CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Plant material: *Garcinia mangostana* Linn. (purchased in Bangkok, Thailand in July, 2004).
2. Standard mangostin: detailed in Appendix A.
3. Acesulfame potassium (Rama production, Thailand, lot no. 0311040).
4. Cetylpyridinium chloride (BDH chemicals, England, lot no. 6152940).
5. Chlorhexidine diacetate (Imperial chemical industries, Great Britain, lot no. 43132314).
6. Clotrimazole (S. Tong Chemicals, Thailand, lot no. 20010605).
7. Disodium hydrogen orthophosphate (Merck, Germany, lot no. F974786 538).
8. Ethanol absolute, AR grade (Merck, Germany, lot no. K32751783 349).
9. Ethyl acetate, AR grade (Labscan Asia, Thailand, lot no. 04 07 0073).
10. Eucalyptus oil (Srichand United Dispensary, Thailand, lot no. 030115).
11. Glycerin (Srichand United Dispensary, Thailand, lot no. 8-03).
12. Hydroxypropyl cellulose (LV) (Pharmaserv, Thailand, lot no. 8019201).
13. Hydroxypropyl methylcellulose (3 cps.) (Shin-etsu, Japan, lot no. 112530)
14. Hydroxypropyl methylcellulose (5 cps.) (Colorcon, USA., lot no. RJ08012405).
15. Commercial product strips A (containing essential oils).
16. Commercial mouth wash solution B (containing essential oils).
17. Commercial mouth wash solution C (containing 0.12% w/v of chlorhexidine gluconate).
18. Menthol (Srichand United Dispensary, Thailand, lot no. 036597).
19. Methanol, HPLC grade (Labscan Asia, Thailand, lot no. 05 03 0064).
20. Mueller Hinton broth (Difco, USA, lot no.4104193).
21. Mueller Hinton agar (Merck, Germany, lot no. VL798237).
22. Polyethylene glycol 400 (Srichand United Dispensary, Thailand, lot no. 419070103).
23. Potassium dihydrogen phosphate (Merck, Germany, lot no. A315973 127).

24. Propylene glycol (Srichand United Dispensary, Thailand, lot no. 1752124024).
25. Sodium chloride (Merck, Germany, lot no. K28555404 049).
26. Sodium lauryl sulfate (S-Tong Chemical, Thailand, lot no. 10420).
27. *Staphylococcus aureus* ATCC 25923 (Department of Microbiology, Faculty of Pharmaceutical science, Chulalongkorn University, Thailand).
28. *Streptococcus mutans* ATCC $KPSK_2$ (Department of Microbiology, Faculty of Dentistry, Chulalongkorn University, Thailand).

Instruments

1. Analytical balance (Model AG285, Mettler Toledo, Switzerland).
2. Autoclave (Model HA-3D, Hirayama, Japan).
3. Botanical grinder (Retsch GmbH SK1, Germany).
4. Centrifuged (model4206, Milano, Italy).
5. Differential scanning calorimeter ($DSC822^e$, Mettler Toledo, Switzerland).
6. Disposable syringe filter nylon 13 mm, 0.45 μm (Chrom Tech, USA, lot no. 0811).
7. Fluorescence analysis cabinet (Model CM-10, Spectroline, USA).
8. High performance liquid chromatography (HPLC) (Perkin Elmer, Germany) equipped with
   - Automatic sample injector (SIL-10A, Shimadzu, Japan).
   - Communications bus module (CBM-10A, Shimadzu, Japan).
   - Liquid chromatography pump (LC-10AD, Shimadzu, Japan).
   - UV-VIS Detector (SPD-10A, Shimadzu, Japan).
   - Column (BDS Hypersil C18, 5 μm, 250 x 4.6 mm, Thermo Electron Corporation England, lot no. 6596).
   - Precolumn (μBondapack C18, 10 μm, $125A^o$, Water Corporation, Ireland, lot no. W2336B1).
9. Hot air oven (Model B40, Memmert, Germany).
10. Incubator (Model B60, Memmert, Germany).
11. Laminar sir flow (Model BV-126, ISSCO, USA).
12. Magnetic stirrer (Stuart, England).
13. Micrometer (Starrett, USA.).

14. Microscope (Nikon eclipse E200, Japan).
15. Modified dissolution tester.
16. pH meter (Orion model 420A, Orion Research Inc., USA).
17. Rotary evaporator (Rotavapor RE-120, Buchi, Switzerland).
18. Stability cabinet (Eurotherm Axyos, Germany).
19. Stopwatch (Heuer, Switzerland).
20. Tensile testing machine (Model H5KS 1509, Tinius Olsen, England).
21. TLC alumina sheet silica gel 60F 254 20 x 20 cm (Merck, Germany, lot no. OB290814).
22. Tube rotary machine (model EW PC 9022T/R/P, Thailand).
23. UV-Visible spectrophotometer (UV-1601, Shimadzu, Japan).
24. Vernier caliper (Macoh, China).
25. Vortex mixer (Vortex Genies-2, Scientific Industries, Inc., USA).

METHODS

A Extraction of Active Constituents from *Garcinia mangostana* fruit hulls

The fruit hulls of *Garcinia mangostana* were cut into small pieces about 1 x 1 inch, and then dried at the temperature 45±0.5 °C in a hot air oven for 48 hours. The dried powder fruit hulls (4345.3 g.) were extracted by maceration method with ethyl acetate at room temperature overnight. The ethyl acetate extract was filtered and evaporated using rotary evaporator at 30°C. Then the crude extract was allowed to crystallize at room temperature. The yield was kept in a desiccator and used for further studies (modified from Hiranras, 2001).

B Identification and Determination of Active Constituents from *Garcinia mangostana*

1. Differential scanning calorimetric (DSC) method

The DSC thermogram was determined by using differential scanning calorimeter (DSC822^e, Mettler Toledo, Switzerland) to detect melting point. An accurately weighed amount 3 mg of the crude extract was placed in an aluminum pan. Then the aluminum pan was sealed with the lid under the pressure of the plunger by using crucible-sealing press. After sealing, the aluminum pan was placed on a DSC sensor in the furnace. The scan rate was performed at 10 °C/min over the temperature range of –50 to 220 °C under the nitrogen atmosphere. The experiment was performed in triplicate.

2. Thin layer chromatographic (TLC) method

TLC is the most versatile and flexible chromatographic method. It is rapid and gives highest sample throughput because many samples and standards can be applied to a single plate and separated at the same time.

The extract was dissolved with ethyl acetate and spotted on TLC alumina sheet, compared with standard mangostin. After that the alumina sheet was placed in a closed chamber saturated with vapor of ethyl acetate: hexane (3:1) as mobile phase. When the mobile phase had moved to an appropriate distance, the alumina sheet was removed and dried. The alumina sheet was detected under UV light at the wavelength of 254 nm (Hiranras, 2001). The basic parameter used to describe the migration is the Rf value, where

$$Rf = \frac{\text{distance moved by solute}}{\text{distance moved by mobile phase front}}$$

3. High performance liquid chromatographic (HPLC) method

The determination of active constituents from *Garcinia mangostana* was performed by HPLC method because of specificity and high sensitivity.

3.1 HPLC condition

From the preliminary study of mangostin assayed with UV spectrophotometer, the scanning spectra of mangostin in 87% v/v methanol in water was obtained. From the spectra, the maximum absorbance was found at the wavelength of 243 nm (Figure 18). Therefore, the detection of mangostin was performed at this wavelength (Hiranras, 2001).

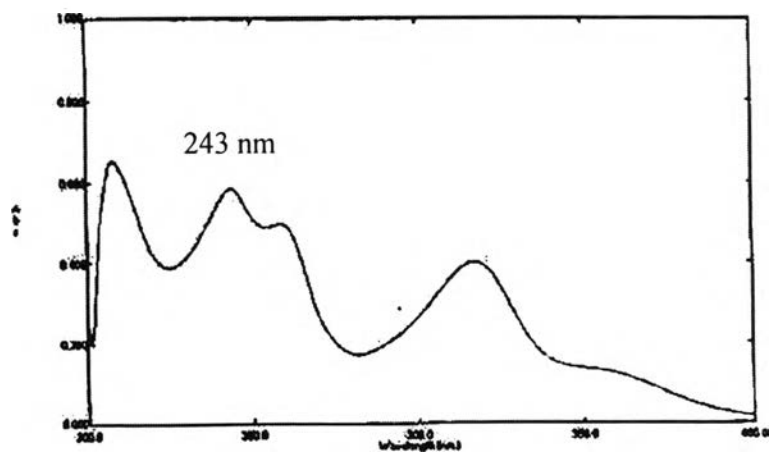

Figure 18 Absorption spectra of mangostin in 87% v/v methanol in water.

The HPLC conditions for the analysis of mangostin were as follows (modified from Hiranras, 2001):

Column	:	BDS Hypersil® C18, 5 μm, 250 x 4.6 mm
Precolumn	:	μBondapack C18, μ10 m, 125A°
Mobile phase	:	methanol: water (87:13)
Injection volume	:	20 μl
Flow rate	:	1 ml/min
Detector	:	UV detector at 243 nm
Temperature	:	ambient
Run time	:	13 min
Internal standard	:	clotrimazole

The mobile phase was freshly prepared by using methanol, HPLC grade and ultrapure water with ratio of 87:13 v/v. The solution was thoroughly mixed, filtered through 0.45 μm membrane filter and then degassed by sonication for 30 min prior to use.

3.2 Standard solutions for HPLC

3.2.1 Preparation of internal standard solutions

In the preliminary of HPLC study, clotrimazole solution (400 μg/ml) was used as an internal standard due to its appropriate retention time and optimal resolution from mangostin peak.

A stock solution of clotrimazole was prepared by accurately weighed 200 mg of clotrimazole into a 50 ml volumetric flask, diluted and adjusted to volume with mobile phase. The final concentration of clotrimazole stock solution was 4,000 μg/ml.

3.2.2 Preparation of standard solutions

An accurate weight 20 mg amount of mangostin was placed into a 10 ml volumetric flask and diluted to volume with mobile phase. This stock solution had a final concentration of 2,000 μg/ml. Then 5.0 ml of this solution was transferred into

a 100 ml volumetric flask and was diluted with mobile phase to give a solution of 100 μg/ml. The solution of 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 ml and 1.0 ml of internal standard stock solution were added into 10 ml volumetric flask. The dilution to volume with mobile phase gave 1, 5, 10, 15, 20, 30, and 40 μg/ml of mangostin, respectively and 400 μg/ml of clotrimazole. Three sets of standard solution were prepared for each HPLC run. As a result, the standard curve of mangostin between concentration and peak area ratio was plotted.

3.2.3 Preparation of sample solutions

The sample stock solution was prepared by accurately weighing 10 mg of *Garcinia mangostana* extract into a 50 ml volumetric flask. Mobile phase was added to dissolve and the solution was adjusted to final volume. This stock solution had a final concentration of 200 μg/ml. Then 1.0 ml of stock solution and 1.0 ml of clotrimazole stock solution were transferred into a 10 ml volumetric flask. The solutions were adjusted to volume with mobile phase to give the concentrations of *Garcinia mangostana* extract and clotrimazole of 20 μg/ml and 400 μg/ml, respectively.

3.3 Validation of the HPLC method

The analytical parameters used in the assay validation for the HPLC method were specificity, linearity, precision and accuracy.

3.3.1 Specificity

Under the chromatographic conditions used, the peak of mangostin must be completely separated from and not be interfered by the peaks of other components in the sample.

3.3.2 Linearity

Three sets of seven standard solutions were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

3.3.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations at 8, 25 and 35 μg/ml were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration multiplied by 100.

3.3.4 Precision

a) Within run precision

The within run precision was determined by analyzing five sets of three concentrations at 8, 25 and 35 μg/ml in the same day. Peak area ratios of mangostin to clotrimazole were calculated and the percent coefficient of variation (%CV) of each concentration was determined.

b) Between run precision

The between run precision was determined by analyzing three concentrations at 8, 25 and 35 μg/ml on five different days. The coefficient of variation (%CV) of mangostin of each concentration was determined.

Acceptance criteria:

For accuracy, the percentage of recovery should be within 98-102 % of each nominal concentration, whereas the percent coefficient of variation for both within run precision and between run precision should be less than 2%.

C Determination of Solubility of *Garcinia mangostana* Extract

1. Solubility study of the extract in ethanol: water system

Excess amount of extract (mangostin) was put into each test tube containing 5 ml of hydroalcoholic mixture 40, 50, 60, 70 and 80 % v/v of ethanol. These suspensions were equilibrated by constant rotating in a tube rotary machine at ambient

temperature for 24, 36, and 48 hours. Then suspensions were centrifuged and the supernatants were filtered through 0.45 μm nylon membrane, appropriately diluted and analyzed by UV spectrophotometry. All solubility determinations were carried out in triplicate.

2. Preparation of calibration curve UV spectrophotometry

A stock solution of mangostin was prepared by accurately weighed 12 mg of mangostin into a 10 ml volumetric flask, then dissolved and adjusted to volume with absolute ethanol. Then pipette 1.0 ml of this standard stock solution into 100 ml volumetric flask. This standard solution had a final concentration of 12 μg/ml. After pipette 2, 3, 4, 5, 6, 7, and 8 ml of this solution into 10 ml volumetric flask, diluted and adjusted to volume with the absolute ethanol. The final concentrations of mangostin were 2.4, 3.6, 4.8, 6.0, 7.2, 8.4, and 9.6 μg/ml, respectively. Then the standard solutions were analyzed using the UV spectrophotometer at 243 nm. The standard curve of mangostin between concentration and absorbance was plotted.

3. Validation of UV spectrophotometric method

The analytical parameters used for the assay validation were specificity, linearity, accuracy and precision.

3.1 Specificity

Under the conditions used, the absorbance of mangostin must not be interfered by the absorbance of other components in the sample.

3.2 Linearity

Three sets of six standard solutions of mangostin ranging from 2.4 to 9.6 μg/ml were prepared and analyzed. Linear regression analysis of the absorbances versus their concentrations was performed. The linearity was determined from the coefficient of variation (R^2).

3.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was

determined from the percentage of recovery. Five sets of three concentrations at 3.0, 5.4 and 7.8 μg/ml were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration multiplied by 100.

3.4 Precision

a) Within run precision

The within run precision was determined by analyzing five sets of three concentrations at 3.0, 5.4 and 7.8 μg/ml in the same day. The percent coefficient of variation (%CV) of mangostin of each concentration was determined.

b) Between run precision

The between run precision was determined by analyzing three concentrations at 3.0, 5.4 and 7.8 μg/ml on five different days. The percent coefficient of variation (%CV) of mangostin of each concentration was determined.

Acceptance criteria:

For accuracy, the percentage of recovery should be within 98-102 % of each nominal concentration, whereas the percent coefficient of variation for both within run precision and between run precision should be less than 2%.

D Determination of Antimicrobial Activities of Extract from *Garcinia mangostana*

The antimicrobial activity of extract was determined by the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) tests against the standard strain of *Staphylococcus aureus* ATCC 25923 and *Streptococcus mutans* ATCC $KPSK_2$.

1. Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) refers to the lowest concentration of an antimicrobial agent that inhibits growth of the microorganism under test and is generally recorded in mg/L or μg/ml (Denyer, Hodges, and Gorman, 2004).

The principle of the broth dilution method is the inhibition of growth of the bacteria by an antimicrobial agent incorporated in the broth medium.

The extract was tested for its activities against *Staphylococcus aureus* ATCC 25923 and *Streptococcus mutans* ATCC $KPSK_2$ using broth dilution method (Lorian, 1991; Mahon and Manuselis, 2000; Hiranras, 2001).

1.1 Preparation of double strength Mueller Hinton broth medium

Double strength Mueller Hinton broth medium was prepared by suspending 21 g of the broth powder to 500 ml of distilled water and warmed to dissolve the medium completely. After that the sterilization was performed by autoclaving at 121±0.5 °C for 15 min.

1.2 Preparation of *Garcinia mangostana* extract stock solution

The working stock solution of extract was prepared by accurately weighed 76.8, 32.0, and 25.6 mg, respectively, of extract into 10 ml volumetric flask diluted to volume with 95% v/v ethanol. The 1.0 ml solution was pipetted into 10 ml volumetric flask and diluted to volume with 95 % ethanol so that the final concentration of this solution was 768, 320, and 256 μg/ml, respectively.

1.3 Preparation of inoculated suspension of bacteria

The inoculum was prepared by picking bacteria colonies on the surface of subculture bacterial agar slant using sterile loop into test tube with sterile normal saline solution. The inoculum was standardized by adjusting turbidity of inoculated suspension with sterile normal saline solution until the turbidity equivalent to McFarland standard No. 0.5, since the concentration of standardized inoculated suspension was 1×10^8 cells/ml. Then the suspension was diluted with sterile normal saline solution to the final concentration of 1×10^6 cells/ml before use.

1.4 Method for the MIC test

The experiment was performed triplicately (using serial two fold-dilution method) by three series of 15 sterile test tubes. Every tube except the first were filled with 1.0 ml of double strength Mueller Hinton broth. Then 1.0 ml of extract solution was pipetted into the first and second tubes, mixed by vortex mixer, and 1.0 ml from the second tube was transferred to the third tube, repeated the procedure to tube no.13. The 1.0 ml in tube no. 13 was discarded, then 1.0 ml of inoculated suspension (1×10^6 cells/ml) was pipetted into tube no. 1-14 and 1.0 ml of sterile normal saline solution was pipetted into tube no 15. Therefore tube no. 14 is a growth control tube (broth with inoculum) and tube no. 15 is an uninoculated control tube (broth with sterile normal saline solution).

All tubes were incubated for a period of time for 24 hr at 37 °C for *Staphylococcus aureus* ATCC 25923 and 48 hr, 37 °C in CO_2 for *Streptococcus mutans* ATCC $KPSK_2$. The MIC was determined visually as the lowest concentration that inhibited bacterial growth, as demonstrated by the absence of turbidity.

2. Determination of minimal bactericidal concentration (MBC)

The minimal bactericidal concentration (MBC) is the lowest concentration of antimicrobial agent that results in $\geq$ 99.9% killing of the bacteria under test (Denyer, Hodges, and Gorman, 2004).

2.1 Preparation of Mueller Hinton agar medium

The MBC of *Garcinia mangostana* extract was determined by using Mueller Hinton Agar. Mueller Hinton Agar medium was prepared by adding 34 g of the agar powder to 1,000 ml of distilled water and boiled to dissolve the medium completely. After that the sterilization was performed by autoclaving at 121$\pm$0.5 °C for 15 min. Each agar plate was prepared by pipetting 25 ml of the medium to Petri dish with diameter of 10 cm and allowed to solidify at room temperature. The depth of agar was approximately 3 mm.

2.2 Method for the MBC test

The MBC was determined by dipping a sterile loop into each tube of no growth broth dilution test from 1.4, then the sterile loop was streaked on the surface of Mueller Hinton agar plate and incubated at the same condition as the MIC test. After incubation, the MBC was determined visually as the lowest concentration that the absence of colony of bacteria occurred on surface of agar.

The MIC and MBC studies for *Staphylococcus aureus* ATCC 25923 were performed in triplicate with another series of 128 μg/ml and 160 μg/ml of extract solution, so that the series were 128 μg/ml to 0.5 μg/ml and 160 μg/ml to 0.625 μg/ml, respectively.

The MIC and MBC studies for *Streptococcus mutans* ATCC $KPSK_2$ were performed in triplicate with another series of 64 μg/ml and 80 μg/ml of extract solution, so that the series were 64 μg/ml to 0.5 μg/ml and 80 μg/ml to 0.625 μg/ml, respectively.

3. The effect of solvent on antimicrobial activity

Because mangostin is a poor water solubility substance, so 95% ethanol was selected as solvent to solubilize the extract. Ethanol is a solvent that has inhibitory and bactericidal effect for many species of bacteria. Therefore, the effect of this solvent for *Garcinia mangostana* extract (95% ethanol) on the inhibition of 2 types of test bacteria were examined using a serial two fold-dilution method.

3.1 Method for the MIC test

The experiment was performed duplicately (using serial two fold-dilution method) by two series of 15 sterile test tubes. Every tube except the first was filled with 1.0 ml of double strength Mueller Hinton broth. Then 1.0 ml of 95% ethanol was pipetted into the first and second tubes, mixed by vortex mixer, and 1.0 ml from the second tube was transferred to the third tube, repeated the procedure to tube no.13. The 1.0 ml in tube no. 13 was discarded, then 1.0 ml of inoculated suspension (1×10^6 cells/ml) was pipetted into tube no. 1-14 and 1.0 ml of sterile normal saline solution

was pipetted into tube no 15. Therefore tube no. 14 is a positive control tube (broth with inoculum) and tube no. 15 is a negative control tube (broth with sterile normal saline solution).

All tubes were incubated in the same manner as in 1.4. After incubation, the MIC of 95% ethanol was determined visually as the lowest concentration that inhibited bacterial growth, as demonstrated by the absence of turbidity.

3.2 Method for the MBC test

The MBC was determined by dipping a sterile loop into each tube of no growth broth dilution test, then the sterile loop was streaked on the surface of Mueller Hinton agar plate and incubated at the same condition as the MIC test. After incubation, the MBC was determined visually as the lowest concentration that the absence of colony of bacteria occurred.

E Preparation of Orally Fast Dissolving Films

1. Preparation of cellulose derivatives film bases

From preliminary studies, some polymers, which could be prepared with appropriate properties including good appearance, integrity, and easy to remove from a glass mold without defect or breakage were explored. These polymers included HPMC 3 and 5 cps, and HPC. The formulas are presented in Table 9. The procedures for preparing were as follows:

1) The polymer was dispersed in hydroalcoholic mixture at 60% v/v of ethanol, sealed the top of beaker with paraffin film to prevent evaporation of solvent. The dispersion was left at room temperature for 12 hours in order to remove entrapped air bubbles and allowed the polymer to completely hydrate and swell.

2) The additives, which are acesulfame potassium, menthol, and eucalyptus oil were added and mixed consequently into the polymer dispersion. The mixture was stirred gently to prevent air entrapment and left until air bubbles were removed.

3) The film was prepared by pouring the mixture into a hollow space of an aluminum box placed on a dry and clean surface of flat glass plate with area 20 x 20 cm^2. The aluminum box was adjusted at height of 0.5 mm. Then it was dragged towards the right side of the flat glass plate. The film was allowed to completely dry on a leveled flat surface in a hot air oven at 45 ^oC for 2 hours (Figure 19).

4) The film was carefully removed from the glass and cut to 2 x 3 cm^2. The final weight and the thickness of the film were measured with an analytical balance (Mettler Toledo, Switzerland) and a micrometer (Starrett, USA), respectively.

In this experiment, the films were prepared in various formulations to study the effect of plasticizer and effect of polymer mixture on mechanical properties. The formulations are depicted in Tables 7 and 8.

Table 7 Composition of cellulose derivatives film bases (HPMC 3 cps) with plasticizers.

Polyethylene glycol 400	Propylene glycol	glycerin
-	-	-
+	-	-
+	+	-
+	+	+
-	+	-
-	-	+
-	+	+
+	-	+

+ quantity of plasticizer 5% w/w of polymer

- quantity of plasticizer 0% w/w of polymer

Table 8 Composition of cellulose derivatives film bases.

composition	Concentration in solution (%w/w)	Formulation codes
HPMC (3 cps)	12	E3
HPMC (5 cps)	12	E5
HPC (LV)	12	HPC
HPMC 3 cps : HPC LV (2:1)	8 : 4	E3HPC (2:1)
HPMC 3 cps : HPC LV (3:1)	9 : 3	E3HPC (3:1)
HPMC 3 cps : HPC LV (4:1)	9.6 : 2.4	E3HPC (4:1)
HPMC 3 cps : HPC LV (5:1)	10 : 2	E3HPC (5:1)
HPMC 3 cps :HPMC 5 cps (1:1)	6 : 6	E3E5 (1:1)
HPMC 3 cps :HPMC 5 cps (2:1)	8 : 4	E3E5 (2:1)
HPMC 3 cps :HPMC 5 cps (3:1)	9 : 3	E3E5 (3:1)
HPMC 3 cps :HPMC 5 cps (5:1)	10 : 2	E3E5 (4:1)
HPMC 5 cps: HPC LV (1:1)	6 : 6	E5HPC (1:1)
HPMC 5 cps: HPC LV (2:1)	8 : 4	E5HPC (2:1)
HPMC 5 cps: HPC LV (3:1)	9 : 3	E5HPC (3:1)
HPMC 5 cps: HPC LV (5:1)	10 : 2	E5HPC (5:1)

All formulations were consisted of 0.3% w/w of acesulfame potassium as sweetener, 1.5% w/w of menthol and 0.6% w/w of eucalyptus oil as flavoring agents.

Table 9 Formulations of orally fast dissolving films (% w/w)

Rx	Formulas	HPMC 3 cps	HPMC 5 cps	HPC LV	PEG 400	PG	Glycerin	Ace- K	Menthol	Eucalyptus oil	60% ethanol
1	PG	12	-	-	-	0.6	-	0.3	1.5	0.6	85.0
2	PEG	12	-	-	0.6	-	-	0.3	1.5	0.6	85.0
3	GLY	12	-	-	-	-	0.6	0.3	1.5	0.6	85.0
4	PG/GLY	12	-	-	-	0.6	0.6	0.3	1.5	0.6	84.4
5	PEG/GLY	12	-	-	0.6	-	0.6	0.3	1.5	0.6	84.4
6	PEG/PG	12	-	-	0.6	0.6	-	0.3	1.5	0.6	84.4
7	PEG/PG/GLY	12	-	-	0.6	0.6	0.6	0.3	1.5	0.6	83.8
8	E3	12	-	-	-	-	-	0.3	1.5	0.6	85.6
9	E5	-	12	-	-	-	-	0.3	1.5	0.6	85.6
10	HPC	-	-	12	-	-	-	0.3	1.5	0.6	85.6
11	E3HPC (2:1)	8	-	4	-	-	-	0.3	1.5	0.6	85.6
12	E3HPC (3:1)	9	-	3	-	-	-	0.3	1.5	0.6	85.6
13	E3HPC (4:1)	9.6	-	2.4	-	-	-	0.3	1.5	0.6	85.6
14	E3HPC (5:1)	10	-	2	-	-	-	0.3	1.5	0.6	85.6
15	E3E5 (1:1)	6	6	-	-	-	-	0.3	1.5	0.6	85.6
16	E3E5 (2:1)	8	4	-	-	-	-	0.3	1.5	0.6	85.6
17	E3E5 (3:1)	9	3	-	-	-		0.3	1.5	0.6	85.6
18	E3E5 (5:1)	10	2	-	-	-	-	0.3	1.5	0.6	85.6
19	E5HPC (1:1)	-	6	6	-	-	-	0.3	1.5	0.6	85.6
20	E5HPC (2:1)	-	8	4	-	-	-	0.3	1.5	0.6	85.6
21	E5HPC (3:1)	-	9	3	-	-	-	0.3	1.5	0.6	85.6
22	E5HPC (5:1)	-	10	2	-	-	-	0.3	1.5	0.6	85.6

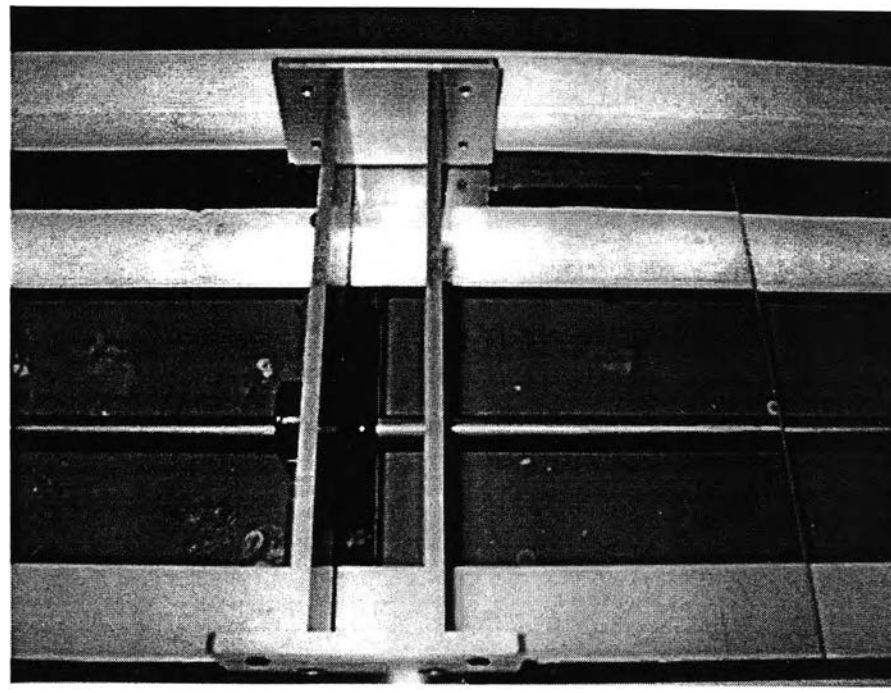

Figure 19 Film casting apparatus (assembled in the laboratory).

F *In vitro* Evaluation of Orally Fast Dissolving Films

1. Physical appearances

Color, transparency, flexibility and integrity of the film bases were visually observed. Ease of detachment from glass in the preparation process was also investigated.

2. Determination of film thickness

The thickness of 2 x 3 cm^2 films was determined using a micrometer (Starrett, USA), which has a sensitivity of 0.001 mm. Thickness was measured at five different points, one point at film center and others around the central point. The results were recorded as the mean of five measurements.

3. Determination of film weight

The weight of film bases was investigated using an analytical balance (Mettler Toledo, Switzerland), that has a sensitivity of 0.00001 g. The measurement was performed in 5 samples and the results were showed in the mean of five measurements.

4. Determination of surface and cross section morphology

The surface and cross section morphology of polymer films was observed by using a scanning electron microscope (Joel, Japan). The films were mounted on a metal stub and coated with gold. The films were imaged with a 15 kV electron beam with an appropriate magnification.

5. Determination of mechanical properties of film bases

Mechanical properties of test films were measured by using a tensile testing machine (Tinius Olsen®, Model H5KS 1509) equipped with 10 N tension load cell. The mechanical properties studied included the tensile strength, percent elongation at break, work of failure, and Young's modulus. The procedure employed was based on the guideline of the American Society for Testing and Material (1995).

The film specimens were cut into small strips 2 x 30 mm by using a standard knife. The specimens were left to expose to room humidity for 1 hour before tested. The thickness of each strip was the average value of five separate measurements taken along the length of the sample by using micrometer. The test specimen was carefully clamped by an upper and lower flat-faced grip and was extended by the test machine with the condition as follows:

Rate of grip separation	=	3 mm/min
Gauge length	=	5 mm
Load range	=	8 Newton
Temperature	=	25 ± 2 °C
Relative humidity	=	40 ± 5 %

In this experiment, at least five specimens were examined for one film formulation. After the specimen was ruptured the breaking force and the change in length at the moment of rupture were analyzed by the software-controlled program, QMat 4.10 S-Series-5K). The acceptable data were only those obtained from the strip that ruptured at the bilateral section.

The tensile stress was plotted against the percent strain to give stress-strain curve, and ultimate tensile strength as well as elongation at break was reported. The mean and standard deviation of the values were obtained from five determinations. The ultimate tensile strength and percent elongation at break were calculated from the following formulas.

$$\text{Tensile strength (MPa)} = \frac{\text{maximum load}}{\text{original minimum cross-sectional area of the specimen}}$$

$$\text{Percent elongation} = \frac{\text{extension at the moment of rupture} \times 100}{\text{initial gauge length of the test specimen}}$$

$$\text{Work of failure (mJ)} = \text{area of a curve plotting between force and extension}$$

$$\text{Young's modulus(MPa)} = \frac{\text{tensile stress}}{\text{elastic strain in tension}}$$

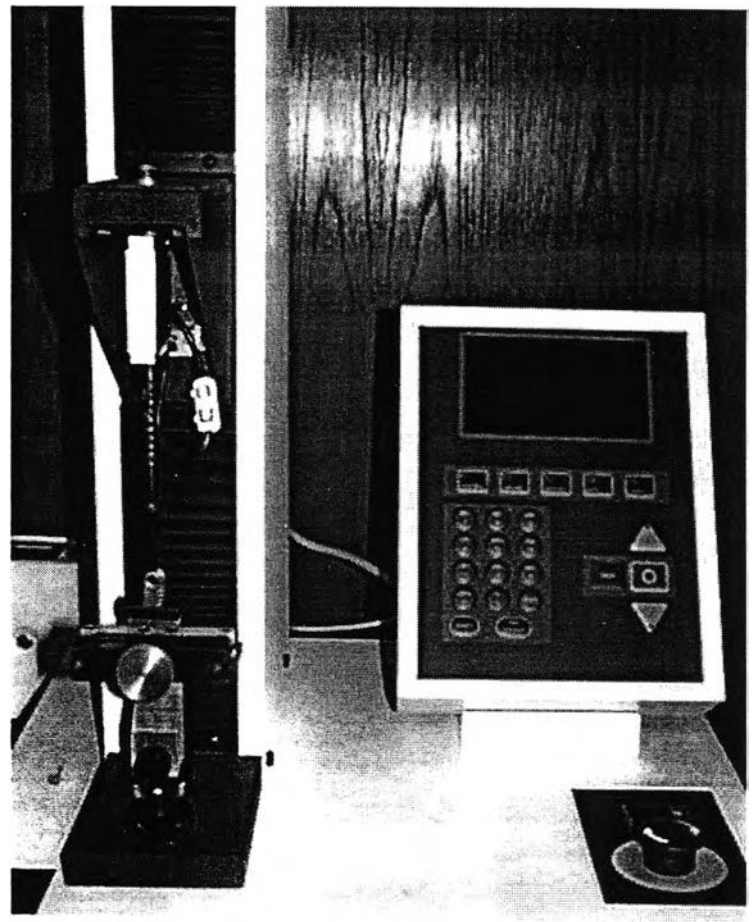

Figure 20 Tensile testing apparatus (H5KS 1509, Tinius® Olsen, England)

6. *In vitro* determination of dissolution time of film bases

The dissolution time of film bases was determined using a modified version of the speed of dissolution of the film in water described by Xu et al. (2002). The apparatus consisted of glass beaker containing isotonic phosphate buffer saline pH 6.2 and stirred with a magnetic bar at 60 $\pm$ 5 cycles per minute, a heating unit equipped with a thermostat controlling the temperature at 37 $\pm$ 1 °C. A fast dissolving film base was secured on the surface of fluid and recorded the dissolution time when the film disappeared or completely disintegrated using stopwatch (Heuer, Switzerland), which

has a sensitivity of 0.2 second. The apparatus employed in this study is displayed in Figure 21-23.

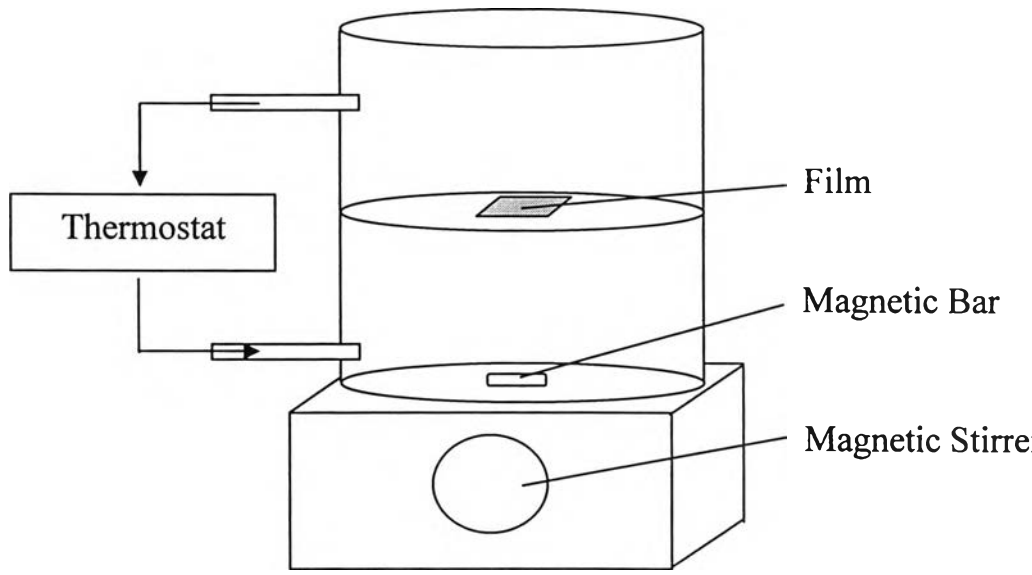

Figure 21 Schematic illustration of dissolution time testing apparatus (assembled in the laboratory).

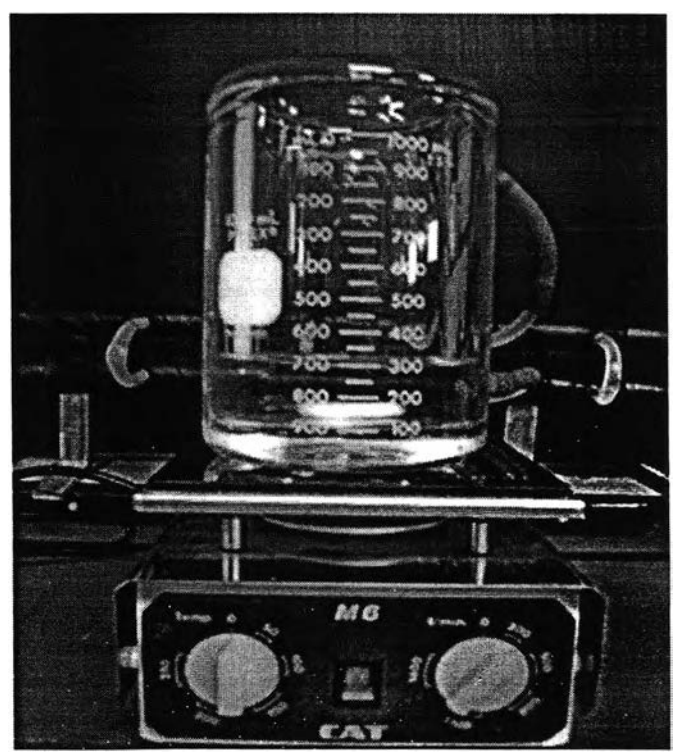

Figure 22 Dissolution time testing apparatus (assembled in the laboratory).

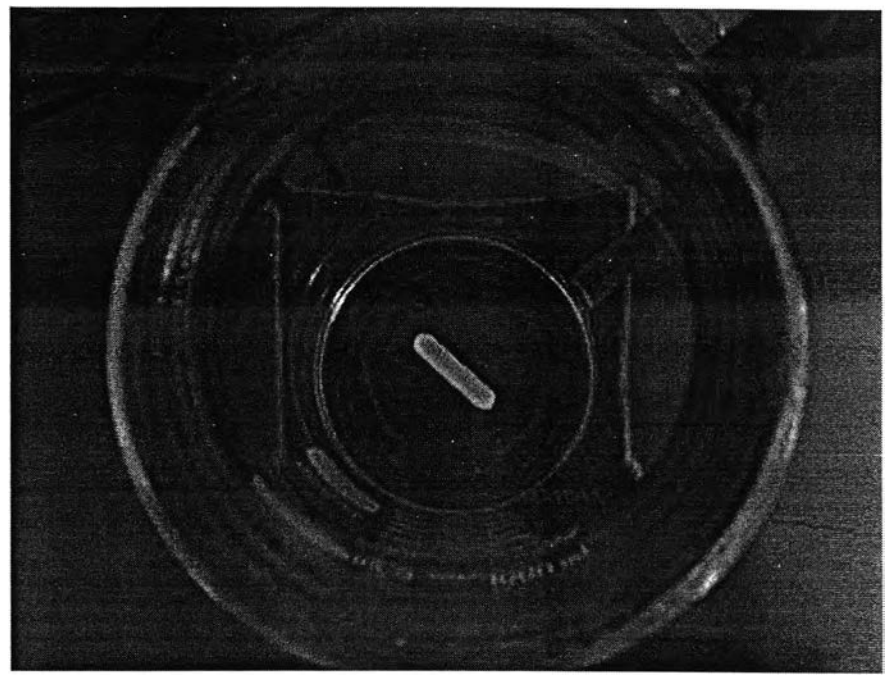

Figure 23 Dissolution time testing apparatus; top view (assembled in the laboratory)

G Formulation of Orally Fast Dissolving Films Containing *Garcinia mangostana* Extract

From the evaluation of film bases (physical, mechanical, and dissolution time), it was found that all formulations of the film consisting of HPMC 3 cps and HPC LV as polymer mixture had appropriate properties both mechanical properties and fast dissolution time and thus were selected to use in the further formulation.

According to the microbiological study of the *Garcinia mangostana* extract, the optimal concentration in the film against *Staphylococcus aureus* ATCC 25923 was obtained from the dose of about 100 times of MIC in a specimen of 2 x 3 cm^2 size. The formulations of orally fast dissolving film containing *Garcinia mangostana* extract are shown in Table 10.

Table 10 The formulations of orally fast dissolving films containing *Garcinia mangostana* extract consisting of HPMC 3 cps and HPC LV as polymers. All substances are shown in % w/w.

Substances (%w/w)	Formulation code			
	E3HPC 2:1	E3HPC 3:1	E3HPC 4:1	E3HPC 5:1
HPMC 3 cps	8	9	9.6	10
HPC LV	4	3	2.4	2
Garcinia mangostana extract	0.216	0.216	0.216	0.216
Acesulfame K	0.3	0.3	0.3	0.3
Menthol	1.5	1.5	1.5	1.5
Eucalyptus oil	0.6	0.6	0.6	0.6
60% v/v ethanol	85.4	85.4	85.4	85.4

H Characterization of Orally Fast Dissolving Films Containing *Garcinia mangostana* Extract

1. Determination of general characteristics of films

Physical appearances, thickness, weight, surface morphology, mechanical properties and dissolution time of the films containing *Garcinia mangostana* extract were investigated as the same apparatus and procedures of film bases.

2. Determination of differential scanning calorimetric thermograms

Differentials scanning calorimetric thermograms were recorded with a differential scanning calorimeter (DSC822^e, Mettler Toledo, Switzerland), which is instrument for thermal analysis. A highly sensitive ceramic sensor in DSC instrument is used to measure the difference between the heat flows to the sample and reference crucibles. The standard type pan for DSC measurement is aluminum crucible standard 40 µl. Indium (melting point 156.6 °C) was used to calibrate the instrument. An accurately weighed amount of 3-5 mg of the sample was placed into an aluminum

crucible. Then the aluminum crucible was sealed with the lid under the pressure of the plunger by using crucible sealing press. After sealing, the aluminum crucible was placed on a DSC sensor in the furnace. Samples were heated ranging from –50 to 220 °C at a heating rate of 10 °C/min. All tests were performed under a nitrogen atmosphere.

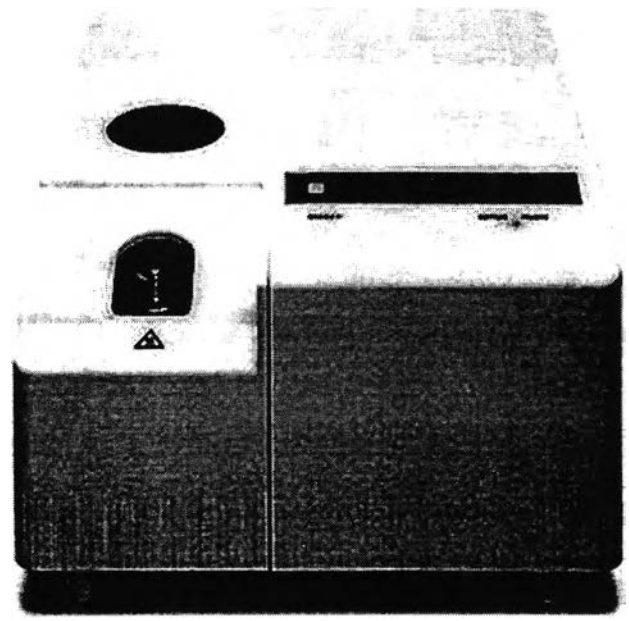

Figure 24 Differential scanning calorimeter (DSC822^e, Mettler Toledo, Switzerland).

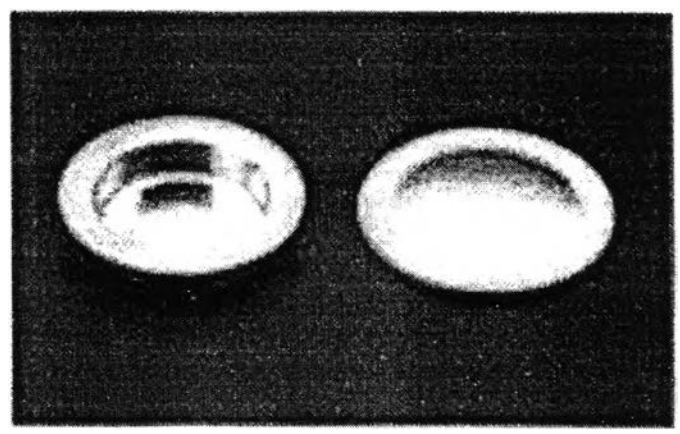

Figure 25 Aluminum crucible standard 40 µl (a) and crucible sealing press (b).

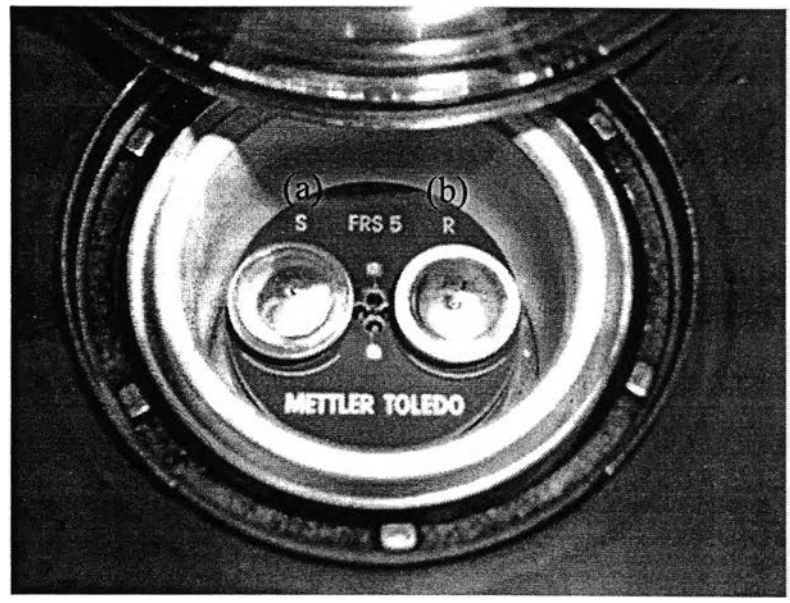

Figure 26 DSC sensor (a) sample pan and (b) reference pan

3. *In vitro* dissolution study of *Garcinia mangostana* extract orally fast dissolving films

3.1 Select medium for dissolution study

Selection of an appropriate dissolution medium is important to maintain sink condition during the dissolution study. Sodium lauryl sulfate was used to enhance drug solubility in dissolution medium. The solubility study of extract was performed by adding excess amount of the extract into each screw-capped tube containing 5 ml of isotonic phosphate buffer pH 6.2 and sodium lauryl sulfate ranging from 0 to 1.0 % w/v. The tubes were continuously rotated using rotary shaker at 37±0.5 °C for 72 hr to achieve equilibrium. Then the suspensions were centrifuged and the supernatants were withdrawn and filtered through 0.45 μm nylon membrane syringe filters. The filtrates were appropriately diluted and analyzed by HPLC. All solubility determinations were carried out in triplicate.

3.2 *In vitro* dissolution study

Dissolution study of orally fast dissolving films containing *Garcinia mangostana* extract was determined using a modified dissolution testing apparatus. The apparatus consisted of a glass beaker containing 20 ml of 1.0% sodium lauryl sulfate in isotonic phosphate buffer pH 6.2 as the dissolution medium and stirred with

a magnetic bar at 100 ± 5 cycles per minute, a heating unit equipped with a thermostat controlling the temperature at 37 ± 1 °C. A film product (2 x 3 cm) was secured by placing in a basket before being immersed into the glass beaker and a 3 ml of the dissolution medium was sampled at 1, 3, 5, 7, 10, 15, 30, 45 and 60 minutes. Then the sample was cooled down to ambient temperature, 2.0 ml of sample was pipetted into test tube containing 0.4 ml of clotrimazole stock solution and 1.6 ml of methanol. The solution was filtered through 0.45 μm nylon membrane and analyzed for mangostin content using HPLC as the same previously described conditions. The apparatus employed in this study is displayed in Figure 27.

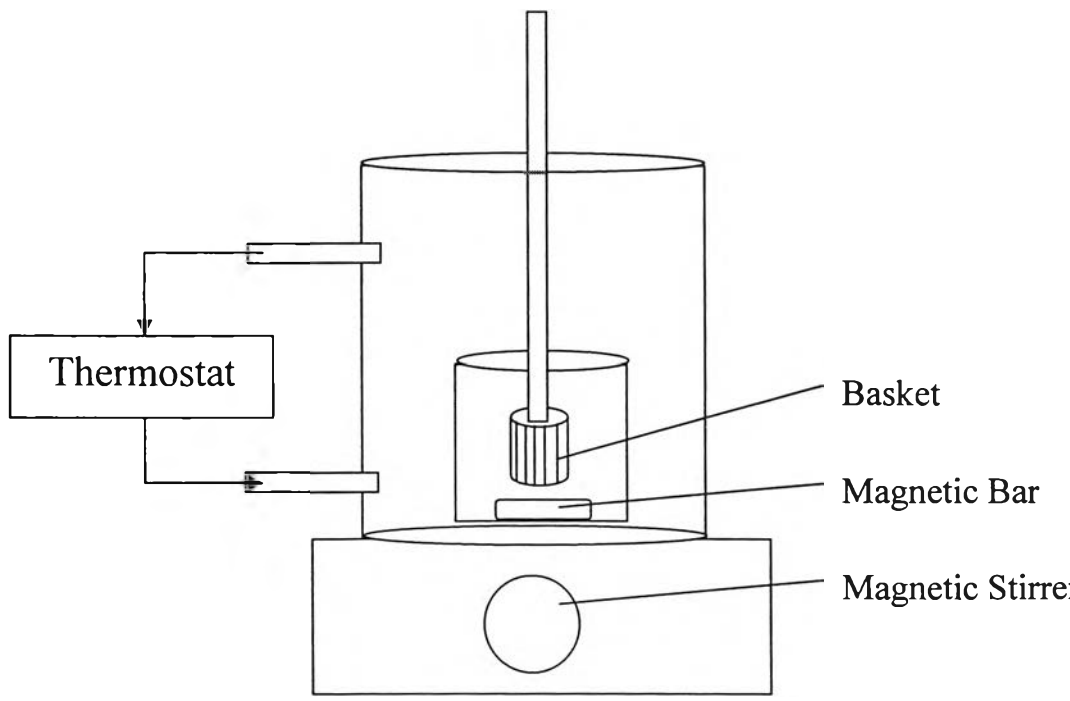

Figure 27 Modified dissolution testing apparatus (assembled in the laboratory)

4. Determination of content uniformity

The content uniformity of *Garcinia mangostana* extract in the orally fast dissolving films was quantitatively determined by mean of peak area ratio by HPLC method. The film was cut into ten small pieces (2 x 3 cm^2) with an accurate weight. Each piece was determined for the amount of mangostin. They are individually tested for their content uniformity. Each piece was analyzed by dissolving in 10 ml volumetric flask containing mobile phase and shaken at room temperature until the film was completely dissolved. Then 1.0 ml of stock internal standard was added into

the flask, finally adjusted volume to 10 ml. The solution was filtered through 0.45 μm nylon membrane and analyzed using the HPLC method described previously. The percentage of active drug within the test films was calculated from the calibration curve.

I Stability Study of Mangostin in Orally Fast Dissolving Films Containing *Garcinia mangostana* Extract

The film preparations (2 x 3 cm^2) were stored in glass vials, which were tightly sealed with rubber closure and aluminum caps at 40±2 °C and the humidity at 75±5 %RH for three months (Carstensen, 1990). The amounts remaining of mangostin were determined in triplicate at initial, 1, 2, and 3 month periods. The analysis of mangostin in film samples followed the previously described HPLC method. Triplicate samples of each test preparation were randomly drawn and analyzed compared with purified extract solution.

J *In Vitro* Antimicrobial Activity of Orally Fast Dissolving Films Containing *Garcinia mangostana* Extract

All formulations of orally fast dissolving films containing *Garcinia mangostana* extract were evaluated for their *in vitro* antimicrobial effect against *Staphylococcus aureus* ATCC 25923 and *Streptococcus mutans* ATCC $KPSK_2$ that are commonly microorganisms found in oral cavity.

1. Antimicrobial activity of films

Antimicrobial activity of films was determined by agar diffusion using Mueller Hinton agars (Difco, USA). The method was previously standardized by adjusting the microbial incubation at 0.5 Mcfarland, 1 x 10^8 cells/ml.

After adjusting the turbidity of the inoculum suspension, a sterile swab was dipped into the suspension and pressed the swab on the inside wall of tube to remove excess inoculum from the swab. The inoculum was swabbed over the entire agar surface in three different directions to ensure an even distribution of the inoculum. Five-mm diameter disks were cut from different films and placed on Mueller Hinton agars using sterile forceps. The agar plates were cooled down at 25 °C for 3 hours to allow diffusion of the extract and then incubated at 37 °C, 24 hours for *Staphylococcus aureus* ATCC 25923 and 48 hr, 37 °C in CO_2 for *Streptococcus mutans* ATCC $KPSK_2$. Each formulation was performed in triplicate. After incubation, the diameters of the zones produced by antimicrobial inhibition of bacterial growth were measured using Vernier caliper (Macoh, China). Data are expressed as growth inhibitory zone diameter in millimeter (Lorian, 1991; Mahon and Manuselis, 2000; Sebti, Broughton, and Coma, 2003).

The negative control groups of this experiment were polymer films without any additives and films base formulations without extract. Commercial product strips A, and the prepared film of cetylpyridinium chloride and chlorhexidine diacetate at the same concentration of extract were the positive control.

2. Antimicrobial activity of solution of films

The films were cut into 2 x 3 cm and dissolved with 2 ml of isotonic phosphate buffer pH 6.2 as simulated saliva. The films solutions were dropped into the sterile stainless steel cups that placed on Mueller Hinton agars swapped with inoculum (1 x 10^8 cells/ml). The plates were cooled down at 25 °C for 3 hours to allow diffusion of the extract and then incubated at 37 °C, 24 hr for *Staphylococcus aureus* ATCC 25923 and 48 hr, 37 °C in CO_2 for *Streptococcus mutans* ATCC $KPSK_2$. Each formulation was performed in triplicate. After incubation, the inhibition zone diameters were determined by Vernier caliper in millimeter.

The antimicrobial activity of orally fast dissolving films containing extract were compared with positive control as commercial product strips A, commercial mouth wash product B, containing essential oils, and commercial mouth wash product C, contained 0.12% w/v of chlorhexidine gluconate, prepared film with

cetylpyridinium chloride and film with chlorhexidine diacetate at the same concentration with extract. The negative control was isotonic phosphate buffer pH 6.2, polymer film without any additives, and film base without *Garcinia mangostana* extract.

K Statistical Analysis

The data of dissolution time, tensile strength, percent elongation, work of failure, percentage of drug dissolution and amount of mangostin remaining after the stability study were analyzed by statistically using one-way analysis of variance (ANOVA). When a significant difference ($p<0.05$) was indicated, the data were subjected to multiple comparison by Duncan test to compare the difference. The statistical package for the social sciences (SPSS) program version 11.0 was used in this study.