# CHAPTER IV

## DISCUSSION

The immune system is a highly complex, intricately regulated group of cells whose integrated function is essential to health. It comprises two systems: the innate immune system and the adaptive immune system. The innate system is the first line of defense, and it is the first to clear non-self antigens such as bacteria and viruses from the body. The adaptive immune system is characterized by specificity and memory. Macrophages and lymphocytes are the main cells involved in the immune system. They may interact in a cell-cell manner and may also respond to intercellular messages, including hormones and cytokines. Compounds that are capable of interacting with the immune system to upregulate or downregulate specific aspects of the host response can be classified as immunomodulators. The immunomodulators include diet, pharmacological agents, environmental pollutants, and naturally occurring food chemicals (Middleton et al., 2000).

Medicinal plants are claimed to have therapeutic efficacy for a variety of immune-related problems, ranging from upper respiratory infections to autoimmune and neoplastic disorders. Based on early studies, some of these plant extracts appear to affect humoral (acquired) immunity, but most appear to enhance cellular (innate) immunity. Changes in humoral immunity may include mitogenic effects on B lymphocytes (increased proliferation) and production of specific types of antibodies. Changes in cell-mediated immunity, the more common outcome in phytochemical studies, are measured in terms of natural killer (NK) cell number and activity, lymphokine-activated killer (LAK) cell activity, macrophage activation, phagocytic activity, and proliferation of specific T-lymphocyte subsets (Block and Mead, 2003). Several herbal products that may enhance the function of the immune system are available. These include Echinacea, ginseng, astragalus, licorice, cat's claw, and garlic. Herbs that are rich in flavonoids, vitamin C, or the carotenoids may enhance immune function. The flavonoid-rich herbs may also possess mild anti-inflammatory action. Thai medicinal plants have been used for a centuries to relieve various symptoms of diseases. They have many pharmacological properties, including anti-cancer and anti-inflammatory activities. However, detailed study on the immunomodulatory activity of Thai

medicinal plants is still lacking. This work has aimed aimed at finding Thai medicinal plants that have immunostimulating activity. The selection of Thai medicinal plants used for screening was partly based on their reputed anti-cancer, anti-mutagenicity, or anti-inflammatory activities. The lymphocyte proliferation assay was used to screen eleven Thai medicinal plants. Lymphocyte proliferation assay measures the ability of lymphocytes placed in short term tissue culture to undergo a clonal proliferation when stimulated *in vitro* by a foreign molecule, antigen or mitogen. Mitogens are substances that promote mammalian mitosis in a non-specific manner and are mostly derived from plant and bacterial extracts (Brown and Hunt, 1978). Mitogens have individual stimulatory effects on lymphocytes, e.g. phytohemagglutinin (PHA) stimulates T-cells while pokeweed mitogen (PWM) stimulates T- and B-cells (Pearson et al., 1979; Rouse and Babiuk, 1974).

From eleven Thai medicinal plants, only water extracts of *C. asiatica* and water and ethanol extracts of *R. nasutus* exerted immunostimulating effects on mitogen-stimulated proliferation of human PBMCs with a dose-dependent relationship. In contrast, ethanol extracts of *C. asiatica* showed a strong immunosuppressive activity. Water and ethanol extracts of Ke-Sorn-Buo, Dee-Buo, *C. rotundus* and *E. alba* showed immunosuppressive effects. These extracts strongly decreased PBMC proliferation in a dose-dependent manner. It should be noted that the inhibitory effects observed in this study could not be considered as toxic effect of the plants, because in each case the viability of cells was determined by trypan blue exclusion test and in all of the experiments the cells showed a high viability. Moreover, ethanol extracts of *C. tinctorius* also showed immunosuppressive activity.

Previous study showed that the methanol extract of *E. alba* which contain 1.6% wedelolactone significantly increased the phagocytic index, antibody titer and WBC count *in vivo* (Jayathirtha and Mishra, 2004). In contrast, our result showed the immunosuppressive activity of water and ethanol extract of this plant. The contradictory data may cause by 1) different active component in each types of extract, 2) the metabolic activation of active component *in vivo*.

The rhizomes of *C. rotundus* have been used in oriental traditional medicines for the treatment of stomach and bowel disorders, and inflammatory diseases. Recent study showed the polyherbal formulation contained four different drugs viz. including *C. rotundus* significant inhibited inflammatory bowel disease induced in acetic acid-induced colitis in mice and

indomethacin-induced enterocolitis in rats. The activity was comparable with the standard drug prednisolone (Jagtap et al., 2004). Nitric oxide and superoxide ($O^{2-}$) are important mediators in the pathogenesis of inflammatory diseases. Seo et al. (2001) reported that the methanol extract of rhizomes of *C. rotundus* showed the inhibition of NO and $O^{2-}$ production in RAW 264.7 cells. The inhibition of NO production by the extract was due to the suppression of iNOS protein, as well as iNOS mRNA expression (Seo et al., 2001). These results showed the immunosuppressive activity of methanol extract of *C. rotundus*, which correlated with our data on inhibition of lymphocyte proliferation by both water and ethanol extracts of rhizomes of *C. rotundus*.

The immunomodulatory activities of *C. tinctorius* have been studied. Our data showed the inhibition of lymphocyte proliferation by ethanol extracts of *C. tinctorius*. The result correlated with the study by Lu et al. (1991), which reported the inhibition of nonspecific and specific immune functions by Safflower yellow (SY) extracted from *C. tinctorius*. *In vitro* studies, the inhibitory effects on [$^{3}$H]-thymidine incorporation during human peripheral T- and B-lymphocyte proliferation, murine mixed lymphocyte culture response and the production of IL-2 were observed. *In vivo* studies, mice received SY decreased serum lysozyme concentration and phagocytosing functions of both peritoneal macrophages and peripheral leukocytes; diminished the production of plaque forming cells, specific rosette forming cells, and antibody; inhibited delayed type hypersensitivity reaction and the activation of T suppressor cells elicited by supraoptimal immunization (Lu et al., 1991).

Other medicinal plants did not show any mitogenic responses. However, at higher concentrations of the 80% ethanol extraction of *M. loriformis* and *M. charantia,* strong decreases in T- and B-cell proliferation were observed, and the effect was more potent than for the respective water extracts. The decrease may be due to toxicity of the extracts, because it was observed both in the presence and absence of mitogen. This *in vitro* study revealed preliminary effects of the extracts on the non-specific cellular immune responses. *C. asiatica* and *R. nasutus,*, both of which showed immunostimulating activity, were selected and used in the further experiments.

### 4.1 Effects of *C. asiatica* and *R. nasutus* extracts on the immune system.

From preliminary results, the extracts of *C. asiatica* and *R. nasutus* showed immunostimulating activities. To confirm those results, plants were collected in three independent batches and tested for their effects on mitogen-stimulated lymphocyte proliferation. The result showed no different effect on lymphocyte mitogenesis (Figure3.5 and Figure 3.6). However, *C. asiatica* extracts from batch 1 and batch 3, which were collected during the winter season, seemed to have stronger effect than batch 2, which was collected in May. This result indicated that the amounts of the active components found in plants might change with the season. However, a different area of planting might also affect the amount of active component found in the same species of plant. Thus, when using crude extracts of medicinal plants one should be concerned about the time of planting and harvest season. The data obtained from this experiment could confirm the consistency of the immunostimulating effects of these plants.

*C. asiatica* and *R. nasutus* are widely used to treat a variety of ailments. However, whether the therapeutic efficacy of these plants may in part be mediated via their influence on the immune response is unclear. There is some evidence that the plants can affect immune reactions through their anti-inflammatory activity. *C. asiatica* extracts have been reported to reduce acute radiation reactions in rats by this mechanism (Chen *et al*, 1999). In this study, a water extract of *C. asiatica* significantly increased PWM-induced lymphocyte proliferation in a dose dependant manner and slightly increased PHA-induced lymphocyte proliferation as shown in Figure 3.7. However, ethanol extracts 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. This result indicated that *C. asiatica* has both immunostimulant and immunosuppressive activities. This bi-functional activity of *C. asiatica* suggested that there may be different active components in water and ethanol extracts. Some purified compounds, asiaticoside and asiatic acid, have been reported to be biologically active ingredients in *C. asiatica*. These compounds were tested for their effect on lymphocyte mitogenesis and no effect was observed. This result indicated that the other components in the extracts might be responsible for their activities. Recent study showed that the degraded derivatives of pectin isolated from *C. asiatica* had

immunostimulating activities (Wang et al., 2003). They enhanced proliferative effects on both T- and B- lymphocytes *in vitro*. Pectin is a water-soluble dietary fiber. Recently it was reported that dietary fiber plays an important role in typical immune indices such as T-cell population, cytokine production and Ig production in rat mesenteric lymph node (Lim *et al.*, 1997). Both water and ethanol extracts of *R. nasutus* significantly increased lymphocyte proliferation induced by either PHA or PWM as shown in Figure 3.8. These results demonstrated the immunostimulating activity of plant extracts on cell-mediated immune responses.

The effect of plant extracts on proliferative responses of human PBMCs to PHA may involve many mechanisms, such as the modulation of cytokine production, direct effect on T-cells and/or macrophages. PHA is a mitogen for T lymphocytes (Janeway and Travers, 1994). It binds to N-acetylgalactosamine glycoproteins expressed on the surface of T cells then activates the cells for proliferation. In the present study, T cells were major proliferating cells in PBMC cultures activated with PHA. The activation and clonal expansion of T cells play important roles in generation of immune responses (Janeway and Travers, 1994). Passage through T lymphocyte activation and proliferation is a highly regulated process. Interaction of T cells with antigens or PHA initiates a cascade of biochemical events and gene expression that induces the resting T cells to enter the cell cycle, and then begin proliferating and differentiating (Ajchenbaum *et al.*, 1993). It has been demonstrated that IL-2 is pivotal in the growth of T lymphocytes induced by antigens or PHA (Robb, 1984). TNF-α is mainly produced by macrophages, however it can be produced by Th2 lymphocytes. Like IL-2, TNF-α promotes cell-mediated immunity. This study found that water extract of *C. asiatica* 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 (Figure 3.9). Both water and ethanol extracts 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 (Figure 3.10). These results indicated that the immunomodulatory activities of plant extracts might be in part by the modulation of cytokine production.

*C. asitica* and *R. nasutus* had an effect on both T- and B- cell responses. However the water extract of *C. asiatica* had a strong effect on B-cell proliferation. The main role of B cells is to produce specific antibodies. 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 non-treated control group (Figure 3.24). *R. nasutus* extract-treated mice also produced higher amounts of secondary antibodies against BSA than the non-treated mice (Figure 3.25). Interestingly, the observed effect was not dose-dependent. Mice fed with higher concentrations of extracts showed no difference in regard to specific antibody production when compared with non-treated control mice. The antibody production to T dependent antigen requires the cooperation of T and B lymphocytes (Eldridge *et al.*, 1985). The stimulation of humoral immune response to BSA could be due to extract-induced enhanced phagocytic functions of macrophages, the cells involved in antigen processing and presentation, or may be influenced by relative amounts of different cytokines produced at the site of T and B cell stimulation (Gregg and Denis, 1991; Vos *et al.*, 2000; Luzzati *et al.*, 1997). The antibody production result correlated with the cytokine production result. These data showed that water extract of *C. asiatica* and *R. nasutus* increased the T lymphocyte proliferation by stimulating cytokine (IL-2 and TNF-α) production, resulting in stimulation of B cell proliferation, and finally increased the specific antibody production.

Another mechanism of plant extracts in stimulating antibody production may involve macrophage functions. Macrophages are involved in many different processes such as tissue remodeling during embryogenesis, wound repair, removal of damaged or senescent cells subsequent to injury or infection, hemopoiesis, and homeostasis. Another function of macrophages is to provide a line of defense against microbial invasion and to recognize and kill tumor cells. Macrophages can accomplish this in a direct manner, involving the release of products such as oxygen and nitrogen radicals and TNF- α that are harmful to microorganisms or cancer cells (Adam and Hamilton, 1992). On the other hand, they play an indirect role in these anti-microbial or anti-tumor activities by secretion of cytokines or by antigen processing and presentation, thereby regulating the immune system (Klimp *et al.*, 2002). In this study, a water extract of *C. asiatica* increased the production of NO and, consistent with this for TNF-α, in a mouse macrophage cell line; this effect was observed both when the extract was used alone and in conjunction with LPS. NO has been identified as a major effecter molecule produced by activated macrophages and is involved in the host defense against microorganisms (Macmicking, 1997) and tumor cells (Gorelik *et al*, 1996). Therefore one possibility is that *C. asiatica* mediates some of its beneficial effects by the stimulation of macrophage function. *C. asiatica* has also been widely

used for wound healing and NO is produced in the early phase of this response by inflammatory cells, mainly macrophages (Maria et al, 2002).

The *R. nasutus* extract increased NO production but only when used in conjunction with LPS. This plant was identified as being potentially useful against herpes virus infections (Sendl *et al*, 1996; Kernan *et al*, 1997). Moreover, the literature reports that this plant extract is used for treatments against ringworm and other fungal skin diseases, as well as eczema, pulmonary tuberculosis and cancer (Farnsworth and Bunyapraphatsara, 1992). The reported antiviral effect of NO (Kernan *et al.*, 1997; Croen, 1993) could again provide a plausible mechanism for some of these observed effects.

Interestingly, an ethanol extract of *C. asiatica* dramatically inhibited NO production through the suppression of TNF-α production, the opposite effect to that of the water extract. Although physiological production of NO plays a role in host defense mechanisms, overproduction of NO has been implicated in the pathogenesis of conditions such as bacterial sepsis and chronic inflammation. Therefore, an agent that inhibits the production of NO might be useful for the treatment of these inflammatory diseases. Identification of the active components in *C. asiatica* extracts that modulate NO and TNF-α production certainly merits further study. We did test one of the known components, namely asiaticoside, in the mouse macrophage system but this did not show any effect on NO production.

The cellular mechanisms by which these plants up or down-regulate NO and TNF-α production were studied. The water extract of *C. asiatica* resulted in an induction of TNF-α gene expression in the presence or absence of LPS but had no effect on iNOS expression suggesting the induction of NO was a result of an increase in TNF-α rather than an induction of iNOS expression. This phenomenon was also observed when macrophages were stimulated with *R. nasutus* extracts in combination with LPS. In contrast, an ethanol extract of *C. asiatica* suppressed iNOS and TNF-α expression and this result was correlated with a reduction in NO and TNF-α at the protein level. In macrophages, nuclear factor-κB (NF-κB) coordinates the expression of genes encoding both iNOS and TNF-α. NF-κB plays a major role in controlling the expression of proteins involved in the immune response (Kopp and Ghosh, 1995). The hypothesis that plant extracts modulate this parameter warrants further study.

Macrophages play an indirect role in anti-tumor activity by secretion of cytokines or by antigen processing and presentation. The earlier studies suggested the potential for cytotoxic and anti-tumor properties of *C. asiatica* (Babu *et al*, 1995; Bunpo *et al*., 2004) and *R. nasutus* (Wu *et al*., 1998). 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 extract of *C. asiatica* in 200 μg/ml or 400 μg/ml doses increased cytolytic activities of macrophages against B16F10 by 12% and 19%, respectively (Figure 3.24). This result indicated that the anti-tumor property of *C. asiatica* might in part arise via the stimulation of macrophage functions. The water extract of *R. nasutus* had no effect on cytolysis activities of macrophages.

There is some evidence that natural immune mechanisms can be modulated to impede the development and progression of certain infectious and neoplastic diseases (Whiteside and Herberman, 1994; Smith, 1994). Although the impact of the immune system on malignancy is questionable, cancer patients commonly seek complementary and alternative medicine to enhance the immune system. The use of chemotherapeutic drugs in cancer therapy involves the risk of life threatening host toxicity. An alternative treatment of cancer is required for patients who fail to respond to current cancer therapies such as radiotherapy. The modification of immune function may be the most promising alternative for controlling cancer, particularly stimulation of nonspecific immune response and cell-mediated immune response, because cancer cells are not recognized as foreign substances by the immune system. The present study revealed the immunostimulating activity of *C. asiatica* and *R. nasutus* extract on non-specific cellular immune response. The mechanism of this effect mediated by interference of cytokine production. Another possible mechanism is that the active components of extract may interact with cell surface molecules or growth factors involved in mitogen activation. Although further investigation is warranted, the data available to date suggest that *C. asiatica* and *R. nasutus* may alleviate symptoms of cancer patients through immunostimulating activity.



### 4.2 Effect of mycotoxin mixtures on the immune system

Information regarding the potential synergistic interaction between $AFB_1$ and $FB_1$ could be important for risk assessment analyses for liver cancer development in humans. A recent study

in growing barrows showed that diets containing both $AFB_1$ and $FB_1$ induce liver disease more effectively than the individual toxins (Harvey *et al.*, 1995). In similar study, $FB_1$ and DON induced a more toxic response regarding clinical performances, serum biochemical, hematological and immunologic values than that induced by either toxin separately (Harvey *et al.*, 1996). Therefore, combined administration of mycotoxins may give additive and sometimes synergistic results.

In this study the effects of mycotoxin mixtures on the immune cells were studied. In order to find the appropriate concentrations of mycotoxins, effect of individual mycotoxins on the immune cells were tested. DON had the strongest effect on lymphocyte proliferation when compared with mycotoxin tested in this study; IC50 was $75 \pm 8.9$ and $37.3 \pm 7.8$ ng/ml for PHA- and PWM-stimulated human PBMC mitogenesis, followed by $AFB_1$, which had IC 50 of $6.0 \pm 1.0$ and $4.5 \pm 0.9$, respectively. For $AFB_1$, without metabolic activating enzymes from the 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. These results are comparable to those of an earlier study by Charoenpornsook *et al.* (1998), who found a 50% inhibition for DON and $FB_1$ at 90 and 18 μg/ml respectively using PHA as a mitogen, and at 40 and 11 μg/ml respectively using PWM as a mitogen. Although using the same assay, Charoenpornsook *et al.* (1998) tested this effect on bovine PBMCs. This study used human PBMCs. The result indicated that human PBMCs were more sensitive to these mycotoxins than bovine PBMCs.

The effects of individual mycotoxins on NO and TNF-α production were also evaluated. $AFB_1$ inhibited the production of NO in dose-dependent manner; IC50 was 5.7 μg/ml (Figure 3.29). DON showed a strong inhibition of NO production; IC50 was 200 ng/ml (Figure 3.30). However, at the lowest concentration of DON, 62.5 ng/ml increased the NO production about 33%. In contrast to $AFB_1$ and DON, $FB_1$ significantly increased NO production only at the highest dose (50 μg/ml). As shown in Figure 3.35, DON strongly increased the production of TNF-α in all concentrations tested. In contrast to DON, at concentration of $AFB_1 \geq 5$μg/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 the highest dose (100 μg/ml). These results were correlated with previous study as discuss in section 1.2.3.

The interaction between DON and $AFB_1$ was observed at the highest dose of $AFB_1$ used (5 μg/ml), and shown by a greater inhibition of lymphocyte proliferation than that caused by DON alone. This effect was found in both PHA- and PWM-induced lymphocyte proliferations. The effect of mycotoxin mixtures has been studied *in vitro*. There were no interactions between mycotoxins (Meky *et al.*, 2001). The contradictory data, however, may be caused by the different techniques used to determine cell proliferation. Meky *et al.* (2001) used the MTT assay, which has lower sensitivity than [$^3$H]-thymidine incorporation assay. Metabolic activation of $AFB_1$ is assumed to be a prerequisite for this toxin to exert its toxic and carcinogenic effects (Eaton et al., 1994). This study used the S9 fraction, which contained various metabolizing enzymes including P450 enzymes. These enzymes metabolize $AFB_1$ into several metabolites, especially the $AFB_1$-8,9-epoxide. In the mouse macrophage model, the DON-$AFB_1$ mixture inhibited NO production in macrophages further than in treatments with either DON or $AFB_1$ alone. This result showed the synergistic effect between these two mycotoxins. At the highest concentration of $AFB_1$ tested (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. These data indicate that although $AFB_1$ diminished the TNF-α level, the potent induction of TNF-α by DON still existed. There have been studies on the effect of DON-$AFB_1$ mixture. However, Li and Guo (2000) evaluated the effect of an $AFB_1$-DON mixture on injury and repair of DNA. Both $AFB_1$ and DON could induce DNA synthesis in primary rat hepatocytes at the S phase of the cell cycle, and an

interaction between them was found. The synergistic effect of these mycotoxins might be caused by the inhibition of DNA synthesis.

The mixture of DON and $FB_1$ showed a synergistic effect on inhibition of lymphocyte proliferation. As $FB_1$ alone did not show any effect on human lymphocytes, co-incubation of $FB_1$ with DON caused more inhibition of lymphocyte proliferation than that of DON alone. In mouse macrophages, the DON-$FB_1$ mixture did not have an effect different from DON alone, except at the lowest dose of $FB_1$ (6.25 mg/ml), which increased NO production when compared to DON alone. Both DON and $FB_1$ increased the production of TNF-α. When combined together, the antagonistic effect was observed. At the concentration of $FB_1$ (50 μg/ml) in combination with DON, the level of TNF-α was lower than in the DON control. However the concentration of TNF-α was still much higher than in the LPS control and $FB_1$ control. These results demonstrated the interaction of these two mycotoxins on both lymphocytes and macrophages.

The $AFB_1$-$FB_1$ mixture did not show any interaction effects on lymphocyte proliferation. In macrophages, treatment with $AFB_1$-$FB_1$ mixture caused NO levels to increase (Figure 3.34). This result showed that $FB_1$ could modulate the effect of $AFB_1$, which suppressed NO production. Combination of these toxins led to 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 was not different from $AFB_1$-$FB_1$ mixture (269 pg/ml). The results from this study were similar to the other studies. In the study of subchronic mycotoxicoses, the body weight of rats was increased by $AFB_1$ treatment, and decreased by $FB_1$ treatment. However, the $AFB_1$-$FB_1$ mixture showed similar behavior to $FB_1$. The serum ALT activity was decreased by $AFB_1$ and increased by $FB_1$ treatment. The mixture of these toxins had no effect on ALT activity, which possibly was caused by self-compensation by individual mycotoxins. Spleen mononuclear cell (SMC) proliferation was also determined in the presence of concanavalin A (Con A). $AFB_1$ increased SMC proliferation. However $FB_1$ and a mixture of $AFB_1$-$FB_1$ had no effect on SMC proliferation. Thus $FB_1$ could be modulating the proliferative effect induced by the $AFB_1$ ingestion through some unknown mechanism (Theumer *et al.*, 2003). All together, these results showed that mixtures of these mycotoxins did not change those effects caused by individual $AFB_1$ or $FB_1$. In a short-term carcinogenesis model in rat, the $AFB_1$ pretreatment enhanced the $FB_1$ initiating potency, presumably by rendering the liver more susceptible to the toxic effects of $FB_1$. So, the co-

occurrence of $AFB_1$ and $FB_1$ in corn consumed as a staple diet could pose an increased risk and should be included in establishing risk assessment parameters in humans (Gelderblom *et al.*, 2002). Another study on the $AFB_1$-$FB_1$ mixture, the toxicological effects of chronic low doses of these toxins in weaning piglets was examined. The reduction of feed consumption, induction of various hematological and biochemical parameters including erythrocyte number, hematocrit, total albumin, total protein, activity of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase, and increase chloresterol level were observed in a group of piglets receiving $FB_1$ or $FB_1$-$AFB_1$ mixture. No additional effect was observed in the mycotoxin mixture group (Dilkin *et al.*, 2003).

### 4.3 Effect of water extract of *C. asiatica* and *R. nasutus* extracts on mycotoxin-induced immunotoxicity

The realization that aflatoxins, deoxynivalenol and fumonisins are immunosuppressive agents has broad implications for the ability of human populations to resist disease. According to the concept of immunosurveillance, immunosuppressive activity of mycotoxins might be one mechanism of cancer susceptibility. Substances that have immunostimulating activity might reduce the toxicity of mycotoxins and subsequcnt risk of development of cancer. Medicinal plants have been used as foods and for medicinal purposes for centuries. Research interest has focused on various medicinal plants that have hypolipidemic, anti-platelet, anti-inflammatory, anti-tumor or immunomodulating properties and which may serve as useful adjuncts in helping reduce the risk of various diseases, including cardiovascular disease and cancer. It is hypothesized that the immune stimulating activity of Thai medicinal plants might protect or reduce the immunotoxicity of these mycotoxins. To test this hypothesis, *C. asiatica* and *R. nasutus* were used for pretreatment of lymphocytes or macrophages following exposure thoses cells to DON or $AFB_1$. 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 increased the production of NO. The level of TNF-$\alpha$ 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. Pretreatment with *R. nasutus* extracts could not modulate the mycotoxin effects of both DON and $AFB_1$ treatments. The results indicated that medicinal plants that had immunostimulating activity could modulate the immunotoxic effect caused by mycotoxins. The mechanisms of plant extract to modulate the mycotoxin-induced immunotoxicity may be mediated by their properties in the induction of cytokines, increase macrophage functions, and increase lymphocyte activities. Further study should be placed on *in vivo* study to evaluate whether these extracts actually show the protective effects on mycotoxin-induced immunotoxicity as *in vitro* study.