The Thai Journal of Veterinary Medicine

Volume 45
Issue 3 *September, 2015*

Article 6

9-1-2015

In Vitro Screening of Crude Extracts from Thai Plants in the Moraceae and Leguminosae Families on Proliferation of Swine Lymphocyte and Maturation of Monocyte-Derived Dendritic Cell

Thaniwan Cheun-Arom

Tullaya Pungchaipat

Dachrit Nilubol

Pornpen Werawatganone

Boonchoo Sritularak

See next page for additional authors

Follow this and additional works at: https://digital.car.chula.ac.th/tjvm

 Part of the Veterinary Medicine Commons

Recommended Citation

Cheun-Arom, Thaniwan; Pungchaipat, Tullaya; Nilubol, Dachrit; Werawatganone, Pornpen; Sritularak, Boonchoo; and Tantituvanont, Angkana (2015) "In Vitro Screening of Crude Extracts from Thai Plants in the Moraceae and Leguminosae Families on Proliferation of Swine Lymphocyte and Maturation of Monocyte-Derived Dendritic Cell," *The Thai Journal of Veterinary Medicine*: Vol. 45: Iss. 3, Article 6.
Available at: https://digital.car.chula.ac.th/tjvm/vol45/iss3/6

This Article is brought to you for free and open access by the Chulalongkorn Journal Online (CUJO) at Chula Digital Collections. It has been accepted for inclusion in The Thai Journal of Veterinary Medicine by an authorized editor of Chula Digital Collections. For more information, please contact ChulaDC@car.chula.ac.th.

In Vitro Screening of Crude Extracts from Thai Plants in the Moraceae and Leguminosae Families on Proliferation of Swine Lymphocyte and Maturation of Monocyte-Derived Dendritic Cell

Authors

Thaniwan Cheun-Arom, Tullaya Pungchaipat, Dachrit Nilubol, Pornpen Werawatganone, Boonchoo Sritularak, and Angkana Tantituvanont

This article is available in The Thai Journal of Veterinary Medicine: https://digital.car.chula.ac.th/tjvm/vol45/iss3/6

Original Article

In Vitro Screening of Crude Extracts from Thai Plants in the Moraceae and Leguminosae Families on Proliferation of Swine Lymphocyte and Maturation of Monocyte-Derived Dendritic Cell

Thaniwan Cheun-Arom[1] Tullaya Pungchaipat[2] Dachrit Nilubol[1] Pornpen Werawatganone[2]

Boonchoo Sritularak[3] Angkana Tantituvanont[2,4*]

Abstract

In this study, nine crude extracts from Thai plants in the Moraceae and Leguminosae families were screened for their immunoadjuvant activities on the proliferation of swine lymphocytes and the maturation of monocyte-derived dendritic cells (MoDCs). The study demonstrated that the total phenolic content was significantly higher in the extract from *Artocarpus gomezianus* (264.17±6.5 mg of gallic acid equivalents/gm of extract) than in the extracts from the other plants. The crude extract from the root bark of *A. gomezianus* significantly increased the proliferative response of peripheral blood mononuclear cells measured using both MTT and CFSE assays ($p < 0.05$). In addition, the *A. gomezianus* extract induced the maturation of MoDCs through major histocompatibility (MHC) Class II and CD80 expression. Constituents of phenolic compounds, in particular tannins and flavonoids in the γ-benzopyrone nucleus group, may be important for the immunoadjuvant effect. The results of this study suggest that the extract from *A. gomezianus* have immunoadjuvant activities that may be developed as vaccine adjuvants.

Keywords: *Artocarpus gomezianus*, immunoadjuvant, monocyte-derived dendritic cells, peripheral blood mononuclear cells, phenolic compounds

[1]*Department of Veterinary Microbiology, Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand*

[2]*Department of Pharmaceutics and Industrial Pharmacy, Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*

[3]*Department of Pharmacognosy and Pharmaceutical Botany, Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*

[4]*Cell-based Drug and Health Product Development Research Unit, Chulalongkorn University, Bangkok 10330, Thailand*

***Correspondence**: tuvanont@gmail.com*

Introduction

Vaccination is one of the most effective ways to induce an immune response to protect the body from diseases. However, although the component that induces immunity in vaccine is a pathogen that claims not to cause the disease after its administration into the body, such substances raise safety concerns in consumers. New generation vaccines, such as subunit, synthetic protein, recombinant and DNA vaccine, have been shown to alleviate the potential problem of back-reversion of the vaccine to virulence. The drawback is that such vaccines have poor immunogenicity and require the use of an adjuvant to accelerate the immune response to the antigen.

An adjuvant is a substance that is added to vaccine preparation to enhance the body's immune response to the pathogen, generally referred to as the antigen, towards the desired immune response. The adjuvant helps to stimulate immune responses through various mechanisms, including extending the antigen deposition time, sustaining the release of antigen from the depot and inducing irritation at the injection site (Coffman et al., 2010; Del Giudice et al., 2001; Fischer et al., 2007; Schijns, 2000). Some adjuvants function directly on the immune cells by targeting the antigen presenting cells (APCs), enhancing the expression of both co-stimulatory molecules and major histocompatibility (MHC) Class II on the surface of APCs, inducing the release of cytokine from lymphocytes and APCs (Cox and Coulter, 1997; Foged et al., 2002) and promoting dendritic cells migration to the T cell area of the lymph nodes (Foged et al., 2002; Martín-Fontecha et al., 2003). Most of the adjuvants available on the market can stimulate the humoral immune response leading to pathogen-specific antibody production, but lack the ability to stimulate the cell-mediated immune response (Reed et al., 2009). A cell-mediated immune response is important to combat infected or abnormal cells such as cancer or tumor cells. Although some adjuvants can stimulate cell-mediated immune responses, these adjuvants usually cause many side effects such as fever, allergy and fatigue (Gupta, 1998; Minutello et al., 1999; Asa et al., 2000; Podda, 2001). Therefore, it is necessary to find new adjuvants that are safe and can stimulate both humoral and cell-mediated immune responses.

The wide use of plant-derived remedies in both traditional and modern medicine has made medicinal plants an interesting source of new therapeutics. Recently, there has been increased attention on finding new adjuvants from plants containing phenolic compounds. Several plant phenolic compounds were reported to enhance the proliferative response of lymphocytes (Brattig et al., 1984); induce the activation of lymphocytes and macrophages; increase antibody production (Brattig et al., 1984; Kong et al., 2004); promote the secretion of the inflammatory cytokines IL-1β, TNF-α, and IL-6 (Barak et al., 2001); increase the mRNA expression of IL-1β, IL-6, iNOS, and TNF-α (Johnson et al., 2003); and stimulate the expression of the IFN-γ coding gene (Nair et al., 2002; Chauhan et al., 2010).

Medicinal plants that contain phenolic compounds are commonly found in the Moraceae and Leguminosae families (Barron and Ibrahim, 1996); within these families, immunoadjuvant properties have been found in *Acacia catechu*, *Artocarpus integrifolia*, *Ficus carica*, *Ficus bengalensis*, *Morus alba*, *Pachyrhizus erosus* and *Pongamia glabra* (Kabir, 1998; Albuquerque et al., 1999; Rosa et al., 1999; Kim et al., 2000; Ismail and Asad, 2009; Venkatachalam et al., 2009; Patil et al., 2010; Sharma et al., 2011; Heroor et al., 2012; Kumalasari et al., 2014). Extracts from these plants were shown to induce both humoral and cell-mediated immunity (Ismail and Asad, 2009; Patil et al., 2010; Sharma et al., 2011); activate the complement system, induce lymphocyte proliferation, stimulate macrophages (Patil et al., 2010), enhance humoral antibody (HA) titers and increase total leukocyte counts (TLC) and differential leukocyte counts (DLC) (Singh et al., 2011). Plants in the Moraceae and Leguminosae families that are very common in Thailand include *Artocarpus altilis*, *Broussonetia papyriferra*, *Morus alba*, *Derris reticulata*, *Derris scandens*, *Erythrina variegata*, *Pithecolobium dulce*, *Artocarpus gomezianus* and *Dalbergia cultrata.* Investigating the effects of these plants on the proliferation of peripheral blood mononuclear cells (PBMCs) and the maturation of monocyte-derived dendritic cells (MoDCs) may lead to the discovery of new adjuvants that can be utilized in future vaccine production and also add value to Thai plants. Therefore, the objective of the study was to investigate Thai herbal plants in the Moraceae and Leguminosae families for their immunoadjuvant activities on the proliferation of swine PBMCs and the maturation of MoDCs.

Materials and Methods

Plant materials: Nine plants in the Moraceae and Leguminosae families, whose local Thai and scientific names are listed in Table 1, were collected and investigated for their phenolic content and immunoadjuvant activities. Authentication of these nine plants was performed by comparison with the herbarium specimens at the Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environment, Bangkok, Thailand. Crude extracts were obtained from these plants using maceration and evaporation techniques. Briefly, the dried plants were ground into coarse powder. The coarse powder was macerated with methanol for 3 days at room temperature and filtered through filter paper (Whatman No. 1). The filtrates were dried in a rotary evaporator (Buchi, Flawil, Switzerland). The crude extracts were kept at -20°C until used.

Total phenolic content assay: The total phenolic content was determined by spectrophotometry. The amount of total phenolic compound in the crude extracts was determined using the Folin-Ciocalteu method (Javan et al., 2003). In brief, 50 µl of each crude extract solution (5 mg/ml in dimethylsulfoxide or DMSO) was transferred into 15 ml test tubes, 2.5 ml of a 10% (v/v) Folin-Ciocalteu reagent was added and the resulting solution was mixed thoroughly. After 5 min, 2 ml of 7.5% (w/v) sodium carbonate was added and mixed. The obtained mixture was incubated in the dark

for 15 min at 45°C before the absorbance was read at 765 nm using UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). Gallic acid was used as the standard and the total phenolic content was expressed as mg/g gallic acid equivalents (GAE). The standard concentrations of gallic acid were 0.2-0.8 mg/ml in DMSO. All the experiments were performed in triplicate.

Preliminary phytochemical studies: The crude extract from the root bark of *A. gomezianus* was used for preliminary phytochemical tests. Preliminary phytochemical testing for the presence of various compounds by standard methods like shinoda test, pew test and anthocyanins test for flavonoids (Peach and Tracey, 1959; Khandelwal, 2000), Mayer's test, Marme's test and Dragendorff's test for alkaloids (Wagner et al., 1996), and ferric chloride test and lead acetate test for tannins (Trease and Evans, 1978) was conducted (Sharanabasappa et al., 2007).

Table 1 Total phenolic content of crude extracts

Local name	Scientific name	Family name	Part	Content (mg GAE/g)
Sake	*Artocarpus altilis* (Parkinson) Fosberg	Moraceae	Stem	48.3±4.5[a]
Hat nun	*Artocarpus gomezianus* Wall. ex Trécul	Moraceae	Root bark	264.2±6.5[e]
Po krasa	*Broussonetia papyriferra* (L.) Vent.	Moraceae	Root bark	92.9±3.1[c]
Mon	*Morus alba* L.	Moraceae	Root bark	190.7±6.2[d]
Kra phi khao khwai	*Dalbergia cultrata* Graham ex Benth.	Leguminosae	Heartwood	173.2±2.0[d]
Cha em nuea	*Derris reticulata* Craib	Leguminosae	Stem	78.5±5.6[b]
Thao wan priang	*Derris scandens* (Roxb.) Benth.	Leguminosae	Stem	163.5±7.3[d]
Thong lang lai	*Erythrina variegate* L.	Leguminosae	Stem bark	106.6±6.8[c]
Ma kham thet	*Pithecolobium dulce* (Roxb.) Benth.	Leguminosae	Stem	77.4±2.0[b]

Isolation of porcine peripheral blood mononuclear cells: Blood samples were collected from the same three pigs throughout this study. PBMCs were isolated from whole blood samples by density gradient centrifugation as previously described (Nilubol et al., 2004). In brief, the blood sample was diluted with an equal volume of phosphate buffer saline (PBS). The diluted blood sample was overlaid on Isoprep (Robbins Scientific, CA, USA) and centrifuged at 400 x g for 30 min at 20°C. The lymphocyte layer was transferred into a new test tube, washed twice with PBS and resuspended in a complete medium; advanced RPMI 1640 media (Invitrogen, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco®, MD, USA), 1% (v/v) L-glutamine (Invitrogen, CA, USA), 1% (v/v) Antibiotic/Antimycotic solution (Sigma-Aldrich®, MO, USA), 2.5% (v/v) HEPES (Gibco®, MD, USA) and 0.1% (v/v) 2-mercaptoethanol (Gibco®, MD, USA). PBMCs were used to determine cytotoxic and proliferation effects of the crude extracts using MTT and Carboxyfluorescein succinimidyl ester (CFSE) assays, respectively.

MTT cytotoxicity assay: The effects of nine crude extracts on the viability of PBMCs were determined using a colorimetric technique, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as previously described (Yeap et al., 2007). In brief, 100 µl of a suspension of 5 x 10^5 cells/ml of PBMCs was seeded in a flat-bottomed 96-well culture plate and cultured for 24 hr at 37°C, 5% CO_2 in a humidified atmosphere. At approximately 70% cell confluence, the PBMCs were treated with the crude extracts at final concentrations of 1, 10, 50 and 100 µg/ml and incubated at 37°C, 5% CO_2 in a humidified atmosphere for 3 days. Following 3 days of incubation, 20 µl of an MTT reagent (Sigma-Aldrich®, MO, USA) at 5 mg/ml was added into each well; the cell cultures were further incubated for 4 more hours. After 4 hours, the medium and MTT solution was removed from every well and 100 µl DMSO (Fisher Scientific, LE, UK) was then added into each well to dissolve formazan crystals. Finally, the culture plate was read at 570 nm using a microplate reader (Perkin Elmer, MA, USA). Cells treated with Phytohemagglutinin (PHA, 5 µg/ml) and cells treated with 0.5% DMSO in complete medium were used as the positive control group and the control group, respectively. The experiments were performed in triplicate. Relative percentage of cell survival was calculated by dividing the absorbance of treated cells by that of the control group in each experiment. Crude extracts that demonstrated no toxicity to PBMCs were further tested for lymphocyte proliferative effects using the CFSE dye dilution assay.

CFSE lymphocyte proliferation assay: The proliferative effect of the crude extracts on PBMCs was performed using CFSE staining, as previously described (Parish and Warren, 2002). In brief, 1 × 10^7 cells/ml of PBMCs in sterile PBS were transferred to a 15 ml test tube, labeled with 2.5 µM CFSE (Invitrogen, CA, USA) in PBS and incubated for 5 min at room temperature with periodic mixing. The reaction was stopped by addition of 0.5 ml fetal bovine serum (FBS). The PBMCs were washed once with sterile PBS, then washed again with the complete medium. The CFSE-labeled PBMCs were seeded in a 96-well flat-bottomed culture plate at density of 5 x 10^5 cells/well in 200 µl of 0.5% DMSO in complete medium (the control group),

5 µg/ml PHA (the positive control group), or each of the crude extract (1, 10, 50 and 100 µg/ml, the treatment groups). The treated cultures were further incubated at 37°C in a 5% CO_2 humidified atmosphere for 3 consecutive days. Lymphocyte proliferation was determined by flow cytometric analysis (FACS Calibur™, CA, USA). Relative proliferative index was calculated by dividing the percentage of proliferating cells in the treatment group by that of the control group in each experiment. Crude extracts that significantly enhanced the proliferation of PBMCs were selected for further investigation into the maturation of porcine MoDCs.

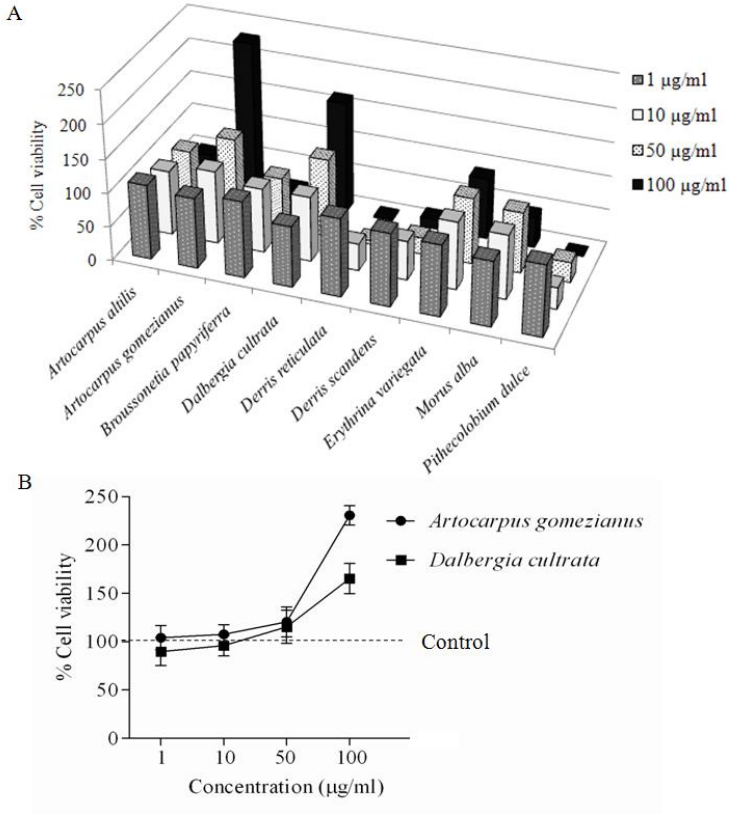

Figure 1 Cell viability was analyzed using MTT assay. (A) Effects of 1, 10, 50, and 100 µg/ml of nine crude extracts on PBMC cytotoxicity. (B) Percentage of viable PBMC on *Artocarpus gomezianus* and *Dalbergia cultrata* treatment.

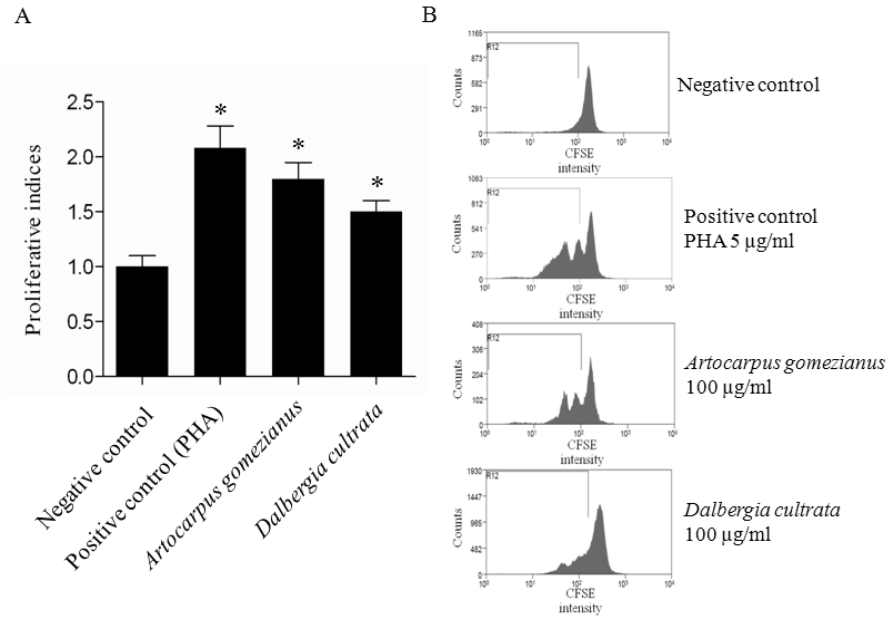

Figure 2 Proliferation of PBMCs was determined by using CFSE dye. (A) Proliferative effect of 100 µg/ml of crude extracts of *Artocarpus gomezianus* and *Dalbergia cultrata* on porcine PBMCs, compared to 5 µg/ml of phytohemagglutinin (PHA). (B) Flow cytometric histograms of CFSE stained PBMCs. * $p < 0.05$ versus non-treated control.

Generation of porcine monocyte-derived dendritic cells: Porcine MoDCs were generated from PBMCs as previously described (Dauer et al., 2003). Briefly, PBMCs at density of 1×10^7 cells/ml were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% FBS (Gibco®, MD, USA), seeded in a 6-well culture plate (Corning, NY, USA) and incubated at 37°C in a humidified atmosphere at 5% CO_2 for 2 hr to allow monocytes adherence. Peripheral blood lymphocytes (PBLs) were then removed and the adhered monocytes were washed gently twice with PBS. The monocytes were cultured in advanced complete RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (Gibco®, MD, USA), 1% (v/v) Antibiotic/Antimycotic (Sigma-Aldrich®, MO, USA), 50 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 ng/ml of recombinant human interleukin-4 (IL-4) (R&D systems®, MN, USA) and incubated at 37°C in a humidified atmosphere at 5% CO_2 for 48 hr to generate immature MoDCs. The immature MoDCs were then harvested, resuspended in the complete medium, and plated into a 24-well culture plate at a concentration of 5×10^5 cells/ml. Quality of the MoDCs generated was monitored visually using microscopy and by flow cytometric comparisons of the expression level of CD80 and MHC Class II molecules on these MoDCs and freshly isolated monocytes.

Determination of monocyte-derived dendritic cell maturation by flow cytometry: The crude extracts selected from the CFSE dye dilution assay were tested for their effects on the maturation of MoDCs. MoDCs are a major component of the cellular immune system and are highly potent in the induction of specific immune responses by using major histocompatibility Class II (MHC II) and costimulatory molecules (CD80, CD86, etc.) which are upregulated in mature MoDCs. To detect the expression of MHC Class II and CD80, immature MoDCs at density of 5×10^5 cells/ml were resuspended in the complete medium and cultured in a 24-well flat-bottomed culture plate with 100 µg/ml crude extracts. Ten µg/ml of lipopolysaccharide (LPS) and the complete medium were used as the positive control group and the control group, respectively. The cells were incubated at 37°C in a humidified atmosphere at 5% CO_2 for 48 hr and then harvested for immunophenotyping. The harvested cells were stained with 1:50 of anti-human CD80 PE (BD Biosciences, CA, USA) and 1:50 of mouse (IgG2a) anti-pig SLA-DR (BD Biosciences, CA, USA) as the primary antibody and incubated at 4°C in the dark for 30 min. Subsequently, the cells were stained with 1:100 of secondary conjugate, goat anti-mouse IgG2a FITC (Invitrogen, CA, USA), diluted in FACS buffer and incubated at 4°C in the dark for 30 min. Washing between each staining step was performed by adding 200 µl of FACS buffer. In the final state, the pellet was resuspended in 400 µl of 2% formaldehyde to fix the MoDCs. Analysis of the expression level of surface antigens was performed by flow cytometry (BD Biosciences, CA, USA). Relative fluorescence intensity of MHC Class II and CD 80 after incubation of the MoDCs with crude extracts in comparison to incubation with LPS was reported.

Statistical analysis: All data were expressed as the mean ± standard deviation of three independent experiments. Difference in the mean values between the groups was analyzed by a one-way analysis of variance (ANOVA) for randomized block design. Homogeneity of variance were analyzed by Levene's test followed by a post-hoc test using the Dunnett test in the case of unequal variance or the Tukey HSD test in the case of equal variance. A $p < 0.05$ was considered statistically significant.

Results

The total phenolic content of the nine crude extracts is shown in Table 1. The amount of phenolic compound in all nine crude extracts varied widely, ranging from 48.3±5.3 to 264.2±6.5 mg of GAE/gm of extract. Crude extracts from *Artocarpus gomezianus* had significantly higher levels of total phenolic compound compared to the other eight crude extracts (264.17±6.5 mg GAE/gm extract). The next highest levels of total phenolic compound were found in the extracts from *Morus alba*, *Dalbergia cultrata* and *Derris scandens* (190.7±6.2, 173.2±2.0 and 163.5±7.3 mg GAE/gm extract, respectively). In contrast, *Artocarpus altilis* had the lowest level of total phenolic compound (48.3±4.5 mg GAE/gm extract).

The effects of the nine crude extracts on the viability of PBMCs are shown in Figure 1. PBMCs were exposed to 1, 10, 50, and 100 µg/ml of the crude extracts for 72 hr in normal culturing condition. In the MTT assay, seven crude extracts, i.e. extracts from *Artocarpus altilis*, *Broussonetia papyriferra*, *Morus alba*, *Derris reticulata*, *Derris scandens*, *Erythrina variegata*, and *Pithecolobium dulce*, decreased cell viability in a concentration-dependent manner compared to the control group (PBMCs incubated with 0.5% DMSO in complete medium). Interestingly, the PBMCs treated with *A. gomezianus* and *D. cultrata* at 100 µg/ml showed 230% and 165%, respectively, increase in the cell viability compared to the control group ($p < 0.05$), showing the potential of these two crude extracts to enhance lymphocyte proliferative responses. Non-toxic concentrations of all nine crude extracts were chosen to investigate the ability of the extracts to stimulate PBMCs proliferation using the CFSE dye dilution assay.

PBMCs were treated with the non-toxic concentrations of all nine crude extracts and cell proliferation was measured using the CFSE staining assay. Only the extracts from *A. gomezianus* and *D. cultrata*, at the concentration of 100 µg/ml significantly enhanced the proliferation of PBMCs compared to the control group ($p < 0.05$). The proliferative index of cell treated with *A. gomezianus* and *D. cultrate* were 1.8 times and 1.5 times, respectively, higher than that of the control group (Fig 2A). The other seven crude extracts from *A. altilis*, *B. papyriferra*, *M. alba*, *D. reticulata*, *D. scandens*, *E. variegata* and *P. dulce* did not induce PBMC proliferation at any of the concentrations tested (data not shown). Flow cytometric analysis also confirmed that the crude extracts from *A. gomezianus* and *D. cultrata* could stimulate the proliferation of PBMCs as observed by the progressive halving of CFSE

fluorescence within daughter cells following cell division (Fig 2B). Therefore, the 100 µg/ml of crude extracts of *A. gomezianus* and *D. cultrata* were further investigated for their ability to induce MoDCs maturation, as dendritic cell maturation is a key event in regulating and amplifying the antigen specific immune response.

The characteristics of the MoDCs were confirmed by the appearance of dendrite-like cells using microscopy in conjunction with the expression of co-stimulatory molecules using flow cytometry. After the incubation of monocytes with IL-4 and GM-CSF for 48 h, the appearance of the monocytes changed from a rounded shape to a dendritic shape (Fig 3A), which is a unique characteristic of dendritic cells. Moreover, flow cytometric immunophenotyping showed that the expression level of MHC Class II and CD 80 was higher in the MoDCs than in the monocytes (Fig 3B), as expected in dendritic cells.

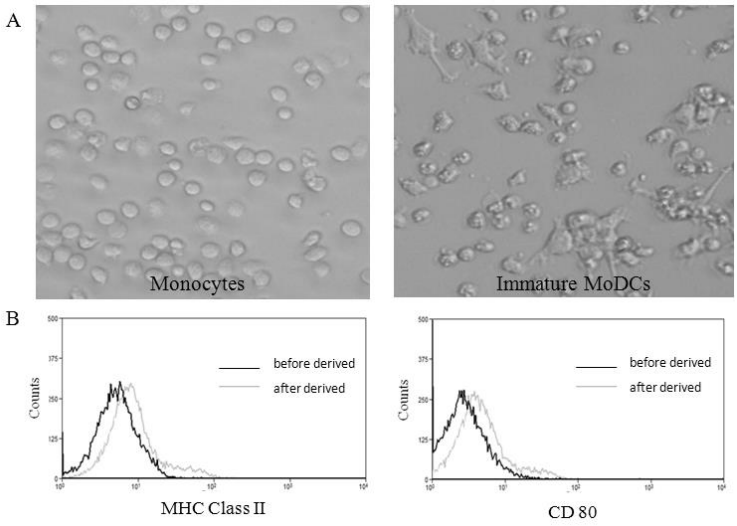

Figure 3 Cell viability was analyzed using MTT assay. (A) Effects of 1, 10, 50, and 100 µg/ml of nine crude extracts on PBMC cytotoxicity. (B) Percentage of viable PBMC on *Artocarpus gomezianus* and *Dalbergia cultrata* treatment.

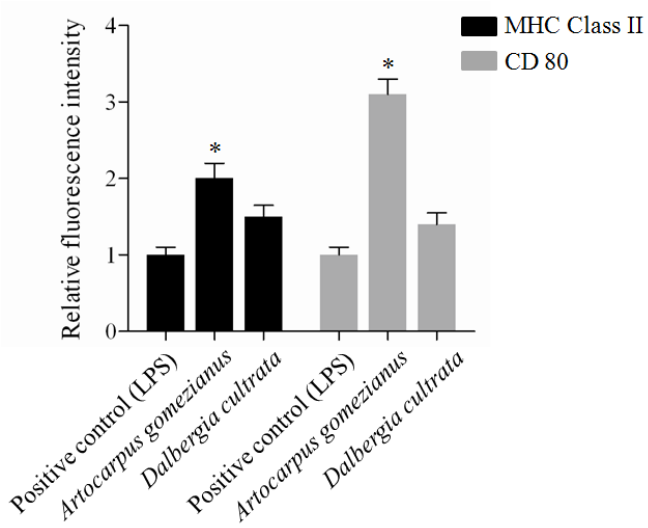

Figure 4 Relative fluorescence intensity of MHC Class II and CD80 expression on surface of MoDCs incubated with crude extracts of *Artocarpus gomezianus* and *Dalbergia cultrata*. * $p < 0.05$ versus lipopolysaccharide (LPS) as a positive control.

Crude extracts from both *A. gomezianus* and *D. cultrata* were tested for their ability to induce the expression of MHC Class II and CD80 on the surface of MoDCs. The immature MoDCs incubated with 100 µg/ml of the crude extract from *A. gomezianus* showed an increase in the number of MHC Class II and CD80 molecules compared to the positive control group (MoDCs treated with 10 µg/ml LPS). The relative fluorescent intensity of MHC Class II and CD80 expressed in PBMCs treated with *A. gomezianus* were 2 times and 3 times higher than that of the control group ($p < 0.05$, Fig 4). Although the expression level of MHC Class II and CD80 surface antigen was slightly increased after the incubation of MoDCs with the crude extract from *D. cultrata* (Fig 4), the effect was not statistically significant when compared to the positive control group.

Discussion

Immunomodulatory substances can stimulate, suppress, or modulate any aspect of the

immune system, including both the adaptive and the innate arms of the immune system. Immunomodulators can be classified into three categories: immunoadjuvants, which enhance the efficacy of vaccines; immunostimulants, which induce activation of the immune response; and immunosuppressants, which are used to treat various types of organ transplant rejection and autoimmune diseases (Sharma et al., 2011; Kumar et al., 2012). Several methods, including antibody production assays, lymphocyte proliferation assays, phagocytic activity assays and assays to measure the maturation of antigen presenting cells have been used to investigate immunomodulators (Chen et al., 2012; Li et al., 2012; Wang et al., 2012). Phenolic compounds, commonly found in many plants, including those from the Moraceae and Leguminosae families, have multiple biological effects, including immunomodulatory properties. This study investigated the immunoadjuvant properties of nine Thai herbal plants in the Moraceae and Leguminosae families on the proliferation of PBMCs and the maturation of MoDCs.

The results of the study demonstrated that two of the nine crude extracts, extracts from the root bark of *A. gomezianus* and the heartwood of *D. cultrata*, showed immunoadjuvant potential. The immunoadjuvant activities of the crude extract of *A. gomezianus* was greater than that of the crude extract of *D. cultrata*. Both crude extracts from *A. gomezianus* and *D. cultrata* could significantly increase the viability and the proliferation of PBMCs. However, only the crude extract from the root bark of *A. gomezianus* could induce the maturation of dendritic cells. The *A. gomezianus* extract increased the expression of MHC Class II and CD80 by 50% compared to the control group, whereas the *D. cultrata* extract had no significant effect on the expression of MHC Class II and CD80.

As expected, high total phenolic content in the extracts of *A. gomezianus and D. cultrata* corresponded to their ability to stimulate PBMCs proliferation and dendritic cells maturation. *D. cultrata and A. gomezianus* are in the Leguminosae and Moraceae families, respectively. Many plants in both families have been reported to have immunomodulatory activities both in vitro and in vivo (Kim et al., 2000; Ismail and Asad, 2009; Patil et al., 2010) and it is hypothesized that their phenolic compounds are the main constituents responsible for this immunomodulation. Although the crude extracts from *Morus alba* and *Derris scandens* showed high phenolic content, these extracts were not included in the study due to their toxicity and limited ability to increase the number of PBMCs observed in the MTT assay.

This is the first study to demonstrate the effect of crude extracts from the heartwood of *D. cultrata* and the root bark of *A. gomezianus* on the proliferation of PBMCs and to show the stimulatory effect of the extract from the root bark of *A. gomezianus* on MoDCs. The phenolic compounds currently identified in *D. cultrata* are neoflavanoids and dalberatins A and B, all classified as flavonoids (Donnelly et al., 1972). Thus, flavonoids may be the main compounds responsible for the proliferative effect of *D. cultrata* crude extract on PBMCs. There are many phytochemical reports on other parts of *A. gomezianus* such as the heartwood (Likhitwitayawuid et al., 2006), the stem bark (Sritularak et al., 2010), the root (Likhitwitayawuid et al., 2000; Prashanth et al., 2014), fruit and aerial parts (Prashanth et al., 2014). However, there are no reports on the secondary metabolites from the root bark of this plant, the immunoadjuvant compounds in the root bark of *A. gomezianus* are therefore unknown. Our preliminary phytochemical screening of the extract from the root bark of *A. gomezianus* showed the presence of tannins by lead acetate, 1% ferric chloride and vanillin-HCl tests and flavonoids in the γ-benzopyrone nucleus group such as flavones, flavanones and dihydroflavonols by the shinoda test. In addition, it was reported that one of the components in the root of *A. gomezianus* was in the group of stilbene compounds (Likhitwitayawuid and Sritularak, 2001; Hakim et al., 2002). Stilbene compounds such as trans-3,5,4′-trihydroxystilbene (resveratrol) found in the root of *A. gomezianus* have been reported to be ligands for Toll-like receptor 4. Activation of Toll-like receptor 4 leads to the maturation of dendritic cell (Thomas et al., 2003; Yusuf et al., 2009). Thus, these groups of compounds may also be found in the root bark of *A. gomezianus* and may be responsible for the proliferation of PBMCs and the maturation of dendritic cells observed in this study. However, the evidence supporting which constituents in the crude extracts are responsible for the immunoadjuvant activity is ambiguous; further studies of the chemical constituents of crude extract from the root bark of *A. gomezianus* are needed.

Based on the results presented in this study, the crude extracts from the root bark of *A. gomezianus* possessed immunoadjuvant properties that caused the proliferation of PBMCs and the maturation of monocyte-derived dendritic cells. Thus, the extracts from the root bark of *A. gomezianus* may potentially be used as an adjuvant in vaccine formulations.

Acknowledgements

This work was supported by the National Research Universities and a postdoctoral fellowship (Ratchadaphiseksomphot endowment fund), Chulalongkorn University. We also thank Mr. N. Eiamsa-ard, Immunology Laboratory, Faculty of Dentistry, Chulalongkorn University, for his insight into the flow cytometry analysis.

References

Albuquerque DA, Martins GA, Campos-Neto A and Silva JS 1999. The adjuvant effect of jacalin on the mouse humoral immune response to trinitrophenyl and *Trypanosoma cruzi*. Immunol Lett. 68: 375-381.

Asa PB, Cao Y and Garry RF 2000. Antibodies to squalene in Gulf War syndrome. Exp Mol Pathol. 68: 55-64.

Barak V, Halperin T and Kalickman I 2001. The effect of sambucol, a black elderberry-based, natural product, on the production of human cytokines:

I. Inflammatory cytokines. Eur Cytokine Netw. 1: 290-296.

Barron D and Ibrahim RK 1996. Isoprenylated flavonoids–a survey. Phytochemistry. 43: 921-982.

Brattig NW, Diao GJ and Berg PA 1984. Immunoenhancing effect of flavonoid compounds on lymphocyte proliferation and immunoglobulin synthesis. Int J Immunopharmacol. 6: 205-215.

Chauhan PS, Satti NK, Suri KA, Amina M and Bani S 2010. Stimulatory effects of *Cuminum cyminum* and flavonoid glycoside on cyclosporine-A and restraint stress induced immune-suppression in *Swiss albino* mice. Chem Biol Interact. 185: 66-72.

Chen Y, Tang J, Wang X, Sun F and Liang S 2012. An immunostimulatory polysaccharide (SCP-IIa) from the fruit of *Schisandra chinensis* (Turcz.) Baill. Int J Biol Macromol. 50: 844-848.

Coffman RL, Sher A and Seder RA 2010. Vaccine adjuvants: Putting innate immunity to work. Immunity. 33: 492-503.

Cox JC and Coulter AR 1997. Adjuvants–a classification and review of their modes of action. Vaccine. 15: 248-256.

Dauer M, Obermaier B, Herten J, Haerle C, Pohl K, Rothenfusser S, Schnurr M, Endres S and Eigler A 2003. Mature dendritic cells derived from human monocytes within 48 hours: A novel strategy for dendritic cell differentiation from blood precursors. J Immunol. 170: 4069-4076.

Del Giudice G, Podda A and Rappuoli R 2001. What are the limits of adjuvanticity? Vaccine. 20: 38-41.

Donnelly DMX, O' Reilly J and Thompson J 1972. Neoflavanoids of *Dalbergia cultrata*. Phytochemistry. 11: 823-826.

Fischer G, Cleff MB, Dummer LA, Paulino N, Paulino AS, de Oliveira Vilela C, Campos FS, Storch T, D'Avila Vargas G, de Oliveira Hübner S and Vidor T 2007. Adjuvant effect of green propolis on humoral immune response of bovines immunized with bovine herpesvirus type 5. Vet Immunol Immunopathol. 116: 79-84.

Foged C, Sundblad A and Hovgaard L 2002. Targeting vaccines to dendritic cells. Pharm Res. 19: 229-238.

Gupta RK 1998. Aluminum compounds as vaccine adjuvants. Advanced Drug Delivery Reviews. 32: 155-172.

Hakim EH, Ulinnuha UZ, Syah YM and Ghisalberti EL 2002. Artoindonesianins N and O, new prenylated stilbene and prenylated arylbenzofuran derivatives from *Artocarpus gomezianus*. Fitoterapia. 73: 597-603.

Heroor S, Beknal A and Mahurkar M 2012. Preliminary investigation for immunomodulation of methanolic extracts of leaves and flowers of *Pongamia glabra* Vent. in mice model. Advances in Life Sciences. 2: 170-173.

Ismail S and Asad M 2009. Immunomodulatory activity of *Acacia catechu*. Indian J Physiol Pharmacol. 53: 25-33.

Javan MJ, Stushnoff C, Locke E and Vivanco JM 2003. Antioxidant activity and total phenolic content of *Iranian ocimum*. Food Chem. 83: 550-557.

Johnson VJ, He Q, Osuchowski F and Sharma RP 2003. Physiological responses of a natural antioxidant flavonoid mixture, silymarin, in BALB/c mice: III. Silymarin inhibits T-lymphocyte function at low doses but stimulates inflammatory processes at high doses. Planta Med. 69: 44-49.

Kabir S 1998. Jacalin: a jackfruit (*Artocarpus heterophyllus*) seed-derived lectin of versatile applications in immunobiological research. J Immunol Methods. 212: 193-211.

Khandelwal KR 2000. Practical Pharmacognosy techniques and experiments. 2nd ed. Pune: Nirali, Prakashan.

Kim H, Han S, Lee K, Lee C, Kim C, Lee E and Huh H 2000. Immunomodulating activity of a polysaccharide isolated from mori cortex radicis. Arch Pharm Res. 23: 240-242.

Kong LD, Yang C, Ge F, Wang HD and Guo YS 2004. A Chinese herbal medicine Ermiao wan reduces serum uric acid level and inhibits liver xanthine dehydrogenase and xanthine oxidase in mice. J Ethnopharmacol. 93: 325-330.

Kumalasari I, Nishi K, Harmayani E, Raharjo S and Sugahara T 2014. Immunomodulatory activity of Bengkoang (*Pachyrhizus erosus*) fiber extract in vitro and in vivo. Cytotechnology. 66: 75-85.

Kumar D, Arya V, Kaur R, Bhat ZA, Gupta VK and Kumar V 2012. A review of immunomodulators in the Indian traditional health care system. J Microbiol Immunol Infect. 45: 165-184.

Li X, Su Y, Sun J and Yang Y 2012. Chicken embryo extracts enhance spleen lymphocyte and peritoneal macrophages function. J Ethnopharmacol. 144: 255-260.

Likhitwitayawuid K, Sritularak B and De-Eknamkul 2000. Tyrosinase inhibitors from *Artocarpus gomezianus*. Planta Med. 66: 275-277.

Likhitwitayawuid K and Sritularak B 2001. A new dimeric stilbene with tyrosinase inhibitiory activity from *Artocarpus gomezianus*. J Nat Prod. 64: 1457-1459.

Likhitwitayawuid K, Chaiwiriya S, Sritularak B and Lipipun V 2006. Antiherpetic flavones from the heartwood of *Artocarpus gomezianus*. Chem Biodivers. 3: 1138-1143.

Martín-Fontecha A, Sebastiani S, Höpken UE, Uguccioni M, Lipp M, Lanzavecchia A and Sallusto F 2003. Regulation of dendritic cell migration to the draining lymph node: Impact on T lymphocyte traffic and priming. J Exp Med. 198: 615-621.

Minutello M, Senatore F, Gecchinelli M, Andreani T, Podda A and Crovari P 1999. Safety and immunogenicity of an inactivated subunit influenza virus vaccine combined with MF59 adjuvant emulsion in elderly subjects, immunized for three consecutive influenza seasons. Vaccine. 17: 94-108.

Nair MP, Kandaswami C, Mahajan S, Chadha KC, Chawda R, Nair H, Kumar N, Nair RE and Schwartz SA 2002. The flavonoid, quercetin, differentially regulates Th-1 (IFNgamma) and Th-2 (IL4) cytokine gene expression by normal peripheral blood mononuclear cells. Biochim Biophys Acta. 1593: 29-36.

Nilubol D, Platt KB, Halbur PG, Torremorell M and Harris DL 2004. The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. Vet Microbiol. 102: 11-18.

Parish CR and Warren HS 2002. Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. Curr Protoc Immunol. 4: 4-9.

Patil VV, Bhangale SC and Patil VR 2010. Studies on immunomodulatory activity of *Ficus carica*. Int J Pharm Pharm Sci. 2: 97-99.

Peach K and Tracey MV 1959. Modern method of plant analysis. 3rd ed. Narosa Publishing House: New Delhi. 64 pp.

Podda A 2001. The adjuvanted influenza vaccines with novel adjuvants: experience with the MF59-adjuvanted vaccine. Vaccine. 19: 2673-2680.

Prashanth SJ, Suresh D, Potty VH and Sadananda Maiya P 2014. In vitro antioxidant activity studies of *Artocarpus gomezianus*. Asian J Bio Sci. 9: 273-283.

Reed SG, Bertholet S, Coler RN and Friede M 2009. New horizons in adjuvants for vaccine development. Trends Immunol. 3: 23-32.

Rosa JC, De Oliveira PS, Garratt R, Beltramini L, Resing K, Roque-Barreira MC and Greene LJ 1999. KM+, a mannose-binding lectin from *Artocarpus integrifolia*: Amino acid sequence, predicted tertiary structure, carbohydrate recognition, and analysis of the β-prism fold. Protein Sci. 81: 13-24.

Schijns VE 2000. Immunological concepts of vaccine adjuvant activity. Curr Opin Immunol. 12: 456-463.

Sharanabasappa GK, Santosh MK, Shaila D, Seetharam YN and Sanjeevarao I 2007. Phytochemical studies on *Bauhinia racemosa* Lam. *Bauhinia purpurea* Linn. and *Hardwickia binate* Roxb. E-J Chem. 4: 21-31.

Sharma R, Rohilla A and Arya V 2011. A short review on pharmacology of plant immunomodulators. Int J Pharma Sci Rev Res. 9: 126-131.

Singh MP, Ahirwar J and Muthal N 2011. Evaluation of immunomodulatory activity of aqueous extract of *Ficus bengalensis* aerial roots in wistar rats. Asian J Pharm Clin Res. 4: 82-86.

Sritularak B, Tantituvanont A, Chanvorachote P, Meksawan K, Miyamoto T, Kohno Y and Likhitwitayawuid K 2010. Flavonoids with free radical scavenging activity and nitric oxide inhibitory effect from the stem bark of *Artocarpus gomezianus*. J Med Plants Res. 4: 387-392.

Thomas PG, Carter MR, Atochina O, Da'Dara AA, Piskorska D, McGuire E and Harn DA 2003. Maturation of dendritic cell 2 phenotype by a helminth glycan uses a toll-like receptor 4-dependent mechanism. J Immunol. 171: 5837-5841.

Trease GE and Evans WC 1978. Pharmacognosy. 11th ed. Bailliere Tindall Ltd: London. 75 pp.

Venkatachalam VV, Kannan K and Ganesh S 2009. Preliminary immunomodulatory activities of aqueous extract of *Morus alba* Linn. Int J Chem Sci. 7: 2233-2238.

Wagner HXS, Bladt Z and Gain EM 1996. Plant drug analysis. Springer Veralag: Berlin, Germany. 360 pp.

Wang J, Shan A, Liu T, Zhang C and Zhang Z 2012. In vitro immunomodulatory effects of an oleanolic acid-enriched extract of *Ligustrum lucidum* fruit (*Ligustrum lucidum* supercritical CO_2 extract) on piglet immunocytes. Int Immunopharmacol. 14: 758-763.

Yeap SK, Alitheen NB, Ali AM, Omar AR, Raha AR, Suraini AA and Muhajir AH 2007. Effect of *Rhaphidophora korthalsii* methanol extract on human peripheral blood mononuclear cell (PBMC) proliferation and cytolytic activity toward HepG2. J Ethnopharmacol. 114: 406-411.

Yusuf N, Nasti TH, Meleth S and Elmets CA 2009. Resveratrol enhances cell-mediated immune response to DMBA through TLR4 and prevents DMBA induced cutaneous carcinogenesis. Mol Carcinog. 48: 713-723.

บทคัดย่อ

การคัดกรองฤทธิ์ในการกระตุ้นการแบ่งตัวของลิมโฟไซต์และการเจริญเติบโตเต็มขั้นของเซลล์เดนไดร์ทติกที่เปลี่ยนมาจากโมโนไซต์ของสุกรในหลอดทดลองของสารสกัดหยาบจากพืชไทยในวงศ์ขนุนและวงศ์ถั่ว

ธนิวรรณ ชื่นอารมณ์[1] ตุลยา พึงไชยพัฒน์[2] เดชฤทธิ์ นิลอุบล[1] พรเพ็ญ วีระวัฒกานนท์[2] บุญชู ศรีตุลารักษ์[3] อังคณา ตันติธุวานนท์[2, 4]*

การศึกษานี้ได้คัดกรองสารสกัดหยาบจากพืชของไทยในสกุล Moraceae และ Leguminosae จำนวน 9 ชนิด ที่มีฤทธิ์เป็นอิมมูโนแอดจูแวนท์ โดยทดสอบการเพิ่มจำนวนของลิมโฟไซต์และการเจริญเติบโตเต็มขั้นของเซลล์เดนไดรท์ติกที่เปลี่ยนมาจากโมโนไซต์ (MoDCs) จากการทดลองพบว่าปริมาณสารประกอบฟีนอลทั้งหมดที่สกัดได้จากเปลือกรากหาดหนุน (*Artocarpus gomezianus*) (264.17±6.5 mg of gallic acid equivalents/gm of extract) มีปริมาณสูงกว่าที่สกัดได้จากพืชชนิดอื่น สารสกัดหยาบจากเปลือกรากหาดหนุนมีผลต่อการเพิ่มจำนวนของโมโนนิวเคลียร์เซลล์ในเลือดอย่างมีนัยสำคัญจากการตรวจวัดด้วยวิธี MTT และ CFSE ($p < 0.05$) นอกจากนี้ยังสามารถกระตุ้นการเจริญเติบโตเต็มขั้นของ MoDCs ผ่านการแสดงออกของ major histocompatibility (MHC) Class II และ CD80 องค์ประกอบของสารประกอบฟีนอลลิก โดยเฉพาะอย่างยิ่งแทนนินและฟลาโวนอยด์ที่มี γ-benzopyrone nucleus ในโครงสร้าง อาจมีความสำคัญต่อการมีฤทธิ์เป็นอิมมูโนแอดจูแวนท์ ผลการศึกษาครั้งนี้แสดงให้เห็นว่าสารสกัดจากเปลือกรากหาดหนุนมีฤทธิ์เป็นอิมมูโนแอดจูแวนท์ซึ่งน่าจะพัฒนาเป็นแอดจูแวนท์ร่วมกับวัคซีนได้

คำสำคัญ: *Artocarpus gomezianus* อิมมูโนแอดจูแวนท์ เซลล์เดนไดรท์ติกที่เปลี่ยนมาจากโมโนไซต์ โมโนนิวเคลียร์เซลล์ในเลือด สารประกอบฟีนอลลิก

[1]ภาควิชาจุลชีววิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330

[2]ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330

[3]ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330

[4]หน่วยปฏิบัติการวิจัยกลไกการออกฤทธิ์ของสารที่จะพัฒนาเป็นยาและและผลิตภัณฑ์สุขภาพในระดับเซลล์ คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330

***ผู้รับผิดชอบบทความ** E-mail: tuvanont@gmail.com*