicating the presence of a Δ^{12}-ursene skeleton.

For ^{1}H-NMR spectrum (300 MHz) of the isolated MA, showed the signal in range 0.7-1.2 (four singlets and two doublets of six methyl group), 5.18 (triplet-like, 1H) and 2.10 (doublet, J = 10.98 Hz, 1H) indicating the presence of a Δ^{12}-ursene skeleton. Furthermore, ^{1}H-NMR spectrum showed additional signal at 4.04 of –C<u>H</u>OH-(doublet of doublet, 1H).

For ^{13}C-NMR spectrum of the isolated AS and MS,

showed the signal around 175 (-COOR), 137 and 124 (-C=C), 76 (C-C=0), 60-70 (3C-O).

4.3.3.3.3 Physical properties

Melting range of isolated compounds, found at 215-217 °C (MS), 231-232 °C (AS), 266-268 °C (MA) and 305-307 °C(AA). They were corresponded to literature review.

4.3.4 Determination of percentage purity of triterpene glycoside and aglycone as working standard

Percentage purity of isolated AS, MS, AA and MA were 101.3, 95.55, 93.09 and 93.98, respectively when calculated by comparing the peak area of the working standard with peak area of the reference standard.

4.4. Development of quantitative analytical method for the determination of AS, MS, AA and MA

4.4.1 Development of sample preparation

4.4.1.1 Extraction solvent and refluxing time

When refluxing the CA plant sample with 90 ml of 60%, 70% and 80% methanol in water for 3 hours, as shown in table 4.2. The mixture of 80 % methanol in water gave the highest amount of triterpenoids in the extract. Therefore, the mixture of 80% methanol in water was chosen as the extracting solvent. However, too more methanol may cause too more chlorophyll and other impurities in the matrix.

To determine the optimum refluxing time as shown in table 4.3 and figure 4.15, the area under the peak of each compound per weight of dried plant sample were increased with the increment of refluxing time and remain constant after refluxing the mixture more than 90 min.

Concluded, The optimum sample preparation was followed; five gram of dried ground sample was refluxed with 90 ml of 80% methanol in water for 90 min.

4.4.1.2 Clean up of sample preparation by solid phase extraction

SPE cartridge containing C18 stationary phase was chosen for clean up the sample preparation before injecting the sample to the HPLC system. To determine the solvent for washing out the impurity from the sample, it was found that only water and the mixture of 10% of acetonitrile-water could wash out impurities without eluting out the interested compounds and the internal standard. The mixture of 10% of acetonitrile-water was more effective solvent than water for washing out the impurities from the sample.

To determine the solvent for eluting the analyte, mixture of 45% acetonitile in phosphate buffer eluted all interested analyte with good recovery (97-103% recovery).

To determine the volume of the eluting solvent, three cycles of 1 ml of eluting solvent was more effective than eluting with the same single total volume (3 ml).

Figure 4.16 represented the HPLC chromatogram of the sample which had been passed through a clean-up process compared to the sample which had not been passed through the clean-up process.

4.4.2 Development of high-performance liquid chromatographic method (HPLC method)

4.4.2.1 Criteria of the method development

A mixture of acetonitrile and phosphate buffer was used as mobile phase because of the lower UV cutoff than 200 nm. A reversed phase HPLC using C-18 column with the mobile phase composed of acetonitrile and 10 mM, pH 7.1 phosphate buffer (29:71) at the flow rate 1 ml/min was found to be suitable for separation of the four compounds (MS, AS, MA and AA) as shown in figure 4.17. Because of resolutions of each interested peak were more than 2 and tailing factors of each interested peak were less than 2. Resolutions of these compounds were 3.63 (MS), 5.59 (AS), 2.11 (MA), 3.86 (PL) and 11.44 (AA). Tailing factor of all compounds were 1.39 (MS), 1.46 (AS), 1.32 (MA), 1.67 (PL) and 1.13 (AA). The numbers of theoretical plate were 2026 (MS), 2717 (AS), 4445 (MA), 4756 (PL) and 4434 (AA).

Figure 4.18 represented 3D chromatogram of standard solution and sample solution; where as the figure 4.19 represent the UV spectrum of reference and the sample compound. It was noticed that the UV spectrum of the standard and the sample

were identical in all analyte peaks.

4.4.2.2 Effect of stationary phase

Phenyl- column had also been studied for separation of MS, AS, MA and AA by using acetonitrile-phosphate buffer (10 mM, pH 6.2)(23:77) as mobile phase was optimized for separation of MS, AS, MA and AA as presentation in figure 4.20.

In general C18 column is popular and more durable than phenyl column, therefore by using acetonitrile-phosphate buffer (10mM, pH7.1) (29:71) as mobile phase was optimized for separation of these compounds as presentation in figure 4.17. The C18 column was chosen in this study.

4.4.2.3 Effect of mobile phase

4.4.2.3.1 Effect of ratio of organic solvent in buffer

The effect of mobile phase composition was investigated by increasing the organic modifier, acetonitrile form 28:72 to 29:71. Table 4.4 represented the retention time (t_R) and capacity factor (k') of each analytes upon changing the

solvent ratio from 28:72 to 29:71. Figure 4.21 showed the HPLC chromatogram effecting composition of mobile phase. It was noticed that increasing the modifier for 1% this effect the retention time of each analyte. The k' of MA, which is more polar than AA, was decreased more than k' of AA. It was also observed that increasing acetonitrile more than 29% caused the overlapping of MA peak to AS peak.

From the experiment, the exact ratio of organic modifier is critical for the resolution of the analyte. To small amount of acetonitrile may lengthen the analysis time due to the longer retention of the aglycone. In contrast, to more amount of acetonitrile, MA may overlap with other AS and MS.

4.4.2.3.2 Effect of concentration and pH of buffer

The effect of changing pH of buffer from 7.0 to 7.1 on the retention time of the analyte was observed as shown in figure 4.22. Table 4.5 represented the retention time and capacity factor of each analytes upon changing the pH of buffer from 7.0 to 7.1. It was observed that only MA was effected by increased the pH more than AA and the other compound.

From the experiment, the pH of buffer was critical factor for the resolution of the analyte. The pH of the buffer less than

7.0 might length the analysis time. The pH of the buffer more than 7.1 might cause overlapping of MA to AS. A more increasing pH of buffer to alkaline might cause deterioration of column by alkaline hydrolysis.

The effect of concentration of buffer was also investigated by using the mixture of acetonitrile and phosphate buffer (pH 7.1, 29:71) at concentration of buffer 10 and 20 mM as mobile phase. The figure 4.22.2 represented the chromatograms of changing concentration of buffer from 10 to 20 mM. It was observed that no effect was observed on the retention time of all analytes. Therefore the concentration of 10 mM of buffer was chosen, in order to minimize the precipitation of inorganic compound by the organic modifier.

4.4.2.4 Selection of internal standard

Of all the ten compounds tested for the internal standard, only the prednisolone showed a peak appearance in between the peak of MA and AA without interference the other compound, as shown in figure 4.21. Therefore, Prednisolone was chosen as internal standard.

4.4.3 Development of thin layer chromatographic method with densitometer (TLC method -densitometer)

TLC combined with densitometer was used to determine MS and AS in the CA plant sample by using chloroform-methanol-water (30:15:2) as developing solvent. Figure 4.24 represented TLC chromatogram of standard (MS and AS) and sample of CA extract in the TLC method.

0.2% anthrone reagent was used as spraying reagent. Because of the color intensity of spot chromatogram was stable when kept longer than 30 min at room temperature after heat at 110 °C for 10 min. While color intensity of spot, developed by Lieberman Burchard's reagent, was unstable. Figure 4.23 showed color faded diagram of color's spot versus time.

Chromatographic condition

TLC plate	Silica plate, GF 254, 10 x 20 cm
Developing solvent	Chloroform-methanol-water (30:15:2)
Detection	Spray with 0.2% anthrone, heat at 110 °C for 10 min and keep at room temperature for 30 min, Densitometer at wavelength 525 nm.

Densitometer parameter

Photo mode : Reflection

Scan mode : Linear

Set zero mode : At star

Beam size : 0.4 x 1.0 mm.

Wavelength : 525 nm

4.5.Method validation

4.5.1 Validation of HPLC method

Following ICH guideline, that recommended to test for 2 sections, method validation and system suitability test.

4.5.1.1 Method validation

Accuracy, precision, specificity, quantitative limit, linearity and range were considered in the method validation study.

4.5.1.1.1 Accuracy

The accuracy of an analytical method is the extent to which test results generated by the method and the true value

agree. The true for accuracy assessment can be obtained in several ways. One alternative is to compare results of the method with results from an established reference method. Secondly, accuracy can be assessed by analyzing a sample with known concentrations, for example, a certified reference material, and comparing the measured value with the true value as supplied with the material. If such certified reference material is not available, no blank-sample matrix of interest can be spiked with a known concentration by weight or volume, which is selected in this study. After extraction of the analyte from the matrix and injection into the analytical instrument, it's recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent.

Accuracy was tested in term of percentage recovery by addition of standard solution in various concentrations. Percent recovery of MS was in the range of 99.12-102.3 and average of percent recovery was 100.2. Percent recovery of AS was in range of 100.1-101.8 and average of percent recovery was 100.6. Percent recovery of MA was in range of 98.97-100.2 and average of percent recovery was 99.68. Percent recovery of AA was in range of 99.29-101.3 and

average of percent recovery was 100.1. As presented in Table 4.6. The accuracy test of this method was in the acceptance criteria (96-104 %R) so it could be used to determine the analytical compounds in CA plant sample.

4.5.1.1.2 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision is usually expressed as the variance, relative standard deviation (%RSD) or coefficient of variation of a series of measurements. Precision was considered at two levels: repeatability (intra-day precision) and intermediate precision (inter-day precision).

Intra-day precision

Relative standard deviation was calculated from five replications of sample preparation in one day at various concentrations. It found that %RSD of MS, AS, MA and AA were in the range of 0.38-1.79, 0.54-1.19, 1.41-2.04 and 0.86-1.78, respectively. Mean %RSD of MS, AS, MA and AA were 1.60, 1.21, 1.75 and 1.51,

respectively. As presented in Table 4.7. These relative standard deviation of intra-day precision were in the range of acceptance criteria (%RSD $\leq$ 2) so it could be used to determine the analytical compounds in CA plant sample.

Inter-day precision

Relative standard deviation was calculated from two replications of sample preparation for three days at various concentrations. It found that %RSD of MS, AS, MA and AA were in range of 0.68-1.42, 0.33-1.52, 0.80-1.81 and 0.57-1.12, respectively. Mean %RSD of MS, AS, MA and AA were 1.51, 1.35, 1.76 and 1.50, respectively. As presented in Table 4.8. These relative standard deviation of inter-day precision were in the range of acceptance criteria (%RSD $\leq$ 2) so it could be used to determine the analytical compounds in CA plant sample.

4.5.1.1.3 Linearity and range

Linearity should be evaluated by visual inspection of a plot of signal as a function of analyte concentration or content. If there is a linear relationship, test results should

be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data might be subjected to mathematical transformation prior to the regression analysis.

The linear equations of MS, AS, MA and AA were obtained as following; y = 1.011x-0.0013 for MS, y = 1.01x-0.005 for AS, y = 0.9957x+2E-05 for MA and y = x for AA. The coefficient of determination (r^2) for MS, AS, MA and AA were 0.9997, 1.000, 0.9999 and 0.9999, respectively. These r^2 showed a good relation of standard addition and amount of analytical found.

Ranges of concentration of MS, AS, MA and AA that show good linear correlation between concentration versus response with the acceptable accuracy (96-104 %R) and precision (%RSD $\leq$ 2) were 0.06-0.40, 0.05-0.33, 0.008-0.048 and 0.004-0.024 mg/ml, respectively as shown in Table 4.9 and Figure 4.25.

4.5.1.1.4 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

Figure 4.19 represented the scanned UV spectrum from photo-diode array detector of each compound in standard solution and sample solution. Spectrums of each compound in sample solution were identical to spectrum in standard solutions.

4.5.1.1.5 Quantitation limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be quantitatively determined with acceptable precision and accuracy.

Quantitation limit of MS, AS, MA and AA were resulted 5.97, 1.84, 2.11 and 2.32 µg/ml, respectively with 10 times signal to noise ratio. Percent relative standard deviation of five replications was less than 3. As shown in table 5.2.

4.5.1.2 System suitability test

Four parameters (precision, number of theoretical plates, tailing factor and resolution) were determined the system suitability test. The table 4.10 represented data from the system suitability test.

Percentage relative standard deviation (%RSD) from the precision determination of five replications of standard solutions, were 0.89 (MS), 0.63 (AS), 1.75 (MA), 1.34 (PL) and 0.87(AA). This precision of system was passed the system suitability test because %RSD of all interested peaks was less than 2.

The average of number of theoretical plates of all peaks were 2026 (MS), 2717 (AS), 4445 (MA), 4434 (PL) and 4750 (AA). Because of the numbers of theoretical plates were more than 2000, this system was passed systems suitability test.

Tailing factor of all compounds were 1.39 (MS), 1.46 (AS), 1.32 (MA), 1.67 (PL) and 1.13 (AA). Resolution for all compounds were 3.63 (MS), 5.59 (AS), 2.11 (MA), 3.86 (PL) and 11.44 (AA). This system was passed the system suitability test because tailing factor of all interested peaks was less than 2.

4.5.2 Validation of TLC-Densitometer method

Accuracy, precision, linearity and range were considered in the method validation study that presented in table 4.11.

4.5.2.1 Accuracy

Percentage recovery of analytical method of MS was in range of 98.35-100.8 (average at 99.81). Percentage recovery of AS was in range of 96.19-101.3 (average at 99.18). The percentage recoveries of two compounds were in acceptance criteria (96-104 %R) so this method was used to determine the MS and AS in CA sample plant.

4.5.2.2 Precision

Percentage relative standard deviation (%RSD) of analytical method of MS from five replications was in range of 0.79-1.47 (average at 1.07). Percentage relative standard deviation (%RSD) of analytical method of AS from five replications was in range of 0.70-1.43 (average at 1.18). Because of Percentage relative standard deviation (%RSD) was in acceptance criteria (%RSD $\leq$ 2), this method could be used

to determine the MS and AS in CA plant sample.

4.5.2.3 Linearity and range

The coefficients of determination (r^2) of MS and AS were 0.9995 and 0.9997, respectively. These r^2 showed a acceptable linearity ($r^2 \geq 0.99$) so this method was used as analytical method. As shown in figure 4.25 and table 4.11.2.

MS and AS that were good relation between amount and response (linearity), accuracy and precision were in range of 1.08 - 21.60 and 0.78 - 15.60 µg respectively.

4.6.Determination of the content of MS, AS, MA and AA in *CA.*

4.6.1 HPLC method

Percentage of content of MS, AS, MA and AA in CA dried plant sample in an annual period were shown in Table 4.12 and Figure 4.26.

MS in source A was found in the range of 0.313 – 1.664 % with average at 0.950 %. The maximum content was observed in June and the minimum content was observed in February. In source B, MS was found in the range 0.149 – 1.941 % with average at 1.031 %. The maximum content

was observed in May and the minimum content was found in February.

AS in source A was found in the range of 0.291 – 1.742 % with average at 0.889 %. The maximum content was observed in May and the minimum content was found in February. In source B, AS was observed in the range of 0.140–1.341 % with average at 0.745 %. The maximum content was in July and the minimum content was found in February.

MA in source A was found in the range 0.007– 0.411 % with average at 0.149 %. The maximum content was observed in February and the minimum content was found in January. In source B, MA was found in the range 0.005 – 0.260 % with average at 0.109 %. The maximum content was observed in February and the minimum content was found in December.

AA in source A was found in the range 0.013 – 0.336 % with average at 0.115 %. The maximum content was observed in November and the minimum content was found in January. In source B, AA was found in the range 0.014 – 0.164 mg % with average at 0.065 %. The maximum content was observed in February and the minimum was found in January.

To determined the content of interested compounds in leaves and stems. Leaves contain each of four active components (MS, AS, MA and AA) 7-24 times more than in stems. In leaves, MS, AS, MA and AA were found in the range of 0.919-1.552%, 0.712-1.006%, 0.027-0.309% and 0.120-0.455%, respectively. In stems, MS, AS, MA and AA were found in the range of 0.029-0.062%, 0.069-0.098%, 0.007-0.010% and 0.008-

0.058%, respectively. As represented in Table 4.12 and Figure 4.27.

The developed methods were also applied to analyze five extract samples as shown in table 4.13. The extract powders contained 37.12-47.60% of MS and 31.56-41.41% of AS, respectively.

Two lots of the CA extract also subject to the stability program. The samples were kept in a stability control chamber at 50 °C and 75%RH for three months. The contents of MS and AS were determined every month as shown in table 4.14. It was observed that the percentage content of MS and AS at each times were insignificantly difference throughout the program. These mean the extract is stable under this condition not less than 3 month and the intensive shelf life of the extract powder is 2 years.

4.6.2 TLC method

MS in source A was found in the range 0.321 – 1.660% with average at 0.876 %. The maximum content was observed in June and the minimum content was found in February. In source B, MS was found in the range 0.258 – 1.678% with average at 0.759 %. The maximum content was found in May and the minimum content was observed in February.

AS in source A was found in the range 0.241 – 1.541% with average at 0.965 %. The maximum content was found in May and the minimum content was observed in February. In source B, AS was found in the range 0.205 – 1.239% with average at 0.733 %. The maximum content was found

in July and the minimum content was observed in February.

As presented in Table 4.15 and Figure 4.28.

4.6.3 Comparison of percent content of MS and AS between HPLC and TLC method

Figure 4.29 represented correlation results of percentage content of MS and AS in CA extract from 2 sources by HPLC and TLC method. It was found the percentage content of each compound from 2 sources were well correlated more than 85%. So, TLC method may be used instantly HPLC method for determination of MS and AS. Because of HPLC method was more complicate and expensive than TLC method, but it was more accurate, precise and sensitive than TLC method.