Original article

IMMUNOMODULATORY EFFECTS OF THAI MEDICINAL PLANTS ON THE MITOGEN STIMULATED PROLIFERATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS IN VITRO

Khanittha Punturee,1 B.Sc., Wachara Kasinrerk,2 Ph.D., Christopher Paul Wild,3 Ph.D., Usanee Vinitketkumnuen,1 Ph.D.

1 Department of Biochemistry, Faculty of Medicine, 2Department of Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, 3Molecular Epidemiology Unit, Epidemiology and Health Services Research, School of Medicine, University of Leeds, Leeds LS2 9JT, UK

Abstract The immunomodulatory effects of Thai medicinal plants, including Murdannia loriformis, Cymbopogon citratus, Momornica charantia, Centella asiatica, Allium sativum, Carthamus tinctorius, Eclipta alba, Cyperus rotundus, lotus pollen (Dee-Buo), and plant embryos in seeds of the lotus (Ke-Sorn-Buo), on the mitogen stimulated proliferation of human peripheral blood mononuclear cells (PBMCs) were investigated. The results obtained from this study indicated that only water extracts from C. asiatica had an immunostimulating effect on mitogen-stimulated proliferation of human PBMCs. In contrast, the ethanol extract from this plant showed immunosuppressive activity. Moreover, the water and ethanol extracts of Ke-Sorn-Buo, Dee-Buo, C. rotundus and E. alba had immunosuppressive effects. These extracts strongly decreased PBMC proliferation in a dose-dependent manner. Ethanol extract of C. tinctorius also showed immunosuppressive activity at a high concentration. Other medicinal plants did not show any mitogenic responses. This in vitro study revealed various effects of Thai medicinal plant extracts on non-specific cellular immune responses. Further investigation should be considered on the effect of extracts on other immune parameters such as macrophage activity, NK cell activity including cell signaling and cytokine production. Chiang Mai Med Bull 2005;44(1):1-12.

Keywords: Thai medicinal plant, immune system, lymphocytes

Many Thai medicinal plants are used for the treatment of various diseases including infection, immunological disorders and cancer.1 They may affect
both the cell-mediated and humoral immune systems. Thus, modulation of the immune response in order to alleviate disease is of primary interest. Anecdotal data from cancer patients who used Thai medicinal plants, and some laboratory data have suggested anticancer or anti-mutagenicity effects of Thai medicinal plants such as *Murdannia loriformis*, *(2)* *Cymbopogon citratus* *(3)* and *Mormodica charantia*. *(4)* However, a biological study of the immunomodulatory activity of Thai medicinal plants is still lacking.

*Centella asiatica* (Linn.) Urban (Umbelliferae) is a pantropical plant in Thailand. It is an effective medicinal plant for development as a wound-healing drug and for dermatological preparations. Its active constituents include pentacyclic triterpene derivatives such as asiatic acid, asiaticoside, madecassic acid and madecassoside. *(1)* At present, clinical studies, which are aimed at investigating the sedative, analgesic, antidepressive, antimicrobial, antiviral, and immunomodulating effects that have been demonstrated experimentally, are still lacking. *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy, which belongs to the family Commelinaceae, has been used in Thailand for pain relief from bronchitis and various types of cancer. *(5)* Both anticarcinogenic and antimutagenic activities of *M. loriformis* have been reported; the level of aflatoxin-albumin adducts in rats receiving *M. loriformis* extract was significantly lower than in non-treated animals. *(6)* The ethanol extract of *M. loriformis* was non-mutagenic to *Salmonella typhimurium* strain TA100 and TA98, with or without metabolic (S9) activation, and it had anticarcinogenic enzyme (DT-diaphorase) inducing activity in the hepa 1c1c7 murine hepatoma cell line. *(4)* Recently, the methanol extract of *M. loriformis* was found to contain the active principles 3-O-β-D-glucopyranosyl-24-ξ-ethyl-5-α-cholesta-5-ene, and a glycosphingolipid, named 1-β-O-D-glucopyranosyl-2-(2’-hydroxy-6’-ene-carboxamide)-sphingosine. The active glycosphingolipid exerted a cytotoxic effect against human colon carcinoma and human breast cancer cell lines. *(7)* *Cymbopogon citratus* (DC.) Stapf (lemon grass) (Gramineae) has long been used in Thailand as a source of medicine as well as a dietary component. Previous studies have found that it had antimutagenic activity toward chemically induced mutation in *Salmonella typhimurium*. *(3)* *Allium sativum* Linn. (Garlic) has been shown to reduce the risk factors for cardiovascular diseases, in way such as lowering of serum cholesterol and triglyceride, and inhibiting blood coagulation. *(8)* Many *in vitro* and *in vivo* studies have suggested possible cancer-preventive effects of garlic. *(9)* In addition, garlic has been shown as a possible biological response modifier. Because some diseases can be caused by immune dysfunction, modification of immune function by garlic may contribute to the treatment and prevention of diseases. Thus, some pharmacological effects of garlic may be mediated through immunomodification. Recently, aged garlic extract showed an immune stimulating activity. *(10)* *Momordica charantia* Linn (Bitter melon), a widely
distributed cucurbitaceous fruit in Asia, is a medicinal plant with antidiabetic, antitumor and immunomodulating properties.\(^{(1)}\) The other medicinal plants used in this study included *Carthamus tinctorius* Linn, *Eclipta alba*, *Cyperus rotundus*, lotus pollen (Dee-Buo) and plant embryos in seeds of the lotus (Ke-Sorn-Buo).

This study aimed to investigate the effects of the Thai medicinal plants mentioned above on spontaneous lymphocyte mitogenic activity and mitogen (phytohemagglutinin or pokeweed mitogen) induced-lymphocyte proliferation *in vitro*.

**Materials and methods**

**Reagents**

\(^{3}\)H]-thymidine was obtained from Amersham, Germany. Phytohemagglutinin (PHA), Pokeweed mitogen (PWM), Trypan Blue, Histopaque and RPMI 1640 medium were obtained from Sigma, U.S.A. Ninety six-well flat bottomed tissue culture plates were obtained from Costar, U.S.A. and fetal calf serum (FCS) from STARTRATE, Australia.

**Plant materials**

Whole fresh *Murdannia loriiformis* and *Centella asiatica* plants, *Momordica charantia* fruits, *Allium sativum* bulbs, *Cymbopogon citratus* stem parts, and *Carthamus tinctorius* flowers were obtained from local markets in Chiang Mai, Thailand. Four types of commercial dried plants, comprising *Eclipta alba*, *Cyperus rotundus*, lotus pollen (Dee-Buo) and plant embryos in lotus seeds (Ke-Sorn-Buo), were obtained from Lampang province, Thailand.

**Preparation of the extracts**

All fresh plants were washed with tap water, sliced into small pieces, dried and ground to a fine powder. One hundred grams of powder were extracted in 1 liter of 80% ethanol for ethanol extraction, or 200 mL of distilled water by stirring for 4 hours. The 80% ethanol and water extracts of all plants were centrifuged at 5,000 rpm for 15 minutes. The supernatants were filtered through Whatman filter paper number 2 and the filtrates were evaporated until dry by using a rotating evaporator (60 °C). The residue was dissolved in dimethylsulfoxide (DMSO) or distilled water, and adjusted to a 5 mg/mL final concentration and sterilized by a Millipore filter membrane (0.22 µm).

**Preparation of peripheral blood mononuclear cells (PBMCs)**

The peripheral blood mononuclear cells (PBMCs) were separated from the whole blood of three healthy donors by Ficoll-Hypaque gradient centrifugation. The PBMCs were prepared under sterile conditions in RPMI-1640 medium containing 10% fetal calf serum. Their viability, as determined by the trypan blue exclusion test, was more than 98%, and their concentration was finally adjusted to 1.0x10^6 cells/mL.

**Lymphocyte activation assay**

One hundred microliters of cell suspension were pipetted into each well of
96-well tissue culture plates; to which 100 μL of media containing different concentrations of 80% ethanol or water extracts, and 10 μL of media containing a sub-optimal concentration of phytohemagglutinin (PHA) to stimulate T-cells or pokeweed mitogen (PWM) to stimulate B- and T-cell mitogenesis were added. One triplicate series of wells was used as a negative control (without extracts and mitogen), and as a positive control with mitogen. The plates were incubated for 3 days at 37 °C in a 5% CO₂ incubator. Cell proliferation was estimated by adding 0.2 μCi of [3H]-thymidine per well during the final 18-hr culture. [3H]-thymidine incorporation into cells was measured as count per minute (CPM) using a liquid scintillation counter (β-counter). The stimulation index (SI) was determined by dividing the mean CPM of treated cultures - the mean CPM of negative controls divided by the mean CPM of positive controls - by the mean CPM of negative controls. The cytotoxicity of extracts on human PBMCs was also determined by using a Trypan blue exclusion test.

Statistical analysis
A Mann-Whitney U test was used to assess the statistical significance of differences. A p value of < 0.05 was considered significant.

Results
The effects of water extracts on lymphocyte mitogenesis
In vitro immunomodulatory activity of Thai medicinal plants was assessed using the lymphocyte activation assay. Comparison of the cell proliferation in non-treated and extract-treated cultures showed no direct mitogenic activity (data not shown). The extract of C. asiatica (50-200 μg/mL) significantly increased both PHA and PWM-induced lymphocyte proliferation. In contrast, the extract of M. loriformis, Ke-sorn-Buo, Dee-Buo and E. alba significantly decreased both mitogen-induced lymphocyte proliferation (Table 1, Fig. 1 and 2). M. charantia and C. citratus extracts decreased only PHA, and not PWM-induced lymphocyte proliferation, suggesting that they affected T cell proliferation (Table 1). Other plant extracts did not show any effect on lymphocyte mitogenesis.

The effects of 80% ethanol extracts on lymphocyte mitogenesis
Comparison of the cell proliferation in non-treated and extract-treated cultures showed no direct mitogenic activity (data not shown). At higher concentrations of C. asiatica; Ke-Sorn-Buo, Dee-Buo, M. loriformis, C. rotundus, C. tinctorius, M. charantia and E. alba extracts significantly decreased both mitogen-induced PBMC mitogenesis (Table 2, Fig 3 and 4). However, the effect of M. loriformis and M. charantia was caused by their toxicity to human PBMCs. Other extracts were not toxic to human PBMCs at higher concentrations. This result indicated that ethanol extracts of C. asiatica, Ke-Sorn-Buo, Dee-Buo, C. rotundus, C. tinctorius, and E. alba have immunosuppressive activity.
Figure 1. The effect of water extracts from Thai medicinal plants on PHA-induced PBMC proliferation. Human PBMCs (1x10^5 cells/well) were treated with various concentrations of water extracts (1-200 g/ml) and stimulated with PHA for 72 hr. Proliferation was evaluated by [^3H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value was 1.00. The results are expressed as the average of three donors.

Figure 2. The effect of water extracts from Thai medicinal plants on PWM-induced PBMC proliferation. Human PBMCs (1x10^5 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 [^3H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value was 1.00. The results are expressed as the average of three donors.
The effect of ethanol extracts from Thai medicinal plants on PWM-induced PBMC proliferation. Human PBMCs (1x10^5 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 [3H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value was 1.00. The results are expressed as the average of three donors.

The effect of ethanol extracts from Thai medicinal plants on PHA-induced PBMC proliferation. Human PBMCs (1x10^5 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 [3H]-thymidine incorporation. The stimulation index (SI) was calculated. Without any extract, the SI value was 1.00. The results are expressed as the average of three donors.
Discussion
The study of the immunomodulatory effects of medicinal plants on both cell-mediated and humoral immune response is a matter of interest for many researchers. Several studies have previously demonstrated the immunomodulating effects of medicinal plants on lymphocyte proliferation in the presence of mitogen, allogenic cells and specific antigens.\(^{(12-14)}\)

This study focused on the influence of Thai medicinal plants that have shown anti-cancer activity on mitogen-induced PBMC proliferation. The results obtained from this study indicated that water extraction of *C. asiatica* exerted an immunostimulating effect on the mitogen-stimulated proliferation of human PBMCs with a dosage-dependent relationship. In contrast, ethanol extract of *C. asiatica* showed a strong immunosuppressive activity. This bi-functional activity of *C. asiatica* suggested the different active components in water and ethanol extract. 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 the toxic effect of the plants, because in each case the viability of cells was determined, and in all of the experiments the cells showed a high viability. Moreover, ethanol extract of *C. tinctorius* also showed immunosuppressive activity. Other medicinal plants did not show any mitogenic responses. However, at higher concentrations of the 80% ethanol extraction of *M. loriformis* and *M. charantia*, a strong decrease of T- and B-cell proliferation was demonstrated and the effect was more potent than that for the respective water extracts. The decrease might be due to toxicity of the extracts, which 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.

The use of chemotherapeutic drugs in cancer therapy involves the risk of life-threatening host toxicity. The research, therefore, goes on to develop the drugs, which selectively act on tumor cells. 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, in particular via stimulation of nonspecific immune response and cell-mediated immune response. This is because cancer cells are not recognized as foreign substances by the immune system. Thai medicinal plants are widely used for treatment of various diseases including cancer. Whether the therapeutic efficacy of these herbs may in part be mediated via their influence on the immune response, is not clear. It has been shown that some of these herbs can affect the immune response through their anti-inflammatory actions. The *C. asiatica* extracts are able to reduce acute radiation reactions by their anti-inflammatory activity\(^{(15)}\). A
recent study showed the potential cytotoxic and anti-tumor properties of *C. asiatica*.\(^{(10)}\) This study revealed the immunostimulating activity of *C. asiatica* extract on non-specific cellular immune response. Although the exact mechanism of this effect is not known, it may be mediated by the interaction between active components of extract and cell surface molecules, or growth factors involving mitogen activation. The other possible action of *C. asiatica* extracts may be interference with cell signaling and cytokine production. Although further investigation is warranted, the data available to date suggest that *C. asiatica* may alleviate symptoms in cancer patients through immune stimulation activity.

In conclusion, this study demonstrated various effects of medicinal plants. The difference in the way these extracts affected lymphocyte stimulation or inhibition perhaps indicated various modes of action. Further investigation should be considered in the effect of extracts on other immune parameters such as macrophage activity, NK cell activity including cell signaling and cytokine production.

**Acknowledgements**

The investigators wish to extend their appreciation to the Professor Dr. Takeo Wada Cancer Research Fund, Faculty of Medicine, Chiang Mai University and the Royal Golden Jubilee Scholarship Thailand for their generous financial support for the project.

**References**


การศึกษาผลของสมุนไพรไทยต่อการแบ่งตัวเพิ่มจำนวนเซลล์ลิมโฟซัยต์ในหลอดทดลอง

ชนิชญา พันธุรี,¹ วท.บ., วธระ กลิ่นถิ่น,¹ Ph.D., Christopher Pual Wild,³ Ph.D.,
อุษณีย์ วิไลสวัสดิ์,¹ Ph.D.
¹ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่, ¹Molecular Epidemiology Unit, Epidemiology and Health Services Research, School of Medicine, University of Leeds, Leeds LS2 9JT, UK

บทคัดย่อ งานวิจัยนี้เป็นการศึกษาผลของสมุนไพรไทยต่อระบบภูมิคุ้มกันชนิดเซลล์ โดยนักการสกัดจากquatกระตุ้นการแบ่งตัวเพิ่มจำนวนเซลล์ลิมโฟซัยต์ร่วมกับไมโตเจน สมุนไพรที่นำมาศึกษาได้แก่เห็ดปูปิ้งต้า, ตะไคร้, ตะไคร้แช่กับงา, กระเทียม, ขมิ้น ทั้งหมดนี้ให้ผลดีและข้อเสนอของสารสกัดจากquatกระตุ้นภูมิคุ้มกันโดยเพิ่มการแบ่งตัวเพิ่มจำนวนเซลล์ลิมโฟซัยต์เมื่อกระตุ้นร่วมกับไมโตเจน ซึ่งฤทธิ์จะเพิ่มขึ้นตามความเข้มข้นของสารสกัดที่มากขึ้น ในทางตรงกันข้ามสารสกัดจากquatกระตุ้นมีฤทธิ์กดภูมิคุ้มกันโดยสารสกัดดังกล่าวลดการแบ่งตัวเพิ่มจำนวนเซลล์ลิมโฟซัยต์เมื่อกระตุ้นร่วมกับไมโตเจนอย่างมีนัยสำคัญ ส่วนสารสกัดจากquatกระตุ้นไม่มีผลต่อการแบ่งตัวเพิ่มจำนวนเซลล์ลิมโฟซัยต์ จากผลการทดลองมีที่สุ่มให้มีสมุนไพรไทยมีผลต่อระบบภูมิคุ้มกันชนิดเซลล์ ครั้งนี้จะมีประโยชน์ในการศึกษาเพื่อหาสมุนไพรไทยที่มีประสิทธิภาพต่อระบบภูมิคุ้มกันชนิดอื่นๆ เช่น การรักษาโควิด-19 หรือการส่งสัญญาณภายในเซลล์และการผลิตไซโตเชนต์ต่างๆ เขียนในเวชศาสตร์ 2548; 44(1):1-12.

คำสำคัญ: สมุนไพรไทย ระบบภูมิคุ้มกัน ลิมโฟซัยต์