CHAPTER 2

RESEARCH DESIGNS AND METHODS

2.1 Research Designs

This project used integrated research methods to collect both qualitative and quantitative data. It was divided into three parts. First, surveys were done to find medicinal plants used by the Mien people in Sancharurn Village, Nan Province, Thailand, along with an examination of the medicinal plant's habitat. Data regarding knowledge of medicinal plants was obtained from interviews of 4 herbalists, 58 villagers, and 30 postpartum women using questionnaires. Second, experimental research was performed to determine the chemical compounds of the medicinal plants (using phytochemical screening) along with the biological activities of selected plants in the recipe with the highest potential. The results of the laboratory research were used to confirm the usefulness of medicinal plants traditionally used by the Mien and improve their conservation methods. The implementation of the conservation project was the last part of the project. This part used participatory action research by triangular validation and was followed by meetings for brainstorming. Data of the medicinal plants habitat from the surveys, and scientific data from both literature reviews and laboratory studies were combined and then provided to the community to find the best way to conserve the knowledge of medicinal plants, their habitat and for sustainable use of medicinal plants in the community.

2.2 Materials, chemicals, and instruments

2.2.1 Materials used in field research and identification procedure

Materials	Companys
Altimeter	Brunton®
Camera	Sony®
Recorder	Soken® F-202
Field work tools: cutting tools, plastic bag, paper labels, string, mounting papers, reused newspaper, bamboo flame, glue, needles and thread, pencils, 95% alcohol	-
Text books: Flora of Thailand, Flora of China	

2.2.2 Chemicals used in experimental research

Chemicals	Companys
Absolute alcohol AR	Merck
Acetic anhydride	Sigma
Anhydrous Na_2SO_4	MDA
Butylated hydroxytoluene, (BHT)	Sigma

2.2.2 Chemicals used in experimental research (continued)

Chemicals	Companys
Chloroform	Labscan
1, 1-diphenyl-2-picrylhydrazyl radical, (DPPH radical)	Sigma
Folin Ciocalteau's phenol reagent	Sigma
Gallic acid	Sigma
Gelatin	Ajax
Glacial acetic acid	Merck
H_2SO_4	Sigma
Iron (II) sulfate heptahydrate	Scharlau
Iron (III) Chloride	Sigma
KOH	Merck
Lead acetate	Labscan
Mg-Ribbon	Ajax
NaOH	Ajax Finechem
NH_4OH	JT baker
Picric acid.	Merck

2.2.2 Chemicals used in experimental research (continued)

Chemicals	Companys
Sodium acetate-3-hydrate	Unila R
TPTZ (2, 4, 6-Tris (2-pyridyl)-s-triazine)	Sigma
Trolox (6-hydroxy-2,5,7,8-tetramethyl chlorman-2-carbox carboxylic acid)	Sigma

2.2.3 Materials and instrument used in experimental research

Materials and instruments	Companys
Analytical balance	Sartorious®
Micropipettes	Pipetman®
Filter paper	Whatman
Glass container	-
Hot air oven	Memmert
Microplate 96 well	BD Primaria™
Microplate reader	Beckman®
Mueller Hinton Agar	BBL Ltd.

2.2.3 Materials and instrument used in experimental research (continued)

Materials and instruments	**Companys**
Needle and loop	-
Petri disk, sterile disk	-
Rotary evaporator	EYELA®
Sterile cotton swab	-
Ultrasonic	Model 889
UV-Visible absorbance detector	HP 1100
Water bath	Memmert®
pH meter	Model pH900
Antibiotic: vancomycin	-
Staphylococcus aureus	ATCC25923

2.3 Methods

The research consisted of three sections, with seven steps contained in the first section (field study: an ethnomedicinal plant survey**)**, four steps contained in the second section (experimental research) and one step in the last section (local conservation and sustainable use of medicinal plants using participatory action research). Their details are shown as followings:

1. The researcher contacted the primary school director and Village leaders in Sancharurn Village to observe the actual situation and get consent to study in the research area.

2. Meetings with primary school teachers, Village leaders, herbalists and primary school students were conducted to inform them of the objectives of the study, the research process, and the subsequent benefits to their community.

3. Open-ended questionnaires (Appendix A) and interviews were used with four key informants (herbalists and collectors) to collect the qualitative and quantitative medicinal plant data. The following details were recorded: the use of medicinal plants, vernacular names of each medicinal plant, the recognition of medicinal plants, the part or parts used, the method of preparation, the method of administration, and the abundance of each medicinal plant within the project area. Recorder also was used.

4. Transecting walks in forest plots were carried out with herbalists, primary school teachers, and primary school students in order to get more information on the ecological status of medicinal plants. Record forms 1 and 2 (Appendix B) were used. The species diversity index by Shanon-Weaver [113] was calculated. Plant samples were collected for making voucher specimens to determine their botanical names by plant taxonomists. Discussions and photographs were recorded during each step.

5. Semi-structured interviews were also conducted with fifty-eight non-specialist informants (aged 15 -75). Questionnaires I (Appendix C) were prepared to obtain information on the knowledge, attitude, and practices of the non-specialist informants. Quantitative data from this section was analyzed to find the actual use.

Ethnobotany indices [115-118]: Use value was utilized to fine the dominant plants that are actually used. Questionnaires II: EuroQOL (EQ-5D) Plus (Appendix D) was used to determine the quality of life of the target people who used medicinal plants for their primary healthcare and also to determine the effectiveness of the medicinal plants used by villagers. These questionnaires were validated content by experts.

6. Qualitative data from herbalists and medicinal plants users was analyzed by content analysis, the medicinal plants themselves (i.e., description of diversity, number of families/species present, etc.), and the usage of the plants (i.e. part/parts used, method of preparation, etc.) were analyzed by descriptive analysis. Ethnobotany indices: Use categories, Relative Cultural Important (RCI), and Informant Agreement Ratio (IAR) [115-118] were used to find the most popular preparations in use and other relevant details. A paired t-test was used to determine the difference in the number of medicinal plants known by male and female, and also between teenagers and the elderly to determine knowledge erosion in community. All of the analyses were conducted with the SPSS 17.0 software package for Windows.

The indices used in ethnobotany for the determination of potential value of recipes and plants were used as following.

6.1 **Use categories** each time a plant was mentioned as "used", it was considered as a single "use-report" [113]. When an informant used a plant to treat more than one disease in the same category, it was considered as a single use-report [114].

6.2 **Relative Cultural Importance (RCI)** these indices are applied in ethnobotany to calculate a value per folk or biological plant taxon [115].

6.2.1 **Use Value (UV)** informant consensus index is used for testing the relative importance of locally known species [116]:

$$UV = Ui / N$$

Ui is the number of use-reports cited by each informant for a given species.

N is the total number of informants.

Data Interpretation

UVs are low (Near 0) when there are few use-reports related to its use.

UVs are high (Near 1) when there are many use-reports for a plant, implying that the plant is important.

6.2.2 **Informant Agreement Ratio (IAR)** this is an index for test homogeneity of knowledge [117]:

$$IAR\ (ICF) = \frac{Nur - Nt}{(Nur - 1)}$$

Nur refers to the number of use-reports for a particular use category.

Nt refers to the number of taxa used for a particular use category by all informants.

Data Interpretation [118]

IAR values are low (Near 0) if plants are chosen randomly or if there is no exchange of information about their use among informants.

IAR values are high (Near 1) when there is a well-defined selection criterion in the community and/or if information is exchanged between informants.

7. The highest potential value recipe was chosen to determine the phytochemicals of plants by phytochemical screening and to determine bioactivities of these plants in accordance with the following criteria:

7.1 Results from interviews which show a high level of agreement on the uses of the medicinal plants by the Mien community.

7.2 The selected plants have obvious indications of the Mien's traditional use and they lack of scientific data support.

7.3 An adequate supply of medicinal plant materials for research and development.

8. The alcoholic and aqueous extracts for phytochemical screening and biological activities were prepared.

8.1 Extraction methods

8.1.1 In order to perform a phytochemical analysis and measure antioxidant activity, one hundred grams of dried plant material was macerated in 70% ethyl alcohol. Flasks containing the extracts were shaken for 5 hrs, and they were kept at room temperature for 24 hrs in closed containers. Extracts were then filtered through vacuum filtration. The extraction was repeated 3 times. The combined filtrate was evaporated under reduced pressure to give concentrated crude extract.

8.1.2 To prepare the extract for antibacterial activity, twenty-five grams of dry plant material was boiled in distilled water for 1 hour and allowed to cool at room temperature, filtered and concentrated at reduced

pressure using a rotary evaporator. The concentrated extracts were kept in the refrigerator at 4 °C until use.

9. Determination of chemical compounds was performed for alkaloids, cardiac glycosides, flavonoids, saponins, coumarins, and tannins using phytochemical screening. Methods of Fransworth *et al.* [119-122], Ramstad [123], Segelman *et al.* [124-125], and Evans [126] were applied to use in this tests. The methods used are shown as the following.

9.1 Screening for alkaloids

9.1.1 Plant extract (equivalent to 25 g. of plant material) were placed into an evaporating dish and evaporated to a syrupy consistency over a water bath.

9.1.2 Twenty milliliters of 5% HCl was added to the syrupy residue and stired with a stirring rod while heated on a water bath for 10 minutes, and was then filtered.

9.1.3 A filtrate was divided to 7 aliquots and dropped onto test tube. To each was added the following reagents, Hager's, Mayer's Valser's, Wagner's Dragendorff's, tannic acid's and Marme's reagents. Turbidity was observed for a) slight turbidity or opacity (+), b) definite turbidity but no flocculation (++), c) heavy precipitate or heavy flocculation (+++). Mayer's and Valser's tests will give white to cream precipitate, Dragendorff's will give an orange precipitate while Wagner's

will give reddish-brown precipitate, Mame's will give off-white precipitate, and Hager's will give yellow color.

9.2 Screening for cardiac glycosides

9.2.1 Ten milliliters of ethanolic extract was put into a flask, 50 ml. of 10% lead acetate solution was then added and mixed well. The mixture was boiled for 15 minutes then allowed to cool and filter.

9.2.2 The filtrate was extracted three times with 25 ml portions of chloroform.

9.2.3 The chloroform phase was drawn off. A small amount of anhydrous sodium sulfate was added to the combined chloroform fraction, stirred and then filtered through dry filter paper.

9.2.4 The filtrate was collected in an evaporating dish, concentrated to 1/10 of the original volume, and then tested following:

9.2.4.1 Determination of the steroidal moiety by Liebermann-Burchard's test

a. Approximately 2 ml of the concentrated sample was transferred to a mall evaporating dish, evaporated almost to dryness on a water bath.

b. The dish was removed from the water bath, 3 drops of acetic anhydride was added into the cooled extract, followed by 1 drop of concentrate H_2SO_4 which was added onto the side wall of the

dish, the two solutions were allowed to mix gently.

c. A change of color was observed immediately and over a period of 1 hour. The change in color will occur from rose through red, violet, and blue to green. The colors are slightly different from compound to compound.

9.2.4.2 Determination of unsaturated lactone of C_{17} (butenolide) by Kedde's test

Two milliliters of the concentrated sample was evaporated to dryness on a water bath. Kedde's reagent (3,5 dinitrobenzoic acid) and a few drops of 1 N NaOH was then added into cooled, dried extract. A positive test was presented by the appearance of a blue or violet color.

9.2.4.3 Determination of deoxy sugar by Keller-Kiliani's reaction

a. Two milliliters of the concentrated sample was placed into a test tube, 3 ml of $FeCl_3$ reagent (0.3 ml of 10% $FeCl_3$ in 50 ml of glacial acetic acid) was added, mixed and allowed to stand for a few minutes.

b. The test tube was held at a 45° angle, 1-2 ml of concentrated H_2SO_4 was carefully added to run

down the wall of the tube and form a separate layer at the bottom. An appearance of a brown color at the interface (due to deoxy sugars) and pale green color in the upper layer (due to the steroid nuleus) form in the presence of cardiac glycosides.

c. Positive reactions in all the above tests are taken to indicate the presence of cardenolides.

9.3 Screening for triterpenes

9.3.1 Five milliliters of 70% ethanolic extract of the sample was placed into a flask. After that, 10 ml of diluted H_2SO_4 was added and mixed well. The mixture was boiled for 15 minutes and was then cooled and filtered.

9.3.2 The filtrate was extracted with 3 successive 10 ml portions of chloroform. The chloroform phase was drawn. The combined chloroform fraction was collected and evaporated in an evaporating dish then the concentrate was evaluated by Liburmann-Burchard's test. Triterpnoid were indicated by an appearance of red, pink or purple-red color.

9.4 Screening for saponins

9.4.1 One hundred milligrams of dried plant material was transferred to a tube of 5 ml of water then boiled on a water bath, which allowed it to cool and then filter.

9.4.2 The clear filtrate was vigorously shaken for 1 minute. The presence of saponins is indicated if a honey- comb shaped frothing occurred; this should be at least 2 cm in height above the surface of the liquid and the persistence should be for 30 minutes.

9.4.3 Diluted H_2SO_4 was added to the filtrate and boiled for 5 minutes. The filtrate was allowed to cool and then the solution was shaken as before. Note the results.

9.4.4 The result was compared with the first one.

9.5 Screening for lactone glycosides (coumarins)

9.5.1 Three grams of the testing sample was transferred into a suitable flask and moistened with distilled water.

9.5.2 The flask was covered with a disk of filter paper moistened with 1 N sodium hydroxide solution and the disk was firmly covered with a piece of aluminium foil. The flask was heated on a water-bath for 15 minutes.

9.5.3 The filter paper was examined and the fluorescing circle appearing under UV-light was observed.

9.6 Screening for flavonoids

9.6.1 Five milliliters of the plant extract 2 drops of concentrated hydrochloric acid and few pieces of magnesium ribbon were added.

9.6.2 After the end of the reaction, a change of color (to green, red, etc.) was carefully observed within ten minutes. Color formation was also recorded in the foaming magnesium surface area.

9.6.3 Colors that definitely occurred (compared with the blank) was recorded.

9.6.4 To test for leucoanthocyanins, 2 drops of concentrated hydrochloric acid were added into 5 ml. of extract and then warmed on a water-bath for five minutes.

9.6.5 The change of color was observed. Development of red-violet color was indicative of the presence of leucoanthocyanins.

9.7 Screening for anthraquinones

9.7.1 Five milliliter of alcoholic extract was evaporated over a stream bath. The syrupy was then transfered to a flask that contained 1 ml of H_2O_2 and 10 ml of 0.5 N KOH then boiled on a water bath for 10 minutes, and allowed to cool and filter.

9.7.2 The clear filtrate was acidified with glacial acetic acid. The acid solution was then extracted in 3 successive of 10 ml of chloroform.

9.7.3 Ammonium hydroxide was carefully added to the chloroform extract. Occurrence of a pink color in ammonium hydroxide phase was indicated by the appearance of anthraquinones.

9.8 Screening for cyanotic glycosides

9.8.1 Two grams of the testing sample was transferred into a suitable flask and moistened with distilled water.

9.8.2 The flask was covered with a cock hung with a disk of sodium picrate paper. The flask was boiled in a water-bath for 15 minutes.

9.8.3 A change of color was observed. The color of sodium picrate paper changed to brick red color when testing sample containing cyanotic glycosides.

9.9 Screening for tannins

9.9.1 Ten milliliters of plant extract as placed into an evaporating dish and evaporated to a syrupy consistency over a water bath.

9.9.2 Four of ten parts were added with 20 ml of water. The filtrate was divided into two portions A and B. Portion A was added with gelatin while B was used as control. The appearance of white turbidity or precipitate indicated a presence of tannins [126].

9.9.3 Six of ten parts were used to identify tannins' types as follows: The filtrate was divided into 3 aliquots, tube

number 1 and 2 the following reagents were added, $FeCl_3$, lime water, and the last tube was used as control.

9.9.4 Results were observed for tube 1, a blue-black precipitates showed positive results of a hydrolysable tannins while the brownish-green showed positive result of condensed tannins (if the extract contains both types of tannins, a positive results will show a blue-green color). Tube 2 showed silver-gray precipitate indicating a positive result with the hydrolysable tannins and tube 3 was preserved as control [125-126].

10. Determination of biological activities

Biological tests were performed as follows: antioxidant activity of these plants was assessed by using DPPH, FRAP, and a antimicrobial activities test by disk diffusion with MIC tests was used. Details are shown as follows:

10.1 Determination of antioxidant activities by DPPH radical scavenging activity

Modified method of Hatano *et al.* [127] was used to measure the free radical scavenging activity of each ethanolic extract (with different dilutions) on DPPH (2, 2-diphenyl-1-picrylhydrazyl from Sigma, Germany). One hundred microliters of extract was added to a well containing 100 μl of DPPH in ethanol and mixed well. After mixing, the mixture was incubated in a dark room, then absorbance was measured at 520 nm after exactly 30 minutes by a microplate reader (DTX880 multimode detector, Beckman Coulter). All

samples were run in triplicate. Scavenging percentage of test samples were calculated as follows:

$$\% \text{ DPPH}^{\bullet} \text{ scavenging} = [(A_0-A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the mixture containing extracts. EC_{50} of reference antioxidant compounds. Butylated hydroxyl toluene (BHT) (Sigma-Aldrich GmbdH, Germany) were used for comparison to EC_{50} of the extracts.

10.2 Determination of antioxidant activities by Ferric-reducing power (FRAP) assay

One hundred microliters of extract (with different dilutions) was added to wells containing 100 μl of FRAP reagent, mixed well, and absorbance was then measured at 595 nm at 5 minutes by a microplate reader (DTX880 multimode detector, Beckman Coulter). All samples were run in triplicate. The FRAP value was expressed as $FeSO_4$, Trolox equivalents in mg per gram extract.

10.3 Determination of antimicrobial activities was performed by NANOTEC, Thailand as follows:

10.3.1 Disc diffusion method

The antimicrobial activity was determined by a disc diffusion method of NCCLS [130]. Briefly, 0.1 ml of 10^8 cells per ml of a suspension of the tested microorganism (*Staphylococcus aureus* ATCC

25923) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 15 µl of the extracts and placed on the inoculated plates. These plates, after staying at 4 °C for 2 hours, were then incubated at 37 °C for 24 hours. The diameters of the inhibition zones were measured in millimeters. Vancomycin was used as positive control.

10.3.2 Minimum inhibitory concentration (MICs)

A microdilution broth susceptibility assay was used as recommended by NCCLS for the determination of MIC [131]. All tests were performed in Mueller Hinton broth (MHB; BBL) supplemented with Tween 80 detergent (final concentration of 0.5 % (v/v). Bacterial strains were cultured overnight at 37 °C in MHA. Test strain was then suspended in MHB to give a final density of 5×10^5 cfu/ml and these were confirmed by viable counts. Geometric dilutions ranging from 0.036 – 72.00 mg/ml of the extracts were prepared in a 96-well microtiter plate, including one growth control (MHB+Tween 80) and one sterile control (MHB+Tween 80 + test extracts). Plates were incubated under normal atmospheric conditions at 37 °C for 24 hrs. The MIC of vancomycin was determined in order to control the sensitivity of the test organism. The bacterial growth was indicated by the presence of a white “pellet” on the well bottom.

11. Determination of Total Phenolic Content (TPC)

This test was performed for finding quantity of total phenolic content of plant extracts. Their results can be used to confirm antioxidant activity of these plants. Test method use the Folin-Ciocalteau assay which was adapted from methods of Kahkonen *et al.* [128] and Waterhouse [129] as following: Samples (500 μl) were introduced into test tubes followed by 5 ml of a Folin-Ciocalteau's reagent (10x dilutions) and 4 ml of sodium carbonate solution. The tubes were kept in a dark room for 2 hours then filled to cuvettes before absorbance at 765 nm was measured. TPC was expressed as gallic acid equivalent (GAE) in mg per gram extract

12. The analyzed community data, the scientific data including data from literature reviews and laboratory results, and international conservation guidelines were provided to the community in order to guide them in conserving medicinal plants for sustainable use in their community. Participatory action research was subsequently performed for brain storming to find the best way to protect the medicinal plant's habitat at a local level and for the sustainable use of their medicinal plants. Collaboration with the community and relevant organizations was also subsequently performed.