CHAPTER II

MATERIALS AND METHODS

Materials

1. Chemicals

- Amerchol L101 (Dow, U.S.A.)
- Betel vine oil (Thai-china flavours and fragrances industry CO., LTD)
- Curcuminoid (Thai-china flavours and fragrances industry CO., LTD)
- Curcuminoid (The Government Pharmaceutical Organization)
- Citric acid (Carlo. ERBA., Germany)
- Brain heart infusion agar (Merk, Germany)
- Brain heart infusion broth (Merk, Germany)
- Calcium chloride (Merk, Germany)
- Cremorphor RH-40 (Dow, U.S.A.)
- Dimethyl sulfoxide (Merk, Germany)
- 2,2-diphenyl-1-picrylhydrazyl (DPPH), HPLC grade (Fluka, Switzerland)
- Di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) (Fluka, Switzerland)
- Ethyl alcohol USP (Merk, Germany)
- Gallic acid monohydrate ($C_7H_6O_5 \cdot H_2O$), HPLC grade (Fluka, Switzerland)
- Glycerin (Carlo. ERBA., Germany)
- Hydrochloric acid (HCl) 36.5-38%, analytical grade (J.T. Baker, USA.)
- Isopropyl alcohol (Merk, Germany)
- Methanol (CH_3OH), analytical grade (J.T. Baker, USA.)
- Methyl paraben (Henkel, Germany)
- Mueller Hinton agar (Merk, Germany)
- Mueller Hinton broth (Merk, Germany)
- Panoxyl 5® gel (Stiefel Laboratories (Pte) Ltd, Singapore)
- Polyoxyethylene 20 sobitan monoleate (Tween 80) (The East Asiatic Co., Ltd., U.S.A.)

- Potassium chloride (Merk, Germany)
- Propyl paraben (Henkel, Germany)
- Propylene glycol (Dow, U.S.A.)
- Oleoresin (The Government Pharmaceutical Organization)
- Sabouraud dextrose agar (Difco, France)
- Sabouraud dextrose broth (Difco, France)
- Sodium hexametaphophate (Carlo. ERBA., Germany)
- Sodium hydrogencarbonate (Merck, Germany)
- Sodium chloride (Merk, Germany)
- Sodium hydroxide pellet, analytical grade (Merck, Germany)
- Triethanolamine (May and Baker, England)
- Tryptic soy agar (Merk, Germany)

2. Equipments

- Anaerobic chamber, Model 1029 S/N (Forma Scientific, U.S.A.)
- Analytical balance (Sartorious Model A200S, Germany)
- Autoclave HA-3D (Hirayama manufacturing coorperation, Japan)
- Beaker (Pyrex, U.S.A.)
- Blender (Moulinex 327, Spain)
- Cylinders (Pyrex, U.S.A.)
- Erlenmeyer flasks (Pyrex, U.S.A.)
- Hammermill (U.S.A.)
- Hot air oven (Mammert, Germany)
- Hot plate (E.G.O., Germany)
- Incubator Model 6 (Thelco)
- Laminar air flow (ISSCO, model BV2225, Thailand)
- Magnetic stirrer (Model SP 46920-26, Barnstead/Hermodyne, USA)
- Micropipette (Pipetman, France)
- Pasteur pipettes (John poulten, England)
- Petri dishes (Pyrex, U.S.A.)
- pH meter (MP230, Mettler Toledo,LE413, ME51340 251, Switzerland)
- Pipettes (HBG, Germany)
- Refrigerator

- Rotary evaporator (Buchi R-200, Switzerland)
- Stirrer (KMO2, Janke & Kenkel GMBA & Co. KG)
- Test tubes (Pyrex, U.S.A.)
- UV/VIS spectrophotometer (Spectroic® Genesys ™ 5)
- Viscometer (Brookfield, Model LVDV-I+, Brookfield Engineering Laboratories INC., USA)
- Volumetric flask (Witeg Diffico, Germany)
- Vortex mixer (Vortex-2 genie, USA)

Methods

1. Preparation of Polysaccharide Gel (PG) from Fruit-Hulls of Durian

Fresh durian fruit-hulls were collected, cleaned, blended and kept at -20 °C. One kilogram of blended fresh durian fruit-hulls was dried by hot air oven at 50 °C for 48 hrs, about 200 grams of dried sample was obtained. Dried sample was kept in cold room (4 °C) until use. The polysaccharide gel was extracted from dried fruit-hull with hot water and purified by the method modified by Pongsamart and Panmaung (1998).

2. Physical properties of Polysaccharide Gel (PG)

2.1 pH

PG solutions were prepared by dissolving PG powder in distilled water to make various concentrations at 1-6% w/v of PG. PG solutions were individually measured using pH meter at ambient temperature. The results of the mean values were calculated from three determinations. Plots of pH against concentrations of PG were presented.

2.2 Viscosity

PG solutions of various concentrations at 1-6% w/v PG were prepared by sprinkled the PG powder into distilled water while stirring. Viscosity of PG dispersion were measured by using Brookfield Viscometer (model LVDV-I+). The presented data were the means of three determinations. Viscosity of PG was then plotted against the concentrations of PG to determine the viscosity concentrations relationship.

3. Compatibility studies of Polysaccharide Gel (PG)

3.1 Acid and Base

In order to determine the effect of acid and base on 3% PG in water. A solution of 5M hydrochloric acid (HCl) and 5 M sodium hydroxide (NaOH) was gradually added to the desired acid and basic pH of PG solution, respectively. The pH and viscosity of PG solution were measured. The mean values were calculated. The data of mean values was plotted between pH and viscosity.

3.2 Electrolytes

In order to evaluate the influence of calcium, magnesium, zinc and iron ions on the viscosity and stability of polysaccharide gel, aqueous stock solutions (1M) of $CaCl_2$, $MgCl_2$, $ZnSO_4$ and $FeSO_4$ were prepared. Into 50 ml of 3% PG solution added an electrolyte stock solution and deionized water and mixed thoroughly to make final concentration of each electrolyte at 0.02, 0.04, 0.06, 0.08 and 0.1M, respectively. The viscosity and pH of the PG solution in each electrolyte at different concentrations were recorded. The mean values were calculated. A graph was plotted between concentration of electrolyte and viscosity to determine the relationship of electrolytes on PG viscosity.

3.3 Organic solvents

In order to study the influence of organic solvents (ethyl alcohol and isopropyl alcohol) on PG solution, viscosity of PG solution in the present of tested organic solvent was measured. Each solvent was added to 3% PG solution in order to obtain final concentrations of 5, 10, 15, 20, 25 and 30% v/v solvent in total volume of 50 ml. The viscosity and pH of the PG solutions were determined. The mean values were calculated. A graph of concentrations of each solvent were plotted against viscosity to determine the relationship of organic solvents on viscosity of PG.

3.4 Humectants

The influence of humectants on PG solutions was studied using different types of humectants (propylene glycol, glycerin and sorbitol) effecting on the viscosity of PG solutions. Each humectant was added to 3% PG solution to make final concentrations of 5, 10, 15, 20, 25 and 30% w/v of humectant, respectively, in a final volume of 50 ml. The viscosity and pH of the PG solutions were recorded. Mean values from each determination were calculated. The result obtained in this study was plotted between concentration of humectant and viscosity.

3.5 Emollient

In order to study the influence of a lanolin derivative on PG solutions, amerchol L101 was added to PG solution and determined its effect on viscosity of PG dispersions. Amerchol L101 was added to 3% PG solution to make final concentrations of 5, 10, 15, 20, 25 and 30% w/v of amerchol L101 in a final volume of 50 ml. The viscosity and pH of PG solution were recoreded. The data reported were the mean values of three determinations. Concentrations of emollient were plotted against viscosity.

3.6 Preservative

In order to study the effect of preservative on PG solutions, paraben concentrate was used to determined its effect on viscosity of PG solutions. Paraben concentrate was added to 3% PG solution to make a final concentration of 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4 and 5% w/v of preservative with a final volume of 50 ml. The viscosity and pH of PG solution were measured. The mean values were calculated from each tested concentration of preservative in PG solution. The data obtained in this study was plotted between concentration of paraben concentrate and viscosity.

4. Effect of buffer on Polysaccharide gel (PG)

In order to test the effect of buffers on the viscosity and stability of polysaccharide gel, the stock solutions (0.2 M) of buffers such as citrate buffer and phosphate buffer were prepared. Citrate buffer at various pH 4, 4.5, 5, 5.5 and 6 were gradually mixed to 3% PG solution. Whereas phosphate buffer at pH 5.5, 6, 6.5, 7 and 7.5 were added to 3% PG solution to make final concentration of each buffer at 0.1 M. The pHs of the buffer were plotted against viscosity.

5. Determination of free radical scavenging activity using DPPH method

DPPH has been widely used to measure the free radical scavenging activity of various samples (Du *et al.*, 2001; Okawa *et al.*, 2001). This method is based on the reduction of DPPH to form colored stable free radical.

Briefly, the reaction mixture contained 2 ml of dilutions of the methanolic solution of sample, 1 ml of 0.5 mM DPPH (in methanol) and 2 ml of distilled water. Absorbance at 517 nm was determined after standing for 30 minutes. After the antioxidant compounds presence in sample interacted completely with DPPH, the percentage of DPPH radical scavenging activity was calculated by the following formula.

$$\% \text{ radical scavenging activity} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \right) X\ 100$$

A_{sample} is the absorbance value of DPPH containing sample solution.

A_{control} is the absorbance value of DPPH.

The DPPH radical scavenging activity (%) was plotted against the concentrations of the samples.

6. Antimicrobial susceptibility tests of betel vine oil

6.1 Test microorganisms

Microorganisms used in the present studies were provided by the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The nine bacterial strains, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 9341 and *Bacillus subtilis* ATCC 6633, represent gram positive bacteria; *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 9721, represent gram negative bacteria; two yeast strains, *Candida albicans* ATCC 10230 and *Saccharomyces cerevisiae* ATCC 9763; and acnes causing bacteria *Propionibacterium acnes* ATCC 6919, provided by Department of Medical Sciences were used for the studies.

6.2 Preparation of samples

Betel vine oil and curcuminoid were obtained from Thai-china flavours and fragrances industry CO., LTD. Oleoresin was obtained from The Government Pharmaceutical Organization. Betel vine oil was prepared in a series of two fold dilutions in 0.1% tween 80 to make the concentrations in a range of 50 to 0.078% v/v. Curcuminoid and oleoresin of curcumin were prepared at concentration 50 and 25% v/v in 20% MeOH. Oleoresin was prepared in a series of two fold dilutions of various concentrations (5, 2.5, 1.25 and 0.63%) in 10% DMSO. The test samples were used in the agar diffusion test.

Betel vine oil was prepared in a series of two fold dilutions in 1% dimethyl sulfoxide (DMSO) to make the concentrations ranging from 5 to 0.0049% v/v. The test sample was used in broth macrodilution test.

6.3 Preparation of agar and broth media

6.3.1 Agar media

Mueller hinton agar (MHA) medium was prepared. The medium solution was sterilized at 121 °C under 15 pound per square inch pressure for 15 min. Twenty milliliters of the sterile MHA was pour into petri dishes of 90 mm diameter. The agar was allowed to solidify on a flat level surface. The plates were left to dry for 1 hours at ambient temperature. All test bacteria were tested on MHA except for *P. acnes* was tested on Brain heart infusion agar (BHIA). Two yeast strains were tested on Sabouraud dextrose agar (SDA).

6.3.2 Broth media

Mueller hinton broth (MHB) was used as a test broth medium for all tested bacteria, except for the *P. acnes* the Brain heart infusion broth (BHIB) was used. Sabouraud dextrose broth (SDB) was used for inoculating two yeast strains.

6.4 Preparation of the inoculum

Preparation of bacterial inocula was performed according to the standard method. Each bacterial strain was grown on Tryptic soy agar (TSA) slant overnight at 37 °C. The bacteria were washed from agar surface and the bacterial suspensions were adjusted to match turbidity of standard Mcfarland no. 0.5 with sterile normal saline (NSS) before used. Two yeast strains, *S. cerevisiae* and *C. albicans*, were cultivated by the same method as described for bacteria except that nutrient agar was replaced with SDA slant.

P. acnnes was grown on BHIA and was incubated under anaerobic condition at 37°C for 48 hours. The bacterial suspension was prepared in BHIB medium by the same procedure previously described.

6.5 Test for antimicrobial susceptibility

The study of antimicrobial activity of betel vine oil was determined by using the agar diffusion method and broth macrodilution method (Lorian, 1996). The procedure was as follows.

6.5.1 Agar diffusion test (Lorian, 1996)

The petri dishes containing agar media were inoculated with 1% microorganism suspension by seed layer method (Leonardo *et al.*, 2000). The inoculated plates were allowed to dry for 5 to 10 minutes. Sterile stainless cups with approximately 8 mm diameters were placed on surface of the inoculum plates using sterile forceps and filled with different concentrations of sample (300 µl). The plates were allowed to stand for 1 hour at room temperature for prediffusion and then incubated at 37 °C overnight. The diameter of inhibition zones was measured, excluding cup size, after incubation overnight. Except for *P. acnes*, the plates were incubated at 37°C for 48 hours under anaerobic conditions. Following incubation, zones of inhibition were measured.

6.5.2 Broth macrodilution test (Lorian, 1996)

A broth macrodilution method was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) as prescribed by Lorian, 1996.

Into 4 ml of nutrient broth added 0.5 ml of various concentrations of betel vine oil dilutions and 0.5 ml of microorganism suspension, and then all bacteria and yeasts were incubated at 37 °C overnight, except for *P. acnes* was incubated under anaerobic condition at 37°C for 48 hours. The MIC is defined as the lowest concentration of the betel vine oil at which the microorganism tested does not demonstrate visible growth. The microorganism growth was indicated by the turbidity observed in broth medium. To determine MBC, broth medium from tubes showed no visible growth was taken and inoculated in Mueller hinton agar and incubated at 37°C overnight in the case of bacteria and yeasts, but *P. acnes* was

incubated for 48 hours. The MBC is defined as the lowest concentration of the betel oil at which no growth occurred on the agar plates.

7. Surfactant optimization

The influence of the surfactant on the antimicrobial PG preparation was studied by using different kinds of surfactant such as Cremophor RH-40, Pluronic F-68 and Tween 80, at various concentrations (1, 5, 10 and 15% w/v). Each surfactant was mixed thoroughly together with betel vine oil, propylene glycol and glycerin to a completed dispersion. The appearances of solutions were observed.

8. Formulation of antimicrobial PG preparation

Antimicrobial PG preparation was formulated. The formula composed of the ingredients and its function as follows:

Ingredients	Function
PG	Gelling agent and antibacterial agent
Propylene glycol	Humectant
Glycerin	Humectant
Betel vine oil	Antimicrobial agent
Amerchol L-101	Emollient
Cremophor RH-40	Surfactant
Triethanolamine	pH adjuster
Paraben concentrate	Preservative
Distill water	Vehicle

The compositions in the formula of antimicrobial PG preparations studied are presented in the following table:

8.1 Preparation of PG gel base

Dissolve the polysaccharide gel (PG) in the distilled water until uniform. Add propylene glycol and glycerin to the powder and mix then add to PG solution. Amerchol L-101 and cremophor RH-40 were measured. Mix them together well and add this mixture to the solution. Paraben concentrate should be gradually added, with mixing, followed by the distill water to make a total weight of 100 gram. Gel viscosity and pH were measured and then formulation was evaluated. Physical appearances flow, air bubbles, colour, pH and viscosity were determined. Stability test was performed.

Ingredients	**Content (%w/w)**
PG	2.5
Propylene glycol	5
Glycerin	5
Amerchol L-101	0.25
Cremorphor RH-40	5-10
Triethanolamine	0.1
Paraben concentrate	1
Distill water to	100

8.2 Preparation of PG gel contained betel vine oil

Firstly, prepare PG dispersion by sprinkling powder of polysaccharide gel (PG) in distilled water while string until uniform. Mixed glycerin with propylene glycol, the mixture was added to the PG dispersion and mixed until homogenous. The liquid ingredients oil phase such as betel vine oil, amerchol L-101 and Cremophor RH-40 were mix well together and then slowly added to the PG dispersion. Added paraben concentrate and mixed thoroughly. Distilled water was added to make a total weight of 100 gram and mix until uniform. Gel viscosity and pH were measured. The final product was evaluated. Physical appearances, flow, air bubbles, colour, pH and viscosity were recorded. Stability test was performed.

Ingredients	**Content (%w/w)**
PG	2.5
Propylene glycol	5
Glycerin	5
Betel vine oil	1-2
Amerchol L-101	0.25
Cremorphor RH-40	5-10
Triethanolamine	0.1
Paraben concentrate	1
Distill water to	100

8.3 Preparation of PG gel contained organic acid

Dissolve the polysaccharide gel (PG) in the distill water and stir until PG has dissolved. Propylene glycol, glycerin and lactic acid were mixed respectively and then add to the PG solution. While if some formulation use salicylic acid, it was to a find powder in a mortar and gradually add propylene glycol, glycerin with mixing, respectively. Then add them to PG solution. The betel vine oil, amerchol L-101 and Cremophor RH-40 were mixed together and added to the mixture of PG dispersion and then slowly added mixture of oil in PG with continuously mixing into salicylic acid and /or lactic acid in the mortar. Paraben concentrate was added add then make up a total weight of 100 gram with distill water. The physical properties of final product were evaluated.

Ingredients	**Content (%w/w)**
PG	2.5
Propylene glycol	5
Glycerin	5
Lactic acid	0.1-1.0
Salicylic acid	0.1-1.0
Betel vine oil	1
Amerchol L-101	0.25
Cremorphor RH-40	5
Paraben concentrate	1
Distill water to	100

8.4 Preparation of PG gel contained antimicrobial agents (insoluble)

Prepare PG solution by stir polysaccharide gel (PG) in distilled water. Grinding precipitate sulphur and/or zinc oxide to a fine powder in a mortar. Added propylene glycol and glycerin with mixing to PG dispersion. Add the mixture to the PG dispersion. The betel vine oil, amerchol L-101 and Cremophor RH-40 were mix together and added to the mixture of PG dispersion, and then added with contained mixing in precipitate sulphur and/or zinc oxide in the mortar. Added paraben concentrate, added the distill water gradually to make a total weight of 100 gram and mix until uniform. Gel viscosity and pH were measured the final product was evaluated. Physical appearances, flow, air bubbles, colour, pH and viscosity were recorded. Evaluation of physical properties of anti acne was determined.

Ingredients	**Content (%w/w)**
PG	2.5
Propylene glycol	5
Glycerin	5
Precipitated sulphur	0.1-1.0
Zinc oxide	0.1-1.0
Betel vine oil	1
Amerchol L-101	0.25
Cremorphor RH-40	5
Paraben concentrate	1
Distill water to	100

8.5 Preparation of PG gel contained antimicrobial agents (insoluble) and HPMC 4000

Prepare PG solution by stir polysaccharide gel (PG) in distilled water and stir until PG has dissolved. HPMC 4000 is dispersed in the distilled hot water and kept in refrigerator until it was dissolved and then add to the PG solution and mixed together. The precipitate sulphur and zinc oxide should be size reduced in a mortar. Gradually add propylene glycol and glycerin with mixing, respectively. Then add them to PG mixture. Betel vine oil, amerchol L-101 and Cremophor RH-40 were measured and mixed thoroughly then gradually add to the mixture. Add paraben concentrate to the mixture and then make up a total weight of 100 gram by distill water. Evaluation of physical properties of anti acne was determined.

Ingredients	**Content (%w/w)**
PG	2.5
Propylene glycol	5
Glycerin	5
Precipitated sulphur	0.5
Zinc oxide	0.5
HPMC 4000	0.5-1.0
Betel vine oil	1
Amerchol L-101	0.25
Cremorphor RH-40	5
Paraben concentrate	1
Distill water to	100

9. Evaluation the physical properties of antimicrobial PG preparation

9.1 pH

All finished products were measured their pH by using a pH meter (Mettler Toledo MP230). The pH values of the final products at ambient temperature were recorded after freshly prepared and after stability tested.

9.2 Viscosity

Viscosity measurements of the finished products were performed at ambient temperature by using a Brookfield LVDV-I+ Viscosimeter (Brookfield, USA). About 8 ml of the antimicrobial PG products were filled in a measuring cylinder and the viscosity was measured at ambient temperature. Viscosity of the finished products were recorded after freshly prepared and after stability tested.

10. Assessment of antimicrobial PG preparation stability

10.1 Storage at ambient temperature

About 25 ml of each formulation of antimicrobial PG preparations was filled in a glass bottle and closed tightly. The preparation products were stored vertically at ambient temperature and observed the resulting products for 30 days. Any change was recorded, pH and viscosity were determined.

10.2 Freeze-thaw cycle

All of formulations of antimicrobial PG preparations were tested for their stability by the method of freeze-thaw cycle. The tested products were incubated in oven at 45 °C for 48 hours and then transfered into a refrigerator at temperature -4 °C for 48 hours to complete 1 cycle. The same procedure was continued for 6 cycles. The products were observed and record if any changes appeared, pH and viscosity were also determined.

11. Antibacterial susceptibility tests of antimicrobial PG preparation

11.1 Agar well diffusion method (Daljit and Jasleen, 1999; Iqbal *et al.*, 1998)

The agar well diffusiton method was used with the following details.

11.1.1 Preparation of test samples

Antimicrobial PG preparations contained 1 and 2% w/v concentrations of betel vine oil was tested. Solutions of the betel vine oil at 1 and 2% v/v were prepared. The PG gel base was used as a negative control. Panoxyl 5® gel was used as positive control.

11.1.2 Preparation of medium

Mueller hinton agar (MHA) and Brain heart infusion agar (BHIA) medium was used and prepared as described in 6.3.1.

11.1.3 Preparation of the inoculum

Each bacterial strain such as *Staphylococcus aureus, Staphylococcus epidermidis* and *Propionibacterium acnes* was prepared as described in 6.4.

11.1.4 Inoculation of test plate

The agar media were inoculated with 1% of microorganism suspension by seed layer method. Wells of 8 mm diameter were punched into the agar plates by using a sterilized stainless steel borer and were filled with various samples. The plates were then incubated at 37°C overnight, except for *P. acnes* was incubated at 37°C for 48 hours under anaerobic conditions. The antibacterial activity was evaluated by observing the clear inhibition zone of no bacterial growth on agar plates and measuring the inhibition zone diameter of each test.

11.2 Broth microdilution assay (Lorian, 1996)

11.2.1 Preparation of test samples

Formulations of antimicrobial PG preparations contained 1 and 2% w/v betel vine oil were prepared. Solutions of the betel vine oil (1 and 2% v/v) in 0.1% tween 80 were standard. The PG base was also used as control. Panoxyl 5® gel was used as positive control. The samples were diluted five-fold.

11.2.2 Preparation of medium

A medium was prepared by weighing of 21 g MHB in an erlenmeyer flasks. Distilled water was added to dissolve the medium and adjusted to 100 ml to make a final concentration 10 times more concentrated than normal use inoculating medium. The medium was sterilized at 121 °C under 15 pounds per square inch pressure for 15 min.

11.2.3 Preparation of the inoculum

Preparation of bacterial inocula was performed according to the standard method. Each bacterial strain was grown on TSA slant overnight at 37 °C. The bacteria were washed from agar surface. The bacterial suspensions were adjusted to match turbidity of standard Mcfarland no. 0.5 with sterile normal saline solution (NSS) and cell suspension of about $1x10^9$ CFU/ml was used.

P. acnnes was grown on BHIA and was incubated under anaerobic condition at 37°C for 48 hours. The bacterial suspension was prepared in BHIB medium by the same procedure previously described.

11.2.4 Broth microdilution

The 96 well plates were prepared by dispensing into each well 20 µl of nutrient broth and 20 µl of the inoculum. The 160 µl of undiluted sample was added into the first wells. Then, 160 µl of serial five fold dilutions of each sample was transferred into consecutive wells. The final volume in each well was 200 µl and then incubated at 37 °C overnight for *S. aureus* and *S. epidermidis*. Except for *P. acnes* was incubated under anaerobic condition at 37°C for 48 hours. The MIC is defined as the lowest concentration of the samples that inhibited visible growth of microorganism. MBC was determined by subculturing inoculated broth medium from wells showing no turbidity of visible growth onto agar plates of MHA and incubated at 37°C overnight, except for *P. acnes* was incubated for 48 hours. The MBC is defined as the lowest concentration of the sample at which no growth obtained on the agar plates.