CHAPTER IV
RESULTS AND DISCUSSIONS

1. Extraction, isolation identification and solubility determination of mangostin

1.1 Extraction

The crude extract of *Garcinia mangostana* Linn. was obtained by macerating the dried fruit rind powder in a percolator with hexane then the marc was extract with ethyl acetate. The ethyl acetate extract was brownish viscous liquid with the yeild of 310.86 g. The extract was further evaporated and recrystallized in ethyl acetate/hexane (3:1) the yellow bright needle shaped crystalline powder of mangostin were obtained the photograph and photomicrographs of mangostin are shown in Figure39.

1.2 Isolation

A portion of the ethyl acetate extract was further isolated and purified using quick column chromatography and eluted with ethyl acetate/hexane (0-25%). Each fraction was monitored by TLC and detected under UV light at 254 nm, then the fraction giving the same TLC characteristic were combined. The fractions eluted with 20% ethyl acetate/hexane were combined and were evaporated. The yellow needle crystalline powders of mangostin were obtained.

1.3 Identification

The identification of mangostin crystals by Nuclear Magnetic Resonance showed that the spectra (Appendix B) were identical and conformed with the structure of mangostin that reported by Mahabusarakam and Wiriyachitra (1987).

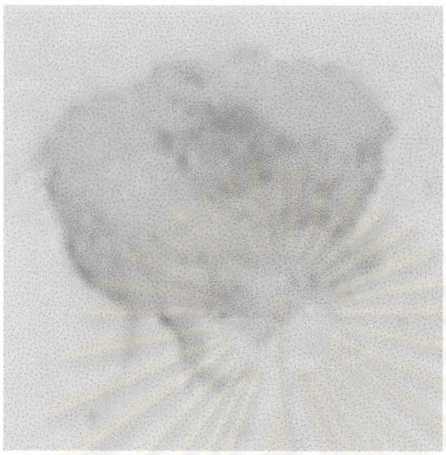
The melting point of mangostin crystalline was obtained by Gallenkamp melting point apparatus was in the narrow range of 180.9-182.0 °C and conformed with the melting point of mangostin (181.6-182.6 °C) (Budavari ed, 1996).

In addition, the molecular weight of mangostin crystals was obtained from mass spectrometry (Appendix B) was 410 nearly equal to the molecular weight of mangostin (410.47) (Budavari ed, 1996).

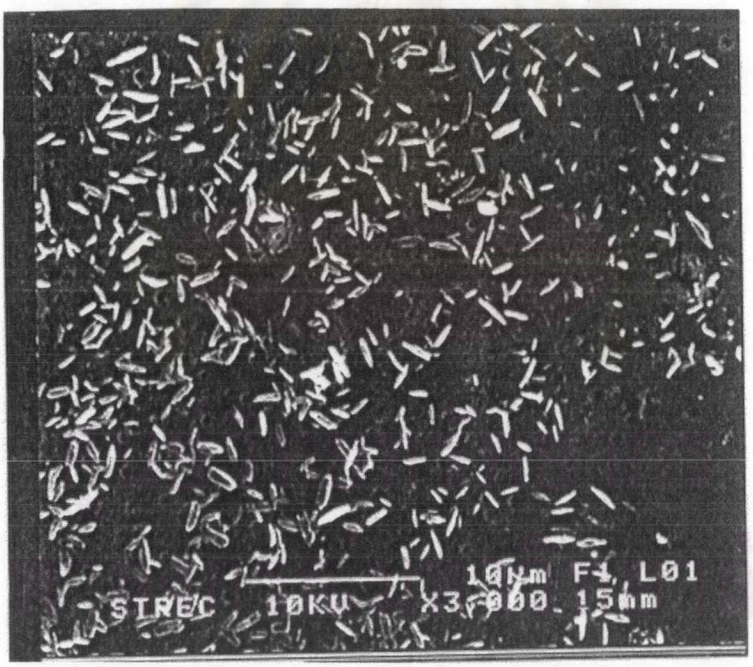
All data from the identification of the extracted compound contributed that the yellow needle crystalline powders obtained from isolated fractions were mangostin.

1.4 Solubility

The solubility of purified extract (mangostin) in deionized water and pH 6.0 isotonic phosphate buffer at 37°C were 0.028 ± 0.0045 mg/ml and 0.020 ± 0.0031 mg/ml, respectively. The solubility of purified extract in the present of 35% v/v ethanol was 1.0225 ± 0.238 mg/ml. The solubility values expressed were the mean ± SD of four determinations. The increase in the solubility of purified extract was observed on adding of absolute ethanol as co solvent to the medium and it was closely related to the amount of co solvent.



a



b

Figure 39: Photograph and photomicrograph of mangostin.

2. Antimicrobial activities of purified extract from *Garcinia mangostana* Linn.

2.1 Disk diffusion method

The paper disks containing purified extract of 12.5, 25, 50 and 100 μg/disk were placed on the surface of agar plates, which were inoculated with *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate). After incubation, the diameters of the inhibition zones were examined (Appendix B). The results were presented as the minimum amount of the purified extract in the disk that produced the inhibition zone (Table 4).

Table 4 The minimum amount of purified extract which presented antimicrobial activity on various types of bacteria.

Types of bacteria	Minimum amount of purified extract (μg/disk)
Staphylococcus aureus ATCC 25923	50
Streptococcus mutans ATCC $KPSK_2$	25
Streptococcus sanguis (a clinical isolate)	25

It was found that the purified extract had a higher activity against *Streptococcus mutans* ATCC $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate) than *Staphylococcus aureus* ATCC 25923.

2.2 Broth dilution method

The preliminary experiment was performed with a. series of purified extract having the concentration range of 256-0.063 μg/ml. After incubation the MIC was determined visually as the lowest concentration that inhibited bacterial growth, which was demonstrated by the absence of turbidity. The result was found to be 1 μg/ml for *Staphylococcus aureus* ATCC 25923 and 0.5 μg/ml for *Streptococcus mutans* ATCC $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate).

Then the experiment for studying MIC and MBC was intensively carried out with the concentration ranged 307.2-0.075 μg/ml for *Staphylococcus aureus* ATCC 25923 and 192-0.09375 μg/ml for *Streptococcus mutans* ATCC $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate). The study was performed in triplicate. The results including MIC, MBC of the purified extract and inhibitory effect of ethanol are presented in Table 5.

Table 5 The MIC and MBC of purified extract and minimum concentration of ethanol with inhibitory effect on various types of bacteria.

Types of bacteria	MIC (μg/ml)	MBC (μg/ml)	MIC of 95% ETOH (%v/v)
Staphylococcus aureus ATCC 25923	1.2	4.8	0.7813
Streptococcus mutans ATCC $KPSK_2$	0.75	3.0	1.5625
Streptococcus sanguis (a clinical isolate)	0.75	3.0	1.5625

Due to the lower MIC and MBC value, it was found that *Garcinia mangostana* extract had a higher activity against *Streptococcus mutans* ATCC $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate) than *Staphylococcus aureus* ATCC 25923.

Since, the minimum concentration of 95% ethanol that showed the inhibitory effect was found to be 0.7813 %v/v against *Staphylococcus aureus* ATCC 25923 and 1.5625 %v/v against *Streptococcus mutans* ATCC $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate) (Appendix B), whereas, the concentration of 95% ethanol in the system was as low as 0.3906 % v/v for *Staphylococcus aureus* ATCC 25923 and 0.1953 % v/v for *Streptococcus mutans* ATCC $KPSK_2$ and *Streptococcus sanguis* (a clinical isolate), thus, the solvent had no inhibitory effect and did not affect the MIC and MBC results.

3. Preparation of mucodhesive free films

3.1 Preparation of cellulose derivatives free films

From the preliminary studies, appropriate type and concentration of polymers were investigated to obtain buccal films with satisfactory appearance, good integrity,

and which were easily detached from the plate after drying with flexibility and no brittleness or breakage. Consequently, six formulations of the mucoadhesive polymers were employed for the study including SCMC, HPMC and either SCMC and HPMC in combination with 10 and 20% w/w of CP 934. The higher concentration of CP 934 over 20% w/w clearly produced more brittleness and less integrity while the film containing only CP 934 gave unsatisfactory film characteristics, brittleness and easy breakage.

3.2 Preparation of chitosan free films

3.2.1 Evaluation of chitosan solution

The physical properties of 1% chitosan solutions were presented in Table 6.

Table 6 Physical properties of chitosan solutions.

Formulas	Yellowness[a]	Transparency[b]	Mean pH	Mean viscosity (m Pas)
LA1	++	+	3.98	124.30
LA2	++	+	3.27	143.84
LL1	++	+	2.96	155.29
LL2	++	++	2.59	167.65
MA1	+	+++	3.94	325.19
MA2	+	+++	3.33	354.98
ML1	+	+++	2.93	435.32
ML2	+	+++	2.63	467.97
HA1	-	+++	3.96	420.92
HA2	-	+++	3.20	454.77
HL1	-	+++	2.99	716.35
HL2	-	+++	2.65	746.64

[a] yellowness - means colorless; + means yellowish; ++ means pale yellow

[b] transparency + means almost clear; ++ means clear; ++ means transparent

All Chitosan solutions were clear and viscous. The viscosity varied in a wide range depending on the molecular weight of chitosan. The higher molecular weight, the higher viscosity resulted. The viscosity also depended on type and concentration

of the solubilizing acid. Lactic acid produced more viscous solution than acetic acid. Additionally, it was found that the chitosan solution prepared with 2% acid had a higher viscosity than that with 1% acid.

It was obvious that the viscosity of chitosan solutions is a function of type and concentration of acid. This finding agrees with previous studies by Begin ,and Calsteren (1999) and Leffler ,and Muller (2000).

The reason was that in acid medium, the protonation of amino groups by hydrogen ion increased charge density along the molecular chain, causing the chain to unfold and elongated the conformation. Moreover, the protonated groups were reactive with water molecule, therefore, the dissolution of chitosan occurred (Lim, and Wan, 1995).

Regarding the effect of type of acid, it was possible that the various counter ions caused the difference in the interaction between counter ions and chitosan molecules. Consequently, the viscosity of chitosan solution was different (Begin, and Calsteren, 1999).

3.2.2 Rheology study of chitosan solutions

As the viscosity of chitosan solutions in 1% w/w acetic and lactic acid was determined at varied shear rates the rheograms of chitosans MMW solution were plotted as depicted in Figures 40 and 41. It was found that the rheograms of the 1% chitosan solution in 1% acetic and 1% lactic acid followed Newtonian flow.

4. Evaluation of mucoadhesive layer free films

4.1 Physical properties

The physical properties of films including color, transparency, flexibility glossiness, stickiness, integrity, ease of detachment and mean thickness are illustrated in Table 7.

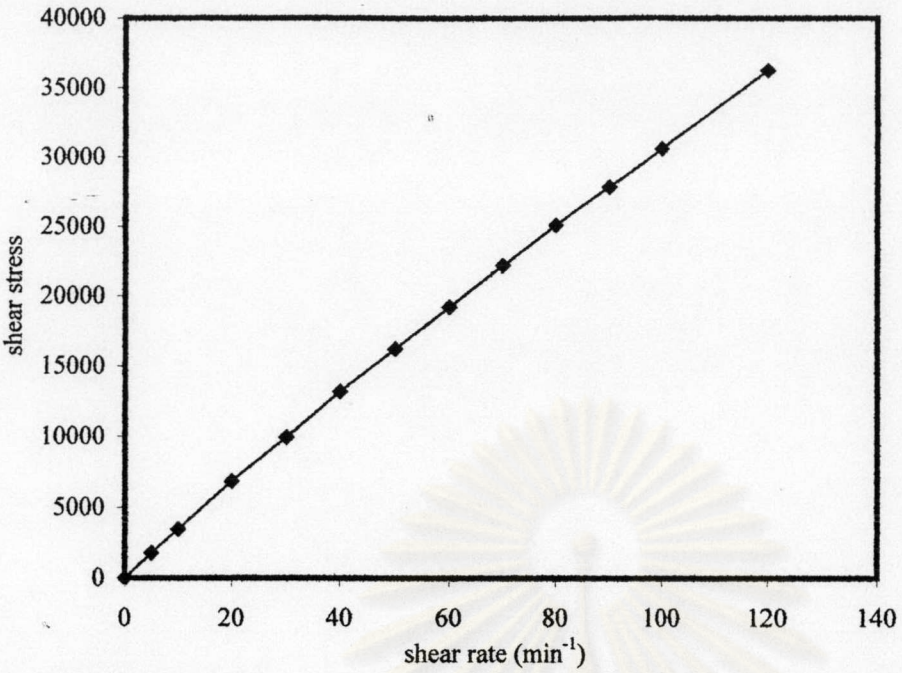


Figure 40 The rheogram of chitosan medium molecular weight solution prepared from 1% acetic acid.

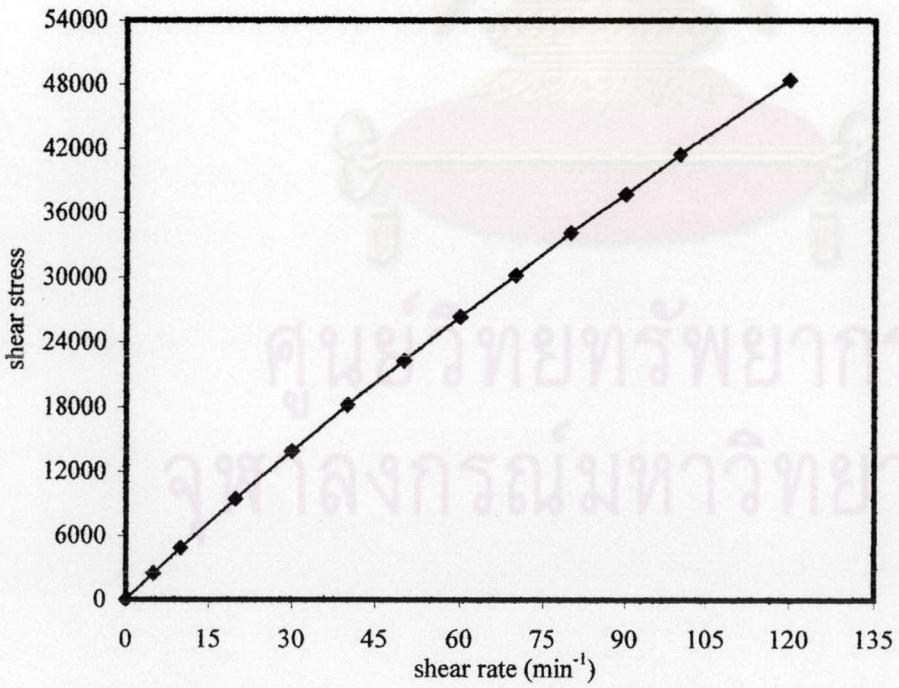


Figure 41 The rheogram of chitosan medium molecular weight solution. prepared from 1% lactic acid.

Table 7 Physical properties of free films.

Formulas	yellowness	transparency	Flexibility	Glossiness	Stickiness	Integrity	Detachability	Mean Thickness (μm)
S0	-	+	-	++	-	++	++	65.67
SC1	-	+	-	++	-	++	++	65.20
SC2	-	+	-	++	-	++	++	66.67
H0	-	+++	+	+++	-	+++	+++	66.53
HC1	-	+++	+	+++	-	+++	+++	66.60
HC2	-	+++	+	+++	-	+++	++	67.73
LA1	++	+++	+	+++	-	+++	+++	66.13
LA2	++	+++	+	+++	-	+++	+++	67.13
LL1	++	+++	++	+++	+	+++	+++	66.80
LL2	+++	++	+++	+	++	++	++	71.67
MA1	+	+++	+	+++	-	+++	+++	65.13
MA2	+	+++	+	+++	-	+++	+++	63.67
ML1	+	+++	++	++	+	++	+++	67.93
ML2	++	++	++++	+	+++	++	+	73.27
HA1	-	+++	+	+++	-	+++	+++	65.13
HA2	-	+++	+	+++	-	+++	+++	65.73
HL1	-	+++	+++	++	++	++	++	67.53
HL2	++	++	++++	+	+++	++	+	71.80

The symbols of (+) and (-) means the appearance and no appearance respectively, and the number of symbols of (+) means a degree of the appearance of the specified property.

Most of free films were transparent whereas S0, SC1 and SC2 were relatively translucent. Chitosan acetate films presented higher degree of transparency, glossiness and ease of detachment than chitosan lactate films. In contrast, chitosan lactate films exhibited higer degree of flexibility, stickiness and breakage than chitosan acetate films. The highest degrees of flexiblity, stickiness, breakage and yellowness were found in chitosan lactate films which prepared from 2% lactic acid.

The characteristics of hard and slight flexiblity were found in H0, HC1, HC2 and chitosan acetate films, while soft and highly flexible ones were found in chitosan lactate films. This maybe due to the plasticizing effect of lactic acid. The free films of S0, SC1 and SC2 were hard and brittle films.

The inclusion of CP934 did not produce the marked differences in the above physical properties in comparison with single films of SCMC and HPMC.

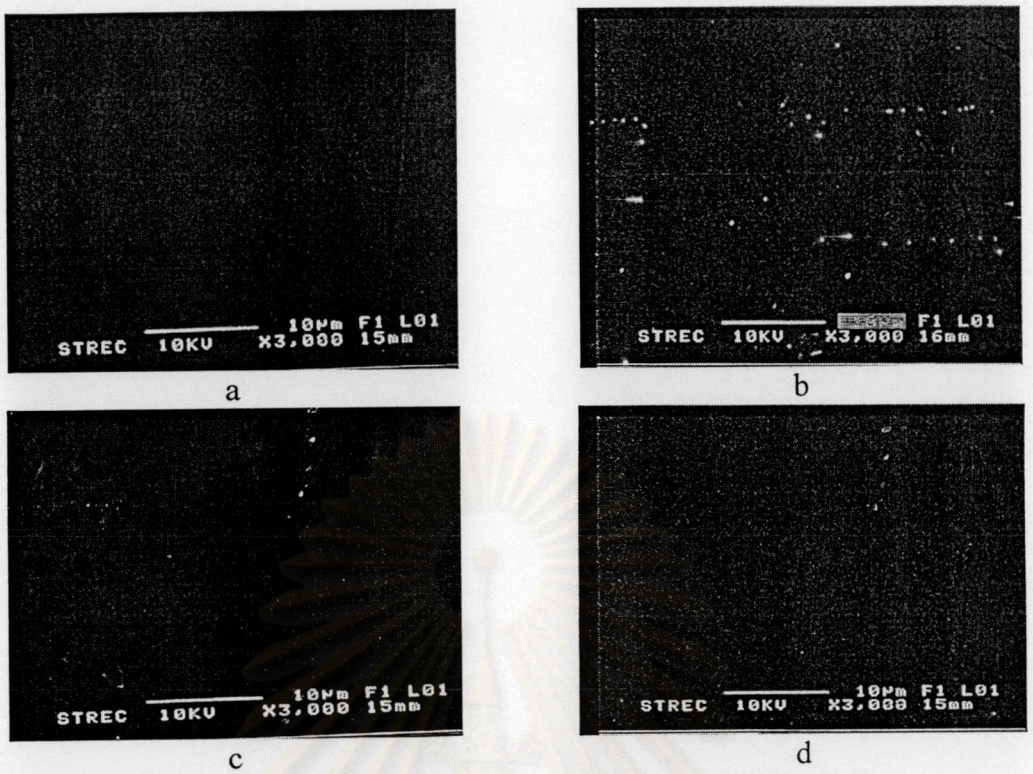


Figure 42 The photomicrographs of surface morphology of chitosan low molecular weight films prepared from 1 and 2 % acid.
(a) 1 % acetic acid (b) 2 % acetic acid (c) 1 % lactic acid (d) 2 % lactic acid

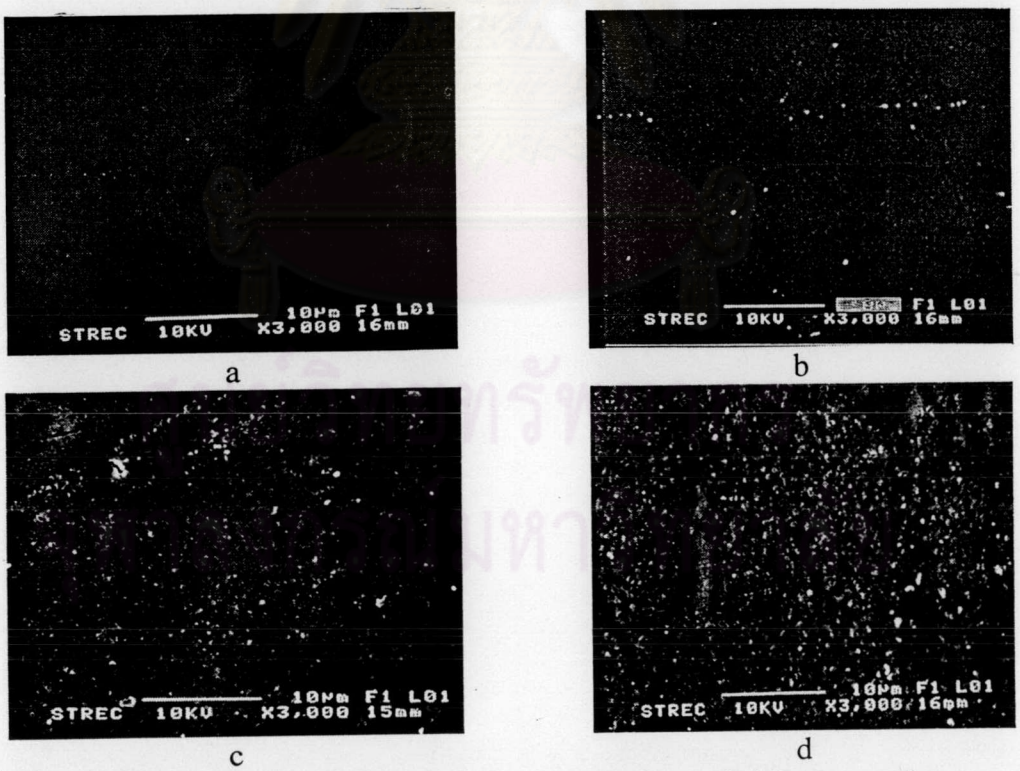


Figure 43 The photomicrographs of surface morphology of chitosan medium molecular weight films prepared from 1 and 2 % acid.
(a) 1 % acetic acid (b) 2 % acetic acid (c) 1 % lactic acid (d) 2 % lactic acid

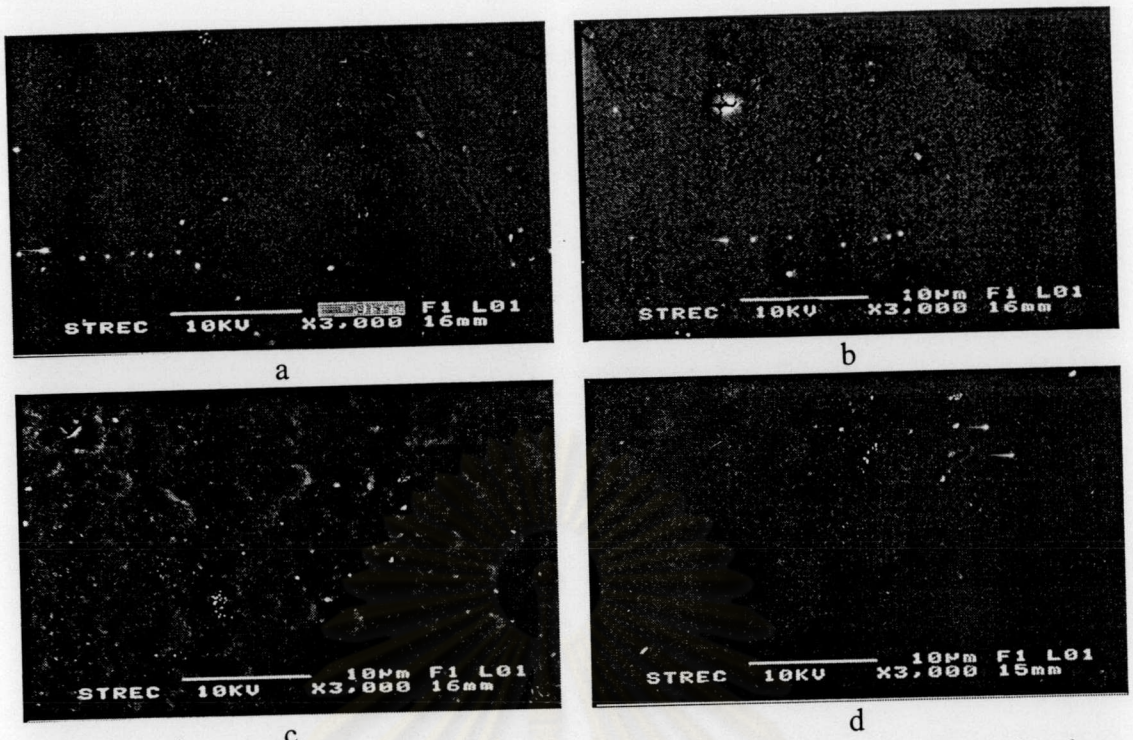


Figure 44 The photomicrographs of surface morphology of chitosan high molecular weight films prepared from 1 and 2 % acid. .
(a) 1 % acetic acid (b) 2 % acetic acid (c) 1 % lactic acid (d) 2 % lactic acid

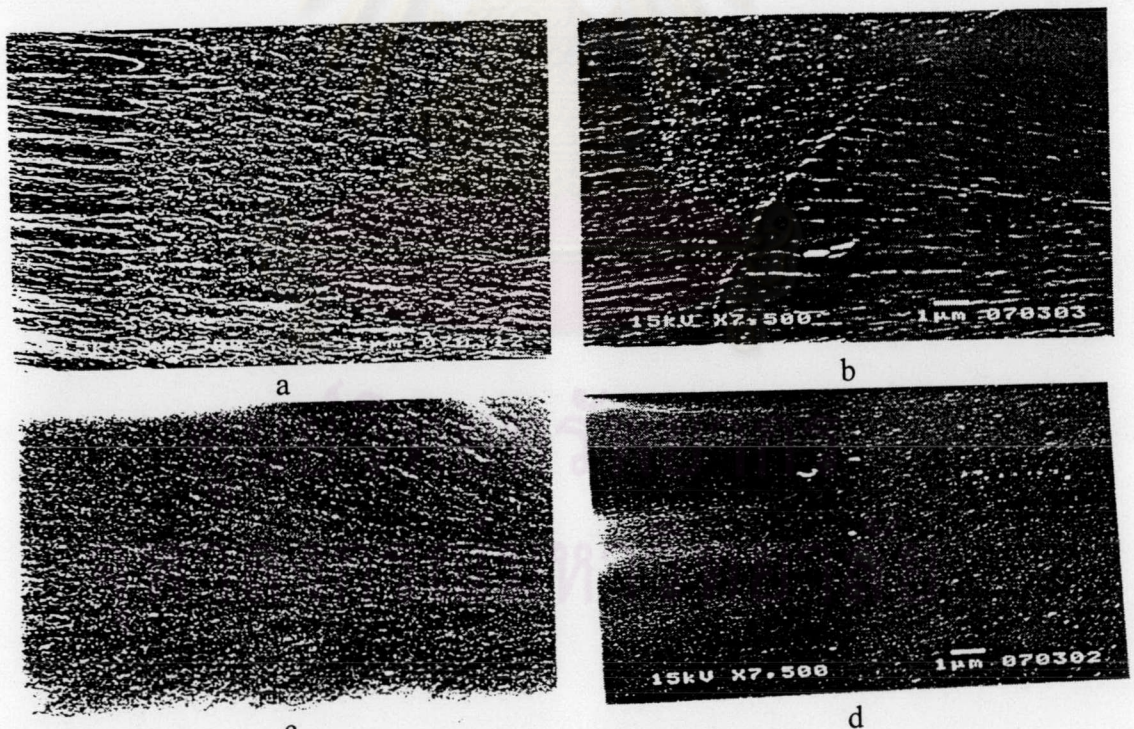
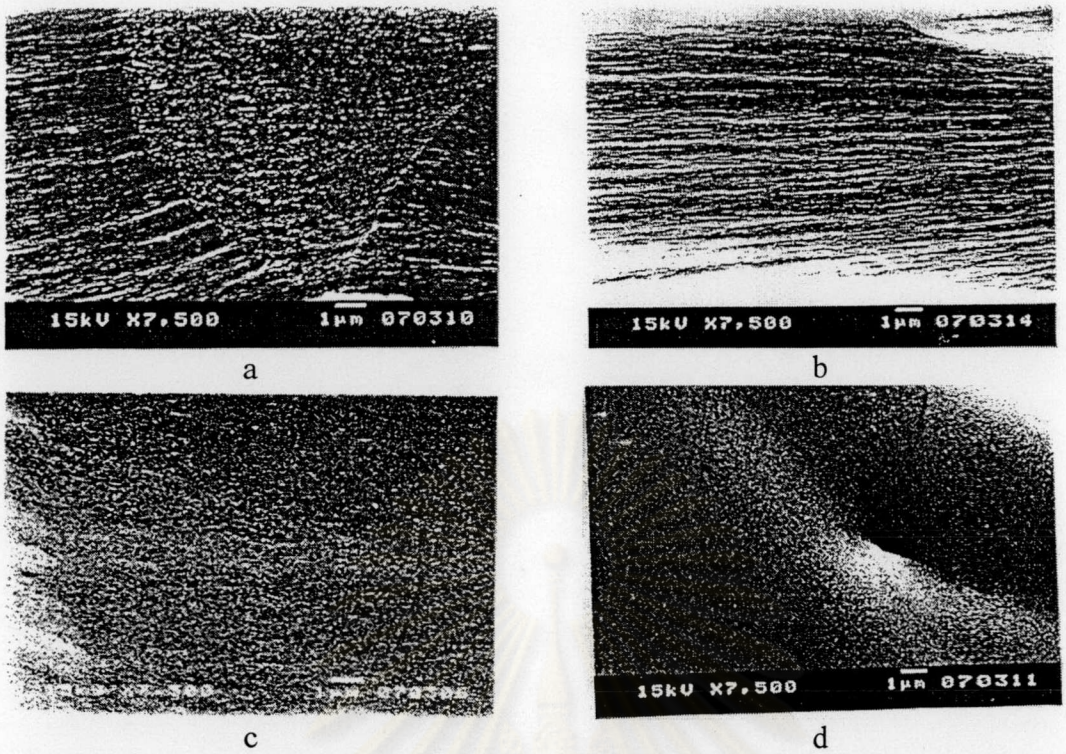
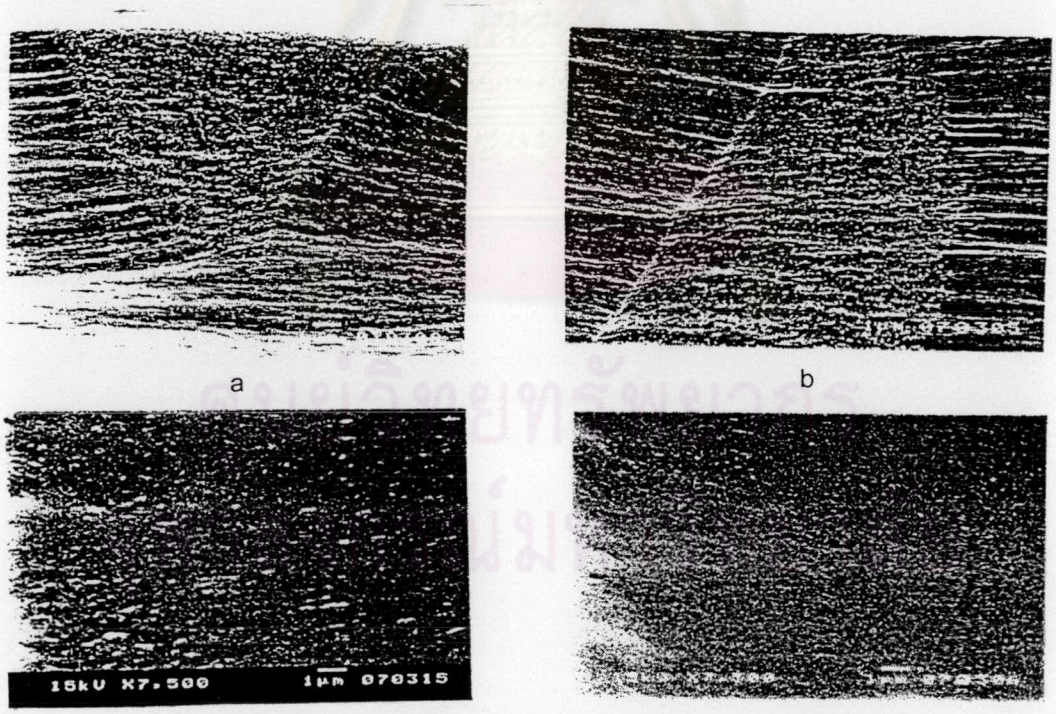


Figure 45 The Photomicrographs of cross section of chitosan low molecular weight films prepared from 1 and 2 % acid.
(a) 1 % acetic acid (b) 2 % acetic acid (c) 1 % lactic acid (d) 2 % lactic acid



a b c d

Figure 46 The Photomicrographs of cross section of chitosan medium molecular weight films prepared from 1 and 2 % acid.
(a) 1 % acetic acid (b) 2 % acetic acid (c) 1 % lactic acid (d) 2 % lactic acid



a b c d

Figure 47 The Photomicrographs of cross section of chitosan high molecular weight films prepared from 1 and 2 % acid.
(a) 1 % acetic acid (b) 2 % acetic acid (c) 1 % lactic acid (d) 2 % lactic acid

4.3 Mechanical properties

An ideal buccal film should be flexible, elastic, durable and adequately strong to withstand breakage due to stress from oral activities. Consequently, mechanical properties are critical and essential to be evaluated.

The ultimate tensile strength, elongation at break point are the parameters commonly used to define mechanical properties and demonstrate tensile characteristic of films. For mechanical propertied evaluation, the free films with uniformity of thickness were selected. The ultimate tensile strength and percentage of elongation at break of prepared films are illustrated in Tables 8-9 and Figures 48-53.

From the ultimate tensile strength data, SCMC films containing 20% of CP 934 (SC2) exhibited the highest value, whereas chitosan (LMW) films with 2% lactic acid (LL2) presented the lowest. The maximum percent elongation at break was obtained from chitosan (MMW) films with 2% lactic acid (ML2), while SCMC films without CP 934 showed the minimum value.

SCMC film was hard and brittle with moderate tensile strength and low elongation. It was found that increasing the content of carbopol tended to increase the tensile strength and elongation as illustrated in Figures 48 and 49. This result might be due to the interaction between SCMC and carbopol molecules in the film and made a contribution to enhance the strength and flexibility of films.

On the contrary, HPMC film was hard and strong with high tensile strength and moderate elongation. Increasing in carbopol content was observed to produce the reduction in tensile strength and elongation as illustrated in Figures 50.and 51 This was in agreement with a previous study of Peh and Wong (1999) who found that carbopol reduced the strength and flexibility of HPMC film. It is probable that the crosslinking between carbopol and HPMC which may related to its conformation and configuration made HPMC film less flexible and weaker.

For chitosan films, increasing in the molecular weight of chitosan caused a raising of tensile strength in both acetate and lactate film (Figures 52-53). This agreed

with a previous study by Nunthanid et al. (2001) who found that the tensile strength increased with the increasing molecular weight of chitosan. The reason may be high molecular weight chitosan can produces a considerable entanglement network which is important for applicable tensile strength. A reverse order was obtained from elongation data of chitosan acetate which indicated that the increasing in molecular weight decreased the stretching of chitosan acetate films. This may be due to the increasing of inter and intramolecular bondings of each chitosan chain which, resulted in the films having more ability to withstand higher stress but less flexibility. Concerning chitosan lactate film, the highest elongation was obtained from MMW chitosan followed by LMW and HMW.

Comparison of chitosan acetate films with chitosan lactate films, chitosan acetate films were hard and brittle with high tensile strength and moderate elongation, whereas, the latters were soft and ductile with low tensile strength and high elongation. It was found that chitosan acetate film showed a considerable higher tensile strength but comparatively lower elongation than chitosan lactate films. This is consistent to their appearance that chitosan lactate films are more flexible and softer than chitosan acetate film. Similar results were obtained from previous studies of Begin, and Calsteren (1999) and Peh, Khan, and Ch'ng (2000). The reason was that the tensile strength and elongation of each salt film were different depending on the difference in counter ions from each type of acid. Moreover, the structure of the counter ions could influence the intramolecular and intermolecular interactions which related to mechanical properties of film (Begin ,and Calsteren, 1999).

There was no significant difference in the ultimate tensile strength and percent elongation at break between chitosan acetate prepared from 1 and 2 % acetic acid ($p < 0.05$, Appendix E). However, the percent elongation at break of 1% acetate films were slightly higher than 2% films.

Regarding chitosan lactate films, it was found that LL1, ML1 and HL1 produced significantly higher tensile strength than LL2, ML2 and HL2, respectively ($p < 0.05$, Appendix E). A reverse order was obtained from elongation data of chitosan lactate films, percent elongation at break of 2% lactate films were marked higher than 1% films. This result might be due to the difference in ionic strength from two concentrations of acid. Increasing the ionic strength resulted in a higher association

Table 8 The rank order of ultimate tensile strength of prepared films.

Formulas	ultimate tensile strength (N/mm2)		
	Mean*	SD	%CV
SC2	68.35	1.33	1.95
H0	60.42	1.11	1.84
HA1	57.80	0.83	1.44
SC1	56.84	1.41	2.48
HA2	56.08	0.52	0.93
HC2	53.68	1.49	2.77
MA1	51.29	0.41	0.81
HC1	49.75	1.51	3.04
MA2	49.10	0.38	0.77
LA1	43.59	0.78	1.79
S0	41.12	2.20	5.34
LA2	40.93	1.58	3.87
HL1	31.55	0.71	2.26
ML1	20.37	0.30	1.46
HL2	10.87	0.26	2.35
LL1	8.35	0.47	5.59
ML2	7.02	0.16	2.25
LL2	3.59	0.12	3.37

*averaged from 5 studies

Table 9 The rank order of percentage of elongation at break of prepared films.

Formulas	Percentage of elongation at break		
	Mean*	SD	%CV
ML2	35.997	4.218	1.17
LL2	32.615	5.616	1.72
HL2	29.967	7.568	2.53
ML1	26.939	2.915	1.08
LL1	24.091	5.104	2.12
HL1	18.792	3.711	1.97
H0	11.784	1.160	0.98
LA1	7.630	0.947	1.24
LA2	7.465	0.939	1.26
MA1	5.697	1.775	3.12
HC1	5.622	0.687	1.22
MA2	4.859	1.191	2.45
HC2	3.527	0.674	1.91
HA1	2.598	0.856	3.29
SC1	2.590	0.590	2.28
HA2	2.510	0.719	2.86
SC2	2.092	0.694	3.32
S0	1.442	0.355	2.46

*averaged from 5 studies

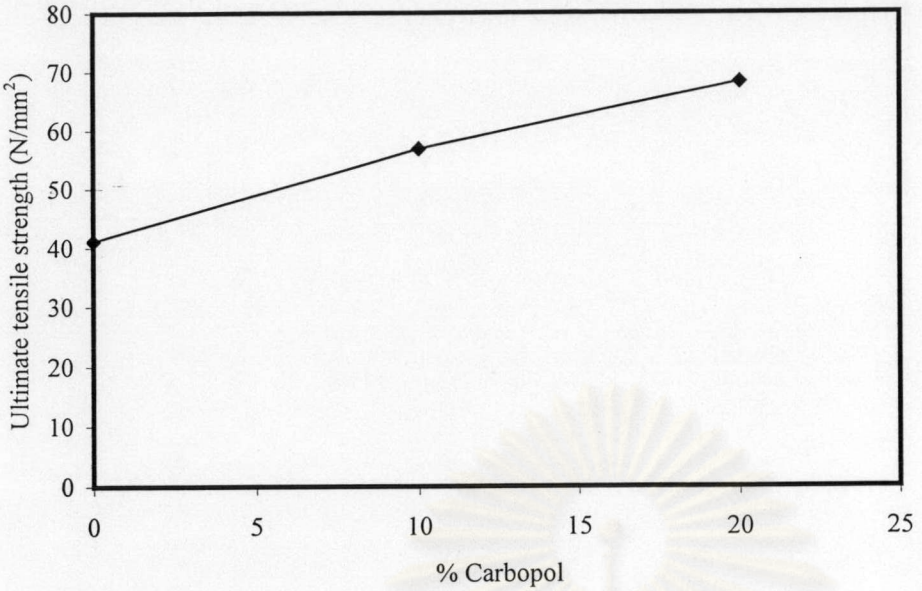


Figure 48 The ultimate tensile strength of SCMC films combined with varied concentrations of CP934.

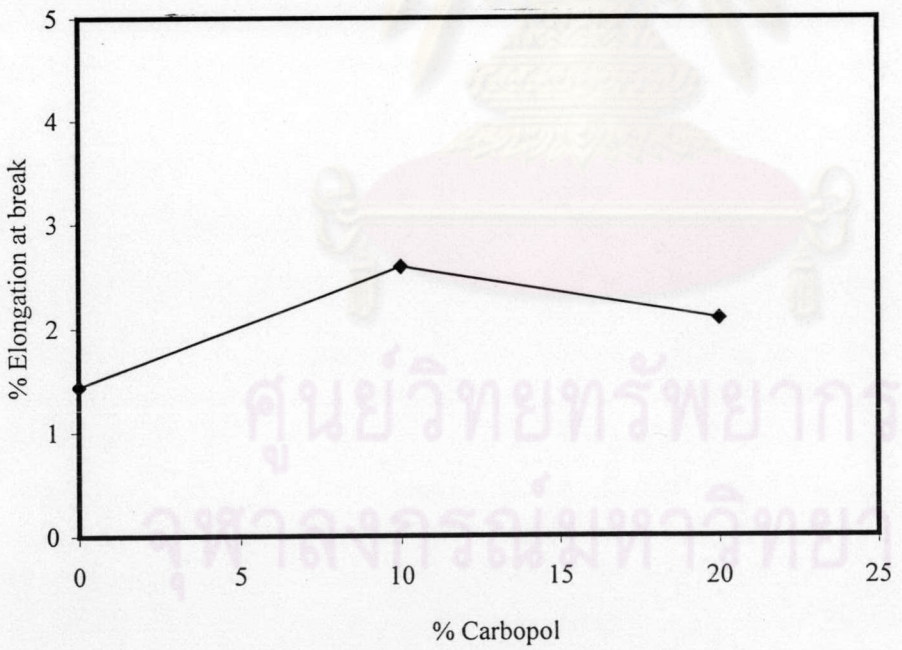


Figure 49 Percentage elongation at break of SCMC films combined with varied concentrations of CP934.

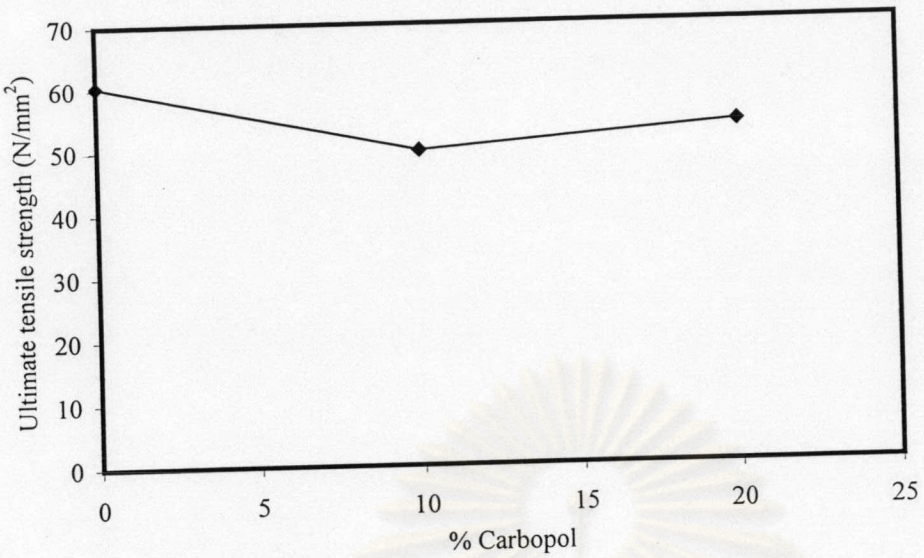


Figure 50 The ultimate tensile strength of HPMC films combined with varied concentration of CP 934.

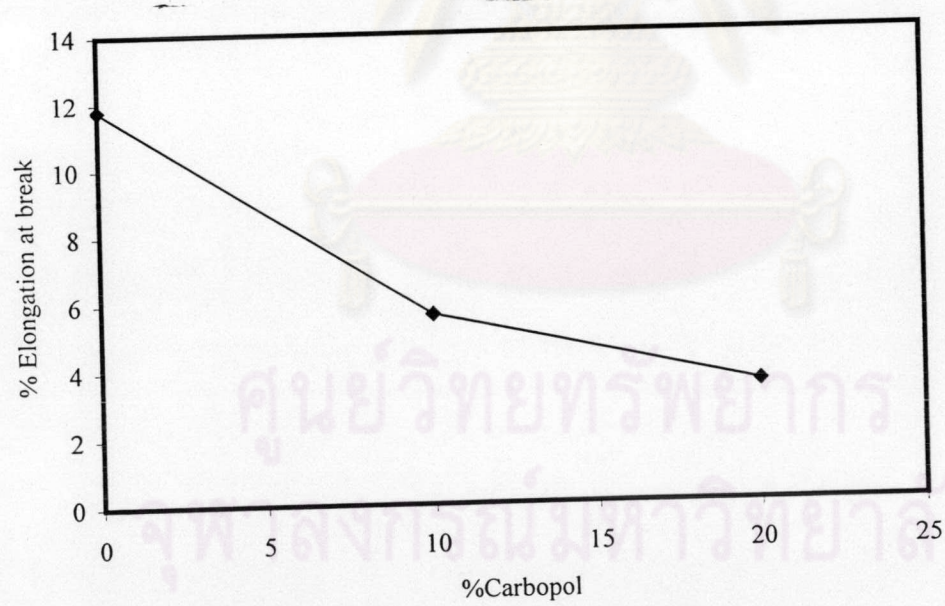


Figure 51 Percentage elongation at break of HPMC films with varied concentration of CP934.

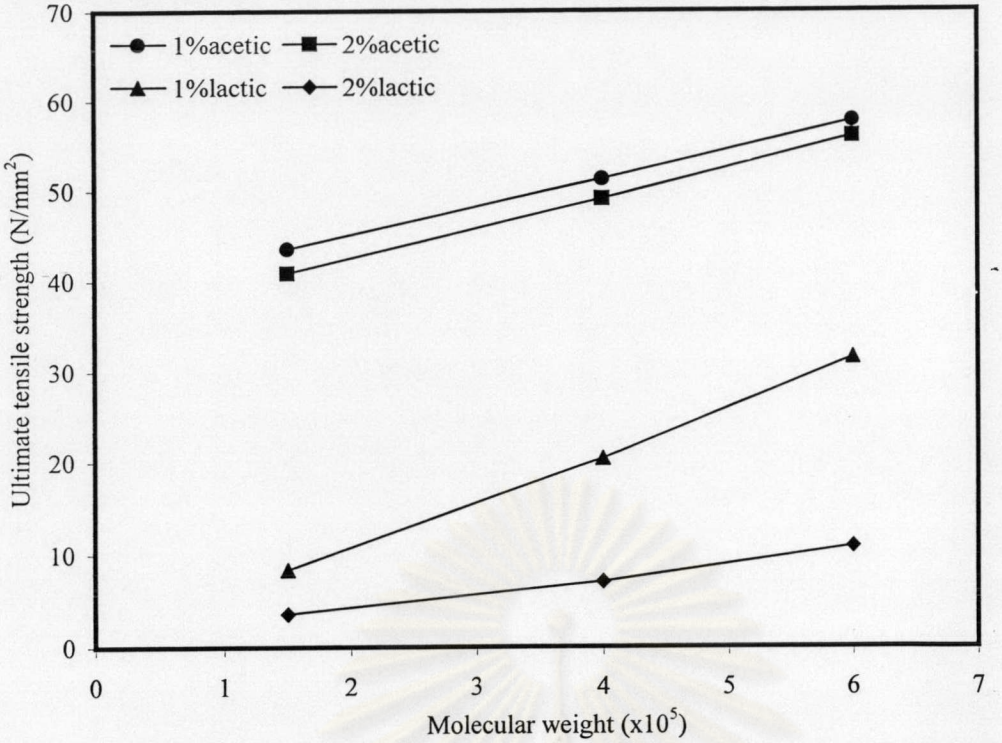


Figure 52 The ultimate tensile strength of chitosan films of different molecular weight with 1 and 2% of acetic acid and lactic acid.

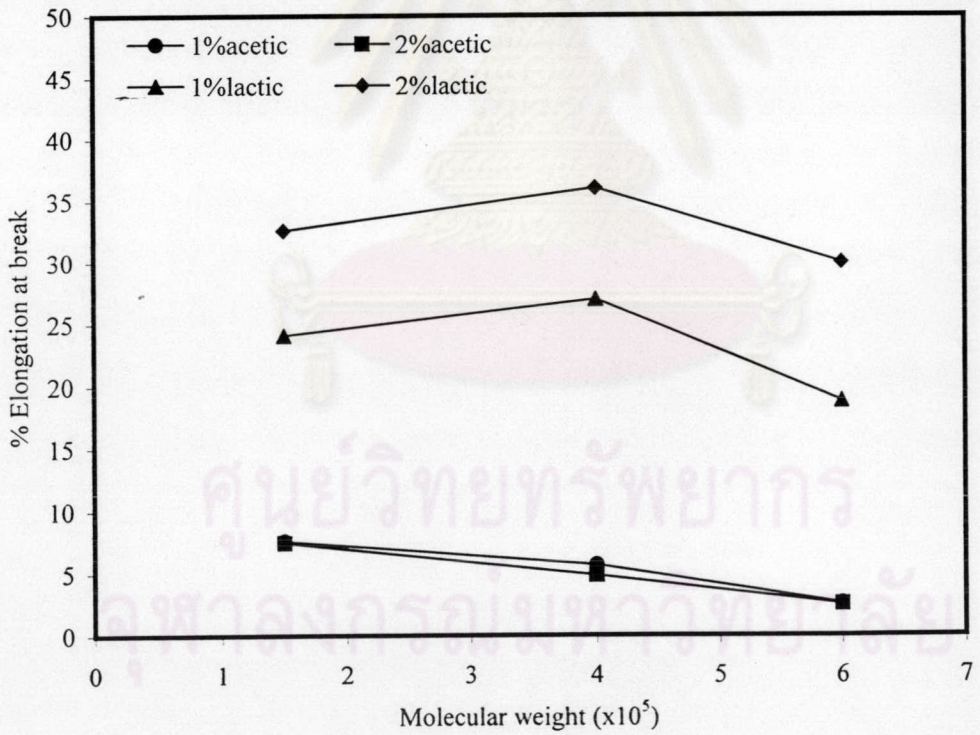


Figure 53 Elengation at break of chitosan films of different molecular weights with 1 and 2 % of acetic acid and lactic acid .

between the polyelectrolyte and the counter ions. Moreover, an increase in the shielding effect of the counter ions might caused the interfering effect on crystalite formation, which related to mechanical properties of chitosan films (Begin, and Calsteren, 1999).

These observations obviously showed that type and concentration of acid had an influence on the mechanical properties of chitosan films.

When comparing 18 formulations of mucoadhesive films by one way analysis of variance, significant diffrences ($P < 0.05$) are observed in the ultimate tensile strength as illustrated in Table 1E; VR (1967.20) > F(1.79) and percent elongation at break as shown in Table 2E.; VR(7834.55) > F(1.79). Then, the average tensile strength and percent elongation were compared by John tukey honestly significant diffrence (HSD) and the result are presented in Table 3E and 4E.

However, it was obvious that the addition of CP 934 in two different films, SCMC and HPMC, improved differently the mechanical properties of films both in tensile strength and elongation. For SCMC, 20% CP934 increased the tensile strength upto 68 N/mm^2 and elongation to 2.1% ; whereas for HPMC, it slightly decreased the tensile strength to 54 N/mm^2 but reduced the elongation from 11.8% to 3.5%.

4.4 Swelling property of free films

Swelling of films is the hydration of the polymer chain due to absorption of water. The hydration is an importance parameter in mucoadhesion due to encouraging entanglement and mobility of polymer chain which contribute the intimate contact for orientation of adhesive sites for interpenetration and bond formation.

After immersed in a medium (deionized water or artificial saliva) for at least one hour, all membranes could be classified into two groups. The first group, which the swelling index value could not be evaluated due to the rupture or unsatisfactory appearance of the films into small pieces of gel and the difficulty in retrieving for weight measurements. This group included S0, H0, HC1 and HC2, all of which were regarded as very hydrophilic polymers. It was possible that these films had lower

water resistance and higher ability to dissolve in water. The lower water resistance may be due to the uncounterbalance between the elastic forces in the network structure acting to hold them together and the ability of the solvent to move the chains apart.

The second group consisted of SC1, SC2 and chitosan films. The data of film swelling determined by weight difference on immersion with media are compiled in Table 10 and illustrated in Figure 54, respectively.

Regarding the films prepared from the combination of SCMC and CP 934, the higher proportion of CP 934 (SC2) caused the lowering swelling index value both in water and artificial saliva. This result is complied with a previous study of Peh and Wong (1999). It was probable that the interaction or crosslinking between SCMC and CP934 blending resulted in less capability of hydrogen bonding with water molecules opposed the expansion of polymer chain and reduced the efficiency of the film to equilibrium degree of swelling (Kim et al. 1992; Lim, and Wan, 1995).

From the swelling profile of SC1 and SC2 (Figure 54), it could be observed that the swelling index value of SC films were rather higher in distilled water than in artificial saliva. These results also conformed with Peh and Wong (1999). This might be due to the viscosity effect of the media. Moreover, it was possible that artificial saliva was composted of many kinds of ions, which could interfere the swelling of film. These ions penetrated the film, which absorbed water and swelled, then cross-linked at ammonium groups of chitosan molecules resulting in the screening electrostatic attractions and the denser films with reduced volumes (Nanthanid et al., 2001).

The swelling index value was increased with increasing molecular weight of chitosan acetate and lactate films in both media (Figures 55-60). HMW films swelled extensively with the highest swelling index value and the maximum increasing in the film area followed by MMW and LMW films, respectively whereas, LMW the latter had a considerably lower swelling index and area changing but higher disintegration and dissolution. The explanation might be that chitosan molecules formed salts during film formation in aqueous acid solution. After immersed in the medium, the residual acid in the film protonated amino groups and formed ammonium ions. This caused the

increasing in the repulsion of charged groups resulting in chitosan molecules unfold, more elongated. Consequently, chitosan of higher molecular weight compromised the accessibility of reactive groups and promoted chain entanglement, had higher degree of swelling. In addition, the amonium ions on chitosan molecules are reactive for the interaction with water thus, after swelling chitosan film disintegrated into small fragments and dissolved in water (Kim et al.,.1992; Lim , and Wan, 1995; Yan, Khor ,and Lim, 2001).

The effects of acid concentration on chitosan films were investigated (HA, MA and LA groups), HA1, MA1 and LA1 exhibited slightly higher swelling index value than HA2, MA2 and LA2, respectively, in both media. Thus, for chitosan acetate film, the concentration of acetic acid (one and two percent) did not produce a marked difference in swelling index value. On the contrary with chitosan lactate films it was found that HL1, ML1 and LL1 showed considerably higher swelling index values than HL2, ML2, LL2, respectively, in both media.

There was a considerable difference in swelling property between chitosan acetate and lactate films. For LMW films, LL1 exhibited highest swelling index value followed by LA1, LA2 and LL2, respectively. In contrast, MMW and HMW films, MA1 and HA1 showed higher swelling index values than ML1 and HL1 in both media, respectively.

Moreover, the scanning electron photomicrogrophs of cross section view of chitosan acetate films showed the porosity inside the films. It was possible that the porosity of chitosan acetate films might promote the rate of water sorption and interaction with water, resulting in higher degree of swelling. These results indicated that ionic strength, carboxyl, hydroxyl and alkyl groups in the acid molecules affected the degree of swelling of chitosan salt film, thus, the difference type of acid-solvent produced the difference in swelling index value (Ritthidej, Phaechamud and Koizumi, 2002).

Table 10 Swelling index value in deionized water (DI) and artificial saliva (AS) of SCMC films combined with CP934.

Formulas		Swelling index					
		2min	5min	10min	15min	30min	60min
SC1(DI)	mean*	11.5597	12.5879	15.4227	17.6844	18.6558	20.3186
	SD	0.4426	0.2602	0.2035	0.1887	0.1522	0.5109
	%CV	3.83	2.07	1.32	1.07	0.82	2.51
SC1(AS)	mean*	10.4206	11.6608	13.4643	14.3707	15.6376	16.9205
	SD	0.1797	0.1356	0.1408	0.2014	0.2400	0.1964
	%CV	1.72	1.16	1.05	1.40	1.53	1.16
SC2(DI)	mean*	6.6745	7.0697	7.8416	8.5495	10.2573	13.3218
	SD	0.1335	0.0664	0.0810	0.2348	0.1155	0.1974
	%CV	2.0006	0.9397	1.0332	2.7463	1.1261	1.4814
SC2(AS)	mean*	5.8301	6.0520	6.4618	7.2485	7.7189	8.5258
	SD	0.0966	0.1229	0.2143	0.3175	0.1506	0.1079
	%CV	1.66	2.03	3.32	4.38	1.95	1.27

* averaged from triplicate studies

Table 11 Swelling index value in deionized water (DI) and artificial saliva (AS) of chitosan (low molecular weight) films prepared with1 and 2% acetic and lactic acid.

		Swelling index					
Formulas		2min	5min	10min	15min	30min	60min
LA1(DI)	mean*	6.8495	8.4375	9.8411	11.1850	12.6490	18.0044
	SD	0.0672	0.2270	0.2198	0.1876	0.2279	0.1026
	%CV	0.98	2.69	2.23	1.68	1.80	0.57
LA1(AS)	mean*	6.6420	8.4456	9.1351	9.7769	10.5011	12.9079
	SD	0.0928	0.4466	0.1066	0.1582	0.2645	0.2674
	%CV	1.40	5.29	1.17	1.62	2.52	2.07
LA2(DI)	mean*	6.8431	7.8732	9.4315	10.7420	12.6109	16.6641
	SD	0.0138	0.1510	0.3312	0.4518	0.3086	0.1924
	%CV	0.20	1.92	3.51	4.21	2.45	1.15
LA2(AS)	mean*	6.4876	7.2831	8.2128	9.4844	11.1381	13.7370
	SD	0.3990	0.3342	0.3473	0.2749	0.2300	0.2885
	%CV	6.15	4.59	4.23	2.90	2.06	2.10
LL1(DI)	mean*	9.3553	10.7506	11.9441	18.1885	19.9255	20.5149
	SD	0.2278	0.3212	0.1058	0.1749	0.0476	0.2436
	%CV	2.43	2.99	0.89	0.96	0.24	1.19
LL1(AS)	mean*	8.4272	10.7053	11.7433	13.5060	13.8147	14.9232
	SD	0.4419	0.1616	0.2012	0.3312	0.1584	0.0566
	%CV	5.24	1.51	1.71	2.45	1.15	0.38
LL2(DI)	mean*	4.3966	4.9978	5.4118	5.7429	5.8995	5.9801
	SD	0.1492	0.1882	0.1639	0.1598	0.0810	0.0444
	%CV	3.39	3.77	3.03	2.78	1.37	0.74
LL2(AS)	mean*	4.3754	4.3866	4.7893	4.9711	5.1411	5.5675
	SD	0.3721	0.2744	0.2229	0.0665	0.1492	0.3286
	%CV	8.50	6.26	4.65	1.34	2.90	5.90

* averaged+B12 from triplicate studies

Table 12 Swelling index value in deionized water (DI) and artificial saliva (AS) of chitosan (medium molecular weight) films prepared with 1 and 2% acetic and lactic acid.

		Swelling index					
Formulas		2min	5min	10min	15min	30min	60min
MA1(DI)	mean*	10.8552	12.7061	21.3911	24.4873	30.3269	50.3249
	SD	0.2115	0.4604	0.4242	0.2628	0.7661	2.0771
	%CV	1.95	3.62	1.98	1.07	2.53	4.13
MA1(AS)	mean*	12.0447	13.0829	18.9193	24.0483	27.0677	37.5037
	SD	0.3443	0.3582	0.4603	0.4751	0.6290	0.4958
	%CV	2.86	2.74	2.43	1.98	2.32	1.32
MA2(DI)	mean*	8.9658	11.9184	19.0366	23.1972	28.0440	46.0558
	SD	0.8136	0.2500	0.7792	0.3434	0.2528	0.2507
	%CV	9.07	2.10	4.09	1.48	0.90	0.54
MA2(AS)	mean*	8.4864	14.3532	20.0842	23.7331	26.1107	35.3859
	SD	0.1659	0.1804	0.7617	1.2374	0.1488	0.3942
	%CV	1.96	1.26	3.79	5.21	0.57	1.11
ML1(DI)	mean*	10.0692	11.5679	13.0348	18.8054	26.2582	39.0661
	SD	0.4431	0.4947	0.6092	0.1658	0.3171	0.1930
	%CV	4.40	4.28	4.67	0.88	1.21	0.49
ML1(AS)	mean*	8.4831	10.1931	12.1831	16.0739	21.0034	31.2517
	SD	0.2736	0.5455	0.6292	0.2531	0.3172	0.3459
	%CV	3.22	5.35	5.16	1.57	1.51	1.11
ML2(DI)	mean*	4.9596	5.7316	6.0565	6.5737	6.9422	7.0467
	SD	0.1141	0.0923	0.0564	0.2157	0.0573	0.0531
	%CV	2.30	1.61	0.93	3.28	0.83	0.75
ML2(AS)	mean*	4.3203	4.7543	4.9455	5.2870	5.7111	6.0950
	SD	0.2183	0.0976	0.0474	0.1782	0.1581	0.1494
	%CV	5.05	2.05	0.96	3.37	2.77	2.45

* averaged from triplicate studies

Table 13 Swelling index value in deionized water (DI) and artificial saliva (AS) of chitosan (high molecular weight) films prepared with 1 and 2% acetic and lactic acid.

Formulas		Swelling index					
		2min	5min	10min	15min	30min	60min
HA1(DI)	mean*	12.0531	14.9064	21.4715	25.3959	37.1322	55.9931
	SD	0.0474	0.0411	0.3732	0.3805	0.1165	0.1241
	%CV	0.39	0.28	1.74	1.50	0.31	0.22
HA1(AS)	mean*	10.9510	13.1261	18.2227	21.9850	30.5409	49.1521
	SD	0.1008	0.2184	0.6209	0.1439	0.3182	0.1980
	%CV	0.92	1.66	3.41	0.65	1.04	0.40
HA2(DI)	mean*	11.0202	13.6781	21.3390	24.8263	33.3243	49.7738
	SD	0.1298	0.2312	0.7775	0.1526	0.3425	0.2522
	%CV	1.18	1.69	3.64	0.61	1.03	0.51
HA2(AS)	mean*	10.4848	11.6102	18.4634	21.5418	29.3133	42.4100
	SD	0.5210	0.5544	0.1725	0.3015	0.3629	0.4680
	%CV	4.97	4.78	0.93	1.40	1.24	1.10
HL1(DI)	mean*	13.1878	14.6723	20.4776	22.9396	29.2054	45.1593
	SD	0.3163	0.1852	0.4270	0.2237	0.1908	0.2068
	%CV	2.40	1.26	2.09	0.98	0.65	0.46
HL1(AS)	mean*	12.4567	14.3799	19.3910	21.1474	25.0833	39.1047
	SD	0.3982	0.3286	0.2243	0.1848	0.2003	0.2152
	%CV	3.20	2.28	1.16	0.87	0.80	0.55
HL2(DI)	mean*	5.4196	5.9283	6.8288	7.3551	7.9445	8.1556
	SD	0.1025	0.0984	0.1268	0.2220	0.0497	0.1418
	%CV	1.89	1.66	1.86	3.02	0.63	1.74
HL2(AS)	mean*	4.7663	5.2218	5.9500	6.2588	6.5588	6.9321
	SD	0.1822	0.0999	0.1002	0.0630	0.0864	0.0696
	%CV	3.82	1.91	1.68	1.01	1.32	1.00

* averaged from triplicate studies

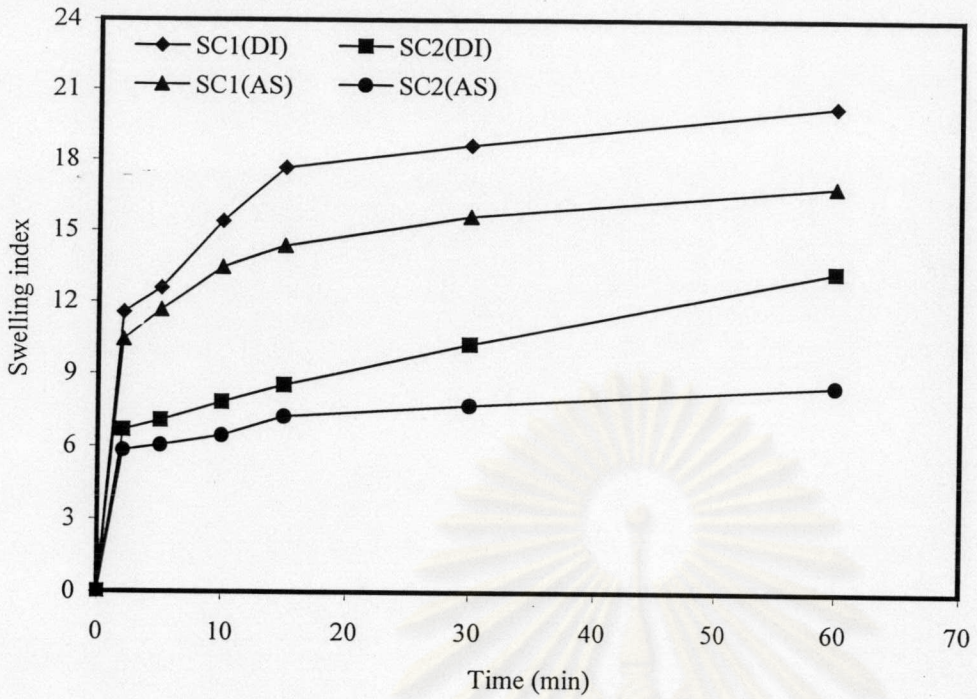


Figure 54 Swelling profiles in deionized water (DI) and artificial saliva (AS) of SCMC combined with CP934 films.

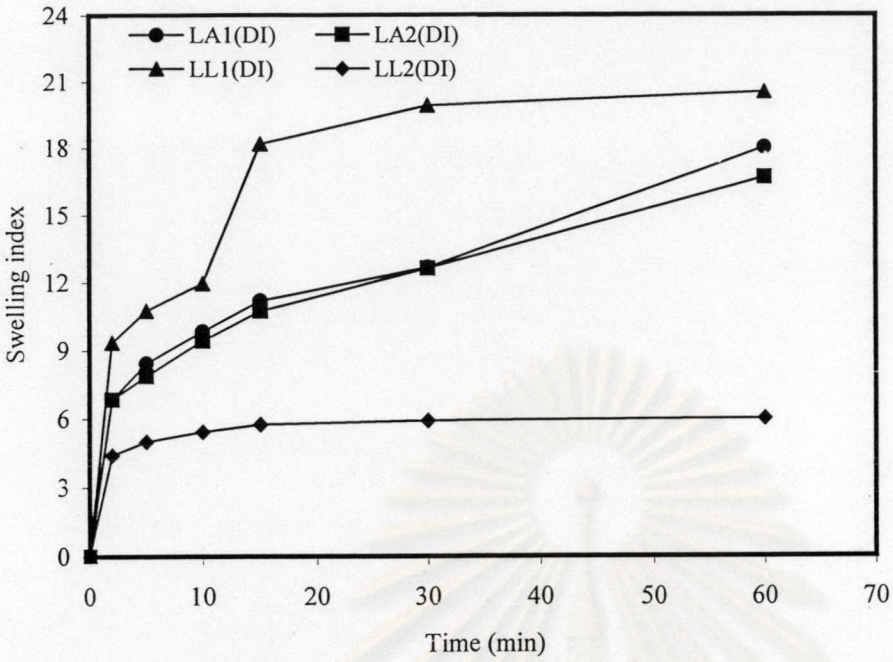


Figure 55 Swelling profiles in deionized water (DI) of low molecular weight chitosan films prepared from 1 and 2 % acetic acid and lactic acid.

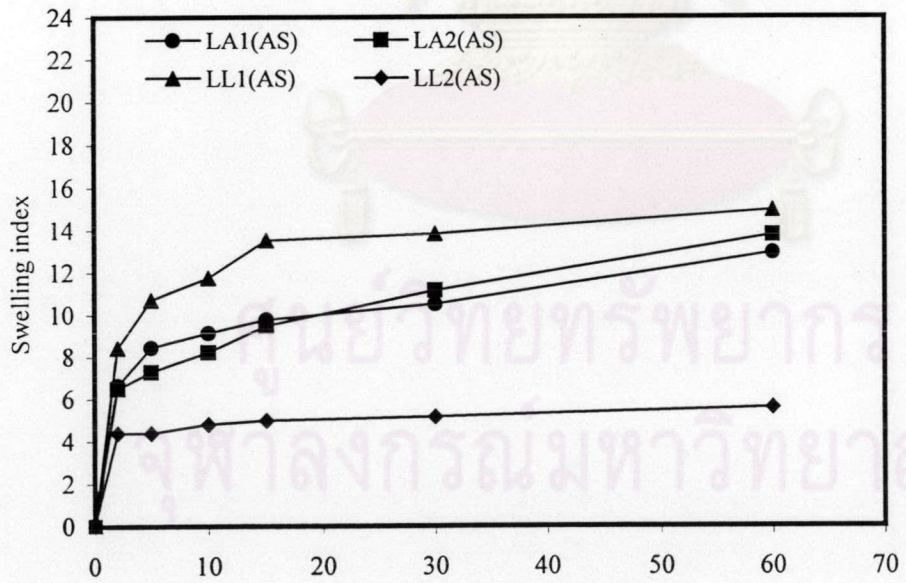


Figure 56 Swelling profiles in artificial saliva (AS) of low molecular weight chitosan films prepared from 1 and 2 % acetic acid and lactic acid.

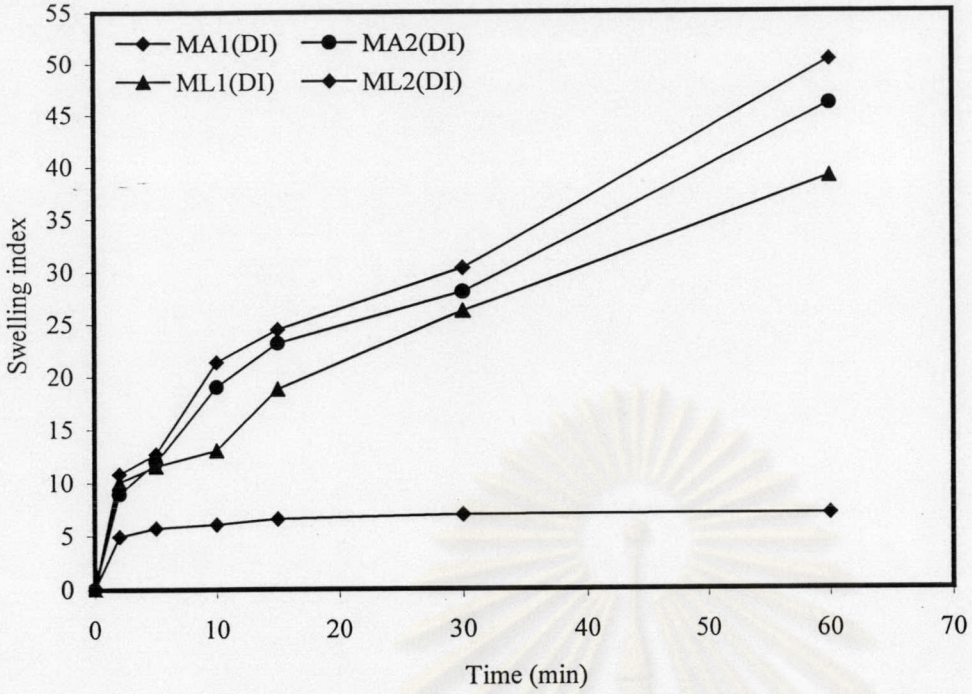


Figure 57 Swelling profiles in deionized water (DI) of medium molecular weight chitosan films prepared from 1 and 2 % acetic acid and lactic acid.

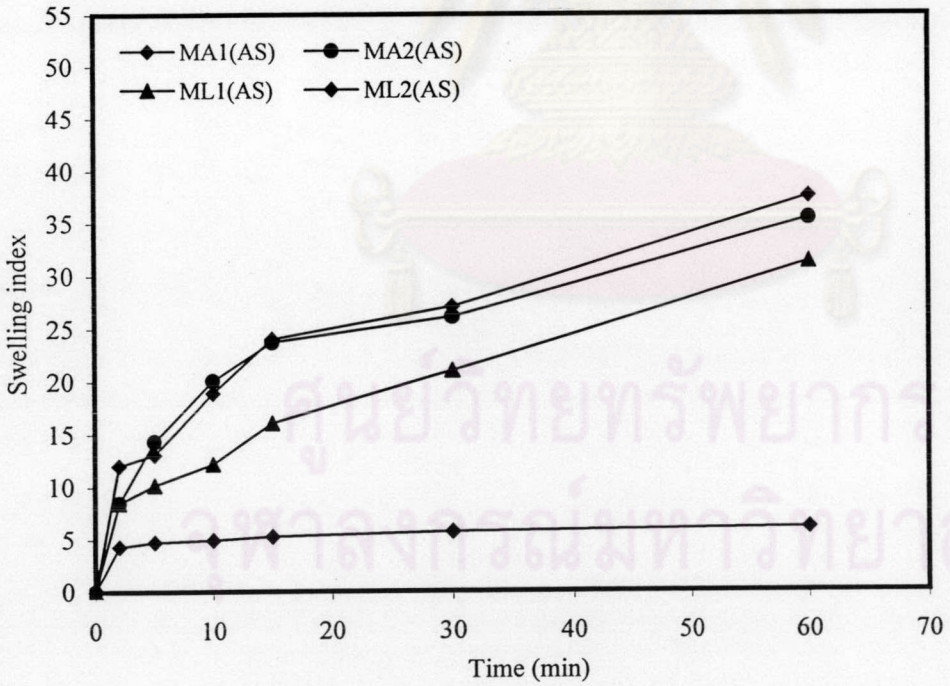


Figure 58 Swelling profiles in artificial saliva (AS) of medium molecular weight chitosan films prepared from 1 and 2 % acetic acid and lactic acid.

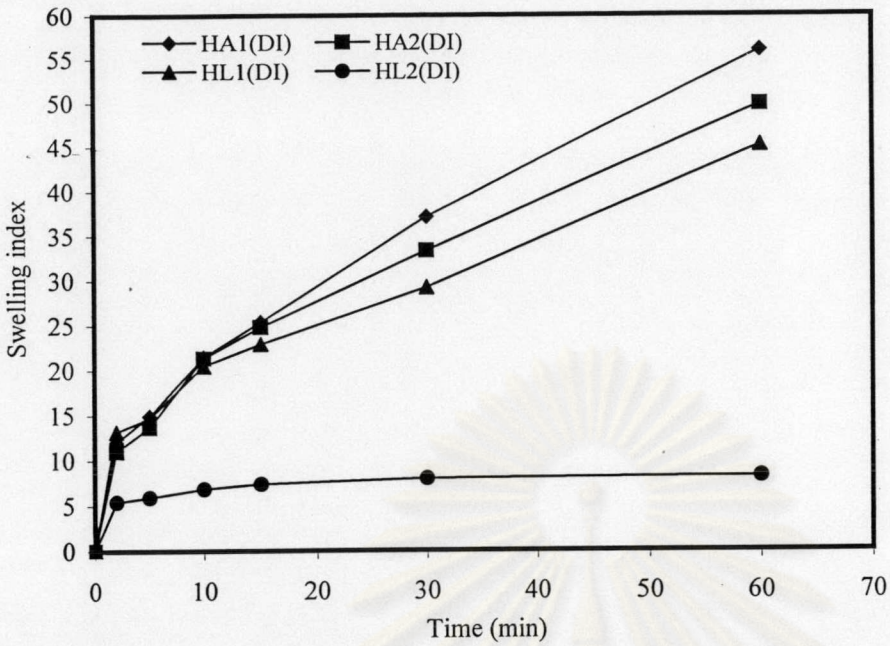


Figure 59 Swelling profiles in deionized water (DI) of high molecular weight chitosan films prepared from 1 and 2 % acetic acid and lactic acid.

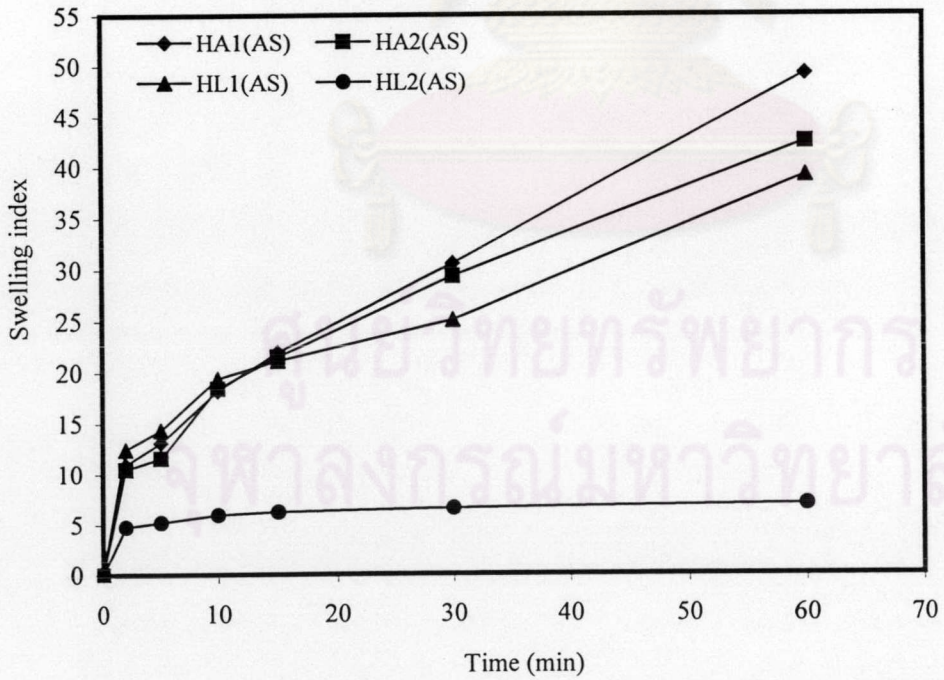


Figure 60 Swelling profiles in artificial saliva (AS) of high molecular weight chitosan films prepared from 1 and 2 % acetic acid and lactic acid.

4.5 In Vitro mucoadhesion test

4.5.1 Mucoadhesive force

Determination of the mucoadhesion strength is important in the development of mucoadhesive dosage form due to satisfactory mucoadhesion is essential for successful application of a buccal mucoadhesive drug delivery. An ideal buccal film should be flexible, elastic, durable and possesses high mucoadhesive strength so that it can be retained in the oral cavity for a desired duration. Several methods have been developed for measuring mucoadhesive force, however, there is still no universal test method for mucoadhesion measurement (Wong, Yuen, and Peh, 1999). In this study the adhesion strength after a predetermined contact time was investigated using rat intestinal mucosa as a model membrane. This kind of biological membrane was commonly used and acceptable results were obtained in the study of Rao, and Buri (1989), Smart (1991) and Mortazavi and Smart (1994).

The rank order of mucoadhesive force is illustrated in Table14 and Figures 61,63 and 65 Among all formulas, HA1 exhibited the strongest adhesive force whereas LA2 showed the weakest force.

For films obtained from formulations of SCMC alone and SCMC combined with CP934, it was found that SC2 exhibited the strongest abhesive force followed by SC1 and S0. This result indicated that increasing carbopol composition led to a significant increase in mucoadhesive strength. Similaly, film of HPMC alone and HPMC combined with carbopol, increasing concentration of carbopol caused an increase in mucoadhesive force. This findings were in agreement with Peh and Wong (1999), who found that an increase in bioadhesion strength was observed with an increase in carbopol content. It is possible that SCMC and HPMC may form complex or crosslink with carbopol which has an excellent mucoadhesive property due to containing large numbers of carboxylic acid groups that promote mucoadhesion. Thus, the inclusion of carbopol produced the additive effects which caused enhancement of the mucoadhesive force. Moreover, the mucoadhesive force increased proportionally to the content of carbopol. This indicated that the adhesive strength depended on ionizable groups and charge density on the polymer chain. Regarding the tensile strength an increase in carbopol content caused SCMC film to become stronger

to withstand the more tensile stress. This showed the correspondent effect of carbopol on the mucoadhesive strength and the tensile strength of SCMC films. Incontrast, the unrelated effect of carbopol on these values of HPMC films was found. The increase of carbopol content decreased the tensile strength of HPMC films.

From Table 14, S0, SC1 and SC2 exhibited higher mucoadhesive strength than H0, HC1 and HC2, respectively. This finding is consistent with previous studies of Sam, Heuij and Tukker (1992) ; Smart, Kellaway and Worthington (1984) ; Peh and Wong (1999) and Wong, Yuen and Peh (1999). In addition, these support the hypothesis that polyanionic molecules seem to have more effective bioadhesion than neutral polymers like HPMC. It could be attributed to the ionizable groups which were reactive for mucoadhesive sites and charge density on the polymer chains (Park and Robinson, 1984).

The mucoadhesive force was increased with increasing molecular weight of chitosan acetate and lactate films. This result supports the theory that high molecular weight possesses a sufficient linear chain length to promote entanglement and ensure interpenetration (related to diffusion coefficients) between polymer and mucin chains (Leung, and Robinson, 1990; Rathbone ed., 1996). Furthermore, high molecular weight chitosan offers numerous interaction sites from reactive, ionized groups for mucin attachment than lower molecular weight, which tends to develop intense mucoadhesive bond (Mathiowitz, 1999). The result was in agreement with Henriksen et al (1996) ; Lehr et al. (1992) and Qaquish and Amiji (1999). In addition, this finding also conformed with ultimate tensile strength tendency which represents the higher ability to withstand the stress of higher molecular weight film.

Regarding HA, MA and LA groups, HA1 and MA1 produced considerable higher adhesive force than HA2 and MA2, respectively, whereas, LA1 exhibited slightly higher adhesive strength than LA2. Similar result, appeared in HL, ML and LL groups. Concurring with swelling data of chitosan lactate prepared from 2% acid, which had a considerable lower degree of swelling than other chitosan films, also presented lower mucoadhesive strength. This finding may be due to the low degree of swelling led to insufficient intimate contact and interpenetration.

This result indicated that concentration of acid used affected mucoadhesive force of chitosan films. It is probable that ionic strength in film preparation may affect mucoadhesive force. There was an evidence stated that, as ionic strength increased, mucoadhesive strength decreased due to a reduction in expanded nature of the polymer network and decreases in the mobility of the polymer chain, resulting in a decrease of the interdiffusion process and extent of entanglement (Leung, and Robinson, 1990).

There was no significant difference ($p<0.05$) between LMW chitosan films, which were prepared from acetic acid and lactic acid. For MMW and HMW films, chitosan acetate films exhibited rather higher mucoadhesive force than lactate films. Similar result was obtained from the ultimate tensile strength data that chitosan acetate films showed higher tensile strength than lactate films.

In general, chitosan acetate and lactate films prepared from 1 % acid exhibited higher adhesive strength than films of SCMC and HPMC both single and combined with carbopol. This result was in agreement with previous study of Lehr et al. (1992) and supported the hypothesis that polycationic polymer had better mucoadhesive properties than polyanionic and neutral polymers due to the strong multivalent electrostatic interaction with mucin which possessed polyanionic chains (Qaquish, and Amiji, 1999).

According to Table 5E (Appendix E), which depicts one way analysis of variance between 18 formulations of mucoadhesive films, VR (1032.02) > F(1.79). Therefore, significant differences in adhesive forces are shown between mucoadhesive films ($p<0.05$) John Tukey's honestly significant diffrence (HSD) of average adhesive force between 18 formulations is depicted in Table 7E)

In summary, the parameters that could affect mucoadhesive strength are type, molecular weight, configuration, complexation or crosslinking density, charge and degree of ionzation and extent of hydration or swelling of mucoadhesive polymer. In addition, condition and method of film preparation are also important factors.

Table 14 The rank order of mucoadhesive forces of prepared free films in artificial saliva.

Formulation	Mucoadhesive force (N/cm^2)		
	Mean*	SD	%CV
HA1	9.418	0.255	2.71
HL1	8.920	0.284	3.18
MA1	8.790	0.376	4.28
ML1	7.551	0.244	3.23
SC2	7.512	0.170	2.31
HC2	6.861	0.186	2.71
SC1	6.052	0.258	4.26
HC1	5.451	0.203	3.73
S0	5.265	0.191	3.63
H0	3.829	0.178	4.65
HA2	3.344	0.179	5.35
HL2	2.878	0.094	3.26
MA2	2.226	0.317	14.26
LL1	1.601	0.163	10.15
ML2	1.509	0.180	11.94
LA1	1.410	0.133	9.46
LL2	0.567	0.066	11.68
LA2	0.373	0.059	15.78

* averaged from 5 determinations

4.5.2 Mucoadhesive time

When formulating a mucoadhesive dosage form, it is important to evaluate not only the adhesive strength but also the duration of adhesion, due to the mucoadhesive dosage should be retained in the oral cavity for a desired duration for effectiveness of treatment. In this study, the duration of adhesion of 18 formulations was evaluated by the same apparatus as mucoadhesive force with applying a constant tensile force of 0.373 N (38 g loading weight) to the adhesive bond and leaving until the bond had been broken, then the duration of adhesion was recorded automatically. The data of the mean mucoadhesive time with the standard deviation are illustrated in Table 15 and Figures 62, 64 and 66.

The rank order of the duration of adhesion is presented in Table 15 MA1, was found to adhere with the longest duration. Whereas, the shortest duration was obtained from LL2.

For the SCMC groups, it was found that S0 exhibited the longest duration followed by SC1 and SC2, respectively ($p<0.05$ Appendix E). This result might be due to when SC1 and SC2 exposed to water, they became thicker and formed slippery film, which led to less adhesive property and a decrease in duration of adhesion.

On the contrary, the duration time of HPMC groups increased with the increasing of carbopol content as follows : HC2 > HC1 > H0 ($p<0.05$). This finding was in agreement with Peh and Wong (1999), who found that increasing in carbopol content caused an increase in duration of adhesion and mucoadhesive strength.

In combination with carbopol, HC films exhibited longer duration of adhesion than SC films. This also agreed with Peh and Wong (1999), who revealed that SCMC combined with carbopol films had shorter residence time than the HPMC combined with carbopol films.

Regarding chitosan acetate and lactate films, the longest duration of adhesion was found in the films prepared from of chitosan of MMW followed by HMW and LMW, respectively.

Although the hydration of the mucoadhesive polymer was essential to initiate the mucoadhesive bonding process, excess of increasing level of hydration was found to reduce adhesive force and duration of adhesion. Since mucoadhesive bonds became over extended, thus, over swelling of the polymer led to a reduction in mucoadhesive properties (Rathbone ed., 1996; Smart, 1991).

Therefore, the adhesion was maximum at a certain degree of swelling and the duration time required optimal swelling. This might be the reason why high molecular weight chitosan film had shorter duration time than medium molecular weight films.

Concerning HA, MA and LA groups, HA1, MA1 and LA1 presented substantial longer adhesive times than HA2, MA2 and LA2, respectively. Similar results could be observed from HL, ML and LL groups. This may be closely related to their unsatisfactory mucoadhesive force as illustrated in Table 14. It was clearly seen that concentration of acid influenced the adhesive time.

Chitosan acetate films exhibited longer duration time than chitosan lactate films. The insignificant result was obtained inLA2 and LL2($p<0.05$). The result indicated that type of acid used in chitosan films had a significant effect on adhesive time.

In general, chitosan acetate and chitosan lactate films prepared from 1% acid presented longer adhesive time than SCMC and HPMC both single and combined with carbopol. It was possible that when exposed to water, S0, H0 and HC films swollen to form gels and eventually a slippery mucilage which caused a decrease in the adhesive force and duration time. Furthermore, the swelling and deformation of films resulted in the gradual reduction in the adhesive propertics and limited the duration of films.

According to Table 5E, which shows one way analysis of variance between 18 formulations of mucoadhesive films, VR (501.822) > F (1.79). Thereby, significant differences of adhesive time were shown between mucoadhesive films ($P < 0.05$). John Tukey's honestly significant diffrence (HSD) of average adhesive time between 18 formulations is presented in Table 6E .

Table 15 The rank order of mucoadhesive time of prepared free films in artificial saliva.

Formulas	Mucoadhesive Time (hour)		
	Mean	SD	%CV
MA1	9.369	0.271	2.90
ML1	7.252	0.127	1.75
HC2	6.827	0.458	6.71
HA1	6.288	0.124	1.97
S0	5.466	0.196	3.59
HL1	4.879	0.107	2.19
HC1	4.299	0.203	4.72
MA2	4.004	0.129	3.23
SC1	3.824	0.290	7.58
LA1	3.303	0.175	5.30
ML2	3.276	0.061	1.88
HA2	3.220	0.067	2.07
SC2	2.898	0.074	2.57
LL1	2.689	0.076	2.83
HL2	2.402	0.084	3.51
H0	1.911	0.164	8.60
LA2	0.666	0.050	7.51
LL2	0.560	0.018	3.20

* averaged from 5 determinations

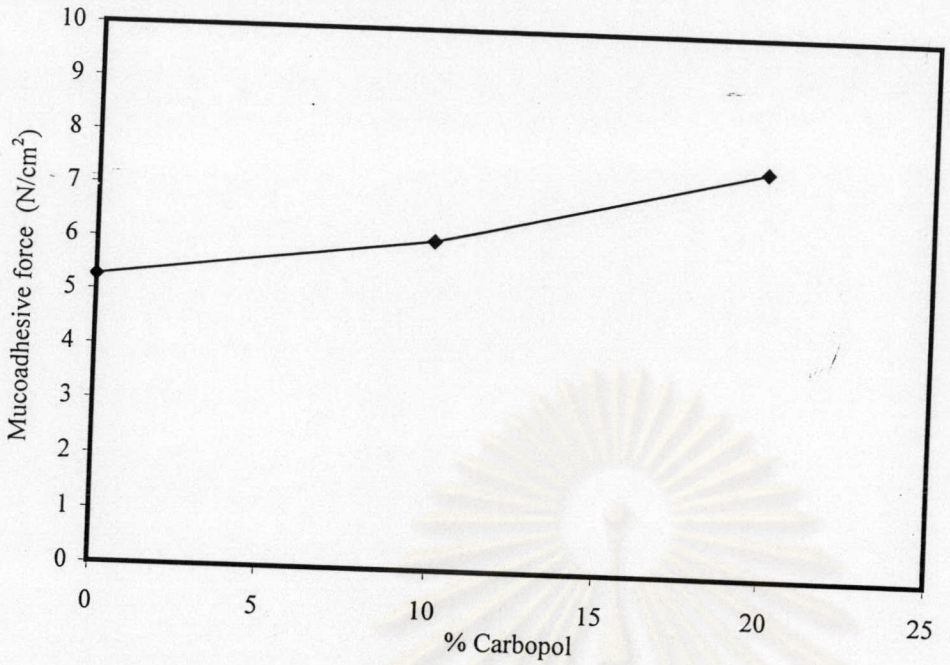


Figure 61 The mucodahesive force of SCMC films combined with varied concentrations of CP934.

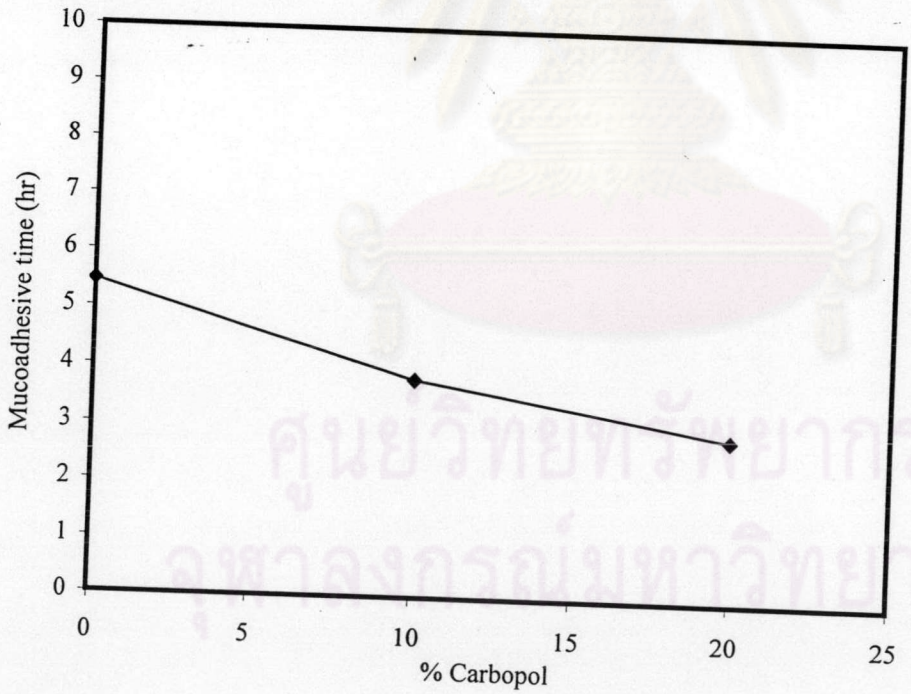


Figure 62 The mucodahesive time of SCMC films combined with varied concentrations of CP934.

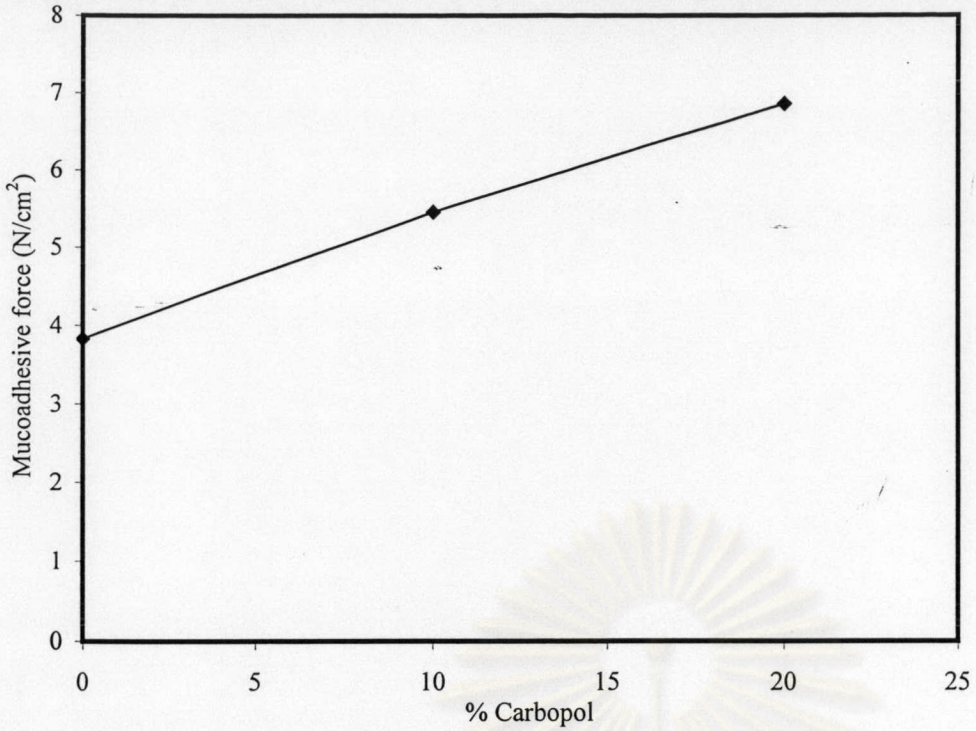


Figure 63 The mucoadhesive force of HPMC films combined with varied concentrations of CP934.

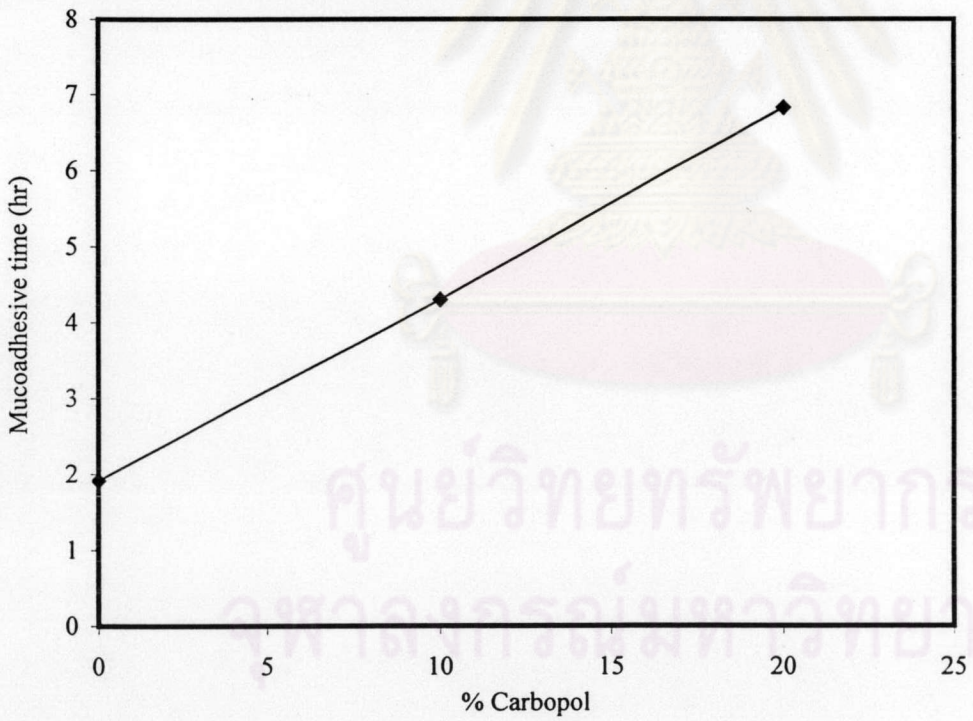


Figure 64 The mucoadhesive time of HPMC films combined with varied concentrations of CP934.

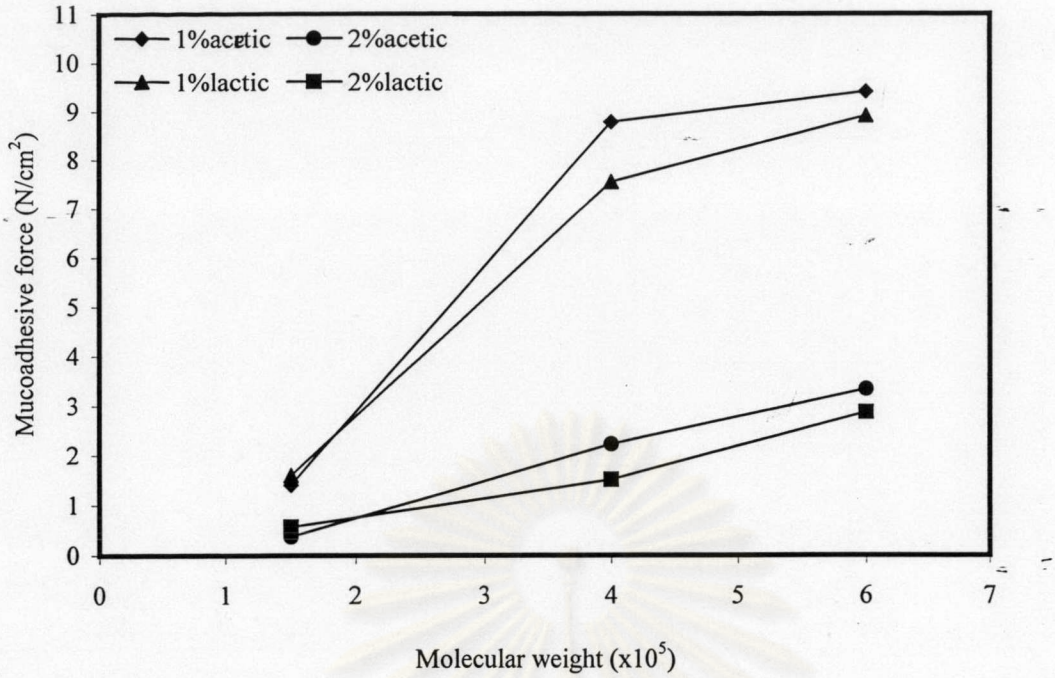


Figure 65 The mucoadhesive force of chitosan films of different molecular weights with 1 and 2% acetic acid and lactic acid.

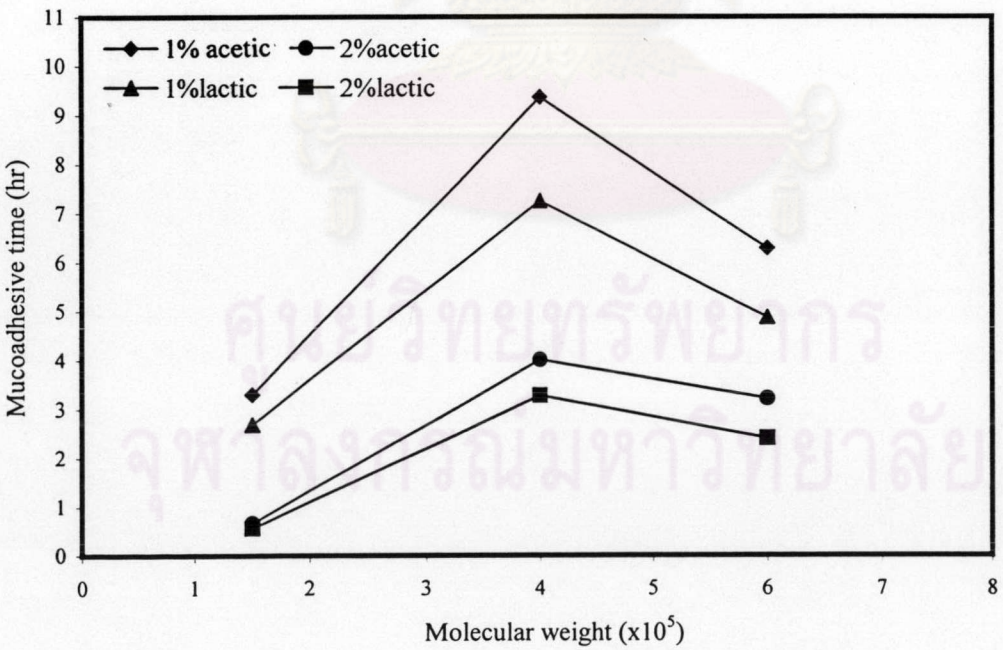


Figure 66 The mucoadhesive time of chitosan films of different molecular weight with 1 and 2 % acetic acid and lactic acid.

In summary, the duration of adhesion is influenced by type and degree of swelling of mucoadhesive polymer, condition and preparation method.

5 Formulation of *Garcinia mangostana* extract buccal mucoadhesive films

5.1 Monolayer films (mucoadhesive layer)

From the results of the evaluation of free film, the optimal formulas wich appropriate properties such as good physical characteristics, suitable mechanical properties, proper hydration and excellent mucoadhesive performance, were selected to formulate of buccal films. These polymers including MA1, ML1 and HC2 were selected in preparation of film containing *Garcinia mangostana* Linn. extracts. The concentration of purified extract employed in the formulations was 0.2133 mg/cm^2. That is the film of 1.5 x 1.5 cm^2 (2.25 cm^2) contained 0.48 mg of the purified extract. The physical properties of three formulations were depicted in Table 16.

As a result, all formulations were clear and their colors were yellow due to the color of purified extract. The other physical appearances of each formulation were similar to free film. Even though the incorporation of the purified extract led to the yellowness of HC2 film, the ease of detachment from the preparing plate was unchanged.

Moreover, the incorporation with the purified extract also decrease transparency of HC2 film. Similar results were also obtained from ML_1 film.

Table 16 Physical properties of three formulations of *Garcinia mangostana* extract buccal mucoadhesive films.

Formulas	Yellowness	Transparency	Glossiness	Stickiness	Flexibility	Integrity	Ease of detachment from plate
MA_1	++++	+++	++++	-	+	+++	+++
ML_1	+++	++	++	++	++	++	+
HC_2	++	+	++	-	+	+++	+++

The symbols of (+) and (-) showed the appearance and no appearance respectively, and the number of the symbols of (+) showed a degree of the appearance

5.2 Bilayered films (mucoadhesive and backing layered)

From preliminary study of backing layer preparation, the optimal backing layers were obtained from 15 ml of 1% w/v ethylcellulose. As the casting technique was performed to prepare bilaminated films, it was found that a complete binding between the mucoadhesive and the backing layers without defect or breakage was achieved.

Thus the three formulations of *Garcinia mangostana* mucoadhesive films were prepared according to the process as described previously. All bilayered films exhibited good physical appearance. The defect or breakage was absent. Moreover, a perfect binding between the backing and the mucoadhesive layers was obtained.

6. Determination of *Garcinia mangostana* extract mucoadhesive films

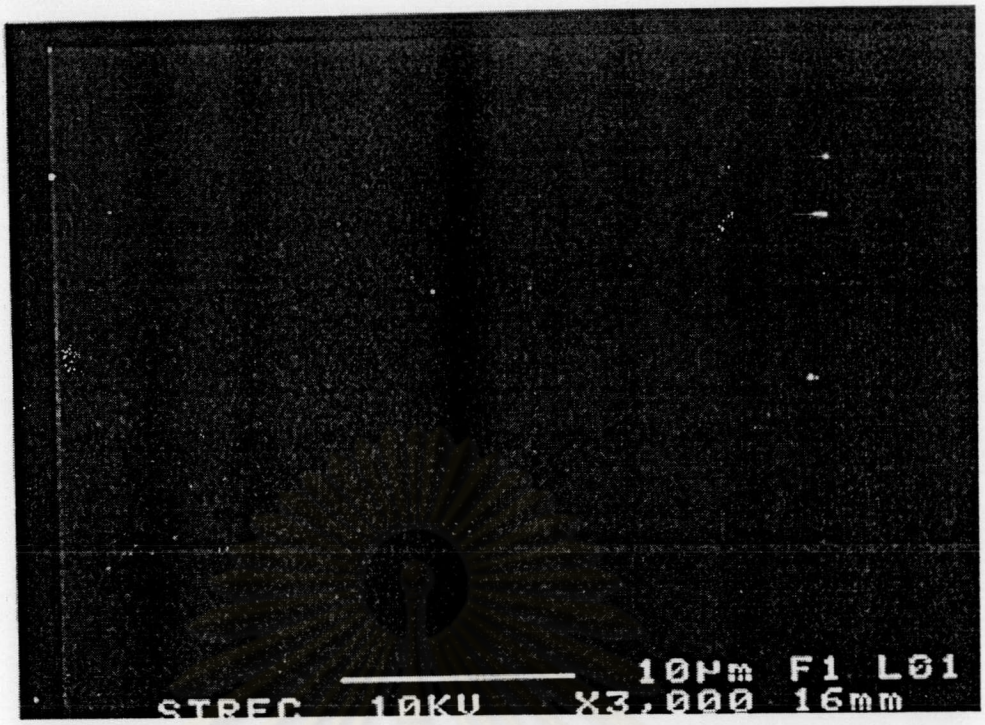
6.1 Surface and cross section morphology

The surface morphology of the three formulations was observed at the same condition as described previously. For MA1 formulation as compared to its free film, it was found that MA1 formulation displayed a similar smooth nonporous structure. In addition, no crystals of mangostin on the surface of film could be observed (Figure 67).

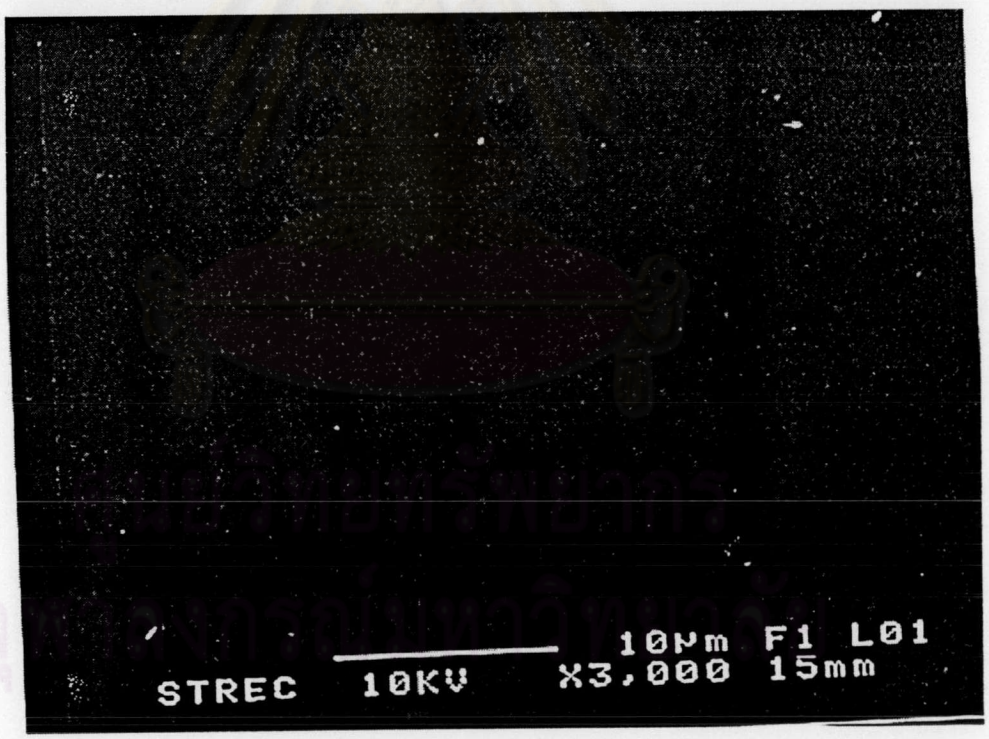
Comparison of ML1 formulation with its free film, it indicated that the incorporation of purified extract into ML1 film caused a slightly increase of surface roughness and a few small particles were observed (Figure 69).

Regarding HC2 formulation in comparison with its free film, the increasing in surface roughness without the crystals of mangostin was observed (Figure 71).

The cross section photomicrographs of the three formulations were examined under the same condition as described previously. There was no crystal of mangostin observed in all film formulations. For MA_1 and ML_1 as compared to their free film, there was no marked difference in cross section morphology (Figures 68 and 70.).

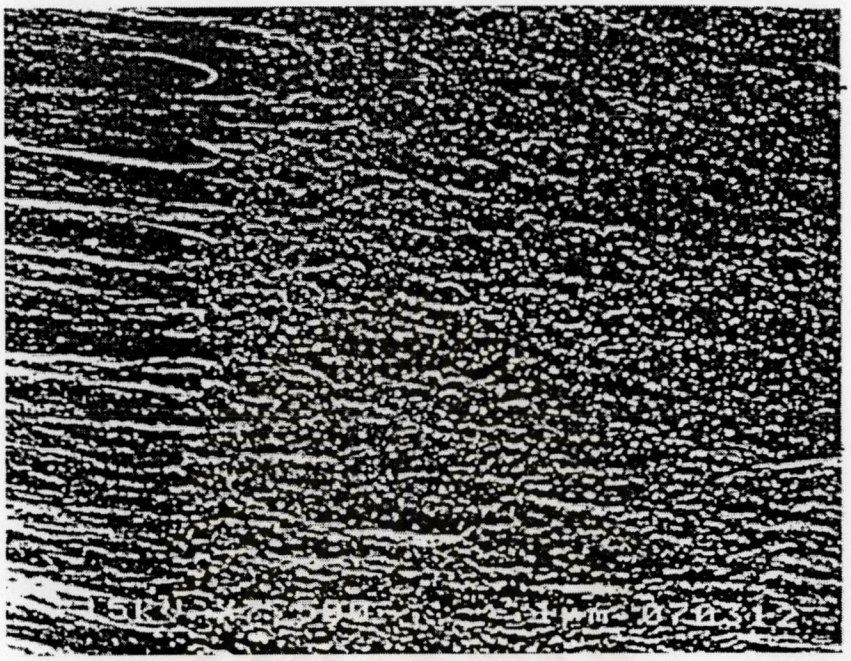


a

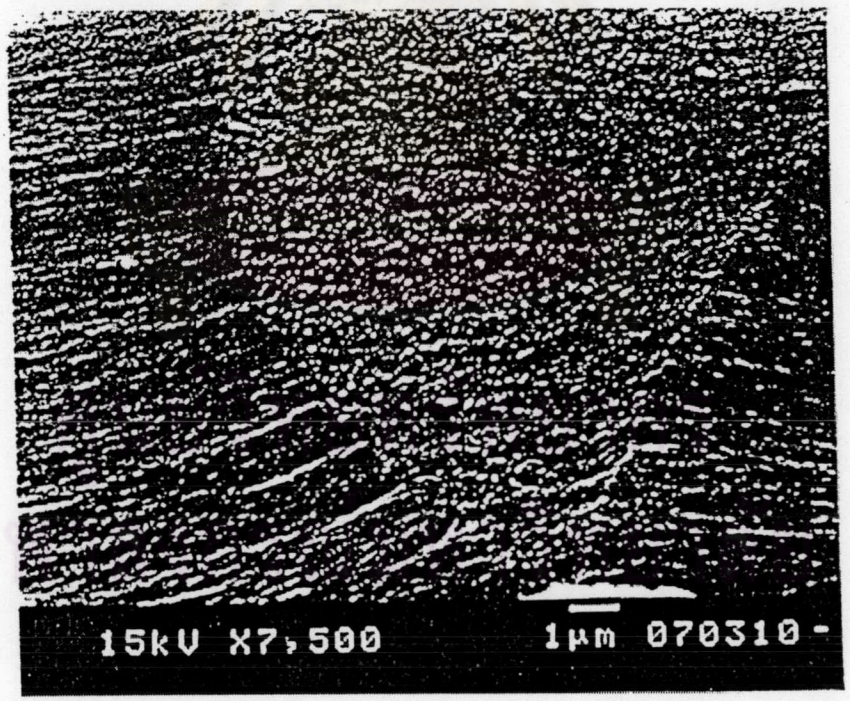


b

Figure 67 The photomicrographs of surface morphology of (a) MA_1 free film and (b) MA_1 film containing *Garcinia mangostana* extract.

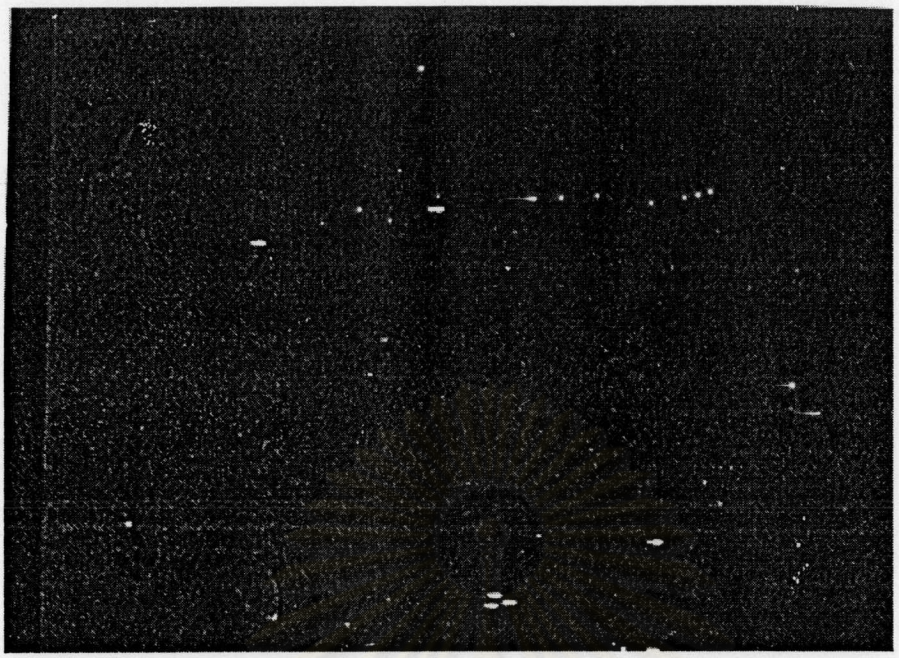


a

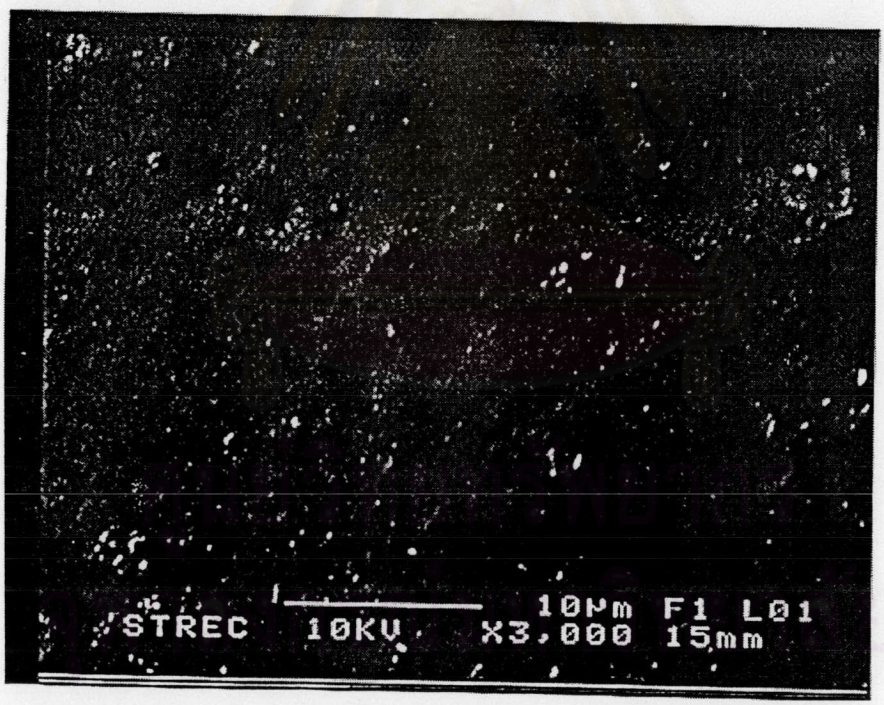


b

Figure 68 The photomicrographs of cross section morphology of (a) MA1 free film and (b) MA1 film containing *Garcinia mangostana* extract.

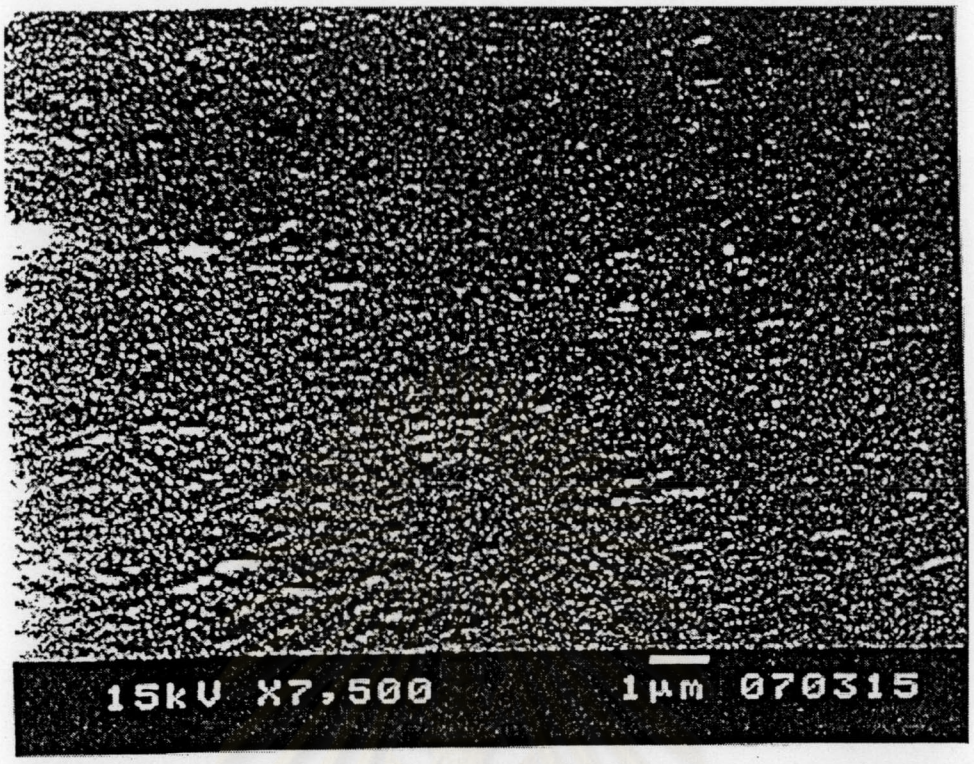


a

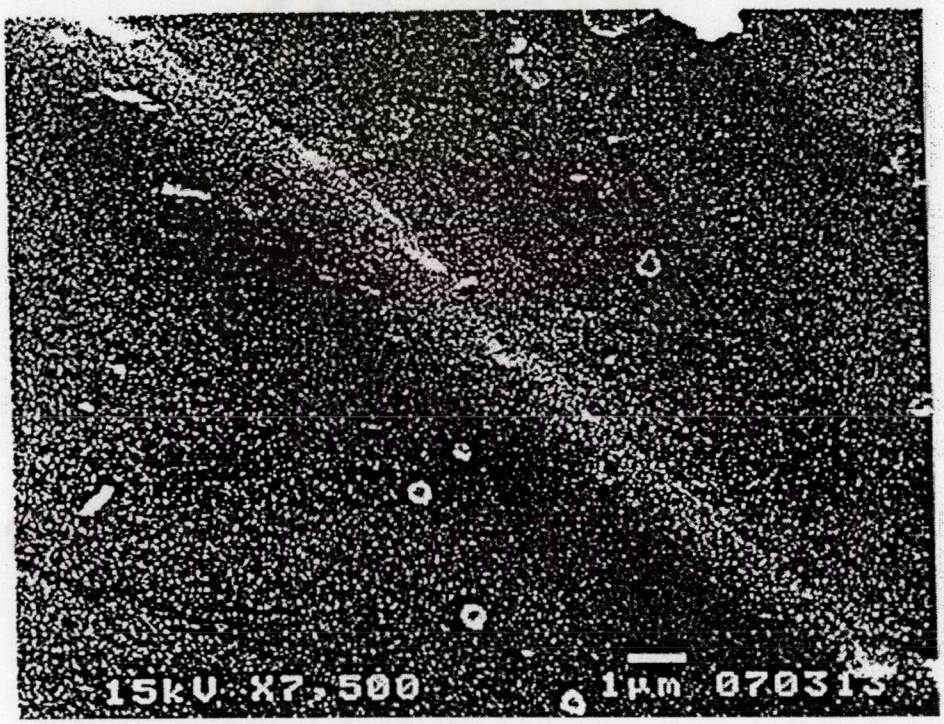


b

Figure 69 The Photomicrographs of surface morphology of (a) ML1 free film and (b) ML1 film containing *Garcinia mangostana* extract.



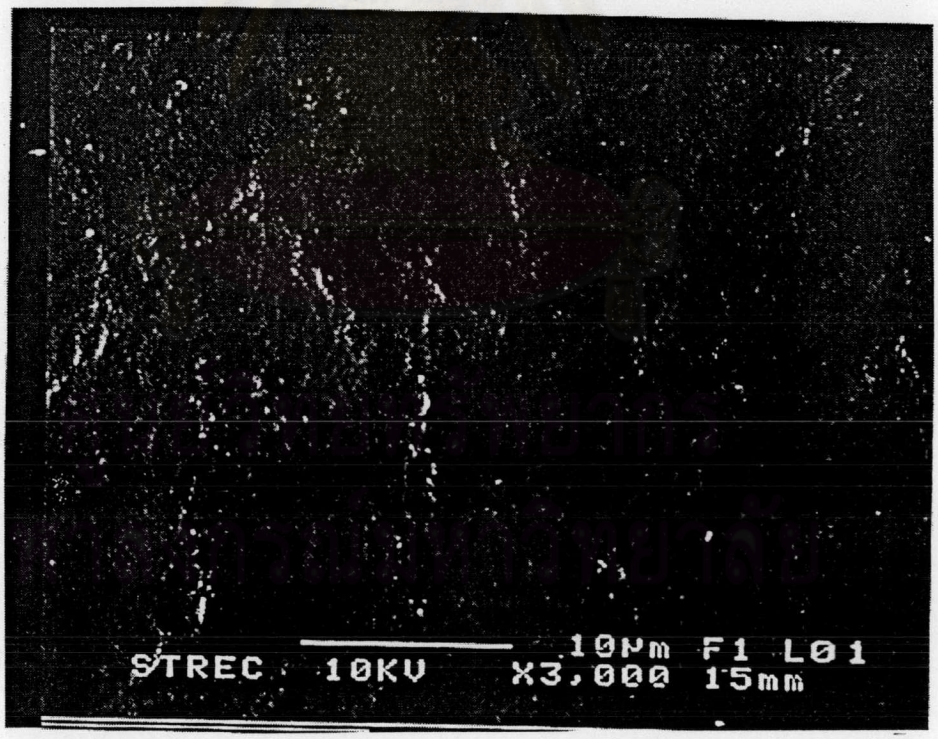
a



b

Figure 70 The photomicrographs of cross section morphology of (a) ML1 free film and (b) ML1 film containing *Garcinia mangostana* extract.

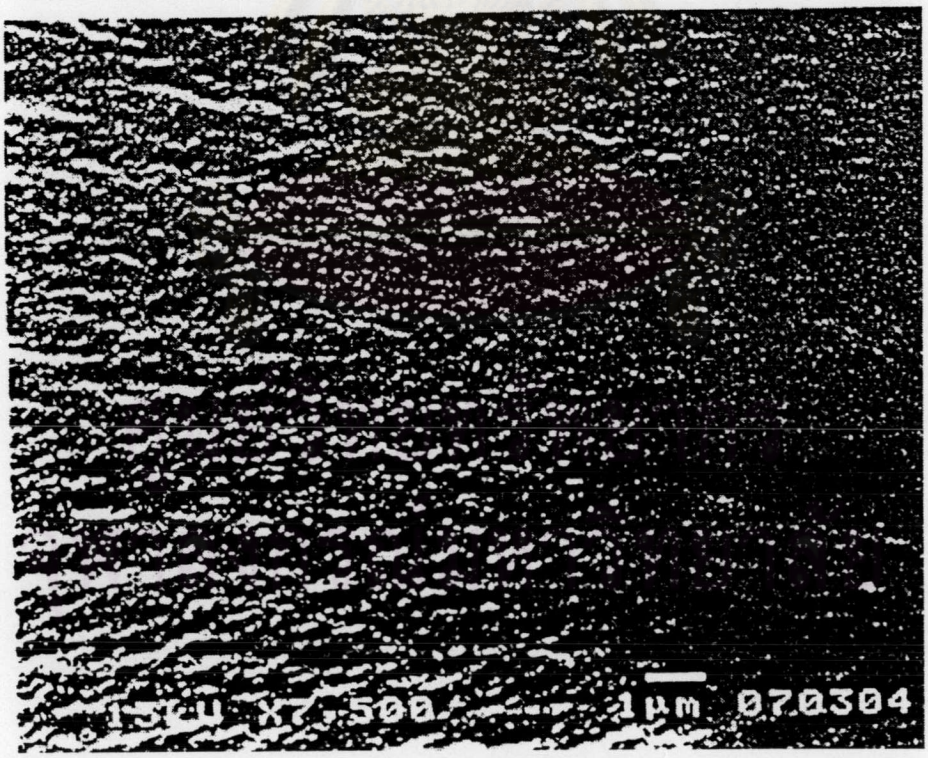
a



b

Figure 71 The Photomicrographs of surface morphology of (a) HC2 free film and (b) HC2 film containing *Garcinia mangostana* extract.

a



b

Figure 72 The photomicrographs of cross section morphology of (a) HC2 free film and (b) HC2 film containing *Garcinia mangostana* extract.

The comparison of HC2 containing purified extract with HC2 free film, it was indicated that addition of purified extract in HC2 film resulted in a slight increase in roughness and porosity of HC2 film (Figure70).

6.2 The x-ray diffractograms

The x-ray diffraction patterns of chitosan and mangostin powders, three formulations and free films of MA1, ML1 and HC2 are illustrated in Figures 74-75.

Powder x-ray diffraction pattern of mangostin showed crystalline presented diffraction peaks at approximately 5.9°, 11.9° and 13.3° (2θ). The substantially high intensity of peak at 5.9° (2θ) and absence of amorphous characteristic indicated that mangostin was in a crystalline state.

The x-ray patterns of chitosan of medium molecular weight flake are shown in Appendix D, showed the halo pattern indicating an amorphous state of chitosan flakes. Similar pattern was obtained from a previous study by Nunthanid et al (2001).

When processing chitosan flakes into free films, the x-ray diffraction patterns were observed. The x-ray patterns presented the transformation from an amorphous state to partially crystalline state. This was consistent to a previous study of Lim and Wan (1995) who found that heat treatment in the process of free films preparation enhanced the crystallinity of the films.

For MA1 free film, the crystilline peaks with rather small intensity was observed at approximately 11.7°, 20.8°, 26.7°, 28.7° and doublet peak at 29° (2θ) (Appendix D). The diffractograms of ML1 film were very similar to that of MA1, however, the additional peaks were observed approximately at 9.5° and 19° (2θ) (Figure 74).

Regarding HC_2 free film, its x-ray diffractograms indicated that it was partially in amorphous form, however, some small intensity peaks were observed at approximately 9.5°, 20.8°, 26.6° and 28.7° (2θ). The rather high intensity peak was found at approximately 29° (2θ) (Figure 75).

For MA1 loaded with purified extract (Appendix E) the small intensity peaks compared with MA1 free film were observed. No diffraction park dul to mangosstin was clearly observed at 5.9°, 8.8°, 9.4°, 13.5°, 15°, 16° and 19° (2θ). This revealed that mangostin may dissolved into molecular level in this film. Concerning ML1 formulation, its diffractogram displayed the substaintially higher intensity of mangostin peak at approximately 5.8° (2θ) than of MA1 formulation. The other diffraction peaks occurred near the same position at that of its free film (Appendix E). This indicated that mangostin in ML1 film were in a crystalline state.

The x-ray pattern of HC2 loaded purified extract is shown in Figure 75 As compared to HC2 free film, the similar pattern was obtained and the peak at 5.9°(2θ) was absent, indicating an amorphous state of mangostin in this film.

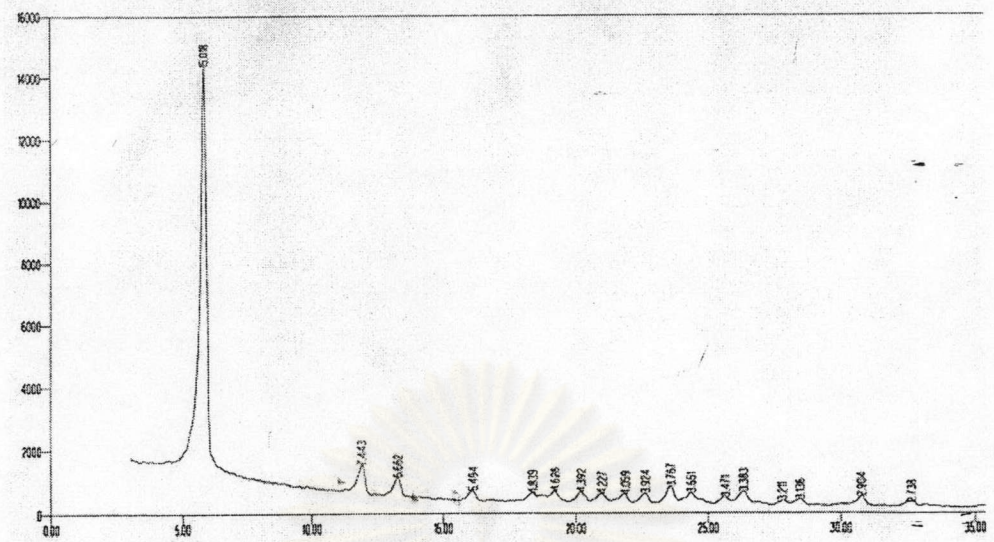


Figure 73 The X-ray diffractogram of mangostin.

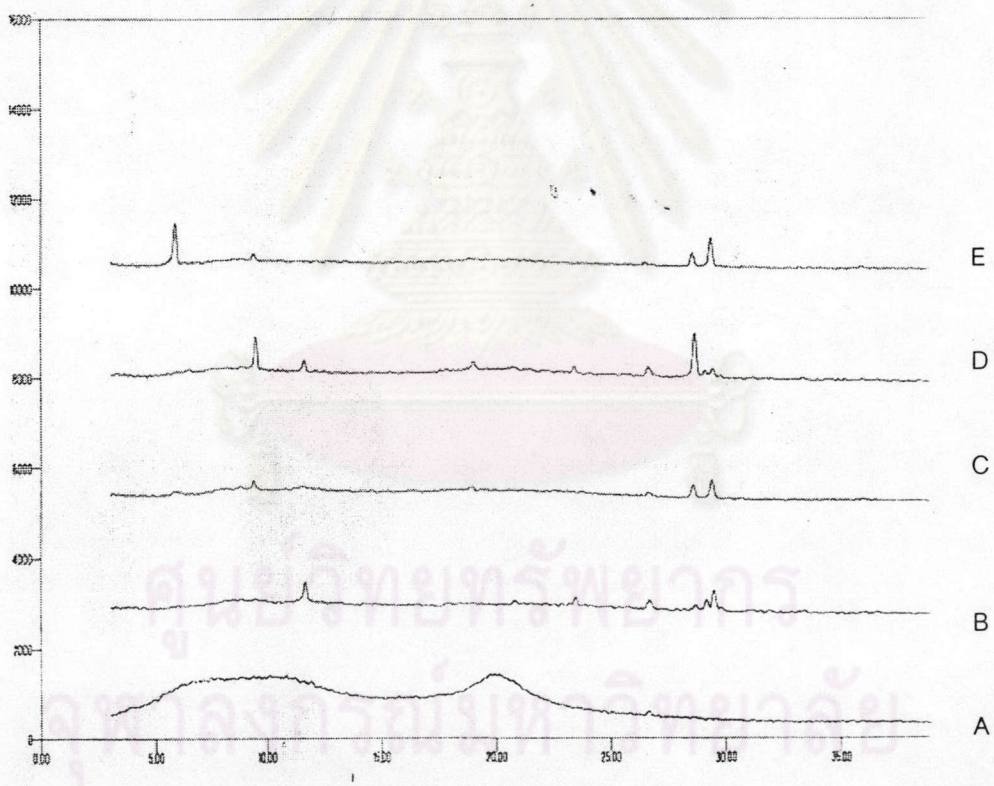
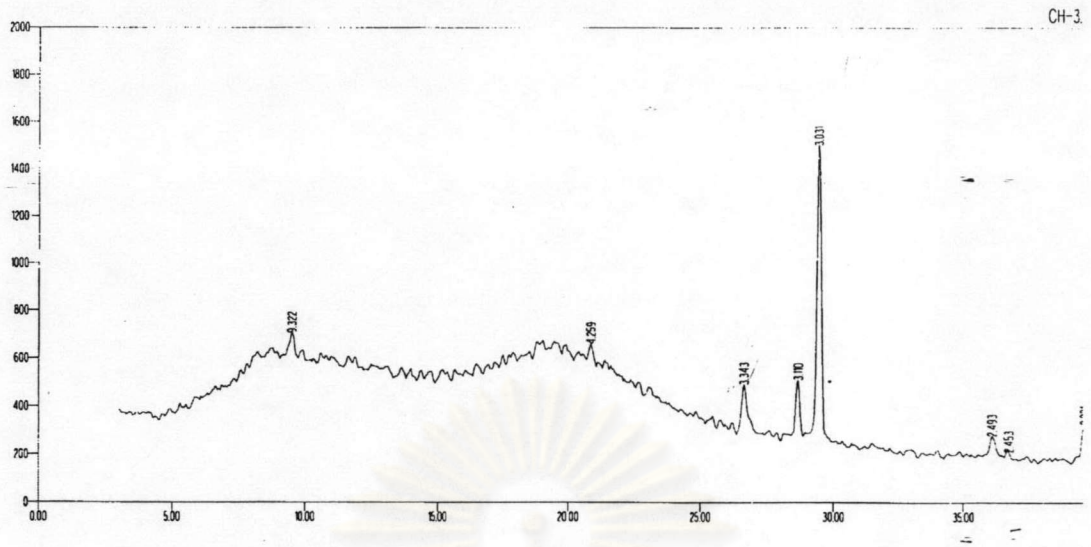
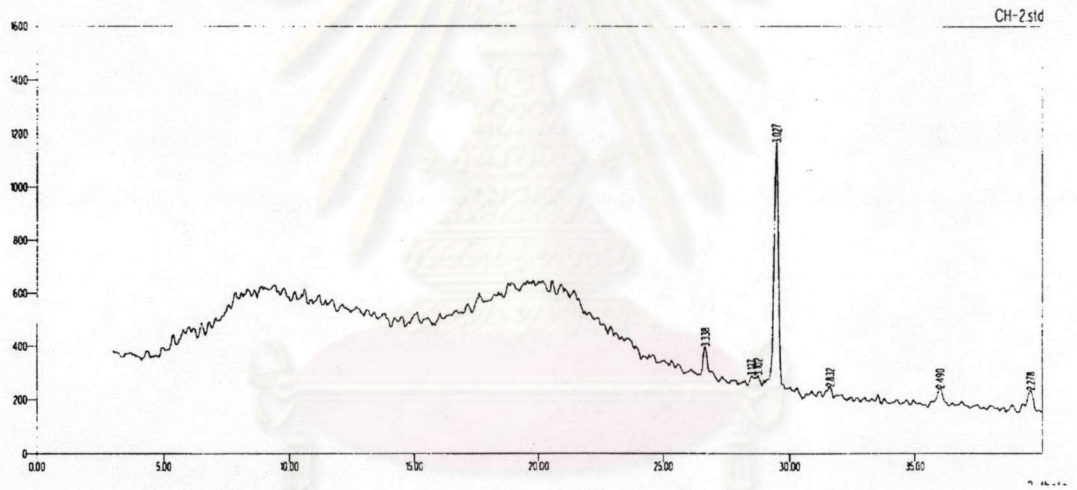


Figure 74 : The overlay x–ray diffractograms of chitosan flake and chitosan films (A) means chitosan flake, (B) means MA1 free film (C) means MA1 film containing *Garcinia mangostana* extract. (D) means ML1 free film and (E) means ML1 containing *Garcinia mangostana* extract.



a



b

Figure 75 The x–ray diffractograms of (a) HC2 free film and (b) HC2 film Containing Carcinia mangostana extract.

6.3 Water repellent and mucoadhesion properties of *Garcinia managostana* bilayered film

In the development of mucodhesive buccal film, application of a water impermeable backing layer has been considerated to restrict the access of saliva to the drug matrices, prevent drug loss from salivary flow, enhance the efficacy of the device by ensuring unidirectional drug release and prolong the residence time of buccal film as a result of the ability to withstand salivary wash off. Furthermore, the device may be conveniently and accurately applied.

Ethylcellulose, a hydrophobic polymer, has been reported to be an excellent backing material due to its low water permeability and moderate flexibility (Guo, and Cooklock, 1996). In this study, ethyl cellulose was selected to be prepared as the backing layer. It showed a good integrity with some hydrophobic films after the drying process.

In this study, water repellent and mucoadhesion properties wer evaluated by the comparison of the duration of adhesion which represented the ability to withstand artificial saliva wash off and to adhere with intestinal mucosa, respectively. The result were illustrated in Table17.

In comparision between monolayer films and bilayer films, it was found that ML1 and HC2 bilayer films exhibited a considerable longer duration time than their corresponding monolayer films significantly. It obviously indicated that the backing layer could increase the duration of adhesion and the resistability of film in resistance to washing off by artificial saliva effectively.

From the selected films for this study, the longest adhesion time could be observed in MA_1, folllowed by ML1 and HC2 from both mono layer and bilayer films (Table 17). This result corresponded with the result from the mucoadhesive test of free film in Tables 14-15. This revealed that the mucoadhesive performance was related to the ability to withstand salivary wash off of the films.

Regarding HC2 showed the lowest adhesion time, it is possible that the low water resistance of the film may be attributable to high water affinity of HPMC.

Table 17 The water repellent and mucoadhesive properties of monolayered and bilayered films of MA1 ,ML1 and HC2 containing *Garcinia mangostana* extract.

		Water repellent time (hr)		
	Formulas	Mean**	SD	%CV
Monolayered	MA1	*	0	0
film	ML1	8.051	0.14	1.79
	HC2	1.536	0.07	4.51
Bilayered	MA1	*	0	0
film	ML1	20.311	0.07	0.34
	HC2	6.050	0.22	3.69

* means more than 24 hr

* *averaged form 6 determinations

For ML1, it could be observed that under the saliva flow, it absorbed water and gradually eroded. Simulatancously, it formed a slippery gel and resulted in a decrease in mucoadhesion property.

Although the difference in adhesion time between the monolayer and bilayer of MA1 could not be determined completely due to their duration time was longer than 24 hr This might be the resulted from the ability to maintain the original shape of the device throughout the experiment of MA1 film. It could be concluded that the inclusion of a impermeable backing layer resulted in the significant increase of the duration of adhesion ($p<0.05$) (Appendix E).

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

The dissolution medium in the in vitro release study was 35% v/v ethanol in pH 6.0 isotonic phosphate buffer due to the ability to maintain the sink condition. Moreover, from the preliminary study, this medium also gave the concentration released which reach to MBC against *Staphylococcus aureus* ATCC 25923. The solubility of purified extract in the presense of 35% v/v ethanol was 1.0225 ± 0.238 mg/ml.

For in vitro release study, the results are presented in Table 24D-26D . The example of chromatogram and the calibration curve are shown in Appendix C. The drug (purified extract) release profiles were plotted according to the following equations to elucidate the mechanism of drug release.

Zero order equation $Q_t = k_0 t + b$

First order equation $Q_t = Q_0 (1\text{-}e^{-kt})$

Higuchi equation $Q_t = k_2 t^{1/2} + C$

where Q_t = amount of drug release at time t, k_0 = drug release rate constant, k_1 = first order rate constant, k_2 = diffusion rate constant, b and C = constant.

It was found that the coefficients of determination (R^2) as plotted of drug release profile according to the first order equation was relatively low for all formulas. It indicated that the drug release mechanism of all formulations was not the first order process.

The release profiles were plotted between the cumulative amount of drug release versus time and the square root of time according to the Higuchi equation. The coefficients of determination (Table 18 and Figure 77) obtained were found highest among all plots. Therefore, the release behavior of drug from the mucoadhesive films tended to be the diffusion-controlled release (Higuchi model).

The mechanism of diffusion-controlled release (Higuchi, 1963) was dominated by the penetration of the medium into the drug matrix phase through microchannels (pores), cracks and intergranular spaces of hydrated polymer, then the drug was presumed to leach out by gradually dissolving into the permeating medium phase and diffusing from the matrix along the cracks and capillary channels filled with the extracting medium. Thus, the release behavior of drug was expected to be governed by the solubility and diffusion coefficient of the drug in the polymer film and by the solubility in the release medium. This mechanism was explained by Higuchi equation as follows :

$$Q = \left[\frac{D\varepsilon}{\tau} (2A - \varepsilon C_s) C_s t \right]^{1/2}$$

where Q is the amount of drug release, D is the diffusion coefficient ; ε is the porosity factor of the matrix; τ is the tortuosity factor of the matrix; A is the total amount of drug in the matrix per unit volume; and Cs is the solubility of the drug.

The highest drug release was obtained from ML1 followed by HC2 and MA1, respectively (Figure 77).

Regarding ML1, the initial drug release was found immediately high due to the burst effect. As presented by the x-ray diffractogram of ML1. It appeared the sharp

and intense peak of drug (mangostin) at 5.84°(2θ). This result could be explained that the drug dispersed in ML1 film in crystalline form at film surface. On exposure to the medium the release occurred immediately. After that, the release was slow, which was consistent to the SEM of the surface and cross section photomicrographs of ML1, which showed the smooth, compact and nonporous structure.

As compared with MA1, the initial release with a very slightly burst effect also observed. This might be attributed to the enhancing property of chitosan salt itself to the dissolution and release of drugs as reported in many investigation .This was supported by the x-ray diffractogram of MA1 that the drug dispersed in the chitosan film in an amorphous form. Moreover, the SEM of the surface and cross section photomicrographs of MA1 presented a porous structure, which resulted in the faster drug release (Thacharodi, and Rao, 1993). This also corresponded to the swelling profile of MA1, which presented the higher rate than ML1 and resulted in the faster drug release. The result was in agreement with the previous researched by Panomsuk et al. (1995) and Lopez et al (1998).

It was obvious that acid type marked influenced drug liberation and release behavior of chitosan films.

Regarding HC2 films, which presented the lowest drug release and substantially different release profile as compared to MA1 and ML1 films. The lower amount of drug release may be due to the complexation or crosslinking effect between HPMC and Carbopol, which, led to a decrease in diffusion coefficient of mangostin in the tight matrix structure. Moreover, the gel layer and controlled release characteristics of carbopol might oppose the release of drug (Nigalaye, Adusumilli and Bolton (1990).

After exposed to the release medium, all formulas swelled. The initial erosion of HC2 and ML1 was observed at the third and fourth hour of the release, respectively. However, it was notable that the erosion could not be observed in MA1. The erosion and partial dissolution of the polymer might cause the change and complexity in the drug release mechanism.

Table 18 The release rate constants of zero order (k_0) , first order (k_1) and Higuchi model (kh) and the coefficients of determination (R^2) of three formulations of *Garcinia mangostana* mucoadhesive films.

Formulas	Sample No.	Zero order plot (k_0)	(R^2)	First order plot (k_1)	(R^2)	Higuchi plot (k_h)	(R^2)
MA1	1	0.0750	0.8273	0.0010	0.7135	1.9748	0.9337
	2	0.0840	0.8549	0.0011	0.7251	2.2023	0.9554
	3	0.0940	0.8813	0.0012	0.7382	2.4272	0.9573
	4	0.1050	0.8500	0.0012	0.6738	2.7568	0.9517
Mean		**0.0895**	**0.8534**	**0.0011**	**0.7127**	**2.3403**	**0.9495**
ML1	1	0.5100	0.9175	0.0041	0.6465	4.4804	0.9543
	2	0.1644	0.6935	0.0011	0.5394	1.4838	0.7609
	3	0.7872	0.9742	0.0049	0.6527	6.8457	0.9927
	4	0.4335	0.9598	0.0033	0.6378	3.7816	0.9842
Mean		**0.4738**	**0.8863**	**0.0034**	**0.6191**	**4.1479**	**0.9230**
HC2	1	0.0835	0.9386	0.0036	0.6985	2.5544	0.9934
	2	0.0938	0.9249	0.0037	0.6836	2.5751	0.9864
	3	0.0855	0.9377	0.0038	0.6910	2.6180	0.9926
	4	0.0824	0.9190	0.0036	0.6866	2.5423	0.9877
Mean		**0.0863**	**0.9301**	**0.0037**	**0.6899**	**2.5725**	**0.9900**

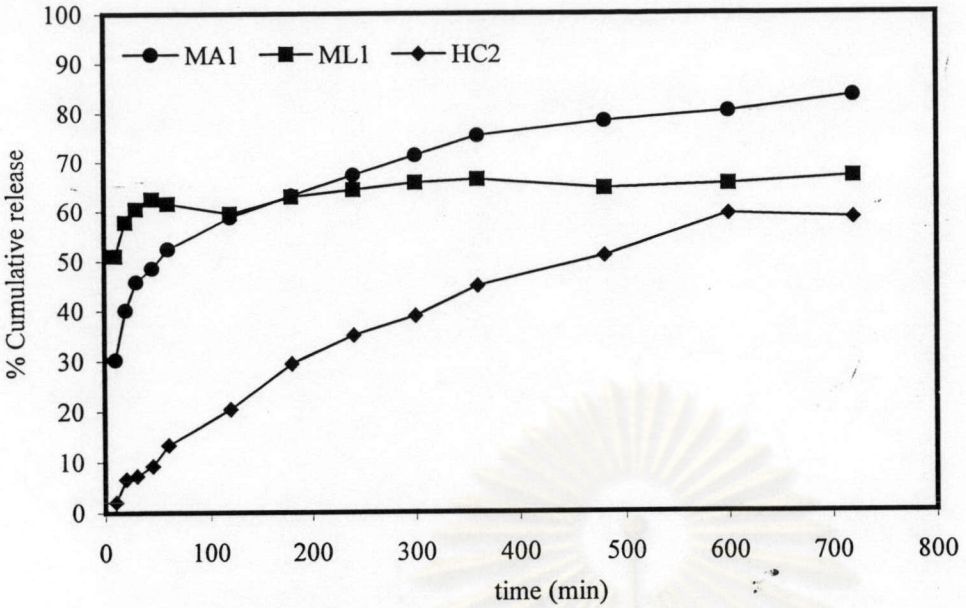


Figure 76 The release profiles of mangostin from mucoadhesive films containing *Garcinia mangostana* extract prepared from HPMC combined with 20% CP934 (HC2) and chitosan medium molecular weight prepared from 1% acetic acid (MA1) and 1% lactic acid (ML1).

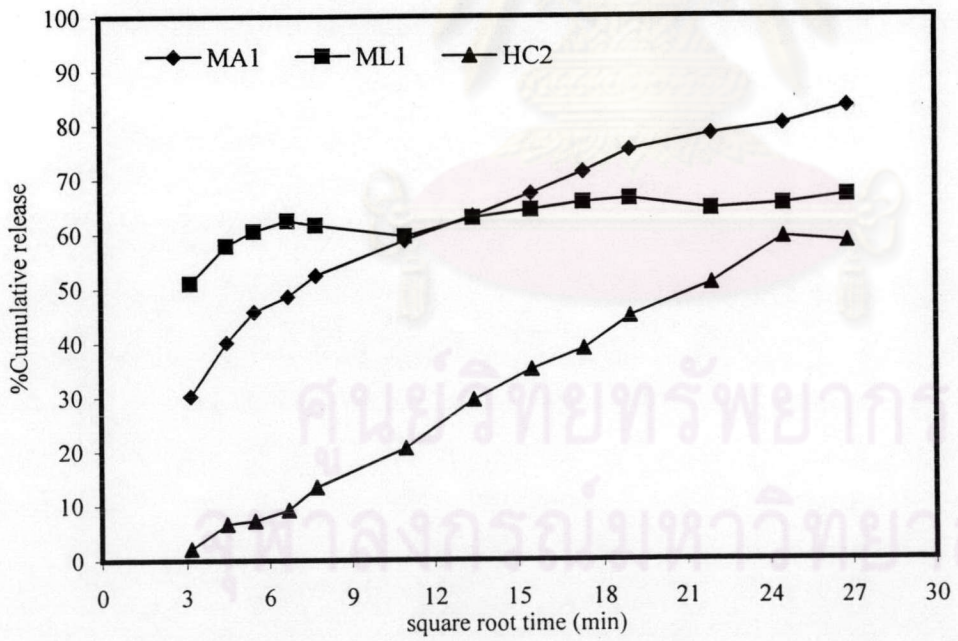


Figure 77 The release profiles of mangostin from mucoadhesive films containing *Garcinia mangostana* extract prepared from HPMC combine with 20% CP934 (HC2) and chitosan medium molecular weight prepared from 1% acetic acid (MA1) and1% lactic acid (ML1) plotted between cumulative percent release versus square root time.

8. Development of quantitative analysis of mangostin in mucoadhesive films by HPLC methed

8.1 HPLC method validation

The validation of an analytical method is the process by which performance characteristics of the method are established to meet the requirements for the intended analytical applications. The performance characteristics are expressed in terms of analytical parameters. For HPLC assay validation, these include specificity, precision, accuracy and linearity.

8.1.1 Specificity

The specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of expected components in the sample matrix.

The UV spectra (Figure 38) indicated that 243 nm was the optimal wavelength giving the highest sensitivity without interference of other constituents in purified extract. The internal standard technique was performed by determining the area ratio of mangostin to clotrimazole (internal standard) to give the complete separation, appropriate resolution and sharp peaks of all components. The methanol-water mixture of 80% by volume was used as the mobile phase. The typical chromatograms of mangostin standard solution, internal standard solution, extracted free films and system suitability data are shown in Figure 4C-10C.

The retention times of mangostin and clotrimazole were at 4.5 and 9.0 min, respectively. In addition, there was no interference from other components in the chromatogram.

8.1.2 Precision

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation).

Tables 19 and 20 illustrated the data of within-run and between-run precision, respectively. All coefficient of variation values were small, as 0.06-0.84% and 0.17-1.08%, respectively, indicating that the HPLC method used were precise for quantitation of mangostin concentrations in the range studied at any time.

8.1.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known, added amounts of analyte. The determination of accuracy of the analysis of mangostin by HPLC method was performed by analyzing the percentage analytical recoveries of three sets of eight standard solutions. The percentages of analytical recovery of each drug concentration are shown in Table 21. All percentages of analytical recovery of all drug concentrations with the mean of 100.57% and the % CV of 1.05%, indicated that this method could be used for analysis of mangostin in all concentrations studied with high accuracy.

8.1.4 Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of the analyte in samples within a given range. The linearity is usually expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte. The calibration curve data of mangostin standard solutions is shown in Table 22. The plot of mangostin concentrations versus the peak area ratios of mangostin and its internal standard (Figure 78) illustrated the linear correlation in the concentration range studied of 5-40 μg/ml. The coefficients of determination (R^2) of this line was 0.999.

Table19 The within-run precision data of mangostin by HPLC method.

Conc	Peak area ratio of mangostin					
(mcg/ml)	Set no.1	Set no.2	Set no.3	Mean	SD	%CV
0.00	0.0000	0.0000	0.0000	0.0000	0.0000	0.00
5.00	0.4977	0.5053	0.5049	0.5026	0.0042	0.84
10.00	0.9585	0.9578	0.9593	0.9585	0.0007	0.08
20.00	1.8931	1.8974	1.9028	1.8978	0.0048	0.25
30.00	2.8495	2.8490	2.8398	2.8461	0.0055	0.19
40.00	3.7945	3.7907	3.7951	3.7934	0.0024	0.06
50.00	4.7403	4.7438	4.7479	4.7440	0.0038	0.08

Table20 The between-run precision data of mangostin by HPLC method.

Conc	Peak area ratio of mangostin					
(mcg/ml)	Day1	Day2	Day3	Mean	SD	%CV
0.00	0.0000	0.0000	0.0000	0.0000	0.0000	0.00
5.00	0.5049	0.4950	0.4977	0.4992	0.0051	1.03
10.00	0.9593	0.9531	0.9497	0.9540	0.0049	0.51
20.00	1.9028	1.9012	1.8769	1.8936	0.0145	0.77
30.00	2.8398	2.7869	2.8398	2.8222	0.0305	1.08
40.00	3.7951	3.7996	3.8075	3.8007	0.0063	0.17
50.00	4.7479	4.7846	4.7952	4.7759	0.0248	0.52

Table21 The percentage analytical recovery of mangostin by HPLC method.

Mangostin concentration (mcg/ml)	(mcg/ml) (mcg/ml)	% Analytical recovery
5.00	5.13	102.65
10.00	9.98	99.82
20.00	19.97	99.87
30.00	30.06	100.21
40.00	40.14	100.35
50.00	50.25	100.51
	Mean	100.57
	SD	1.05
	%CV	1.05

Table 22 Data for calibration curve of mangostin by HPLC method.

Conc	Peak area ratio of mangostin					
(mcg/ml)	Set No.1	Set No.2	Set No.3	Mean	SD	%CV
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.00
5	0.4977	0.5053	0.5049	0.5026	0.0042	0.84
10	0.9585	0.9578	0.9593	0.9585	0.0007	0.08
15	1.4327	1.4367	1.4383	1.4359	0.0029	0.20
20	1.8931	1.8974	1.9028	1.8978	0.0048	0.25
25	2.3918	2.3929	2.3933	2.3927	0.0008	0.03
30	2.8495	2.8490	2.8398	2.8461	0.0055	0.19
35	3.3324	3.3416	3.3247	3.3329	0.0085	0.25
40	3.7945	3.7907	3.7951	3.7934	0.0024	0.06

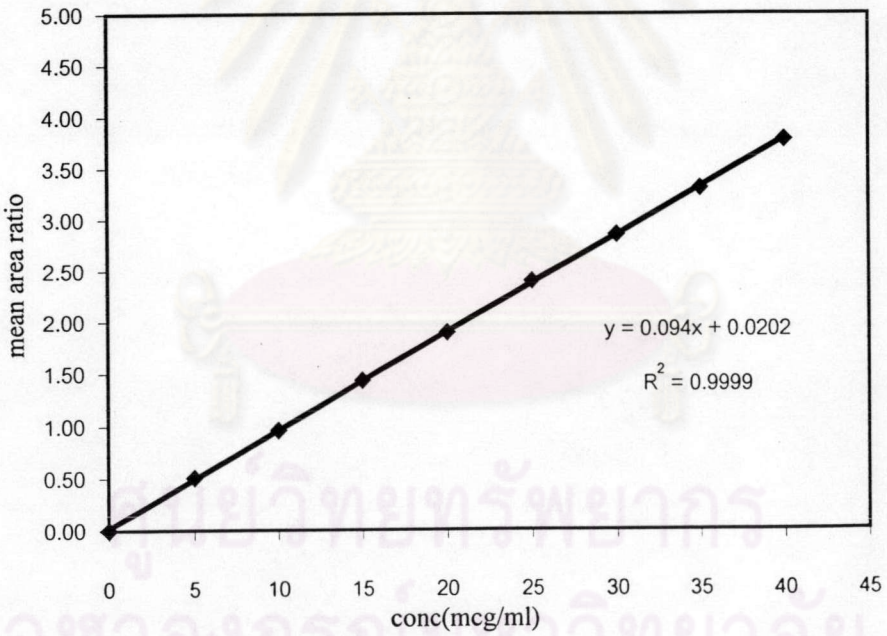


Figure78 Calibration curve of mangostin assayed by HPLC method.

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

The stability study of *Garcinia mangostana* Linn. extract buccal mucoadhesive films was performed by triplicate samples of three formulations, placed into amber glass vials which tighly sealed with rubber closures and aluminium caps. The storage condition is accelerated at 40°C and 75%/RH (Cartensen, 1990). It was claimed that the product which showed good stability in the accelerated condition should possess 2 years of shelf-life.

The analytical method, which employed in this investigation was HPLC method as previously described. The amount of mangostin containing in mucoadhesive films were analysed at the initial time, the first, the second and the third month period and calculated as the average percentage labeled amounts as shown in Table 23. In addition, the percentage loss of mangostin after the exposure to heat and high humidity at the end of the storage was also calculated.

As a result, all mucoadhesive films appeared to be stable due to their percentage loss after storage was less than 10% of the initial value. It was found that MA_1 degraded with the highest extent, followed by ML_1 that showed a slightly higher percentage loss than HC2. During the storage period, MA1 exhibited a relatively greater difference of loss between months. However, its percentage loss was only 8.48% at the end of the storage.

Since there has never been investigation on degradation kinetics of mangostin before, the interpretation of the results is limited. Moreover, its degraded products have never been reported.

Therefore, it might be postulated from the obtained data that pH, ionic strength and counter ions of the various acids using in the film preparation might influence on the stability of mangostin. Moreover, the crystalline state of mangostin in the film and the interaction between mangostin and chitosan salt molecule might play a certain role.

However, films produced from chitosan are more stable in high humidity than other polymers (Skaugrud, 1989).

As regarding to HC2 formula, the interaction between HPMC and carbopol and the interaction between mangostin molecule and these polymers might affect the degradation of mangostin, which resulting in the most stable formulation of HC2 film.

Table23 The percentage labeled amount of mangostin mucoadhesive films before and after the stability test.

Formulation	% Labeled amount of mangostin											
	MA1				ML1				HC2			
	Time (month)				Time (month)				Time (month)			
Sample No	0	1	2	3	0	1	2	3	0	1	2	3
1	99.1616	96.0098	90.5376	87.0575	98.8811	94.4084	94.0370	89.6762	100.6065	99.8793	96.8255	95.7226
2	97.0594	97.6384	95.3350	89.0664	100.1736	97.0533	95.0121	95.5683	98.4252	97.3103	94.4117	93.1350
3	95.2499	92.9318	90.3225	90.6416	97.1601	98.8645	96.9176	94.1812	101.8940	98.7329	99.4640	97.8037
Mean	97.1570	95.5266	92.0651	88.9218	98.7382	96.7754	95.3222	93.1419	100.3086	98.6408	96.9004	95.5538
SD	1.9577	2.3902	2.8339	2.6470	1.5118	2.2410	1.4651	3.0805	1.7534	1.2869	2.5270	2.6596
%CV	2.6625	2.5021	3.0782	2.9583	1.5312	2.3157	1.5370	3.3073	1.7481	1.3047	2.6078	2.7893
% Loss of mangostin		8.47609				5.667836				4.740184		

$$\% \text{ Loss of mangostin} = \frac{\text{Initial \%labeled amount - Final \%labeled amount}}{\text{Initial \%labeled amount}} \times 100$$