CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

1. Ethanol (Labscan Asia, Ireland)
2. Methanol (HPLC grade, Labscan., Thailand)
3. Tween 80 (VIDHYASOM Co., LTD., Thailand)
4. Mineral oil (VIDHYASOM Co., LTD., Thailand)
5. Cetiol HE (VIDHYASOM Co., LTD., Thailand)
6. Glycerin (VIDHYASOM Co., LTD., Thailand)
7. Conc. Paraben (VIDHYASOM Co., LTD., Thailand)
8. Propylene glycol (VIDHYASOM Co., LTD., Thailand)
9. Gentamicin (Schering-Plough., LTD., Indonesia)
10. Sodium metabisulfite (Namsiang Co., Thailand)
11. Hydroxyethylcellulose (O.V. chemical and supplied Co., Thailand)
12. Gallic acid (Sigma Aldrich Co., U.S.A.)
13. Quercetin (Sigma chemical Co., USA)
14. Tran-cinnamamide (Sigma chemical Co., USA)
15. Morin (Sigma chemical Co., USA)
16. Rutin (Sigma chemical Co., USA)
17. Ellagic acid (Sigma chemical Co., USA)
18. Chlorogenic acid (Sigma chemical Co., USA)
19. Pinocembrin (Sigma chemical Co., USA)
20. Chysin (Sigma chemical Co., USA)
21. Hesperidine (Sigma chemical Co., USA)
22. Acetonitrile (HPLC grade, Labscan, Thailand)
23. Natriumdihydrogenphosphate-2-hydrate (Riedel-de Haen)

24. Phosphoric acid (Labscan, Thailand)

3.1.2 Instruments

1. Analytical balance 2 position (A&D. Co., LTD. Japan)
2. Analytical balance 4 position (Precisa XT220A, Switzerland)
3. Evaporator (Buchi, Switzerland)
4. Water bath (Memmert®, CMbH Co., Ltd., Germany)
5. pH Meter (Horiba Model EX-20, Korea)
6. Vortex mixer (Scientific industry. USA)
7. Autoclave (HICLAVE® HVE-50, Hirayama Manufacturing Co.Ltd., Japan)
8. Centrifuge (Zigma® 2-16, Scientific Promotion Co., Ltd., Germany)
9. Rheometer (Brookfield® Model DV-III, Brookfield Co. Ltd.)
10. HPLC (hp 1100 series, Hewlett Packard, USA)
11. Auto pipett 1-200 µl, 1-1000 µl (Pipetman®, Gilson Co. Ltd., France)
12. Incubator (Napco® 332, National Appliance Co., Ltd., Germany)
13. Sonicator (Elma®, Elma GmbH & Co KG, Germany)
14. Heating mantle (KIKA®, Yellow line, Germany)
15. Homogenizer (KIKA® Yellow line, Germany)
16. Hot air oven (Memmert®, CMbH Co., Ltd., Germany)
17. Lamina air flow hood (Mauire®, USA)

3.1.3 Microorganisms

The bacterial strains were obtained from the Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University.

Gram- positive bacteria

1. *S. aureus* ATCC 25923 (for screening antibacterial activity)
2. *S. aureus* ATCC 29213 (for determination of MIC and MBC)
3. MRSA
4. GAS

Gram- negative bacteria

1. *P. aeruginosa* ATCC 27853
2. *E. coli* ATCC 25922

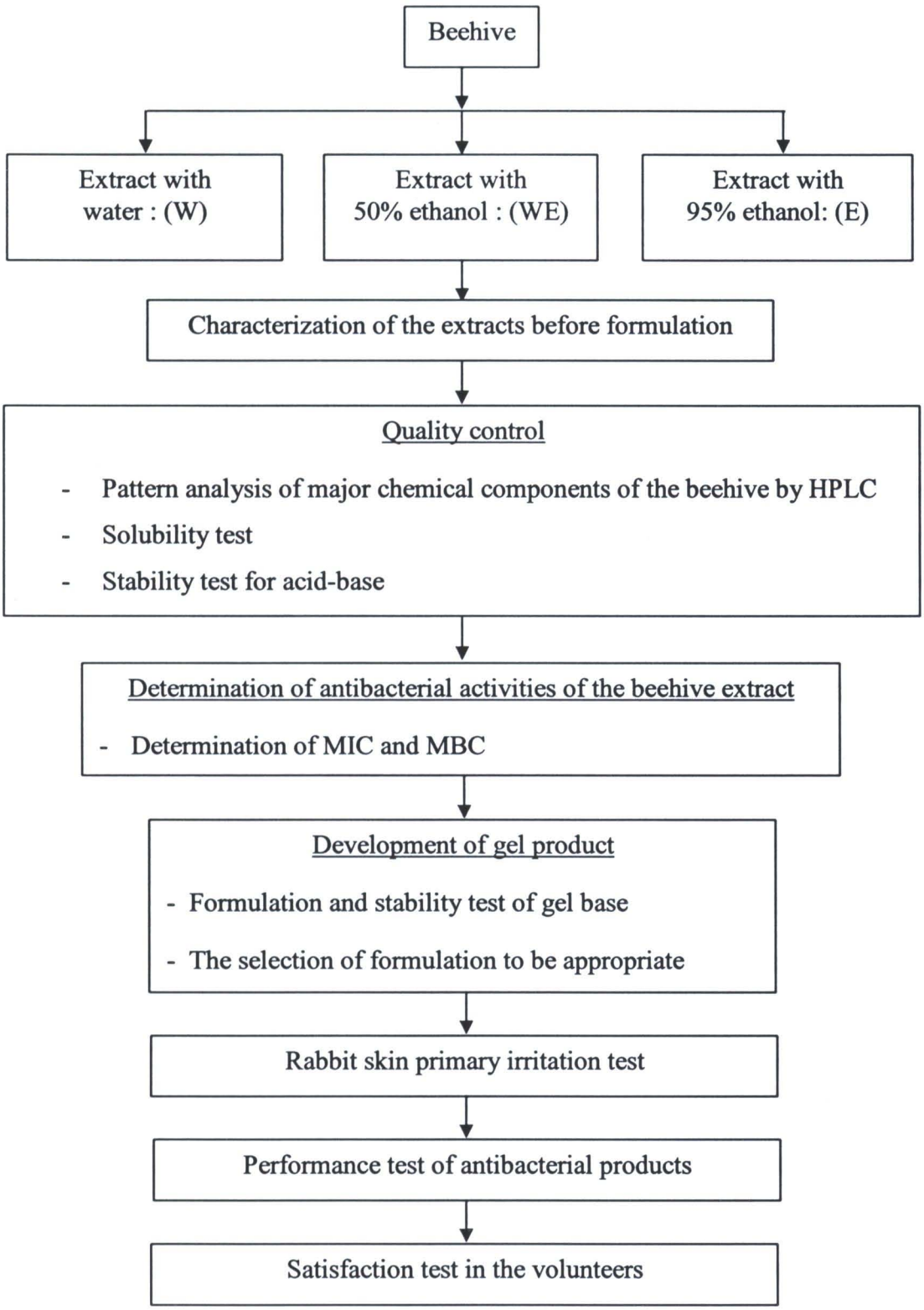
3.1.4 Culture media

1. TSA (Merck Ltd. Germany)
2. TSB (Merck Ltd. Germany)
3. THB (Merck Ltd. Germany)

3.1.5 Animal experiences

Three amount rabbits of the male (species of New Zealand white) weigh between 2.0 to 2.5 kg, age 8-9 months, from the Faculty of Agricultural, Chiang Mai University.

Research designs



3.2 Methods

3.2.1 Extraction of beehive from various methods, physical and chemical characterization

The beehives after removing the honey off, were obtained from Chiang Mai Healthy Products Company Limited, Sarapee district, Chiang Mai. The beehives that kept no longer than a month were cleaned and extracted using different solvents. The extraction processes are as follows

3.2.1.1 Extraction of beehive

Extracted by water

- Weigh the beehive and then cut into small pieces
- Put into the beaker, then added the water (for flooded the beehive)
- Beakers close to prevent evaporation and to set the water bath temperature of 80 ° C for 20 minutes
- Filter the extracts with thin white cloth 2 times
- Leave to cool at room temperature for 1 hour (solution to segregation)
- Separate the wax, then filter the extracts using filter paper whatman No.1 and evaporated by vacuum rotary evaporator at 65 °C
- Weighing the extracted obtain

Extracted by 50% ethanol

- Weigh the beehive and then cut into small pieces
- Put into the beaker, then added 50% ethanol (for flood the beehive) and beaker closed to prevent evaporation
- Leave for 1 hour
- The beaker set in the water bath temperature of 50 °C for 1 hour
- Filter the extracts with thin white cloth 2 times
- Filter the extracts using filter paper whatman No.1
- Evaporated the extracts by vacuum rotary evaporator temperature at 45°C

- Separate the wax, then filtrate extracts by paper filter
- Evaporated the extracts by vacuum rotary evaporator 45 °C
- Weighing the extracted

Extracted by 95% ethanol

- Weigh the beehive and then cut into small pieces
- Put into the beaker, then add 95% ethanol (for flood the beehive) and closed beaker to prevent evaporation
- The beaker set in the water bath temperature of 50 °C for 1 hour
- Filter the extracts with thin white cloth 2 times
- Evaporated the extracts by vacuum evaporator temperature at 45 °C
- Added hot water to them (Wait to cool at room temperature)
- Separate the wax, then filter the extracts using filter paper
- Evaporated the extracts by vacuum rotary evaporator at 65 °C
- Weighing the extracted obtain

3.2.1.2 Quality control of the beehive extracts by HPLC

The chromatographic fingerprint of beehive extract was determined by reverse phase high performance liquid chromatography. The separation was performed on a HPLC series hp 1100 (Hewlett Packard, USA) equipped with CHEM STATION software, a degasser G1322A, a binary gradient pump G1311A. a thermoautosampler G1313A. The column was an Zorbax Eclipse XDB - C18 4.6 x 250 mm 5 microns and a C18 guard column (Eclipse XDB 4.6 x 12.5 mm, 5 micron). The mobile phase consisted of Acetonitrile (eluent A) and 3 mM of phosphate buffer solution pH 3 in water (eluent B). The gradient program was as follows: 10% A to 40% A (40 min), 40% A to 55% A (10 min). The injection volume for all samples was 50 µl. Simultaneous monitoring was performed at 210 nm at a flow-rate of 1.5 ml/ min.

3.2.1.3 Physical and physicochemical characterization

Solubility test

The solubility of beehive extracts in various vehicles were tested as DI water, ethanol, glycerin, propylene glycol, cetiol HE, mineral oil and tween 80, in the concentration of 1:20. Then the solutions were mixed with vortex mixer at room temperature and observed for their solubility and compatibility.

The stability test for acid-base

The solution of HCl (1N) or NaOH (10% w/v) was added into the beehive extract solution for adjusting the pH to 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 (the beehive extract solution was used as a control). Then observed the physical changes that occur immediately and after storage at various conditions: room temperature, room temperature in the dark, 4 °C and 45 °C for 1 month and at accelerated test: heating/cooling cycling method which defined as the alternation of storage conditions from 45 °C for 48 hr to 4 °C for 48 hr (1 cycle) for 6 cycles.

3.2.2 Determination of antibacterial activity of beehive extracts [84]

3.2.2.1 Preparation and standardization of the inoculums

Four standard strains of bacteria as *E. coli*, *P. aeruginosa*, *S. aureus* and MRSA were cultured on TSA plate agar. At least three well-isolated colonies of the same morphologic type from culture plate were picked and suspended in TSB, GAS was suspended in THB. The turbidity of the actively growing culture was adjusted with broth to be a turbidity standard at 0.5 McFarland standard (10^8 CFU/ml).

3.2.2.2 Preparation of antibacterial drug and beehive extracts

W, WE and E extracts were diluted in steriled water at the concentration 1%, 5% and 10% and gentamicin cream was used as an antibacterial standard (positive control) while steriled water and ethanol was used as negative control.

3.2.2.3 Antibacterial activity by Agar well diffusion method [85]

10 mL TSA was melted and inoculated with 1 mL of *E. coli* *P. aeruginosa*, *S. aureus* and MRSA suspension which were already prepared from 3.2.2.1 after the temperature of the medium was about 50 °C. The seeded medium was thoroughly mixed and poured on petri dish. The medium was standed for a while until turned to solid form. In order to make the well on the medium, five steriled 12 mm diameter of stainless rings were placed on the surface of seeded medium as shown in Figure 3.1 the other 10 ml of melted seeded medium was overlaid on the first layer outside the rings. The rings were gently removed after the media was solid and then the wells were filled with the tested samples. For GAS, the bacterial suspensions were spread on the surface of blood agar. The seeded medium was standed for a while until the surface was dried, the medium was punctured by the hollow tube and by applying slight negative pressure to remove the plug of medium. Each well was filled with 200 µL of tested sample. The inoculated plates were incubated at 35-37 °C 18-24 hrs. After incubation, the plates were examined for the diameter of the inhibition zone (the values were the average of three measurements per well as shown in Figure 3.2 , taken at three directions in order to minimize errors).

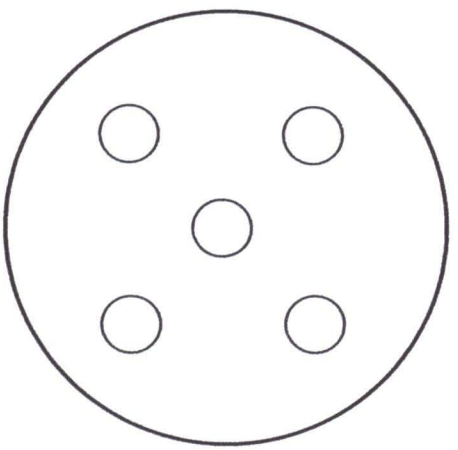


Figure 3.1 Placement of stainless rings on agar plates

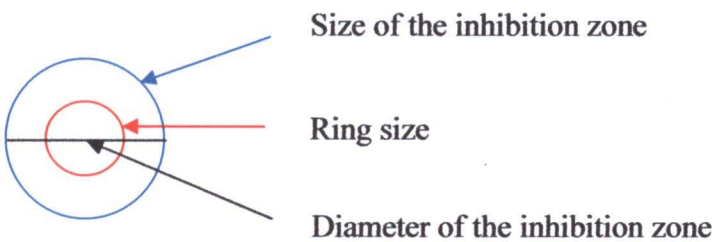


Figure 3.2 Measurement the diameter of the inhibition

3.2.3 Determination of MIC and MBC of the beehive extracts

3.2.3.1 Preparation of the extracts

Beehive extracts were dissolved in steriled distilled water in the ratio of 1:1 (1 g extract per 1 ml of steriled distilled water.)

3.2.3.2 Preparation of Gentamicin

Gentamicin was diluted in steriled distilled water at the concentration of 75 μg/ ml.

3.2.3.3 The culture medium

Broth : The medium using for determination of MIC against *E. coli, S. aureus,* MRSA and *P. aeruginosa* were TSB and for GAS was Todd Hewitt broth (THB).

Solid medium : The TSA was used for determination of MBC against *E. coli, S.aureus,* MRSA and *P. aeruginosa.* Blood agar plate was used for GAS MBC determination.

3.2.3.4 Determination of MIC by broth dilution method [86]

The working antimicrobial solution of gentamicin and the extracts were diluted by serial two-fold dilutions method. This method was performedly filling 0.5 ml TSB (Todd Hewitt broth was used for GAS) into the 13x100 mm steriled test tube number 2-12 (one set of MIC experiment consisted of 12 tubes). The amount of 0.5 ml of extract was filled into each tube of number 1 and 2. The mixture in tube number 2 was mixed and transferred to the third tube for 0.5 ml. This was soon until tube number 11 which the mixture was not transferred further and the volume still 1.0 ml. The equal volume of the adjusted bacterial inoculum was add to every tubes except number 11. The bacterial inoculum was prepared to be 10^5–10^6 CFU/mL by diluting the bacterial suspension from McFarland no 0.5 for 100 times. So, the final concentration of antimicrobial agents were 1:2, 1:4, 1:8 up to 1:1024 as shown in Figure 3.3. All experiments were performed in triplicate and the experiment was incubated at 35-37 ℃ for 24 hrs. The MIC was considered as the minimum concentration of the substance that inhibited the bacterial growth, after incubation as shown in Figure 3.4. The results were expressed in mg/mL.

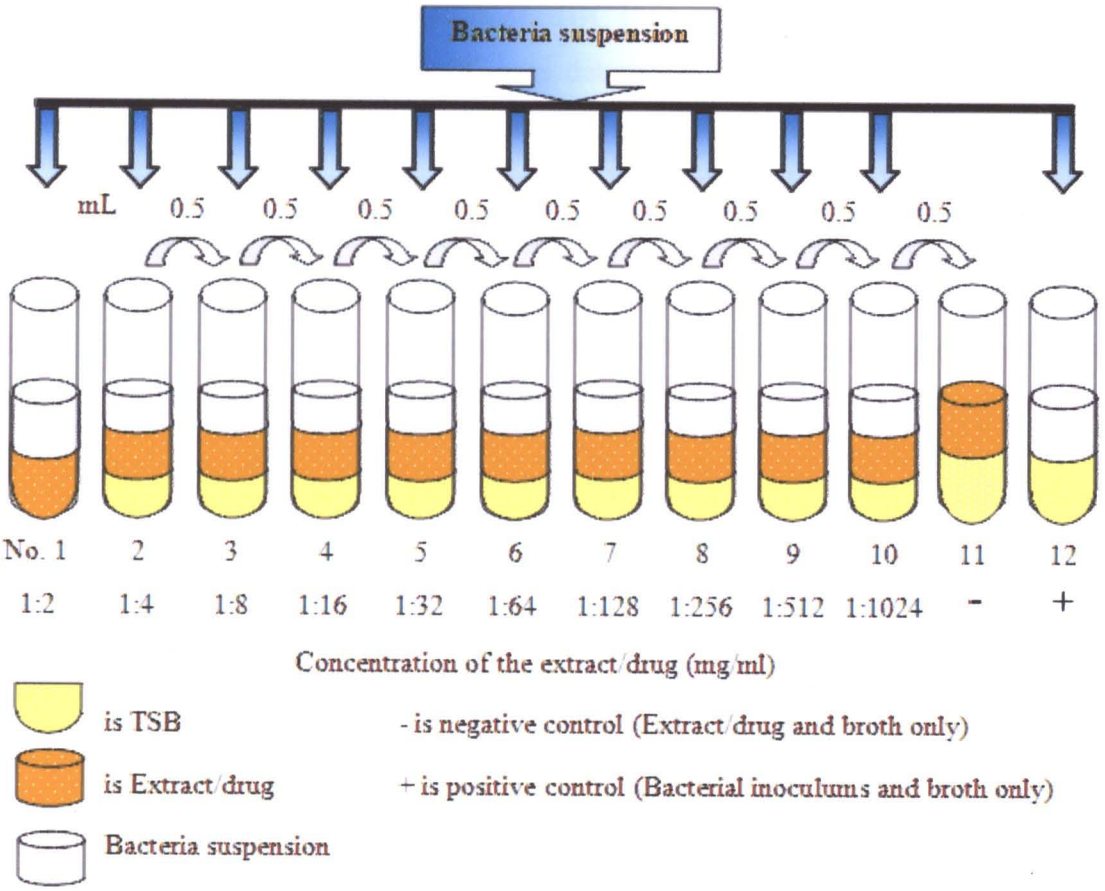


Figure 3.3 Twofold dilution methods for MIC

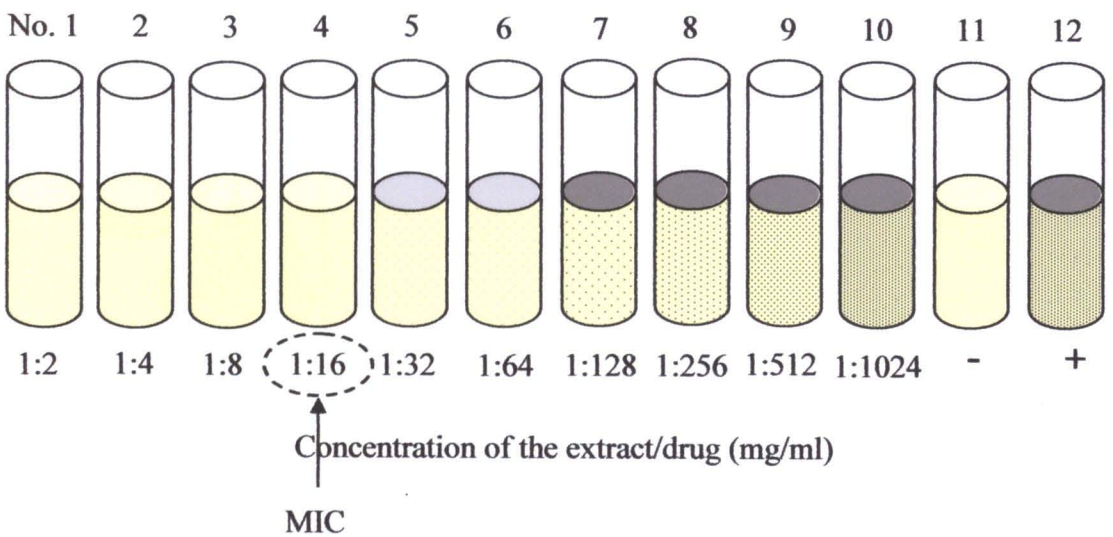


Figure 3.4 Determination of MIC

3.2.3.5 Determination of MBC [86]

After the MIC was determined the MBC was then observed. Each tube that showing no visible evidence of microbial growth was then subcultured on a quadrant of TSA (as shown in Figure 3.5) by pick up1 loop full (standard loop 4 mm in diameter or about 10 µl of the suspension) and streak on the TSA (blood agar for GAS) and observed for the bactericidal colonies on TSA after incubated at 35-37 ° C for 18-24 hr. The MBC was determined as the lowest concentration of the drug or sample which showing less than 0.1% viable bacteria.

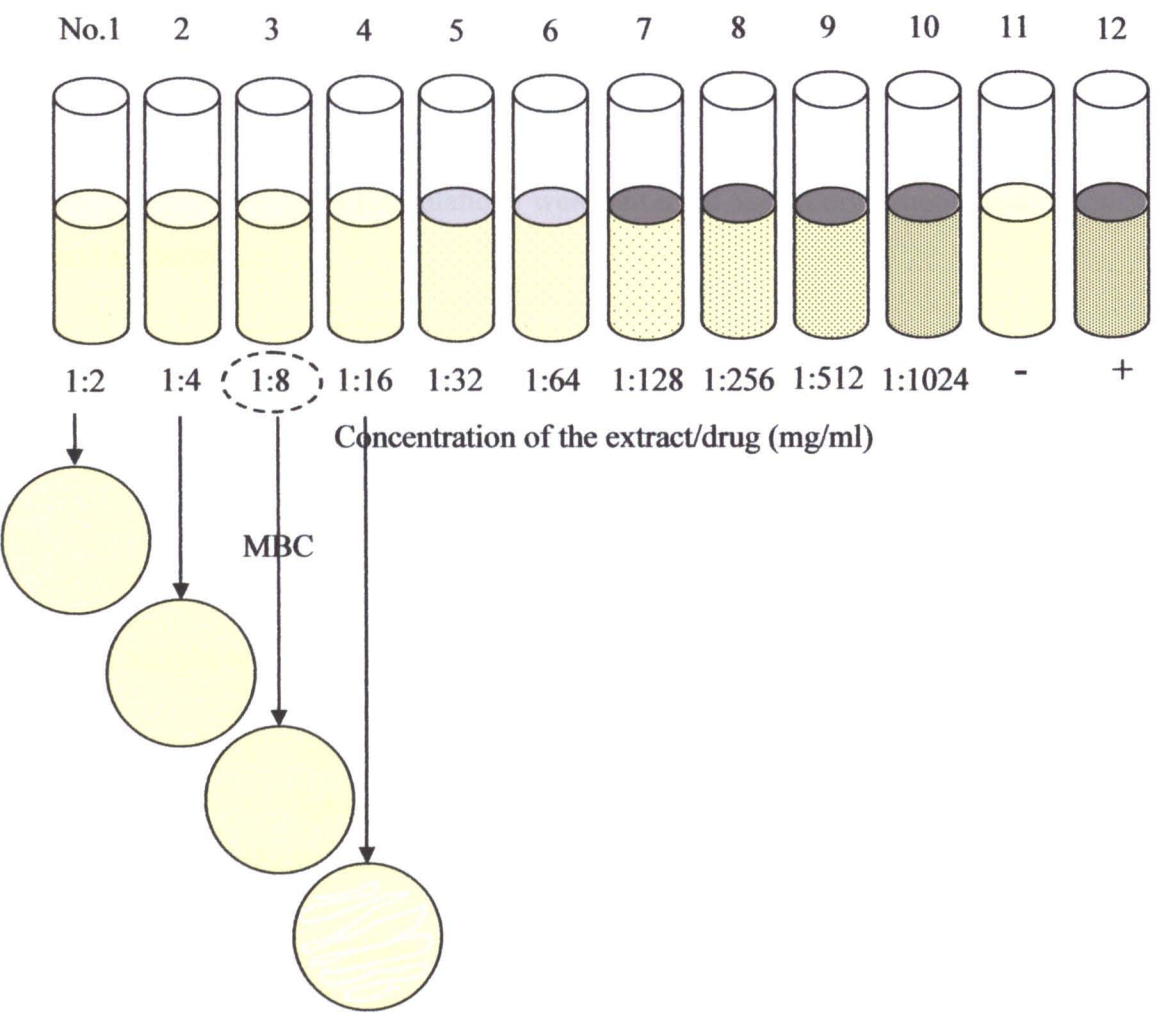


Figure 3.5 Determination of MBC

3.2.4 Formulation and stability test of gel base

3.2.4.1 Formulation of gel base

The gel bases using different gelling agents such as Carbopol, HEC and HPMC. The gelling agents was prepared by disperse in distilled water while the mixture and was stirred for Carbopol 941, and disperse in hot distilled water for HEC and HPMC. The ingredients : propylene glycol, conc. paraben, sodium metabisulfite, TEA, ethanol and DI water were weighed and placed into gelling agent and mix until homogeneous. The obtained gel base was packed in tight container and kept in a cool place.

Determination of physical properties of the gel base

- **Appearance**

All formulations were observed for colors, smoothness, viscosity and air bubble.

- **Viscosity measurement**

The viscosity of all formulations were measured by Bookfeild Rheometer Each sample was triplicate measurement.

- **pH measurement**

The pH of all formulations were measured by digital pH meter. Each sample was triplicate measurement.

- **Spread ability and feel on skin**

Spread ability and feel on skin of all gel bases were tested. Each sample was triplicate measurement.

- **Stability test of gel base**

The stability of gel base were tested at room temperature, room temperature in the dark, 2-8 °C, 45 °C for 1 month and accelerated test : heating-cooling cycling method which defined as alternation of storage conditions from 45°C for 48 hr to 2-8 °C for 48 hr (1 cycle) for 6 cycles.

3.2.4.2 Selection of good gel base

The appearance, pH of physical changing, spread ability and feel on skin of all gel bases formulas were compared and selected as gel base to incorporate with the W extract (W gel).

3.2.5 Formulation and stability test of beehive gel (W gel)

The W gel was prepared by the incorporation of W extract in the selected gel base at the concentration of 62.50 % w/w. The obtained gel was packed in tight container. After that the stability test of W gel was tested at room temperature, room temperature in the dark, 2-8 °C, 45 °C for 5 months and accelerated test: heating-cooling cycling method which defined as alternation of storage conditions from 45 °C for 48 hr to 2-8 °C for 48 hr (1 cycle) for 6 cycles.

3.2.6 Rabbit skin primary irritation test [87-88]

Three albino rabbits were used for skin irritation by modified Draize model as shown in Table 3.1. The Draize model and its modification are commonly used to assay skin irritation. Draize used this scoring system to calculate the PII. This is calculated by averaging the erythema scores and the edema scores of all sites (abraded and no abraded). These two averages are then added together to give the PDII value. A value of less than 0.5 was considered nonirritating, 0.5 to 2.0 slightly irritating, 2.1 to 5.0 moderately irritation and more 5 severely irritation as shown in Table 3.3.

Although the Draize scoring system does not include vesiculation, ulceration, and severe eschar formation, all of the Draize-type tests are used to evaluate corrosion as well as irritation. Therefore, Draize assays continue to be recommened by regulatory bodies for drugs and industrial chemicals.

Table 3.1 Modified Draize-FHSA Model used in this research

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

Table 3.2 Draize-FHSA Scoring System

Topics	Score
Erythema and eschar formation	0
No erythema	1
Very slight erythema (barely perceptible)	2
Well-defined erythema	3
Moderate to severe erythema	4
Severe erythema (beef redness) to slight eschar formation (injuries in depth)	

Table 3.2 (Continued) Draize-FHSA Scoring System

Topics	Score
Edema formation	0
No edema	1
Very slight edema (barely perceptible)	2
Slight edema (edges of area well defined by definite raising)	3
Moderate edema (raised > 1 mm)	4
Severe erythema (raised > 1 mm and extending beyond the area of exposure)	

Table 3.3 Classification of skin irritation

Primary Dermal Irritation Index (PDII)	Classification of skin reaction
0 – 0.4	No irritation
0.5 – 1.9	Slightly irritation
2.0 – 4.9	Moderately irritation
5.0 – 8.0	Severe irritation

3.2.7 Antibacterial activity of the W gel

The W gel were stored at room temperature , room temperature in the dark , 2-8 °C, 45 °C for 1, 3 and 5 months and was accelerated test. All samples were tested for antibacterial activity by agar well diffusion method. The experiments were carried out in triplicate.

3.2.8 Satisfaction test of volunteers by questionnaire

Twenty Thai volunteers (aged 20–50; n = 20). Subjects were instructed to apply the test gel subjects used the right-hand fore and middle fingers to apply test gel to left forearm by gentle circular massaging motion. Applied approximately amount (0.2 g) of each assigned test formulation. After testing was finished, all volunteers were asked some questions.

Table 3.4 The rate and degree of satisfaction of volunteers for Wgel

Score	Satisfaction
1	Improve
2	Middle
3	Good
4	Very good