CHAPTER III

RESULTS

3.1 Screening for immunostimulating activity from Thai medicinal plants

In order to find medicinal plants that have immunostumulating properties, the lymphocyte activation assay was used to screen eleven Thai medicinal plants that have been shown to have anti-mutagenicity, anti-cancer or anti-inflammatory activities. Human PBMCs were co-cultured with extracts and mitogen. The concentration of extract used in this screening test ranged from 1 μg/ml to 200 μg/ml final concentration. The proliferation of human PBMCs was determined by the [^{3}H]-thymidine incorporation technique. PHA was used to stimulate T and B cell proliferation, whereas PWM was used to stimulate B cell proliferation. The sub-optimal concentrations of both mitogens were used in the following experiments in relation to testing of increase or decrease effects. Sub-optimal concentrations of PHA and PWM were 0.15 μg/ml and 0.1 μg/ml, respectively (appendix D). Comparison of the cell proliferation in non-treated and extracts-treated cultures showed no direct mitogenic activity. Figure 3.1, Figure 3.2, Table 3.1 and Table 3.2, show the effect of water extracts of Thai medicinal plants on lymphocyte mitogenesis. Figure 3.2, Figure 3.3, Table 3.4, and Table 3.5 show the effect of ethanol extracts of Thai medicinal plants on lymphocyte mitogenesis.

3.1.1 Effect of water extracts on lymphocyte mitogenesis

The extracts of *C. asiatica* and *R. nasutus* significantly increased both PHA and PWM-induced lymphocyte proliferation. The lower concentration (1-10 μg/ml) of *E. alba* extract also increased PHA-induced human PBMCs proliferation. In contrast, the extract of *M. loriformis*, Ke-som-Buo, Dee-Buo and *E. alba* significantly decreased both mitogen-induced lymphocyte proliferation. *M. charantia* and *C. citratus* extracts decreased only PHA but not PWM-induced lymphocyte proliferation, suggesting that they affected T cell proliferation (Figure 3.1 and Figure 3.2). Table 3.1 and Table 3.2 showed the actual value of [^{3}H]-thymidine incorporation (cpm) from one of three donors. Other plant extracts did not show any effect on lymphocyte mitogenesis.

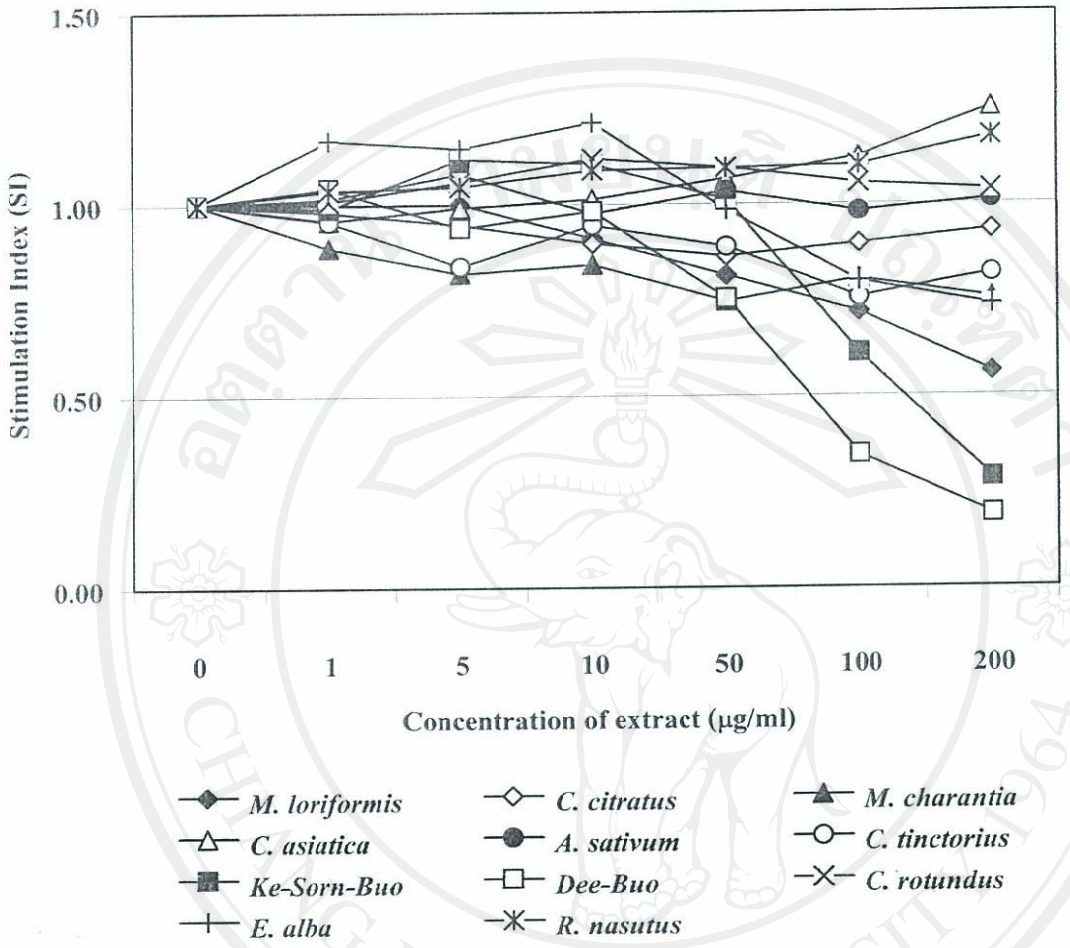

Figure 3.1 Effects of water extracts of Thai medicinal plants on PHA-induced PBMC proliferation. Human PBMCs ($1.0x10^5$ cells/well) were treated with various concentrations of water extracts (1-200 µg/ml) and stimulated with PHA for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of three donors.

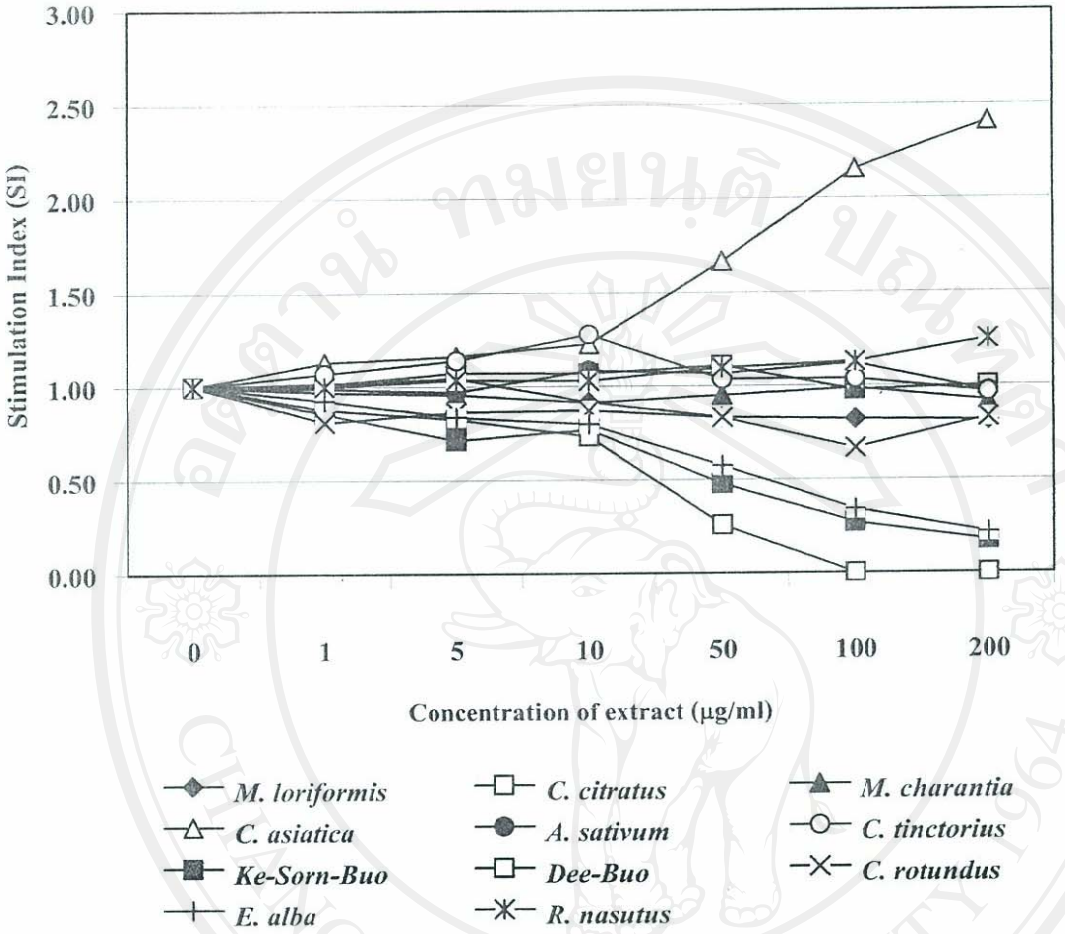

Figure 3.2 Effects of water extracts of Thai medicinal plants on PWM-induced PBMC proliferation. Human PBMCs ($1.0x10^5$ cells/well) were treated with various concentrations of water extracts (1-200 µg/ml) and stimulated with PWM for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of three donors.

Table 3.1 Influence of water extract of Thai medicinal plants on proliferative responses to the PHA stimulation of human PBMCs. Human PBMCs were incubated during 72 hr in the presence of different concentrations of the extract in the absence (negative control) or presence of PHA. Proliferation was determined by [^{3}H]-thymidine incorporation. Each value represents the mean ± S.D. of triplicate cultures from one donor out of three donors.

Plant	[^{3}H]-thymidine incorporation CPM ± S.D.				
	Negative Control	Positive Control	Extract 50 μg/ml	Extract 100 μg/ml	Extract 200 μg/ml
M. loriformis	219 ± 39	13011 ± 553	11844 ± 133*	11663 ± 506*	12468 ± 121
C. citratus	200 ± 43	10142 ± 583	8762 ± 470*	9097 ± 354*	9444 ± 30*
M. charantia	108 ± 13	9876 ± 939	7209 ± 480*	7228 ± 673*	5510 ± 638*
C. asiatica	95 ± 13	13580 ± 341	15203 ± 143*	15994 ± 423*	19223 ± 143*
A. sativum	162 ± 41	10209 ± 1083	10248 ± 215	10023 ± 752	10768 ± 115
C. tinctorius	339 ± 49	8633 ± 268	7718 ± 398	6629 ± 720	7117 ± 799
N. lotus (Ke-Sorn-Buo)	138 ± 40	8331 ± 565	7012 ± 984*	2574 ± 219*	439 ± 35*
N. lotus (Dee-Buo)	138 ± 40	8331 ± 565	5409 ± 716*	1657 ± 216*	207 ± 86*
C. rotundus	206 ± 71	9394 ± 778	10422 ± 864	9760 ± 450	9695 ± 2238
E. alba	156 ± 21	9683 ± 180	7652 ± 448*	6306 ± 700*	5136 ± 386*
R. nasutus	147 ± 48	9173 ± 245	10026 ± 565*	10084 ± 423*	10759 ± 1078*

* Statistical significance versus positive control in each mitogen group: $P<0.05$.

Table 3.2 Influence of water extract of Thai medicinal plants on proliferative responses to the PWM stimulation of human PBMCs. Human PBMCs were incubated during 72 hr in the presence of different concentrations of the extract in the absence (negative control) or presence PWM. Proliferation was determined by [^{3}H]-thymidine incorporation. Each value represents the mean ± S.D. of triplicate cultures from one donor out of three donors.

Plant	[^{3}H]-thymidine incorporation CPM ± S.D.				
	Negative Control	Positive Control	Extract 50 μg/ml	Extract 100 μg/ml	Extract 200 μg/ml
M. loriformis	219 ± 39	4781 ± 555	3323 ± 753*	2749 ± 218*	2342 ± 550*
C. citratus	200 ± 43	2331 ± 253	2543 ± 28	2267 ± 25	2289 ± 339
M. charantia	108 ± 13	2256 ± 192	2040 ± 98	2117 ± 107	1967 ± 221
C. asiatica	95 ± 13	2282 ± 45	3834 ± 259*	5387 ± 258*	6267 ± 191*
A. sativum	162 ± 41	2713 ± 68	2884 ± 171	3015 ± 158*	2968 ± 99*
C. tinctorius	339 ± 49	2709 ± 182	2780 ± 191	2771 ± 460	2617 ± 522
N. lotus (Ke-Sorn-Buo)	138 ± 40	2096 ± 181	1381 ± 47*	806 ± 610*	948 ± 176*
N. lotus (Dee-Buo)	138 ± 40	2069 ± 181	787 ± 11*	345 ± 185*	134 ± 26*
C. rotundus	206 ± 71	2055 ± 119	1875 ± 130*	1223 ± 91*	1270 ± 131*
E. alba	156 ± 21	2504 ± 39	1764 ± 71*	1533 ± 110*	1126 ± 126*
R. nasutus	147 ± 48	1667 ± 1232	2671 ± 227*	2688 ± 253*	2951 ± 60*

* Statistical significance versus positive control in each mitogen group: $P<0.05$.

3.1.2 Effect of ethanol extracts on lymphocyte mitogenesis

At higher concentrations of *C. asiatica*, *N. lotus* (Ke-Sorn-Buo), *N. lotus* (Dee-Buo), *M. loriformis*, *C. rotundus*, *C. tinctorius*, *M. charantia* and *E. alba* extracts significantly decreased both mitogen-induced PBMCs mitogenesis (Figure 3.4 and Figure 3.5). However, the effect of *M. loriformis* and *M. charantia* was caused by their toxicity to human PBMCs (Table 3.3). Other extracts were not toxic to human PBMCs at higher concentration. This result indicated that ethanol extracts of *C. asiatica*, *N. lotus* (Ke-Sorn-Buo), *N. lotus* (Dee-Buo), *C. rotundus*, *C. tinctorius*, and *E. alba* have immunosuppressive activity.

Table 3.3 Effect of plant extracts on viability of human PBMCs. Human PBMCs ($1.0x10^5$ cells/well) were cultured with plant extracts (200 μg/ml). After 3 days, cell survival was assessed by trypan blue exclusion test. The results are mean ± SD of three independent experiments. In control (without extract), % cell viability was 96 ± 5.

Plant	% Cell viability	
	Water extraction	Ethanol extraction
M. loriformis	92 ± 2	50 ± 9
C. citratus	96 ± 1	91 ± 3
M. charantia	93 ± 3	59 ± 6
C. asiatica	96 ± 1	96 ± 1
A. sativum	95 ± 2	94 ± 2
C. tinctorius	96 ± 2	87 ± 4
N. lotus (Ke-Sorn-Buo)	92 ± 3	87 ± 3
N. lotus (Dee-Buo)	91 ± 3	85 ± 4
C. rotundus	95 ± 2	88 ± 3
E. alba	90 ± 2	90 ± 2
R. nasutus	97 ± 2	91 ± 3

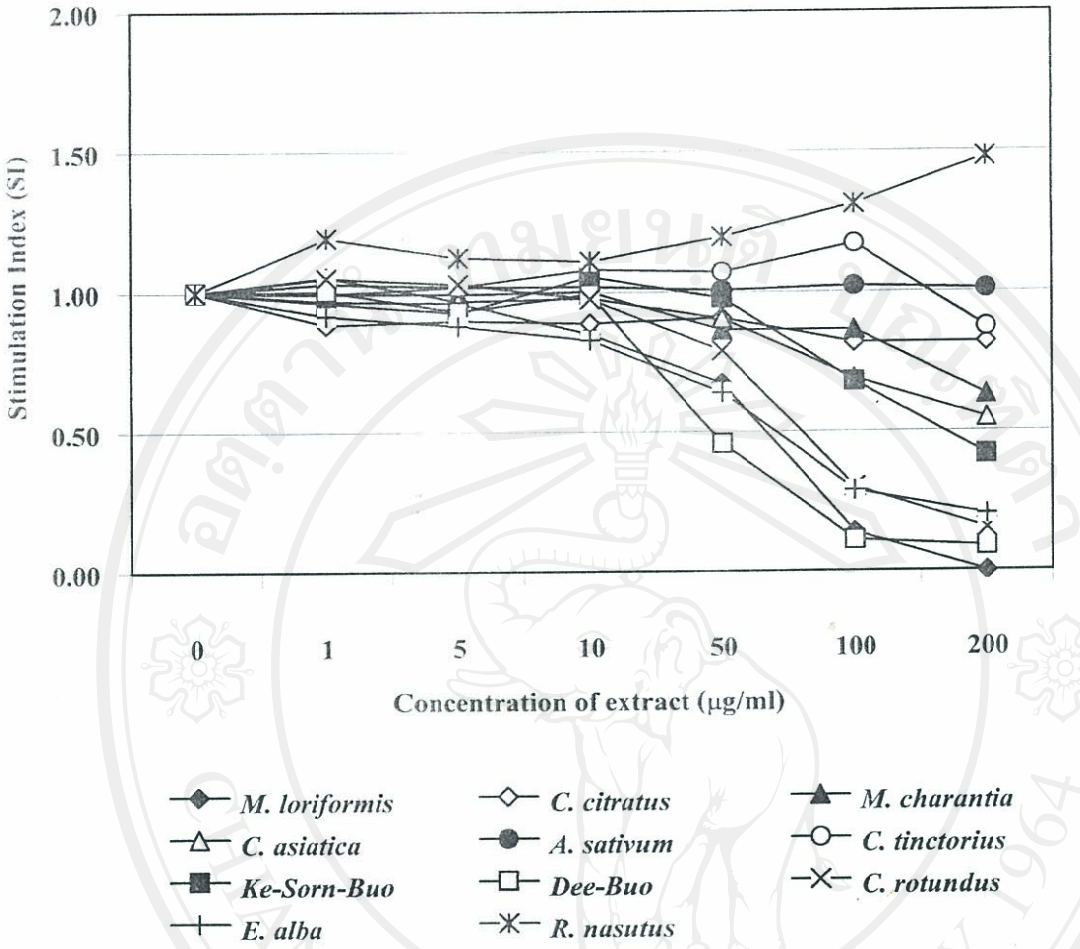

Figure 3.3 Effects of ethanol extracts of Thai medicinal plants on PHA-induced PBMC proliferation. Human PBMCs ($1.0x10^5$ cells/well) were treated with various concentrations of ethanol extracts (1-200 µg/ml) and stimulated with PHA for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of three donors.

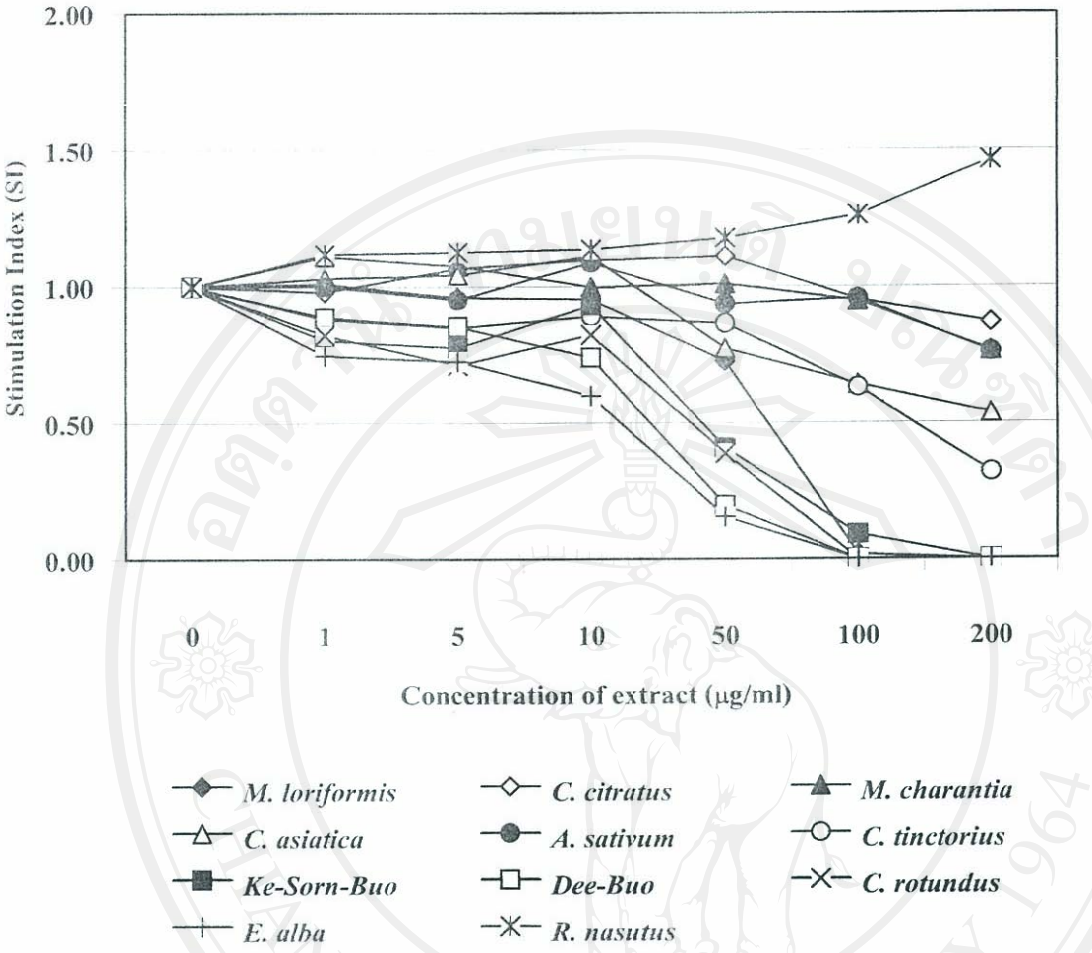

Figure 3.4 Effects of ethanol extracts of Thai medicinal plants on PWM-induced PBMC proliferation. Human PBMCs ($1.0x10^5$ cells/well) were treated with various concentrations of ethanol extracts (1-200 μg/ml) and stimulated with PWM for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of three donors.

Table 3.4 Influence of ethanol extract of Thai medicinal plants on proliferative responses to the PHA stimulation of human PBMCs. Human PBMCs were incubated during 72 hr in the presence of different concentrations of the extract in the absence (negative control) or presence PHA. Proliferation was determined by [^{3}H]-thymidine incorporation. Each value represents the mean ± S.D. of triplicate cultures from one donor out of three donors.

Plant	[^{3}H]-thymidine incorporation CPM ± S.D.				
	Negative Control	Positive Control	Extract 50 μg/ml	Extract 100 μg/ml	Extract 200 μg/ml
M. loriformis	219 ± 39	13011 ± 553	9705 ± 3080*	847 ± 40*	18 ± 3*
C. citratus	200 ± 43	10142 ± 583	8838 ± 481*	7145 ± 1051*	9233 ± 576
M. charantia	108 ± 13	9876 ± 939	8505 ± 125*	8831 ± 787	6136 ± 690*
C. asiatica	230 ± 52	11113 ± 713	8265 ± 782*	6568 ± 478*	5587 ± 225*
A. sativum	162 ± 41	10209 ± 1083	9456 ± 903	10486 ± 773	10103 ± 137
C. tinctorius	157 ± 52	9997 ± 710	10796 ± 511	10626 ± 651	8619 ± 622*
N. lotus (Ke-Sorn-Buo)	138 ± 40	9854 ± 820	7761 ± 260*	7389 ± 983*	2897 ± 40*
N. lotus (Dee-Buo)	120 ± 20	9854 ± 820	4411 ± 450*	1025 ± 223*	123 ± 221*
C. rotundus	206 ± 71	9394 ± 778	8340 ± 201*	3643 ± 1336*	559 ± 221*
E. alba	156 ± 21	9683 ± 180	4650 ± 376*	2672 ± 273*	406 ± 201*
R. nasutus	184 ± 15	9700 ± 1005	11618 ± 940*	12699 ± 702*	14304 ± 843*

* Statistical significance versus positive control in each mitogen group: $P<0.05$.

Table 3.5 Influence of ethanol extract of Thai medicinal plants on proliferative responses to the PWM stimulation of human PBMCs. Human PBMCs were incubated during 72 hr in the presence of different concentrations of the extract in the absence (negative control) or presence PWM. Proliferation was determined by [^{3}H]-thymidine incorporation. Each value represents the mean ± S.D. of triplicate cultures from one donor out of three donors.

Plant	[^{3}H]-thymidine incorporation CPM ± S.D.				
	Negative Control	Positive Control	Extract 50 μg/ml	Extract 100 μg/ml	Extract 200 μg/ml
M. loriformis	219 ± 39	4781 ± 555	3461 ± 1474	435 ± 168*	159 ± 74*
C. citratus	200 ± 43	2331 ± 253	2343 ± 278	2166 ± 119	1564 ± 226*
M. charantia	108 ± 13	2256 ± 192	2011 ± 333	2075 ± 274	1735 ± 124*
C. asiatica	230 ± 52	2081 ± 197	1479 ± 164*	1293 ± 137*	1207 ± 54*
A. sativum	162 ± 41	2713 ± 68	2557 ± 444	2600 ± 285	2435 ± 372
C. tinctorius	157 ± 52	2482 ± 233	1671 ± 288*	1316 ± 312*	593 ± 183*
N. lotus (Ke-Sorn-Buo)	138 ± 40	2087 ± 318	785 ± 45*	340 ± 89*	145 ± 42*
N. lotus (Dee-Buo)	120 ± 20	2087 ± 318	408 ± 9*	80 ± 8*	56 ± 6*
C. rotundus	206 ± 71	2055 ± 119	942 ± 177*	213 ± 14*	85 ± 12*
E. alba	156 ± 21	2504 ± 39	1429 ± 45*	1157 ± 12*	855 ± 57*
R. nasutus	184 ± 15	3479 ± 289	4081 ± 126*	4319 ± 103*	5008 ± 321*

* Statistical significance versus positive control in each mitogen group: $P<0.05$.

3.2 Effects of *C. asiatica* and *R. nasutus* extracts on lymphocytes

From the screening results, only *C. asiatica* and *R. nasutus* extracts, which showed immunostimulating activity, were used for further experiments. To confirm the immunomodulating activity of *C. asiatica* and *R. nasutus* extracts, plants were collected in three independent batches and tested on lymphocyte mitogenesis. Batch 1 and Batch 3 of plants were collected during the winter season (November to January), whilst batch 2 was collected on May. The yield of each batch of plant extraction was similar (Table 3.6).

All extracts from three batches showed similar immunomodulating results as shown in Figure 3.5 and Figure 3.6. However, *C. asiatica* extracts from batch 1 and batch 3 seemed to have stronger immunomodulation effects than batch 2.

Table 3.6 The % yield of *C. asiatica* and *R. nasutus* after water and ethanol extraction. Three different batches of *C. asiatica* and *R. nasutus* were extracted as described in section 2.2.2. The % yield was calculated from gram of dried weight plant powder (100g).

Plant	% Yield	
	Water extraction	Ethanol extraction
C. asiatica		
Batch 1	5.42	5.26
Batch 2	5.62	5.43
Batch 3	5.16	5.05
R. nasutus		
Batch 1	8.20	7.04
Batch 2	7.74	6.79
Batch 3	8.43	7.20

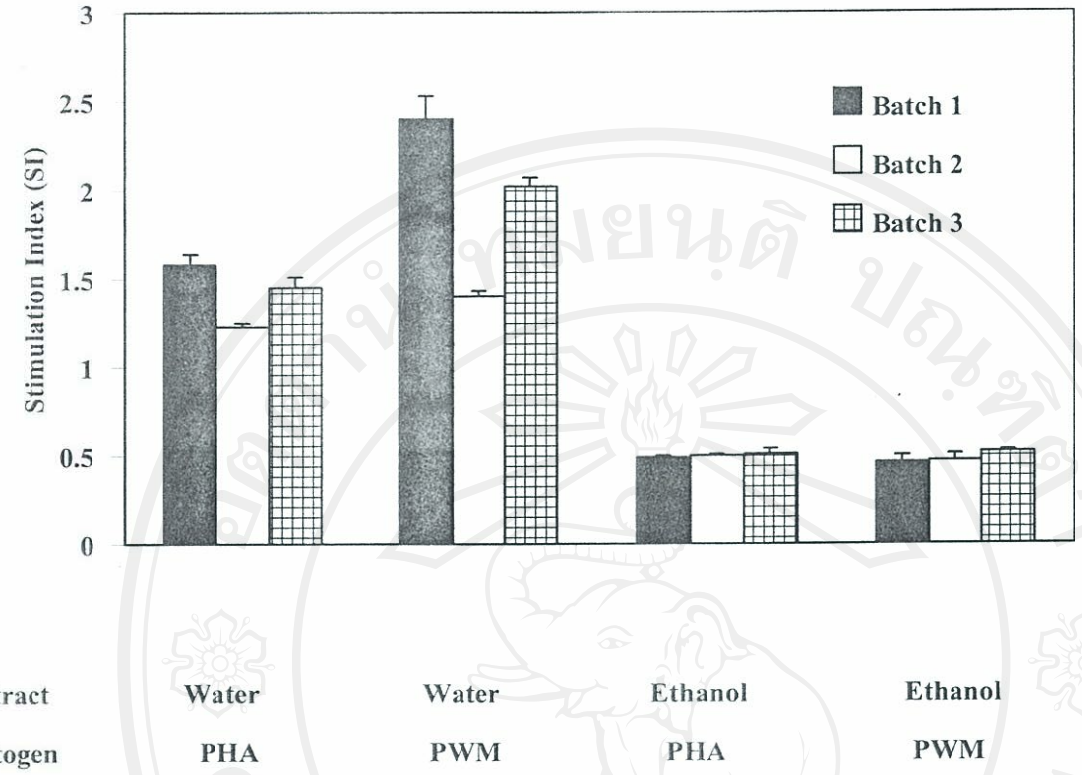

Figure 3.5 Effects of three different batches of *C. asiatica* on lymphocyte mitogenesis. Human PBMCs were cultured with three different batches of *C. asiatica* extracts (200μg/ml) with or without PHA or PWM for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of three independent experiments from the same donor.

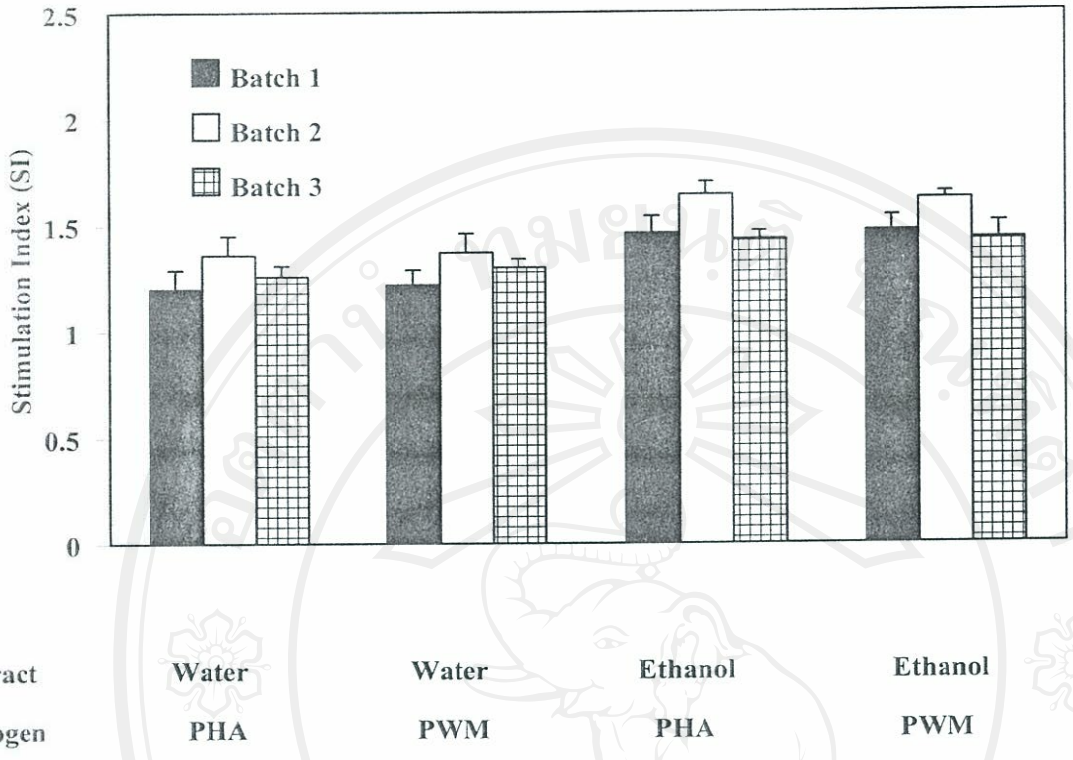

Figure 3.6 Effects of three different batches of *R. nasutus* on lymphocyte mitogenesis. Human PBMCs were cultured with three different batches of *R. nasutus* extracts (200µg/ml) with or without PHA or PWM for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of three independent experiments from the same donor.

3.2.1 Effects of *C. asiatica* and *R. nasutus* extracts on lymphocyte mitogenesis

The water extract of *C. asiatica* significantly increased PWM-induced lymphocyte proliferation with a dose-response manner, and slightly increased PHA-induced lymphocyte proliferation as shown in Figure 3.7. Although a big variation of lymphocyte proliferative response among donors was observed, an increase cell proliferation by water extract of *C. asiatica* was found in all donors (Figure 3.8). Ethanol extract of *C. asiatica* inhibited mitogen-induced lymphocyte mitogenesis as shown in Figure 3.7. The viability of lymphocytes was also determined by trypan blue exclusion to confirm that this inhibition effect was not caused by the cytotoxicity of ethanol extract itself. As shown in Table 3.5, human PBMCs treated with ethanol extract of *C. asiatica* showed high viability. This result indicated that *C. asiatica* has both immunostimulant and immunosuppressive activities. Both water and ethanol extracts of *R. nasutus* significantly increased lymphocyte proliferation induced by either PHA or PWM as shown in Figure 3.9. These results showed immunomodulating activity of plant extracts on cell-mediated immune response.

Some purified compounds, asiaticoside and asiatic acid, have been reported to be a biological active ingredients in *C. asiatica*. These compounds were tested an effect on lymphocyte mitogenesis. Both asiaticoside and asiatic acid had no effect on lymphocyte proliferation (Figure3.10).

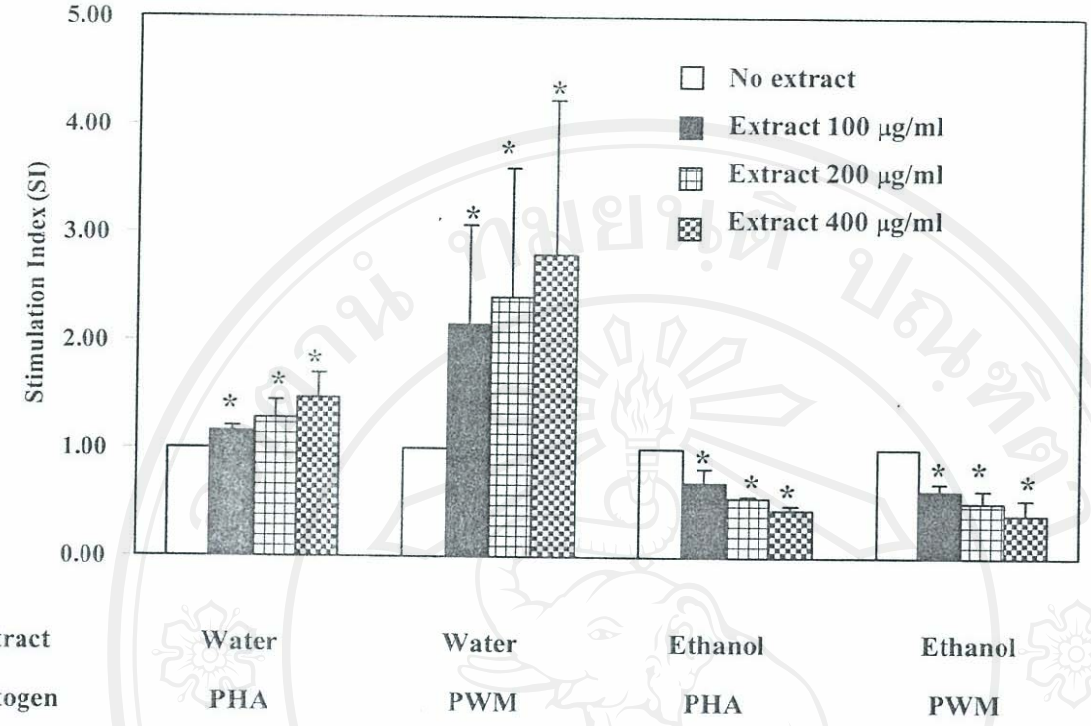

Figure 3.7 Effects of *C. asiatica* extracts on lymphocyte mitogenesis. Human PBMCs ($1.0x10^5$ cells/well) were cultured with various concentrations of *C. asiatica* extracts (100-400 µg/ml) with or without PHA or PWM for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of three donors.

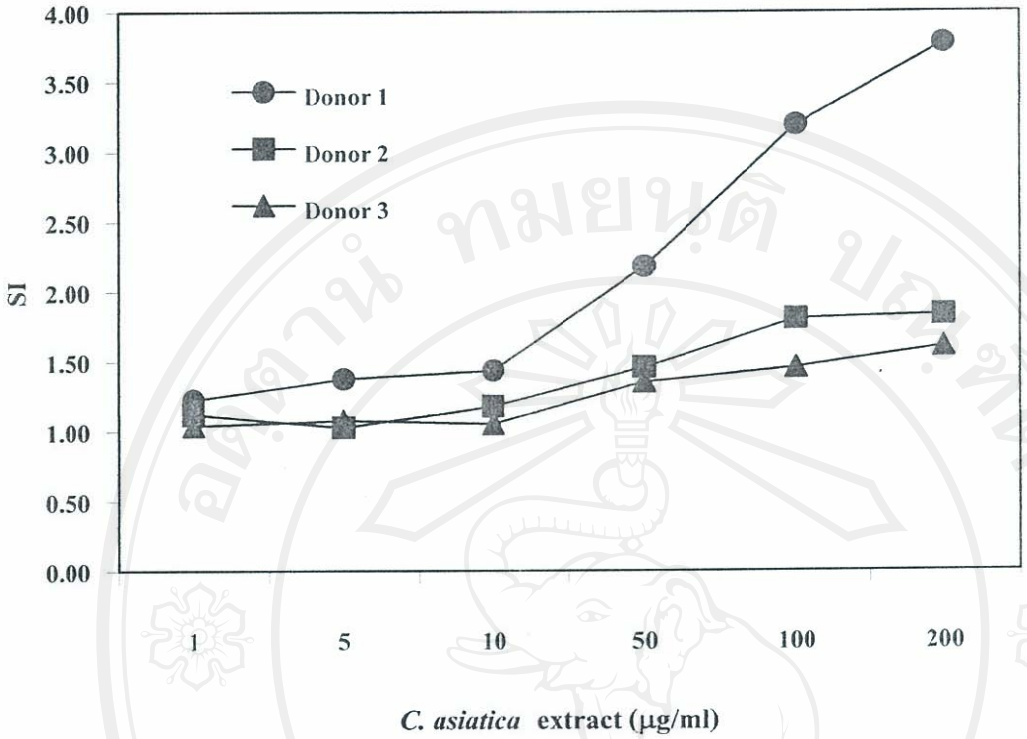

Figure 3.8 Variation of PWM-induced lymphocyte mitogenesis in response to *C. asiatica* treatment. Human PBMCs ($1.0x10^5$ cells/well), isolated from three independent donors were cultured with water extract of *C. asiatica* (1-200 μg/ml) with or without PWM for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of triplicate cultures. Standard deviation of all values was less than 0.1.

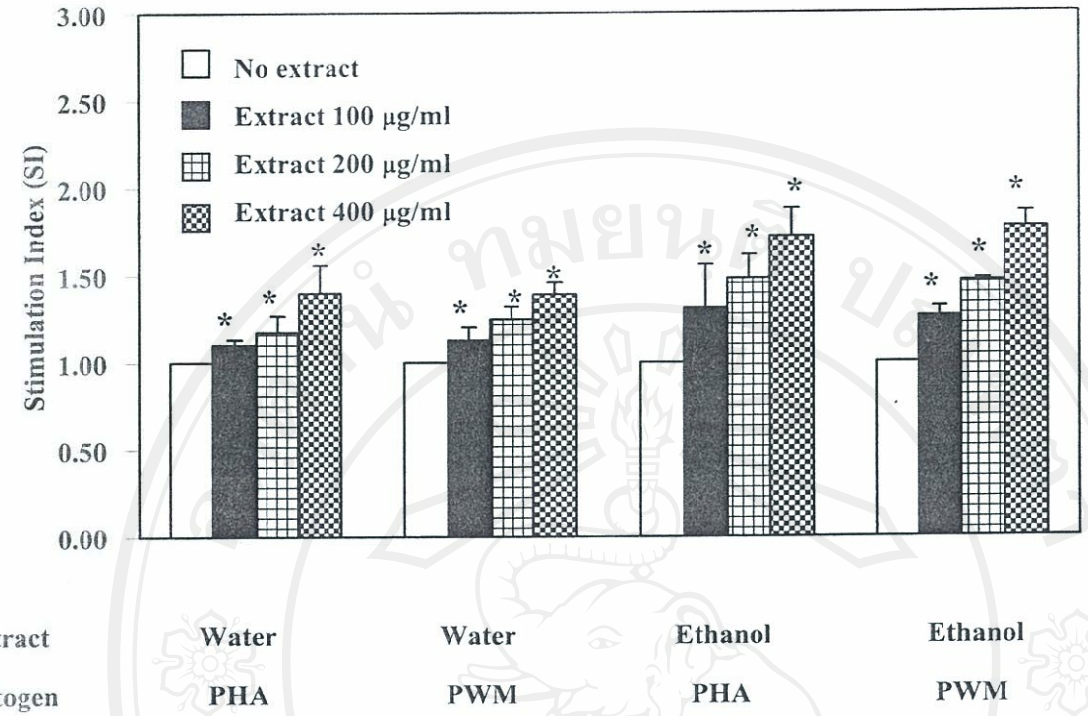

Figure 3.9 Effects of *R. nasutus* on lymphocyte mitogenesis. Human PBMCs ($1.0x10^5$ cells/well) were cultured with various concentrations of *R. nasutus* extracts (100-400 µg/ml) with or without PHA or PWM for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of three donors.

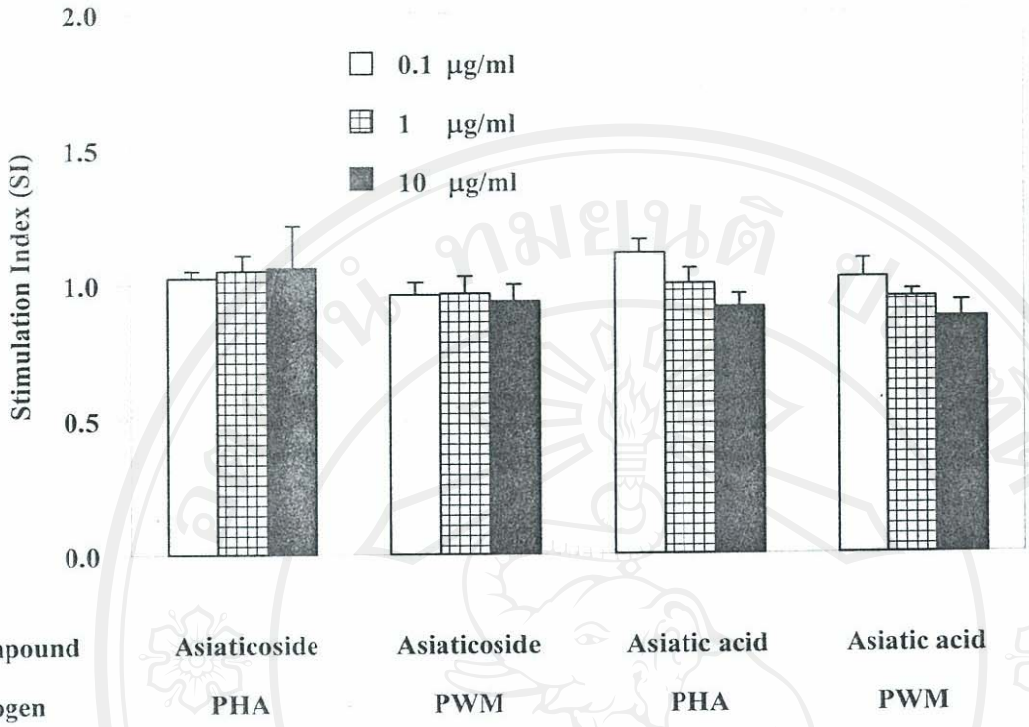

Figure 3.10 Effects of asiaticoside and asiatic acid on lymphocyte mitogenesis. Human PBMCs ($1.0x10^5$ cells/well) were cultured with various concentrations of asiaticoside or asiatic acid (0.1-10 µg/ml) with or without PHA or PWM for 72 hr. Proliferation was evaluated by [^{3}H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value is 1.00. The results are expressed as the average of three donors.

3.2.2 Effects of *C. asiatica* and *R. nasutus* extracts on cytokine production

Human PBMCs were used for the production of IL-2 and TNF-α. The water extract of *C. asiatica* at concentration 500 μg/ml increased PHA-stimulated IL-2 as well as LPS-stimulated TNF-α production. In contrast, the ethanol extract of *C. asiatica* inhibited IL-2 and TNF-α production as shown in Figure 3.11 and Figure 3.12. Both water and ethanol extract of *R. nasutus* increased PHA-stimulated IL-2 as well as LPS-stimulated TNF-α production in human PBMCs when cells were exposed to the extract for 18 h as shown in Figure 3.13 and Figure 3.14.

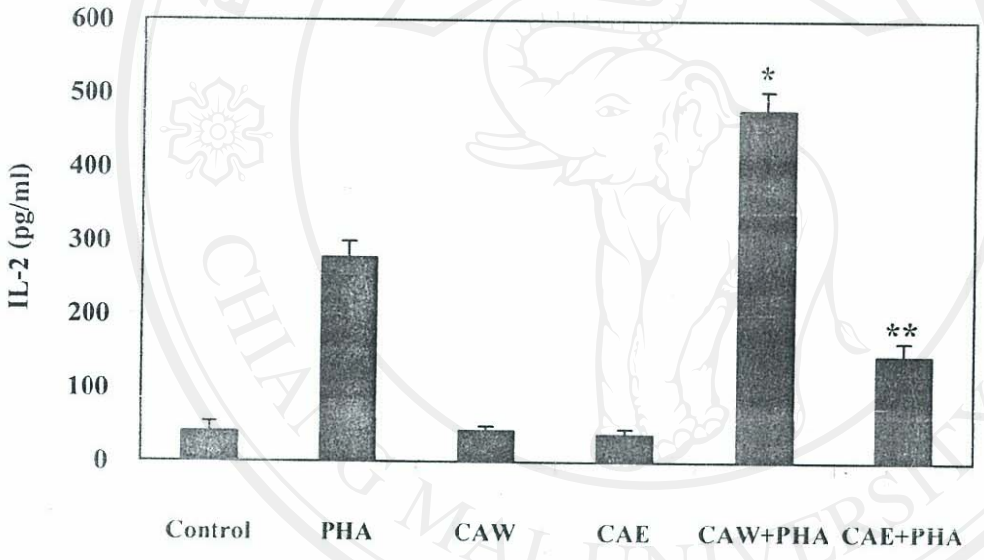

Figure 3.11 Effects of *C. asiatica* extracts on IL-2 production in human PBMCs. Human PBMCs ($5x10^5$ cells/ml) were cultured in the presence or absence of 500 μg/ml of *C. asiatica* extracts, water extract (CAW) or ethanol extract (CAE) for 18 hr. PHA (5 μg/ml) was used as a positive control to stimulate IL-2. * $p<0.05$: a significance increase versus PHA control, ** $p<0.05$: a significance decrease versus PHA control.

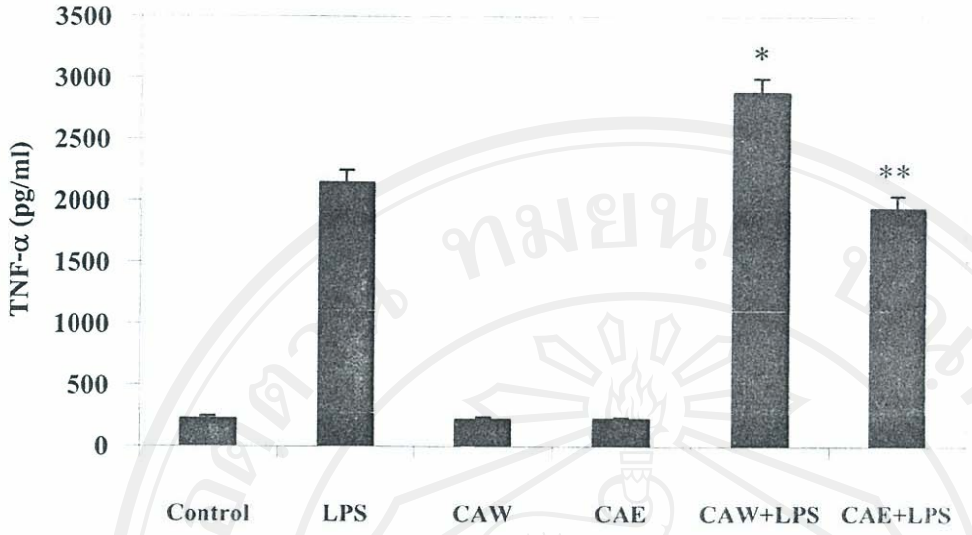

Figure 3.12 Effects of *C. asiatica* extracts on TNF-α production in human PBMCs. Human PBMCs ($5x10^5$ cells/ml) were cultured in the presence or absence of 500 μg/ml of *C. asiatica* extracts, water extract (CAW) or ethanol extract (CAE) for 18 hr. LPS (10 ng/ml) was used as a positive control to stimulate TNF-α. * $p<0.05$: a significance increase versus LPS control. ** $p<0.05$: a significance decrease versus LPS control.

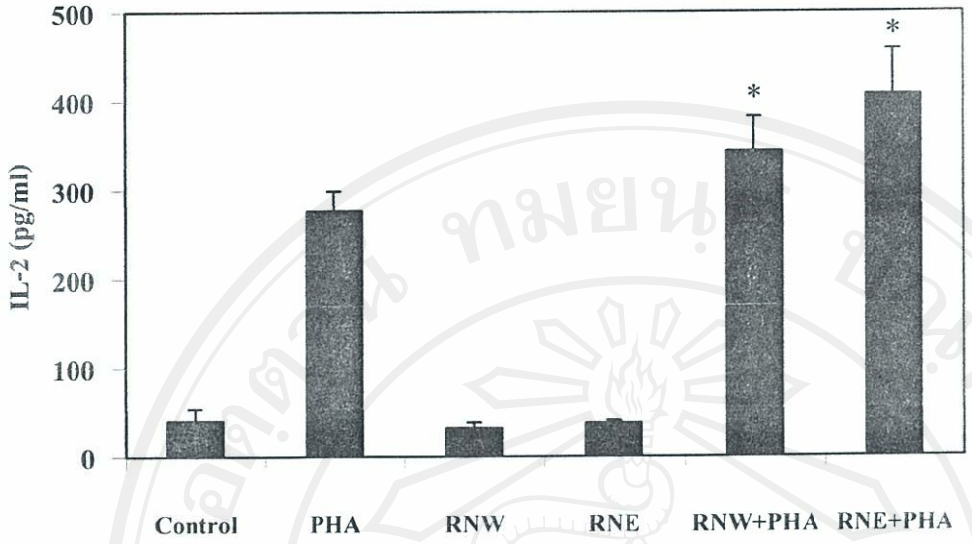

Figure 3.13 Effects of *R. nasutus* extracts on IL-2 production in human PBMCs. Human PBMCs ($1x10^5$ cells/ml) were cultured in the presence or absence of 500 μg/ml of *R. nasutus* extracts, water extract (RNW) or ethanol extract (RNE) for 18 hr. PHA (5 μg/ml) was used as a positive control to stimulate IL-2. Statistical significance versus PHA control: * $p<0.05$.

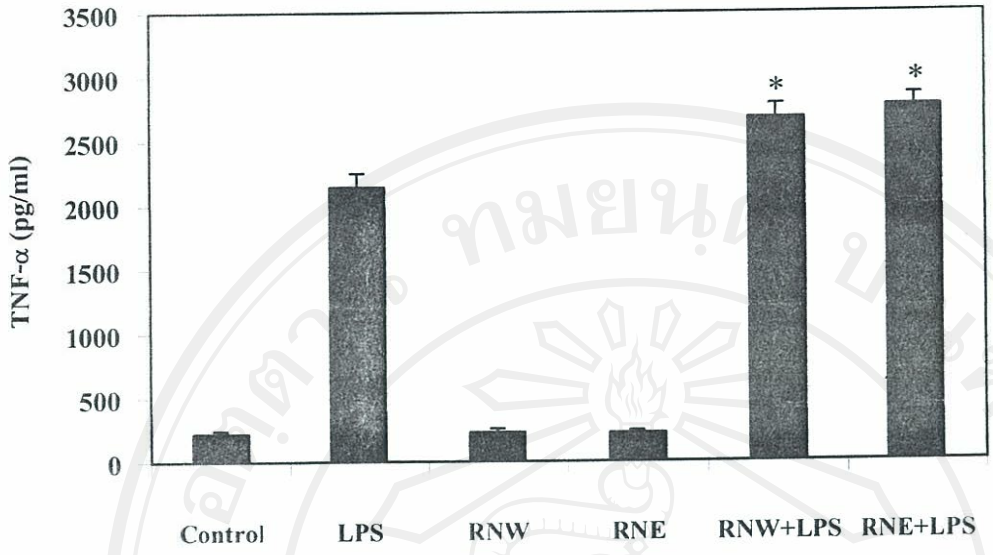

Figure 3.14 Effects of *R. nasutus* extracts on TNF-α production in human PBMCs. Human PBMCs ($5x10^5$ cells/ml) were cultured in the presence or absence of 500 μg/ml of *R. nasutus* extracts, water extract (RNW) or ethanol extract (RNE) for 18 hr. LPS (10 ng/ml) was used as a positive control to stimulate TNF-α. Statistical significance versus LPS control: * $p<0.05$.

3.3 Effects of *C. asiatica* and *R. nasutus* extracts on macrophages

3.3.1 Cytotoxicity of *C. asiatica* and *R. nasutus* extracts on J774.2 mouse macrophages

The MTT assay was used to assess the cytotoxicity of plant extracts on the J774.2 mouse macrophage cells. As shown in Figure 3.15, there was no detectable effect with any of the extracts at concentrations between 62.5-500 μg/ml. At a dosage of 1 mg/ml of extracts, viability of cells was still higher than 80%, except for the ethanol extract of *C. asiatica* showed mild cytotoxicity to the cells. The ethanol extract of *C. asiatica* showed a strongly cytotoxic effect at a dosage of 2 mg/ml. The concentration of the extracts in the following experiments was up to 1 mg/ml.

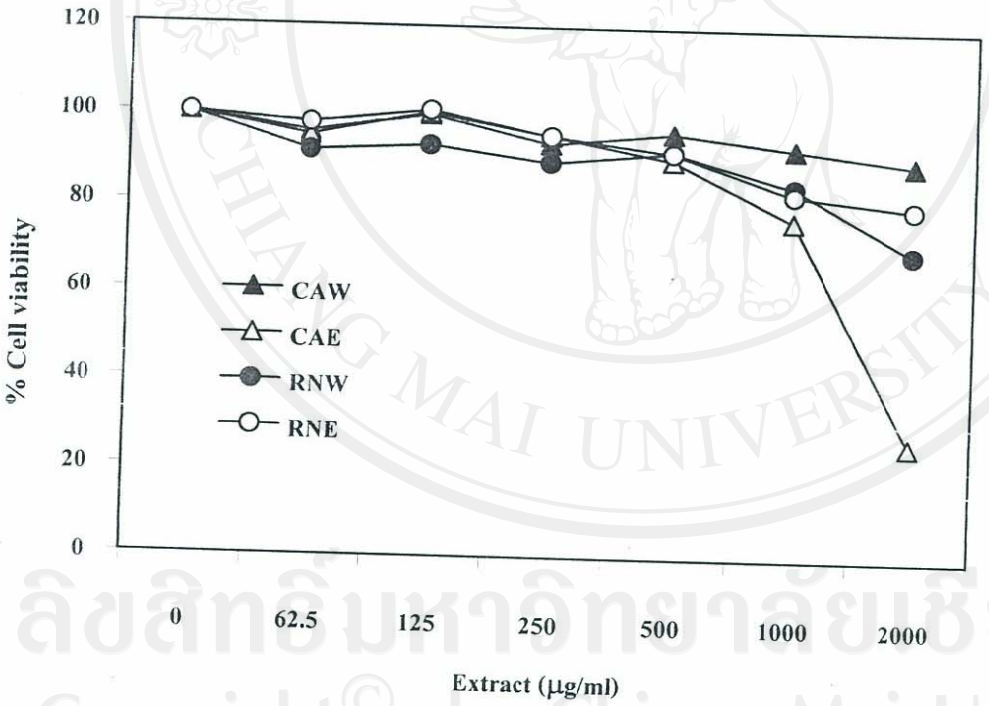

Figure 3.15 Effects of *C. asiatica* and *R. nasutus* extracts on viability of J774.2 mouse macrophages. J774.2 mouse macrophages were treated with various concentrations of *C. asiatica*, water (CAW) and ethanol (CAE) extract, and *R. nasutus*, water (RNW) and ethanol (RNE) extract for 48 hr. Cell viability was determined by the colorimetric MTT cleavage assay. Data are the mean of triplicate cultures. The result of one typical experiment out of three independent experiments is depicted.

3.3.2 Effects of *C. asiatica* and *R. nasutus* extracts on nitric oxide production

To determine whether macrophages could be stimulated by plant extracts, either alone or in combination with LPS, to alter NO and TNF-α production. J774.2 mouse macrophages were cultured with various concentrations of extracts in the presence or absence of LPS. The water extract of *C. asiatica* itself increased NO production at concentrations above 62.5 μg/ml in a dose dependent manner; the maximum NO concentration produced was 1.6 μM at 1000 μg/ml of extract as shown in Figure 3.16. This stimulation with the water extract of *C. asiatica* was confirmed with a different batch of *C. asiatica*. As shown in Figure 3.17, all three batches of water extract of *C. asiatica* (1 mg/ml) showed the stimulation of NO production in mouse macrophages. However, macrophages can be induced to produce NO and TNF-α by LPS, a main component of bacterial endotoxins (Stuehr and Maeletta, 1985). To confirm that the induction of NO by the *C. asiatica* water extract was not due to LPS contamination, polymyxin B (10 μg/ml), an LPS inhibitor, was co-incubated with the extract and did not diminish NO induction as shown in Figure 3.18. The level of NO in LPS (10 ng/ml) stimulated macrophage was 4 μM and this level was completely inhibited (0 μM) when co-incubating with 10 μg/ml of polymyxin B. No significant effect was observed with the ethanol extraction of *C. asiatica*, or with either water or ethanol extracts of *R. nasutus* (Figure 3.16).

Stimulation of cells with LPS resulted in a greatly increased generation of NO to a level of around 4 μM compared to <0.1 μM in untreated cells. The effect of plant extracts on this LPS-induced NO production was then examined. In the presence of LPS, the water extract of *C. asiatica* still enhanced NO synthesis, although there was no clear dose-response relation. In contrast, an inhibition of the LPS simulated production of NO was found with the *C. asiatica* ethanol extract at concentrations of 500 and 1000 μg/ml. Although the ethanol extract of *R. nasutus* itself had no effect on NO, we actually observed a relatively strong stimulation when the extract was incubated with the cells and LPS (Figure 3.19); the water extract of *R. nasutus* had little effect.

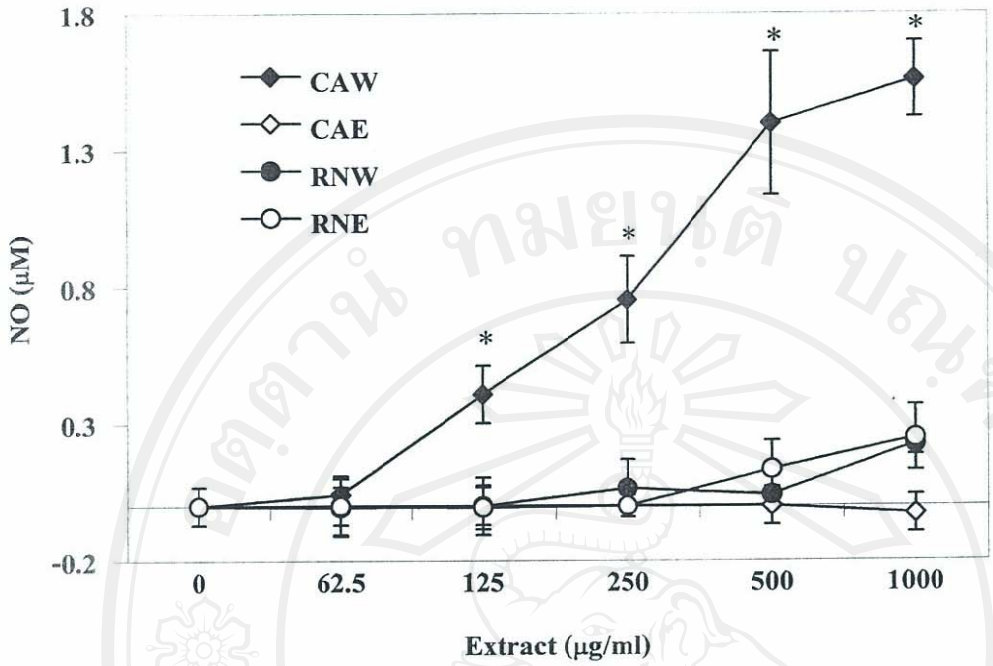

Figure 3.16 Effects of plant extracts on NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were cultured with various concentrations of water and ethanol extract of *C. asiatica* (CAW and CAE, respectively) or of *R. nasutus* (RNW and RNE, respectively). Supernatants were collected after 24 hr and nitrite level was determined by the Griess reaction. The data represent the mean ± SD of triplicate cultures. The result of one typical experiment out of three independent experiments is depicted. LPS (10 ng/ml) was used as a positive control. NO level in positive control was 5μM. * $P<0.05$, significantly different from the control.

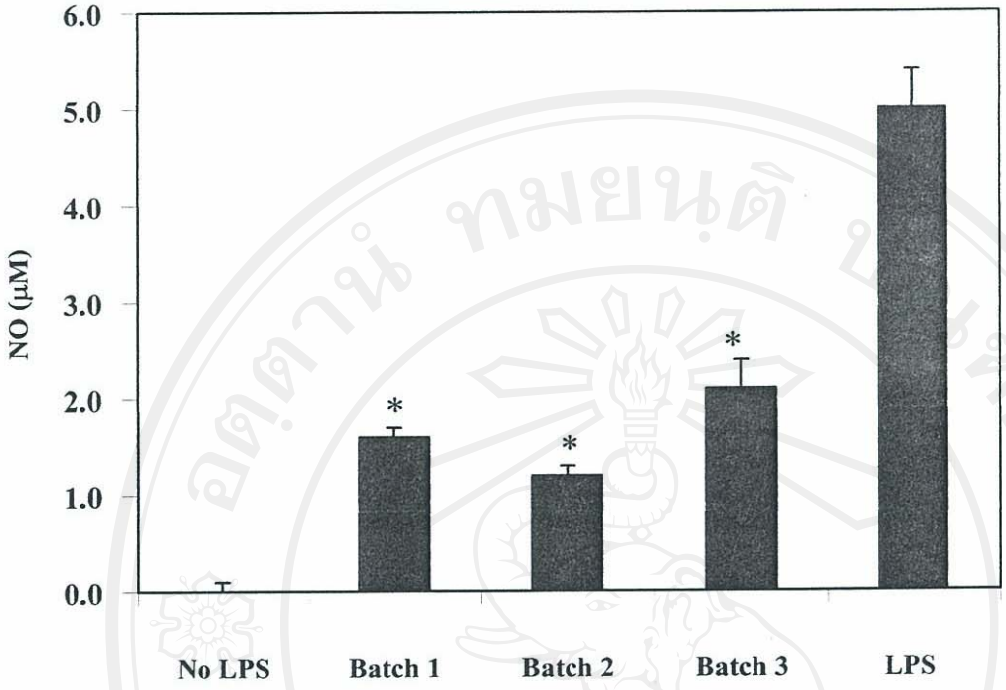

Figure 3.17 Effects of three different batches of *C. asiatica* water extracts on NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were cultured with 1 mg/ml of three different batches of water extract of *C. asiatica*. Supernatants were collected after 24 hr and nitrite level was determined by the Griess reaction. The data represent the mean ± SD of triplicate cultures. The result of one typical experiment out of three independent experiments is depicted. LPS (10 ng/ml) was used as a positive control. * $P<0.05$, significantly different from the control (No LPS).

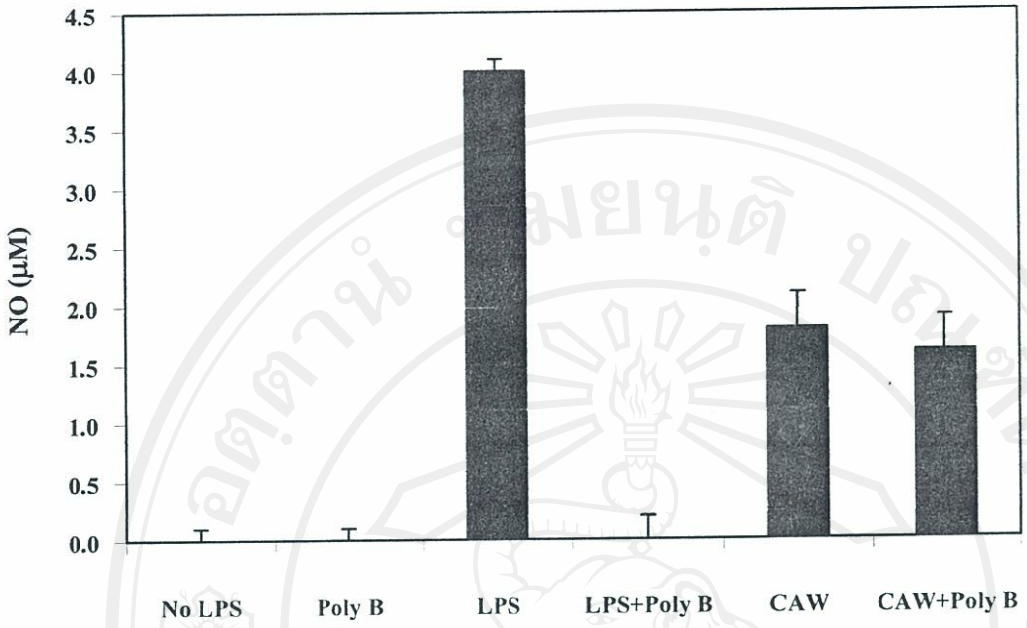

Figure 3.18 Effects of polymyxin B on water extracts of *C. asiatica* induced-NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were cultured with 1 mg/ml of water extract of *C. asiatica* (CAW) in absence or presence of 10 μg/ml polymyxin B mix (Poly B). Supernatants were collected after 24 hr and nitrite level was determined by the Griess reaction. The data represent the mean ± SD of triplicate cultures. The result of one typical experiment out of three independent experiments is depicted. LPS (10 ng/ml) was used as a positive control.

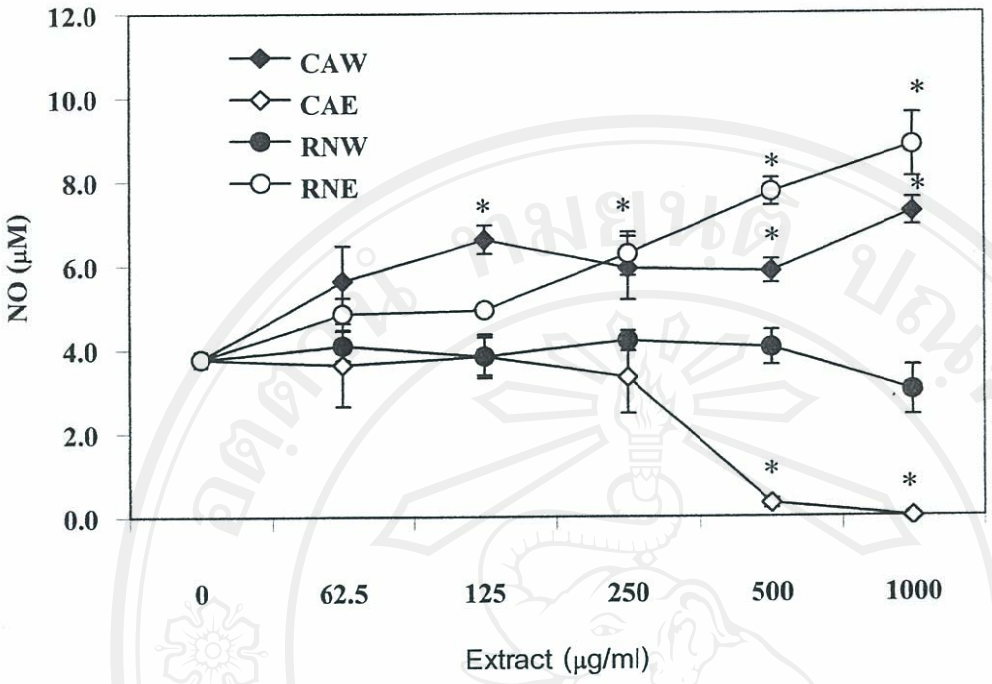

Figure 3.19 Effects of plant extracts on LPS- induced NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were cultured with various concentrations of water and ethanol extract of *C. asiatica* (CAW and CAE, respectively) or of *R. nasutus* (RNW and RNE, respectively) and 10 ng/ml of LPS. Supernatants were collected after 24 hr of LPS stimulation and nitrite level was determined by Griess reaction. The data represent the mean ± SD of triplicate cultures. The result of one typical experiment out of three independent experiments is depicted. * $P<0.05$, significantly different from the control.

3.3.3 Effects of *C. asiatica* and *R. nasutus* extracts on TNF-α production

TNF-α, one of the pro-inflammatory cytokines, is a key mediator of macrophage function, and can induce the production of NO. Thus the modulation of NO production by these extracts might be mediated via an effect on TNF-α and therefore the influence of plant extracts on TNF-α was examined. The extracts themselves had no effect except for the water extract of *C. asiatica*, which resulted in a significant increase in TNF-α, consistent with the increase in NO production (Figure 3.20). When the various extracts were incubated in the presence of LPS, the water extract of *C. asiatica* and ethanol extract of *R. nasutus* significantly increased the production of TNF-α, again correlating with the previously observed effects on NO production (Figure 3.21). In contrast, the ethanol extract of *C. asiatica* actually suppressed the production of TNF-α at the concentration >62.5 μg/ml (Figure 3.21) as it did the production of NO (Figure 3.19).

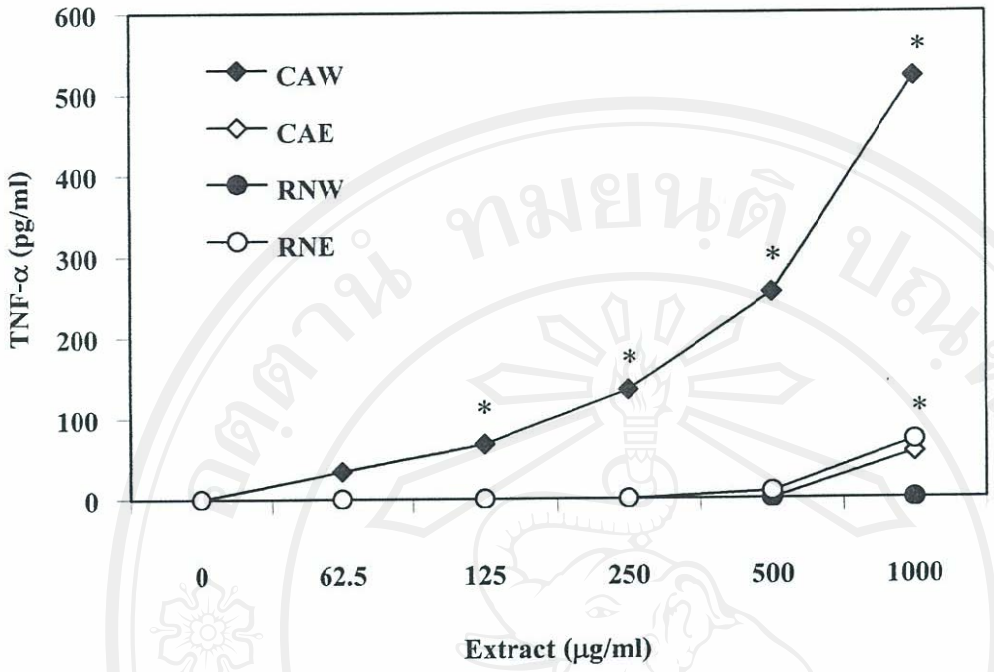

Figure 3.20 Effects of plant extracts on TNF-α production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were cultured with various concentrations of water and ethanol extract of *C. asiatica* (CAW and CAE, respectively) or of *R. nasutus* (RNW and RNE, respectively). Supernatants were collected after 24 hr and TNF-α level was determined by ELISA. The data represent the TNF-α level from pooled triplicate cultures. The result of one typical experiment out of three independent experiments is depicted. LPS (10 ng/ml) was used as a positive control. TNF-α level in positive control was 200 pg/ml. * $P<0.05$, significantly different from the control.

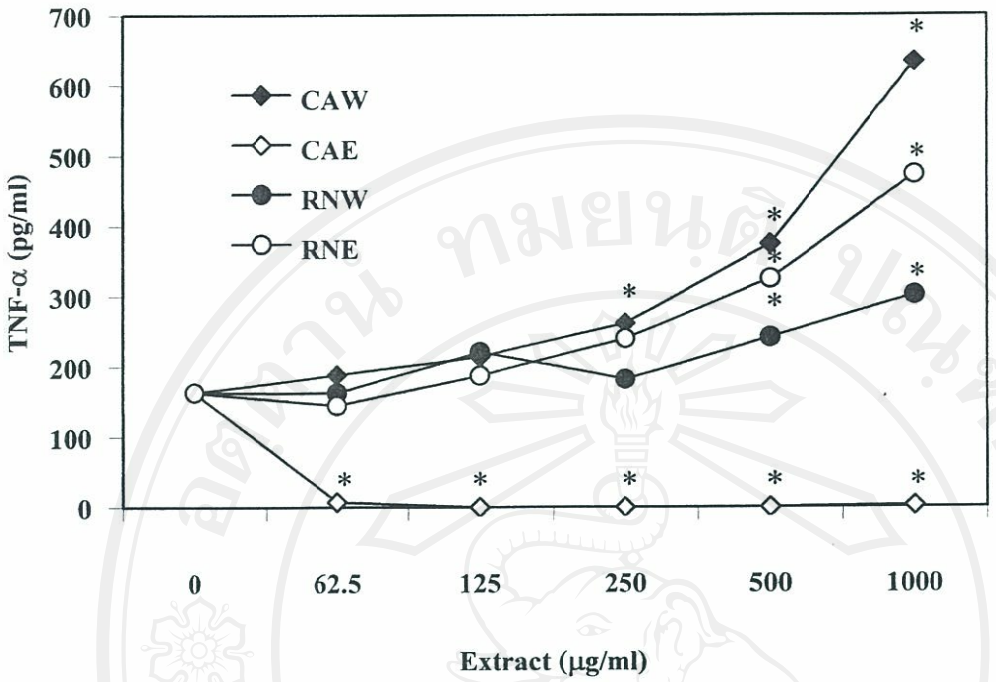

Figure 3.21 Effects of plant extracts on LPS- induced TNF-α production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were cultured with various concentrations of water and ethanol extract of *C. asiatica* (CAW and CAE, respectively) or of *R. nasutus* (RNW and RNE, respectively) and 10 ng/ml of LPS. Supernatants were collected after 24 hr of LPS stimulation and TNF-α level was determined by ELISA. The data represent the TNF-α level from pooled triplicate cultures. The result of one typical experiment out of three independent experiments is depicted. * $P<0.05$, significantly different from the control.

3.3.4 Effects of *C. asiatica* and *R. nasutus* extracts on iNOS and TNF-α gene expression

The modulation of NO and TNF-α protein levels by the plant extracts could be a result of altered iNOS and TNF-α gene expression. Therefore, the mRNA level of both these genes following extract treatments was examined. The water extract of *C. asiatica* alone, without LPS stimulation, induced TNF-α gene expression at high doses (500 and 1000 μg/ml extract) but had no effect on iNOS gene expression (Figure 3.22). Other extracts alone altered expression of neither gene (data not shown). In the presence of LPS, the ethanol extraction of *C. asiatica* inhibited iNOS expression in a dose-dependent manner whilst TNF-α expression was inhibited only at the high dose of CA (400 μg/ml) (Figure 3.23). The water extract in the presence of LPS induced TNF-α expression but not iNOS. For *R. nasutus*, ethanol and water extracts both led to modest increases in TNF-α expression but did not change iNOS gene expression (Figure 3.24).

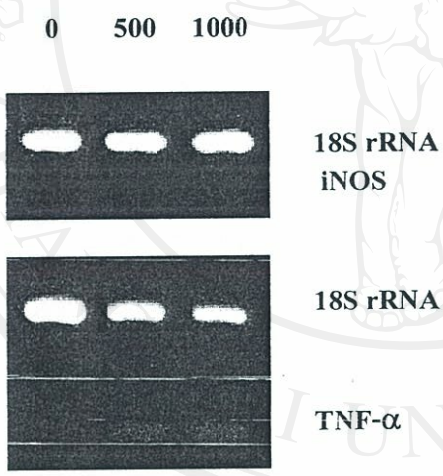

Figure 3.22 Effect of water extract of *C. asiatica* on TNF-α gene expression. J774.2 macrophages ($2x10^6$ cells/well) were cultured with the water extract of *C. asiatica* alone for 12 hr. Cells were lysed and total RNA was prepared for RT-PCR. The housekeeping gene, 18S ribosomal RNA (rRNA), was amplified as an internal control. The result of one typical experiment out of three independent experiments is depicted.

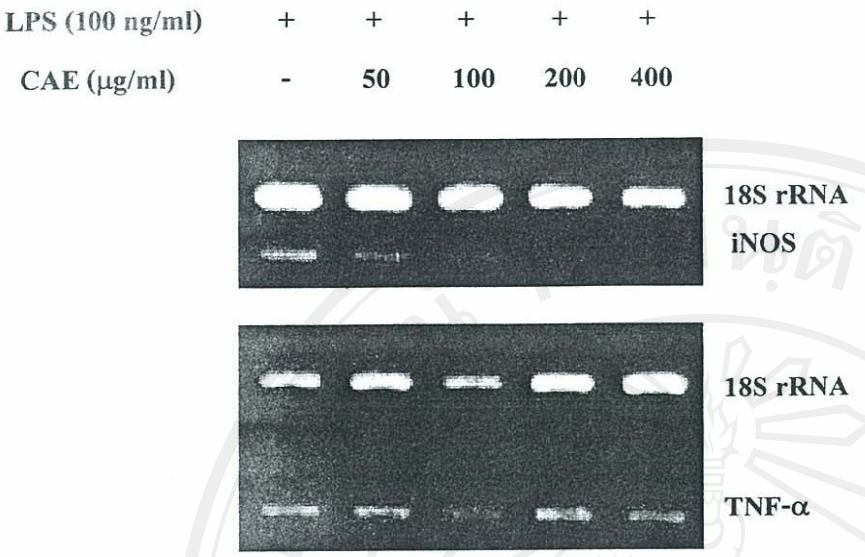

Figure 3.23 Effect of ethanol extract of *C. asiatica* on iNOS gene expression. J774.2 macrophages ($2x10^6$ cells/well) were cultured with an ethanol extract of *C. asiatica* in the presence of 100 ng/ml LPS for 12 hr. Cells were lysed and total RNA was prepared for RT-PCR. The housekeeping gene, 18S ribosomal RNA (rRNA), was amplified as an internal standard. The result of one typical experiment out of three independent experiments is depicted.

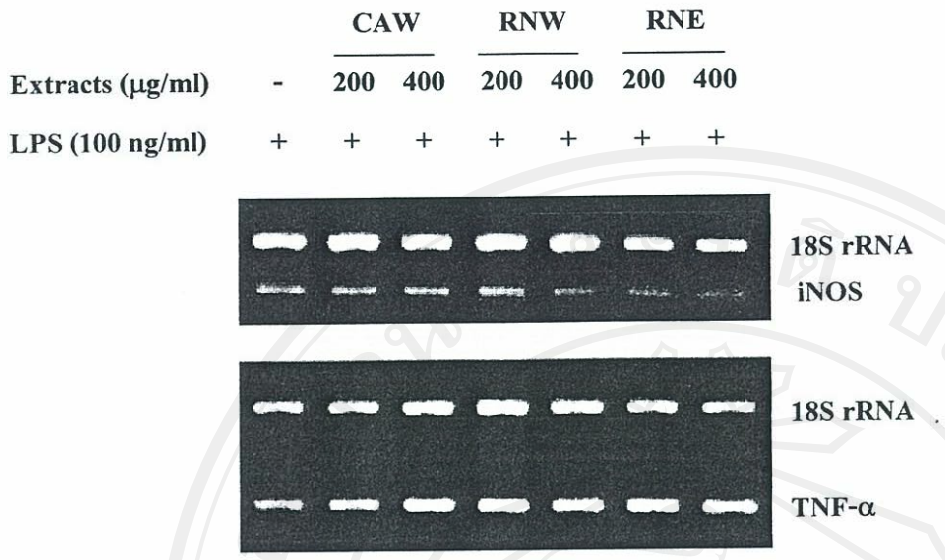

Figure 3.24 Effects of plant extracts on iNOS and TNF-α gene expression. J774.2 macrophages ($2x10^6$ cells/well) were cultured with water extract of *C. asiatica* (CAW), water and ethanol extract of *R. nasutus* (RNW and RNE, respectively) in the presence of 100 ng/ml LPS for 12 hr. Cells were lysed and total RNA was prepared for RT-PCR. The housekeeping gene, 18S ribosomal RNA (rRNA), was amplified as an internal control. The result of one typical experiment out of three independent experiments is depicted.

3.3.5 Effect of *C. asiatica* and *R. nasutus* extracts on macrophage-mediated cytolytic activity.

To evaluate the effect of plant extracts on anti-tumor activity of macrophages, the cytolytic activities of mouse macrophages pretreated with extracts were measured by staining the cells with crystal violet containing 10% formaldehyde. The water extracts of *C. asiatica* in 200 μg/ml or 400 μg/ml doses increased cytolytic activities of macrophages against B16F10 by 12% and 19%, respectively. The water extract of *R. nasutus* had no effect on cytolysis activities of macrophages (Figure 3.25). This result indicated that *C. asiatica* extract enhanced macrophage-mediated tumor cell killing activity.

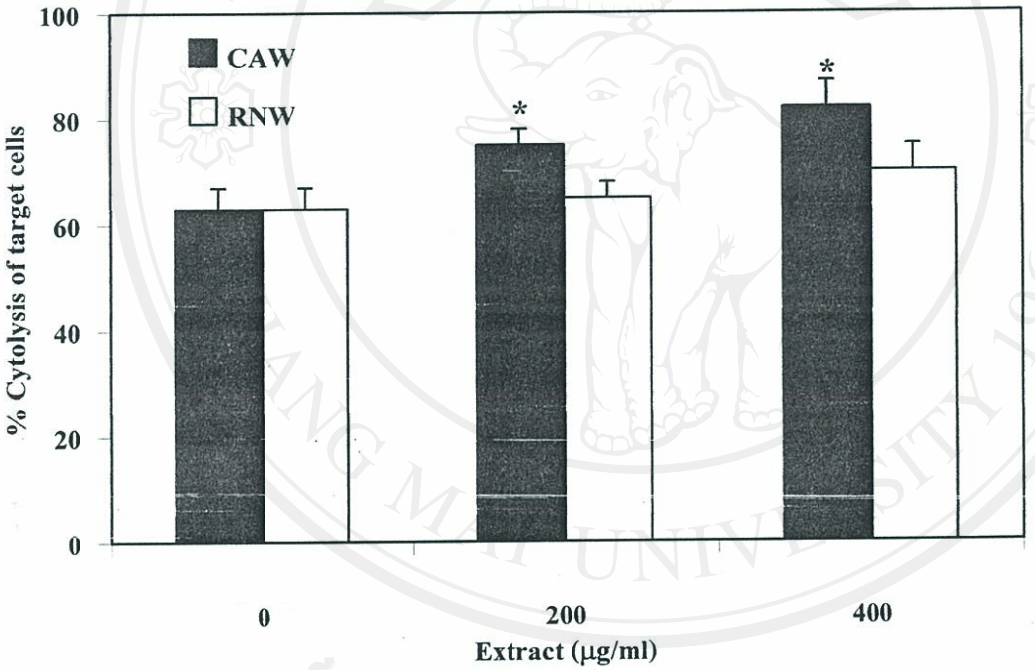

Figure 3.25 Effects of water extracts of *C. asiatica* and *R. nasutus* on cytolytic activities of J774.2 mouse macrophages. J774.2 mouse macrophages were pretreated with water extract of *C. asiatica* (CAW) or *R. nasutus* (RNW) and co-incubated with B16F10 for 18 hours in the presence of 1 mg/ml LPS. Cytolytic activities were expressed as a percentage cytolysis of target cells, B16F10 after staining the cells with crystal violet containing 10% formaldehyde. * $P<0.05$, significantly different from the control (no extract).

3.4 Effects of *C. asiatica* and *R. nasutus* extracts on specific antibody production *in vivo*

Water extracts of *C. asiatica* and *R. nasutus* increased lymphocyte proliferation induced by PWM (Figure 3.7 and Figure 3.9, respectively). PWM is a mitogen that can induce the proliferation of a specific group of cells called B-lymphocytes. B-lymphocytes are responsible for the specific antibody production in the immune system. The evaluation of effects of water extracts of *C. asiatica* and *R. nasutus* on *in vivo* antibody production was performed. BALB/c mice treated with water extract of *C. asiatica* (100 mg/kg bw) significantly increased both primary (IgM) and secondary (IgG) antibody responses to BSA when compared with a non-treated control group (Table 3.7). *R. nasutus* extract-treated mice produced higher amounts of secondary antibodies against BSA than the non-treated mice (Table 3.9). Interestingly, the observed effect was not dose-dependent. Mice fed with higher concentrations of extracts showed no difference in the specific antibody production when compared with non-treated control mice.

Table 3.7 Effects of water extract of *C. asiatica* and *R. nasutus* on antibody response in mice. BALB/c mice were fed with extract and immunized twice with 1 mg BSA. Sera were collected 2 weeks after each immunization. Primary antibody (IgM) and secondary (IgG) antibody against BSA were determined by ELISA method. Values are mean antibody titer log2 ± SD of six animals in each group. Statistical significance versus mitogen control: * $p<0.05$.

Treatment	Primary response	Secondary response
Control	3.7 ± 0.12	6.8 ± 0.58
C. asiatica extract 10 mg/kg bw/day	3.8 ± 0.57	7.0 ± 0.80
C. asiatica extract 100 mg/kg bw/day	5.7 ± 0.38*	9.7 ± 0.89*
C. asiatica extract 300 mg/kg bw/day	4.0 ± 0.35	7.3 ± 0.76
Control	4.2 ± 0.41	7.2 ± 0.67
R. nasutus extract 10 mg/kg bw/day	4.7 ± 0.92	9.8 ± 0.71*
R. nasutus extract 100 mg/kg bw/day	4.3 ± 0.55	7.5 ± 0.85

3.5 Effect of mycotoxin mixtures on the immune cells

Our knowledge concerning the effects of complex mixtures of mycotoxins is still limited. To evaluate the effects of complex mixture of mycotoxins on lymphocytes and macrophages, the combination of DON with either AFB_1 or FB_1 and the combination of AFB_1 and FB_1 were studied.

3.5.1 Effect of individual mycotoxin on lymphocytes

The result of an optimization study aimed at finding a suitable concentration of mitogens for use in the lymphocyte activation assay is shown in appendix D. The concentrations of PHA and PWM mitogen used in this study were 5 μg/ml and 1 μg/ml, respectively. The 50% inhibition of human PBMCs proliferation (IC50) by individual mycotoxin is shown in Table 3.8. IC50 of three different donors were similar. DON had the strongest effect on lymphocyte proliferation when compared with mycotoxin tested in this study; IC50 was 75 ± 8.9 and 37.3 ± 7.8 ng/ml for PHA- and PWM-stimulated human PBMC mitogenesis, following by AFB_1 which had IC50 of 6.0± 1.0 and 4.5 ± 0.9, respectively. For AFB_1, without metabolic activating enzymes from S9 mix, the IC50 was more than 10 μg/ml. FB_1 had no effect on human PBMC proliferation as the IC50 of PHA-stimulated condition more than 10 μg/ml, which was the highest concentration of FB_1 used in this study.

Table 3.8 Effect of individual mycotoxins on cell proliferation assay. The concentrations of mycotoxins after 3 days incubation causing 50% inhibition of proliferation of human PBMCs stimulated by the mitogens PHA and PWM determined by the incoporation of [^{3}H]-thymidine. Data are shown as mean ± SD of three donors.

Mycotoxin	IC50	
	PHA	PWM
AFB_1 (μg/ml)	6.0± 1.0	4.5 ± 0.9
DON (ng/ml)	97.3 ± 12.5	55.3 ± 12.5
FB_1 (μg/ml)	> 10	>10

3.5.2 Effect of mycotoxin mixtures on lymphocytes

As the natural co-occurring of mycotoxin mixtures was observed, there is a potential for synergistic or antagonistic effects between mycotoxins. Therefore the effects of mycotoxin mixtures were tested on human lymphocyte proliferation. Figure 3.26 shows the effect of DON-AFB_1 mixture on human PBMC proliferation. Various concentrations of AFB_1 (0-5 μg/ml) were co-cultured with 100 or 50 ng/ml or of DON in regarding to stimulate the proliferation of human PBMC by PHA or PWM, respectively. The interaction between DON and AFB_1 was observed at the highest dose of AFB_1 used (5 μg/ml). This effect was found in both PHA- and PWM-induced lymphocyte proliferations. Using PWM, in combination with DON, AFB_1 significantly inhibited lymphocyte proliferation at dose ≥ 2.5 μg/ml higher than DON treated alone (Figure 3.26). The mixture of DON and FB_1 also showed synergistic effects on inhibition of lymphocyte proliferation. As FB_1 alone did not show any effect on human lymphocytes (Table 3.8), co-incubation of FB_1 with DON caused more inhibition of lymphocyte proliferation than that of DON alone (Figure 3.27). The AFB_1-FB_1 mixture did not show any interactions (Figure 3.28).

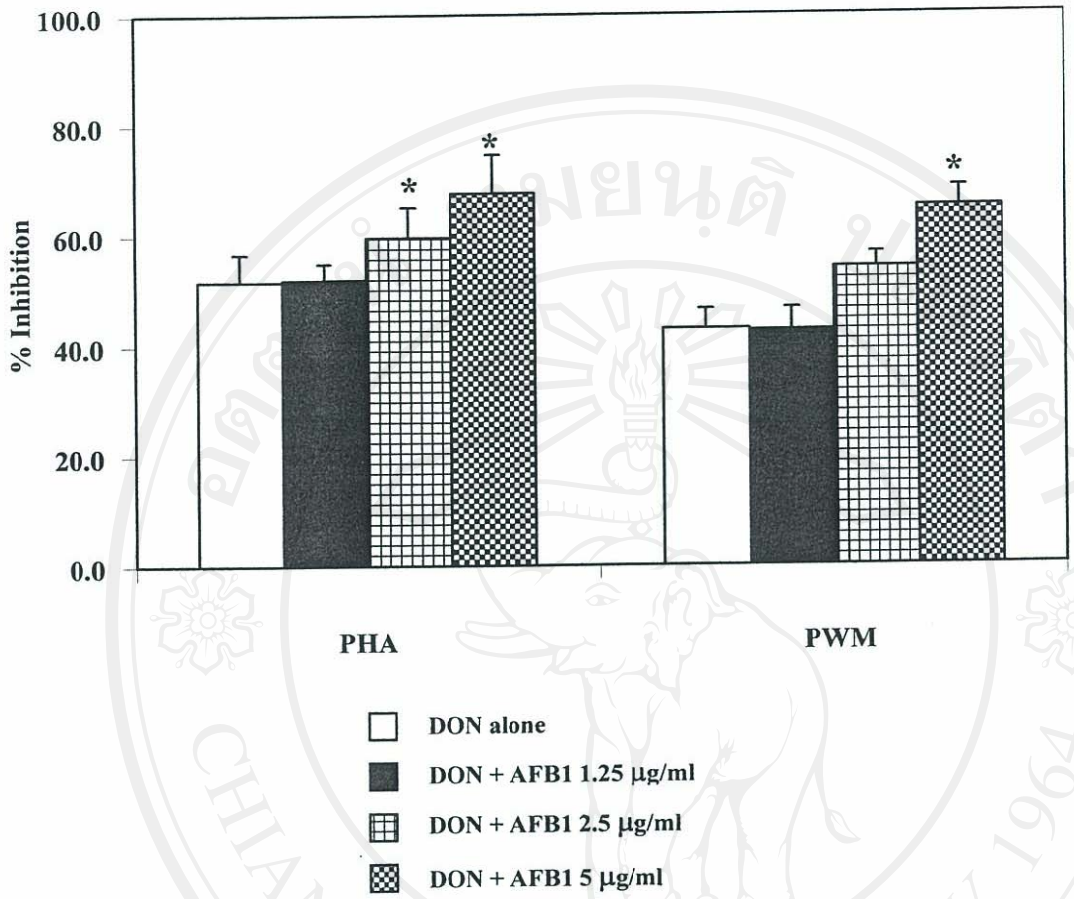

Figure 3.26 Effect of DON-AFB_1 mixture on the proliferation of human PBMCs. Human PBMCs were cultured with various concentrations of AFB_1 with either fixed concentration of DON (100 or 50 ng/ml in the presence of PHA or PWM, respectively). The proliferation of human PBMCs was determined after 3 days incubation by [^{3}H]-thymidine incoporation technique. The % inhibition of cell proliferation was calculated and expressed as mean $\pm$ SD of three individual donors. Cells treated with AFB_1 (5µg/ml) alone, the % inhibition of cell proliferation were 41$\pm$3.2 (PHA stimulation) and 45$\pm$4.1 (PWM stimulation). Statistical significance versus DON control (without AFB_1): * p<0.05.

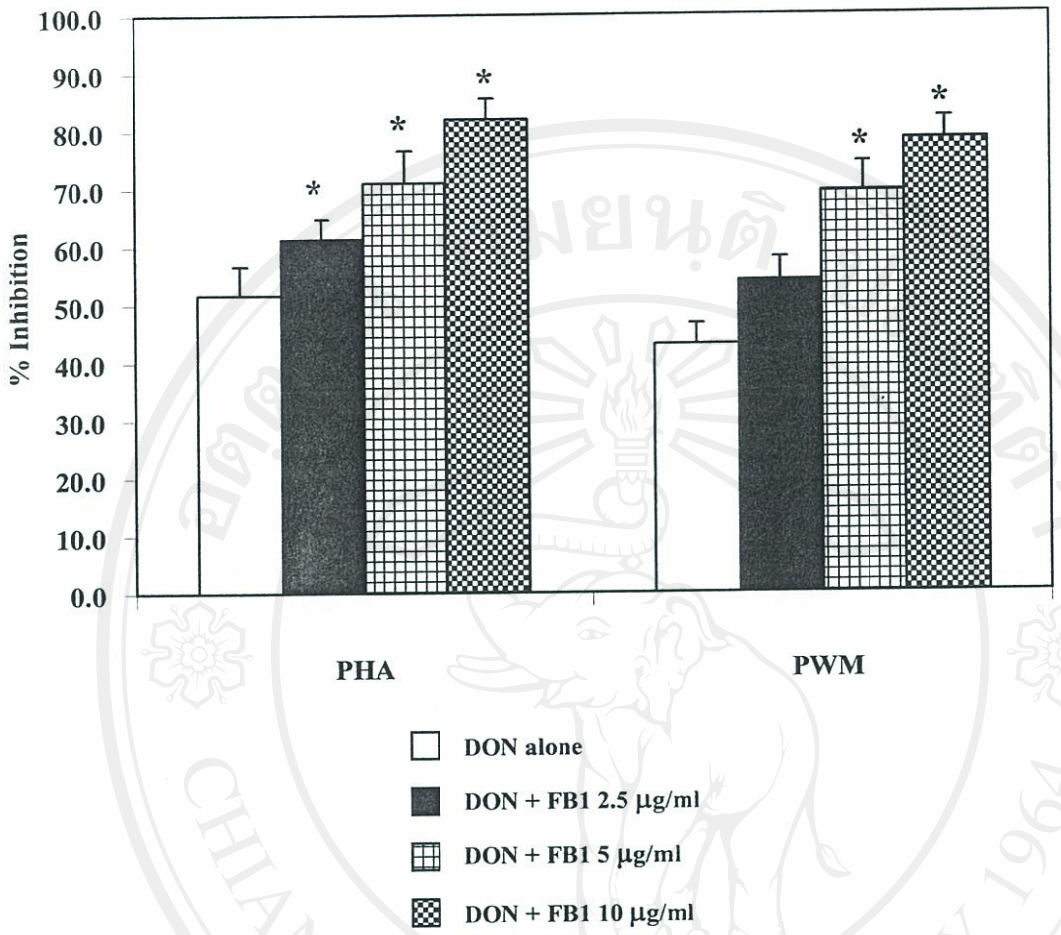

Figure 3.27 Effect of DON-FB_1 mixture on the proliferation of human PBMCs. Human PBMCs were cultured with various concentrations of FB_1 with either fixed concentration of DON (100 or 50 ng/ml in the presence of PHA or PWM, respectively). The proliferation of human PBMCs was determined after 3 days incubation by [^{3}H]-thymidine incoporation technique. The % inhibition of cell proliferation was calculated and expressed as mean ± SD of three individual donors. FB_1 (10µg/ml) alone did not inhibit cell proliferation. Statistical significance versus DON control (without FB_1): * $p<0.05$.

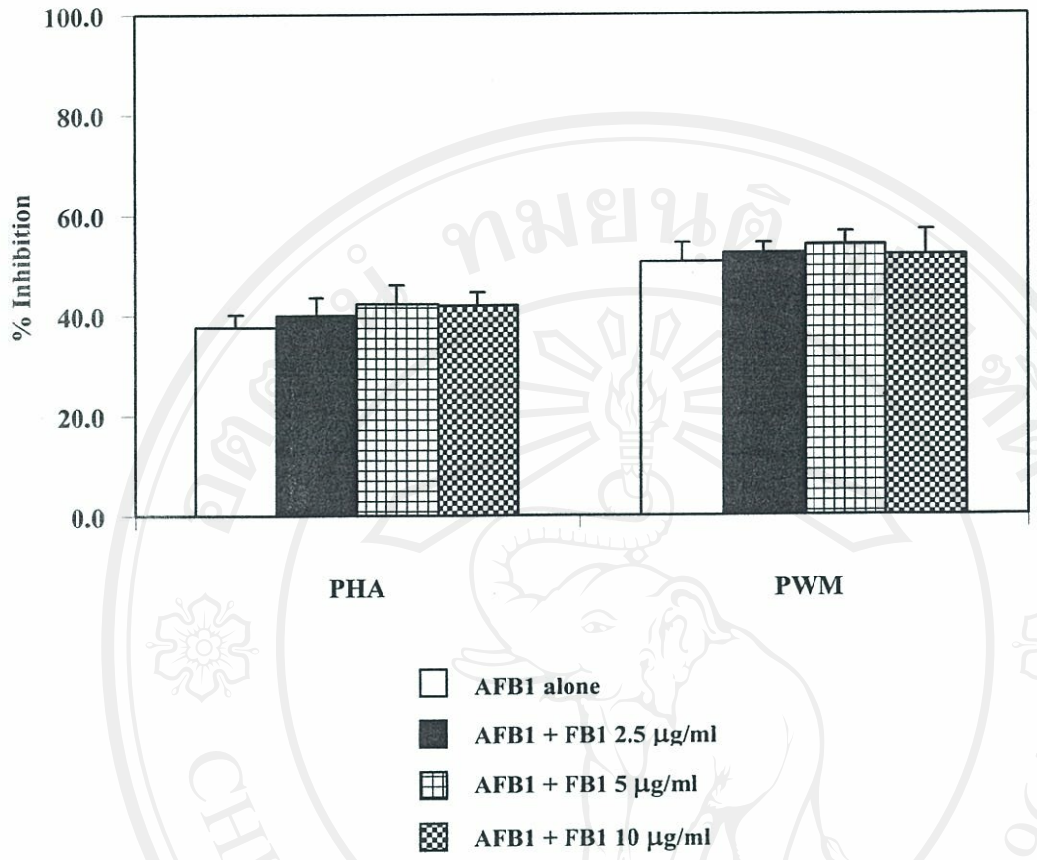

Figure 3.28 Effect of AFB_1-FB_1 mixture on the proliferation of human PBMCs. Human PBMCs were cultured with various concentrations of FB_1 with either fixed concentration of AFB_1 (5 µg/ml) in the presence of PHA or PWM. The proliferation of human PBMCs was determined after 3 days incubation by [^{3}H]-thymidine incorporation technique. The % inhibition of cell proliferation was calculated and expressed as mean ± SD of three individual donors. FB_1 (10µg/ml) alone did not inhibit cell proliferation.

3.5.3 Effect of mycotoxin mixture on macrophages

3.5.3.1 Effect of individual mycotoxins on nitric oxide production

The effects of individual mycotoxins on NO production were evaluated. AFB1 inhibited the production of NO in a dose-dependent manner; IC50 was 5.7 μg/ml (Figure 3.29). As shown in Figure 30, DON also strongly inhibited NO production; IC50 was 200 ng/ml. However, the lowest concentration of DON, 62.5 ng/ml increased the NO production about 33%. In contrast to AFB1 and DON, FB1 significantly increased NO production only at the highest dose (50 μg/ml).

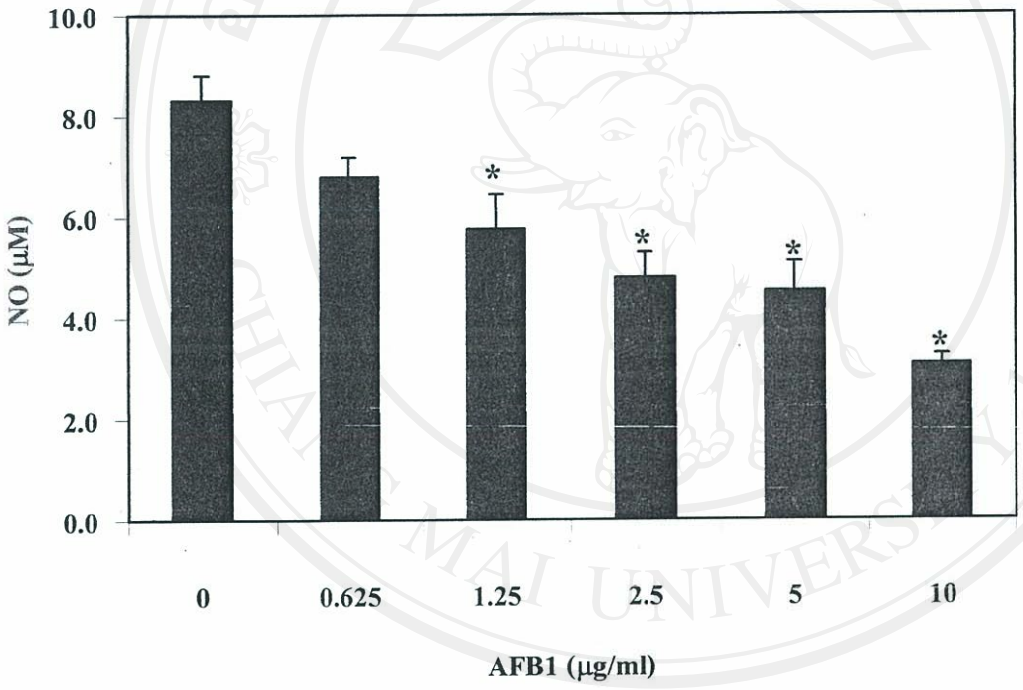

Figure 3.29 Effect of AFB_1 on NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were co-cultured with various concentrations of AFB_1 and LPS (50ng/ml). Supernatants were collected after 24 h and nitrite level was determined by the Griess reaction. The data represent the mean ± SD of three independent experiments. * $P<0.05$, significantly different from the control.

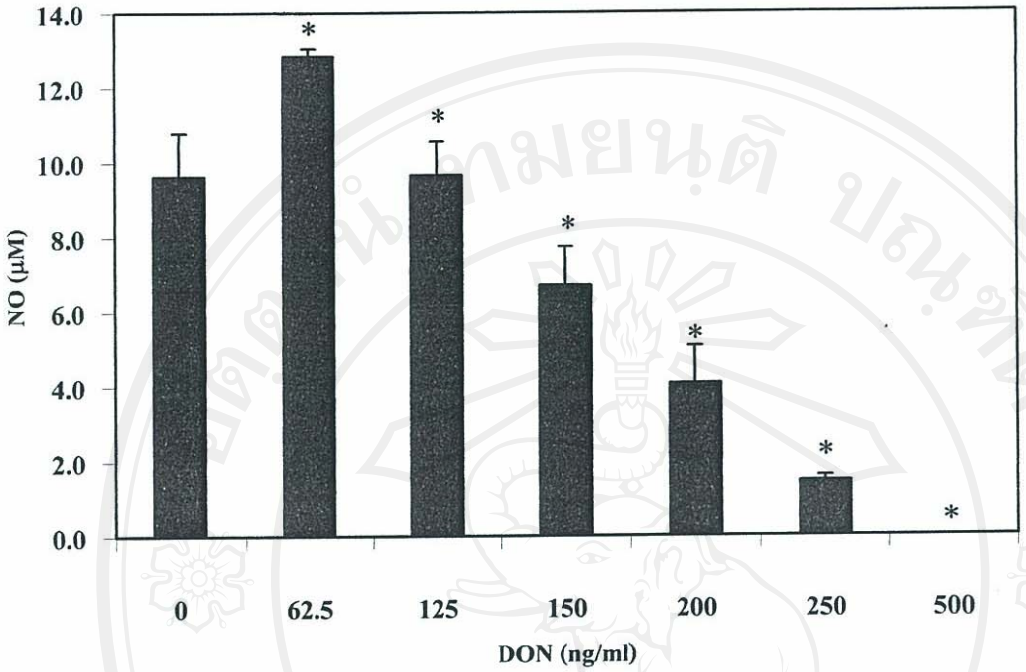

Figure 3.30 Effect of DON on NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were co-cultured with various concentrations of DON and LPS (50ng/ml). Supernatants were collected after 24 h and nitrite level was determined by the Griess reaction. The data represent the mean ± SD of three independent experiments. * $P<0.05$, significantly different from the control.

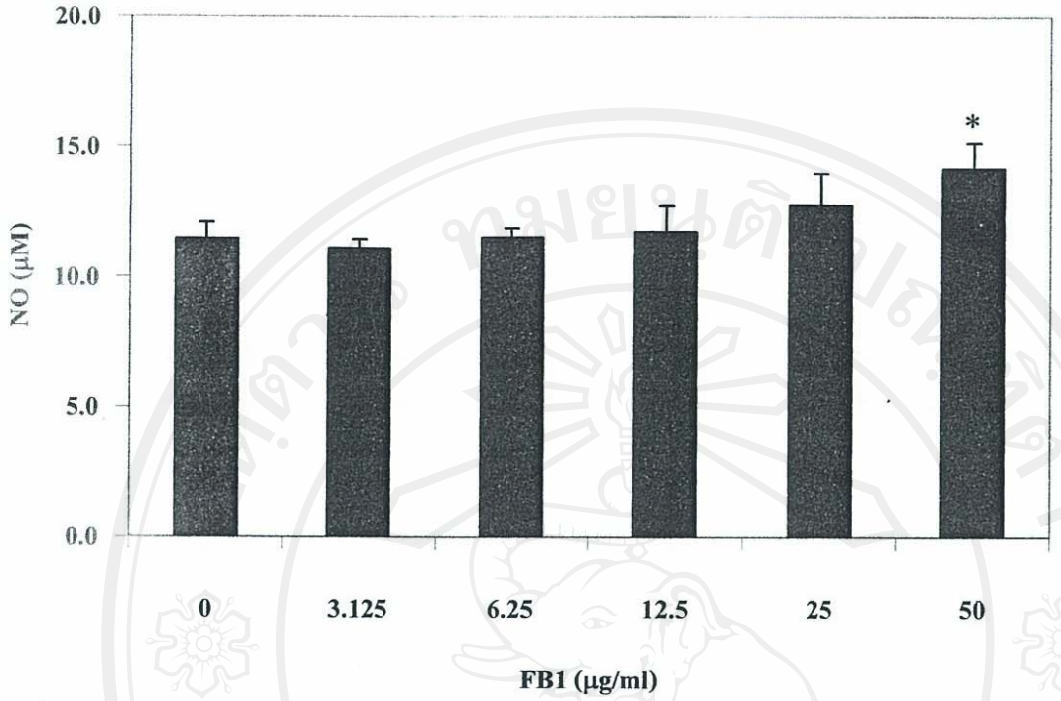

Figure 3.31 Effect of FB_1 on NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were co-cultured with various concentrations of FB_1 and LPS (50ng/ml). Supernatants were collected after 24 h and nitrite level was determined by the Griess reaction. The data represent the mean ± SD of three independent experiments. * $P<0.05$, significantly different from the control.

3.5.3.2 Effects of mycotoxin mixtures on nitric oxide production

Study of individual mycotoxins on NO production gave the IC50 values of DON and AFB1 on the inhibition of NO synthesis. To study the combined effect of mycotoxins, macrophages were co-treated with DON (200 ng/ml) and either AFB_1 or FB_1 in the presence of LPS (50ng/ml) for 24 hours. Treatment of cells with LPS alone was used as a positive control, which gave the NO value of 7 μM. DON (200ng/m) alone inhibited the production of NO about 57% (3μM). The NO value was 4 μM for the AFB_1 (5μg/ml) treated alone. The DON-AFB_1 mixture inhibited the NO production in macrophages further than treatment of either DON or AFB_1 alone. This result showed the synergistic effect between these two mycotoxins. This effect could be observed in all dosages of AFB_1 treated. However, the effect was not dose-dependent (Figure 3.32). FB_1 (100 μg/ml) alone increased NO production to 10 μM, while DON (200 ng/ml) alone decreased NO level to 3 μM. The NO level in the DON-FB_1 mixture treatment was not significantly different from DON alone, except at the lowest dose of FB_1 (6.25 mg/ml), which increased the NO production when compared to DON alone (Figure 3.33). To study the effect of AFB_1-FB_1 mixture, macrophages were treated with various concentrations of FB_1 in the presence of AFB_1 (5μg/ml) and LPS (50ng/ml). In the LPS control (without any mycotoxins), the NO level was 10.3 μM. FB_1 (100 mg/ml) alone increased the NO level to 12.6 μM, while AFB_1 (5μg/ml) decreased NO production to 5.6 μM. In the mixture of AFB_1-FB_1, treatment with FB_1 caused the NO level increase (Figure 3.34). This result showed that FB_1 could modulate the effect of AFB_1, which suppressed NO production.

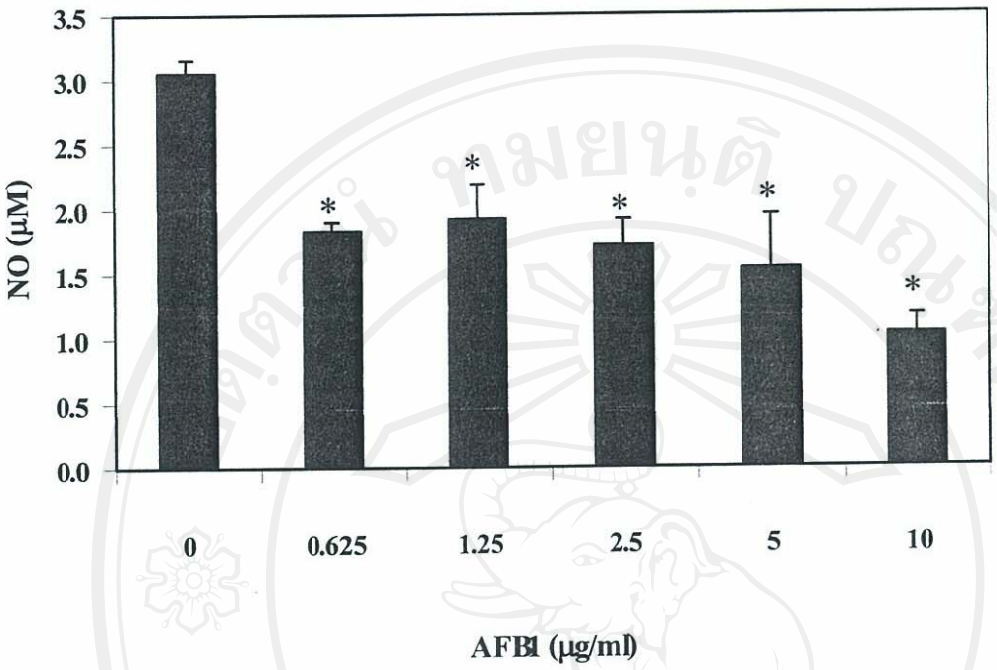

Figure 3.32 Effect of DON-AFB_1 mixture on NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were co-cultured with DON (200ng/ml) and various concentrations of FB_1 in the presence of LPS (50ng/ml). Supernatants were collected after 24 h and nitrite level was determined by the Griess reaction. NO level were 7 μM and 4 μM in LPS control and AFB_1 control (5μg/ml), respectively. The data represent the mean ± SD of three independent experiments. * $P<0.05$, significantly different from the control (DON alone).

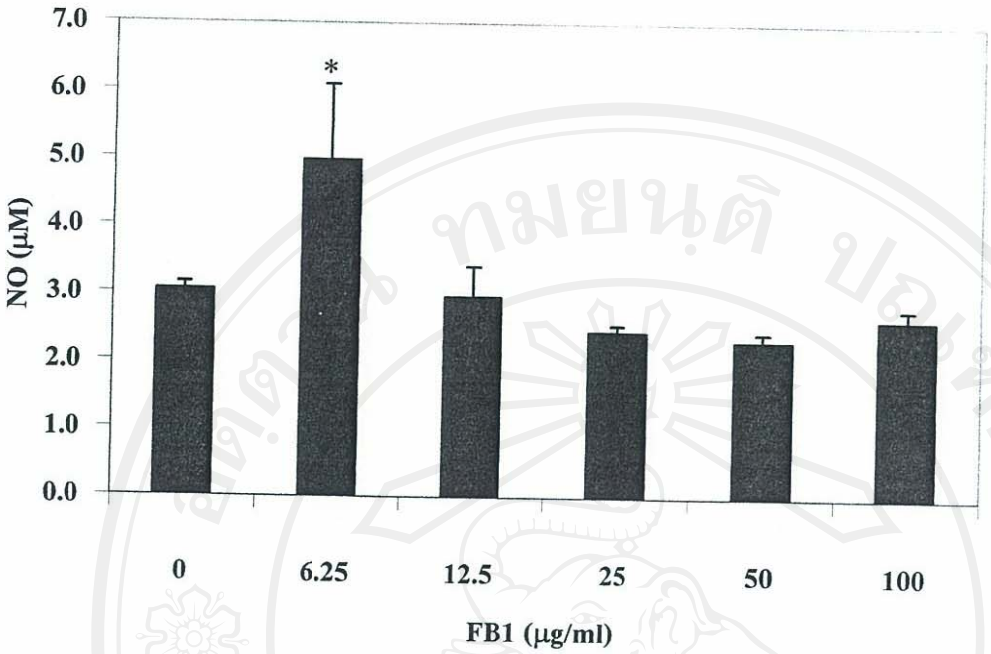

Figure 3.33 Effect of DON-FB_1 mixture on NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were co-cultured with DON (200ng/ml) and various concentrations of FB_1 in the presence of LPS (50ng/ml). Supernatants were collected after 24 h and nitrite level was determined by the Griess reaction. NO level were 7 μM and 10 μM in LPS control and FB_1 control (100 μg/ml), respectively. The data represent the mean ± SD of three independent experiments. * $P<0.05$, significantly different from the control (DON alone).

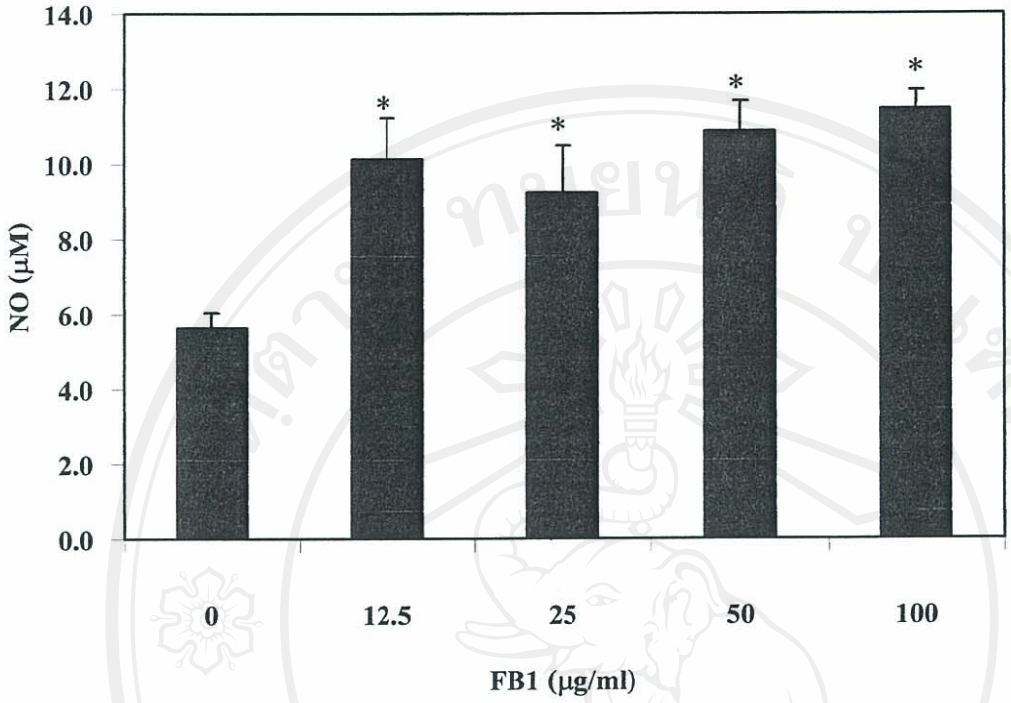

Figure 3.34 Effect of AFB_1- FB_1 mixture on NO production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were co-cultured with DON (200ng/ml) and various concentrations of FB_1 in the presence of LPS (50ng/ml). Supernatants were collected after 24 h and nitrite level was determined by the Griess reaction. NO level were 10.3 μM and 12.6 μM in LPS control and FB_1 control (100 μg/ml), respectively. The data represent the mean ± SD of three independent experiments. * $P<0.05$, significantly different from the control (AFB_1 alone).

3.5.3.3 Effect of individual mycotoxins on TNF-α production

In order to find an appropriate concentration of each mycotoxins for further experiments, the effect of individual mycotoxins on the production of TNF-α was evaluated. As shown in Figure 3.35, DON strongly increased the production of TNF-α in every concentration tested. In contrast to DON, concentrations of $AFB_1 \geq 5$mg/ml decreased TNF-α production, as shown in Figure 3.36. Again with FB_1 treated cells, the production of TNF-α was slightly increased, except at the highest dose (100 μg/ml) (Figure 3.37).

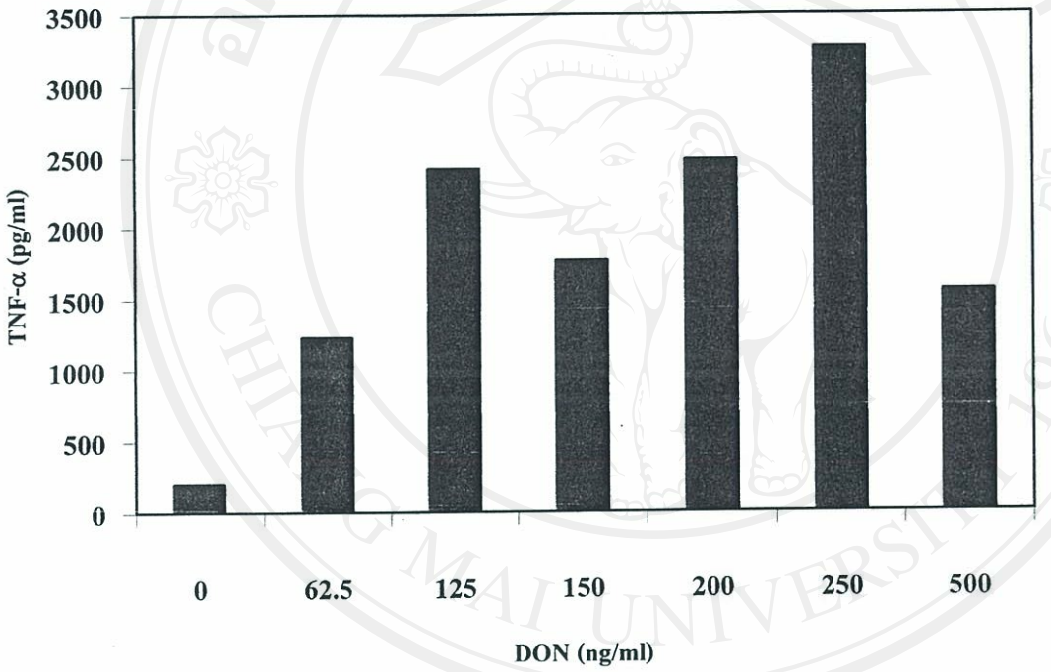

Figure 3.35 Effect of DON on TNF-α production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were cultured with various concentrations of DON and 100 ng/ml of LPS. Supernatants were collected after 24 h of LPS stimulation and TNF-α level was determined by ELISA. The data represent the TNF-α level from pooled triplicate cultures.

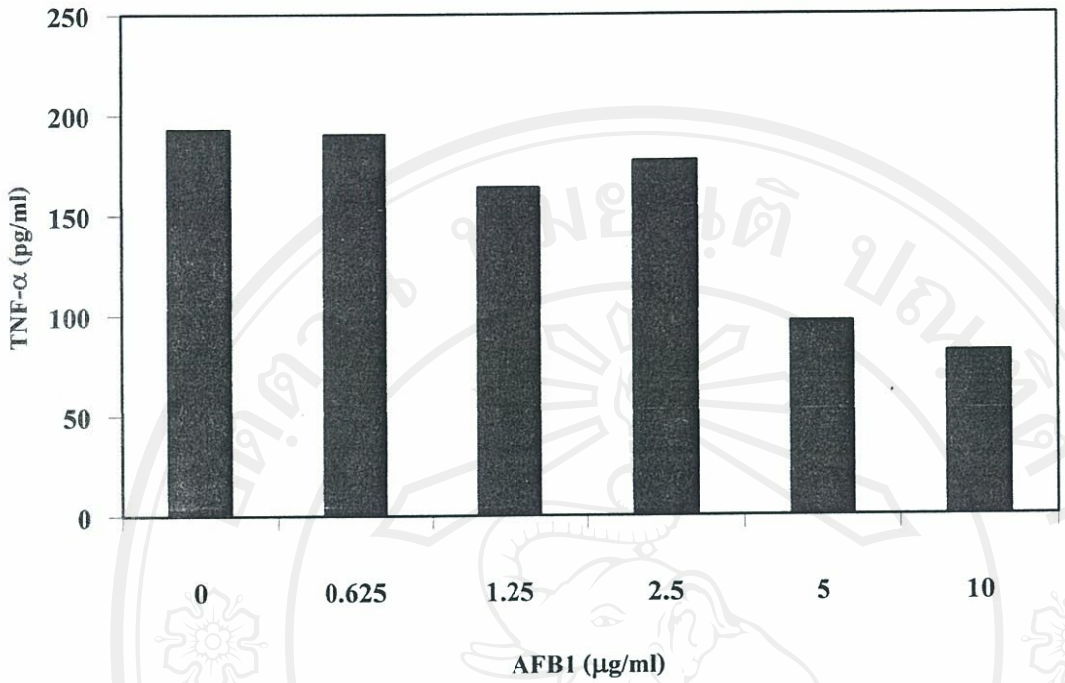

Figure 3.36 Effect of AFB_1 on TNF-α production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were cultured with various concentrations of AFB_1 and 100 ng/ml of LPS. Supernatants were collected after 24 h of LPS stimulation and TNF-α level was determined by ELISA. The data represent the TNF-α level from pooled triplicate cultures.

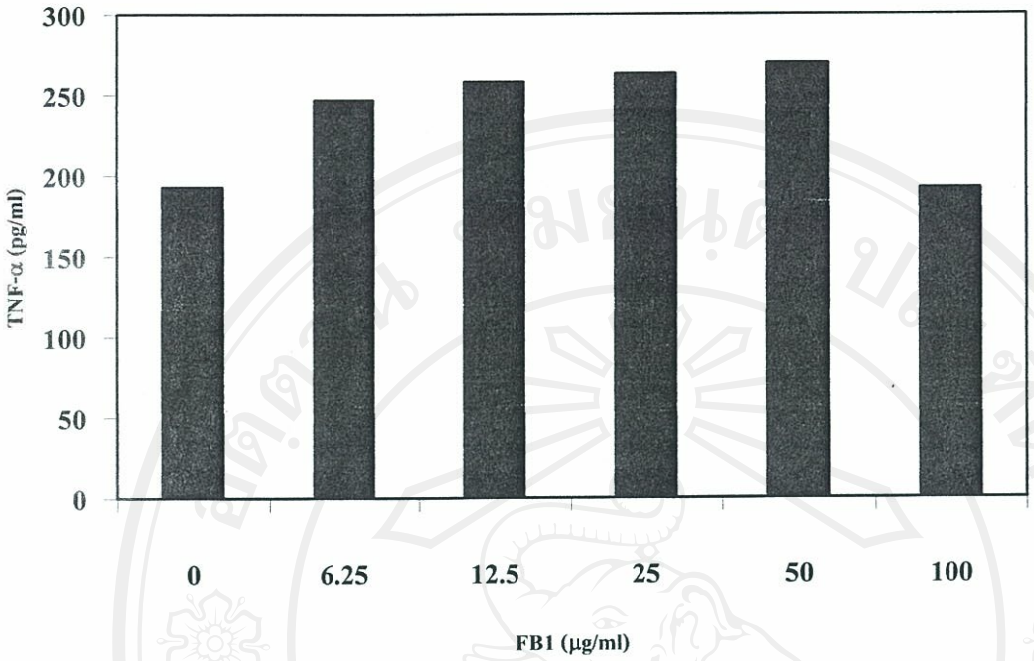

Figure 3.37 Effect of FB_1 on TNF-α production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were cultured with various concentrations of FB_1 and 100 ng/ml of LPS. Supernatants were collected after 24 h of LPS stimulation and TNF-α level was determined by ELISA. The data represent the TNF-α level from pooled triplicate cultures.

3.5.3.4 Effect of mycotoxin mixtures on TNF-α production

The concentration of mycotoxin used in this study was based on the previous study of the effect of individual mycotoxins on TNF-α production. For DON-AFB_1 mixture, J774.2 mouse macrophages were cultured various concentrations of AFB_1 in the presence of DON (200ng/ml) and LPS (100ng/ml). At the highest concentration of AFB_1 (10 μg/ml) in combination with DON, TNF-α level was lower than in the DON control. However, the TNF-α level was still higher than in the AFB_1 treatment alone (112 pg/ml).

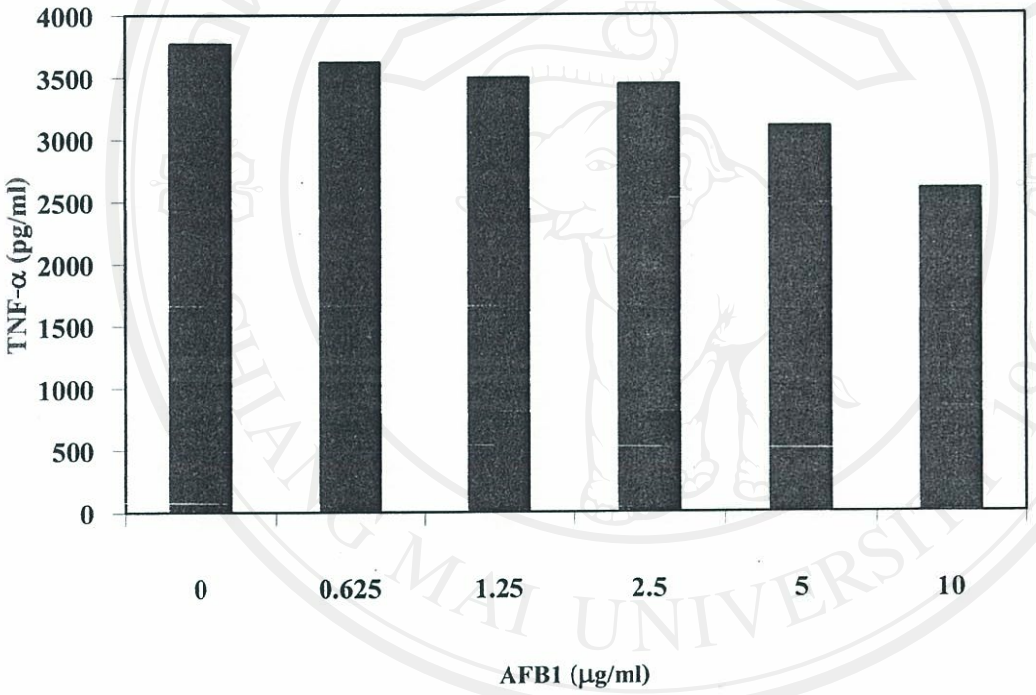

Figure 3.38 Effect of DON-AFB_1 mixture on TNF-α production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were co-cultured with DON (200ng/ml) and various concentrations of AFB_1 in the presence of LPS (100ng/ml). Supernatants were collected after 24 h of LPS stimulation and TNF-α level was determined by ELISA. TNF-α levels were 227 pg/ml and 112 pg/ml in LPS control and AFB_1 control (10μg/ml), respectively. The data represent the TNF-α level from pooled triplicate cultures.

For DON-FB_1 mixture, J774.2 mouse macrophages were cultured at various concentrations of FB_1 in the presence of DON (200ng/ml) and LPS (100ng/ml). As shown in Figure 3.36 and 3.37, both DON and FB_1 increased the production of TNF-α. When combined them together, the antagonistic effect was observed. At the concentration of FB_1 (50 μg/ml) in combination with DON, TNF-α level was lower than DON control. However the concentration of TNF-α was still much higher than LPS control and FB_1 control (Figure 3.39). This result suggested that FB_1 could inhibit the DON effect.

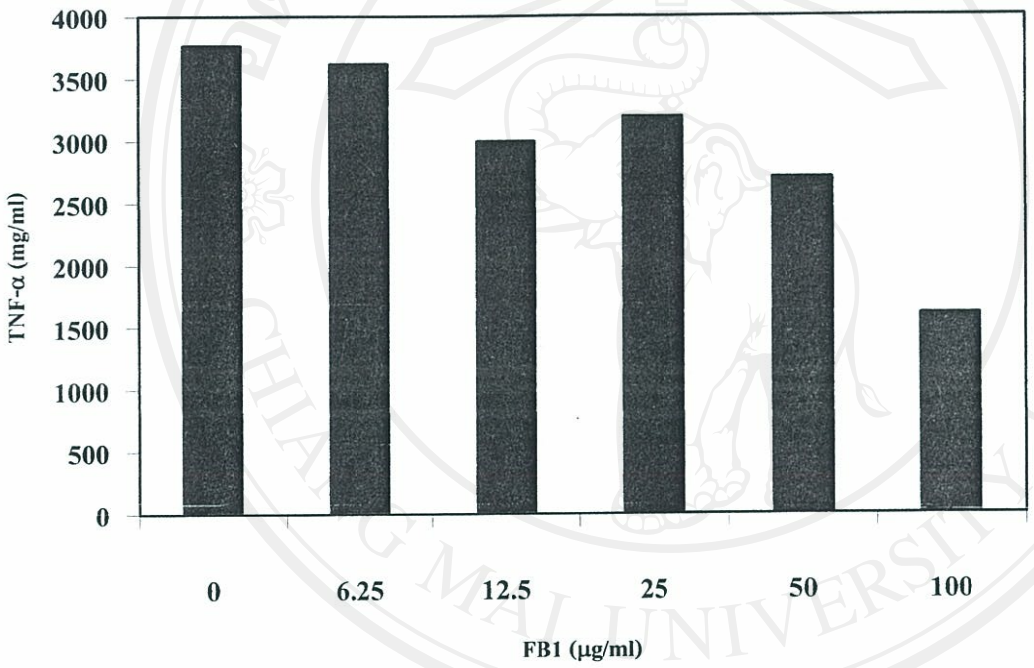

Figure 3.39 Effect of DON-FB_1 mixture on TNF-α production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were co-cultured with DON (200ng/ml) and various concentrations of FB_1 in the presence of LPS (100ng/ml). Supernatants were collected after 24 h of LPS stimulation and TNF-α level was determined by ELISA. TNF-α levels were 227 pg/ml and 385 pg/ml in LPS control and FB_1 control (50 μg/ml), respectively. The data represent the TNF-α level from pooled triplicate cultures

For AFB_1-FB_1 mixture, J774.2 mouse macrophages were cultured at various concentrations of FB1 in the presence of AFB_1 (5ng/ml) and LPS (100ng/ml). The combination of these toxins resulted in an increase in TNF-α level (Figure 3.40). This seemed to be an effect of FB_1 alone. At 50 mg/ml of FB_1, TNF-α level was 289 pg/ml which not different from AFB_1-FB_1 mixture (269 pg/ml).

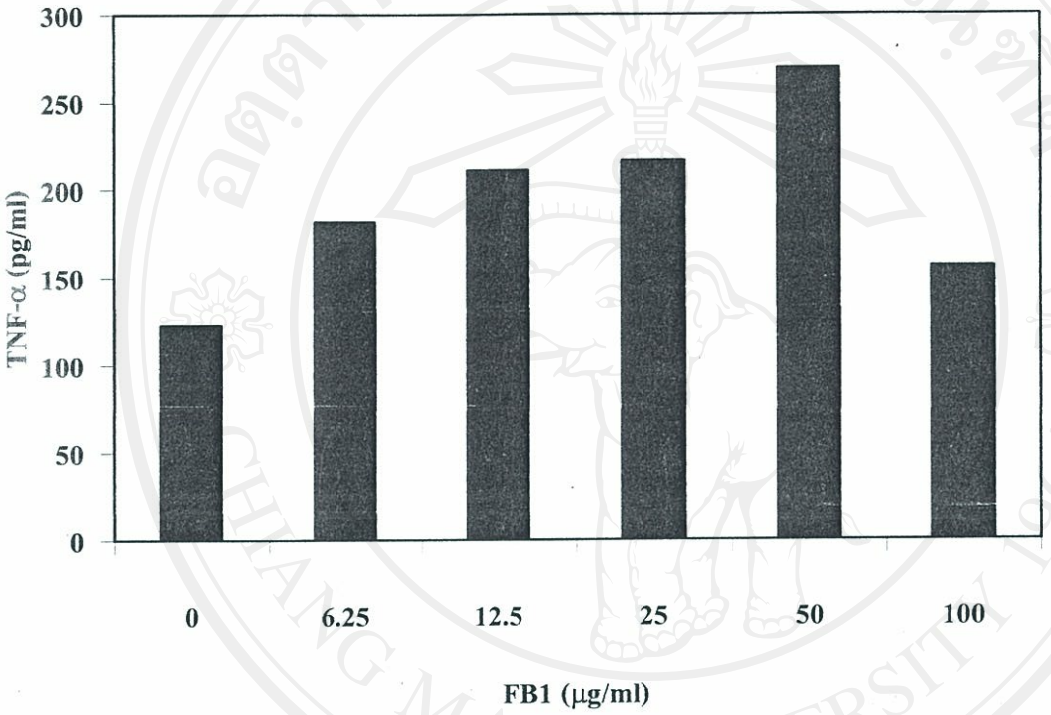

Figure 3.40 Effect of AFB_1-FB_1 mixture on TNF-α production in J774.2 mouse macrophages. J774.2 macrophages ($1x10^5$ cells/well) were co-cultured with DON (200ng/ml) and various concentrations of FB_1 in the presence of LPS (100ng/ml). Supernatants were collected after 24 h of LPS stimulation and TNF-α level was determined by ELISA. TNF-α levels were 197 pg/ml and 289 pg/ml in LPS control and FB_1 control (50 μg/ml), respectively. The data represent the TNF-α level from pooled triplicate cultures.

3.5.4 Effects of *C. asiatica* and *R. nasutus* extracts on mycotoxin-induced immunomodulation

From the previous results, the water extracts of *C. asiatica* and *R. nasutus* possessed immunostimulating activity. The immunotoxicity of AFB_1 and DON were also observed previously. This experiment aimed to evaluate whether the extracts could modulate the immunotoxicity caused by DON and AFB_1.

3.5.4.1 Effect on lymphocytes

To test whether plant extracts could modulate the immunotoxicity caused by mycotoxins, human PBMCs were pretreated with various concentrations of plant extracts (100-400 μg/ml) for 4 hours, then mycotoxins (DON or AFB_1) and mitogens (PHA or PWM) were added. Lymphocyte proliferation was determined by [^{3}H]-thymidine incorporation assay. As shown in Figure 3.41 and 3.42, DON at 100 ng/ml decreased the mitogen (PHA and PWM)-induced lymphocyte proliferation. Pretreatment with the water extract of *C. asiatica* increased the proliferation of human PBMCs in a dose-dependent manner when compared with the mitogen control. Using PHA as a mitogen, *C. asiatica* extract ≥ 200 μg/ml could modulate the DON effect (Figure 3.41). With PWM, only the highest concentration of *C. asiatica* extract showed the modulation effect (Figure 3.42). The water extract of *R. nasutus* could modulate the immunotoxic effect caused by DON only at the highest dose tested (400 μg/ml) in the condition using PHA as a mitogen. The extract had no effect when using PWM as a mitogen. These data indicated that plant extracts could modulate T cell responses more than B cell responses.

Modulation effects of plant extracts on AFB_1 treatment were also observed (Figure 3.43 and Figure 3.44). *C. asiatica* extracts pretreatment could reverse the AFB_1-suppressed lymphocyte proliferation at doses ≥ 200 μg/ml. This effect was observed in the condition using both PHA and PWM to stimulate lymphocyte proliferation. *R. nasutus* extracts also showed the modulation effect in both conditions. However, this effect was stronger using PWM than using PHA. With PWM, all concentrations of *R. nasutus* increased the AFB_1-suppressed lymphocyte proliferation in a dose-dependent manner (Figure 3.44) while only the highest concentration of extract showed the modulation effect with PHA (Figure 3.43).

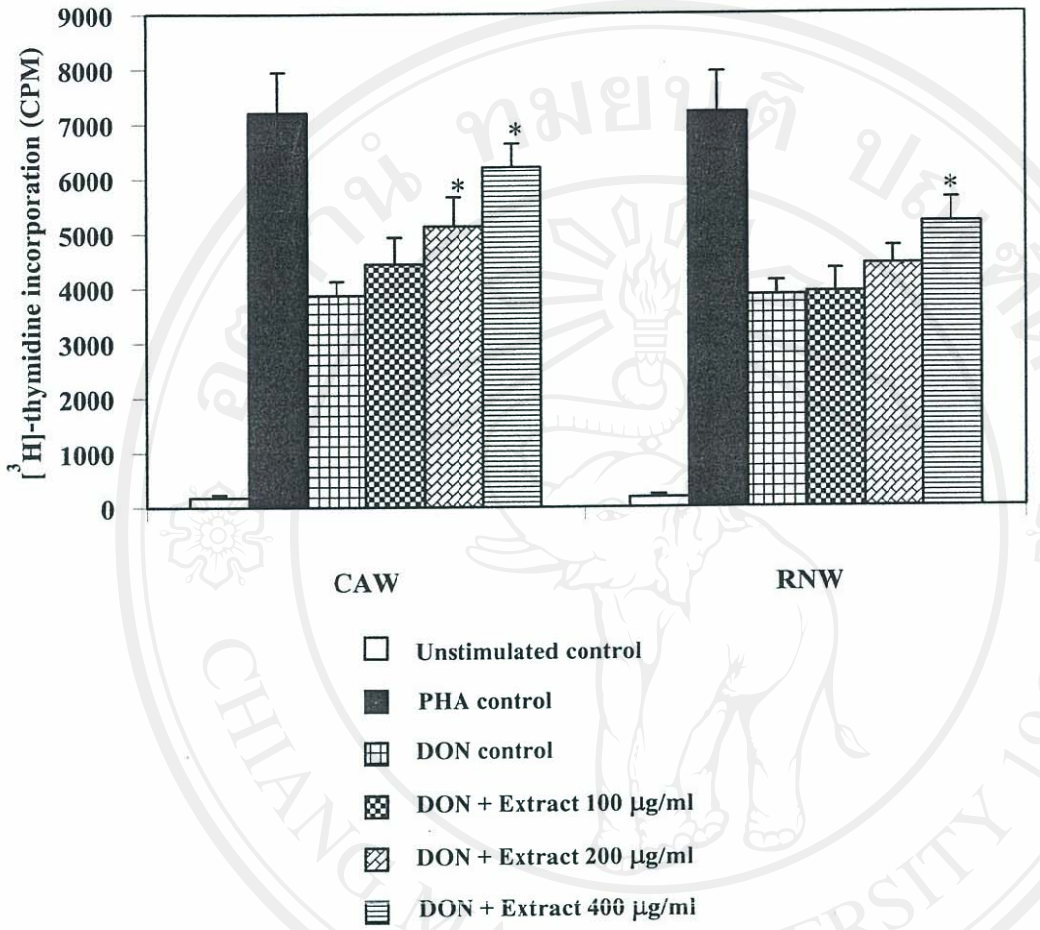

Figure 3.41 Modulation effect of water extracts of *C. asiatica* (CAW) and *R. nasutus* (RNW) on DON-inhibited PHA induced-lymphocyte mitogenesis. Human PBMCs were cultured with various concentrations of extracts in the presence of DON (100 ng/ml) and PHA. The proliferation of human PBMCs was determined after 3 days incubation by [^{3}H]-thymidine incoporation technique. Data represent the mean ± SD of three independent experiments. Statistical significance versus DON control: * $p<0.05$.

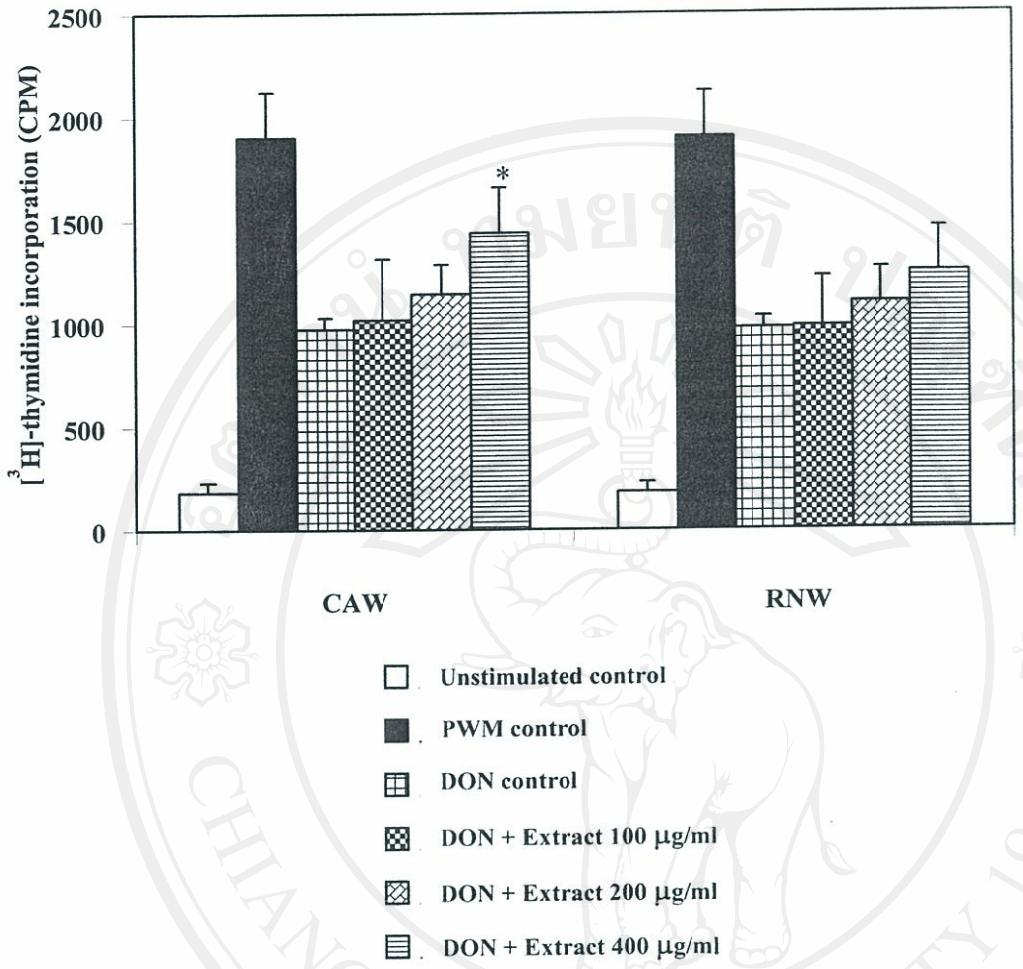

Figure 3.42 Modulation effect of water extracts of *C. asiatica* (CAW) and *R. nasutus* (RNW) on DON-inhibited PWM induced-lymphocyte mitogenesis. Human PBMCs were cultured with various concentrations of extracts in the presence of DON (50 ng/ml) and PWM. The proliferation of human PBMCs was determined after 3 days incubation by [^{3}H]-thymidine incoporation technique. Data represent the mean ± SD of three independent experiments. Statistical significance versus DON control: * $p<0.05$.

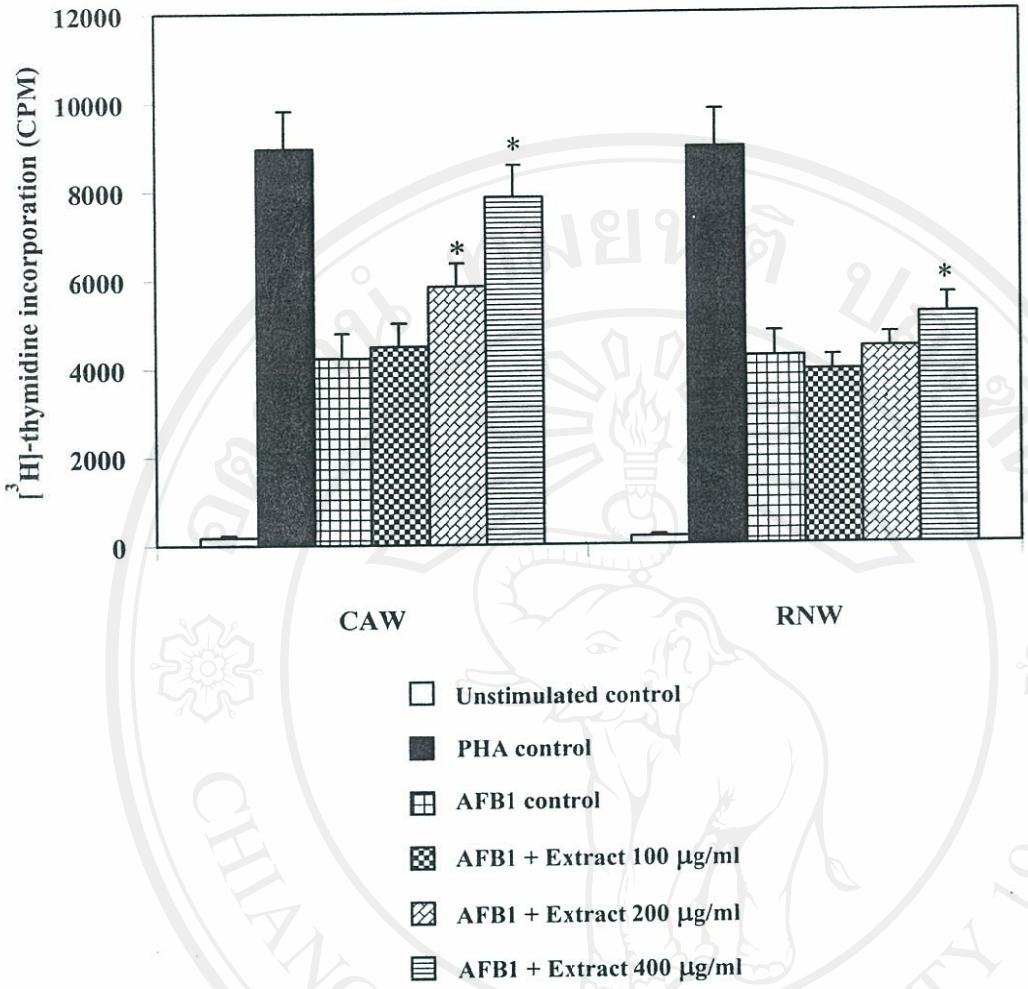

Figure 3.43 Modulation effect of water extracts of *C. asiatica* (CAW) and *R. nasutus* (RNW) on AFB_1-inhibited PHA induced-lymphocyte mitogenesis. Human PBMCs were cultured with various concentrations of extracts in the presence of AFB_1 (5 µg/ml) and PHA. The proliferation of human PBMCs was determined after 3 days incubation by [^{3}H]-thymidine incoporation technique. Data represent the mean ± SD of three independent experiments. Statistical significance versus AFB_1 control: * p<0.05.

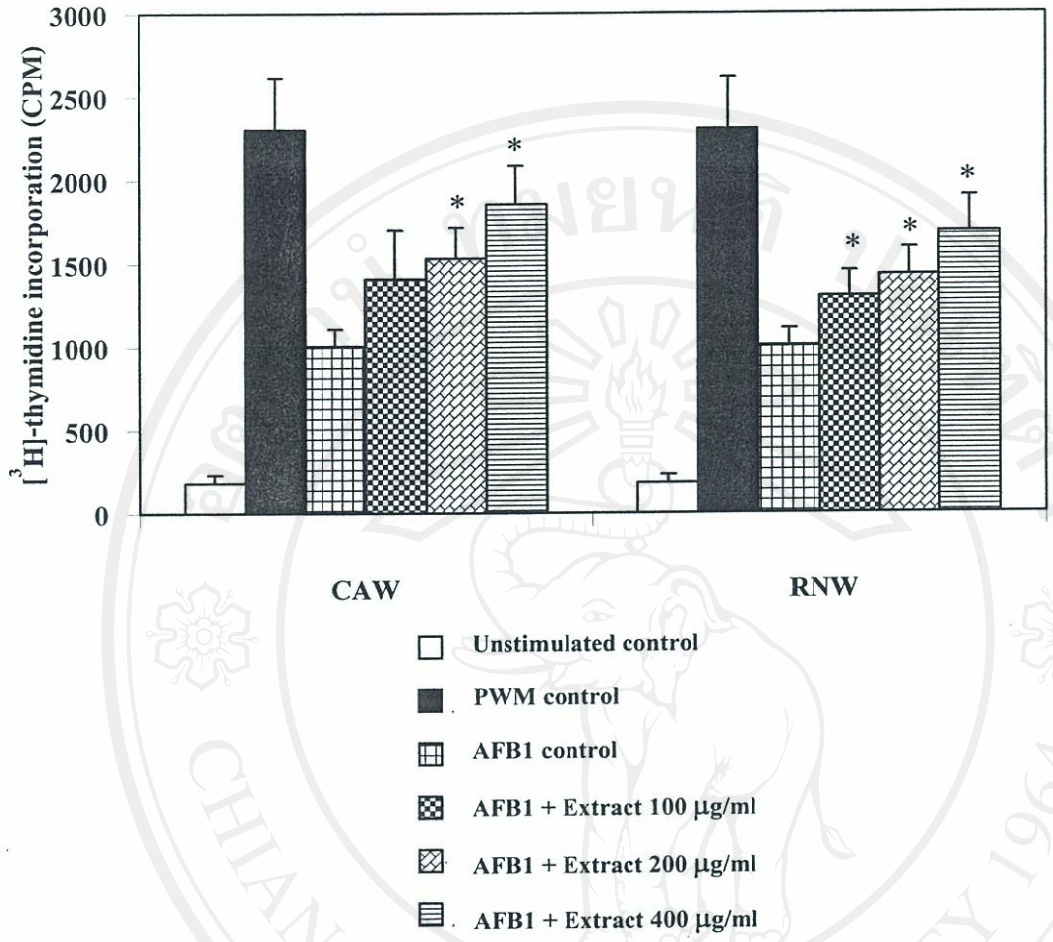

Figure 3.44 Modulation effect of water extracts of *C. asiatica* (CAW) and *R. nasutus* (RNW) on AFB_1-inhibited PWM induced-lymphocyte mitogenesis. Human PBMCs were cultured with various concentrations of extracts in the presence of AFB_1 (5 µg/ml) and PWM. The proliferation of human PBMCs was determined after 3 days incubation by [^{3}H]-thymidine incoporation technique. Data represent the mean ± SD of three independent experiments. Statistical significance versus AFB_1 control: * $p<0.05$.

3.5.4.2 Effect on macrophages

J774.2 mouse macrophages were pretreated with various concentrations of plant extracts (250-1000 μg/ml). For NO production, pretreatment with *C. asiatica* could reverse the NO level that was inhibited by either DON or AFB_1. *C. asiatica* extract could modulate the AFB_1 effect more than DON effect as the modulation effect was found in all concentrations of extracts tested. Pretreatment with *R. nasutus* extract also could increase the production of NO. However, the highest concentration of *R. nasutus* could not change the NO level which inhibited by both mycotoxins (Figure 3.45).

The level of TNF-α was increased by DON treatment, and was decreased by AFB_1 treatment alone. Pretreatment with *C. asiatica* extract showed a modulation effect on both DON and AFB_1 treated cells. In DON treated cells, the level of TNF-α was increased in cells pretreated with extract compared to cells treated with DON alone. However at high doses of *C. asiatica* (1 mg/ml), the TNF-α level was not changed from that of the DON treatment. With AFB_1, *C. asiatica* actually increased the production of TNF-α when compared with AFB_1 treated cells and this effect was dose-dependent (Figure 3.47). Pretreatment with *R. nasutus* extracts could not modulate the mycotoxin effects of both DON and AFB_1 treatments (Figure 3.46 and Figure 3.47).

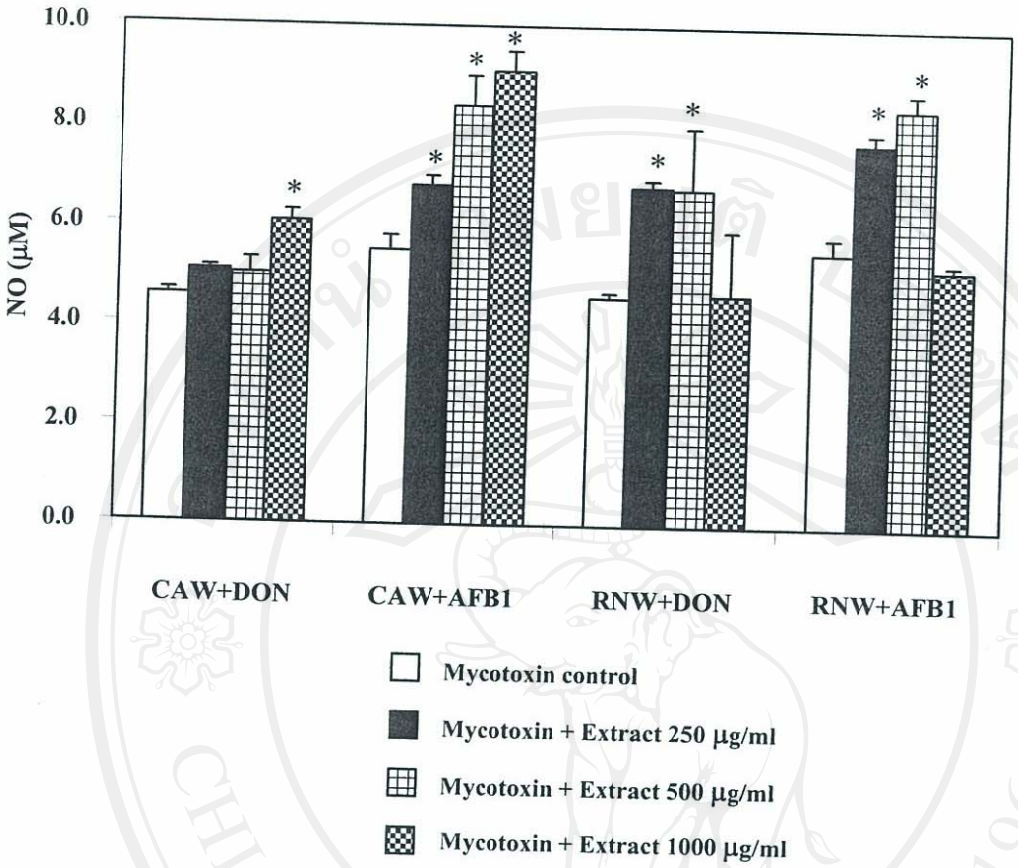

Figure 3.45 Modulation effect of water extracts of *C. asiatica* (CAW) and *R. nasutus* (RNW) on mycotoxin-inhibited NO production. J774.2 macrophages ($1x10^5$ cells/well) were pretreated with various concentrations of extracts for 24 hours. Cells were washed with DMEM medium and cultured with new medium containing either DON (200 ng/ml) or AFB_1 (5 μg/ml) in the presence of LPS (50ng/ml). Supernatants were collected after 24 hours and nitrite level was determined by the Griess reaction. In LPS control, NO level was 9.7 μM. The data represent the mean ± SD of three independent experiments. * $P<0.05$, significantly different from the mycotoxin control.

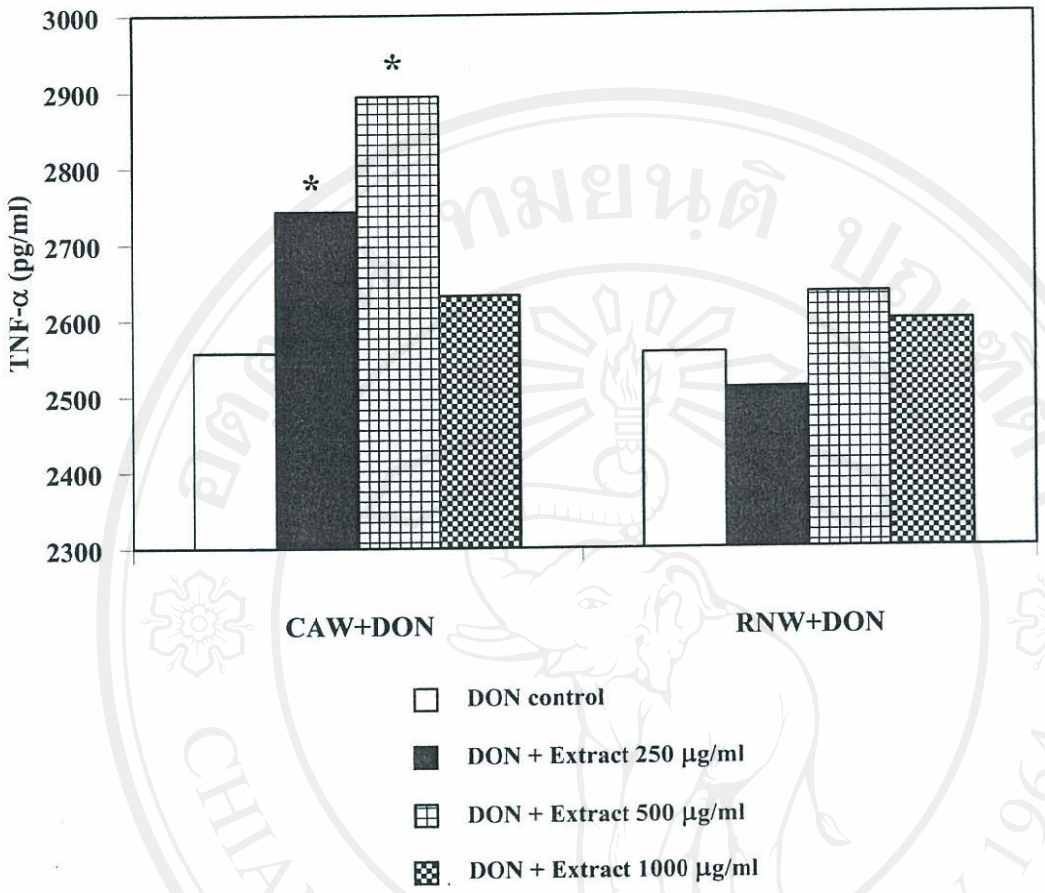

Figure 3.46 Modulation effect of water extracts of *C. asiatica* (CAW) and *R. nasutus* (RNW) on DON-induced TNF-α production. J774.2 macrophages ($1x10^5$ cells/well) were pretreated with various concentrations of extracts for 24 hours. Cells were washed with DMEM medium and cultured with new medium containing either DON (200 ng/ml) in the presence of LPS (50ng/ml). Supernatants were collected after 24 hours. The level of TNF-α was determined by ELISA. In LPS control, TNF-α level was 240 pg/ml. The data represent the TNF-α level from pooled triplicate cultures. * $P<0.05$, significantly different from DON control.

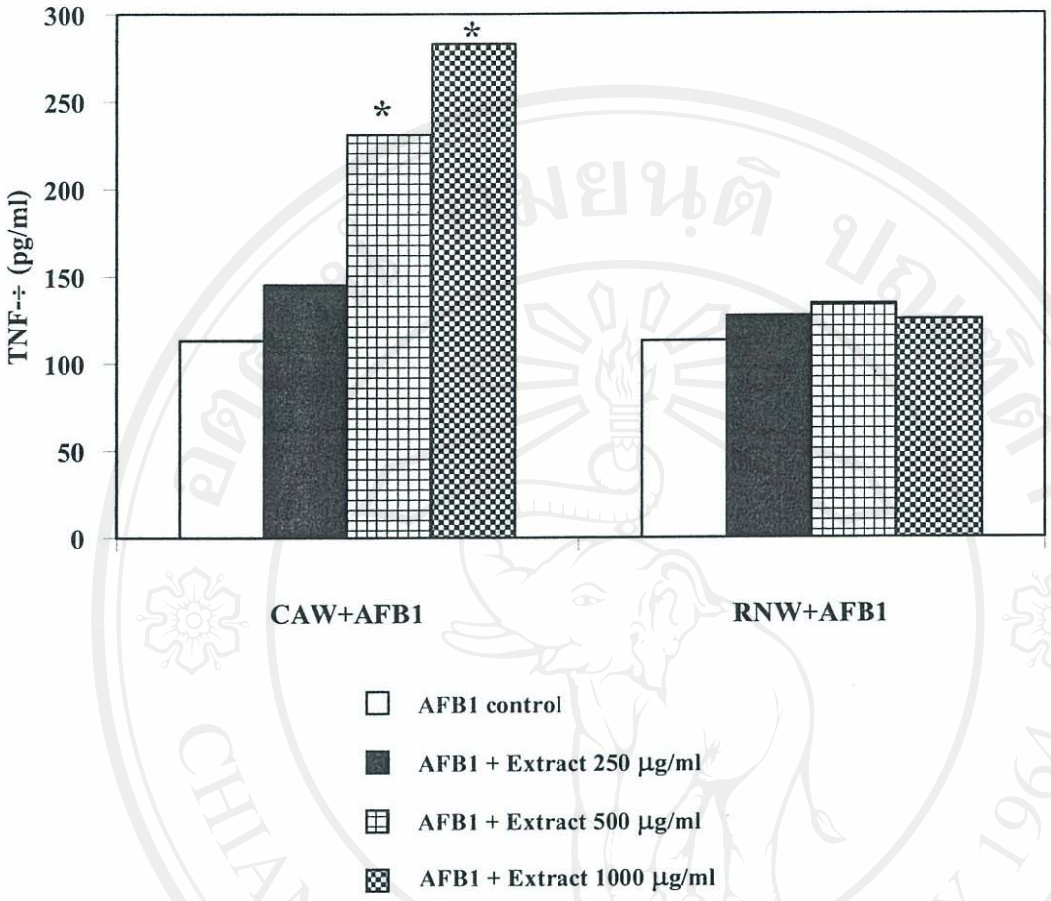

Figure 3.47 Modulation effect of water extracts of *C. asiatica* (CAW) and *R. nasutus* (RNW) on AFB_1-inhibited TNF-α production. J774.2 macrophages ($1x10^5$ cells/well) were pretreated with various concentrations of extracts for 24 hours. Cells were washed with DMEM medium and cultured with new medium containing either AFB_1 (5 µg/ml) in the presence of LPS (50ng/ml). Supernatants were collected after 24 hours. The level of TNF-α was determined by ELISA. In LPS control, TNF-α level was 280 pg/ml. The data represent the TNF-α level from pooled triplicate cultures. * $P<0.05$, significantly different from AFB_1 control.