การประเมินฤทธิ์ปรับภูมิคุ้มกันของสมุนไพรไทยบางชนิด ด้วยวิธีการวัดการเพิ่มจำนวนของลิมโฟซัยท์

Evaluation of Immunomodulating Activity of Selected Thai Medicinal Plants by Lymphocytes Proliferation Assay

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บทคัดย่อ

การทดสอบฤทธิ์ปรับภูมิคุ้มกันของสารสกัดสมุนไพรจำนวน 40 ชนิด ซึ่งเป็นสมุนไพรที่หมอพื้นบ้านในจังหวัดอุบลราชธานีใช้รักษาโรคเรื้อรังและโรคที่เกี่ยวข้องกับระบบภูมิคุ้มกัน โดยวัดการเพิ่มจำนวนของลิมโฟซัยท์จากม้ามหนู ด้วยวิธีการทดสอบด้วย MTT ในสภาวะที่มีและไม่มีตัวกระตุ้น ผลการทดสอบพบสารสกัดสมุนไพร 3 ชนิด มีฤทธิ์ดีในการกระตุ้นภูมิคุ้มกัน คือ *Dendrophthoe pentandra, Croton cascarilloides, Bauhinia penicilliloba* และสารสกัดสมุนไพรจำนวน 3 ชนิดมีศักยภาพในการกดภูมิคุ้มกัน คือ *Crateva adansonii, Polygala chinensis, Chionanthus ramiflorus*ซึ่งผลการวิจัยนี้สอดคล้องกับข้อมูลของการใช้สมุนไพรในการรักษาโรคของหมอพื้นบ้าน

คำสำคัญ : สมุนไพร, ฤทธิ์ปรับภูมิคุ้มกัน, การเพิ่มจำนวนของลิมโฟซัยท์, วิธีการทดสอบด้วย MTT

Abstract

Forty extracts of indigenous Thai medicinal plants having ethnomedical application in the treatment of chronic diseases and immunological disorders by local practitioners of Ubon Rathathani were investigated for their immunobiological activity. They were studied for mouse spleenic lymphocyte

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proliferation in the absence and presence of mitogens by colorimetric MTT assay. Among these extracts, three plants displayed strong immunostimulating activities: *Dendrophthoe pentandra, Croton cascarilloides, Bauhinia penicilliloba.* Three plants showed potential to be immunosupressors: *Crateva adansonii, Polygala chinensis, Chionanthus ramiflorus.* These results are discussed in relation with traditional medicine.

Key words : medicinal plants, immunomodulating activity, lymphocyte proliferation, MTT assay

Introduction

Medicinal plants have been used to treat human illness since time immemorial. Some medicinal plants are believed to promote positive health and maintain resistance to infection by re-establishing body equilibrium and conditioning the body tissue. A number of plants used in the traditional medicinal systems of chronic ailments and rejuvenating therapy have been shown to possess immunostimulating activity acting at different levels of the immune system (Atal et al., 1986; Neelam et al., 2001).

Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reactions it is named as an immunostimulator which primarily implies stimulation of non-specific system, i.e. granulocytes, macrophages, complement, certain T-lymphocytes and different effector's substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may occur on account of environment or chemotherapeutic factors (Neelam et al., 2001). Immunostimulation and immunsuppression both need to be controlled in order to regulate normal immunological functioning. Hence both immunostimulating agents and immunsuppressing agents have their own standing and search for better agents exerting these activities is becoming a field of major interest all over the world (Patwardhan et al., 1990).

Today, the concept of immunomodulation has begun to find acceptance in medicine (Masihi, 2000). The potential uses of immunomodulators in clinical medicine include the reconstitution of immune deficiency and the suppression of normal or excessive immune function (Patwardhan et al., 1990). Immunomodulators are being used as immunotherapy in some diseases such as cancer, infectious diseases, immunodeficiency disorders, autoimmune disease and inflammation. Many of the presently available synthetic immunomodulators are not free from side effects. (Labadie et al., 1989; Daisio and LoBuglio, 1996). Hence many researchers are interested in finding new immunomodulators from plants.

An ethnobotanical survey had previously been conducted on the utilization of medicinal plants as immunomodulators in Ubon Ratchathani Province, Thailand (Prasitpuriprecha et al., 2005). This present study aimed to evaluate the immunomodulatory activity of the aqueous extracts of the forty plants in order to relate the efficacy of those plants with their ethnomedical uses.

Material and methods

Plant materials

Forty medicinal plants traditionally used as immunomodulators for the treatment of immune diseases in Ubon Ratchathani Province were investigated for their immunomodulating activities (Table 1). The plants were collected under the guidance of local traditional practitioners and were verified for their botanical identities. Voucher specimens were deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University.

Preparation of plant extracts

As described by the local traditional practitioners, a decoction was generally made except two plants. *Molineria latifolia* Herb.ex kurz (F.Hypoxidaceae) was extracted by alcoholic maceration. *Prismatomeris griffithii* Ridl. (F.Rubiaceae) extract was made by aqueous infusion. The laboratory method of extraction was performed accordingly, with slight modifications. To be more scientific and reliable, the collected parts were washed and dried at 50-60 °C until constant weight. They were powdered and extracted by methods corresponding to those practiced by the local traditional practitioners. For decoction, the aqueous extract was obtained by boiling 100 g of dried ground plant material for 30 min in 300 ml of distilled water. For alcoholic maceration, the extract was obtained by macerating 100 g of dried ground plant material for a day in 300 ml of 50% ethanol. For infusion, the aqueous extract was obtained by infusing 100 g of dried ground plant material for a day in 300 ml of distilled water. All extracts were filtered and freeze dried. They were all kept at -80 °C in tight and light-protected containers until use.

Animals

Inbred male BALB/c mice, 6-8 weeks old with a weight range of 20-25 g were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed under standard conditions at 25 °C and fed with standard pellets and tap water.

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and pokeweed mitogen (PWM) were purchased from Sigma, USA. Phytohemagglutinin (PHA), RPMI-1640 medium and fetal bovine serum were all purchased from Gibco, USA.

Preparation of cells

Mice were sacrificed by cervical dislocation and spleens were removed aseptically. A single cell suspension was prepared in complete RPMI-1640 medium (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum). After centrifugation at 1,000 rpm for 10 min at 4 °C (Hettich/ Universal32R, Germany), erythrocytes were lysed by hypotonic solution and the cell pellets were washed twice with RPMI-1640. The cells were re-suspended in completed medium and the cell number was adjusted to $2x10^6$ cell/ml. The viability of splenocytes was determined by

the trypan-blue dye exclusion technique (Brousseau et al., 1998).

Cell proliferation assay by modified colorimetric MTT assay

Splenocytes (spleenic lymphocytes) proliferation activity was measured by using a modified colorimetric MTT assay described by Mosmann (1983), Hansen (1989) and Gerlier et al. (1986). Briefly, fresh splenocytes were prepared aseptically into single cell suspension. There were suspended at $2x10^6$ cell/ml in complete medium. Extracts were diluted into 2-fold, starting from 800 µg/ml to 12.5 µg/ml. 50 µl of serially diluted extracts, 50 µl of mitogen (PHA 1:100 or PWM 1 µg/ml) and 100 µl of cells were added to flat-bottom 96-well plate (TPP, Switzerland) for each extract in triplicate wells. Plates were incubated in a 5% humidified CO_2 incubator (Shel Lab, USA) at 37°C for 48 h. After incubation, cell growth was quantitated with MTT by adding 50 µl of 1 mg/ml MTT solution to each well and the plates were again incubated for 4 h after which the plate was centrifuged. The supernatants were carefully removed and discarded by aspiration. 100 µl of 0.04 M HCl in isopropanol was added and mixed thoroughly. Shaking the plate with a plate shaker (ELMI/ ST3, USA) to dissolve the dark blue formazan crystals occurring from this reaction. The plates were read immediately on an ELISA reader (Bio-tek/ ELX 808, USA) using a test wavelength of 562 nm and reference wavelength of 620 nm. Experiments were repeated at least 3 replications for each extract. The data were expressed in term of lymphocyte proliferation index (PI).

$$PI = \frac{\text{mean absorbance of sample}}{\text{mean absorbance of control}}$$

Whereas control means the media without any extract and mitogen. Sample means the extract with and without mitogen.

Data and statistical analysis

The results were reported as mean ± standard error of mean (S.E.M). The data was compared to the control of each group (with and without mitogen). The differences were estimated by one-way ANOVA analysis, followed by Dunnett's Multiple Comparison Test. Significance level was set at $P<0.05$.

Results

Forty aqueous extracts were investigated for splenocyte proliferation with mitogens and without mitogens by colorimetric MTT assay. Mitogens were phytohemaglutinin (PHA) and pokeweed mitogen (PWM). T-lymphocytes proliferation was specifically activated by PHA (Nakamura et al., 1986) and B-lymphocytes proliferation was also specifically activated by PWM (Schreck et al., 1982).

Among these extracts, we selected only the extract having tendency to be an immunomodulator for further study. The first selection criterion was the extract giving high activity on lymphocyte proliferation in dose-dependence. The second criterion

was the extract with non-cytotoxic effect. Six medicinal plants showed interested results. (Figure 1-6) *Dendrophthoe pentadra* and *Croton cascarilloides* could stimulate lymphocyte proliferation both with and without mitogen in dose-dependence. They stimulated both T-lymphocytes and B-lymphocytes. *Bauhinia peniciloba* also stimulated lymphocyte proliferation both with and without mitogen. It gave higher activity in the presence of PHA than that of with PWM, suggesting specificity toward T cell proliferation. However, the extract at high concentration suppressed cell proliferation.

Crateva adansonii and *Chionanthus ramiflorus* both with and without mitogen could suppress lymphocyte proliferation in dose-dependence. They suppressed both T-lymphocytes and B-lymphocytes proliferation. It should be noted that the inhibitory effects observed in this study did not come from toxic effect of the plants. Because in each case the viability of cells was determined. *Polygala chinensis* also suppressed lymphocyte proliferation in dose-dependence both with and without mitogen. The concentration of plant extract with PWM gave higher activity than that of with PHA, suggesting specificity toward B cell proliferation more than T cell proliferation.

Table 1 List of plants screened for immunomodulating activity

Plants	Family	Part tested	Traditional uses	Yield(%w/w)*
Alyxia reinwardtii Blume	Apocynaceae	stem	hepatopathy	6.59
Ampelocissus martinii Planch	Vitaceae	rhizome	breast cancer	10.07
Ardisia helferiana Kurz	Myrsinaceae	bark, leaf	hepatopathy, tonic	6.58
Aristolochia pothieri Pierre ex Lecomte	Aristolochiaceae	rhizome	anti-aging	7.20
Bauhinia penicilliloba Pierre ex Gagnep.	Fabaceae	root	tonic, appetizer	3.33
Butea superba Roxb.	Fabaceae	stem	anti-aging, tonic	2.37
Canthium berberidifolium Geddes	Rubiaceae	root, stem	cirrhosis, hepatopathy	3.24
Capparis micracantha DC	Capparidaceae	stem	breast cancer	4.70
Chionanthus ramiflorus Roxb.	Oleaceae	stem	cancer	9.68
Crateva adansonii DC.	Capparidaceae	bark	cancer, anti-aging	5.79
Croton cascarilloides Raeusch.	Euphorbiaceae	root	fever, viral infection	3.25
Dendrophthoe pentandra (L.)Miq.	Loranthaceae	whole	vaginal cancer	7.89
Desmodium styracifolium (Osbeck)Merr.	Fabaceae	root, stem	fever, allergic dermatitis	5.18
Desmodium triflorum (L.)DC.	Fabaceae	whole	tonic	2.09
Dischidia major	Asclepiadaceae	bulb	hepatopathy	12.90
Dischidia nummularia R.Br.	Asclepiadaceae	bulb	hepatomegaly, cirrhosis	10.01
Echinochloa crus-galli (L.)Pal	Gramineae	whole	fever, dengue	15.35
Gnetum macrostachyum Hook.f.	Gnetaceae	stem	pain, inflammation	4.98
Gnetum montanum Markgr.	Gnetaceae	stem	fever, inflammation	11.15
Holarrhena pubescens Wall.ex G.Don	Apocynaceae	bark	wound, abscess	7.26
Hoya pachyclada Kerr	Asclepiadaceae	stem, leaf	hepatomegaly	7.65
Hoya parasitica (Roxb.) Wall. ex Traill	Asclepiadaceae	stem, leaf	hepatomegaly	7.99
Hymenocardia wallichii Tul.	Euphorbiaceae	stem	fever, chickenpox	3.64
Leptocarpus disjunctus	MastRestionaceae	whole	aphrodisiac, restorative	3.57
Molineria latifolia Herb.ex kurz	Hypoxidaceae	rhizome	anti-aging, tonic	8.74
Murdannia loriformis (Hassk.)R.S.Ras&Kammathy	Commelinaceae	whole	hepatomegaly	17.09
Myxopyrum smilacifolium Blume	Oleaceae	stem, leaf	liver cancer	9.59
Ophiopogon intermedius D.Don	Liliaceae	whole	tonic	4.55
Phyllodium longipes (Craib) Schindl.	Fabaceae	root	hepatopathy	6.22
Polygala chinensis L.	Polygalaceae	whole	aphrodisiac, restorative	6.92
Prismatomeris griffithii Ridl.	Rubiaceae	root	wound from snake bite	6.22
Salacia chinensis L.	Celastraceae	stem	hepatomegaly	3.73
Salacia verrucosa Wight	Celastraceae	stem	hepatomegaly	2.83
Scleropyrum wallichianum (Wight&Arn.)	Santalaceae	stem	tonic	9.01
Suregada multiflorum (A.Juss)Braill	Euphorbiaceae	stem	cancer	7.23
Tinospora crispa (L.) Miers ex Hook.f.&Thomson	Menispermaceae	stem	jaundice, anti-aging	8.55
Vanilla aphylla Blume	Orchidaceae	stem	hepatopathy	5.65
Vangueria spinosa Roxb.	Rubiaceae	stem	cancer	4.96
Xanthonnea parviflora (O.Ktze.)Craib	Rubiaceae	stem	AIDs	10.18
Zornia diphylla (L.)Pers.	Fabaceae	whole	tonic	10.18

* percentage of dried extracts after lyophilized compared with dried plant weights.

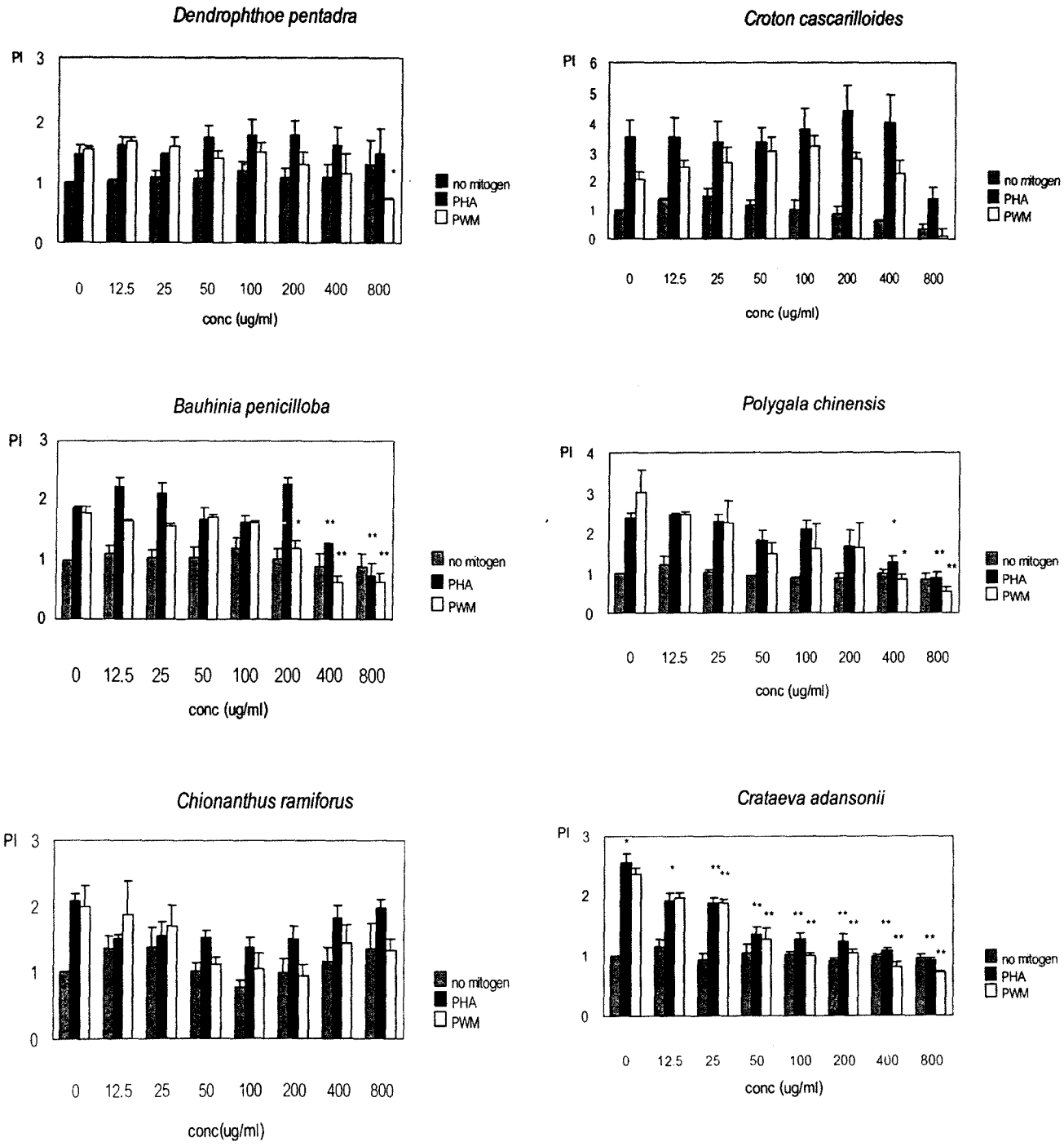

Figure 1-6 : Effects of six aqueous extracts on lymphocytes proliferation activity with and without Mitogens (PHA, PWM).

1 = *Dendrophthoe pentandra* 2 = *Croton cascarilloides*

3 = *Bauhinia penicilliloba* 4 = *Polygala chinensis*

5 = *Chionanthus ramiflorus* 6 = *Crateva adansonii*

* = $P < 0.05$, ** = $P<0.01$

Discussion

New scientific strategies for the evaluation of natural products with biological activity required the implementation of large-scale screening programs. Colorimetric MTT assay was used for assessment of splenocytes proliferation since the cleavage of MTT has several desirable properties for assaying cell survival and proliferation. MTT is cleavaged by all living, metabolically active cells and the amount of MTT formazan generated is directly proportional to the cell number (Mossmann, 1983). This assay can permit to evaluate dose-dependent-effect.

Lymphocytes are the principal cells of the immune system involving in health and disease. They comprise of two major types: B-lymphocytes and T-lymphocytes. B-lymphocytes, the cells of the humoral immunity, recognize specific antigens on the surface of extracellular pathogenic organisms through surface immunoglobulin and differentiate into plasma cells, which can produce large quantity of specific antibody that can bind to the pathogen and their toxins, signaling its degradation by macrophages and other cells. T-lymphocytes, the cells of the cellular immunity, have a wide range of activities. Some are involved in the control of B-cells development and antibody production. Another group of T-cells interacts with phagocytic cells to help them destroy pathogens they have taken up. A third set of them directly attack antigens such as viruses, fungi, cancer or transplanted tissues, and act as regulators of the immune system to eliminate intracellular pathogens (Roitt, 2000; Abbas, 2003; Janeway, 2005).

The obtained results are correlated to the knowledge on traditional medicine using by folklore people in the northeastern part of Thailand. D. *pentadra* (Kafak noina) had been used in vaginal cancer treatment. C. *cascarilloides* (Plaunamgnern) is used for viral infection and fever. B. *peniciloba* (Seawdang) is used as a tonic and appetizer. All of these plants exhibit immunostimulating activity for splenocyte proliferation in mice. These results supported the remedies of herbal medicine practitioners.

The findings that C. *adansonii* (Kam) and C. *ramiflorus* (Plumalee) exhibiting immunosuppressant activity also supported the application of these plants in the folklore remedies of cancer since some kinds of cancer needed immunosupressant effects (Labadie et al., 1989). Moreover, P. chinensis (Ma-e-kum) used for increasing aphrodisiac and restorative power, showed immunosuppressant activity.

Conclusion

The present study revealed the immunomodulating activity on lymphocyte proliferation in mice of six plant aqueous extracts, which could explain the traditional use of these plants in human. Among these extracts, three plants displayed immunostimulating activities: *D. pentandra, C. cascarilloides* and *B. penicilliloba.* The others showed potential to be immunosuppressors: *C. adansonii, P. chinensis, C. ramiflorus.* Further study on the effects of these plants on the expressions of various factors of the immune system such as complement, polymorphonuclear leukocytes,

cytokines, macrophages is suggested. These are necessary for the clinical treatment of immune diseases. Furthermore, these preliminary observations suggest further investigations such as chemical isolation & identification, formulation, clinical trials for future discovery of new immunomodulating drugs from plant origins which might have the potential to treat chronic immune disease with less side-effect.

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References

Abbas, K.; Lichtman, A. 2003. Cellular and molecular immunology. fifth ed. Saunders, Philadelphia, USA, pp.16-40.

Atal, C.K.; Sharma, M.L.; Kaul, A.; Khajuria, A. 1986. Immunomodulating agents of plant origin I: Preliminary screening. Journal of Ethnopharmacology. 18: 133-41.

Brousseau, P; Payette, Y.; Tryphonas, H.; Blackley, B.; Boermans, H.; Flipo, D.; Fournier, M. 1998. Assessment of cell viability. In: Manual of Immunological methods. CRC Press, USA, pp.27-28.

Diasio, R.B.; LoBuglio, A.F. 1996. Immunomodulators: Immunosuppressive agents and Immunostimulants. In: Goodman and Gilman's (Eds.), The Pharmacological Basis of Therapeutics, ninth ed. McGraw-Hill, New York, pp.1291-1307.

Janeway, C.; Travers, P. Walport, M.; Shlomchik, M. Immunobiology : the immune system in health and disease. Sixth ed., Garland Science Publishing, UK, pp.1-102.

Gerlier, D.; Thomasset, N. 1986. Use of MTT colorimetric assay to measure cell activation. Journal of Immunological Methods. 94: 57-63.

Hansen, M.B.; Neilsen, S.E.; Berg, K 1989. Re-examination and future development of a precise and rapid dye method for measuring cell growth/ cell kill. Journal of Immunological Methods. 119: 203-10.

Labadie, R.P.; van der Nat, J.M.; Simons, J.M.; Kroes, B.H.; Kosasi, S.; van den Berg, A.J.; Hart, L.A.; van der Sluis, W.G.; Abeysekera, A. and Bamunuarachchi, A. 1989. An ethnopharmacognostic approach to the search for immunomodulators of plant origin. Planta Medica. 55: 339-48.

Masihi, N. 2000. Immunomodulatory agents for prophylaxis and therapy of infections. International Journal Antimicrobial Agents. 14: 181-91.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 65:55-63.

Nakamura, A.; Nagai, K.; Suzuki, S.; Ando, K.; Tamura, G. 1986. A novel method of screening for immunomodulating substances, establishment of an assay system and its application to culture broth of microorganisms. Journal of Antibiotics. 8: 1148-54.

Neelam, M.; Subhash, B.; Vinod, R. 2001. Immunomodulatory activity of alcoholic extract of Mangifera indica L. in mice. Journal of Ethnopharmacology. 78: 133-37.

Patwardhan, B.; Kalbag, D.; Patki, P.S.; Nagsampagi, B.A. 1990. Search of Immunomodulatory agents: a review. Indian Drugs. 28 (2): 348-58.

Prasitpuriprecha, C.; Sripanidkulchai, B.;Lulitanond, V.; Jaratbhan, S. 2005. Studies on the utilization of medicinal plants as immuno-modulators in Ubon Ratchathani Province, Thailand. KKU Research Journal. 10(1): 1-11.

Roitt, I.; Brostoff, J.; Male, D. 2000. Immunology, fifth ed. Mosby, London, pp.1-24.

Schreck, C.; Lamberson, H.; Davey, F. 1982. Characterization of the B lymphocytes response to pokeweed mitogens. Annals of Clinical and Laboratory Science. 12(6): 455-62.