CHAPTER 3
MATERIALS AND METHODS

3.1 Equipments and materials use in experiments

3.1.1 Apparatus and Instruments

1. Ultrasonicator, model 889, cole Parmer, U.S.A.
2. Analytical balance, Meter PE 300, Switzerland.
3. Analytical balance, Precisa XT220A, Switzerland.
4. Oven, Kan Seng Lee Machinery, modal HA-20, Bangkok, Thailand
5. Rotary vacuum evaporator, EYELA, Japan
6. pH-meter, model pH 900, Precisa, Switzerland.
7. Water bath, Memmert, Germany
8. Anaerobic jar, BBL Ltd., France
9. Brookfield meter, Sunwa YX-360 TRD, Thailand
10. Micropipettes 1-200µl, 100-1000 µl, Pipetman®, Gilson Co. Ltd., France
11. Autoclave, Memmert, Germany
12. Hot air oven, Memmert, Germany
13. Incubator
14. Magnetic stir plate and stir bar

3.1.2 Plant extracts

The commercial plant extracts used in this study were claimed for anti-acnes property. The *Garcinia mangostana* powder extract (MG-1) and liquid extract (MG-2) were from SNP Co. Ltd., the *Psidium guajava* extracts (GU) from TCFF Co. Ltd. and the combination of Capryloyl Glycine, Sarcosine and *Cinnamonum zeylanicum* extract (SP) were obtained from Adinoph Co. Ltd.

1. Mangosteen rind extract, MG-1 and MG-2 (SNP Co. Ltd.)
2. Guava leaf extract, GU (TCFF Co. Ltd.)
3. Cinnamon bark extract SEPICONTROL or SP (Adinoph Co. Ltd.)

3.1.3 Microorganisms

1. *Staphylococcus aureus* (ATCC 25923)
2. *Propionibacterium acnes* (Clinical strain)
3. methicillin resistant *Staphylococcus aureus* (Clinical strain)

3.1.4 Media

1. Tryptic Soy Agar (TSA, Oxoid Ltd, England)
2. Tryptic Soy Borth (TSB, Oxoid Ltd, England)

3.1.5 Animals

Male New Zealand white Rabbits, aged 8-9 months with a weight of 2.0-2.5 kg were purchased from Faculty of Veterinary Medicine, Chiangmai University. All animals were kept in a room maintained under environmentally controlled conditions of 24±1˚C. They were acclimatized at least 1 week before starting the experiments.

3.1.6 Chemicals

1. 95% Ethanol (Commercial grade, Liquor Distillery Organization, Thailand)
2. De-ionized water (Millipore, U.S.A.)
3. Clindamycin (AR, RPC international,Thailand)
4. Propylene glycol (Commercial grade, United Chemical & Trading, Thailand)
5. Glycerin (Commercial grade, Union science, Thailand)
6. Jojoba oil (Commercial grade, United Chemical & Trading, Thailand)
7. Tween 20 (Commercial grade, United Chemical & Trading, Thailand)
8. Sodium lauryl sulfate (AR, United Chemical & Trading, Thailand)
9. Methyl paraben (AR, Union science, Thailand)
10. Propyl paraben (AR, Union science, Thailand)
11. Mineral oil (Commercial grade, United Chemical &Trading,Thailand)
12. Hydroxyethylcellulose (Commercial grade, Union science, Thailand)
13. Triethanolamine (AR, SRICHAND UNITED, England)

14. Azelaic acid (AR, UNIVAR, Germany)
15. sodium metabisulfite (AR, RCI LABSCAN, Thailand)
16. Dimethyl sulfoxide (AR, RCI LABSCAN, Thailand)
17. Carbomer 940 (Commercial grade, Union science, Thailand)
18. Benzoyl peroxide (AR, RPC, Thailand)
19. Salicylic acid (AR, UNIVAR, Germany)
20. Absolute ethanol (AR, Merck, Germany)

3.2 Antibacterial activity test

Propionibacterium acnes, methicillin resistant *Staphylococcus aureus* and *Staphylococcus aureus* (ATCC 25923) were obtained from the Department of Microbiology Faculty of Medicine, Chiangmai University.

3.2.1 Culture Media

The medium using for determination of antibacterial activity against were Tryptic Soy Agar (TSA, Oxoid Ltd, England) and Tryptic Soy Borth (TSB, Oxoid Ltd, England).

3.2.2 Inoculum preparation

3.2.2.1 Preparation and standardization of the inoculums[25]

S. aureus (ATCC 25923), *P. acnes* and MRSA and were cultured on TSA. At least three well-isolated colonies of the same morphologic type from culture plate were picked and suspened in TSB. The turbidity of the actively growing culture was adjusted with broth to a turbidity standard at 0.5 McFarland standard (10^8 CFU/mL).

3.2.2.2 Preparation of antibacterial drug and extracts

The MG-1 extract and GU extract were weighted and suspended in water whereas MG-2 extract and SEPICONTROL® were weighted and suspended in 95%ethanol, to concentration of 2% w/v and 5% w/v. Benzoeyl peroxide, Azelaic acid, Salicylic acid were used as reference antibacterial standard agents. All Standard solutions were made up to 5% except clindamycin standard solution was 0.01%.

3.2.3 Antibacterial activity by agar cup diffusion method [13, 26]

Ten mL of TSA was melted and poured into a Petri dish. The medium was standed still until it turned to solid form. Four sterile 12 mm diameter of aluminium rings were placed on the surface of agar. Then 10 mL of melted TSA kept in 50˚C water bath was mixed with 0.1 mL of *S.aureus* or MRSA or *P.acnes* suspension inoculum. The seeded agar was thoroughly mixed and was overlaid on the first layer. After the top layer was solidified, rings were gently removed and wells were filled with the tested sample and positive control (benzoyl peroxide 5%, clindamycin 0.01%, salicylic acid 5% and azelaic acid 5%). The plates were incubated up right at 37˚C, 18 hrs for *S.aureus* and 72 hrs in anaerobic jar for *P.acnes*. After incubation, the plates were examined and the inhibition zone were measured in diameter (mm).

3.2.4 Determination of minimum inhibitory concentration (MIC) [13, 26]

The working antimicrobial solution of Benzoyl peroxide, Salicylic acid, Azelaic acid and Clindamycin were varied from 25 µg/mL to 0.048 µg/mL for all standard bacteria strains except Clindamycin varied from 0.25 µg/mL to 0.000048 µg/mL for *P.acnes*. The concentration of plant extracts were varied from 62.5 mg/mL to 0.12 mg/mL for all standard bacteria strains. The final concentrations of antimicrobial agents were half of the working solution after an equal volume of the adjusted bacterial inoculum was added. The inoculum was prepared to $10^5 – 10^6$ CFU/mL by diluting the bacterial suspension from McFarland no.0.5 for 100 times. The MIC against *S.aureus* and MRSA were observed after 35 – 37 ˚C for 18 hrs of incubation. The MIC against *P.acnes* were observed after 72 hrs of incubation at 35-37˚C in an anaerobic jar. The MIC was the lowest concentration of the antimicrobial agent containing tube that showed no bacteria growth, as shown in Figure 3.1.

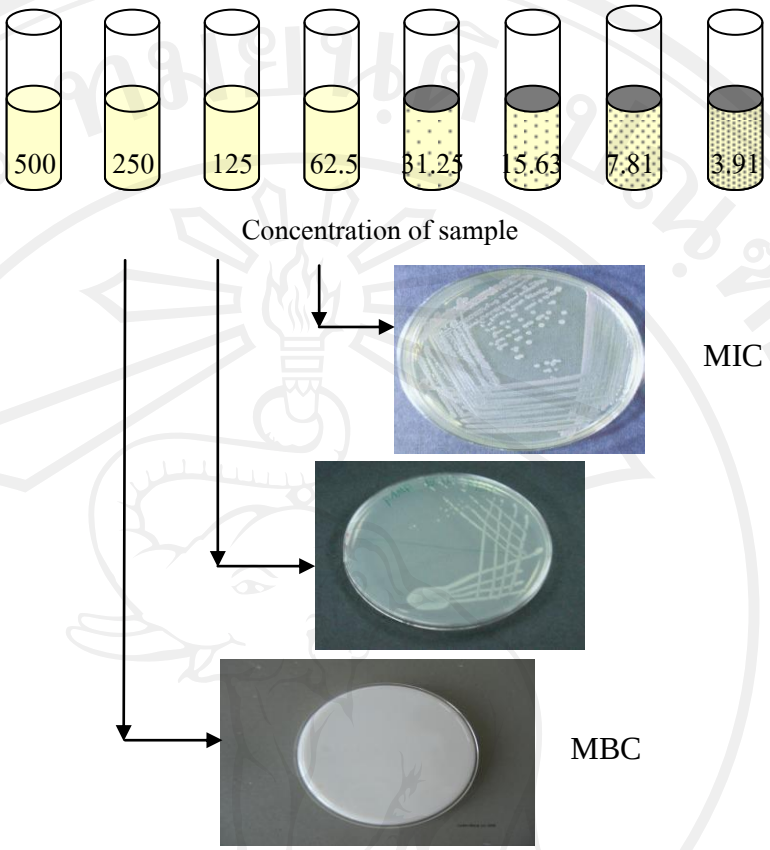

Figure 3.1 Two fold dilution method for MIC (minimum inhibitory concentration) and Determination of MBC (minimum bactericidal concentration)

3.2.5 Determination of minimum bactericidal concentration (MBC) [13,26]

After the MIC was determined, the MBC (minimum bactericidal concentration) was then observed. Each tube that showed no visible of bacterial growth was then subcultured 0.01 ml on a quadrant of TSA plate. The MBC was determined as the lowest concentration of the drug or samples having less than 0.1% viable bacteria, as shown in Figure 3.1.

3.3 Experiments

3.3.1 Solubility test of the MG-2 extract

The solubility of each extract was tested in various solvents, for example, double distilled water (DI), 95%ethanol, glycerine, propylene glycol, jojoba oil, mineral oil, DEP 96, DMSO and Tween 20 by adding 10 volumes of the solvent in a separate extract to give 1:10 condensed intensity weight by volume, then shaked with vortex mixer at room temperature and observe the solubility character immediately.

3.3.2 Stability in acid-base test

Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were used for adjusting pH value of the MG-2 extract equals to 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, then observe physical change immediately. Substance solutions were kept separately at room temperature (with and without light protection), 4˚C for 1 month and the system Heating/Cooling at 4˚C for 48 hrs and 45˚C for 48 hrs, alternately 6 cycles.

3.4 Formulation and stability test of gel base

3.4.1 Formulation of gel base

Fourteen gel base were developed using various types and concentration of gelling agent (HEC, HPMC, CAP 940, CAP EDT 2020 and MC). Each gel base prepared by disperse gelling agent in distilled water while the mixture was left to hydrate. Liquid germal plus was added as preservative, then added sorbitol, EDTA, sodium metabisulfite, triethanolamine, ethanol and DI water. The obtained gel base was packed in a tight container and kept in a cool place until used. All of the developed gel base, were characterized for their physical properties, pH as well as stability. The most suitable gel base was them selected for the incorporation with MG-2 extract.

3.4.2 Characterization of Gel bases

3.4.2.1 Visualization of physical appearances

The physical characteristics of gel bases were observed such as odor, color, smoothness.

3.4.2.2 Spreadability and feel on skin

Spreadability and feel on skin of all gel bases were evaluated. They were also compared at before and after storage at various conditions.

3.4.2.3 pH

The pH of all gel bases were measured. For the stability test, They were also compared at before and after storage at various conditions.

3.4.2.4 Stability test of gel base

The stability of all gel bases were tested under various temperatures (room temperature, with and without light protection, 2-8 ˚C, 45˚C). Accelerated test: heating-cooling cycling method which defined as alternation of storage conditions at 45˚C for 48 hours and 4˚C for 48 hours (1 cycle) for 6 cycles.

3.5 Formulation and stability test of extract gel [24]

The MG-2 extract with the highest antibacterial activity was added in the selected gel base make MG-2 extract gel. These were then determined for pH, stability, physical properties, spreadability and feel on skin. Stability test was also conducted to storage at various temperatures (room temperature, with and without light) for 6 months and 2-8˚C, 45˚C for 4 months and accelerated test: heating – cooling cycling method which defined as alternation of storage conditions at 45˚C for 48 hours and 4˚C for 48 hours (1 cycle) for 6 cycles. After that, the antibacterial activity was tested again by agar cup diffusion method.

3.6 Skin irritation test in Rabbits [9]

Three albino rabbits were used for skin irritation by modified Draize model. Draize model and its modification are commonly used for assaying skin irritation. Draize used this scoring system to calculate the primary irritation index (PI). This is calculated by averaging the erythema scores and the edema scores of all sites (abraded and nonabraded). These two averages are then added together to give the PII value. A value of less than 2 was considered nonirritating, 2 to 5 mildly irritating, and greater than 5 severely irritating. A value of 5 defines an irritant by Consumer Product Safety Commission (CPSC) standards.

Table 3.1 Modified Draize-FHSA Model used in this research

Topics	Descriptions
1. Number of animals	3 albino rabbits (clipped)
2. Test sites	2×1 $inch^2$ sites on dorsum
3. Test material	Applied diluted to the test sites, liquids: 0.5 g
4. Occlusion	1 $inch^2$ surgical gauze over each test site
5. Occlusion period	Rubberized cloth over entire trunk
6. Assessment	4 hours
	1, 24, 48 and 72 hours
	Visual scoring system

Table 3.2 Draize-FHSA Scoring System

Topics	**Score**
Erythema and edema formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well- defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised > 1 mm)	3
Severe erythema (raised < 1 mm and extending beyond the area of exposure)	4

3.7 Skin irritation test in volunteers

Before test for antibacterial activity, the gel base and MG-2 gel were tested for skin irritation in volunteer.

3.7.1 Subjects of study

Thirty-two Thai volunteer (aged 25 – 60; n=32) were selected by using inclusion and exclusion criteria.

Exclusion criteria

1. Subjects who did not or could not sign an informed consent form, unable to comply with the requirements of the protocol
2. Subjects who were participating in other clinical study

Inclusion criteria

1. Healthy skin, no skin diseases such as dermatitis
2. Unnecessary using, receiving or taking any preparation such as antihistamin drug or any other drugs
3. Non-atopic, with no past or present history of skin diseases
4. No any scar, wound, blemish, and any skin diseases
5. No irregular skin color at test site
6. Subjects agree to sign an informed consent form
7. Comfortable involve in this study

Discontinuation criteria

1. Have skin irritation
2. Subjects who want to quit from the experiment for any reason
3. Subjects who couldn't practice following instruction criteria of study

3.7.2 Test substance application protocol [9]

Thirty-two Thai volunteers were used for irritation test. The back of volunteer was divided into four portions along backbone line (the back on the left, the back below the left, the back on the right, the back below the right). Each part was applied by patch test that contain sample (gel base, MG-2 gel and 1% sodium lauryl sulfate) in each point. (Figure 3.1). After 48 hours, the irritation in volunteer was evaluated up to 7 days.

Figure 3.2 Profile of test site on patch test

3.8 Clinical study of MG-2 gel for anti-acnes

3.8.1 Subjects of study

Twenty-one Thai volunteers (aged 14-30; n=21) were selected by using inclusion and exclusion criteria.

Exclusion criteria

1. Subjects who did not or could not sign an informed consent form, unable to comply with the requirements of the protocol
2. Subjects who were participating in other clinical study
3. Subjects were pregnant woman, brea feeding mother
4. Subjects who had a previous history of hypersensitivity to herbal drugs

Inclusion criteria

1. Subjects had mild to moderately severe acne exhibited a minimum of 5 inflammatory lesions i.e. papules and pustules and minimum of 5 non- inflammatory lesions i.e. blackheads and whiteheads.
2. Unnecessary using, receiving or taking any preparation such as antihistamin drug or any other drugs
3. Do not use the products in the fix tests area
4. Non-atopic dermatitis, with no past or present history of skin diseases
5. No any scar, wound, blemish, and any skin diseases
6. No irregular skin color at test site
7. Subjects agree to sign an informed consent form
8. Comfortable involve in this study

Discontinuation criteria

1. Have skin irritation
2. Subjects who want to quit from the experiment for any reason
3. Subjects who couldn't practice following instruction criteria of study

3.8.2 Test substance application protocol [18]

Subjects were advised to wash their face with mild soap regularly. Subjects will be divided into 2 group (Figure 3.3).

- Group 1 receives gel on left side face (MG-2 gel=gel A) and gel base (gel B) on right side face
- Group 2 receives gel on right side face (MG-2 gel=gel A) and gel base (gel B) on left side face

Subjects were instructed to apply the test gel twice daily (once in the morning, once in the evening) for 2 weeks. In the beginning of the study and every weeks, each subject will be attended for clinical evaluation and measured facial skin oiliness with sebumeter.®

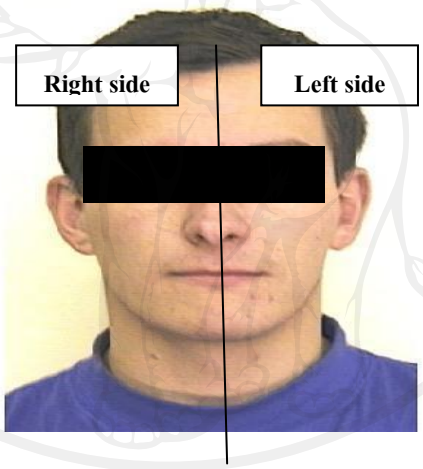

Figure 3.3 The profile of test side on volunteers

3.8.3 Clinical evalution

Two parameters were evaluated:

3.8.3.1 The exact number of each lesion type present on the face from ear to ear and above the mandibular line was counted separately for each side of the face.

3.8.3.2 For visits after the first examination, a clinical evaluation of the subjects's overall change in facial acne compared with the appearances at the beginning of the study.

3.8.4 Statistical analyses

% decreasing of acnes after the test was calculated comparing with the numbers of acnes before the test.