CHAPTER IV
RESULTS AND DISCUSSION

4.1 Weight

The effects of immersion time of sodium alginate solution (Na-AG) on coated gauze were investigated. In the method, gauze was pre-dipped in 3% w/v $CaCl_2$ 5 seconds and immersed in Na-AG solution with varied immersion time (5, 10, 20, 40, 60, 120 and 300 seconds). Eventually, the coated gauze was again immersed in 3% w/v $CaCl_2$ to crosslink the alginate. The weight of coated gauzes was estimated. The result shows in Fig. 4.1, it shows relationship between immersion times and percentage of increasing weight of coated gauze. The weight of coated gauze increased with the increase of immersion time. However, the weight was constant after 120 seconds. The immersion time also affected on surface of coated gauze (Fig. 4.2). At the lower immersion time, the coated gauze surface was smooth. In contrast, the surface had the rough surface when the coated gauzes were soaked in Na-AG more than 60 seconds. From the results, the immersion time at 60 seconds was used for the further study.

The effect of sodium alginate concentration on physical properties was also examined. In a similar method, gauze was pre-dipped in 3% w/v $CaCl_2$ 5 seconds and immersed in varied concentrations of Na-AG solution at 0.0625%, 0.25%, 0.5%, 1.0% and 3.0% w/v. Finally, the coated gauze was again immersed in 3% w/v $CaCl_2$. The result shows in Fig. 4.3. Likewise, the concentration of Na-AG affected on surface of coated gauzes. The coated gauze with Na-AG concentrations more than 1% w/v showed the rough surfaces. In contrast, low concentration of Na-AG solution (less than 1.0% w/v Na- AG solution) had smooth and homogenous coated gauzes' surfaces.

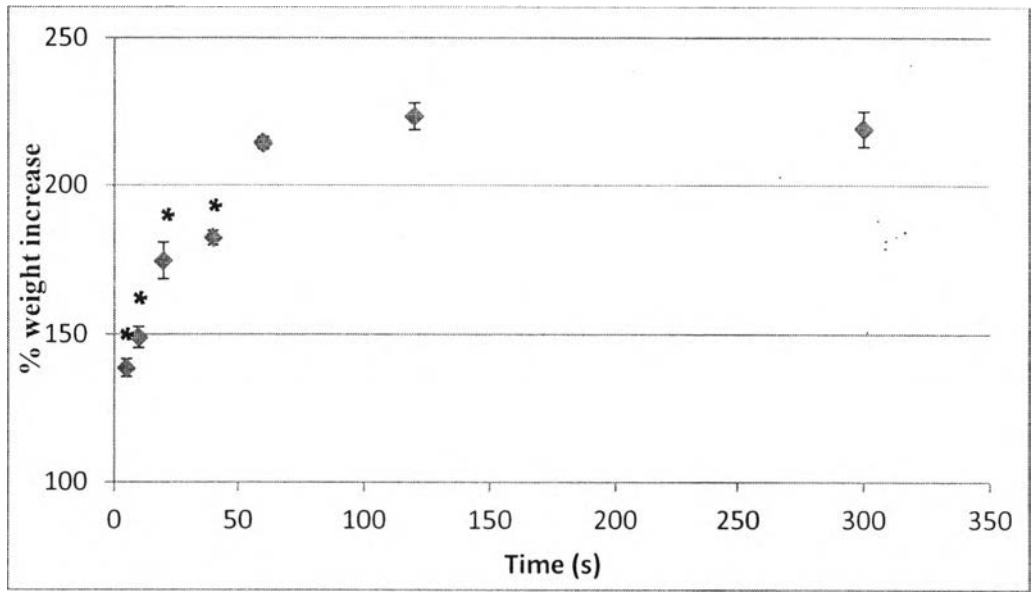


Figure 4.1 The percentage of sample weight after coating by different immersion times. *Significance at $p < 0.05$with respect to coated gauze with AG solution at 60 seconds.

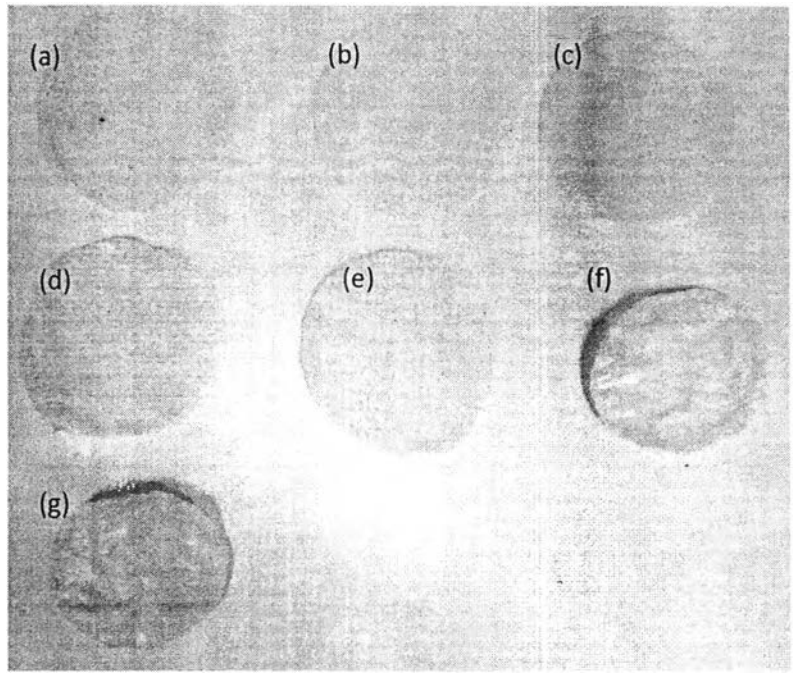


Figure 4.2 The appearance of coated gauze with difference of immersed time; 5 seconds (a), 10 seconds (b), 20 seconds (c), 40 seconds (d), 1 minute (e), 3 minutes (f), and 5 minutes (g).

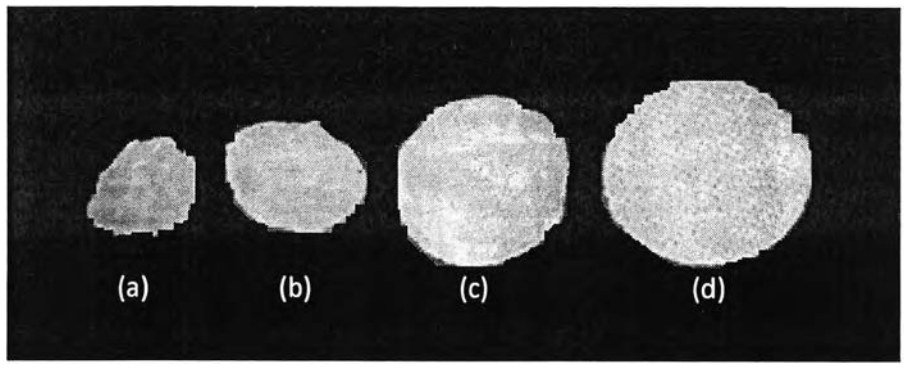


Figure 4.3 The appearance of coated gauze with difference of Na-AG concentration at 5.0% (a.), 3.0% (b.), 1.0%(c) and 0.5% w/v (d.).

4.2 Swelling Behaviors

Sodium alginate can form gel structure by using different ways. In this research, we used ionic crosslink to get alginate gel because of low toxicity. The gel formation was occurred by interaction between alginate and divalent cations. The divalent cations were complex with alginate chains; $CaCl_2$ was as a crosslink agent. The effect of sodium alginate concentration on swelling behaviors was investigated. The coated gauzes were soaked in DI water at 10, 20, 40, 120, 420 and 1440 minutes. After that the dried coated gauze and coated gauze weight was measure at any swelling time and the percentages of swelling was calculated following the equation 1. The percentages of swelling shows in Fig. 4.4, it shows the swelling kinetics of calcium alginate coated gauzes. The result shows that initial gauze absorbed water about 1000% weight of material and Na-AG concentrations affected on swelling behaviors of coated gauze. The swelling of coated gauze increased with the increase concentrations of Na-AG. However, the coated gauzes with 0.5%, 1.0%, and 3.0% w/v displayed significantly different swelling from initial gauze. In addition, the coated gauze with 1.0% Na-AG was not significantly different from coated gauze with 0.5% Na-AG. From the result, the maximum swelling was observed at 40 minute. Afterwards, the swelling ratio was slightly decreased and then remained stable. A little of alginate dissolved in water thus amount of alginate had affected

with swelling. Therefore the swelling ratio slightly decreased because of dissolving of alginate. This result was similar to J. Kim *et al.* (2008) that is noted that the maximum swelling increased with increase concentration of alginate. Another factor that affected with lower swelling behavior was "syneresis". The syneresis is a macroscopic phenomenon that relate with reduction of gel's volume with release water. Some study, Straccia *et al.* (2014), reported that synersis was strongly related to the amount of calcium ions within the gel. By the way the slower swelling rate of 3% w/v alginate solution was observed at 60 min, but against only 20 min in the case of 0.0625% w/v alginate solution.

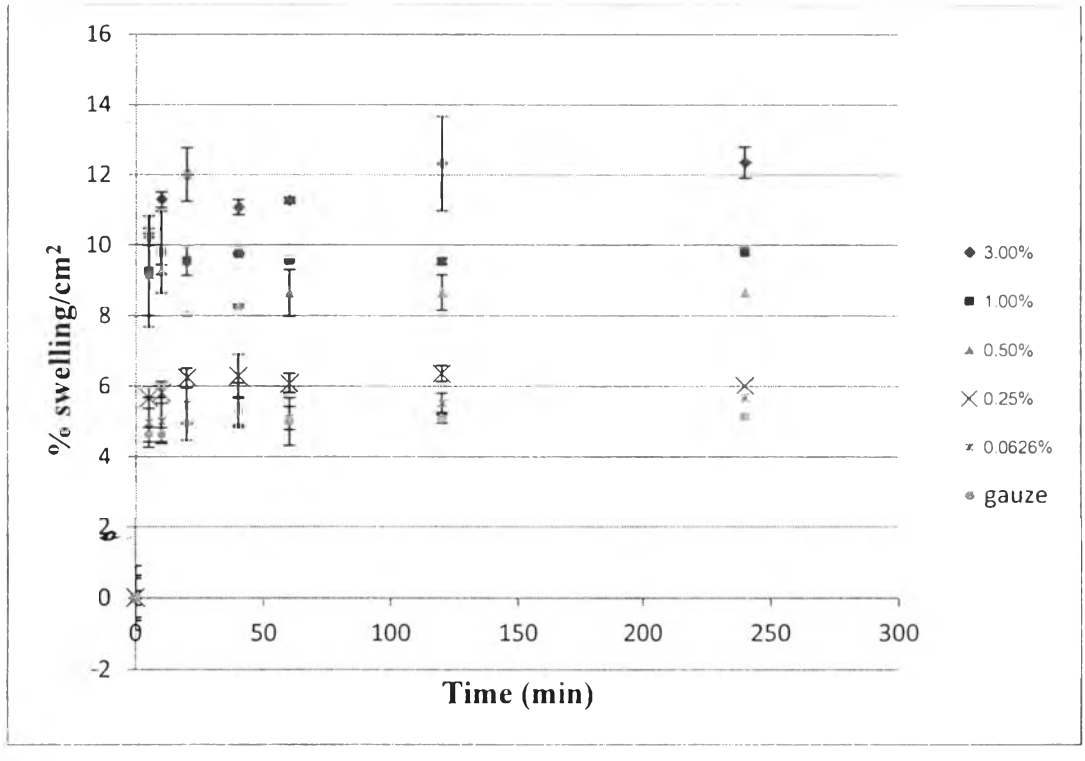


Figure 4.4 Swelling behaviors of coated gauzes with varied alginate concentration of initial gauze (●), 0.0626% (✳), 0.25% (×), 0.50% (▲), 1.0% (■) and 3.0% (♦) w/v alginate solution.

4.3 Oxygen Permeability

Oxygen permeability is one of important factor for wound dressing. Because, it helps to enhance body's healing process, tissue homoeostasis, energy production, cell membrane maintenance, mitochondrial function and cellular repair (Dias *et al.*, 2013). The oxygen permeability was determined by estimating the dissoloved oxygen. That followed by Winkler's method, 1888. In our experimentation, negative control was the flask without infiltrating oxygen. The negative control use to determine initial oxygen in boiled DI water. About positive control, it was an opened flask. Each of wound dressing sample covered flask, which comprise boiled DI water. All of flasks were kept in an opened environment for 24 hours. The amount of dissolve oxygen showed in Fig. 4.5. All of samples showed dissolved oxygen that deduced by a negative control. From the result, the amount of dissolved oxygen represented oxygen-permeable efficiency of wound dressing. The initial gauze had higher oxygen permeability compared with coated gauze with any Na-AG concentration. The increase concentration of Na-AG displayed significantly decreased of the oxygen-permeable efficiency.

The effect of mangosteen extracts on oxygen permeability of coated gauzes was also investigated. The result shows in Fig. 4.6. When coated gauze combined with MT, the oxygen permeability slightly decreased, the amount of oxygen dissolved in water of 0.01%, 0.02% and 0.06% MT was 0.43, 0.39 and 0.43, respectively. However, the decreases of oxygen permeability were not significant. Thus, the increase of MT did not affect on oxygen permeability of coated gauze dressing.

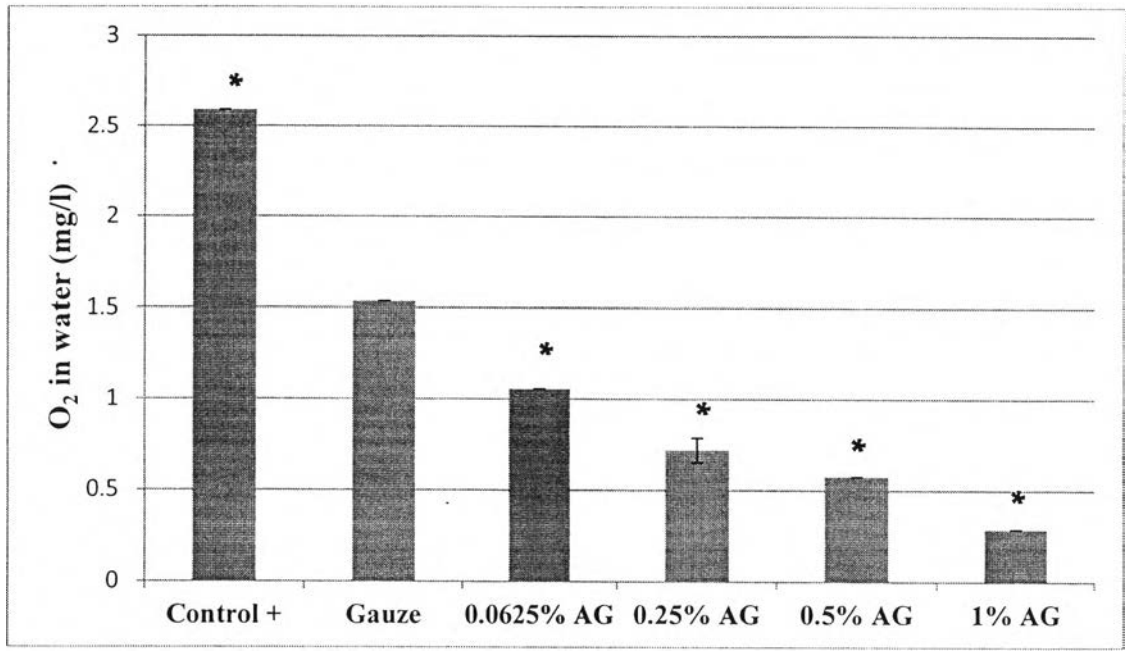


Figure 4.5 The effect of concentrations of calcium alginate solution on oxygen permeability. *Significance at $p < 0.05$with respect to initial gauze.

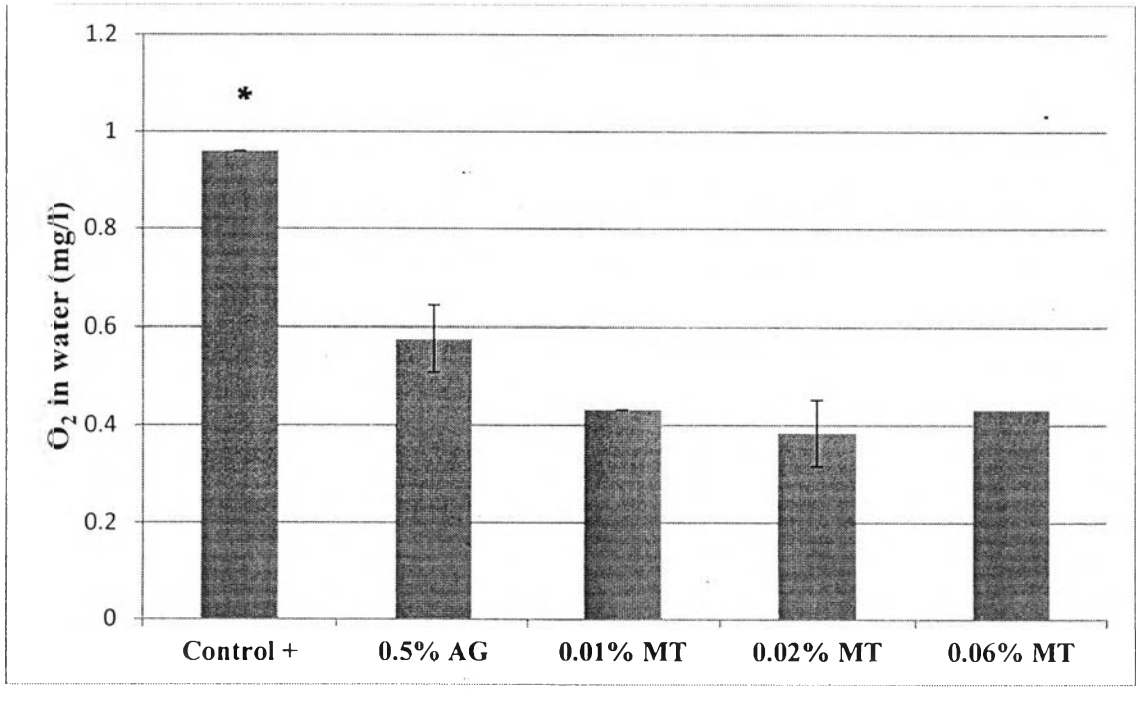


Figure 4.6 The effect of coated gauzes with mangosteen extracts on oxygen permeability. *Significance at $p < 0.05$with respect to coated gauze by 0.5% w/v AG.

4.4 In *Vitro* Blood Clotting Test

Homeostasis, blood coagulation, is a physiological process, that involving in coagulation factors. The factors finally lead to activate factor X to Xa. The factor Xa acts as a catalyst, which converse prothrombin to thrombin. The thrombin activates fibrinogen to fibrin monomer. Then the polymerization of fibrin monomer produces fibers that help to hold platelets and form haemostatic clots (Shih *et al.*, 2006).

The blood clotting was investigated by the action of antithrombogenic activity of a material on human blood (Shih *et al.*, 2006). The antithrombogenic was represented from the blood clotting index (BCI). A lower BCI value indicates a blood clotting. Fig 4.7 shows the effect of calcium alginate on BCI value. A decrease in BCI value was found for the increase of calcium alginate concentration. The BCI value was significantly decrease compared with initial gauze, BCI value was reduced from 36% (initial gauze) to 11% (coated gauze with 3% Ca-AG). Therefore calcium alginate enhanced the haemostatic clots. The result was similar to Ding *et al.,* 2013 and Blair *et al.*, (1990). In additions, previous researchers (Nelsestuen *et al.*, 1983) report the calcium can improve the haemostatic clots because it acts as a catalyst to active the clot factors.

The effect of mangosteen extracts on blood clotting was studied. The result shows in Fig. 4.8, compared BCI value of a calcium alginate dressing and the dressing with MT. An increase mangosteen extracts did not show the significant decrease of the BCI value.

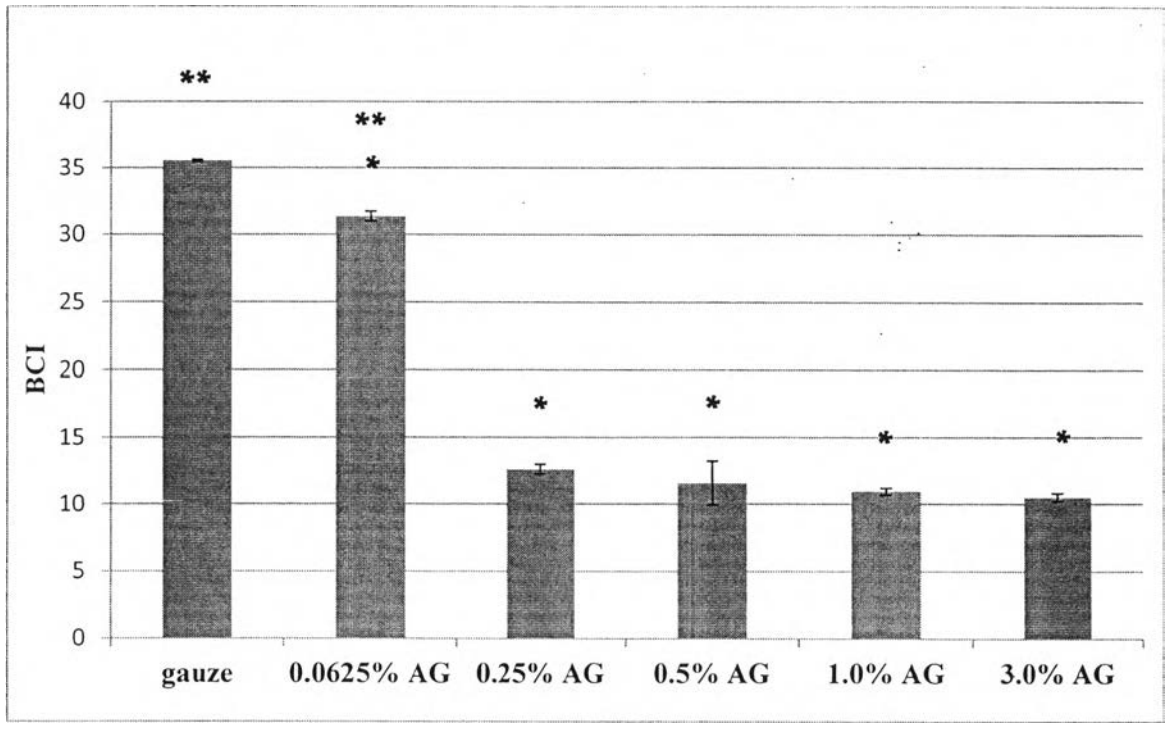


Figure 4.7 The effect of concentrations of calcium alginate solution on blood clotting index (BCI). *Significance at $p< 0.05$with respect to initial gauze. **Significance at $p< 0.05$with respect to coated gauze by 0.5% w/v AG.

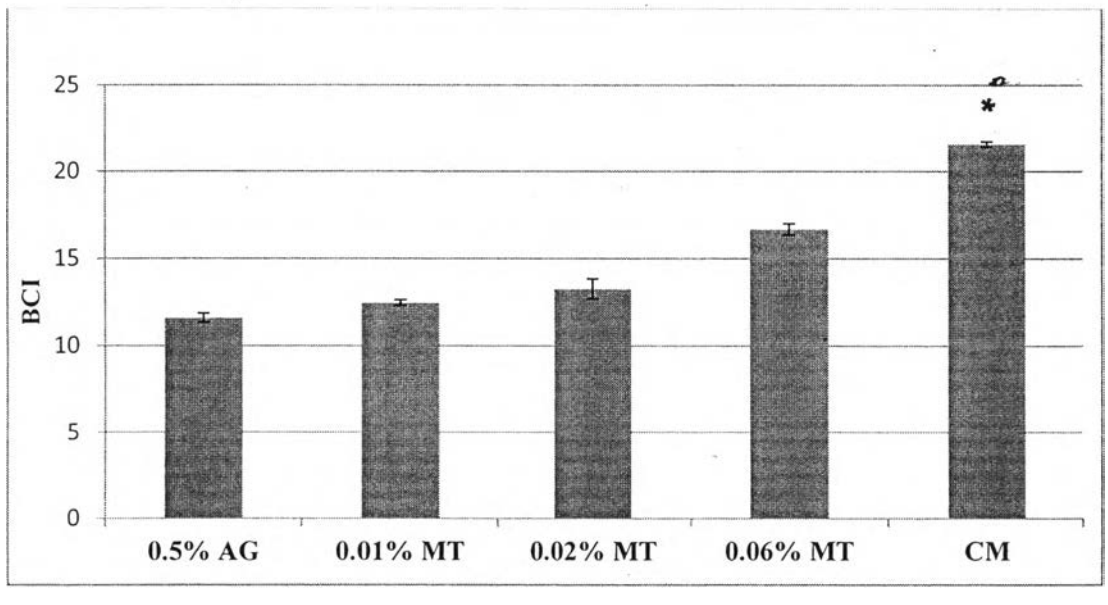


Figure 4.8 The effect of coated gauzes with mangosteen extracts on blood clotting index (BCI). *Significance at $p< 0.05$with respect to coated gauze by 0.5% w/v AG.

4.5 In *Vitro* Drug Release Test

UV-Vis spectrophotometer was used to monitor of the amount of mangosteen extracts at λ_{max} =380 nm^{-1}. The wound dressings which coated by calcium alginate and mangosteen extracts were immersed in PBS and acetate buffer. The PBS solution, pH 7.4, represented solution body of human's blood and the acetate buffer, pH 5.5, represented human's skin. Both solutions needed tween 80 and methanol to dissolve the extracts because of a non-water solubulity of mangosteen extracts. Fig 4.9 displays the relationship between released mangosteen extracts and time in acetate buffer and PBS at a temperature of 37 °C. It can be clearly observe that released amount of mangosteen extracts increased with the increase of time but after 50 hours the released MT remained constant. In a similar way, the released amount of mangosteen extracts significantly increased with increase % MT on coated gauze dressing. As demonstrated, all dressing (both acetate buffer and PBS solution) showed a quickreleased rate at first 6 hours. After that, the rate slightly decreased and remained constant after 50 hours. At the first 1 day, the results showed the cumulative release of mangosteen extract acetate buffer were 36, 41 and 43 g/ml (0.01%, 0.02% and 0.06 %w/w, respectively) and the concentrations in PBS solution were 68, 71 and 83 g/ml (0.01%, 0.02% and 0.06 %w/w, respectively). After 50 hours, the released mangosteen extracts were constant at 50, 52 and 54 g/ml (0.01%, 0.02% and 0.06 %w/w, respectively) in acetate buffer, and 86, 93 and 98 g/ml (0.01%, 0.02% and 0.06 %w/w, respectively) in PBS solution.

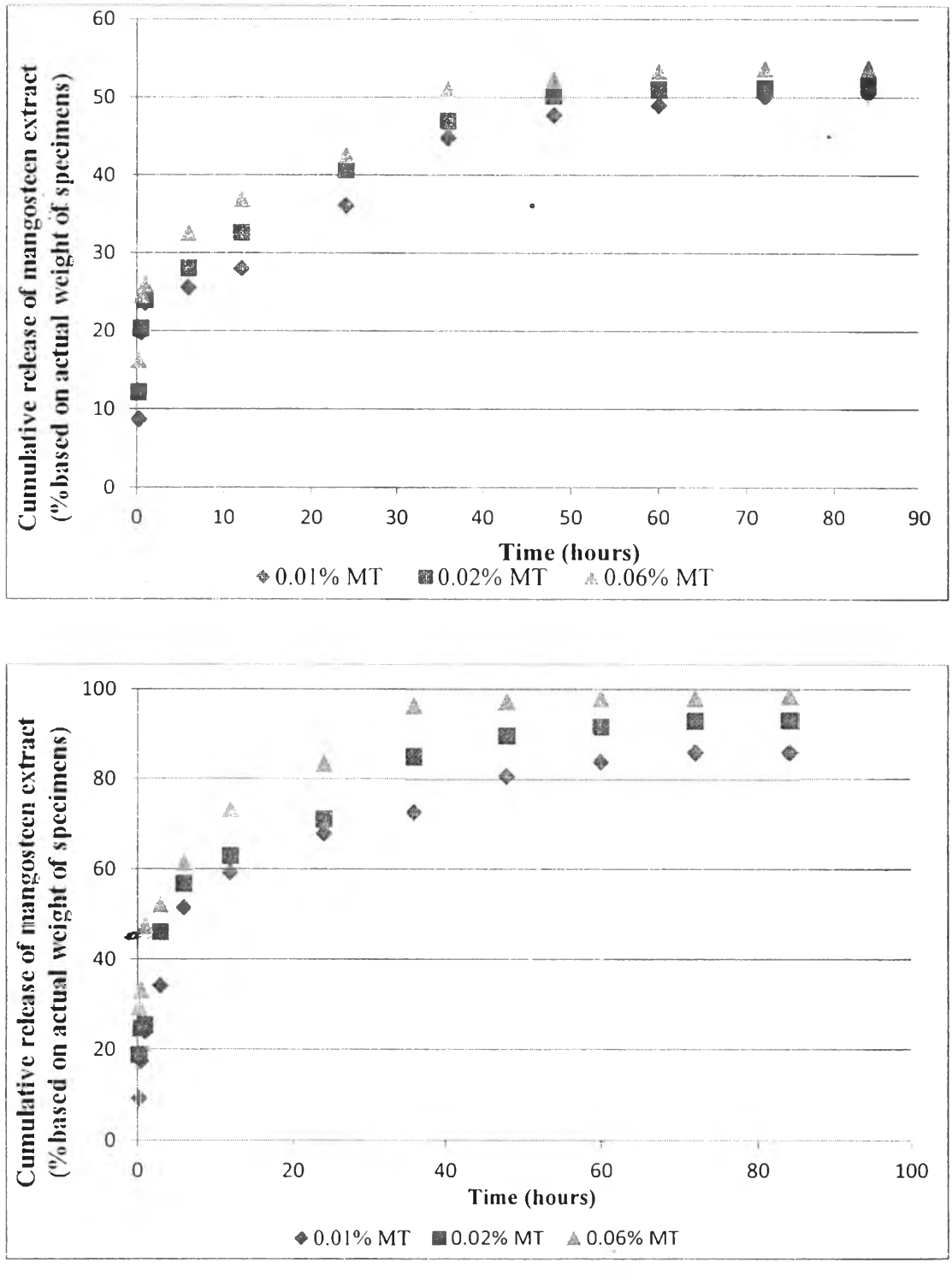


Figure 4.9 The kinetic of released mangosteen extract of coated gauze with 0.01% (♦), 0.02% (■) and 0.06% MT (▲) in acetate buffer (a) and PBS solution (b).

4.6 MIC and MBC

Antibacterial properties were investigated by using MIC and MBC method. Generally, the MIC and MBC use to present the efficiency of antibacterial agents. However, the extracts of mangosteen from several sources did not have standardization. So, the efficiency of the extract in antibacterial properties was examined. The MIC is a lowest concentration that antimicrobial can inhibit the visible growth of bacteria and MBC is a lowest concentration that antimicrobial can kill bacteria. MIC and MBC value show in table 4.1. The MIC for the stain *S. aureus,* MRSA, *S. epidermidis, E. faecalis, E. coil,* MDR *A. beau* and VRE was 0.391, 1.563, 1.563, 12.5, 1.563, 12.5 and 25 ug/ml, respectively. The MBC for the stain *S. aureus,* MRSA, *S. epidermidis, E. faecalis, E. coil,* MDR *A. Baumannii* and VRE was 12.5, 12.5, 1.563, 50, 100, 50 and 100 ug/ml, respectively. This result was similarly found in Mullika *et al.*, (2009). They reported the MIC for the stain *S. aureus,* MRSA and *S. epidermidis* was 0.39, 0.39 and 0.39 ug/ml, respectively. The MBC for the stain *S. aureus,* MRSA and *S. epidermidis* was 1.56, 0.39 and 1.56 ug/ml, respectively.

From the vitro drug release test, the released MT concentration was higher than MIC and MBC values. Thus the coated gauze with MT can inhibit the bacteria and the antibacterial efficiency of the gauzes shows in next parts.

Table 4.1 The MIC and MBC value of *Garcinia Mangostana* extract

	Positive Bacterial				Negative Bacterial		
	S. aureus	MRSA	*S. epidermidis*	*E. faecalis*	*E. coil*	MDR *A. Baumannii*	VRE
MIC (μg/ml)	0.391	1.563	1.563	12.5	1.563	12.5	25
MBC (μg/ml)	12.5	12.5	1.563	50	100	50	100

4.7 Disk Diffusion Method

In order to investigate the antibacterial properties, all of coated gauze was tested by dish diffusion method. The coated gauzes with 0, 0.01%, 0.02% and 0.06% MT were plated on bacterial agar plates. The clean zone of each sample presented the efficiency of coated gauze against the bacteria. In our experiment, the representatives of Gram-positive bacteria were *S. aureus*, MRSA, *S. epidermidis* and *E. faecalis* and Gram-negative bacteria were *E. coil*, MDR *A. beau* and VRE.

The results showed the coated gauze with pure Ca-AG displayed the inhibition only gram positive bacteria (Fig. 4.9); *S. aureus*, MRSA and *S. epidermidis*. The average diameters of clear zone were 3, 2 and 6 mm, respectively. This result was similar to Chomnawang *et al.*, 2009(Chomnawang *et al.*, 2009). When the coated gauze coordinated with MT, we noticed all of the dressing agented the bacteria. From the Fig. 4.9, the mean clear zone diameters of 0.01% MT for the stain S. aureus, E. coil, MRSA S. epidermidis and MDR A. *Baumannii* were 13, 5, 10.8 and 11.5 mm, respectively. We noticed that dressing with 0.01% MT did not inhibit all types of bacteria. Thus, we increased concentration of the extract to improve their efficiency. The mean diameters of 0.02% MT for the stain *S. aureus, E. coil,* MRSA, *S. epidermidis,* MDR *A. Baumannii,* VRE and *E. faecalis* were 16, 6.5, 12, 17, 6, 2 and 1.5 mm, respectively and the diameter of 0.06% MT significantly increased to 30.5, 9.5, 14.5, 35, 8.5, 4 and 3 mm, respectively. The results showed the increase of extract could significantly enhance size of clear zones. Thus, the antibacterial efficiency of dressing was improved by mangosteen extracts.

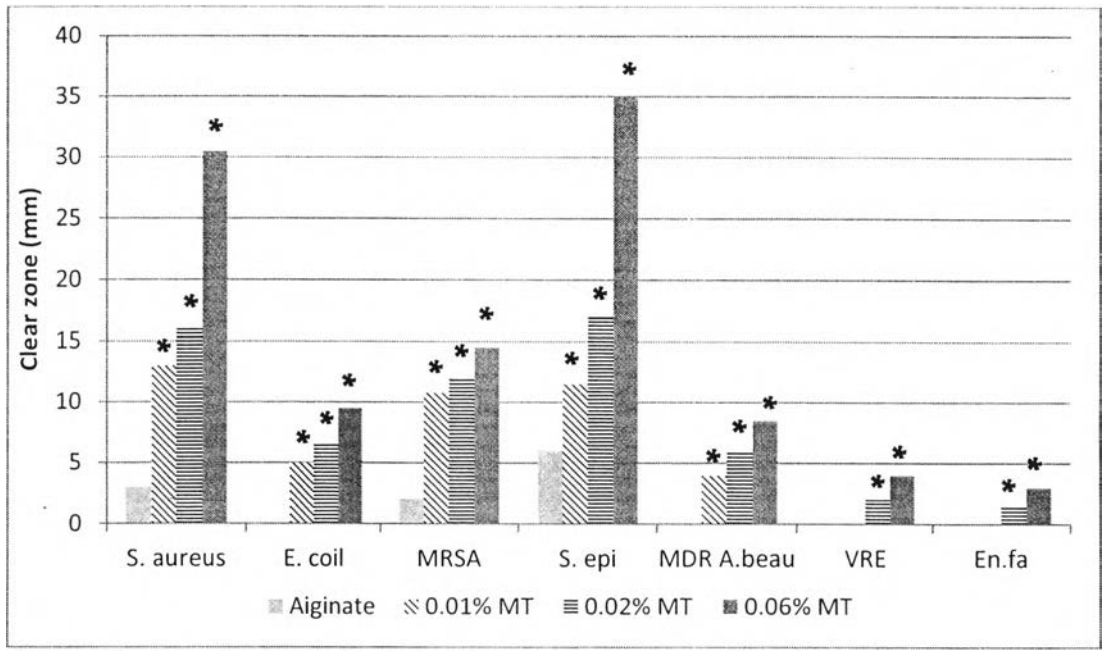


Figure 4.9 The mean diameter clear zones of coated gauze with varied MT agented all of the bacterial: *S. aureus, E. coil,* MRSA, *S. epidermidis,* MDR *A. Baumannii,* VRE and *E. faecalis.* *Significance at $p < 0.05$with respect to coated gauze by 0.5% w/v AG.

4.8 Bacterial Reduction by Counting the Colony

The bacteria reduction method used to confirm the antibacterial efficiency of the mangosteen extracts on the coated gauze dressing. All of the dressings were soaked in an active bacterial solution. Then, the solution was sampled and dropped the sampling onto the bacterial agar plates. Finally, the colony of any bacteria was counted. The amount of bacterial colony indicated the antibacterial efficiency of the wound dressing.

In Fig 4.10 (a-e) displays the amount of the colony of MRSA, *E. Faecalis, E. Coil, P. Aeruginosa* and MDR *A. Baumannii*, respectively. As shown, some control-samples had higher bacteria's colony than at the initial time (0 hour) because these bacteria could growth in experimental conditions. For the initial gauze, it was detected the colony of all the bacteria. That mean the initial gauze dressing did not against all of the bacteria. From the results, pure calcium alginate did not completely

inhibit positive bacteria, however, the negative bacteria was fully inhibited (after 24 hours) by pure calcium alginate. This result was similar to Wiegand *et.al* (2009). They reported that alginate wound dressing fully inhibited the growth of negative bacteria (*E. Coil and P. Aeruginosa*) after 24 hour. However, the alginate was not fully inhibited the growth of positive bacteria (*S. aureus*). The reason of this result was reported by Qin *et.al* (2005). They reported the calcium alginate can absorb exudates by exchanging ion between calcium ion and sodium ion so alginate transforms water-insoluble to water-soluble form. Thus, calcium alginate wound dressing absorbed exudate and swelled. It may trap any bacteria in closed chains. This can help to reduce bacterial growth. Another factor which calcium alginate shows the decrease of bacterial colony involved in absorption. Alginate absorbed bacteria-solution that caused the condition was not appreciate for bacteria. Consequently, calcium alginate dressing showed the decrease of the colony.

To improve the efficiency of coated gauze dressing, the mangosteen was incorporated in the dressing. No bacterial growth was detected after 24 hours for coated gauze with MT. The increase of MT displays enhancing the efficiency. For the alginate dressing, the colony was still detected after 12 hours. But there were not bacterial colony were detected for coated gauze combined with MT.

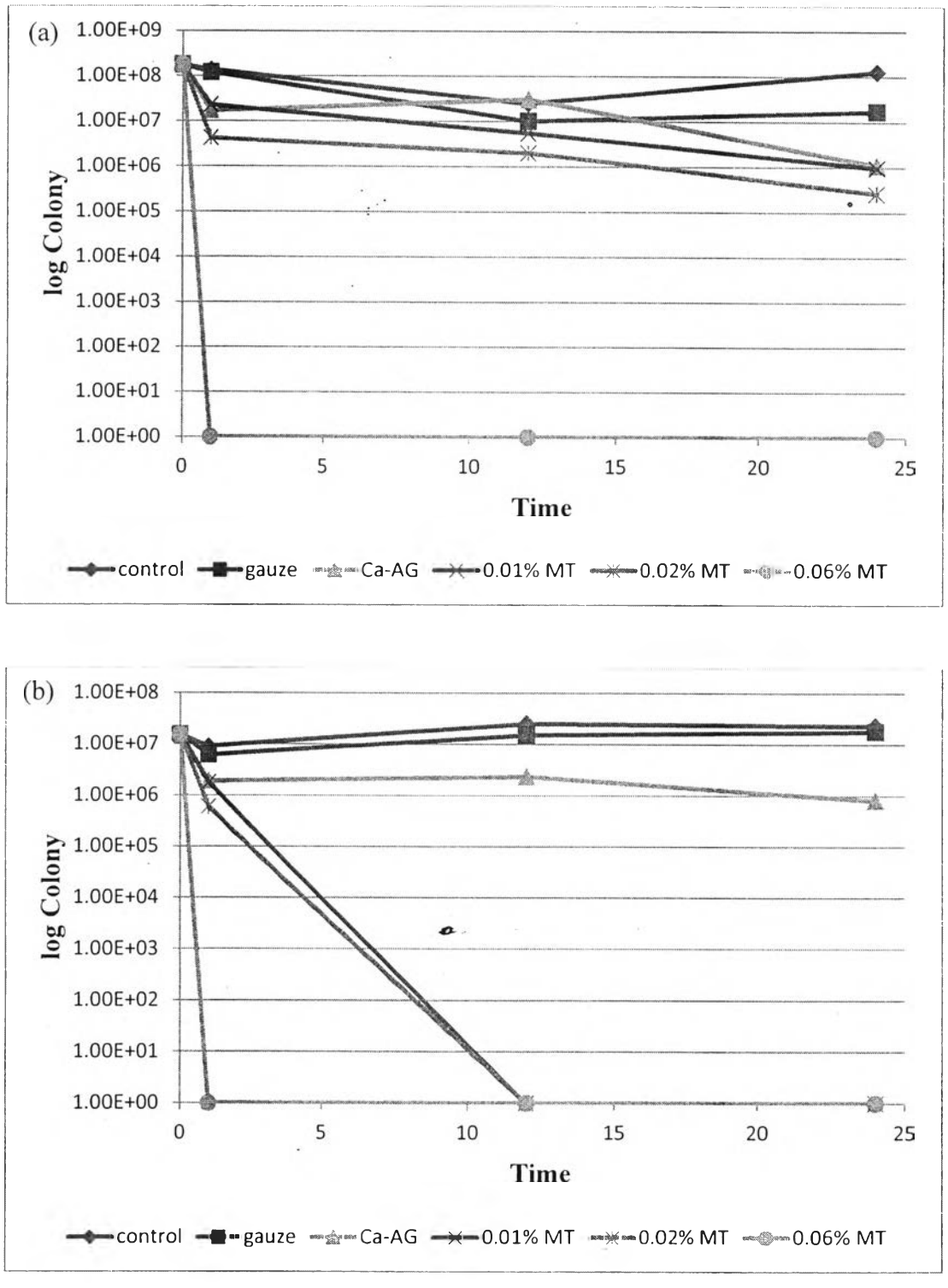
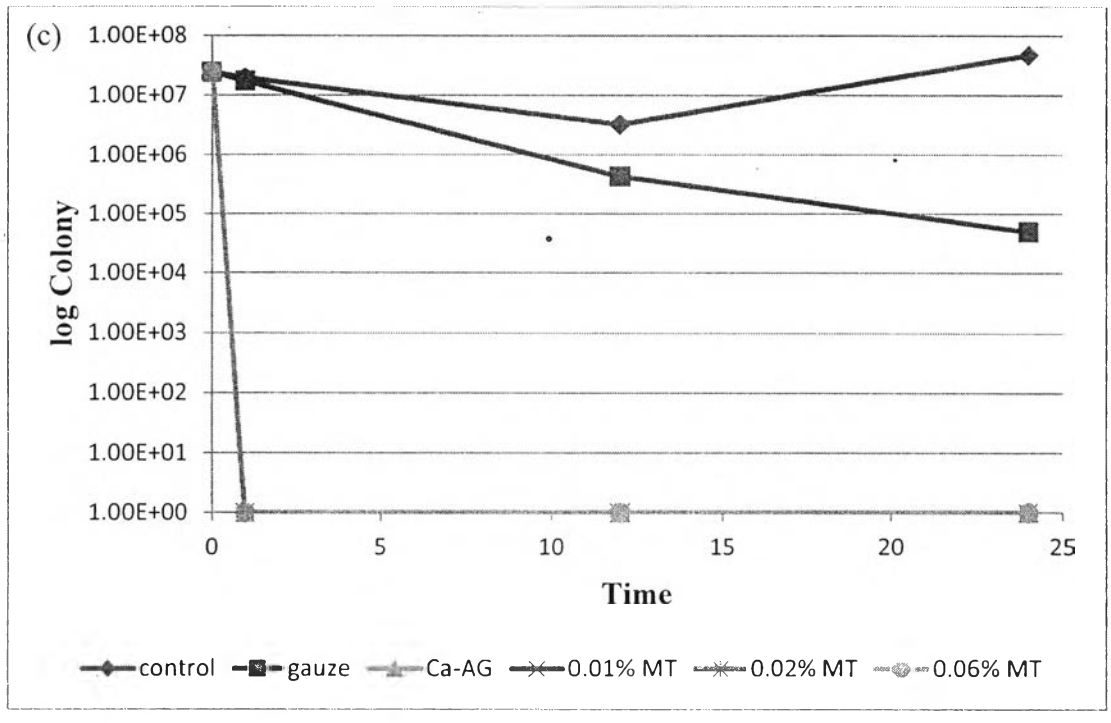
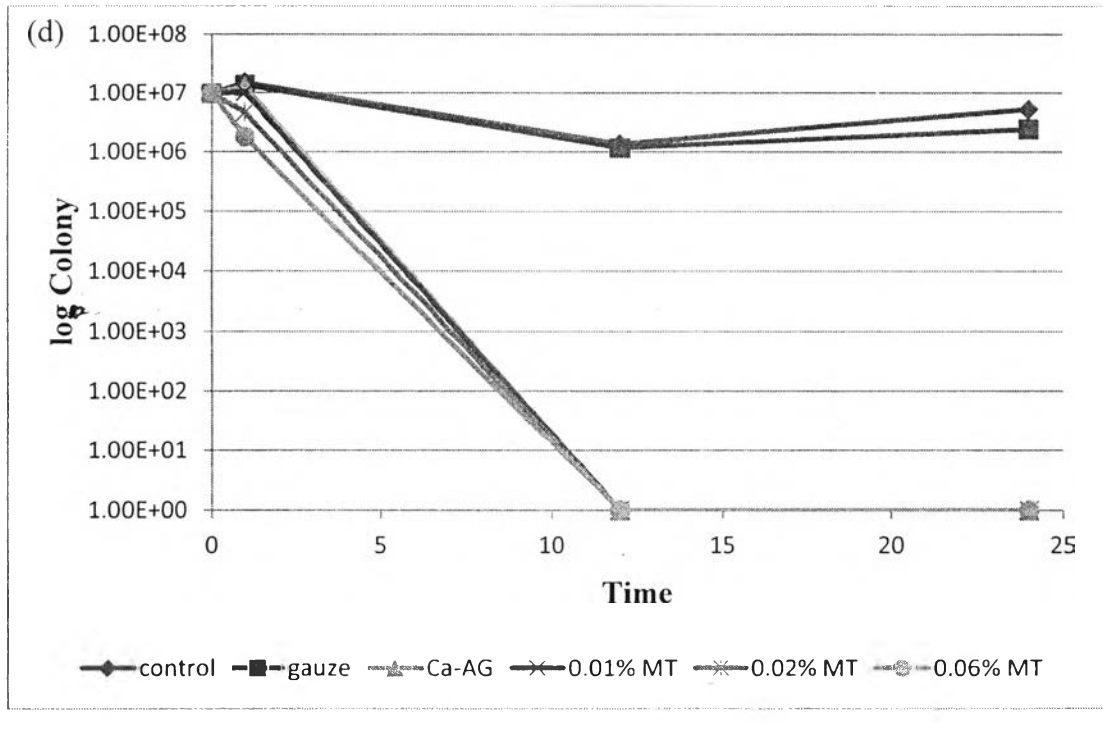


Figure 4.10 The inhibition of MARS (a), *E. Faecalis* (b), *E. Coil* (c), *P. Aeruginosa* (d) and MDR *A. Baumannii* (e) by control(♦), initial gauze (▪), gauze with Ca-AG(▲), and coated gauze with MT 0.01% (×), 0.02% (✳) and 0.06% (●).





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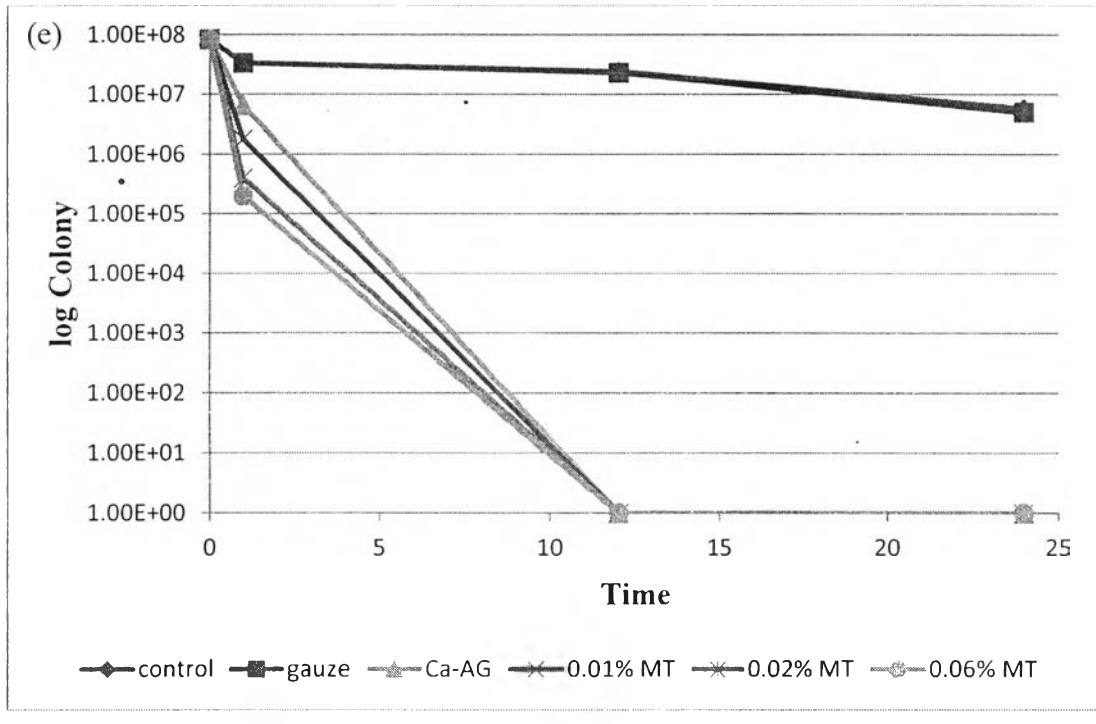

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4.9 Cytotoxicity Test

The cytotoxicity test is a method that uses to determine the surviving cells and cytotoxic effects. In our experiment, the coated gauzes combined with MT were soaked in a media that used to raise cells. Thus, the cytotoxicity was examined by the viability of the cells. For standard testing, non-toxic material that can use with human must have percentage of the cell viability more than 80%. The low percentage of the cell viability is a toxic material. In this research, we used hyperproliferant keratinocytes (HaCat) and fibroblast human celsl (FB), to be representatives for cytotoxic testing.

In order to estimate the amount of released mangosteen extract from coated gauze dressing may cause toxic effect. The toxicity of the dressing was examined by MTT assay (Fig 4.11). From the results, it shows that amount of the extracts clearly

effected on the cytotoxicity of the dressing. The cell viability of HaCat at 1 day significantly decreased from 89% (coated gauze with pure Ca-AG) to 86%, 84% and 57% (0.01% MT, 0.02% MT and 0.06% MT). Like a FB cells, the cell viability also decreased from 89% (coated gauze with pure Ca-AG) to 53% (0.06% MT). It was very clear that the coated Ca-AG gauze with 0.06% MT was toxic with human's cells. However, the coated Ca-AG gauze with 0.01% MT and 0.02% MT displayed an enchanting of cell viability (from 89% to 90% and 91 %, 0.01% MT and 0.02% MT). There are many reports about mangosteen involve in proliferation of cells; Pedro *et al.* (2002) and Jing *et al.* (2011). Thus, a few of MT extracts can enhance antibacterial wound dressing properties and encourage proliferation of both HaCat and FB cells.

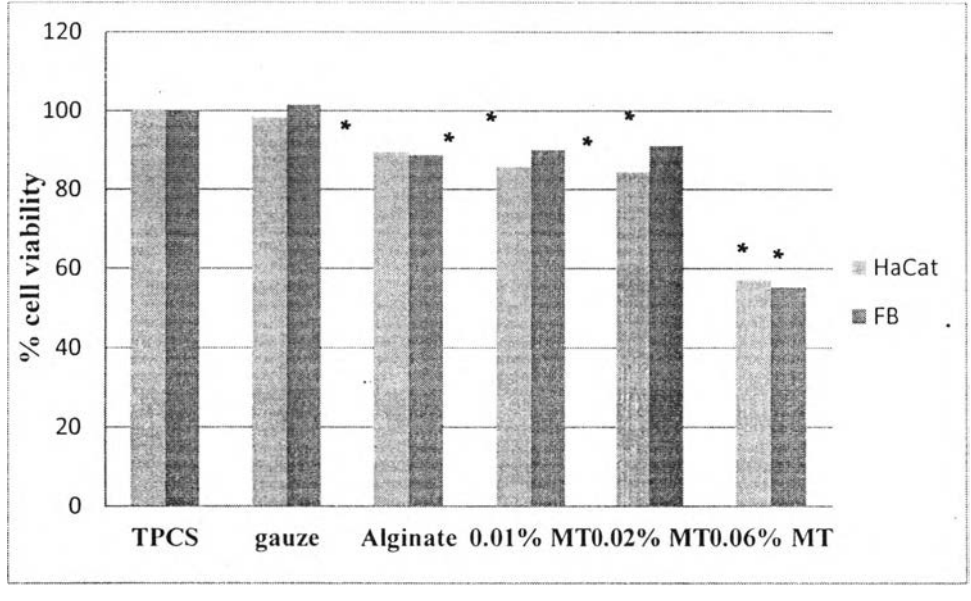

Figure 4.11 The effect of the mangosteen extracts on cell viability; HaCat and FB cells. *Significance at $p < 0.05$with respect to coated gauze by 0.5% w/v AG.