CHAPTER III
MATERIALS AND METHODS

Materials

- Plant material : *Garcinia mangostana* Linn.

The Fruit rinds of *Garcinia mangostana* Linn. were collected and dried at 40 °C in a hot air oven for 24 hr. The dried fruit rinds were cut and pulverized into powder using Bticino AEG type AMEB 80Fx2.

- *Staphylococcus aureus* ATCC 25923
- *Streptococcus mutans* ATCC $KPSK_2$
- *Streptococcus sanguis* (a clinical isolate)
- Sodium carboxymethylcellulose (MV) (lot no. E31032294, distributed by Srichand United dispensary, Thailand.
- Hydroxypropyl methylcellulose (4000) (lot no. 4017, distributed by S.Tong Chemical, Thailand.)
- Carbopol 934 (distributed by S.Tong Chemical, Thailand.)
- Ethylcellulose (10 cps) (distributed by Rama Production, Thailand)
- Chitosan low molecular weight (lot no. 4075682/1, molecular weight 150,000, Fluka, Switzerland).
- Chitosan medium molecular weight (lot no.395416/1, molecular weight 400,000, Fluka, Switzerland).
- Chitosan high molecular weight (lot no. 407490/1, molecular weight 600,000, Fluka, Switzerland).
- Mueller Hinton broth (lot no B238088, Oxoid, England)
- Sodium dihydrogen phosphate (lot no 33093021, Fluka, Switzerland)
- Disodium hydrogen phosphate (lot no 34869481, Fluka, Switzerland)
- Sodium sulfide (lot no TA782138 032, Merck, Germany)
- Potassium chloride (lot no. TA 822436 03, Merck, Germany)
- Sodium chloride (lot no. 383392/1, Fluka, Switzerland)
- Urea (distributed by S.Tong Chemical, Thailand.)
- Mucin (lot no. 70K0700, Sigma, Germany)

- Magnesium chloride (lot no.TA811082, Merck, Germany)
- Acetone (lot no. 98091020, Labscan, Thailand)
- Ethanol absolute (lot no. K28410083, Merck, Germany)
- Glacial acetic acid, AR grade (lot no. K143930, Merck, Germany)
- Lactic acid 90%, AR. grade (lot no. K20704866, Merck, Germany)
- Methanol, HPLC grade (lot no 01030082, Labscan, Thailand)
- Deionized water

Instruments

- Magnetic stirrer (Stuart, England).
- Variable speed stirring motor fitted with a four blade stirring shaft (model R30, Gmg H & Co., France).
- pH meter (Orion model 420A, Orion Research Inc., USA).
- Rotation viscosimeter (Rheology International Shannon,Ireland)
- Centrifuge (model 4206, Milano, Italy).
- Analytical Balance (MC1 research model RC210P, Sartorius, Germany).
- Analytical Balance (model 1615 MP, Sartorius , Germany).
- Electronic dry cabinet (model dry-100 no.5858, Weifo, USA).
- Incubator 37°C (Memmert, Western, Germany).
- Hot air oven (model B40, Memmert , Western Germany).
- Ultrasonicator (Transsonic Digital, Elma Ultrasound, Germany).
- Tube Rotary machine (model EW PC 9022T/R/P, Thailand).
- Shaker bath (Julabo JW1, Western Germany).
- Franz diffusion cell (Crown Glass Company, U.S.A.).
- UV-Visible recording spectrophotometer (UV-160A, Serial No. A113 31034483, Shimadzu, Japan).
- UV-Visible spectrophotometer (Spectro 22RS Digital Spectra Laboned,U.S.A.
- High performance liquid chromatography (HPLC) (Perkin Elmer, Germany) equipped with
  - LC work station (Serics gos interface) (Perkin Elmer, Germany)
  - Automatic sample injector (Series 200 Autosampler) (Perkin Elmer, Germany).

  - Solvent delivery module (Series 200 LC pump) (Perkin Elmer, Germany)
  - Detector (785A Programmable absorbance UV-Visible detector) (Perkin Elmer, Germany).
  - Communicator bus module (Series 600 link) (Perkin Elmer, Germany).
  - Software (Turbochrom Navigator 41) (Perkin Elmer, Germany).
- Column Hi-Q-Sil® C18 (5μm) 4.5 x 150 mm (Japan)with guard column
- Scanning Electron Microscope (model JSM-6400, Jeol, Japan).
- X–ray diffractometer (model JDX-3530, Jeol, Japan).
- Tensile tester (model LR 100K, Lloyd, Segensworth Farehano, England).
- Dial thickness gauge (Peacock, Ozaki, Japan).
- Thermohygrometer (model 608 H1, Testo, USA).
- Gallenkamp melting point apparatus.
- Mucoadhesion tester apparatus.
- Water repellent and mucoadhesion tester apparatus.

METHOD

1. Extraction, isolation, identification and solubitity determination of mangostin

1.1 Extraction

Dried and ground fruit rinds (3 kg) of *Garcinia mangostana* Linn. were extracted by maceration method overnight at room temperature in a percolator with hexane after that, the marc was extracted with ethyl acetate by the same method. The ethyl acetate extract was evaporated using rotary evaporator at 40 °C. Then the crude extract was recrystallized in the mixture of ethyl acetate / hexane (3 : 1) mixture: The yield was kept in a desiccator and used for further studies.

1.2 Isolation

The crude ethyl acetate extract was chromatographed on siliga gel using quick column chromatographic method and eluted with ethylacetate/hexane (0-25%) mixtures of increasing polarity to give 24 fractions. Each fraction was evaporated using rotary evaporator and was monitored by TLC (siliga gel 60 F 254) using ethyl acetate/hexane (3 : 1) mixture as the developing solvent and detected under UV light at wavelength 254 nm. The fractions which showed the same TLC characteristic were combined and allowed to crystallize.

1.3 Identification

The isolated mangostin was identified and characterized by spectroscopic method including mass spectrometry and nuclear magnetic resonance (NMR) and its melting point was also confirmed using Gallenkamp melting point apparatus.

1.4 Solubility of mangostin

Excess amount of purified extract (mangostin) was put into each test tube containing 5 ml of deionized water, pH 6.0 isotonic phosphate buffer and 35% v/v ethanol in pH 6.0 isotonic phosphate buffer. These suspensions were equilibrated by

constant rotating in a tube rotary machine at 37 °C for 48 hours. Then suspensions were centrifuged and the supernatant was withdrawn, appropriately diluted and analyzed by UV spectrophotometry. All solubility determinations were carried out in triplicate.

2. Determination of antimicrobial activities of purified extract from *Garcinia mangostana* Linn.

The purified extract obtained from recrystallization of ethyl acetate crude extract was tested for its activities against a normal strain of *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate) using disk diffusion method and broth dilution method. The efficacy was determined by the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) in μg/ml.

2.1 Disk diffusion method (Mahon and Manuselis, 2000)

The principle of disk diffusion method is dependent upon the inhibition of the growth of bacteria on the surface of an inoculated agar plate, by the antimicrobial agent that diffuses into the surrounding medium from an impregnated disk on the surface of the medium.

The petri dishes of which poured with melted Mueller Hinton Agar to a depth of approximately 4 mm, were allowed to form agar.

The inoculum was then prepared by a McFarland No.0.5 standardized suspension of each subcultured bacterial and swabbed over the surface of the agar plate evenly in three directions. The plate was allowed to dry at room temperature for 3-5 minutes. Paper disks containing various concentrations of purified extract (12.5, 25, 50 and 100 μg/30 μl in each 13 mm paper disk) were applied with a sterile forceps onto the inoculated surface. Each concentration was performed in triplicate. Then the plates were incubated at 37 °C 24 hr for *Staphylococcus aureus* ATCC 25923 and 48 hr in CO_2 for *Streptococcus mutans* ATCC $KPSK_2$ and *Streptococcus sanguis* (a

clinical isolate). After incubation, the diameters of the inhibition zones produced by purified extract were measured using the caliper.

2.2 **Broth dilution method** (Mahon and Manuselis, 2000)

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

The inoculum was prepared by rinsing the surface of subcultured bacterial agar slant with sterile normal saline solution and standardized the inoculum 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 x 10^8 cells/ml. Then the suspension was diluted with sterile normal saline solution to the final concentration of 1 x 10^6 cells/ml before use.

The working stock solution of purified extract was prepared by accurately weighed 51.2 mg of purified extract into a 10 ml volumetric flask diluted to volume with 95% v/v ethanol. The 1.0 ml solution was pipetted into a 10 ml volumetric flask and diluted to volume with 95% ethanol so that the final concentration of this solution was 512 µg/ml.

The experiment was performed duplicate (using serial two fold-dilution method) by two series of 14 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 purified extract solution was pipetted into the first and second tubes, mixed, and 1.0 ml from the second tube was transfered to the third tube, repeated the procedure to tube no. 12. The 1.0 ml in tube no. 12 was discarded, then 1.0 ml of inoculated suspension (1x10^6 cells/ml) was pipetted into tube no. 1-13 and 1.0 ml of sterile water was pipetted into tube no. 14. Therefore tube no. 13 is a growth control tube (broth with inoculum) and tube no. 14 is an uninoculated control tube (broth with sterile water).

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 $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate). After incubation, the MIC was determined visually as the lowest concentration that inhibited bacterial growth, as demonstrated by the absence of turbidity.

The MBC was determined by dipping a sterile cotton swab into each tube of broth dilution test started from the lowest concentration of inhibition (MIC) until the first tube, then the swab 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.

The MIC and MBC studies were performed in triplicate with another series of 192 μg/ml of purified extract solution so that the series of 192 μg/ml to 0.09375 μg/ml were tested.

The effect of solvent (95% ethanol) on the inhibition of test bacteria were examined using serial two fold–dilution method. The experiment was performed duplicate (using serial two fold-dilution method) by two series of 11 sterile test tubes. Every tube except the first was filled with 2.0 ml of double strength Mueller Hinton broth, then 2.0 ml of 95% ethanol was pipetted into the first and second tubes, mixed, and 2.0 ml from the second tube was tranfered to the third tube, repeated the procedure to tube no. 9. The 2.0 ml in tube no. 9 was discarded, then 2.0 ml of inoculated suspension ($1x10^6$ cells/ml) was pipetted into tube no. 1-10 and 2.0 ml of sterile water was pipetted into tube no. 11. Therefore tube no. 10 is a growth control tube (broth with inoculum) and tube no. 11 is an uninoculated control tube (broth with sterile water).

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 $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate). After incubation, the MIC of 95% ethanol was determined by spectrophotometry (Spectro 22 RS digital spectra Laboned).

3. Preparation of mucoadhesive free films

3.1 Mucoadhesive layer

3.1.1 Preparation of cellulose derivatives free films

From preliminary studies, some mucoadhesive polymers, which could be prepared with appropriate properties including good appearance, transparency, integrity, appropriate strength and flexibility to remove from a mold without defect or breakage were explored. These polymers included SCMC, HPMC and inclusion of 10 and 20% w/w CP 934. The formulas are presented in Table 1. The procedures for preparing were as follows :

1) The polymer was dispersed in purified water and the dispersion was left at room temperature for 12 hours in order to remove entrapped air bubbles and allow the polymer to completely hydrate and swell.

2) The predetermined quantity of ethanol was added into the aqueous polymer dispersion and the mixture was stirred gently to prevent air entrapment and left until air bubbles were removed.

3) The film was prepared by pouring 45 g of the mixture on a dry and clean surface of glass plate with a diameter of 90 mm, and allowed to dry on a leveled flat surface in a hot air oven at 45 °C over 24 hours until the film was completely dried.

4) The film was carefully removed from the glass plate The final weight and the thickness of the film were measured with an analytical balance and a micrometer dial thickness gauge

5) The film was stored in a tightly sealed container maintained at relative humidity of 30±1% and at ambient temperature until further analysis.

Table 1 Composition of mucoadhesive layer film formulas using cellulose derivatives.

Composition	Concentration in solution (%w/w)	Concentration in dried film (%w/w)	Formulas
SCMC	1	100	S0
SCMC : CP 934 9 : 1	1	100	SC1
SCMC : CP 934 8 : 2	1	100	SC2
HPMC	1	100	H0
HPMC : CP 934 9 : 1	1	100	HC1
HPMC : CP 934 8 : 2	1	100	HC2
Ethanol	30%	-	
Purified water	69%	-	

3.1.2 Preparation of chitosan free films.

1) Preparation of chitosan solutions.

Chitosan of low, medium and high molecular weights were used in the study. Some physical properties of these materials are given in Table 2.

Table 2 Some properties of chitosan.

Chitosan	Molecular weight	Reference viscosity 1% in 1% acetic acid (mPas)	color
Low molecular weight (LMW)	150,000	100	Yellowish
Medium molecular weight (MMW)	400,000	200	White
High molecular weight (HMW)	600,000	400	White

An accurate weight of chitosan to give 1% w/w of chitosan solution was gradually dispersed into a half of the required volume of water for 20 minutes until all particles were thoroughly wetted. The amount of 1 or 2% w/w acid in the final solution was dissolved in the another part of water and then added into the previous dispersion (the formulas are illustrated in Table 3). The mixture was stirred with a mechanical and a magnetic stirrer for 36 hours. The chitosan solution was filtered to remove insoluble impurities.

Table 3 Formulas of mucoadhesive layer films using chitosan.

Chitosan (1%w/w)	Acid	Concentration of acid solution (%w/w)	Formulas
Low molecular weight (LMW)	Acetic	1	LA1
		2	LA2
	Lactic	1	LL1
		2	LL2
Medium molecular weight (MMW)	Acetic	1	MA1
		2	MA2
	Lactic	1	ML1
		2	ML2
High molecular weight (HMW)	Acetic	1	HA1
		2	HA2
	Lactic	1	HL1
		2	HL2

2) Evaluation of chitosan solutions

(1) Viscosity determination

The viscosity of all formulas of 1% w/w chitosan solution were determined with a rotation viscosimeter (Rheology International Shannon, Ireland) using the spindle ASTM No. 2. The viscosity was monitored at a speed of 100 rpm. The reported data were averaged from three determinations. The rheogram of chitosan solution was determined at varied shear rate at room temperature.

(2) pH determination

All formulas of chitosan solutions were determined pH value using pH meter (Orion model 420A,U.S.A). The result was the mean of three determinations.

3) Preparation of chitosan free films

The chitosan film was prepared by casting the chitosan solution (30 g for acetic acid formula and 25 g for lactic acid formula) on a dry and clean surface of glass plate with a diameter of 90 mm then placed on a leveled flat surface in a hot air oven at 45 °C over 24 hours until, the film was completely dried.

The film was carefully removed from the glass plate. The final weight and thickness were measured and the films were stored in a tightly sealed container maintained temperature and humidity until further investigation.

3.2 Protective / backing layer

The backing layer was prepared using ethylcellulose, which was accurately weighed and gradually dispersed into the required volume of acetone to give a 1% w/v of ethyl cellulose solution. The dispersion was stirred until the clear solution was obtained.

The film of backing layer was produced by pouring 15 ml of the solution over the dried mucoadhesive layer laying in the glass plate and standed to dry on a leveled flat surface in a hot air oven at 60 °C over 1 hour. Then the completely dried bilayered film was carefully removed from the glass plate and kept in a tightly closed container.

4. Evaluation of mucoadhesive layer free films

4.1 Physical appearances

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

4.2 Determination of film thickness

Film thickness was determined with a micrometer dial thickness gauge (Peacock, Japan), which has a sensitivity of 0.01 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 three measurements.

4.3 Determination of surface and cross section morphology

The surface and cross section morphology of chitosan free 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 10 and 15 kV electron beam, respectively with an appropriate magnification.

4.4 Determination of mechanical properties of free films

The ultimate tensile strength and percent elongation at break of test films were measured by using a tensile tester machine (Lloyd, England). The procedure employed was based on the guidelines of the American Society for Testing Material method (ASTM D882-97). A film specimen was cut into small strips 5x60 mm using a standard template. The thickness of each strip was the average value of five separate measurements taken along the length in the middle 4 cm section of each specimen using a micrometer. The test specimen was carefully clamped by an upper and lower pneumatic flat–faced grip and was extended by the test machine with the condition as following :

Rate of grip separation	=	12.5 mm/min
Gauge length	=	3.5 cm
Loading weight	=	10 Newton
Temperature	=	25 ± 2 °C
Relative humidity	=	45 ± 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 recorded by the digital system. The acceptable data were only ones obtained from the strip which ruptured at the bilateral section.

The ultimate tensile strength and percent elongation at break were calculated from the following formulas.

$$\text{Ultimate tensile strength} = \frac{\text{breaking force}}{\text{cross section area of the test specimen}}$$

$$\text{Percent elongation at break} = \frac{\text{difference in length at breaking point} \times 100}{\text{initial length of the test specimen}}$$

4.5 Evaluation of swelling property

The method to determine the swelling of free films was modified from the technique of Nunthanid et al. (2001) and Peh and Wong (1999). The specimens were

carefully cut into 1 cm x 1 cm strips and kept in the container at 30%.RH The initial weight (W_1) of each strip was measured. Each film was immersed in a medium (deionized water and artificial saliva) which maintained at temperature of 37±1 °C. At the determined time intervals (2, 5, 10, 15, 30 and 60 minutes), the film remnants were retrieved and wiped off excess medium on surface with filter paper, then the swollen film was weighted (W_2)

The experiments were performed in triplicates and the swelling index was calculated by the following equation:

$$\text{Swelling index (Wi)} = \frac{W_2 - W_1}{W_1}$$

The result was recorded as the average of three measurements and the swelling profile was performed.

4.6 In vitro mucoadhesion test

The experiment was a modified version of the in vitro method for assessment the duration of mucoadhesion described by Mortazavi and Smart (1994). The apparatus employed in this study is depicted in Figure 36

4.6.1 The Preparation of model mucosal membrane

The middle sections, discarding the first 40-50 mm at either end, of fresh small intestine were removed from male Wistar rats (approximately 200g weight and 7-8 weeks old). Before use the tissue was cut into 1.5 cm lengths which were, then opened longitudinally to expose the inner mucosal surfaces. Each mucosal surface was washed gently with pH 6.0 isotonic phosphate buffer to remove any loose material. This was then mounted securely in place, mucosol side upwards, on a platform (6.2 mm diameter) within a jacketed water bath containing artificial saliva (Appendix A) at 37 °C . The mucosa was then allowed to equilibrate for 1 min before test.

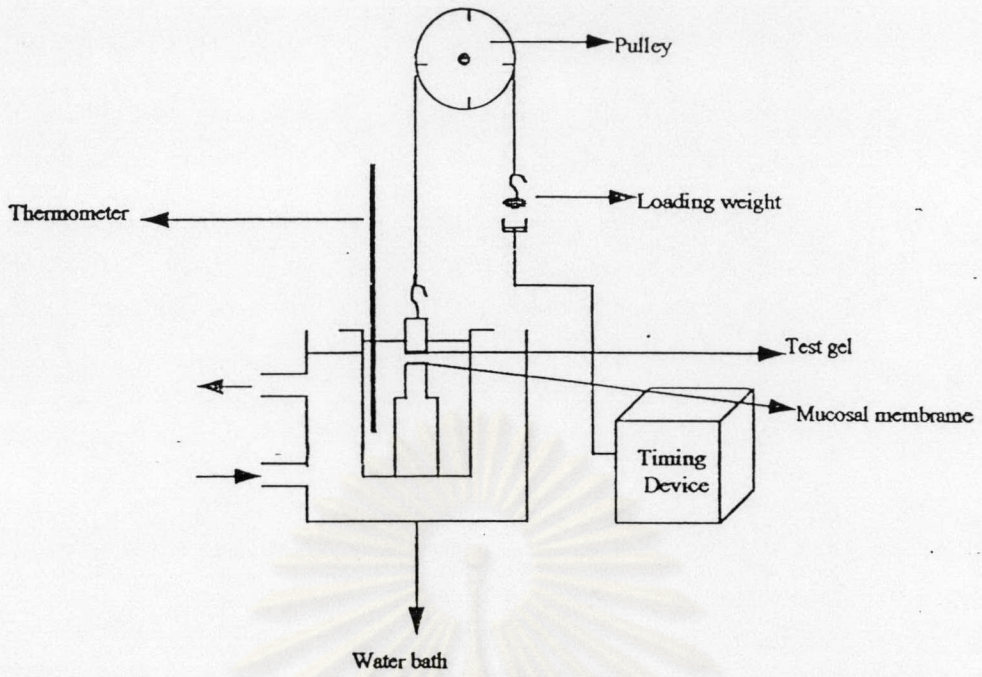

Figure 36 Schematic illustration of mucoadhesion tester apparatus (assembled in the laboratory).

4.6.2 Evaluation of mucoadhesive force

The test film (1.5 x 1.5 cm^2) was attached to a cylindrical weight (1.957g) which has a 6.2 mm diameter with cyanoacrylate adhesive. The cylindrical weight was then placed on platform in order to maintain a contact between the test film and the mucosal surface, giving a contact area of 0.3019 cm^2. After 2 min of contact time, the increment of applied force were achieved by placing loading weights on the balance pan at the other end of a cord that was attached, via a pulley to the hook of the cylindrical weight. When the attached film was completely pulled out, the detachment force which accomplished separation of the two surfaces was recorded and calculated in term of N / cm^2 from the following equation.

$$\text{Detachment force} = \frac{\text{loading weight at adhesion joint fail (g)} \times 0.0098}{\text{contact area } (cm^2)}$$

Each film formula was performed on at least five specimens and the mean force was calculated.

4.6.3 Evaluation of mucoadhesive time

From adhesive force determination, it was found that the minimum detachment force of the mucoadhesive films was 0.373 N (38.06g). Therefore, in the assessment of the duration of mucoadhesion, a constant 38 g of loading weight was placed on the balance pan and a timer mechanism was started. As soon as the adhesive joint failed, this balance pan with loading weight dropped onto a switch which automatically stopped the timing device (which recorded the elapsed time to 1 second). At least five specimens were subjected to the test for each film formula. Then the mean adhesion time was calculated.

5. Formulation of *Garcinia mangostana* extract buccal mucoadhesive films

From the evaluation of free films (physical, mechanical, swelling and mucoadhesive properties), it was found that MA_1, ML_1 and HC_2 had appropriate properties and were selected to use in the further formulation.

According to the preliminary release study of purified extract from the mucoadhesive film, the optimal concentration which reaches to MBC against *Staphylococcus aureus* ATCC 25923 was obtained from the dose of 400 times MIC in a specimen of 1.5 x 1.5 cm^2 size. This result was employed to determine the amount of purified extract in the formulation of *Garcinia mangostana* mucoadhesive film was determined from the basis mentioned above and the formulas were as follows :

5.1 Mucoadhesive layer

B1

HPMC : CP 934 8:2

Purified extract	13.7229	mg/plate(64.326 cm^2)
HPMC	0.8	% w/w
CP 934	0.2	% w/w
Ethanol	50	% w/w
Purified water q.s ad.	100	% w/w

R2

Chitosan medium molecular weight

Purified extract	13.7229	mg/plate (64.326 cm^2)
Chitosan	1	% w/w
Ethanol	50	% w/w
Acetic acid 1% w/w q.s ad.	100	% w/w

R3

Chitosan medium molecular weight

Purified extract	13.7229	mg/plate (64.326 cm^2)
Chitosan	1	% w/w
Ethanol	50	% w/w
Lactic acid 1% w/w q.s ad.	100	% w/w

Purified extract was obtained from recrystallization of ethyl acetate crude extract which presented mangostin (active compound) when examined and compared with mangostin standard solution in the identification process. The following procedure was employed for preparing *Garcinia mangostana* extract buccal mucoadhesive film. An aliquot to give 13.7229 mg of purified extract was pipetted from the stock solution into the ethanol portion of the formula and added to the polymer portion. Then the mixture was stirred using mechanical stirrer until clear solution was obtained using the process as described in 3.1.

5.2 Protective / Backing layer

R

Ethylcellulose		1	% w/v
Acetone	q.s ad.	100	% w/v

The method of preparing the backing layer was described in 3.2.

6. Determination of *Garcinia mangostana* extract mucoadhesive films

6.1 Determination of surface and cross section morphology

The surface and cross section morphology of *Garcinia mangostana* mucoadhesive film was observed by using a scanning electron microscope. The films were imaged with a 10 and 15 kV electron beam at an appropriate magnification.

6.2 Determination of X-ray diffraction

The X-ray diffraction patterns of *Garcinia mangostana* mucoadhesive film were examined using X-ray diffractometer (JEOL, Japan) with nickle-filtered Cu radiation generated at 30 kV and 30 mA as the X-ray source in the ω-2θ scanning mode between 4° and 40°.

6.3 Determination of water repellent property

This experiment was modified from the in vitro method developed by Gaserod et al.(1998) and Rao and Buri (1989) to evaluate mucoadhesive properties of polymer coated microparticles. The apparatus employed in this study is illustrated in Figure 37.

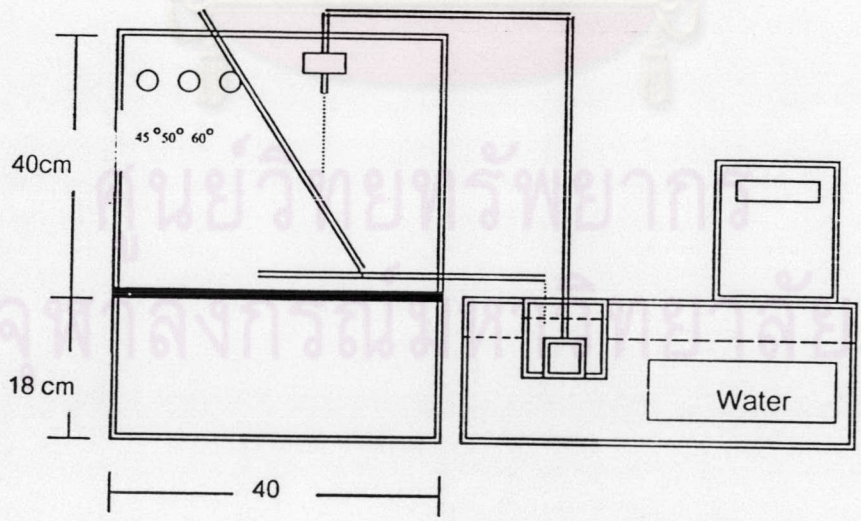

Figure 37 : Schematic illustration of water repellent tester apparatus (assembled in the laboratory).

6.3.1 The preparation of mucosal membrane

The middle sections, discarding the first 40-50 mm at either end, of fresh small intestine were removed from male Wistar rats (approximately 200g weight and 7-8 weeks old) and cut into 20 cm length. Then it was opened longitudinally and gently washed mucosal surface with pH 6.0 isotonic phosphate buffer to remove any loose material. The tissue was placed on a plastic plate (3 x 20 cm) with the mucosal surface facing up from the plate and attached to the plate with cyanoacrylale adhesives and stretched around all the four sides of the plate to make the tissue surface as planar as possible. Then the mucosal surface was wetted with artificial saliva.

6.3.2 Evaluation of water repellent and mucoadhesive property

The twelve specimens (1.5 x 1.5 cm^2); six bilayered films and six monolayered films of purified extract were placed on the tissue and pressed to maintain an intimate contact between test films and mucosal tissue for 2 min. Then the plate was fixed at middle of the slope in an angle of 60° relative to the horizontal plane. The artificial saliva, which was kept in a chamber at 37 °C was pumped and the flow was adjusted at a rate of 12 ml/min from the height of 12 cm above the deposited films. Then a timer machine was started. As soon as the adhesive joint failed and the film was washed off, adhesive duration of each film was recorded and the mean of each six specimens were calculated.

7. In vitro release study of *Garcinia mangostana* extract mucoadhesive films

7.1 The in vitro release study using modified Franz diffusion cells

The monolayer film was fixed with a plastic sheet and placed on the cellophane membrane (Cellu-Sep®), which was placed on a receiver chamber of the modified Franz diffusion cells with 14 ml receptor volume. The receiving compartment contained the solution of 35% v/v of ethanol in pH 6.0 isotonic phosphate buffer which was maintained at 37 °C by a circulating water jacker, which was connected to a constant temperature water bath. Uniform mixing of the receiving solution was

provided by magnetic stirrer at 300 ± 5 rpm through out the time of release study. Any air bubbles formed under the preparation had necessarily been removed before the experiment was started. Four specimens of each formulation were examined.

Samples of 5 ml were taken from the receiver medium at certain time intervals (10, 20, 30, 45, 60, 120, 240, 300, 360, 480, 600 and 720 min) via the side arm sampling port of diffusion cell. The entire receiving solution was removed using a syringe fitted with a piece of flexible tubing and the receiver compartment was immediately replaced with the same amount of medium and the run was continued.

All receiver solutions taken were analyzed using the UV spectrophotometer (Shimadzu, Japan) at 243 nm and the drug concentration was then determined from the calibration curve (Appendix C). The amount release was calculated by multiplying the drug concentration with the receiver volume.

7.2 Preparation of calibration curve UV spectrophotometry

A stock solution of mangostin was prepared by accurately weighed 12 mg of purified extract into 10 ml volumetric flask, then diluted and adjusted to volume with absolute ethanol. Then pipetted 5.0 ml of this standard stock solution into 50 ml volumetric flask diluted and adjusted to volume with 35%v/v ethanol in pH 6.0 isotonic phosphate buffer. This standard solution had a final concentration of 12 μg /ml. After pipetted 2, 3, 4, 5, 6, 7, 8 and 9 ml of this solution into 10 ml volumetric flask, diluted and adjusted to volume with the same solvent the standard solutions concentrations 2.4, 3.6, 4.8, 6.0, 7.2, 8.4 and 9.6 μg/ml respectively were obtained. Then the standard solutions were analyzed using the UV spectrophotometer at 243 mm. The standard curve of mangostin between concentration and absorbance was plotted.

8. Study and development of quantitative analysis method of mangostin in mucoadhesive films

8.1 HPLC method

From the preliminary study of mangostin assayed with UV spetrophotometer, the scanning spectra of mangostin in 50% v/v MeOH in water was obtained. From the

spectra, the maximum absorbance was found at the wavelength of 243 nm (Figure 38). Therefore, the detection of mangostin was perfomed at this wavelength.

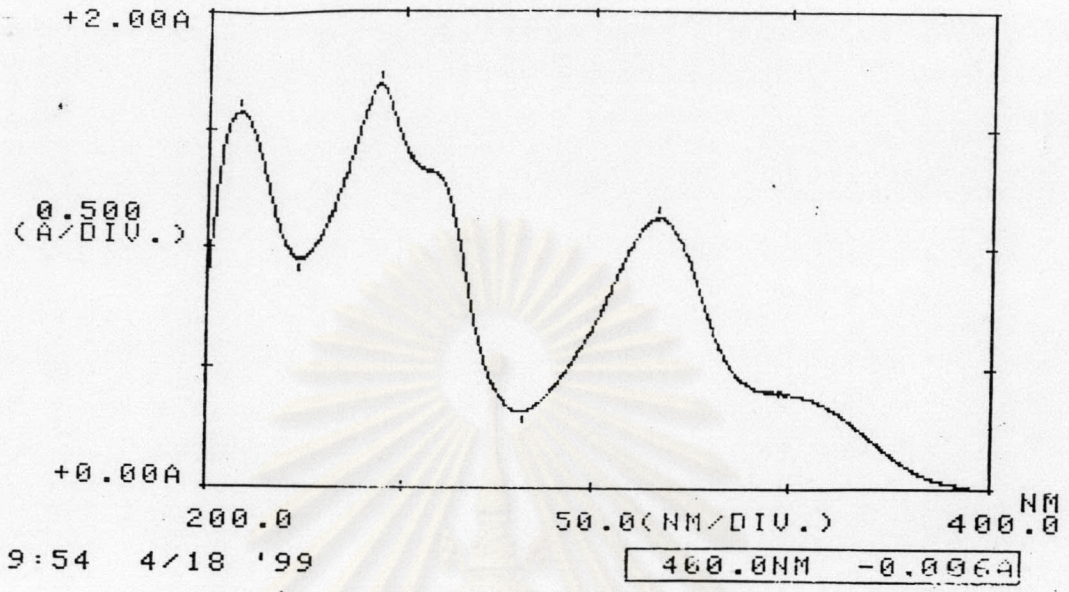

Figure 38 : Spectra of mangostin in 50%v/v MeOH in water.

In the preliminary of HPLC study clotrimazole solution (0.25 mg/ml) was used as an internal standard due to its appropriate retention time and optimal resolution from mangostin peak. The HPLC conditions for the analysis of mangostin were as follows :

Column	:	Hi-Q-Sil C18, 5μm, 4.5 x 150 mm
Mobile phase	:	MeOH : H_2O = 85 : 15
Flow rate	:	1 ml/min
Run time	:	15 min
Detector	:	UV detector
Wavelength	:	243 nm
Temperature	:	ambient
Internal standard	:	clotrimazole

8.2 Preparation of internal standard solutions

A stock solution of clotrimazole was prepared by accurately weighed 125 mg of clotrimazole into 100 ml volumetric flask, diluted and adjusted to volume with absolute ethanol. Then 5.0 ml of this solution was transferred to a 25 ml volumetric flask and diluted with 35 % v/v ethanol to volume to give 50% v/v ethanol. The final concentration of clotrimazole was 0.25 mg/ml.

8.3 Preparation of standard solutions

An accurate weight 25 mg amount of purified extract was placed into a 10 ml volumetric flask and diluted to volume with absolute ethanol. This stock solution had a final concentration of 2.5 mg/ml. Then 5.0 ml of this solution was transferred into a 100 ml volumetric flask and was diluted with 45% v/v ethanol to give a solution of 12.5 μg/ml of purified extract in 50% v/v ethanol. The above solution of 1.0 to 8.0 ml and 5.0 ml of stock internal standard solution were added into 25 ml volumetric flask. The dilution to volume with 50% v/v ethanol gave 5, 10, 15, 20, 25, 30, 35 and 40 μ g/ml of mangostin respectively, and 0.25 μg/ml clotrimazole. Three sets of standard solutions were prepared for each HPLC run. As a result, the standard curve of mangostin between concentration and peak area ratio was plotted.

8.4 Validation of the HPLC method

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

Preparation of standard solution for validation:

An accurately weighed 125 mg amount of purified extract was put into a 50 ml volumetric flask and diluted to volume with absolute ethanol to give 2.5 mg/ml concentration. This stock solution had a final concentration of 2.5 mg/ml. Then 5.0 ml of this solution was transferred into a 100 ml volumetric flask and was diluted with 45% v/v of ethanol to give a concentration of 12.5 μg/ml in 50% v/v of ethanol in water. The above solution of 1.0, 2.0, 4.0, 6.0, 8.0 and 5.0 ml of the stock internal standard were transferred into 25 ml volumetric flask and diluted to volume with 50% v/v ethanol to give the solution of 5, 10, 20, 30, 40 and 50 μg/ml mangostin and 0.25 mg/ml of clotrimazole, respectively. Three sets of standard solutions were prepared for each HPLC run.

8.4.1 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.

8.4.2 Precision

1) Within run precision

The within run precision was determined by analyzing three sets of the six standard solutions of purified extract in three intervals of time in the same day. Peak area ratios of mangostin to clotrimazole were compared and the percent coefficient of variation (%CV) for each concentration was determined.

2) Between run precision

The between run precision was determined by comparing each concentration of three sets of standard solutions prepared and injected on different days. The coefficients of variation (% CV) of mangostin to its internal standard peak area ratios from three sets of standard solutions having the same concentration were calculated.

8.4.3 Accuracy

Three sets of the eight standard solutions were prepared and injected. The percentage of analytical recovery of each standard solution was calculated.

8.4.4 Linearity

Eight standard solution were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed by the method of least square.

8.5 Quantitative analysis method

8.5.1 Purified extract standard solution preparation

An accurate weighed 25 mg amount of purified extract was placed into a 10 ml volumetric flask and diluted with absolute ethanol to volume in the same manner as described in 8.3. The final concentrations obtained were 5, 10, 15, 20, 25, 30, 35 and 40 μg/ml, respectively.

8.5.2 Assay of *Garcinia mangostana* extract mucoadhesive film

The film sample was cut into small pieces (1.5 x 1.5 cm^2) placed in a 125 ml Erlenmeyer flask. Then 12.5 ml of water was pipetted into the flask, closed with

Parafilm® and allowed the film to dissolve completely in a shaker bath for 2 hr. Then 5.0 ml of stock internal standard and 7.5 ml of ethanol were added into the flask, and the shaking was for 1 hr until the clear solution of 25 ml was obtained. The solution was filtered through 0.45 μm nylon membrane and was analyzed using the HPLC method.

9. Stability study of *Garcinia mangostana* extract buccal mucoadhesive film

The test preparations were stored in amber glass vials, which were tightly sealed with rubber closures and aluminium caps at 40 °C and 75% RH for three months. For maintaining the specified relative humidity in closed chambers, a saturated salt solution was used. The saturated solution of sodium chloride gives 74.7%RH at 40 °C (Nyqvist, 1983). In this study, the sodium chloride saturated solution was prepared at 60 °C and then equilibrated for 48 hours in a desiccator which was maintained at 40 °C until the water pressure over the system became constant. The temperature and relative humidity were examined using a digital hygrothermometer. Then the glass vials of the preparations were kept in the desiccator. The amount remaining of mangostin and percent labeled amount were determined in triplicate at the initial, 1, 2 and 3 month periods. The analysis of mangostin in film samples followed the HPLC method described in 8.5.2. Triplicate samples of each test preparation were randomly drawn and analyzed compared with purified extract solution.