CHAPTER 3
MATERIALS AND METHODS

3.1 Materials and packaging

- Pennywort samples were purchased from 5 local markets and Chaingmai farms (Doi Pa Mon), Thailand, in 2007.

- Sugar: Mitr Phol (Mitr Phol Sugar Co. Ltd., Thailand).

- Pouch: flexible pouch (aluminium foil), size 6.5 x 5.5 inc., 4 layers from pouch outer to inner were PET 12 μm, nylon 15 μm, aluminium 9 μm and polypropylene 80 μm (Royal Can Industries Co., Ltd., Thailand)

- Whatman paper (Whatman International Ltd., England)

- 0.20 μm filter, non-pyrogenic hydrophillic (Minisart; Sartorius, Germany)

- 0.20 μm filter, nylon (Whatman, UK.)

- LDPE Stomacher 3500 standard bags (Seward; Seward Ltd., Sussex, UK.)

- 40 ml Amber vial which was capped with a PTFE septum

-75 μm Supleco SPME fibre Carboxen/Polydimethylsiloxane (PDMS) (Supleco, Bellefonte, PA, U.S.A)

- Syringe 5-10 ml (Terumo, Belgium)

3.2 Standards and chemicals

Standards

- Asiaticoside 99.5% HPLC purify (Chengdu Biopurify Phytochemicals Ltd.; Sichuan, China)

- Madecassoside 99.4% HPLC purify (Chengdu Biopurify Phytochemicals Ltd.; Sichuan, China)

- Madecassic acid (Guangxi Changzhou Natural Products Development Co. Ltd.; Guangxi, China)

- Asiatic acid (Sigma, USA)

- *L*-ascorbic acid 99% (Sigma-Aldrich; St. Louis, USA)
- *β*-carotene 95% (Sigma-Aldrich; St. Louis, USA)
- Chlorophyll *a* and *b* from spinach (Sigma-Aldrich; Gillingham, UK.)
- 1,2-Dichlorobenzene 99% (Sigma-Aldrich; Poole, UK.)

Chemicals

- Acetronitrile HPLC grade (Fisher Scientific; Leicestershire, UK.)
- Methanol HPLC grade (Fisher Scientific; Leicestershire, UK.)
- Water HPLC grade (Fisher Scientific; Leicestershire, UK.)
- Hexane HPLC grade (Fisher Scientific; Leicestershire, UK.)
- Tetrahydrofuran (THF) HPLC grade (Sigma- Aldrich, Switzerland)
- Ethanol HPLC grade (Fisher Scientific; Leicestershire, UK.)
- 2,4,6-tri (2-pyridyl)-*s*-triazine (TPTZ) 99% (Fluka; Buchs, Switzerland)
- Folin-Ciocalteu phenol reagent (Fisher Scientific; Leicestershire, UK.)
- Sodium acetate analytical grade (Sigma, USA)
- Glacial acetic acid analytical grade (Sigma, USA)
- Hydrochloric acid (HCl) analytical grade (Sigma, USA)
- Ferric chloride analytical grade (Sigma, USA)
- Ferrous sulphate analytical grade (Sigma, USA)
- Gallic acid monohydrate 98% analytical grade (Fluka; Buchs, Switzerland)
- Triethylamine (TEA) (Fluka, Belgium)
- Sodium carbonate analytical grade (Sigma, USA)
- Sodium hydroxide (NaOH) analytical grade (Sigma, USA)
- *meta*-phosphoric acid analytical grade (Panreac, Spain)
- Sulfuric acid (H_2SO_4**)** analytical grade (Fisher Scientific; Leicestershire, UK.)
- Acetone analytical grade (Fisher Scientific; Leicestershire, UK.)
- Petroleum ether (40-60˚C) analytical grade (Fisher Scientific; Leicestershire, UK.)
- Calcium carbonate ($CaCO_3$) (Merck; Darmstadt, Germany)
- Potassium hydroxide (Fisher Scientific; Leicestershire, UK.)
- Potassium permanganate ($KMnO_4$) (Vechavit,Thailand)
- Methanol analytical grade (Fisher Scientific; Leicestershire, UK.)
- Sodium sulfate (Fisher Scientific; Leicestershire, UK.)

- Butylated hydroxytoluene (BHT) analytical grade (Sigma, USA)
- Triethylamine (TEA) analytical grade (Sigma, USA)
- Plate count agar (PCA) (Merck; Darmstadt, Germany)
- Potato dextrose agar (PDA) (Merck; Darmstadt, Germany)
- Peptone (buffered) (Merck; Darmstadt, Germany)

3.3 Instruments

- Meat chopper, model RS 20 (Meissner GmbH. & Co. Ltd.; Bieenkopf-Wallau, Germany)
- High pressure rig (Food Lab; Stansted Fluid Power, Essex, UK.)
-Brookfield-programmable DV-П$^+$ viscometer, LVDV-П$^+$ (Brookfield Engineering labs, Germany)
- Hand-refractometer (ATAGO, Japan)
- Colour, chroma and hue (HunterLab; Model Color Quest XE, USA)
- pH-meter (Sartorius PB-20, Germany)
- Balance (Sartorius A120S, Germany)
- UV-Vis spectrophotometer (model Lambda Bio-20; Perkin Elmer, USA)
- HPLC (Agilent model 1050 HPLC; Agilent Technologies, USA)
- HPLC (Shimadzu LC-10AD HPLC, Japan)
- HPLC (Agilent model 1200 HPLC; Agilent Technologies, USA)
- YMC S5 ODS-AM, 5 µm, 4.6 mm ID x 250 mm column
- Waters S5 ODS2 guard column
- Inersil C_8-3, 5 µm, 4.6 mm ID x 250 mm column
- Inersil ODS-3, 5 µm, 4 mm ID x 10 mm guard column
- Water spherisorb S5 ODS2 4.6 mm ID x 250 mm column
- GC-MS (Hewlett-Packard 5890 series П GC connected to a 5972 series MS: GMI, Inc.; Minnesota, USA)
- 60 m VF-5 ms capillary column (0.25 mm ID, 0.25 µm film thickness: Varian: Crawford Scientific Ltd.; Scotland, UK.).

3.4 Research design and methods

3.4.1 Research design

Phase 1 Analysis of the composition of raw material

The physical and chemical properties of fresh leaves and petioles of pennywort were determined as follows:

- Determination of physical properties including colour and the percentage yield. The yield of raw material was calculated by weight (kg) per weight (kg) for whole plant to leaves and petioles by stripping and by weight (kg) per volume (liter) for juice extract. For the colour measurement, it was measured by a colourimeter and the derived functions of hue angle (˚h) and chroma (C) value were determined.

- Determination of chemical properties including proximate analysis according to the methods published by AOAC (2000). The AOAC methods were used for analysis of crude protein content, fat content, crude fiber content, moisture content and total solids. The total soluble solids were determined by a hand-refractometer. Carbohydrates were measured by difference methods, based on the total solids and total ash measurements and the pesticide residue. The active compounds (asiaticoside, asiatic acid, madecassoside and madecassic acid), carotenoid content, chlorophyll content and ascorbic acid were determined by HPLC.

Phase 2 Optimization of different processes based on microbiological quality

The original extracted pennywort juice was used as a control. Ten percent of sucrose was added to pennywort juice. The juice sample was subjected to 3 treatments.

1) Ultra-high pressure treatment at 2 pressure levels (400 and 600 MPa), at room temperature for 20 and 40 min (2^2 factorial in complete randomized design (CRD))

2) Juice was pasteurized by heating at 90˚C for holding time of 3, 5 and 7 min. (CRD experimental design)

3) Juice was sterilized at 121˚C for 4 and 6 min (CRD experimental design)

The finished products were assessed for microorganism following Thai Food Regulation-Standard (2003), Ministry of Industry, Thailand.

- Total plate count (AOAC, 2000)
- *Staphylococcus aureus* (BAM, 2001, ch.12)
- *Clostridium perfringens* (BAM, 2001, ch.16)
- *Escherichia coli* (MPN method) (BAM, 2002, ch.4)
- Yeasts & molds (AOAC, 2000)

Optimum conditions for each processing technique were selected to use in phase 3.

Phase 3 Optimization of different processes based on chemical quality

The optimum condition of each process from phase 2 was selected for further chemical assessment.

Chemical analysis

The treated juice samples were analyzed for triterpenes, active compounds including asiaticoside, madecassoside, chlorophyll content, ascorbic acid, total phenolic compounds, ferric reducing antioxidant potential (FRAP) assay, pH and total soluble solids.

Phase 4 Shelf life study of the processed pennywort juice

The experiment of this study was designed to understand the quality of pennywort juice during storage. Processed juice was selected to produce the finished products using conditions from phase 2.

The juice was filled, processed and stored at 4˚C for products treated by high pressure and pasteurization as well as stored at 40˚C for product treated by sterilization. All storage samples were kept for 4 months and/or until the products degraded due to microorganisms exceeding Thai Food Regulation-Standard (2003). The products were determined for physical properties at 1 month intervals, chemical properties at 15 days intervals and microbiological qualities at 1 week intervals during the storage time.

Physical analysis

On the each month of storage, the products were analyzed for colour and viscosity.

Chemical analysis

Chemical analysis was done at every 15 days of storage. The samples were analyzed for total soluble solids, pH value, total phenolics, ferric reducing antioxidant power (FRAP) assay, active compounds (asiaticoside and madecassoside), ascorbic acid content (vitamin C) and chlorophyll content by HPLC.

Microbial analysis

The products were analyzed at every 7 days for microorganism following Thai Food Regulation-Standard (2003).

Phase 5 Study the flavour profile of pennywort juice

The flavour profile of fresh and processed pennywort juice selected from phase 2 was studied by the GC-MS method.

3.4.2 Analysis methods

Analysis of the composition of the raw material

Chemical properties including proximate analysis were determined according to the methods published by AOAC (2000). The AOAC methods that were used in the analyses include no.955.04 for crude protein content, no. 905.02 for fat content, no.978.10 for crude fiber content and no.990.19 for moisture content and total solids. The total soluble solids were determined by a hand-refractometer. Carbohydrates were measured by difference methods, based on the total solids and total ash measurements. The pesticide residue was determined using method no.991.07 (AOAC, 2000).

The proximate analysis was determined by the staff of Food Science and Technology Department, Faculty of Agro-Industry, Chiang Mai University. The pesticide residues including organochlorine and organophosphate were determined by Laboratory Center for Food and Agricultural Products, Chiang Mai.

Preparation of raw materiaM

The plant sample was freshly harvested, from Chiang Mai farm, Thailand with commercial maturity (2-4 months) and identified by Thai Herbal Pharmacopoeia (volume 3) methods (THP, 2007). Upon arrival at the laboratory, the sample was washed with running tap water 4 times to remove debris and damaged portions were removed. The leaves and petioles of pennywort were stripped from the plant and

soaked in potassium permanganate solution (300 ppm) for 15-30 min before being rinsed with running tap water then extracted with water (1:1 w/v) at room temperature for 15 min by meat chopper. The juice was prepared as two types, original juice, and juice with 10% sucrose added. This juice was filled into a double layer of LDPE Stomacher pouch before HPP and filled into retort pouch (PET/nylon/aluminium/PP) but all samples were not vacuum-sealed before pasteurization and sterilization. The samples were subjected to pasteurization (90˚C for 3, 5 and 7 min) in a cooker, sterilization (121˚C for 4 and 6 min) in a retort and high pressure processing (400 and 600 MPa for 20 and 40 min at room temperature (< 30˚C)).

Analysis of bioactive compounds (madecassoside, asiaticoside, madecassic acid and asiatic acid)

Sample preparation for the analysis of bioactive compounds was carried out using a modified method described by Inamdar *et al*. (1996). One ml of juice sample was mixed with 1 ml of 90% methanol and stirred for 2 hr at room temperature. The solution was filtered through a 0.20 μm filter and the clear filtrate was used for HPLC analysis.

The Agilent 1050 HPLC system consisted of a pressure flow pump and a UV spectrophotometric detector. Chromatographic separation was performed with YMC S5 ODS-AM 5 μm, 4.6 mm ID x 250 mm column with Waters S5ODS2 guard column compartment (for asiaticoside and madecassoside). C_{18} Column with a water-acetonitrile mobile phase, with UV detection at 220 nm for madecassoside and asiaticoside. Therefore, madecassic acid and asiatic acid were using a Shimadzu LC-10AD HPLC with Inersil C_8-3 5 μm, 4.6 mm ID x 250 mm column and ODS-3 5 μm, 4 mm ID x 10 mm as guard column. The flow rate was 1.4 ml/min and the gradient was water 80% decreased within 30 min to 45%, held for 5 min and increased to 80% within 10 min. A constant volume (20 μl) of each sample was injected into the column. The peak area of each component was determined and converted to concentration.

Asiaticoside, madecassoside, asiatic acid and madecassic acid were dissolved in methanol (HPLC grade). The concentration of these standards in the juice was determined by a standard addition process. Pennywort juice samples were prepared by

addition of the stock solution of madecassoside and asiaticoside to obtain concentrations of 10-400 µg/ml. For Shimadzu HPLC, asiatic acid and madecassic acid was dissolved in methanol to obtain concentrations of 0.25-20 µg/ml and 3.125-100 µg/ml, respectively, for the calibration curve. The standard solutions were filtered through a 0.20 µm filter.

Analysis of ascorbic acid

One ml of juice sample was mixed with 2 ml of 80% methanol, 19.9% water, 0.1% HCl and stirred for 2 hr. It was then diluted with 2 ml of 5% *meta*-phosphoric acid. The solution was filtered through a 0.20 µm filter and the clear filtrate was used for HPLC assay.

Ascorbic acid content was determined using the method described by Rodriguez-Comesana *et al*. (2002). The Agilent 1050 HPLC system consisted of a pressure flow pump and a UV spectrophotometric detector. Chromatographic separation was performed with YMC S5 ODS-AM column 5 µm, 4.6 mm ID x 250 mm column. Acetic acid in water (0.1 M) as a mobile phase, with UV detection at 250 nm. The flow rate was 1.5 ml/min and analysis was by a single run isocratic mode at room temperature with 20 µl injection volume.

L-ascorbic acid was dissolved in methanol to obtain concentrations of 1-300 µg/ml for the calibration curve. The standard solution was filtered through a 0.20 µm filter and stored at 4˚C.

Analysis of *β*-carotene

Ten ml of juice sample was mixed with 20 ml of cold acetone. Twenty ml of petroleum ether (40-60˚C) was added, and the organic layer was separated and washed with 40 ml phosphate buffer, pH 7. The organic layer was separated and stirred overnight with 20 ml of 10% potassium hydroxide in methanol and washed successively with 40 ml water, 40 ml phosphate buffer, 2 times, 40 ml saturated NaCl and dried with sodium sulfate. Solvent was evaporated and the residue was dissolved in 5 ml methanol. The solution was filtered through a 0.20 µm filter and the clear filtrate was used for HPLC assay.

β-Carotene content was determined using the method described by Lefsrud *et al.* (2007). A HPLC unit with photo diode array detection (model 1200, Agilent Technologies) was used. All samples were analyzed using a Water spherisorb S5

ODS2 4.6 mm ID x 250 mm column with Waters S5ODS2 guard column compartment. The column was maintained at 16˚C using a thermostatic column compartment. Eluents were A: 75% acetronitrile, 20% methanol, 5% hexane, 0.05% BHT and 0.013% TEA and B: 50% acetronitrile, 25% THF, 25% hexane and 0.013% TEA. The flow rate was 0.7 ml/min and the gradient was 100% eluent A for 30 min; a change to 50% A and 50% B over the next 2 min; a change to 100% B over the next 2 min; followed by a change to 50% A and 50% B for the next 2 min. The eluent was instantly returned to 100% A for 10 min prior to the next injection. Components eluted from a 20 µl injection were detected at 452 nm (*β*-carotene), 652 nm (chlorophyll *b*) and 665 nm (chlorophyll *a*).

β-Carotene was dissolved in methanol to obtain concentrations of 2.60-333.33 µg/ml for the calibration curve. The standard solution was filtered through a 0.20 µm filter.

Analysis of chlorophylls

One ml of juice sample was mixed with 2.5 ml of tetrahydrofuran. The mixture was homogenized before being placed into a centrifuge for 3 min at 500xg. The supernatant was filtered through a 0.20 µm filter.

Chlorophyll contents were determined using the method described by Lefsrud *et al.* (2007) by HPLC.

Standards of chlorophyll *a* and *b* were dissolved in methanol to obtain concentrations of 2.60-100 and 6.25-250 µg/ml, respectively for the calibration curve. The standard solutions were filtered through a 0.20 µm filter.

Total phenolics content determination

Total phenolics content (TPC) was determined using modified Folin-Ciocalteu assay described by Zainol *et al*. (2003). An aliquot of 1 ml of the extract was added to 10 ml deionized water and mixed with 2 ml of Folin-Ciocalteu phenol reagent. The mixture was then allowed to react for 5 min and 2 ml of saturated Na_2CO_3 solution was added to the mixture. The resulting blue complex was then determined at 680 nm with gallic acid as a standard. The total phenolics content of the extract was expressed as mg gallic acid equivalents per 100 ml of sample.

Gallic acid stock solution

In a 100-ml volumetric flask, dissolve 0.5 g of dry gallic acid in 10 ml of ethanol and dilute to 100 ml with water.

Sodium carbonate solution

Dissolve 200 g of anhydrous sodium carbonate in 800 ml of water and bring to boil. After cooling, add a few crystals of sodium carbonate, and after 24 hr, filter and add water to 1 L.

Ferric reducing antioxidant potential (FRAP) assays

The ability to reduce ferric ions was measured using a method described by Benzie and Strain (1996), with some modifications. An aliquot (l ml) of juice was added to 10 ml water and 3 ml of FRAP reagent (10:1:1 of 300 mM sodium acetate buffer at pH 3.6, 10 mM TPTZ solution and 20 mM $FeCl_3.6H_2O$ solution) and the mixture was incubated in a water bath at 37˚C for 30 min. Absorbance was measured at 593 nm. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as µmol Fe (Π) per liter of sample.

Measurements of TPC and FRAP were performed in disposable cuvettes using a UV-Vis spectrophotometer.

Flavour profile analysis

Standard solutions

1,2-Dichlorobenzene was used as an internal standard (IS) for GC analysis, as this compound was not found in the pennywort juice. 1,2-Dichlorobenzene (10 µl) was added to juice samples (20 ml) prior to headspace concentration.

Head space concentration

The sample (20 ml) was transferred to a 40 ml amber vial which was capped with a PTFE septum. The vial was incubated for 5 min at 35˚C, before a 75 µm Supleco SPME fibre Carboxen/Polydimethylsiloxane (PDMS) was introduced and exposed to the sample headspace for 30 min. The headspace volatiles were adsorbed onto the SPME fibre for 30 min. The SPME fibre was then inserted into the GC-MS injection port and held for 15 min with the adsorbed components being effectively desorbed. Each sample extraction was repeated 4 times.

GC-MS analysis

Analysis was carried out with a Hewlett-Packard 5890 series Π GC connected to a 5972 series MS and a 60 m VF-5 ms capillary column (0.25 mm ID, 0.25 μm film thickness). The injector port was in splitless mode, and split flow was programmed to turn on after 0.5 min. The temperature of the injector was 250˚C. The oven temperature was kept at 50˚C for 3 min and programmed to increase at 4˚C/min. The final temperature was set at 240˚C and held for 5 min.

The LRI values of detected compounds were determined by comparison of retention times with those for a series of *n*-alkanes (C_5-C_{25}), which were analyzed in a separate analysis under the same conditions.

The relative concentrations of the investigated compounds were calculated by relating the area of the internal standard to the area of the compound of interest, defined as:

$$\text{Relative concentration} = \frac{\text{Peak area of particular compound}}{\text{Peak area of IS}} \times \text{IS conc.}$$

Physical analysis

Viscosity

Viscosity was determined with Brookfield-programmable DV-Π$^+$ viscometer at temperature range 25-28˚C, speed 40 rpm and probe no.4.

Colour measurement

CIELAB parameters were determined with Color Quest XE (Hunter Lab) using the illuminant D65 diffused illumination. The parameters gained were +*a** for redness, -*a** for greenness, +*b** for yellowness and -*b** for blueness. These calculations of *C** for chroma and ˚h for hue angle, were made with the following equations:

$$C^* = [(a^*)^2 + (b^*)^2]^{1/2}$$

$$h = \arctan (b^*/a^*)$$

Mode TTRAN (Total transmission)

Scale CIELCh

Illuminant D65

Procedure none

Observer 10˚

MI Illuminant D65 (ASTM)

Indices APHA-20 mm

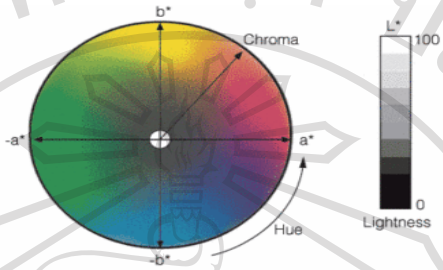

Figure 3.1 Colour system

Source: http://www.visionsystems.com

Microbials analysis

S. aureus, C. perfringens and *E. coli* were determined by Laboratory Center for Food and Agricultural Products, Chiang Mai.

Statistical analysis

Comparison of 4 experimental processes was by completely randomized design (CRD). The data was subjected to One way Analysis of Variance (ANOVA) by Minitab release 14 (www.minitab.com). Significant ($p \leq 0.05$) differences between means were identified using Tukey's test.