CHAPTER 3

MATERIAL AND METHODS

3.1 General Procedures

Three accessions of Bua Bok, namely Nakhon Si Thammarat, Rayong and Ubon Ratchathani available in different locations in Thailand were used in this study. The characteristics of three accessions of Bua Bok are described in Figure 3.1.

Nakhon Si Thammarat accession had light green, big and thickest blades of all others, wavy leaf margin and light green midribs. Its short petioles were also green light as its stolon while the fibrous roots were 8-10 cm. long.

Rayong accession had small, dark green blades, short and light green petioles and green and reddish purple stolons. The 6-8 cm. long fibrous roots were the biggest of all others.

Ubon Ratchathani accession had medium, dark green blades, small, long and light green petioles, reddish purple stolon and 6-8 cm. long fibrous roots.

All three Bua Bok accessions were experimentally grown in the organic farming system at TISTR using their stolons to propagate. 2x2 sq. m. land plots were used for plantation with medium fertility and fine soil of pH 4.5. These experiment land plots of 20x20 cm. plantation spacing were isolated from chemical-used plantation. All experiments were carried out during November, 2007 to September 2008. All Bua Bok accessions in the experiments were regularly watered twice daily (in the morning and the evening) using natural water source nearby. The removal of weeds was conducted by human labour

meaning no chemicals interfered in the study. All necessary climatic data such as temperature, relative humidity and precipitation in the experimental areas were recorded. The trial was planned according to RCBD to explore information about growth, yield, and the quantity of asiaticoside in all Bua Bok accessions. The experiments were divided into 3 main parts as follows :-

Nakhon Si Thammarat accession Rayong accession Ubon Ratchathani accession

Fig. 3.1 The characteristic of three accessions of Bua Bok

3.2 Experiment I Effect of Leaf Maturity on Asiaticoside and Nutritional Content in Bua Bok

This experiment was commenced in March, 2008, using Bua Bok stolons of three accessions from the organic farming system. The growing conditions such as size, type, and soil fertility were treated as described in 3.1 in which three Bua Bok accessions were grown under full sun light. After Bua Bok leaves had emerged from soil, their leaves (the blade and petiole parts) were collected at the ages of 7, 14, 21, and 28 days. Then, the leave samples were cleaned to remove some diluted substances and dried in the shaded area for 24 hrs which was followed by drying by using incubation oven at 50°C for the next 24 hrs. The dried leave samples were then used for the analyses of the quantity of fiber, amounts of protein, calcium, beta-carotene and asiaticoside

Fig. 3.2 The character of leaf at count for day one after emerging of Bua Bok

3.3 Experiment II Effect of Light Conditions During Growing on Asiaticoside and Nutritional Contents in Bua Bok

This experiment was commenced in August, 2008 whereas the same growing conditions were also treated as in 3.1 and 3.3, only light intensity was completely differentiated. Three Bua Bok accessions were cut from the organic farming system and grown zero (full sun light), 50, and 80% black photo selective shading cloths which were fixed at 1.5 m. high above the ground and 1 m. long for each projecting part. The intensities of light under these black photo selective shading cloths were measured using Luxmeter which were 933.07, 362.55 and 93.30 $\mu mol/m^2/s$ from 0, 50, and 80% shading cloths, respectively. After the leaves had emerged from soil, Bua Bok plants were harvested at 28-35 days of age, cleaned with tap water to remove dirt, soil and other solid particles. The fresh weights of all Bua Bok plant samples were taken while leaf and petiole sizes were determined. Then, the leaves were dried in incubation oven at 50°C for 24 hrs. The

dried samples were used for the analyses of fiber, protein, calcium, beta-carotene and asiaticoside contents.

3.4 Experiment III The Influence of Time and Temperature on The Storage of The Asiaticoside Content of Bua Bok

This experiment was also commenced in August, 2008 with the same growing conditions as previously described in 3.1, 3.3 and 3.4. Alternately, only 2 Bua Bok accessions of Nakhon Si Thammarat and Ubon Ratchathani were cut from the organic farming system and used in this experiment. The maturity leave and petiole parts of Bua Bok accessions were collected, cleaned in tap water, then, dried in the shaded area for 24 hrs, followed by drying in incubation oven at 50°C for another 24 hrs. The dried Bua Bok samples kept in plastic bags were stored at 4°C and room temperature for one and four months. Then, the asiaticoside contents of dried Bua Bok samples were analyzed at these various storage conditions of temperature and time. The CRD with three replicates were performed in this experiment.

3.5 Analysis of Parameters

3.5.1 Leaf area

The Bua Bok leaf areas were determined using leaf area meter LI-3100, LI-COR Inc., USA and reported in square centimeter.

3.5.2 Petiole length

The petiole length of Bua Bok was measured using calibrated ruler and reported in centimeter.

3.5.3 Chlorophyll content

The chlorophyll contents of Bua Bok samples were analyzed by using chlorophyll extraction technique following AOAC method (1990) as following. 1 g of fresh Bua Bok leaf was totally ground in a mortar with a pestle, and then, extracted in 10 ml. of 80% acetone solution. The extracted solution was filtered through Whatman No. 1 filter paper and 80% acetone was gradually poured onto the filter paper till the green colour of the filter paper disappeared. The volume of this filtrate was finally adjusted to 20 ml. The light absorbance of each Bua Bok filtrate was measure using Spectrophotometer at 645 and 663 nm wavelengths. The total chlorophyll contents in Bua Bok samples were calculated from the followed equation.

Total Chlorophyll content (mg/g fresh weight) = [20.2 A645+8.02 A663] x [(V/1,000) x W]

Whereas, A = Wavelength V = The final volume solution in ml.

W= Weight of samples in g.

3.5.4 Fresh weigh

The fresh weights of Bua Bok accessions harvested in 1 sq. m. land plot were determined using 2 decimal balance and reported in kg/m2.

3.5.5 Dry weigh

A kilogram of each fresh Bua Bok accession was dried at 50 C in the oven for 24 hrs, then the dried weight was determined and reported in g/kg fresh weight.

3.5.6 Fiber content

Fiber content was determined by AOAC (2000) at the Biochemical and Microbiological laboratory, Industrial Metrology and Testing Service Center of Thailand Institute of Scientific and Technological Research.

The procedure was one gram of dried sample (W) placed on defatted extraction thimble and extract with diethyl ether by the Soxhlet extractor for 4-6 hours. Place the thimble at 100°C, and thus transfer the residue in the thimble to 600 ml beaker. Add 200 ml boiling 1.25% H_2SO_4, and 0.5-1.0 ml amyl alcohol. Bumping chips or granules may also be added. Place beaker on digestion apparatus with preadjusted hot plate and boil exactly 30 minutes at 100°C. Remove beaker, and filtrate contents in beaker immediately through Buchner funnel (pre-coated with linen cloth) using suction. Rinse beaker with 70-75 ml boiling H_2O, and wash through Buchner. Repeat with three 50 ml portion H_2O, and suck dry. Remove residue and replace in the same beaker. Add 200 ml boiling 1.25% NaHO and boil exactly 30 minutes. During the acid and base digestion, rotate beaker periodically to keep solids from adhering to sides. Remove beaker, filter as above. Wash with 25 ml boiling 1.25% H_2SO_4, three 50 ml portions H_2O, 25 ml ethanol, and suck dry. Remove residue and transfer to crucible. Place in oven at 100 °C for 2 hours. Cool in desiccators and weigh (W_1). Ash in furnace at 550°C for 1 hour, cool in desiccators and reweigh (W_2). The crude fiber calculated by formulation below.

$$\% \text{ crude fiber} = [(W_1 - W_2)/W] \times 100$$

Whereas W = Weigh in grams of sample

W1 = Weigh in grams of sample on drying

W2 = Weight in grams of sample on ashing

3.5.7 Protein content

Protein content was determined by AOAC (2000) the Biochemical and Microbiological laboratory, Industrial Metrology and Testing Service Center of Thailand Institute of Scientific and Technological Research.

Determination of protein used the Kjeldahl method. The Kjeldahl method for nitrogen analysis is composed of three distinct steps. There are digestion, distillation and titration. The digestion step, accurately weight 1 g of well ground and mixed sample and transfer to Kjeldahl flask. Add 5 g of selenium reagent mixture and 20 ml conc. sulphuric acid (3-4 boiling chips should be added to prevent superheating). Shake until thoroughly mixed, place flask in inclined position and heat over low flame in the fume hood until frothing ceases. Boil the mixture briskly until solution clears, then about 1 ½ -2 hours longer. Remove tubes and let cool about 10 minutes. The distillation step, add few Zn granules into flask containing cool diluted digest to prevent bumping. Add enough NaOH solution (about 80 ml) without agitation to make contents receiving flask containing 50 ml H_3BO_3 solution mixed indicator. Steam distils until 300 ml collects (absorbing solution turn green from librated NH_3). Remove digestion flask and received from unit. The titration step, titrate absorbing solution with hydrochloric acid standard solution to neutral gray end point and record volume acid required to 0.01 ml. Titrate reagent blank similarly. The total nitrogen calculated by formulation below and the percent protein is calculated as follows:

Kjeldahl nitrogen, % = $[(V_S-V_B) \times M \times 14.01] / (Wx10)$

Crude protein, % = % Kjeldahl N x F

Where as V_S = Volume (ml) of standardized acid used to titrate a test

V_B = Volume (ml) of standardized acid used to titrate reagent blank

M = Molarity of standard HCl

W = Weight (g) of test portion or standard

F = Factor to convert N to protein, are 6.25 for plant material

14.01 = Atomic weight of N

10 = Factor to convert mg/g to percent

3.5.8 Calcium content

Calcium content was determined by atomic absorbance spectrophotometric (AAS) method by Nwokolo (1987) and Ron et al. (1984), at the Biochemical and Microbiological laboratory, Industrial Metrology and Testing Service Center of Thailand Institute of Scientific and Technological Research. It used 0.1% w/v lanthanum chloride was added to each sample which is also contained in the vegetable. Absorbance was read at 422 nm with 5A cathode lamp for calcium

3.5.9 Beta-carotene content

Beta-carotene contents were determined at the Biochemical and Microbiological laboratory, Industrial Metrology and Testing Service Center of Thailand Institute of Scientific and Technological Research. It was analyzed using AOAC (1980) spectrophotometric method involving extraction of pigment with 1:1 v/v acetone-n-hexane solution, saponification and isolation of unsaponified extract using methanolic potassium hydroxide. The saponified extract was dried over anhydrous sodium

hydroxide, evaporated to dryness and then made up to 10ml with acetone-n-hexane. The mixture was chromatograph in a column of manganese oxide-hydro super-gel using 3.5% acetone-n- hexane. This separates carotene from other pigments that were not removed by saponification. The carotene extract was concentrated to dryness dissolved in 100ml acetone-n-hexane; this gave the test solution. The spectrophotometer was set at 436nm and zero absorbance. The acetone-n-hexane was used to standardize the spectrophotometer. The test extract was poured into a 1cm cuvette and absorbance level read (T). The concentration of beta-carotene was thus calculated by the following expression:

Beta-carotene content (mg/100g) = - log T x V x 100 / ($E^{\%}_{1cm}$ x W),

Where T = absorbance,

V = volume of eluate = 100, L = depth of cuvette = 1 cm,

W = original weight of sample,

$E^{\%}_{1cm}$ = 43,336 nm.

3.5.10 Asiaticoside content

The content of asiaticoside was determined by high performance liquid chromatography technique according to the method of Somwong (2006), at The Department of Pharmaceutical Chemistry Laboratory, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

The procedure for the determination of asiaticoside content containing 5 g of powder tissue sample was mixed with 90 ml of 80% methanol for 1.5 hour after the extract solution was cooled down to room temperature, the extract solution was filtered and

was adjusted to volume 100 ml with deionized water. The 5 ml of extract solution and 1.0 ml of internal standard solution (Prednisolone) were mixed together and adjusted volume to 10.0 ml with the mobile phase of HPLC condition. The sample solution was filtered through 0.45 μm Nylon-membrane and was injected onto the HPLC system and then compared the retention time and peak area ratio of each compound to standard asiaticoside, madecassoside, asiatic acid and madecassic acid. Chromatographic separation was performed using a Alltech Alltima column (C18, 5 μm diameter particle size; 4.6 x 150 mm) with acetonitrile-phosphate buffer (29:71) as the mobile phase. Separation was carried out with a flow rate of 1 ml/min. The sample injection volume was 20 μl at 25 °C.

3.6 Statistical Analysis

Three or ten (depending on types and the number of parameters) replicates per treatment were performed in all experiments. Therefore, all values reported were the mean ± (SE) of three or ten replications. Analysis of variance (ANOVA) followed by Duncan's Multiple Range Test with a significance level of $p<0.05$ was performed on data using the statistic software, SAS.