CHAPTER IV

RESULTS

1. Determining the Mid-Exponential Phase of Growth

The tested bacteria were grown in the liquid media to observe their growth pattern over time. The growth curve was plotted as the logarithm of the number of viable cells (log_{10}CFU/ml) versus time.

The growth pattern of *S. mutans* and *A. actinomycetemcomitans* was similar to a typical bacterial culture (Figure 7). After inoculated in a fresh culture media, the bacteria were in the lag phase for one hour, as presented by the relatively flat graph. After the first hour, they reached the log phase, as shown by a rise of graph in exponential progression. The growth curve began to level off, and the stationary phase began after the sixth hour for *S. mutans* and the seventh hour for *A. actinomycetemcomitans*.

The optimal time to reach mid-exponential phase of growth for *S. mutans* and *A. actinomycetemcomitans* was 4 and 5 hours, respectively.

For *P. gingivalis*, the growth curve could not be determined. It was estimated that 24-hour culture was appropriate for antimicrobial testing. When the bacterial culture from an earlier time point was used, the bacteria didn't grow well when subsequently inoculated into fresh medium for testing.

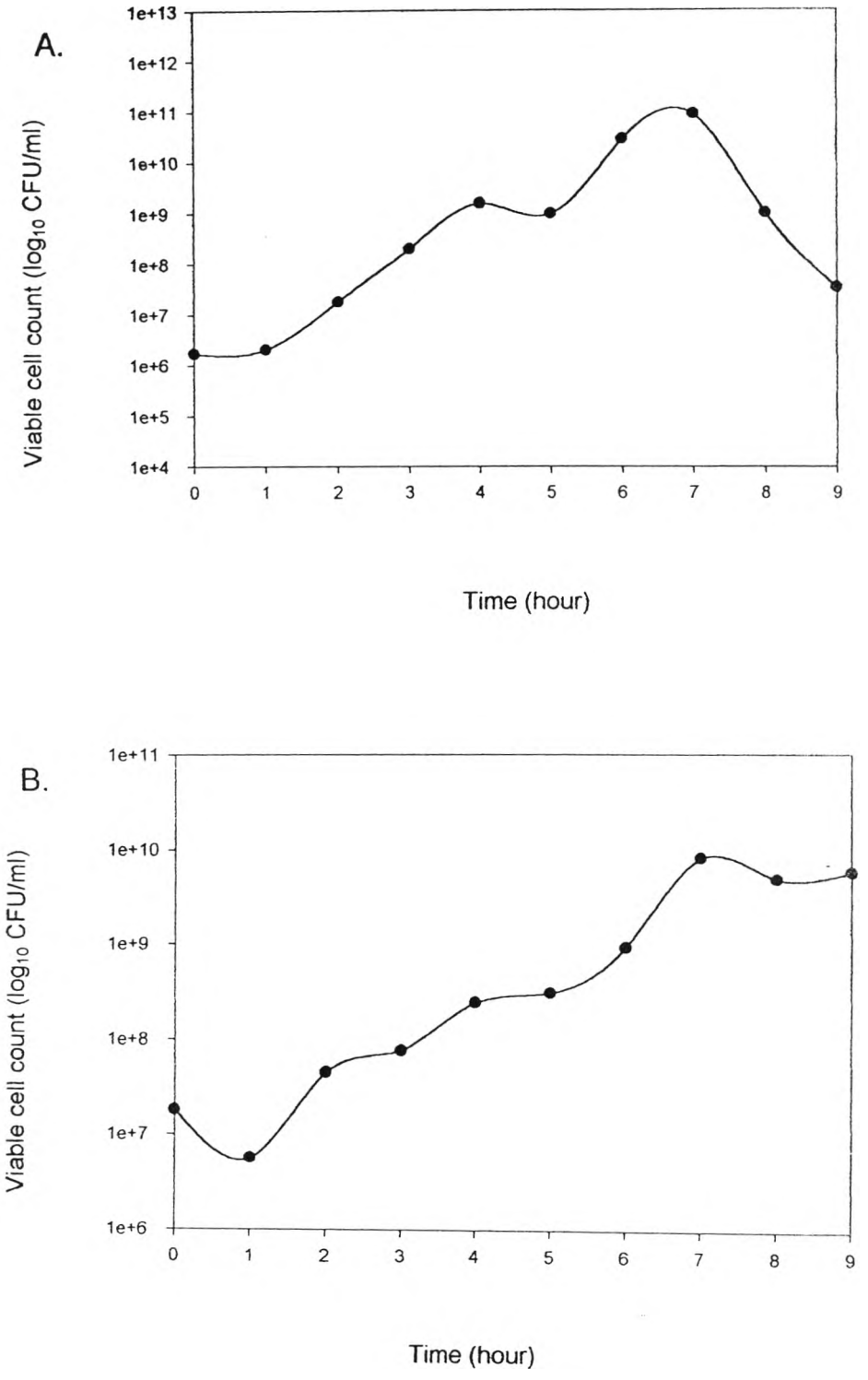

Figure 7. Growth curve of (A) *S. mutans* and (B) *A. actinomycetemcomitans*.

2. Plotting the Standard Curve

The standard curve was plotted between $\log_{10}$CFU/ml and the absorbance at 600 nm for *S. mutans* and *A. actinomycetemcomitans*. From this curve, an estimate of viable bacterial count can be predicted from the absorbance readings so that the desired amount of bacteria can be obtained when testing antimicrobial activity.

Only the values from the exponential phase of growth were used. During this phase, dead cells are minimal. Therefore, the absorbance at 600 nm (reading both dead and live cells) can give the closet estimate of viable cell count. (Ingraham and Ingraham, 2004)

The standard curves for both bacteria were shown in Figure 8. There was a significant linear relationship between $\log_{10}$CFU/ml and the absorbance at 600 nm ($P < 0.05$ for *S. mutans* and $P < 0.01$ for *A. actinomycetemcomitans*). At mid-exponential phase of growth, the absorbance reading for *S. mutans* was approximately 0.15, which was estimated to have viable bacterial count around 1×10^8 CFU/ml. At the same absorbance reading, *A. actinomycetemcomitans* appeared to have more viable cells. The reading at mid-exponential phase was approximately 0.10, which was estimated to have viable bacterial count around 5×10^8 CFU/ml.

For *P. gingivalis*, 24-hour culture gave absorbance reading around 0.5. The bacterial inoculum at this time point was spread onto agar plate, and viable bacterial count was directly determined. The number of bacteria at this reading was approximately 5×10^9 CFU/ml.

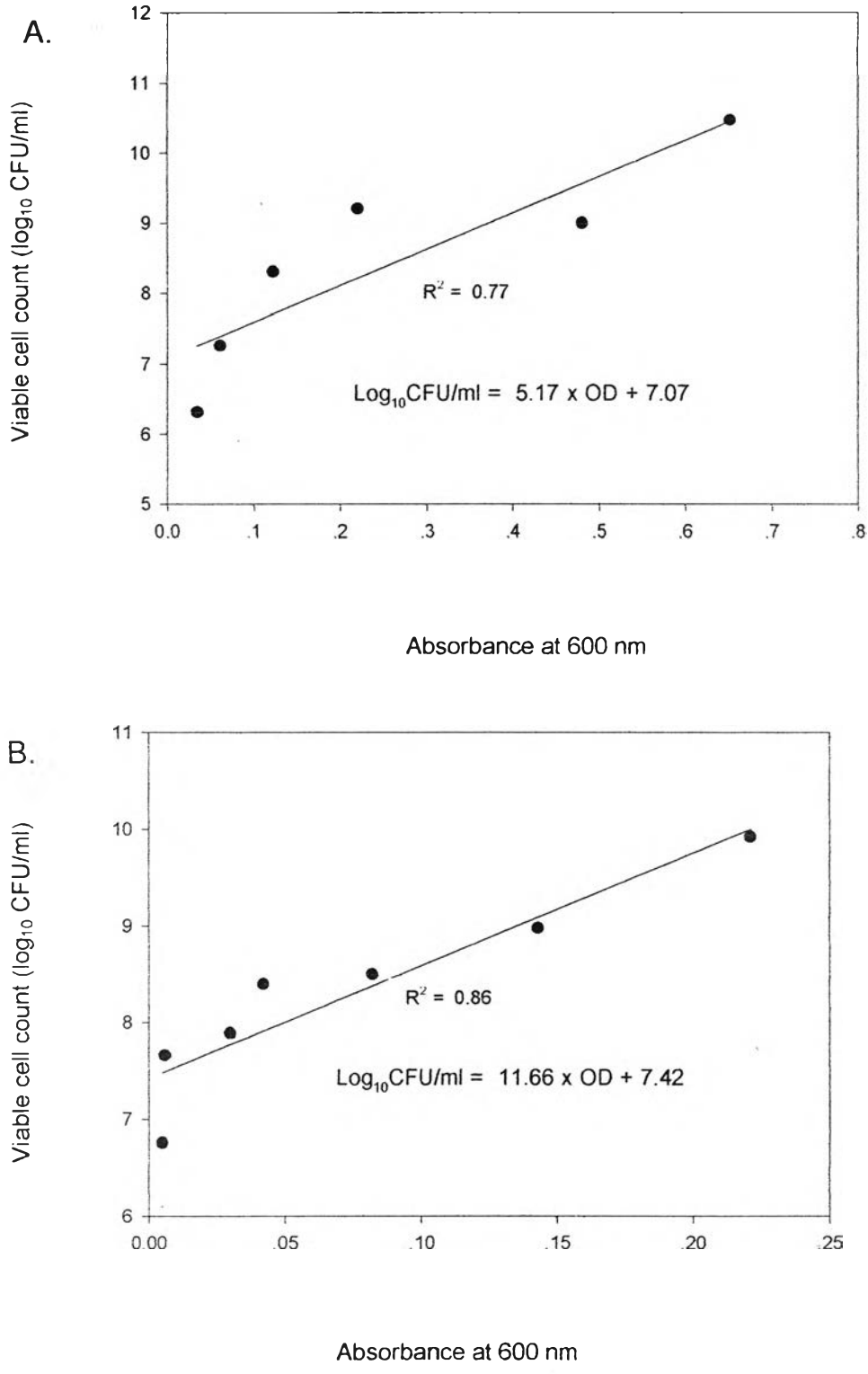

Figure 8. Standard curve of (A) *S. mutans* and (B) *A. actinomycetemcomitans*.

3. Identifying MIC and MBC

The antimicrobial activity of mangosteen pericarp extract was investigated in terms of MIC and MBC (Table 2). Four pathogenic bacterial strains from mid-exponential phase of growth were tested.

The MIC and MBC of mangosteen crude extract against *S. mutans* ATCC 25175 were equal at 0.625 μg/ml, and for *S. mutans* $KPSK_2$ were 0.625 and 1.25 μg/ml respectively. The MIC and MBC for *P. gingivalis* were slightly higher at 20 and 40 μg/ml, respectively. Nevertheless mangosteen crude extract at a concentration as high as 640 μg/ml did not have effect on the growth of *A. actinomycetemcomitans*.

The antimicrobial activity of α-mangostin, a major component of the extract, was compared to that of the crude extract. The results showed that the MIC and MBC values were the same for all tested bacteria.

Chlorhexidine, a commonly used antiseptic, was used as a positive control. The MIC and MBC against both strains of *S.mutans* were comparable with those of mangosteen crude extract. However, the MIC and MBC of chlorhexidine against *P. gingivalis* were much lower at 1.25 and 2.5 μg/ml respectively. *A. actinomycetemcomitans* was not killed by mangosteen crude extract whereas the MIC and MBC of chlorhexidine against this organism were 1.25 and 2.5 μg/ml respectively.

Table 2. MIC and MBC of mangosteen crude extract, α-mangostin and chlorhexidine against oral pathogens (μg/ml).

oral pathogens	crude extract		α-mangostin		chlorhexidine	
	MIC	MBC	MIC	MBC	MIC	MBC
S. mutans ATCC 25175	0.625	0.625	0.625	0.625	0.312	0.625
S. mutans $KPSK_2$	0.625	1.25	-	-	0.625	0.625
P. gingivalis	20	40	20	40	1.25	2.5
A. actinomycetemcomitans	>640	>640	>640	>640	1.25	2.5

4. Time-kill Kinetics

Time-kill kinetics was determined by the number of remaining viable bacteria at varying time after exposed to the mangosteen extract at the concentrations of two or four times of MBC. Only *S. mutans* ATCC 25175 and *P. gingivalis* were tested.

4.1 *Time-kill kinetics for S. mutans*

The mangosteen crude extract at concentrations of 1.25 (2x MBC) and 2.5 (4x MBC) μg/ml were used (Table 3, Figure 9). At the time point earlier than 30 minutes, there were no significant changes in viable cell count in the extract group as compared to the broth control (data not shown). At 30 minutes, the group treated with the extract at 4x MBC showed a slight decrease in viable cell count, which did not reach statistical significance. At 60 minutes, the group treated with the extract at 2x MBC showed a decrease in viable cell count by a half order, while the extract at 4x MBC decreased viable cell count by almost two orders. Only the latter group reached statistical significance ($P < 0.05$). At 90 minutes, the group treated with the extract at 2x MBC showed a significant decrease in viable cell count by one order ($P < 0.01$), while the extract at 4x MBC completely killed the bacteria ($P < 0.01$).

The time-kill kinetics of the extract against *S. mutans* was also compared to that of chlorhexidine at the same concentrations (Table 3, Figure 9). Surprisingly, chlorhexidine did not significantly change viable cell count at the time point earlier than 90 minutes. At 90 minutes, the group exposed to chlorhexidine at 2x MBC showed a slight decrease in viable cell count, while chlorhexidine at 4x MBC decreased viable cell count by half an order. Only the latter group reached statistical significance ($P < 0.05$).

Table 3. The number of remaining viable bacteria (log_{10} CFU/ml) of *S. mutans* ATCC 25175 after exposed to mangosteen extract and chlorhexidine for 30, 60 and 90 minutes. The results are presented as means ± standard deviations.

time (minutes)	control	2xMBC (1.25 μg/ml)		4xMBC (2.5 μg/ml)	
		mangosteen	chlorhexidine	mangosteen	chlorhexidine
30	6.49±0.21	6.43±0.19	6.44±0.17	6.10±0.38	6.29±0.16
60	7.06±0.26	6.40±0.60	6.86±0.20	5.21±0.87*	6.75±0.29
90	6.94±0.29	6.04±0.15**	6.61±0.17	0**	6.49±0.29*

*P < 0.05, Significance differences from the control group at the same time point.

**P < 0.01, Significance differences from the control group at the same time point.

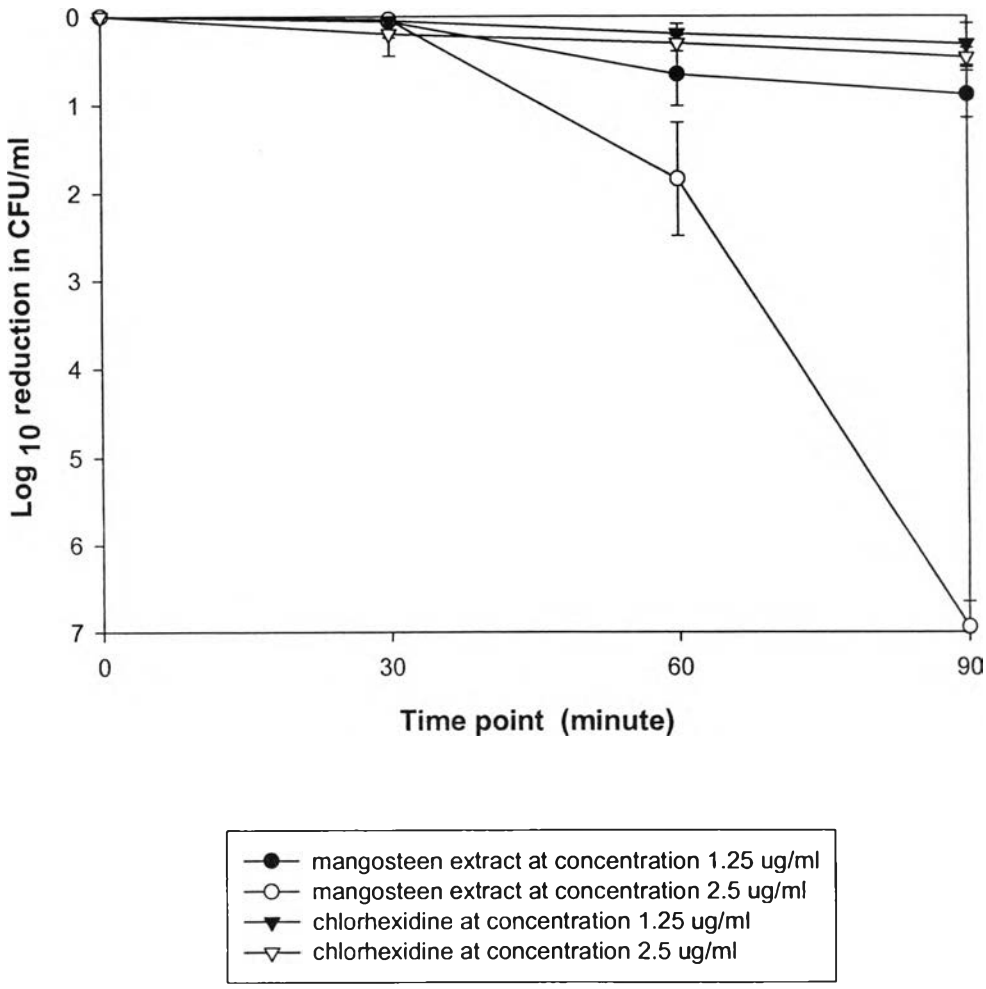

Figure 9. Time kill kinetics of S. *mutans* ATCC25175 when treated with mangosteen crude extract at 2x and 4x MBC compared to chlorhexidine at the same concentrations. The data are presented as $\log_{10}$ reduction in CFU/ml compared to the broth control at each time point (means $\pm$ standard deviations).

4.2 *Time-kill kinetics for P. gingivalis*

The mangosteen crude extract at concentrations of 80 (2x MBC) and 160 (4x MBC) μg/ml were used (Table 4, Figure 10). At 5 minutes, the group treated with the extract at 2x MBC showed a slight decrease in viable cell count, while the extract at 4x MBC decreased viable cell count by one order. Only the latter group reached statistical significance ($P < 0.05$). At 15 minutes, the group treated with the extract at 2x MBC showed a significant decrease in viable cell count by almost two orders ($P < 0.01$), while the extract at 4x MBC completely killed the bacteria ($P < 0.01$). At 15 minutes or longer, both concentrations at 2x and 4x MBC completely killed the bacteria ($P < 0.01$).

The time-kill kinetics of the extract against *P. gingivalis* was also compared to that of chlorhexidine at the same concentrations (Table 4, Figure 10). At 5 minutes, the group exposed to chlorhexidine at 2x MBC showed a slight decrease in viable cell count by half an order, while chlorhexidine at 4x MBC significantly decreased viable cell count by almost two orders ($P < 0.01$). At 15 minutes, the group treated with chlorhexidine at 2x MBC showed a further decrease in viable cell count by over two orders ($P < 0.01$), while chlorhexidine at 4x MBC completely killed the bacteria ($P < 0.01$). At 15 minutes or longer, both concentrations at 2x and 4x MBC completely killed the bacteria ($P < 0.01$).

Table 4. The number of remaining viable bacteria (log_{10} CFU/ml) of *P. gingivalis* after exposed to mangosteen extract and chlorhexidine for 5, 15 and 30 minutes. The results are presented as means ± standard deviations.

time (minutes)	control	2xMBC (80 μg/ml)		4xMBC (160 μg/ml)	
		mangosteen	chlorhexidine	mangosteen	chlorhexidine
5	6.40±0.10	6.12±0.30	5.85±0.33	5.21±0.31*	4.69±0.74**
15	6.71±0.31	4.96±0.31**	4.34±0.67**	0**	0**
30	6.91± 0.15	0**	0**	0**	0**

*P < 0.05, Significance differences from the control group at the same time point.

**P < 0.01, Significance differences from the control group at the same time point

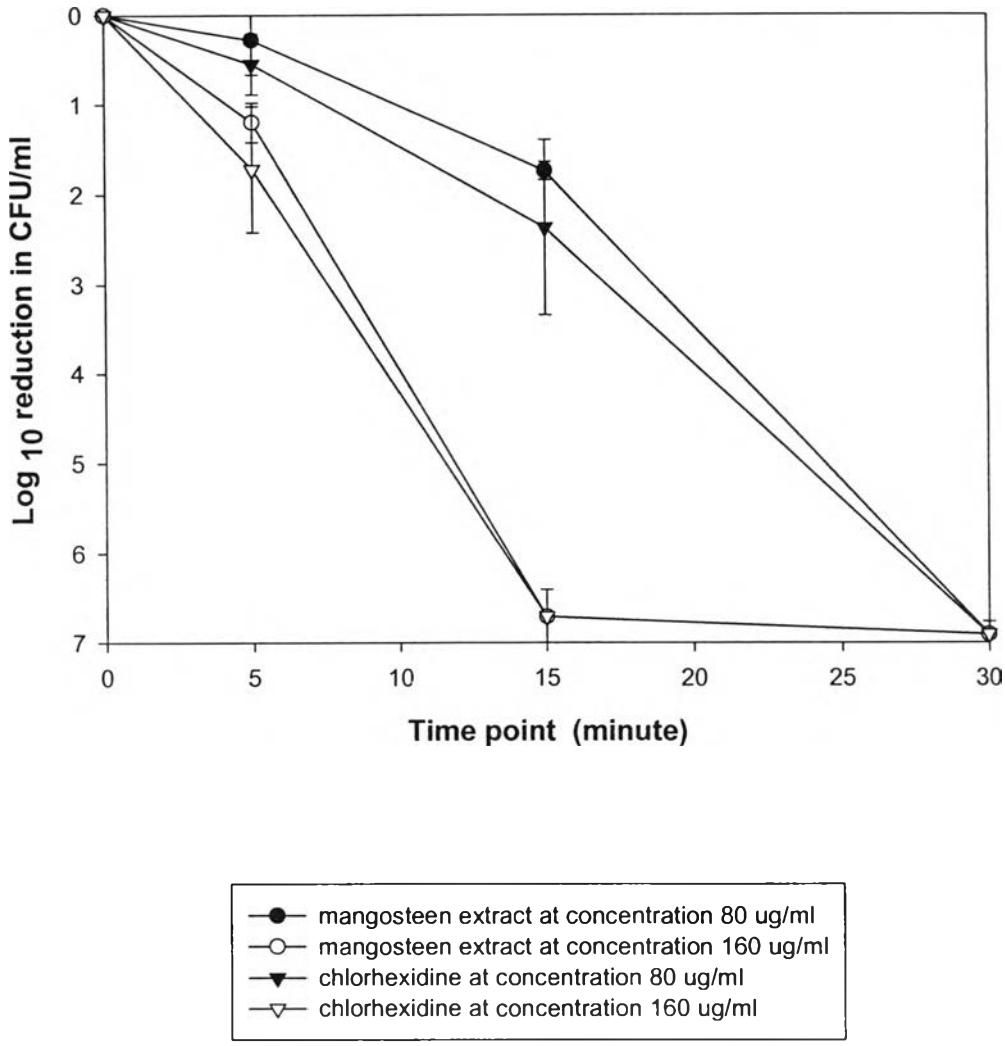

Figure 10. Time kill kinetics of *P. gingivalis* when treated with mangosteen crude extract at 2x and 4x MBC compared to chlorhexidine at the same concentrations. The data are presented as $\log_{10}$ reduction in CFU/ml compared to the broth control at each time point (means $\pm$ standard deviations).