CHAPTER III

MATERIALS AND METHODS

1. Preparation of Mangosteen Pericarp Extract

Extract from mangosteen pericarp was kindly provided by Associate Professor Dr. Suchada Chutimaworapan and her graduate student, Miss Vorratai Tan (the Department of Pharmacy, the Faculty of Pharmaceutical Science, Chulalongkorn University). The methods of extraction, purification and identification were previously described. (Hiranras, 2001, Karnjanachotdamrong, 2000)

1.1 *Preparation of crude extract and purified α-mangostin*

Mangosteens were obtained in July 2003 from the Thewate market in Bangkok. Ground and dried mangosteen pericarps were extracted successively with hexane and ethyl acetate under reflux. The extract was then recrystallized and ground into powder.

The crude extract was further purified by a quick column chromatography to obtain α-mangostin. On a silica gel column, the extract was gradually eluted with hexane and ethyl acetate. The fraction that showed the characteristic of α-mangostin on a thin layer chromatography was recrystallized and ground into powder. The isolated α-mangostin was identified and characterized by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy, and its melting point was confirmed by using Gallenkamp melting point apparatus.

High performance liquid chromatography (HPLC) demonstrated that the crude extract prepared by this method contained approximately 80% α-mangostin. The yield of crude extract was 2.75% w/w, while the yield of α-mangostin was 0.41% w/w of the dried mangosteen pericarp. The extract was kept in a dessicator at room temperature.

1.2 *Testing solubility of mangosteen pericarp extract*

Extract from mangosteen pericarp was soluble in alcohol, ether, acetone, ethyl acetate, chloroform and dimethylsulfoxide (DMSO) but practically insoluble in water. DMSO was selected as a solvent for all experiments in this study.

2. Bacterial Culture

The bacterial strains used in this study included *S. mutans* ATCC 25175 and $KPSK_2$, *P. gingivalis* ATCC 53978 (W50) and *A. actinomycetemcomitans* ATCC 43718 (Y4, serotype b). The bacterial stocks were kept in 15 % glycerol (Asia Pacific Specialty Chemicals Ltd., NSW, Australia) and frozen at -80°C. The same bacterial stock was used for all experiments to ensure the consistency of the results. All experiments were performed in the laminar airflow biological safety cabinets (Class II) (Model BH 2000 series, CLYDE-APAC, NSW, Australia).

2.1 *Bacterial growth condition*

S. mutans and *A. actinomycetemcomitans* were cultured in a CO_2 incubator containing 5-7% carbon dioxide at 37 ^{o}C (Model 3121, Forma Scientific, Inc., Marietta , OH, USA). *P. gingivalis* was grown anaerobically in a GasPak 100 system (BBL Microbiology Systems) at 37 ^{o}C. The broth culture of *P. gingivalis* was maintained in a shaker water bath (Model WB001, Media Science and Supply Ltd., Bangkok, Thailand) at 90 rpm, 37 ^{o}C for optimal growth.

2.2 *Growth on liquid media*

All tested bacteria were cultured in trypticase soy broth (BBL Microbiology Systems). The broth for *P. gingivalis* was supplemented with 5% fetal bovine serum (Life Technology, Paisley, Scotland), 5 mg/L hemin (Sigma Chemical Co., St. Louis, MO, USA) and 0.1 mg/L vitamin K (10 mg/ml vitamin K_1 for injection, Atlantic Laboratories, Corp.,Ltd., Bangkok, Thailand).

2.3 *Growth on solid media*

S. mutans was cultivated on bacteriological agar (Oxoid Ltd., Basingstoke, Hampshire, England) containing trypticase soy broth. *P. gingivalis* was grown on brucella agar (BBL Microbiology Systems) supplemented with 7% human whole blood (Thai Red Cross Society, Bangkok, Thailand), 5 mg/L hemin and 0.1 mg/L vitamin K. *A. actinomycetemcomitans* was grown on bacteriological agar containing brain heart infusion broth (Difco Laboratories, Detroit, MI, USA).

3. Optimizing Bacterial Growth Condition

To determine the mid-exponential phase of growth for the tested bacteria, they were grown in the liquid media to observe their growth pattern over time.

Isolated 48-hour colonies were transferred into trypticase soy broth and grown for 12 hours. The bacterial inoculum was then diluted 10 to 20 folds and grown for another 10 hours. Every one hour, the number of bacteria was indirectly measured by the degree of turbidity of the broth culture. The bacterial inoculum was also spread onto an agar plate and incubated to directly determine the number of live bacteria.

3.1 *Degree of turbidity*

Turbidity was measured using a spectrophotometer (Ultrospec 3000 pro.UV / Visible spectrophotometer, Pharmacia Biotech, England). The absorbance at 600 nm of bacterial culture was substracted by a blank (uninoculated media) to account for the background absorbance by the broth media.

3.2 *Viable bacterial count*

The number of viable bacteria was determined by a spread plate method. The bacterial inoculum at each time point was serially diluted every 10 folds. One hundred μl of each diluted bacterial inoculum were spread onto an agar plate in duplicate. The plates were then incubated for 48 hours for *S. mutans* and *A. actinomycetemcomitans*, and for 96 hours for *P. gingivalis*. Only plates with 30-300 colonies were counted. The numbers of colonies from duplicate plates were averaged. If more than one dilution was in range, all

countable dilutions were averaged. To ensure the accuracy of the results, the average count must not differ from the number of colonies from each plate by more than 20%.

The growth curve was plotted as the logarithm of the number of viable cells (log_{10}CFU/ml) versus time. The time point that each microorganism reached mid-exponential growth phase was selected for testing antimicrobial activity in this study.

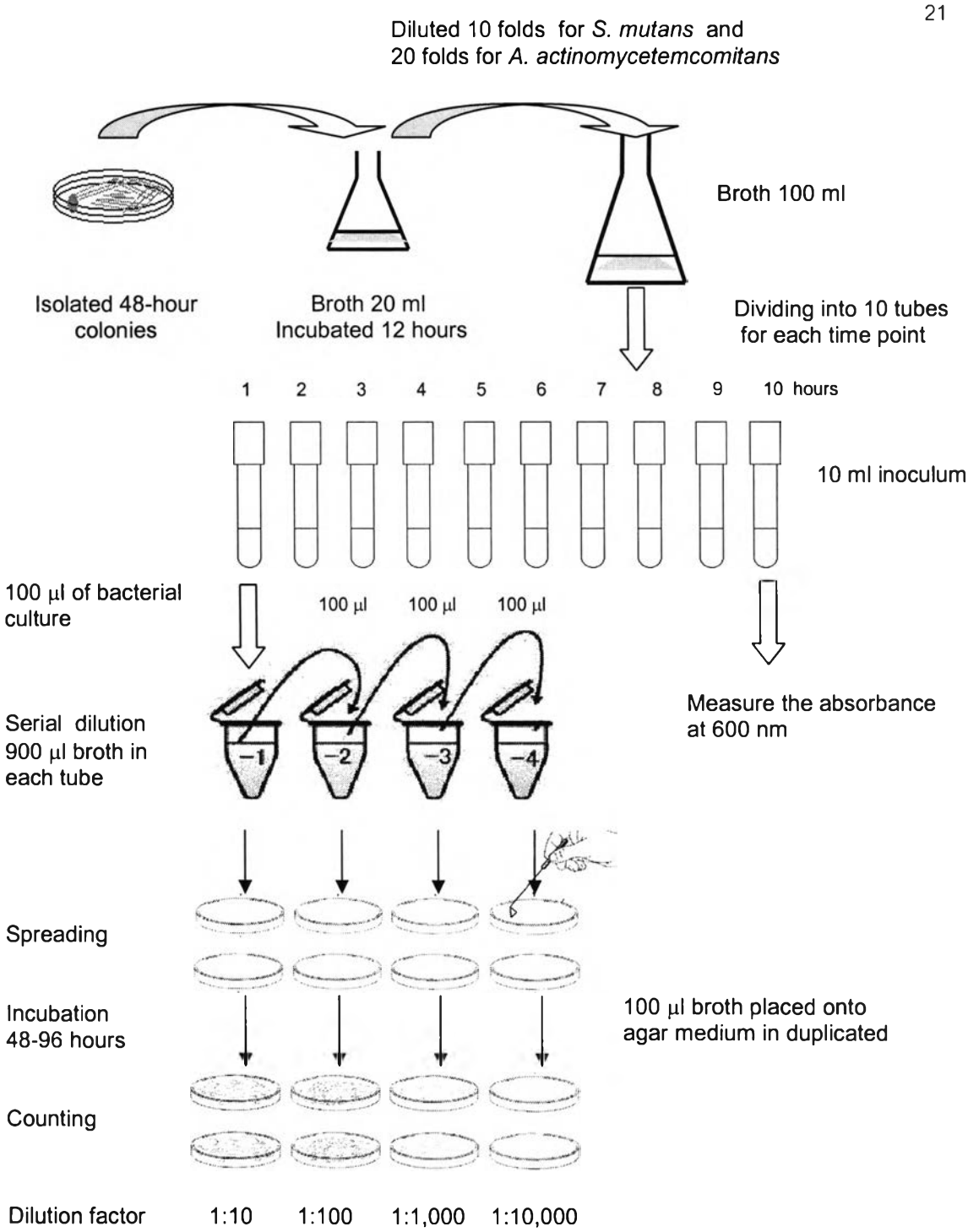
3.3 *Plotting a standard curve*

The standard curve of bacteria was drawn by plotting log_{10}CFU/ml from the exponential phase of growth against the absorbance at 600 nm. Linear regression equation was used to fit the data. From this equation, an estimate of viable bacterial count can be predicted from the absorbance readings so that the desired amount of bacteria can be obtained when testing antimicrobial activity.

3.4 *Optimizing growth condition for P. gingivalis*

For *P. gingivalis*, the growth curve could not be determined. We used the Gaspack 100 system for growing this organism. Re-opening the anaerobic jar every 1 hour could affect the gas content and subsequently changed the bacterial growth. Instead we incubated the bacterial inoculum for a certain period of time varying from 4 to 24 hours. At the end of incubation period, the bacterial inoculum was subcultured (1:5 dilution), and observed for their ability to grow further in a new broth.

Figure 5 summarized the method of determining bacterial growth pattern by viable cell count and degree of turbidity.



CFU/ml = (Average number of colonies / Volume plated in ml) X Dilution factor

Figure 5. Determining bacterial growth pattern by viable cell count and degree of turbidity. Bacterial colonies were transferred into broth and grown for 10 hours. Every 1 hour, the degree of turbidity was measured. The number of viable cell were also counted by a spread plate method, and further calculated to CFU/ml.

4. Preparation of Test and Control Media for Antimicrobial Testing

Test media containing mangosteen crude extract or α-mangostin were prepared. The extract were initially dissolved in DMSO (GC 99.5%, Sigma Chemicals) and further diluted in broth media. The degrees of solubility of the extract were compared among various media including trypticase soy broth, brain heart infusion broth and Muller-Hinton broth. Trypticase soy broth was chosen because it gave the highest solubility even at a high concentration of the extract (1,000 μg/ml). The test media was serially diluted every two folds with the media to reach the testing concentration. The final concentrations of the extract after adding bacterial inoculum varied from 640, 320, 160 μg/ml and so on to 0.156 μg/ml. The corresponding concentrations of DMSO in the test media varied from 26.4, 13.2, 6.6 g/L and so on to 0.0064 g/L.

Chlorhexidine (20% w/v) was used as a positive control. It was serially diluted in the broth media in the similar fashion to the mangosteen extract. Another tube containing only the broth media without extract or chlorhexidine was used as a control for bacterial growth.

5. Identifying MIC

MIC was identified by a broth dilution method. The bacteria were grown to reach mid-exponential phase of growth as previously described. The absorbance reading was recorded, and viable bacterial count was estimated using the standard curve. The bacterial inoculum was further diluted 40-200 folds in the new broth to obtain the desired initial CFU. One ml of diluted bacterial inoculum was added to 4 ml of serially diluted test or control media. The initial density of bacteria was approximately 1-4 x 10^6 CFU/ml for *S. mutans* and *A. actinomycetemcomitans*, and approximately 5 x 10^7 to 1 x 10^8 CFU/ml for *P. gingivalis*.

After adding the bacterial inoculum to the control broth, 100 μl of the inoculum were serially diluted, and spread onto agar plates to directly determine the initial CFU.

All tubes were incubated at 37 ^{o}C for 24 hours and the MIC value for each tested bacteria was recorded. The MIC was defined as the lowest concentration of the extract or

chlorhexidine that limited the turbidity of the broth to < 0.05 at the absorbance of 600 nm. Before adding bacterial inoculum, the broth containing mangosteen extract gave a certain degree of turbidity. Therefore, the absorbance at 600 nm measured after 24-hour incubation had to be subtracted from the value measured before adding bacterial inoculum to give the true turbidity caused by bacterial growth.

DMSO, a solvent for mangosteen extract, has been known for its cytotoxicity at a high concentration. Therefore, a preliminary study was performed to determine the effect of DMSO on bacterial growth. The broth containing serially diluted DMSO was prepared, and bacterial inoculum was added in a similar fashion as the mangosteen extract. The results showed that the amount of DMSO used in this study did not affect the growth of bacteria as compared to the control broth without DMSO.

6. Identifying MBC

MBC was determined by comparing the number of remaining viable bacteria with the initial number of bacteria after exposed to the extract or chlorhexidine for 24 hours. One hundred μl aliquots from test tubes showing no visible bacterial growth from MIC experiments were serially diluted and spread onto agar plates for viable cell counting. The plates were incubated at 37°C for 48 hours for *S. mutans* and *A. actinomycetemcomitans* and 72-96 hours for *P. gingivalis*. The MBC was recorded as the lowest concentration that killed at least 99.99% of the initial number of bacteria. The MIC and MBC experiments were repeated at least four times for each microorganism.

Figure 6 summarized the method of mangosteen extract preparation for determining MIC and MBC.

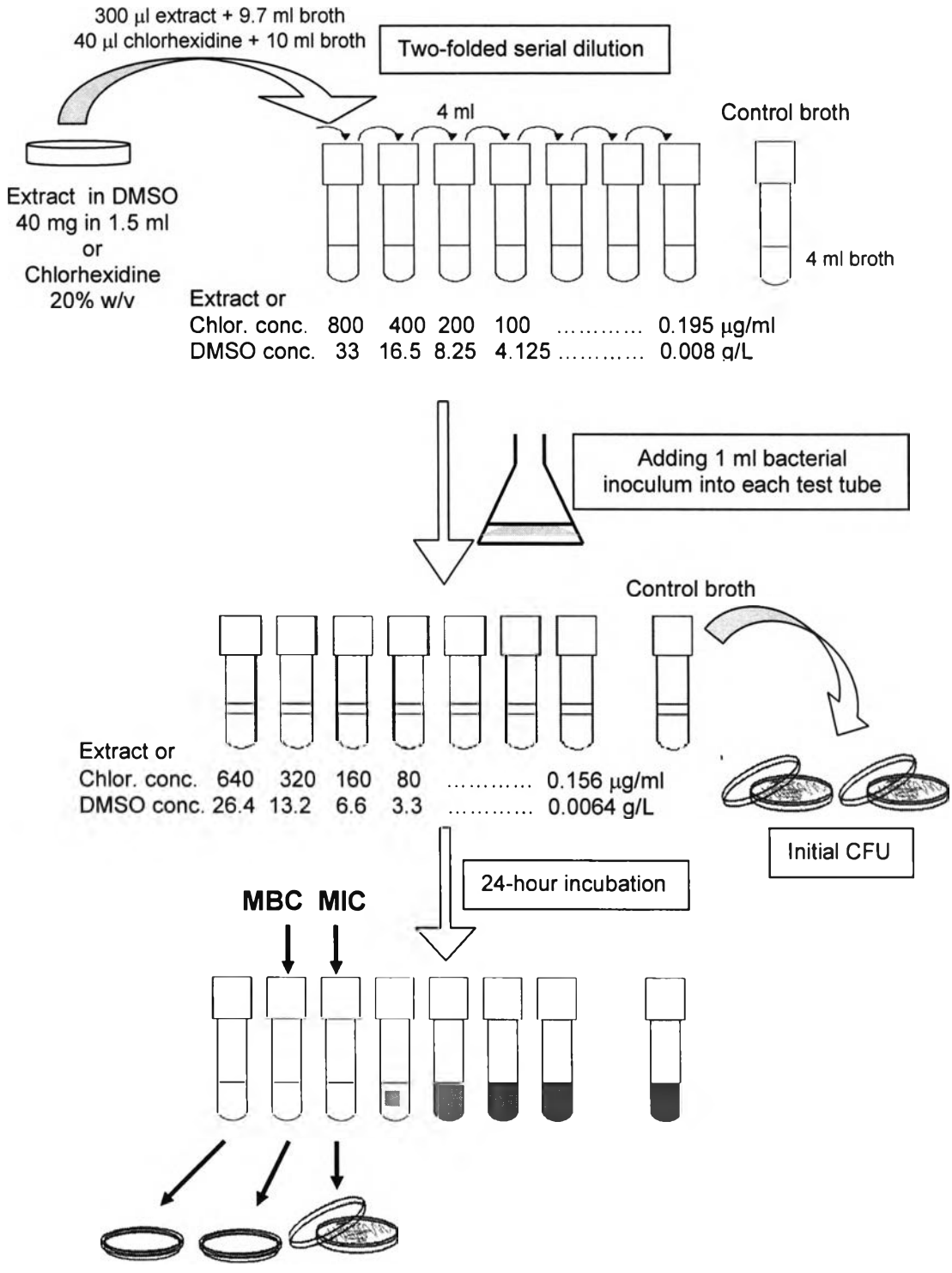


Figure 6. Preparation of mangosteen extract for determining MIC and MBC. The MIC was defined as the lowest concentration of the test that limited the turbidity < 0.05 comparing with the value measured before adding bacterial inoculum. The MBC was recorded as the lowest concentration that killed at least 99.99% of the initial number of bacteria.

7. Time-kill Kinetics

Time-kill kinetics was determined by the number of remaining viable bacteria at varying time after exposed to the mangosteen extract at the concentrations of two or four times of MBC.

Only bacteria that were sensitive to mangosteen extract from the MIC and MBC experiments were studied for time-kill kinetics. After exposed to the mangosteen extract for 5, 15, 30, 60 and 90 minutes, the number of remaining viable bacteria was determined by a spread plate method. The tests were diluted at least 10 folds, and aliquot of 100 μl were transferred to agar plates. Only plates with more than 10 colonies were count, so the limit of detection was 10^3 CFU/ml. The control broth without extract was served as a control for bacterial growth at each time point. Antimicrobial activity was expressed in term of $\log_{10}$ reduction in CFU/ml, which was a logarithm of the ratio of the number of bacterial colonies in the absence of mangosteen extract to the number in its presence. Time-kill curve was plotted as the $\log_{10}$ reduction in CFU/ml against time. The time-kill kinetics of the extract was also compared to that of chlorhexidine at the same concentration. All assays were performed four to five times, with the means and standard deviations presented as results.

8. Statistical Analyses

All statistical computations were performed by SPSS for Windows program version 10. Data were presented as means and standard deviations. The relationship between number of viable cells and the absorbance at 600 nm was determined by linear regression analysis. The differences in means between two groups were analyzed by Student's T test. The differences in means among three groups or more were analyzed by analysis of variance (ANOVA). When the equal variance was assumed, Scheffe's test was used for post hoc analyses. When the equal variance was not assumed, Tamhane's test was chosen. The chosen level of significance was $P < 0.05$.