CHAPTER 3

RESEARCH METHODOLOGY

There are two main parts in this chapter including of materials and testing instruments, and experimental details. Figure 3.1 displayed experimental frameworks for this research.

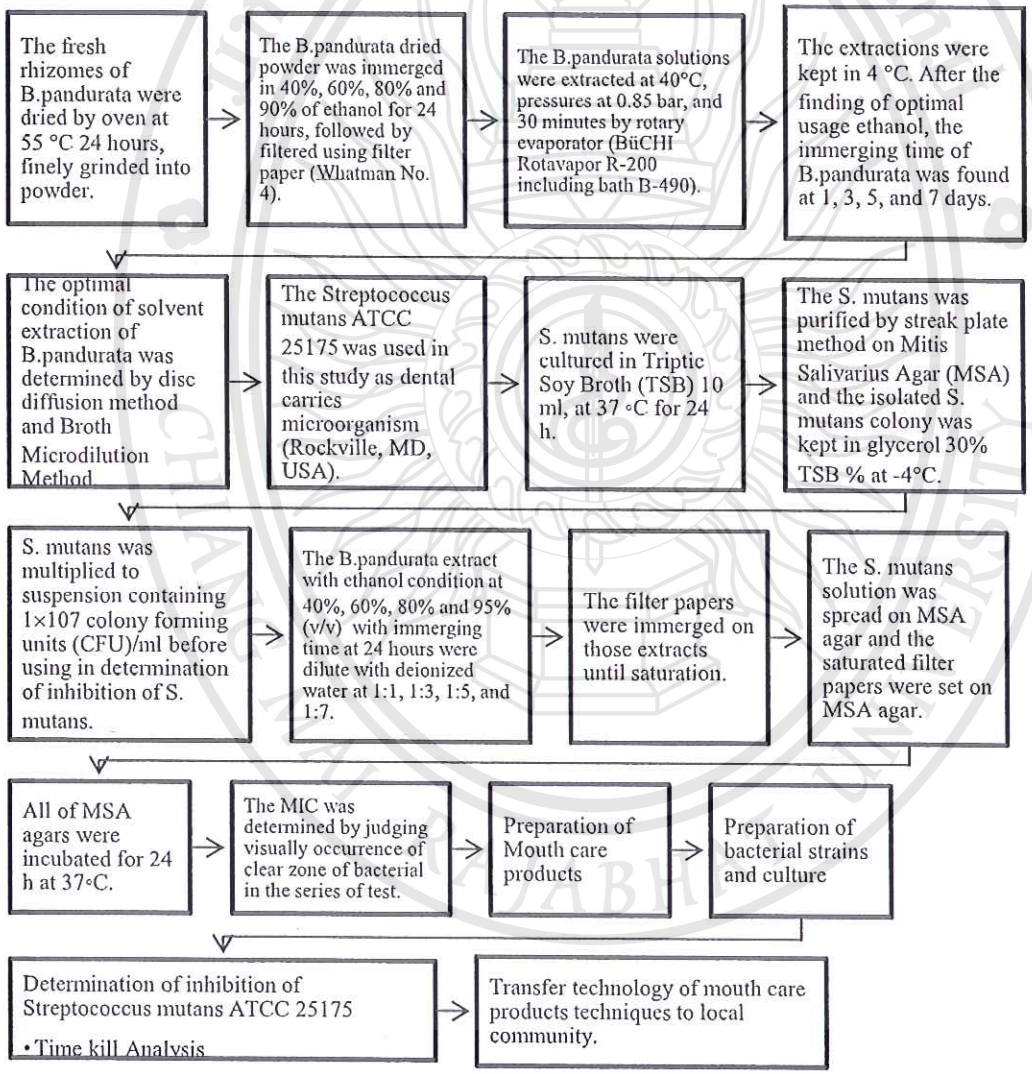

Figure 3.1 Experimental frameworks

3.1 Materials and Testing Instruments

3.1.1 Materials and Chemicals

The extract solution on solvent extraction was used Ethyl alcohol 95% for all experiments. The others materials was applied for bacteria testing such as Glycerol, *Streptococcus mutans* ATCC 25175, Mitis Salivarius Agar (MSA), and Triptic Soy Broth (TSB).

Table 3.1 Composition of Mitis Salivarius Agar (MSA)

Materials	Weight or Volume
Enzymetic Digest of casein	15 g
Enzymetic Digest of animal tissue	5 g
Sucrose	50 g
Dextrose	1 g
K_2HPO_4	0.075 g
Trypan Blue	0.0008 g
Agar	15 g
Distilled water	1 L
The mixture was boiled gently melted. Divide the apple shaped bottle 500 ml bottle 250 ml autoclave sterilized at 121 ° C for 15 minutes, pH 7.3 ± 2.	

Table 3.2 Composition of Triptic Soy Broth (TSB)

Materials	Weight or Volume
Tripticase peptone	17 g
Phytonepepton	3 g
NaCl	5 g
K_2HPO_4	2.5 g
Dextrose	2.5 g
Distilled water	1L
The mixture was boiled gently melted. Divide the apple shaped bottle 500 mL bottle 250 mL autoclave sterilized at 121 ° C for 15 minutes, pH 7.3 ± 2.	

3.1.2 Testing Instruments

Testing instruments were as follows:

- Glassware
- Vacuum Filter
- Oven
- Biological Safety Cabinet
- Autoclave
- Evaporator BuchiR200
- Incubators Shaker DNP Electric Thermostatic Incubator
- Freezer
- Filter paper Whatman No.4
- Microcentrifuge tube 1.5 ml

- Micropipette 200 ul and 1000 ul

3.2 Experimental Procedure

3.2.1 Preparation of *Boesenbergia pandurata* powder

Fresh rhizomes of *B. pandurata* were used for extraction. The cleaned rhizomes were sliced and separated into fresh and dry form. Fresh rhizomes of *B. pandurata* were bought from the Talat Thai market, Bangkok, Thailand in January 2012. The *B. pandurata* rhizomes were cut around 2-3 centimeters. The fresh rhizomes of *B. pandurata* were dried by oven at 55 °C 24 hours, finely grinded into powder for increment of surface area. The high surface area will support the extraction of *B. pandurata* oil. Figure 3.2 was showed the cutting of area for of *B. pandurata* oil extraction.

Figure 3.2 Cutting area for *B. pandurata* oil extraction

3.2.2 Solvent extraction process of *B. pandurata*

The powders of fresh rhizomes of *B. pandurata* were dried at 55°C for 24 hours before extraction processes. The powders of dried rhizomes of *B. pandurata* were immersed 24 hours at room temperature in 40%, 60%, 80%, and 95 %v/v (v/v) of ethyl alcohol at 1:10 of powders of dried rhizomes of *B. pandurata* per ethyl alcohol. The immersed *B .pandurata* solutions were filtered by filter paper No. 4. The *B. pandurata* solutions were extracted at 40°C, pressures at 0.85 bar, and 30 minutes by rotary evaporator (BüCHI Rotavapor R-200 including bath B-490). The extractions were kept in 4 °C. After the finding of optimal usage ethanol, the immerging time of *B. pandurata* was found at 1, 3, 5, and 7 days. The optimal condition of solvent extraction of *B .pandurata* was determined by disc diffusion method and Broth Microdilution Method. The *B. pandurata* extraction by solvent extraction and hydrodistillation were mixed in powdered toothpaste and mouthwash products. The extracts were collect into Amber glass bottle at 4°C.

3.2.3 Hydrodistillation process of *B. pandurata*

The fresh rhizomes of *B. pandurata* were mixed with water at ratio 1:2 (*B. pandurata :* water) and distillated on varied temperature at 30°C, 60°C and 90°C. The hydrodistillation samples were collected in three parts including *B. pandurata* oil (Top product), distillates (Bottom product) and residue (Crude). All hydrodistillation samples were test inhibition of *S. mutans* analyzed by disc diffusion method. All samples were collect into Amber glass bottle at 4°C.

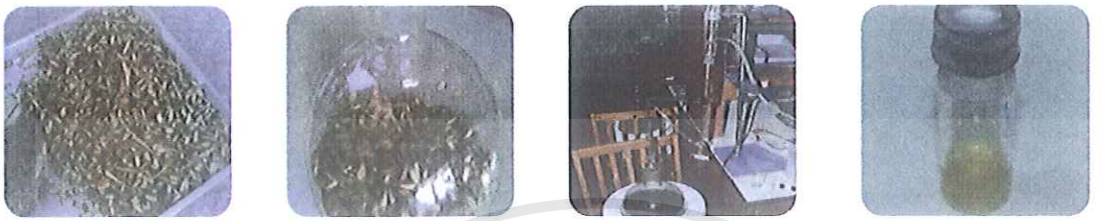

Figure 3.3 Hydrodistillation process of *B. pandurata*

3.2.4 Bacterial strains and culture conditions

The *Streptococcus mutans* ATCC 25175 was used in this study as dental carries microorganism (Rockville, MD, USA). *S. mutans* were cultured in Triptic Soy Broth (TSB) 10 ml, at 37 ◦C for 24 h. The *S. mutans* was purified by streak plate method on Mitis Salivarius Agar (MSA) and the isolated *S. mutans* colony was kept in glycerol30% TSB % at -4°C. *S. mutans* was multiplied to suspension containing 1×10^7 colony forming units (CFU)/ml before using in determination of inhibition of *S. mutans*.

3.2.5 Determination of inhibition of *Streptococcus mutans*

The solvent extraction samples were performed on Disc diffusion method and Broth Microdilution in duplicate. The hydrodistillation samples were only analyzed on Disc diffusion method in duplicate ATCC 25175 by Disc diffusion method and Broth Microdilution method (Barry, 1991)

3.2.5.1 Disc diffusion method

The *B. pandurata* extract with ethanol condition at 40%, 60%, 80% and 95% (v/v) with immerging time at 24 hours were dilute with deionized water at 1:1,

1:3, 1:5, and 1:7. The filter papers were immerged on those extracts until saturation. The *S. mutans* solution was spread on MSA agar and the saturated filter papers were set on MSA agar. All of MSA agars were incubated for 24 h at 37◦C. The MIC was determined by judging visually occurrence of clear zone of bacterial in the series of test.

3.2.5.2 Broth microdilution method

A bacterial suspension (0.1ml) containing 1×10^7 colony forming units (CFU)/ml was added to TSB broth and incubated for 24 h at 37◦C. The *B. pandurata* extract with ethanol condition were added into TSB broth. The MBC was the concentration in which S. mutans was unable to remain viable.

3.2.6 Preparation of Mouth care product

Two mouth care products were prepared into powder toothpaste and mouthwash. The major composition of powder toothpaste contained 18.86% Siam rough bush, 18.86% Liquorice, 18.86% Clove tree, 18.86% Cuttlebone, 18.86% Marl limestone, 4.14% Cinnamomum camphor, 0.06% menthol and 1.50% *B. pandurata* extract. The *B. pandurata* extract were used one optimal condition of solvent extraction and one optimal condition of hydrodistillation. Table 3.3 showed the ratio of composition of powder toothpaste samples. The mixed powder toothpaste showed in Figure 3.4. The preparation of mouthwash mixed many aqueous chemicals such as Surfactant (Polysorbate 20), Sorbitol, Eucalyptus oil, Tartrazine, Menthol, ethanol, salt, deionized water, and *B. pandurata* extract. The ratio of mouthwash compositions were shown in Table 3.4. The mixed mouthwash showed in Figure 3.5. Two mouth care product were analyzed the product quality by time kill analysis for determination of inhibition of *S. mutans*.

Figure 3.4 Powder toothpaste with *B. pandurata* extract

Figure 3.5 Mouthwash with *B. pandurata* extract

Table 3.3 Main composition of powder toothpaste

Compositions	Recipe (% by weight)	
	A1	A2
Siam rough bush	18.86%	18.86%
Clove tree	18.86%	18.86%
Liquorice	18.86%	18.86%
Cuttlebone	18.86%	18.86%
Marl Limestone	18.86%	18.86%
Comphanone	4.14%	4.14%
Menthol	0.06%	0.06%
B. pandurata extract by Solvent Extraction	1.50%	-
B. pandurata extract by Hydrodistillation	-	1.50%
Total	100	100

Table 3.4 Main composition of mouthwash

Compositions	Recipe (% by Volume)	
	B1	B2
Sufactant (Polysorbate 20)	0.07	0.07
Sorbitol	10	10
Eucalyptus oil	0.07	0.07
Tartazine	0.07	0.07
Menthol	0.07	0.07
Ethanol	1	1
Salt	0.07	0.07
Deionized water	87.67	87.67
B. pandurata extract by Solvent Extraction	1	-
B. pandurata extract by Hydrodistillation	-	1
Total	100	100

3.2.7 Time kill Analysis

The *Streptococcus mutans* ATCC 25175 suspension containing 1×10^7 colony forming units (CFU)/ml was added to TSB broth and incubated for 24 h at 37◦C. The powder toothpaste and mouthwash put into all suspension at ratio 1:1. Appropriate

volumes of the suspensions were diluted with 0.9% of NaCl. Test solutions and growth controls were then placed into a shaker and incubated at 37°C. Samples were withdrawn at 5, 15, 30, 50, 75, 105, 140, and 180 minutes serially diluted (when necessary), and plated onto MSA agar using spread plate method. The plates were incubated at 37°C for 24-48 hours, and colony counts were determined. All kill curves were performed in duplicate.

3.2.8 Community Technology transfer

3.2.8.1 Analysis of the community

Approach to analyze community is to study the composition of the population relying on secondary data and survey population and the statistical analysis. For the survey area will be a community surrounding sugar company, the study area consists of five areas of study is the Tambon Phai Lom, Krathum, Norpeahmak, and Tambon Noen Kum. The data will be used to store data in the form of technology transfer. A guideline of transfer technology was developed in the form of preparation of posters and make community to attend the workshop. The guideline is divided into theoretical study and educational workshops. The number of participants is not less than 30 people to allow for the data used in the statistical analysis of the results further.

3.2.8.2 Analysis of technology transfer with statistical principles

Research Instruments for research consist of qualitative and quantitative research tools, questionnaires, interview form, poster and transfer technology to community by 5 level evaluation;

Most = 5

Much = 4

Medium = 3

Little = 2

Least = 1

The range of average value at 1.00 to 1.50 defined to the demand of people at lowest level.

The range of average value at 1.51 to 2.50 defined to the demand of people at low level.

The range of average value at 2.51 to 3.50 defined to the demand of people at moderate level.

The range of average value at 3.51 to 4.50 defined to the demand of people at high level.

The range of average value at 4.51 to 5.00 defined to the demand of people at highest level.

Technology transfer to community in Phisanulok province used both of qualitative and quantitative research tools which were as follows:

- Analysis of qualitative data were study from secondary data, related research, interview data, observation data and focus group
- Analysis of quantitative data were personnel information, technology transfer evaluation to frequencies and percentages by SPSS
  - Personnel information analysis; sex, age, marital status, income and career by frequencies and percentages
  - Technology transfer analysis for career by frequencies and percentages and standard deviation calculator
  - Technology transfer evaluation analysis evaluation to frequencies and percentages