CHAPTER IV

RESULTS

1. Cytotoxic effect of the *S.venosa* tuber extracts

The cytotoxic effect of the water and the ethanol extracts of *S.venosa* tuber on PBMCs and Jurkat cells were determined by trypan blue dye exclusion method and alamarBlue reduction assay. The results in Figure 3 demonstrated the cytotoxic effects of the extracts on PBMCs by alamarBlue reduction assay. The IC_{50} of the water and the ethanol extracts were 200 μg/ml and 40 μg/ml, respectively. The potency of the ethanol extract was five folds higher than that of the water extract. The cytotoxic effects of both extracts on PBMCs were confirmed by using trypan blue dye exclusion methods. The results in Figure 4 showed the similar cytotoxic pattern to the results Figure 3 with the IC_{50} at 100 μg/ml and 10 μg/ml for the water and the ethanol extracts. The potency of the ethanol extract was ten folds more than the water extract. All of the results above indicated that trypan blue dye exclusion method was more sensitive than alarmarBlue reduction method. Thus, trypan blue dye exclusion method was selected for evaluating the cytotoxic activities of the both extracts on Jurkat cells. The data in Figure 5 presented that both extracts also had cytotoxic effects on Jurkat cells in dose dependent pattern with IC_{50} at 200 μg/ml and 100 μg/ml for the water and the ethanol extracts, respectively. The potency of the ethanol extract was two folds when compared with the water extract.

The cytotoxic concentrations of the water and the ethanol extracts at about IC_{50}, two fold lower and higher than IC_{50} were used in the next studies. These concentrations were 200, 100 and 400 μg/ml for the water extract; and 40, 20 and 80 μg/ml for the ethanol extract.

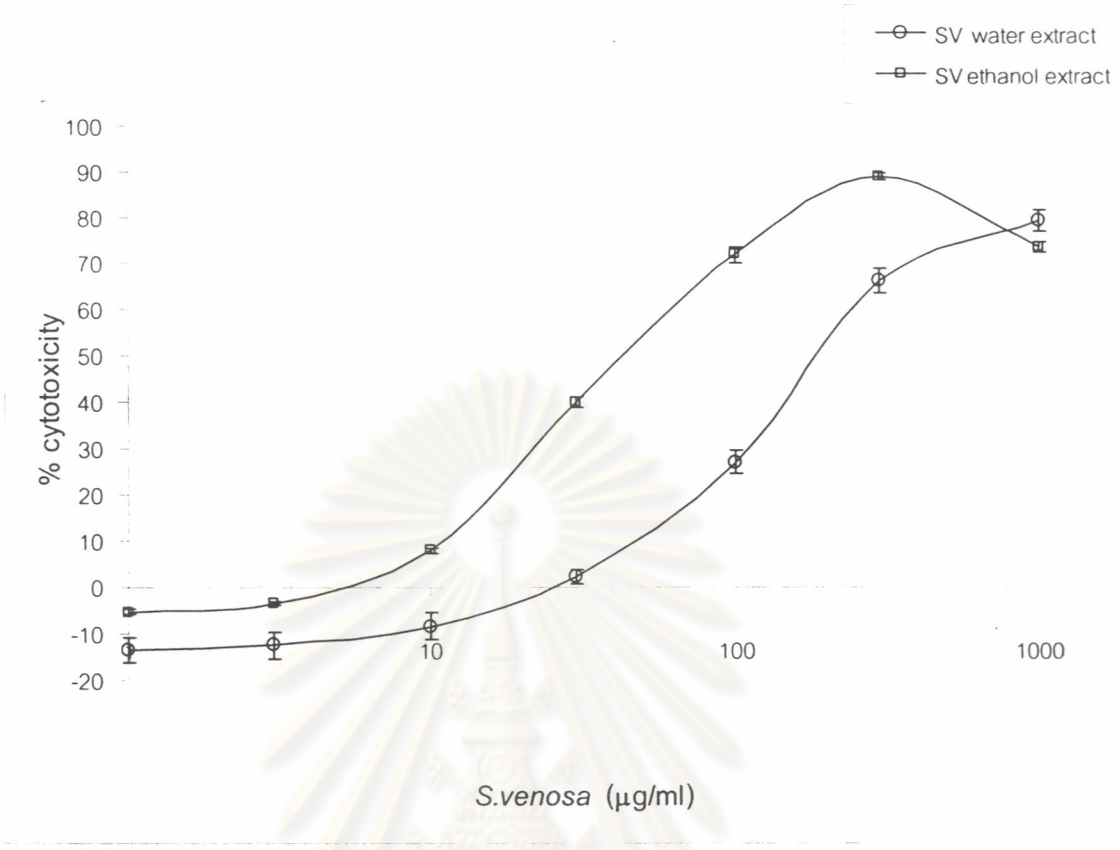

Figure 3. Cytotoxic effect of the water and the ethanol extracts of *S.venosa* tuber at various concentrations by alamarBlue reduction assay on PBMCs. Doxorubicin (1.5 µg/ml) was used as a positive control with the percentage of cytotoxicity 43.85 ± 2.83. The data were represented as mean ± S.E.M (n=10).

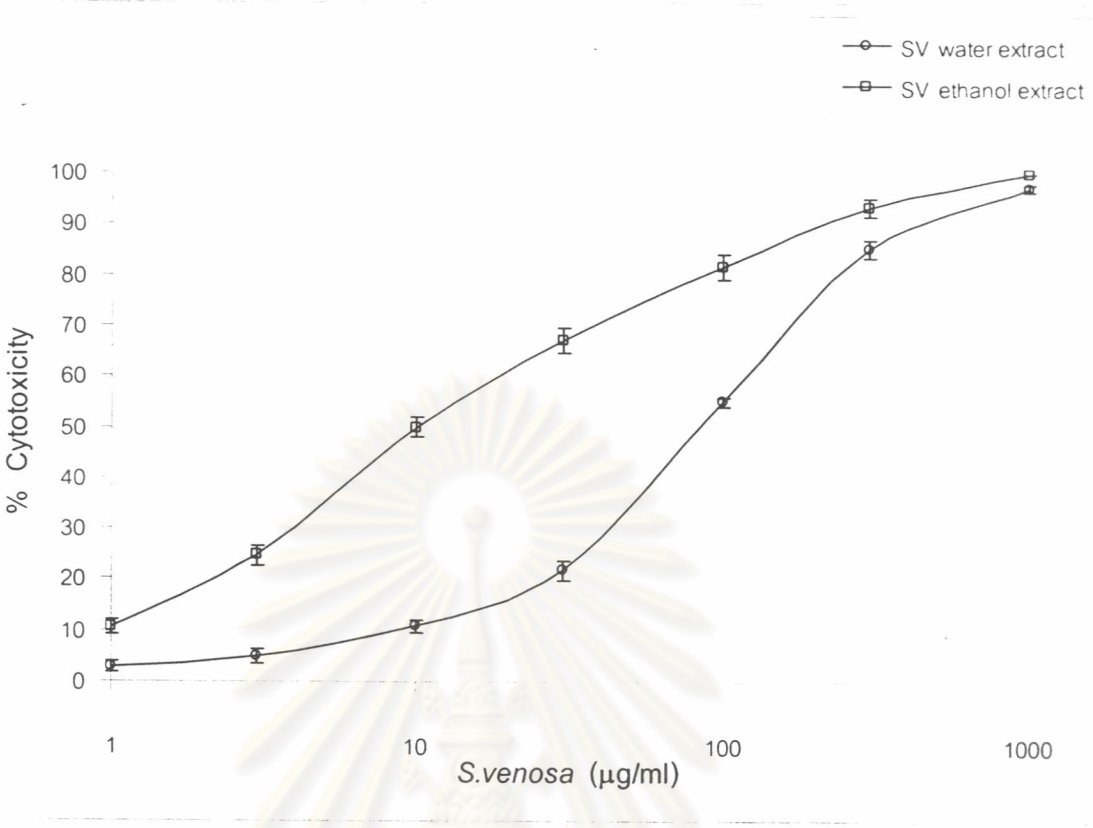

Figure 4. Cytotoxicity effect of the water and the ethanol extracts of *S.venosa* tuber at various concentrations by trypan blue dye exclusion assay on PBMCs. Doxorubicin (1.5 µg/ml) was used as a positive control with the percentage of cytotoxicity 81.20± 3.63. The data were represented as mean ± S.E.M (n=5).

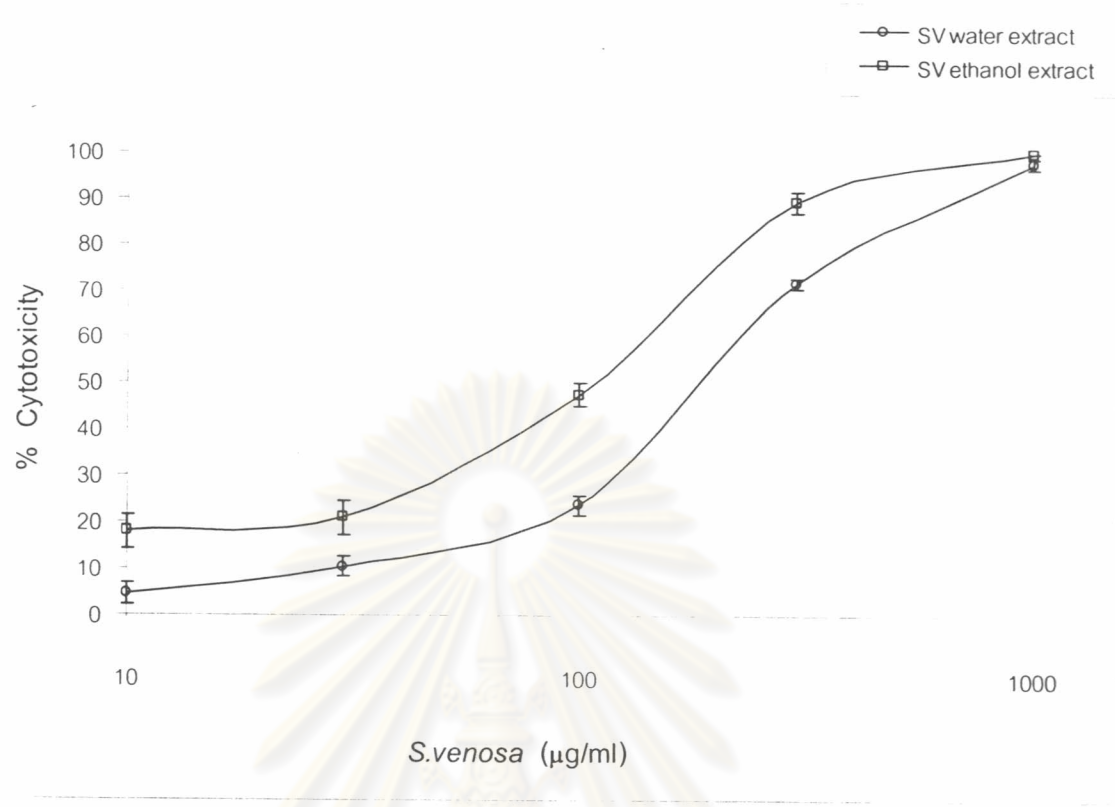

Figure 5. Cytotoxicity effect of the water and the ethanol extract of *S.venosa* tuber at various concentrations by trypan blue dye exclusion methods on Jurkat cells. Doxorubicin (1.5 µg/ml) was used as a positive control with the percentage of cytotoxicity 79± 2.30. The data were represented as mean ± S.E.M (n=5).

2. Antiproliferative effect of the *S.venosa* tuber extracts.

Antiproliferative effect of *S.venosa* tuber extracts on activated PBMCs was investigated by MTT reduction colorimetric method. PHA, SPA, and PWM were used as mitogens in this study. The concentrations of the water extract at 100, 200 and 400 μg/ml and those of the ethanol extract at 20, 40 and 80 μg/ml were used in this study.

Results in Figure 6 showed that the water and the ethanol extracts clearly exhibited antiproliferative effect in a concentration dependent manner on PHA activated PBMCs. The percentage of antiproliferative effect of the water extract was 58±2.90, 69.28 ± 3.21 and 90.41± 4.13 at the concentrations of 100, 200 and 400 μg/ml, respectively (Figure 6A). The percentage of proliferation inhibition of the ethanol extract was 57±2.98, 70.26 ± 3.15 and 86.26 ± 3.48 at the concentrations of 20, 40 and 80 μg/ml, respectively (Figure 6B). The antiproliferative effect of each concentration of the water and the ethanol extracts was significant difference when compared with the concentrations of 100 and 20 μg/ml of the water and the ethanol extracts, respectively ($P<0.05$).

Results in Figure 7 presented that the water and the ethanol extracts also inhibited SPA stimulated PBMCs proliferation in a concentration dependent manner. The percentage of inhibition proliferation of the water extract was 51±1.48, 63.58±1.84 and 79.06 ± 3.49 at the concentrations of 100, 200 and 400 μg/ml, respectively. There were no significant difference at the concentrations between 100 μg/ml and 200 μg/ml, but other concentrations were significant difference (Figure 7A). The percentage of proliferation inhibition of the ethanol extract was 50.45±1.57, 63.85±2.18 and 86.36±2.08 at the concentrations of 20, 40 and 80 μg/ml, respectively. The antiproliferative effect of the ethanol extract was significant difference among at the concentrations of 40 and 80 μg/ml when compared with the concentration of 20 μg/ml ($p<0.05$) (Figure 7B).

Results in Figure 8 also demonstrated that the water and the ethanol extracts clearly exhibited antiproliferative effects in a concentration dependent manner on PWM activated PBMCs. The percentage of antiproliferative effect of the water extract was 65.33±5.88, 77.36 ± 4.20 and 92.29± 1.08 at the concentrations of 100, 200 and 400 μg/ml, respectively (Figure 8A). The percentage of proliferation inhibition of the ethanol extract was 57±2.98, 70.26 ± 3.15 and 86.26 ± 3.40 at the concentrations of 20, 40 and

80 μg/ml respectively (Figure 8B). The antiproliferative effect was significant difference of each concentration when compared with the concentrations of 100 and 20 μg/ml of the water and ethanol extracts, respectively.

By comparison between both extracts, the ethanol extract was more potent than the water extract in inhibiting mitogen stimulated PBMCs. As shown in Figure. 6-8, the ethanol extract could inhibit the cells at the lower concentrations than the water extract at the same degree. This degree of inhibition was observed in all mitogens stimulated conditions.

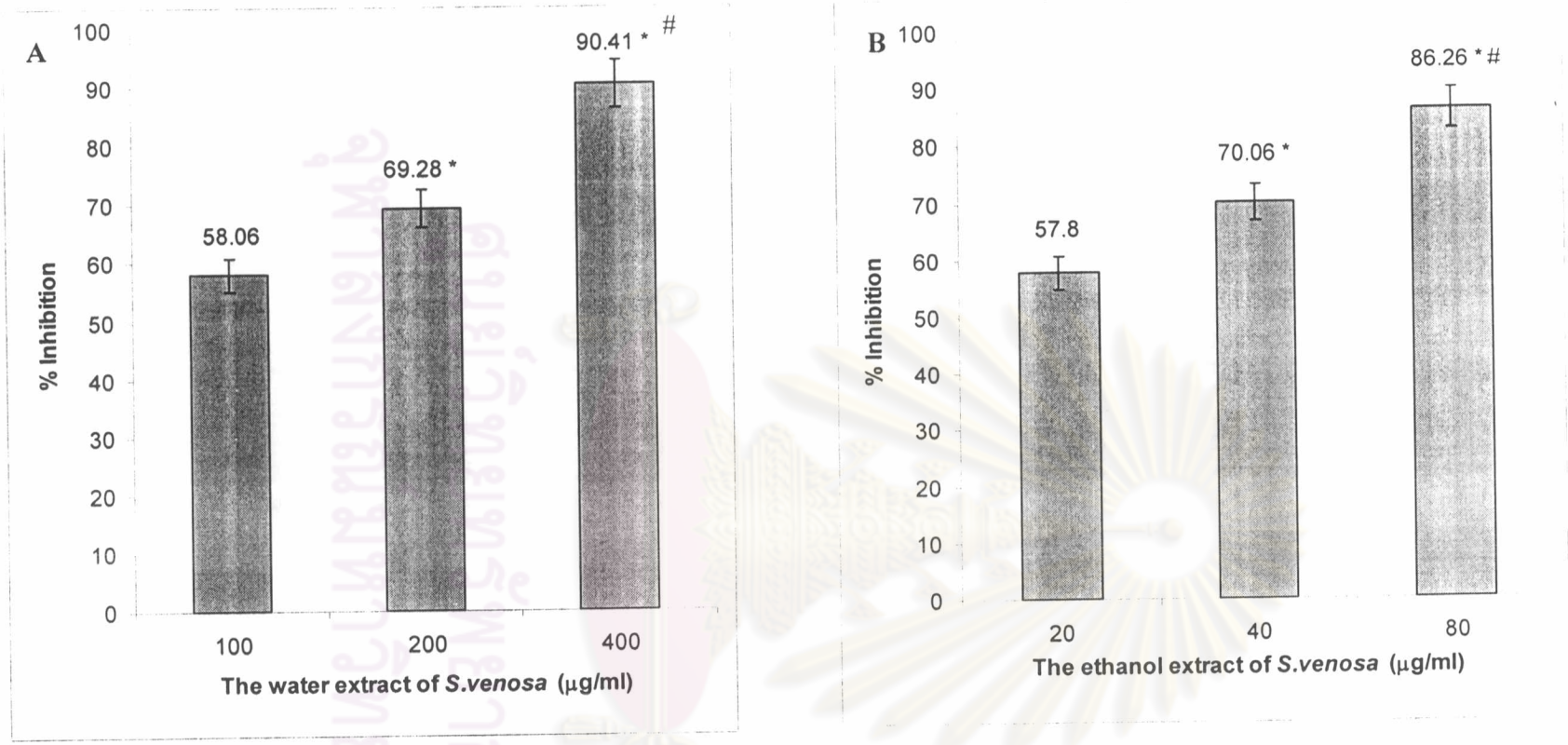

Figure 6. Antiproliferative effect of the water (A) and the ethanol (B) extracts of *S.venosa* tuber on PHA stimulated PBMCs by MTT assay. This cell was treated with the indicated concentrations of the extracts (µg/ml) for 48 hrs. Antiproliferarive effect was expressed as percentage of inhibition of the untreated PHA-stimulated cell. PHA at the concentration of 10 µg/ml was used in this study. The data were represented as mean ± S.E.M (n=5).

* Significant difference when compared to the *S.venosa* concentration 100 µg/ml of the water and 20 µg/ml of the ethanol extract $p<0.05$

Significant difference when compared to the *S.venosa* concentration 200 µg/ml of the water and 40 µg/ml of the ethanol extract $p<0.05$

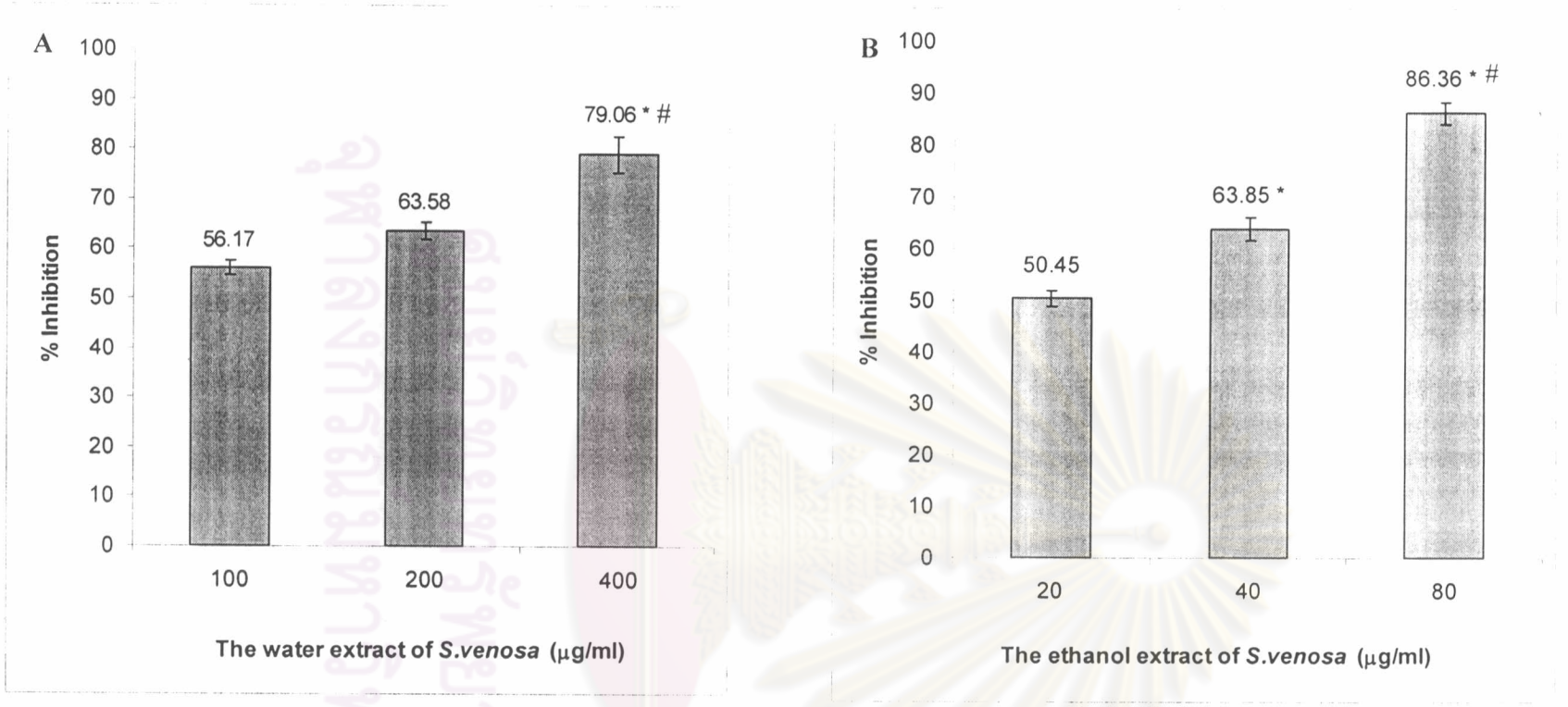

Figure 7. Antiproliferative effect of the water (A) and the ethanol (B) extracts of *S.venosa* tuber on SPA stimulated PBMCs by MTT assay. This cell was treated with the indicated concentrations of the extracts (µg/ml) for 48 hrs. Antiproliferarive effect was expressed as percentage of inhibition of the untreated SPA-stimulated cell. SPA at the concentration of 25 µg/ml was used in this study. The data were represented as mean ± S.E.M (n=5).

* Significant difference when compared to the *S.venosa* concentration 100 µg/ml of the water and 20 µg/ml of the ethanol extract $p<0.05$

\# Significant difference when compared to the *S.venosa* concentration 200 µg/ml of the water and 40 µg/ml of the ethanol extract $p<0.05$

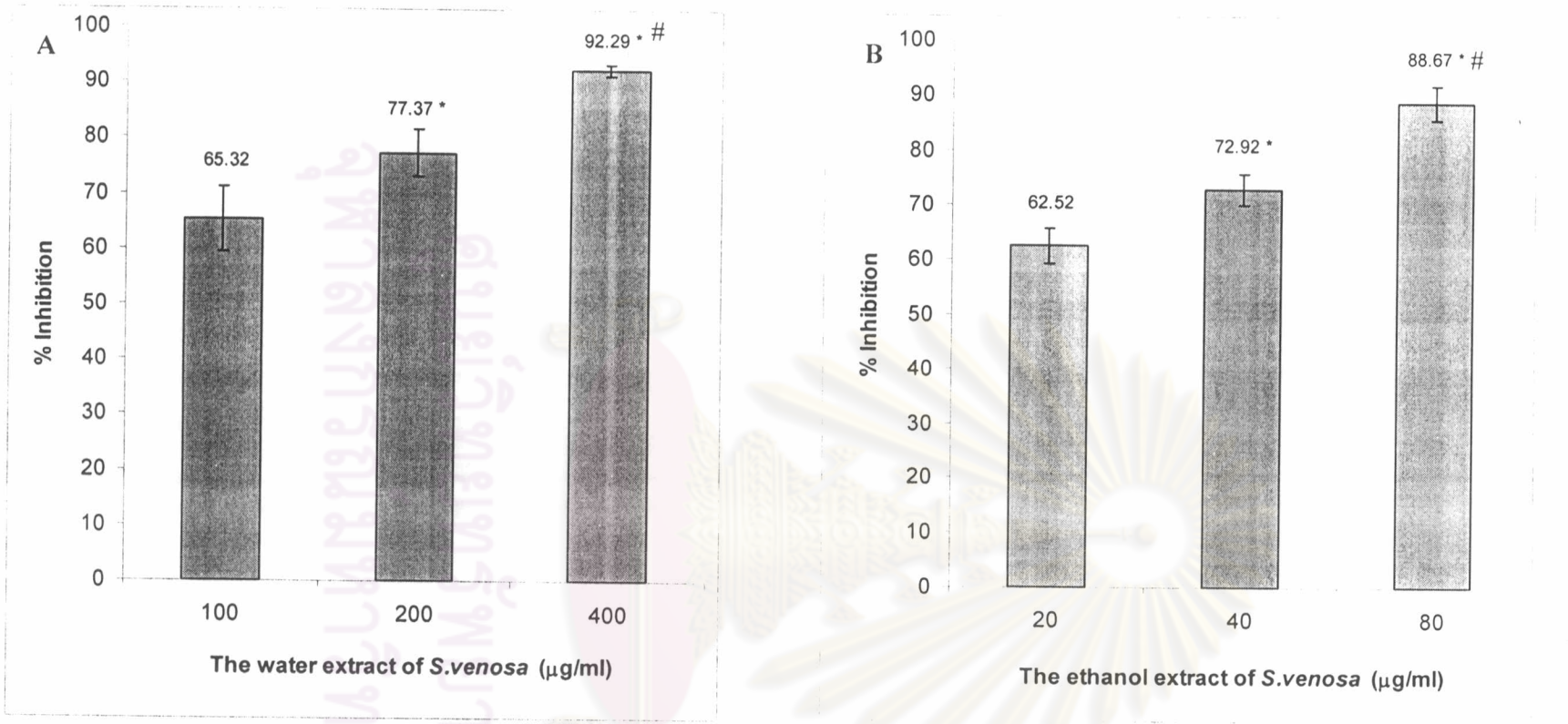

Figure 8. Antiproliferative effect of the water (A) and the ethanol (B) extracts of *S.venosa* tuber on PWM stimulated PBMCs by MTT assay. This cell was treated with the indicated concentrations of the extracts (µg/ml) for 48 hrs. Antiproliferarive effect was expressed as percentage of inhibition of the untreated PWM-stimulated cell. PWM at the concentration of 50 µg/ml was used in this study. The data were represented as mean ± S.E.M (n=5).

* Significant difference compared to the *S.venosa* concentration 100 µg/ml of the water and 20 µg/ml of the ethanol extract $p<0.05$

Significant difference compared to the *S.venosa* concentration 200 µg/ml of the water and 40 µg/ml of the ethanol extract $p<0.05$

3. Apoptotic effect of *S.venosa* tuber extracts

The water extract of *S.venosa* at the concentrations of 100, 200 and 400 μg/ml and the ethanol extract of that at the concentrations of 20, 40, 80 and 160 μg/ml were used in this study. Etoposide (10 μg/ml) was used as a positive control. Apoptotic and necrotic cells were detected by staining the cells with Annexin V-FITC and PI and using FACS analysis (example diagram as shown in Figure 9).

The results in Figure 10 and Table 1 showed that the percentage of apoptotic cells (Annexin V-FITC$^+$/PI$^-$) induced by the water extract at concentration 400 μg/ml was 21.70. The percentages of secondary necrosis or late apoptosis cells (Annexin V-FITC$^+$/PI$^+$) and necrosis cells (PI$^+$) were increased in a concentration dependent manner at the concentrations of 200 and 400 μg/ml. The percentage of the apoptotic cells was significant difference ($p<0.05$) at the concentration of 400 μg/ml while the percentages of secondary necrosis or late apoptotic cells and necrotic cells were significant difference ($p<0.05$) at the concentrations of 200 and 400 μg/ml when compared with negative control, respectively.

The results in Figure 11 and Table 2 demonstrated that the ethanol extract that induced apoptosis, secondary necrosis or late apoptosis and necrosis cells in a concentration dependent manner. The percentages of apoptotic cells were 14.09 and 25.69 at concentrations of 80 and 160 μg/ml, respectively. The percentages of secondary necrosis or late apoptosis cells were 5.74 and 17.00 at the concentrations of 80 and 160 μg/ml, respectively. The percentage of necrosis cells were 2.67, 2.34 and 7.80 at concentrations of 40, 80 and 160 μg/ml, respectively. The percentages of the apoptosis and secondary necrosis or late apoptotic cells were significant difference ($p<0.05$) at the concentrations of 80 and 160 μg/ml. The percentage of necrosis cells were significant difference ($p<0.05$) at concentrations of 40, 80 and 160 μg/ml when compared with negative control, respectively.

Similar to cytotoxic and antiproliferative effects, the ethanol extract was more potent than the water extract in apoptotic induction on human PBMCs. The similar degree of induction was observed at the difference concentrations of both extracts. It was observed when the ethanol extract was used at the lower concentrations than the water extract (Figure 10-11 and Table 1-2). Moreover, both extracts induced difference

patterns of cells death. As presented in Figure 10-11 and Table 1-2, apoptotic was the major pattern of cell death induced by the ethanol extract. Each concentration of this extract cause more than 50% apoptotic cell death (Table 4). On the contrary, the water extract induced apoptotic less than 50 % (Table 3). While late apoptosis or secondary necrosis (Annexin V-FITC^{+}/PI^{+}) seem to be predominate pattern.

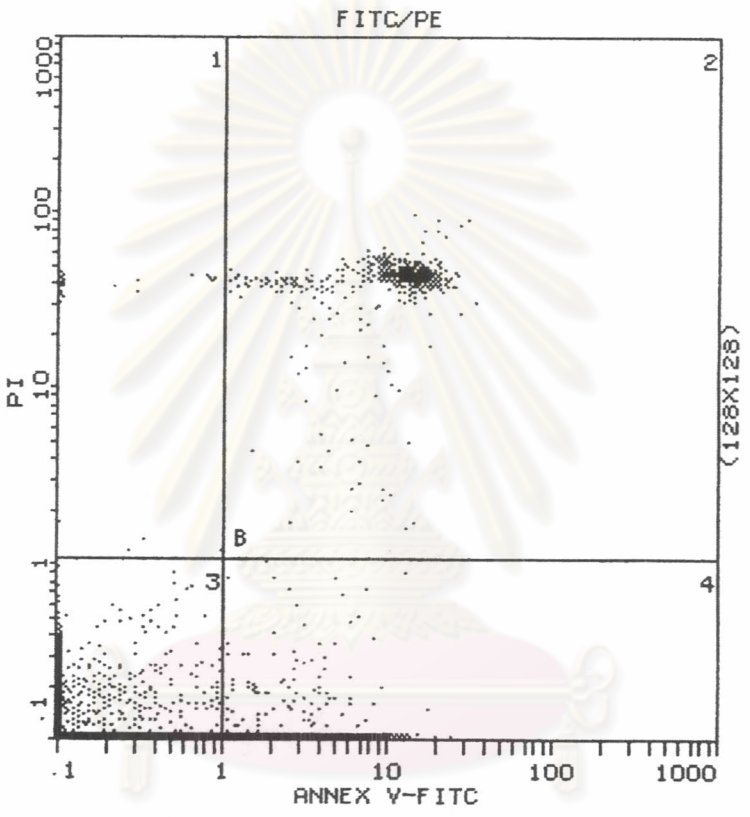

Figure 9. Contour diagram of Annexin V-FITC/PI flow cytomety of PBMCs. The lower left quadrants show the viable cells which exclude PI and are negative for Annexin V-FITC binding. The upper left quadrants show necrotic cells which are positive for PI. The upper right quadrants show the late stage of apoptosis or secondary necrosis for both Annexin V-FITC and PI positive. The lower right quadrants show apoptosis at an earlier stage for positive Annexin V-FITC.

Table 1. Apoptotic effect of the water extract of *S.venosa* on PBMCs. The percentages of cell death induced by the water extract were determined by staining with Annexin V-FITC and PI and using FACS analysis. The data were represented as mean ± S.E.M (n=5).

* Significant difference when compared to the control group at $p<0.05$

Conditions	% Cell death		
	Annexin V-FITC$^+$	Annexin V-FITC $^+$/PI$^+$	PI$^+$
Negative control	5.70 ± 0.36	1.60 ± 0.49	1.81 ± 0.44
S.venosa 100 μg/ml	5.69 ± 0.48	5.00 ± 1.92	4.64 ± 0.83
S.venosa 200 μg/ml	8.56 ± 0.81	9.78 ± 1.51 *	7.01 ± 1.67 *
S.venosa 400 μg/ml	21.70 ± 2.37 *	27.27 ± 4.73 *	8.44 ± 0.90 *
Etoposide 10 μg/ml	12.87 ± 1.12 *	13.89 ± 1.57 *	3.04 ± 0.87

Table 2. Apoptotic effect of the ethanol extract of *S.venosa* on PBMCs. The percentages of cell death were determined by staining with Annexin V-FITC and PI and using FACS analysis. The data were represented as mean ± S.E.M (n=5).

* Significant difference when compared to the control group at $p<0.05$

Conditions	% Cell death		
	Annexin V-FITC$^+$	Annexin V-FITC $^+$/PI$^+$	PI$^+$
Negative control	6.11 ± 0.68	1.18 ± 0.18	0.59 ± 0.37
S.venosa 20 μg/ml	7.88 ± 0.74	2.54 ± 0.15	1.89 ± 0.56
S.venosa 40 μg/ml	9.51 ± 0.72	3.69 ± 0.14	2.67 ± 0.59 *
S.venosa 80 μg/ml	14.09 ± 0.26 *	5.74 ± 0.49 *	2.34 ± 0.34 *
S.venosa 160 μg/ml	25.69 ± 2.52 *	17.00 ± 1.86 *	7.80 ± 0.93 *
Etoposide 10 μg/ml	13.28 ± 1.15 *	8.93 ± 1.30 *	1.1 ± 0.38

Table 3. The percentage of each cell death pattern induced by the water extract of *S.venosa* on PBMCs. The data were represented as mean ± S.E.M (n=5).

Conditions	Total cell death	Pattern of cell death (%)		
		Apoptosis	Late apoptosis or secondary necrosis	Necrosis
Negative control	9.1 ± 0.22	60.55 ± 1.20	16.82 ± 2.02	20.63 ± 2.45
S.venosa 100 μg/ml	15.31 ± 0.86	38.95 ± 2.00	29.03 ± 3.46	31.96 ± 2.89
S.venosa 200 μg/ml	25.35 ± 0.90	34.73 ± 1.79	38.19 ± 1.83	27.08 ± 2.34
S.venosa 400 μg/ml	57.37 ± 2.47	38.50 ± 1.70	46.17 ± 1.88	15.36 ± 1.11
Etoposide 10 μg/ml	30.02 ± 0.34	42.92 ± 1.20	46.09 ± 2.31	10.99 ± 1.16

Table 4. The percentage of each cell death pattern induced by the ethanol extract of *S.venosa* on PBMCs. The data were represented as mean ± S.E.M (n=5).

Conditions	Total cell death	Pattern of cell death (%)		
		Apoptosis	Late apoptosis or secondary necrosis	Necrosis
Negative control	7.89 ± 0.42	78.30 ± 1.63	14.75 ± 0.61	6.96 ± 1.56
S.venosa 20 μg/ml	12.31±0.36	63.79 ± 1.53	21.14 ± 0.92	15.08 ± 1.65
S.venosa 40 μg/ml	15.88±0.29	59.78 ± 1.43	23.55 ± 0.89	16.67 ± 1.47
S.venosa 80 μg/ml	22.17±0.15	63.60 ± 0.28	25.87 ± 0.94	10.58 ± 0.75
S.venosa 160 μg/ml	47.85±0.35	49.71 ± 1.36	33.65 ± 1.69	16.44 ± 0.60
Etoposide 10 μg/ml	23.32±0.9	57.61 ± 1.98	37.88 ± 1.59	4.50 ± 0.60

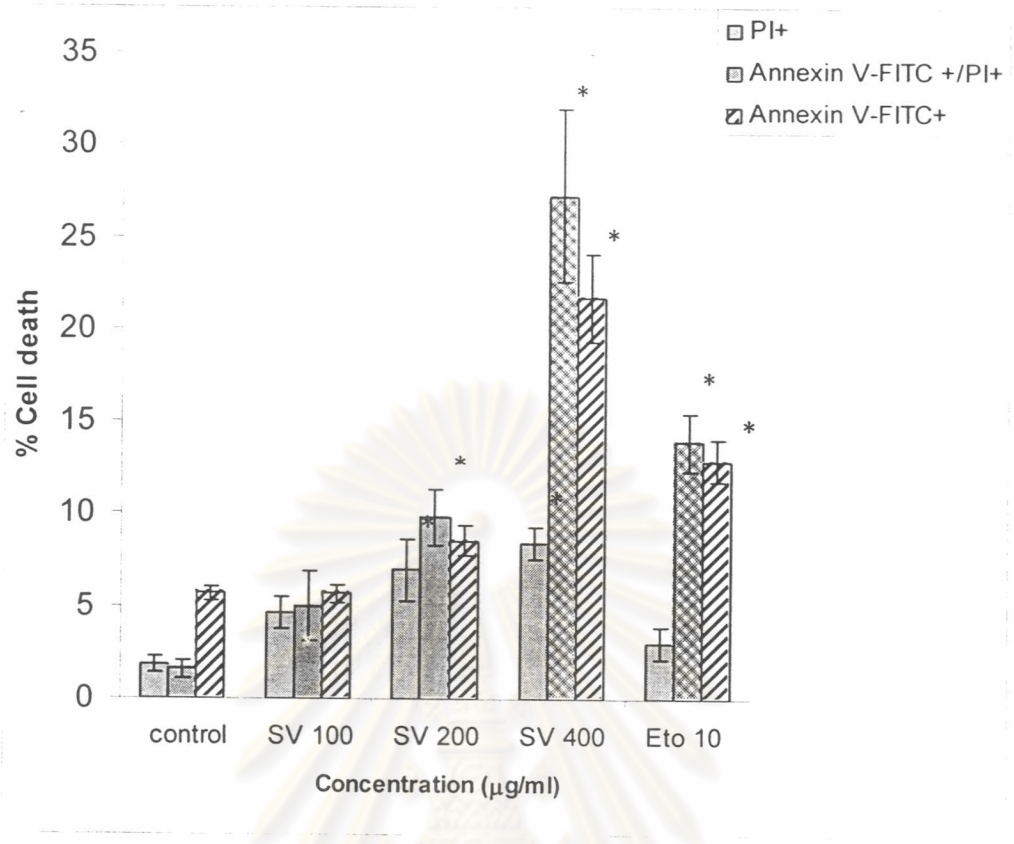

Figure 10. The apoptotic effect of the water extract of *S.venosa* on PBMCs. The percentages of cell death were determined by straining with Annexin V-FITC and PI and using FACS analysis. The data were represented as mean ± S.E.M. (n=5).

* Significant difference when compared to the control group at $p<0.05$

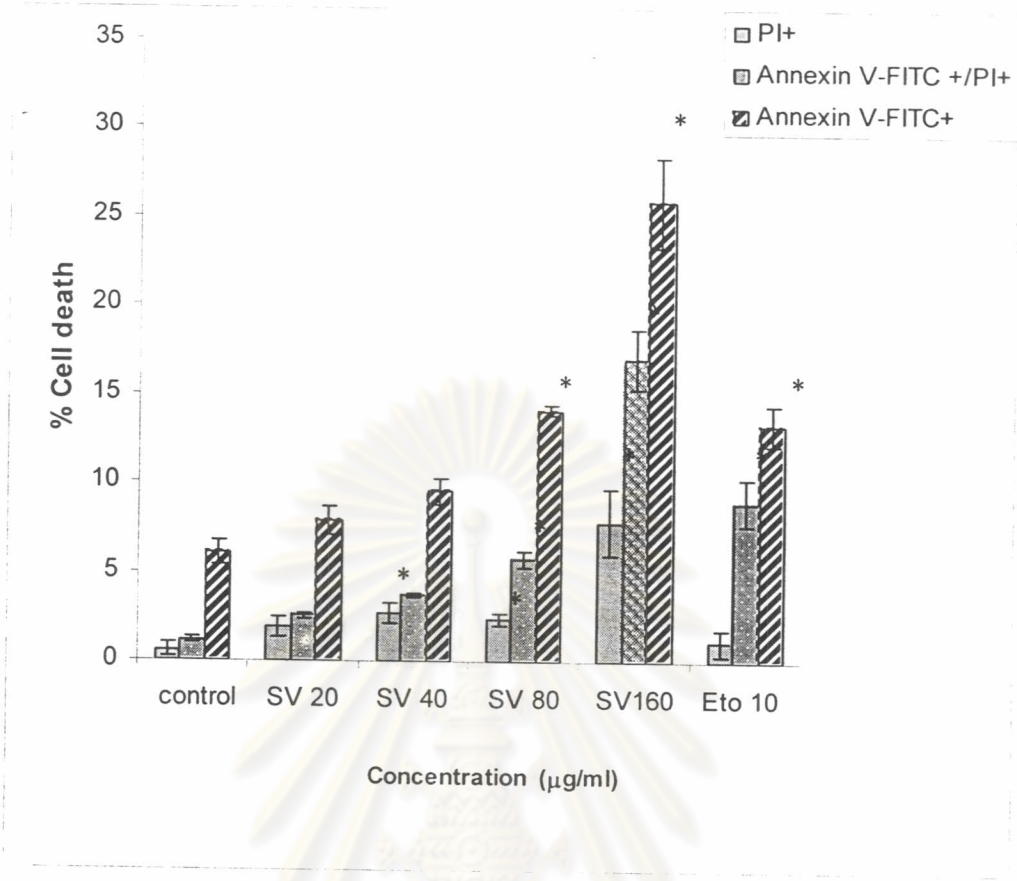

Figure 11. Apoptotic effect of the ethanol extract of *S.venosa* on PBMCs. The percentages of cell death were determined by straining with Annexin V-FITC and PI and using FACS analysis. The data were represented as mean ± S.E.M (n=5).

* Significant difference when compared to the control group at $p<0.05$