CHAPTER IV

RESULTS AND DISCUSSION

4.1 Extraction and isolation of triterpene glycosides

A 2 kg of ground dried CA, collected from the commercial crops of Nakhon Pathom province was extracted and isolated of the triterpene glycosides as mention in the method as Scheme 3.1. The extract was crystallized with ethyl acetate to obtain 20 g (1%, w/w) the yellowish crystal. After determined by the HPLC, it was found that crystal consisted mainly AS (38.8%) and MS (42.9%) as shown in Figure 4.2.

4.2 Preparation of MS, AS, MA and AA

4.2.1 Optimization of conditions for separation of AS and MS

4.2.1.1 Solubility of AS and MS

Both AS and MS compounds can be dissolved in methanol, ethanol, 2-propanol, pyridine, propylene glycol, dimethylformamide, and dimethylsulfoxide. However, both compounds were insoluble in water.

According to their chemical structures, both compounds

have a different on the number of hydroxyl groups. The chemical structure of MS has more hydroxyl groups than AS; thus can be dissolved in the polar-solvent better than the other. The solubilities of MS and AS in methanol were found to be 29.0 mg/ml and 19.3 mg/ml, respectively.

4.2.1.2 Effect of solvent polarity on separation

In this study, AS and MS were dissolved in methanol, and separated by fractional crystallization using drowning-out technique. Several miscible solvents such as acetone (0.56, ε^{*}), ethyl acetate (0.58, ε^{*}) acetonitrile (0.65, ε^{*}) and 2-propanol (0.82, ε^{*}) have also been studied as the drowning-out solvent.

From the data obtained, it was found that some solvents such as acetone, ethyl acetate and acetonitrile can be used as the drowning-out solvent. However, the polar solvent, 2-propanol can not be used as the drowning-out solvent. Although the non-polar solvents can be used to force the drowning-out, there were not separated AS and MS from the extracts.

The crystals obtained by using acetone and ethyl acetate as the drowning-out solvent were found to be the mixture of AS and MS. Therefore more polar solvent than these solvents but less polar than methanol may be used to separate both compounds.

Finally, acetonitrile was chosen as the drowning-out solvent, in order to separate AS and MS from the extracts by fractional

crystallization.

4.2.1.3 Effect of ratio of solvent on fractional crystallization

AS and MS from the extracts were separated by fractional crystallization, with the solvent ratio of methanol and acetonitrile.

The effect of drowning-out solvent was investigated by increasing the ratio of acetonitrile to methanol, acetonitrile form 0.5:1 to 2:1. It was found that increasing acetonitrile ratio this effect the separation of AS from mixtures. AS can be separated first when using acetonitrile: methanol at least as ratio 0.5:1. When AS was completely separated, MS was found as the major content in the residual filtrate.

According to their chemical structures and solubility, MS is more polarity than AS; and thus be separated last. In this study, it was observed that MS can be separated from the extracts with the increase of amount of acetonitrile as ratio 2:1. However, it was also noted that using acetonitrile solvent as ratio more than 2:1 on first steps of crystallization caused the contamination of MS in AS separation.

Conclusively, the separation of AS and MS from the extracts was based on the solubility of each compound due to the different of their chemical structures.

From the experiment, the exact acetonitrile to methanol ratio of 0.5:1 was considered as the optimal ratio of solvent for the fractional crystallization process.

4.2.2 Preparation of AS and MS

Each 100 mg of triterpenoid glycosides was isolated to obtain AS and MS by fractional crystallization as mention in the method as Scheme 3.2.

Finally, AS fraction and MS fraction were purified by recrystallization with acetonitrile-methanol (ratio0.5:1), which yielded 23.5 mg (%R=78) of AS and acetonitrile-methanol (ratio2:1), which yielded 20 mg (%R=67) of MS, respectively.

4.2.3 Preparation of AA and MA

Preliminary investigation revealed that source of CA contain very small amount of the aglycone compounds (MA and AA). Therefore, the aglycones (MA and AA) were prepared by the transformation from the abundant glycosides (MS and AS) *via* an alkali hydrolysis.

The alkali hydrolysis was performed as mention in the method as Scheme 3.3. Pure AA and MA were obtained by recrystallization with methanol, which yielded 1.14 g (%R=89.6) of AA and 0.82 g (%R=63.1) of MA, respectively.

4.2.4 Identification of the isolated triterpene glycoside and its aglycone

The isolated triterpene glycoside and its aglycone can be identified by both chromatographic and spectrophotometric 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.

4.2.4.1 TLC method

TLC method was performed to screen the isolated MS, AS, MA and AA by the system as mention in 3.2.3.1

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

4.2.4.2 HPLC method

HPLC method was performed to confirm the isolated MS, AS, MA and AA by the system as mention in 3.2.3.2

The retention times of isolated MS (3.1 min), AS (4.9 min), MA (6.7 min) and AA (19.3 min) were corresponded to theirs standards and based on their polarities, as shown in Figure 4.3-4.4.

4.2.4.3 Spectrophotometric methods

Three spectrophotometric methods (UV-spectrophotometry,

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

4.2.4.3.1 UV-spectrophotometry

Both separated glycosides (MS and AS), and their aglycones (MA and AA) were identified the purity by the HPLC method as in 3.2.3.2. In this study, each chromatogram was obtained the UV-spectrum by the UV-detector of HPLC instrument. The maximum UV absorbances of MS, AS, MA and AA were 205.6, 205.0, 205.2 and 206.2 nm, respectively as shown in Figure 4.5 (a-d).

4.2.4.3.2 Infrared spectrometry (IR)

Both glycosides, MS and AS, showed the characteristic absorption band in the IR spectrum as shown in Figure 4.6 and 4.7; broad band at 3411 cm^{-1} and 3401 cm^{-1} for OH stretching, 1726 cm^{-1} and 1735 cm^{-1} for ester carbonyl stretching, 1637 cm^{-1} for C=C stretching, 1067 cm^{-1} and 1065 cm^{-1} for C-O stretching, respectively.

Both aglycones, MA and AA, showed the characteristic absorption band in the IR spectrum as shown in Figure 4.8 and 4.9; broad band at 3429 cm^{-1} and 3418 cm^{-1} for OH stretching,

1694 cm^{-1} for carboxylic carbonyl stretching, 1048 cm^{-1} and 1050 cm^{-1} for C-O stretching, respectively.

4.2.4.3.3 Nuclear magnetic resonance spectrometry (NMR)

^{1}H-NMR spectrum of MS and AS showed in Figure 4.10 and 4.11 and ^{13}C-NMR spectrum of MS and AS showed in Figure 4.14 and 4.15, respectively.

For ^{1}H-NMR spectrum (300 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 (300 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-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 (δ5.15, 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 4.12 and 4.13 and ^{13}C-NMR spectrum of MA and AA were shown in Figure 4.16 and 4.17, respectively.

For ^{1}H-NMR spectrum (300 MHz) of the isolated AA, showed the signal in range 0.5-1.2 (four singlets and two doublets of six methyl group), 5.13 (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), 5.14 (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 AA and MA, showed the signal around 178 (-COOR), 138 and 124 (-C=C), 76 and 60-70 (C-O), and 48 (<u>C</u>-C=O).

4.2.4.4 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 **(11, 58)**.

4.2.5 Determination of chromatographic purity of triterpene glycoside and aglycone as working standard

Chromatographic purity of isolated AS, MS, AA and MA were 96.02, 95.55, 96.22 and 95.25, respectively when compared the peak area of these substances to peak area of their standards and calculated by the HPLC software.

4.2.6 Accelerated stability of isolated AS, MS, AA and MA

From the data obtained, AS, MS, AA and MA remain unchanged throughout the stability program, which performed under accelerated condition (45 °C and 75 %RH) during 4 months. The degradation products were not found in each sample by detected with the photodiode array of HPLC system as in 3.2.3.2.

The remained percentage contents of these compounds were calculated as shown in Table 4.1.

4.2.7 Stability of AS and MS in sample solution

AS and MS were spiked into the mixture of acetonitrile and phosphate buffer solution as ratio (29: 71) as the mobile phase of HPLC condition prior to quantitative determination. These samples were evaluated for stability program as followed; under the room temperature, cooling condition in refrigerator (4°C) and the autosampler of HPLC instrument.

From the data obtained, AS and MS in sample solution remain unchanged throughout the stability program as followed; under the room temperature and cooling condition (4°C) during 24 hours, and the autosampler of HPLC instrument during 8 hours. The degradation products were not found in each sample by detected with the photodiode array of HPLC system as in 3.2.3.2.

The stability of these compounds was revealed as the remained percentage contents as shown in Table 4.2 (a-c).

4.3 Development of extraction methods

4.3.1 Heat reflux extraction

Heat reflux extraction was performed by using 80 % methanol in water as the extracting solvent **(58)** and the temperature of water bath was controlled at 70°C during the extraction. The triterpene contents were shown in Table 4.3 and Figure 4.18 (a-e).

The optimum condition of reflux was followed; five gram of dried ground sample was extracted with 90 ml of 80% methanol in water for 60 min.

4.3.2 Development of ultrasonic-assisted extraction (UAE)

4.3.2.1 Optimization of UAE condition

The mixture of 80 % methanol in water gave the highest amount of triterpenoids in the extract **(58)**. Therefore, the mixture of 80% methanol in water was chosen as the extracting solvent. However, too long extraction time by refluxing may cause the degradation of triterpene glycosides in the sample matrix; thus the other extraction methods such as UAE were developed.

The ultrasonic bath was performed to extract and carried out with the three series of 10%, 50% and 100% of power at 70°C to determine the optimum extraction time versus the extraction power as shown in Table 4.4 (a-d) and Figure 4.19 (a-d). The area under the peak of each compound per weight of dried plant sample were increased with the increment of extraction time and remain constant after extracting the mixture more than 3 min. The maximum percentage contents of MS, AS, MA and AA were reached 3.34, 2.83, 0.42 and 0.14 %w/w, respectively. Although the maximum power (100%) was increased the cavitation effect during the extraction; thus should be obtained the highest extraction yields with shortened times but it was obtained the less contents of MS, AS, MA and AA than the

50% of power. However, the percentage contents of MS, AS, MA and AA that obtained by 10%, 50% and 100% of UAE power were not significantly difference at 90% Confidence Interval (p-value>0.1).

Therefore, the optimum UAE condition was followed; five gram of dried ground sample was extracted with 90 ml of 80% methanol in water for 3 min using the 50% of ultrasonic power at 70°C.

4.3.2.2 Effect of ultrasound on the active compounds

When the extraction was performed by using a high frequency of ultrasound, the extraction time was decreased significantly. However, the degradation of the active contents may be occurred.

The stability of AS and MS standards during the UAE was studied and calculated in term of percentage contents and recovery as shown in Table 4.5.

The result showed that the frequency of ultrasound do not induced degradation during the extraction. The photodiode array chromatograms of AS and MS standards during approached an ultrasound do not show any degradation product as shown in Figure 4.21.

4.3.3 Development of microwave-assisted extraction (MAE)

4.3.3.1 Optimization of MAE condition

MAE was performed to extract the triterpene compounds of CA by using focused-microwave assisted extraction (FMAE), which operated the extraction at the boiling point of the solvents. In order to investigate the effects of microwave on the compounds yields and different irradiation times. The experiment was performed with 80% methanol in water, which produced the best components yields with reflux. The results of extraction yields were shown in Table 4.6 and Figure 4.20 (a-e). From the data obtained, the maximum percentage contents of MS, AS, MA and AA were reached 3.29, 2.95, 0.43 and 0.14 %w/w, respectively.

The optimal extraction condition for active triterpene compounds in CA was succeeded irradiation within 3 min.

4.3.3.2 Effect of microwave on the active compounds

Generally, MAE was elevated temperatures within shorter times and improved extraction efficiencies. However, for the extraction of thermolabile compounds, high temperatures that produced by microwave may cause the degradation of extracts. Therefore, the optimal extraction was set by the boiling point of solvent at atmospheric pressure using the FMAE.

The stability of AS and MS standards during the FMAE was studied and calculated in term of percentage contents and recovery as shown in Table 4.7.

Finally, the short extraction time required by FMAE, allowed a good extraction of active triterpene compounds in CA without any degradation problem as shown as the photodiode array chromatograms of AS and MS standards in Figure 4.22.

4.4 Determination of MS, AS, MA and AA contents in various CA samples

4.4.1 Collection and storage of CA samples

CA plant samples were collected on July, September and November of the year 2005 from 12 accessions, cultivated by Thailand Institute of Scientific and Technological Research, as shown in Table 3.4.

CA plant samples were collected during the second week of each month of the year 2005-2006 from the commercial crops in Nakhon Pathom province, which labeled as CA13 as shown in Table 3.5.

CA plant samples were collected during the second week of each month of the year 2006-2007 from the commercial crops in Ubon Ratchathani and Nakhon Si Thammarat province, as shown in Table 3.5.

Furthermore, CA plant samples were collected during their life cycle as followed; 7 days, 14 days, 20 days and 28 days for determined the content profiles of MS, AS, MA and AA.

Finally, each 5 kg of collected fresh CA sample, obtained 500 mg of ground dried plant.

4.4.2 Optimization of SPE condition prior to HPLC determination

SPE cartridge (Bond-elute C18) was chosen as the stationary phase for clean up the sample preparation before injecting the sample onto the HPLC system. It was found that the mixture of 10% of acetonitrile-water could wash out impurities without eluting out the triterpenes and the internal standard.

The optimal solvent for eluting the triterpenes and the internal standard was followed; 3 ml of the mixture of 45% acetonitile in phosphate buffer. Next, eluting with six cycles of 0.5 ml in the vacuum manifold was more effective than eluting with the same single total volume (3 ml).

4.4.3 Contents of MS, AS, MA and AA in various CA

In this study, the 12 accessions of CA sample that collected from various locations of Thailand and cultivated by Thailand Institute of Scientific and Technological Research were prepared to determine the contents of their active compounds.

Each CA dried plant sample was quantitative determined for MS, AS, MA and AA by the HPLC system as in 3.2.3.2 and the percentage contents of these compounds were shown in Table 4.8 and Figure 4.23 (a-e).

From the data obtained, MS in each CA dried sample was found in the range of 1.988 – 5.477 % with average at 4.22 %. The maximum content was observed in CA11 that collected from Phitsanulok province and the minimum content was observed in CA2 that collected from Ban Bo Lo (local growth), Nakhon Si Thammarat province.

AS in each CA dried sample was found in the range of 1.471 – 3.474 % with average at 2.577 %. The maximum content was observed in CA6 that

collected from Rayong province and the minimum content was found in CA2 that collected from Ban Bo Lo (local growth), Nakhon Si Thammarat province.

MA in each CA dried sample was found in the range of 0.021– 0.915 % with average at 0.179 %. The maximum content was observed in CA10 that collected from Chiang Mai (green petioles) and the minimum content was found in CA8 that collected from Pak Chong, Nakhon Ratchasima province.

AA in each CA dried sample was found in the range of 0.026 – 0.391 % with average at 0.115 %. The maximum content was observed in CA4 that collected from Trat province and the minimum content was found in CA8 that collected from Pak Chong, Nakhon Ratchasima province.

Although, 12 accessions of CA in this study were observed the variation in the contents of MS, AS, MA and AA. However, the difference of CA sampling time that collected during on July-November 2005 was also noted.

4.4.4 Contents of MS, AS, MA and AA in annually study

In this study, CA samples that collected annually from the commercial crops of Nakhon Pathom province (CA13), Ubon Ratchathani province (CA3) and Nakhon Si Thammarat province (CA5) were also determined and evaluated the content profiles of their active compounds.

Each CA 13, CA3 and CA5 that collected annually was quantitative determined for MS, AS, MA and AA by the HPLC system as in 3.2.3.2 and

the percentage contents of these compounds were shown in Table 4.9, 4.10 and 4.11, and Figure 4.24 (b), 4.25 and 4.26, respectively.

From the data obtained, the contents of active compounds that observed in CA13 were followed; MS (0.529-1.921 %), AS (0.378-1.551 %), MA (0.628-1.090 %) and AA (0.645-0.959 %). The maximum content of MS and AS was observed in sample that collected on May and the minimum content was observed in sample that collected on September. However, the variation of aglycone contents was also noted. The maximum and the minimum content of MA were observed in sample that collected on May and January, respectively. The maximum and the minimum content of AA were observed in sample that collected on March and July, respectively. As presented in Figure 4.27 (a), 4.28 (a), 4.29 (a) and 4.30 (a).

The contents of active compounds that observed in CA3 were followed; MS (0.443-1.766 %), AS (0.306-1.761 %), MA (0.151-0.645 %) and AA (0.173-0.475 %). The maximum content of MS and AS was observed in sample that collected on March and the minimum content was observed in sample that collected on November. The maximum content of MA and AA was observed in sample that collected on March and July, respectively. The minimum content of MA and AA was also observed in sample that collected on September. As presented in Figure 4.27 (b), 4.28 (b), 4.29 (b) and 4.30 (b).

The contents of active compounds that observed in CA5 were followed; MS (0.551-2.309 %), AS (0.416-1.997 %), MA (0.284-1.354 %) and AA (0.219-1.534 %). The maximum content of MS and AS was observed in sample that collected on May and the minimum content was observed in sample that collected on January. The maximum content of MA and AA was

observed in sample that collected on January and the minimum content was observed in sample that collected on March. As presented in Figure 4.27 (c), 4.28 (c), 4.29 (c) and 4.30 (c).

The content profiles of CA13 and CA5 were also found the maximum contents of glycosides in sample that collected on May as presented in Figure 4.27 and 4.28. However, the maximum content of glycosides of CA3, CA5 and CA13 that collected from original sources and presented in annually in this study was lower than other CA samples that cultivated by Thailand Institute of Scientific and Technological Research.

4.4.5 Comparison of percent contents of MS, AS, MA and AA in annually study between the years

Bungon Kongthong **(58)** reported the percentage contents of MS, AS, MA and AA in CA samples that collected annually on the year 2003 from the commercial crop of Nakhon Pathom province as shown in Figure 4.24 (a)

In this study, the contents of MS, AS, MA and AA of CA sample that collected annually on the year 2006 from the commercial crop of Nakhon Pathom province were determined as shown in Table 4.9 and Figure 4.24 (b) and the variation of triterpene contents between the year 2003 and 2006 was investigated and also compared.

From the data obtained, there were similar maximum contents of both glycosides in CA sample that collected on May between the year 2003 and 2006 (no significantly different; 95% Confidence Interval, p-value>0.05) but there were differences in minimum contents of both glycosides between the

year 2003 and 2006. The minimum contents of both glycosides on the year 2003 and 2006 were observed in CA sample that collected on February and September, respectively. However, the variation of both aglycones was also noted.

4.4.6 Contents of MS, AS, MA and AA during the growth period of CA

CA3 (Ubon Ratchathani), CA5 (Nakhon Si Thammarat) and CA6 (Rayong) that collected from Thailand Institute of Scientific and Technological Research were also determined the contents of MS, AS, MA and AA during their growth period as followed; 7 days, 14 days, 20 days and 28 days.

From the data obtained, the glycosides contents of CA3, CA5 and CA6 were perceptible increased to reach the maximum follow their growth period as presented in Table 4.12 (a-c) and Figure 4.31 (a-c). The maximum glycosides of CA3 were found in sample that collected on 20 days and the maximum glycosides of CA5 and CA6 were found in sample that collected on 28 days. The maximum aglycones of CA3 were found in sample that collected on 20 days and the maximum aglycones of CA5 and CA6 were found in sample that collected on 28 days.

MS and AS contents in whole plant of CA that observed on 7 days and 28 days were significantly difference at 95% Confidence Interval (p-value<0.05). However, MS and AS contents that observed on 20 days and 28 days were not significantly difference at 95% Confidence Interval (p-value>0.05).