การคัดกรองพืชสมุนไพรที่มีสารฟีนอลิกเพื่อใช้เป็นสารเสริมฤทธิ์ในวัคซีนและการเตรียมสารละลายของสารสกัดหยาบ

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SCREENING OF MEDICINAL PLANTS CONTAINING PHENOLIC COMPOUNDS AS A VACCINE ADJUVANT AND PREPARATION OF CRUDE EXTRACT SOLUTION

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ตุลยา พึงไชยพัฒน์ : การคัดกรองพืชสมุนไพรที่มีสารฟีนอลิกเพื่อใช้เป็นสารเสริมฤทธิ์ในวัคซีนและการเตรียมสารละลายของสารสกัดหยาบ. (SCREENING OF MEDICINAL PLANTS CONTAINING PHENOLIC COMPOUNDS AS A VACCINE ADJUVANT AND PREPARATION OF CRUDE EXTRACT SOLUTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ภญ. ดร. อังคณา ตันติธุวานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ภญ. ดร.พรเพ็ญ วีระวัฒกานนท์, ผศ. นสพ. ดร.เดชฤทธิ์ นิลอุบล, 86 หน้า.

วัตถุประสงค์ของการศึกษานี้ คือ การคัดกรองสารสกัดหยาบจากพืชสมุนไพรที่มีฤทธิ์เป็นสารกระตุ้นภูมิคุ้มกันและการเตรียมสารละลายผสมของแอนติเจนต้นแบบและสารสกัดหยาบในระบบตัวทำละลายที่สามารถฉีดได้ สารสกัดหยาบจากพืช 11 ชนิด ที่ความเข้มข้น 1, 10, 50 และ 100 ไมโครกรัม/มิลลิลิตร (ยกเว้น สารสกัดหยาบจากลำต้นชะเอมเหนือ ซึ่งถูกทดสอบที่ความเข้มข้น 1, 10, 50 และ 74.25 ไมโครกรัม/มิลลิลิตร) สารสกัดหยาบถูกบ่มเพาะร่วมกับเซลล์เม็ดเลือดขาวชนิดนิวเคลียสเดี่ยว (พีบีเอ็มซี) เป็นระยะเวลา 72 ชั่วโมง ที่สภาวะการเลี้ยงเซลล์ปกติ ผลของสารสกัดหยาบทุกชนิดต่อความมีชีวิตของเซลล์พีบีเอ็มซีถูกประเมิน สารสกัดหยาบ 3 ชนิด ได้แก่ สารสกัดหยาบจากเปลือกรากหาดหนุน สารสกัดหยาบจากแก่นกระพี้เขาควาย และสารสกัดหยาบจากเปลือกต้นทองหลางด่าง ไม่เป็นพิษต่อ พีบีเอ็มซีที่ทุกความเข้มข้นที่ทำการทดสอบ สารสกัดหยาบ 4 ชนิด ได้แก่ สารสกัดหยาบจากลำต้นสาเก สารสกัดหยาบจากแก่นขนุน สารสกัดหยาบจากเปลือกรากปอกระสา และสารสกัดหยาบจากเปลือกรากหม่อน เป็นพิษต่อ พีบีเอ็มซีที่ความเข้มข้น 100 ไมโครกรัม/มิลลิลิตร สารสกัดหยาบ 4 ชนิด ได้แก่สารสกัดหยาบจากดอกสะเดา สารสกัดหยาบจากลำต้นชะเอมเหนือ สารสกัดหยาบจากลำต้นเถาวัลย์เปรียง และสารสกัดหยาบจากลำต้นมะขามเทศ เป็นพิษต่อ พีบีเอ็มซี ที่เกือบทุกความเข้มข้นที่ทำการทดสอบและถูกคัดออกจากการศึกษา ผลของสารสกัดหยาบ 7 ชนิดต่อการแบ่งตัวของพีบีเอ็มซีถูกประเมิน สารสกัดหยาบ 2 ชนิด ได้แก่ สารสกัดหยาบจากเปลือกรากหาดหนุนและสารสกัดหยาบจากแก่นกระพี้เขาควายแสดงให้เห็นถึงฤทธิ์ในการกระตุ้นการแบ่งตัวของพีบีเอ็มซีที่ความเข้มข้น 100 ไมโครกรัม/มิลลิลิตร สารสกัดหยาบ 2 ชนิด ได้แก่ สารสกัดหยาบจากเปลือกต้นทองหลางด่าง (100 ไมโครกรัม/มิลลิลิตร) และสารสกัดหยาบจากแก่นขนุน (50 ไมโครกรัม/มิลลิลิตร) มีแนวโน้มลดการแบ่งตัวของพีบีเอ็มซี โดยไม่ทำให้เกิดการลดลงของความมีชีวิตของพีบีเอ็มซี ผลของสารสกัดหยาบต่อการเจริญเต็มวัยของเซลล์เดนไดรติคที่กลายมาจากโมโนไซต์ (เอ็มโอดีซี) ถูกประเมิน สารสกัดหยาบจากเปลือกรากหาดหนุนเหนี่ยวนำให้เกิดการเจริญเต็มวัยของ เอ็มโอดีซี ในการเตรียมสารละลายของสารสกัดหยาบจากเปลือกรากหาดหนุนและแอนติเจนต้นแบบ สารสกัดหยาบสามารถละลายในระบบตัวทำละลายที่ประกอบด้วย เอธานอล 10 เปอร์เซ็นต์ โพรไพลีนไกลคอล 8 เปอร์เซ็นต์ พีอีจี 400 30 เปอร์เซ็นต์ และ ทวีน 80 2 เปอร์เซ็นต์ในน้ำที่ความเข้มข้น 29.72 มิลลิกรัม/มิลลิลิตร ในระบบตัวทำละลายนี้แอนติเจนต้นแบบเกาะกลุ่มเป็นอนุภาคแขวนตะกอนขนาดเล็กกว่า 50 ไมโครเมตร อย่างไรก็ตามอนุภาคที่เกาะกลุ่มสามารถถูกทำให้กระจายตัวได้อีกด้วยการเขย่า

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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TULLAYA PUNGCHAIPAT : SCREENING OF MEDICINAL PLANTS CONTAINING PHENOLIC COMPOUNDS AS A VACCINE ADJUVANT AND PREPARATION OF CRUDE EXTRACT SOLUTION. ADVISOR: ASST. PROF. ANGKANA TANTITUVANONT, Ph.D., CO-ADVISOR : ASST. PROF. PORNPEN WERAWATGANONE, Ph.D., ASST. PROF. DACHRIT NILUBOL, Ph.D., 86 pp.

The objectives of this study were to screen the medicinal crude extracts for an immunopotentiators and to prepare the mixed solution of the model antigen and the crude extract in injectable solvent system. The eleven crude plant extracts at the concentration of 1, 10, 50 and 100 μg/ml (except for the crude extract from stems of *Derris reticulata*, which was tested at the concentration of 1, 10, 50 and 74.25 μg/ml). The crude extracts were incubated with the peripheral blood mononuclear cells (PBMCs) for 72 hrs at normal culturing condition. The effect of all crude extracts on the PBMCs viability was determined. Three crude extracts i.e. crude extract from root barks of *Artocarpus gomezianus*, crude extract from heartwood of *Dalbergia cultrata* and crude extract from stem barks of *Erythrina variegata* were not toxic to the PBMCs at all concentration tested. Four crude extracts i.e. crude extract from stems of *Artocarpus altilis*, crude extract from heartwood of *Artocarpus heterophyllus*, crude extract from root barks of *Broussonetia papyriferra* and crude extract from root barks of *Morus alba* were toxic to PBMCs at the concentration of 100 μg/ml. Four crude extracts i.e. crude extract from flowers of *Azadirachta indica*, crude extract from stems of *Derris reticulata*, crude extract from stems of *Derris scandens* and crude extract from stems of *Pithecolobium dulce* were toxic to PBMCs at almost all of the concentrations tested and were excluded from the study. The effect of seven crude extracts to the proliferation of PBMCs was determined. Two crude extracts i.e. crude extract from root barks of *Artocarpus gomezianus* and crude extract from heartwood of *Dalbergia cultrata* showed the stimulatory effect on the proliferation of PBMCs at the concentration of 100 μg/ml. Two crude extracts i.e. crude extract from stem barks of *Erythrina variegata* (100 μg/ml) and crude extract from heartwood of *Artocarpus heterophyllus* (50 μg/ml) tended to decrease the proliferation PBMCs without causing a decrease in viability of PBMCs. The effect of crude extracts on the maturation of monocyte-derive dendritic cells (MoDCs) was determined. The crude extract from root barks of *Artocarpus gomezianus* induced the maturation of MoDCs. In the preparation of crude extract from root barks of *Artocarpus gomezianus* and model antigen solution, the crude extract could be dissolved in the solvent system consisting of 10 % ethanol, 8% propylene glycol, 30 % PEG 400 and 2 % tween 80 in water at the concentration of 29.72 mg/ml. In this solvent system, the model antigen aggregated to be a suspension with the particle size smaller than 50 μm. However, the aggregates could be redispersed by agitation.

Department : Pharmaceutics and Industrial Pharmacy Student's Signature .....

Field of Study : Pharmaceutics Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

Co-advisor's Signature .....

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ε	=	dielectric constant
μl	=	microlitre
mg	=	milligram
ml	=	millilitre
APCs	=	antigen presenting cells
CD 80	=	clusture of differentiation 80
CFSE	=	carboxyfluorescein succinimidyl ester
DMSO	=	dimethyl sulfoxide
GAE	=	gallic acid equivalent
GM-CSF	=	granulocyte macrophage colony-stimulating factor
IL-4	=	interleukine 4
IMDM	=	Iscove's Modified Dulbecco's Medium
MHC	=	major histocompatibility
MoDCs	=	monocyte-derived dendritic cells
MTT	=	methythiazolyldiphenyl-tetrazolium
OVA	=	ovalbumin
PBMCs	=	peripheral blood mononuclear cells
PEG 400	=	polyethylene glycol 400
PG	=	propylene glycol
PAMPs	=	pathogen associated molecular patterns
SRBC	=	sheep red blood cell
TLR	=	toll-like receptor
Th1	=	T helper 1

Th2	=	T helper 2

CHAPTER I
INTRODUCTION

Vaccine is one of the most successful tools for the eradication of several life threatening diseases (de Veer and Meeusen, 2011; Pulendran and Ahmed, 2011). Vaccine can be categorized into two major categories, the traditional ones and the new generation vaccine. The traditional vaccines are live attenuated vaccines, killed vaccines and toxoids. The new generation vaccines are subunit vaccines, synthetic proteins vaccines, deleted live vaccines, live recombinant vaccines and DNA vaccines (Sanchez-vizcaino, 2001). The ability of each vaccines to induce immune response is different, depending on the type and source of antigen. For traditional vaccines, the antigen produced from live attenuated microbe can stimulate the immune response better than the antigen produced from dead microbe or toxoid and can induce live-long immunity (Capron et al., 1994; Wilhelm et al., 2006; Reed et al., 2008; ประวรรณ สุนทรสมัย, 2546). However, live vaccine has a risk to become virulent and limit to non-immunocompromise patients. The killed vaccines and toxoids are safer than live vaccines but require adjuvant to increase their immunogenicity (Petrovsky and Aguilar, 2004). New generation vaccines such as subunit vaccines, synthetic antigen vaccine and DNA vaccine are generated for fighting both infectious diseases and non-infectious diseases (Leroux-Roels, 2010). These vaccines composed of purified protein that can induce the immune response against the causative molecules. However, the immunogenicity of the new generation vaccines is very low and sometimes could not elicit the protection. The poor immunogenicity is a result of the high purity of the antigen. Thus, these vaccines need the adjuvant.

Vaccine adjuvant is the substance used in vaccine formulation as the additive to strengthen and improve the primary immune response of the body (O'Hagan, 2007). Vaccine adjuvant increases the immune response to a supplied antigen by extending the antigen deposit time in the body therefore the immune cells can contact antigen for a long time. Moreover, the adjuvant may cause irritation at the injection site, leading to an increase in immune cells migration to the site. Some adjuvants help increase immune response by targeting the antigen to antigen presenting cells (APCs),

enhance the costimulatory molecules (CD80, CD83 and CD86) and major histocompatibility class II expression on the surface of APCs, and inducing the releasing of cytokine from lymphocyte and APCs (O'Hagan, 2000; Marciani, 2003; Alving, 2009).

The vaccine adjuvants approved for use in human are aluminium compound, MF59 and AS04 (Baylor et al., 2002). The vaccine adjuvants that are under the development are Toll-like receptor agonist (MPL, CpG DNA, Imidazoquinolines etc.), Saponins (QS21) and immune stimulating complexes (ISCOMs) (Leroux-Roels, 2010). Aluminium compound (alum) is the oldest traditional vaccines adjuvants. The advantage of alum is the safety profile (Jefferson et al., 2004). Alum has no evidence in causing the serious or long-lasting adverse effect. However, the alum fails to induce the immune protection in many diseases such as whooping cough and toxoid. Moreover, alum cannot enhance the immunogenicity of the new generation vaccines. The MF59, an oil in water emulsion, came across the alum. This adjuvant stimulates strong antibody response to subunit antigen. Now, the MF59 is used in combination with the commercial influenza subunit vaccine (Tritto et al., 2009). However, aluminium compound adjuvant and MF59 have limitation. They can stimulate only humoral immune response (Ott et al., 2000; Reed et al., 2008). With this limitation, these adjuvants cannot be used in the vaccine prepared for intracellular infection diseases. In addition, MF59 was reported to cause pain, redness, swell, allergy or auto-immune disease (Gupta, 1998; Minutello et al., 1999; Asa et al., 2000; Podda, 2001; Vega, 2005). ASO4, an adjuvant composed of aluminium compound and monophosphoryl lipid A, can stimulate both types of immune response, humoral immune response and cell-mediated immune response. However, there are some suspicions about its safety. It was reported that AS04 was related to paralysis, seizure and visual problems (Ulrich, 2000; Reed et al., 2008; Gordon, 2009).

The current vaccine adjuvants can induce only humoral immune response but lack of the ability to induce cell mediated immune response. Therefore, it is necessary to find new substances for vaccine adjuvant which are safe and can stimulate both types of immunity especially cell-mediated immune response. It will be better if the new substances come up with low price, high stability, can be used with several antigens and induce long term immunity with low boosted doses.

To find the new adjuvants, the good source of the candidates may come from the immunopotentiators (Ribeiro and Schijns, 2010; Wolf et al., 2013). The immunopotentiators are defined as any substances that can increase or stimulate the immune response. The immunopotentiators that show the feasibility to be developed into vaccine adjuvant should possess the key activity related to the mechanism of adjuvant that is the ability to induce the maturation of dendritic cells (Banchereau and Steinman, 1998). From the current knowledge, the effective adjuvants usually affect the dendritic cell functions such as the antigen presentation (Stubbs et al., 2001; Steinman and Pope, 2002). So, the method of dendritic cell maturation determination was used for the screening of immunopotentiators. Moreover, the lymphocyte proliferation assay will be used as the *in vitro* screening of immunopotentiators.

Medicinal plants are the interesting sources of vaccine adjuvants due to their immunostimulatory activities. Many studies reveal that the abilities to stimulate the immune system in the plants belong to the poly-phenolic compounds (Sakagami et al., 2012). The poly-phenolic compound can be categorized into two categories, flavonoids and non-flavonoid compounds. Non-flavonoid compounds such as resveratrol, cinnamic acid, caffeic acid and tannin in various medicinal plants showed the immunostimulatory activity. They can increase the recovery rate from virus infection (Ivanova et al., 2005), enhance movement of macrophage in early infection (Ivanova et al., 2005), stimulate the secretion of IL-1, IL-6, TNF-α, IL-12, IFN-γ from macrophages and lymphocytes (Falchetti et al., 2001; Vetvicka et al., 2007; Yusuf et al., 2009; Francisco et al., 2012), increase antibody titers in mice (Park et al., 2004; Li et al., 2007), increase functions of phagocytes and natural killer cells (Kolodziej and Kiderlen, 2005; Morazzoni et al., 2005), increase lymphocyte proliferation and activate Toll-like receptor 4 (Yusuf et al., 2009).

Flavonoids, which is the subset of phenolic compounds, are also famous in their immunomodulatory activities (Vajdy, 2011). They can stimulate division of lymphocytes (Brattig et al., 1984), increase antibody production (Brattig et al., 1984; Kong et al., 2004), and stimulate the expression of IFN-γ coding gene (Nair et al., 2002; Chauhan et al., 2010). The IFN- γ is the key cytokine that bias the pathway of immune response to cell-mediated immune response (Chiang et al., 2003; Kong et al., 2004; Chauhan et al., 2010). Moreover, in the study of Fischer et al. in 2007, which is the study about adjuvanticity of high flavonoid crude propolis extract in bovine herpes

virus type V in cow, show the good activity of that crude propolis extract in protection of that virus infection. And there are similar studies that used propolis crude extract as the vaccine adjuvant in inactivated suid herpesvirus type 1 vaccine and inactivated porcine parvovirus vaccine in mice (Fischer et al., 2007a; Fischer et al., 2007b; Ma et al., 2011) and they suggested that the high protection is a result from that mixed of prenylate-polyphenolic compounds and flavonoids in the propolis.

Structures of flavonoids are concerned as the key part that affects immune response. In general, only large molecules, infectious agents, or insoluble foreign matter can elicit an immune response in the body. So, a huge hindrance flavonoids might better stimulate the immune system than the small lineage structure (Kensil and Kammer, 1998; Abbas et al., 2000). Furthermore, the lipophilic functional group may increase the potency in stimulate immune response (Hunter, 2002). From this information, prenylated flavonoids is considered to be tested as the adjuvant because they have a huge hindrance flavonoid structure and the prenyl group makes the structure more lipophilic. Prenylated flavonoid can be commonly found in Moraceae and Leguminosae (Barron and Ibrahim, 1996). Moreover, *Azadirachta indica* (Meliaceae), a plant outside those families also contains the prenylated flavonoid.

Crude extracts from plants usually have low solubilities that impede the development of injectable preparations. Because an injectable preparation has a limit volume of administration, a substance that has a low solubility cannot be administrated in a suffice dose. Besides, the low solubility may precipitate during storage and cannot be redispersed, implying physically unstable system. So, there are attempts to find the solubilizing systems that suit for crude extract used in this study and follow the requirement of injectable vehicle.

There are many techniques to improve the solubility of poorly water soluble substances for examples, pH adjustment, cosolvency, micellization, particle size reduction and complexation (Savjani et al., 2012). The pH adjustment is suitable for the acidic or basic compounds. The crude extracts contain various types of chemicals which their structures are undefined. The complexation employs cyclodextrin to form inclusion complex that is more aqueous soluble. This technique limits to small molecules that can be included in the cavity of cyclodextrain. Cosolvency and micellization are the most common techniques to improve the solubilities of any substances. Cosolvency uses one or more organic solvents to modify the polarity of

solvent system. The polarity of solvent system can be reduced by organic solvents until it reaches a suitable level for dissolving the substances. Micellization uses surfactants to form micelles. The micelles can improve the solubilities of any non polar compounds. Thus, the cosolvency and micellization were used in this study.

Studying the effect of Thai medicinal plants containing flavonoids and polyphenol on the proliferation of PBMCs and the maturation of dendritic cells may lead to the exploration of new immunopotentiators which might be utilized in vaccine production in the future. Moreover, it can add value and fulfill the information about immunostimulatory activity to Thai plants.

The objectives of this study were

1. Screening the medicinal plants containing flavonoids and polyphenols for an immunopotentiator.
2. Finding the appropriate solubilizing system for the crude extracts.

CHAPTER II
LITERATURE REVIEW

I. Immune response

The immune system has an ability to protect body from exogenous pathogens, foreign particles and abnormal cells such as tumor cells and death cells. Once the pathogens, foreign bodies or abnormal molecules appear in the body, the immune system works coordinately to eliminate them. The action of immune system to those alien bodies is called “the immune response”. There are two types of immune responses, the innate immune response and the adaptive immune response.

The innate immune response reacts to invaded pathogens instantly when the pathogens get into the body in contrast to the adaptive immune response that works later with more specificity. These two systems work coordinately to produce the fine effector cells for completely eradicating the virulent pathogens from the body (O'Hagan and Valiante, 2003).

The innate immune response exists before the pathogen infringement and it is being like a patrol for inspecting of the abnormal events within the body. The physical barriers such as skin and epithelium are the members of the innate immunities. They enwrap our body for preventing of the pathogen intrusion and produce antimicrobials for pathogen elimination. Phagocytes such as neutrophils, macrophages and dendritic cells are another parts of the innate immune cells that firstly attack the invaded pathogens. These cells will engulf those pathogens into the intracellular compartment of the cell. Within the cell, the pathogens were digested to small peptide fragments, containing epitopes or antigen determinants which will bind to major histocompatibility molecules (MHC) for the presentation of the antigenic peptides to antigen-specific T lymphocytes to generate the antigen-specific immune response. The phagocytic cells that present the peptide antigens to T lymphocyte also named the antigen presenting cells (APCs).

The adaptive immune system does not response as fast as the innate immune system but it is very powerful to eradicate the pathogens from the body completely.

Antigen presentation by APCs is the first step to generate adaptive immune response. The APCs present antigen to the specific T lymphocytes. In the milieu of presenting procedures, additional signals beside the antigen-MHC complex are needed. Those additional signals are co-stimulatory molecules on APCs' surface such as CD80, CD86 and CD40, and pro-inflammatory cytokines such as IFN-γ, IL-1, IL-2 and IL-6. Some studies showed that without those signals, the T lymphocytes were completely unresponsive (Jenkins and Schwartz, 1987). There are two types of effector T cells, including T helper cells and cytotoxic T cells. T helper cells have capability to synthesize and release the cytokines that are necessary for other immune cell functions. Cytotoxic T cells are the cells that kill virus infected cells. Once the antigen presentation is successful, the T lymphocytes will proliferate and differentiate to T helper 1 (Th1) or T helper 2 (Th2). Th1 secrete cytokines that lead to cellular immune response whereas Th2 secrete cytokines that lead to humoral immune response. Cellular immunity or Cell-mediated immunity (CMI) has the ability to eliminate the intracellular pathogens such as viruses and mycobacterium.

For humoral immune response, the mechanism of induction is initiated by the binding of antigen and B cell receptor. B cell receptor is an antibody that anchors in cell membrane of B cells. When antigen binds to the receptor, the intracellular compartment of the receptor will send signals into the cell. B cells will proliferate and differentiate to plasma cells, which have capability to secrete antibody. To differentiate to plasma cells, B cells need cytokines from helper T cells.

Both cellular immunity and humoral immunity do not only fight against the invaded pathogen, but also memorize the characteristics of pathogen. The characteristics of pathogen can be recorded by the function of memory T cells and memory B cells. Both memory cells are long lived and responsible for secondary immune response that will be active instantly when they confront the same pathogen in the next time. The speed and efficacy of pathogen eradication by secondary immune response will increase as much as the number of subsequent exposure times. This concept of secondary immune response leads to the origin of vaccine.

II. The efficacy and mechanism of action of vaccine adjuvant

The old style vaccines usually have no problems in stimulating the immune response because they consist of the "natural adjuvant". The natural adjuvants of the antigen are the structural molecules on the surface of pathogens such as peptidoglycan, lipopolysaccharide and muramyldipeptide. These molecules called pathogen associated molecular patterns (PAMPs). PAMPs bind to pattern-recognition receptors (PRRs) of phagocytic cells, especially macrophages and dendritic cells, resulting in high expression of MHC molecules on their surface and vigorously release the stimulating cytokine such as IL-2 and IFN-γ leading to high efficiency of antigen presentation and regulation of the T cell response. The new generation of vaccines consist of pure protein antigens that lack of PAMPs, so they need these adjuvants.

Dendritic cells are the most important antigen presenting cells. They have high efficacy of inducing immune response. They can highly express MHC and CD80/CD86 costimulatory protein and they can migrate from the site of pathogen invasion to lymph node for presenting the antigens. At lymph node, many differentiated T lymphocytes are conglomerated and wait for the antigen that specific to them.

The mechanisms of action of vaccine adjuvant may be categorized into two groups (Marciani, 2003). One is the physical mechanism that is the sustain release of antigen from depot. Another is the immunological mechanism which is the mechanism that modulates the activity of antigen presenting cell. The physical mechanism for example the sustain release of antigen from alum precipitates in depot, can be provided by the pharmaceutical technology. For the second mechanism, immunological mechanism is the mechanism of PAMPs.

Many adjuvants under experimental study are those molecules that similar to PAMPs. The molecules that were recognized by immune system as a foreign body usually stimulate the immune responses.

III. Methods for screening of the immunopotentiator

Initially, the finding of new candidates for use as vaccine adjuvant, is the finding of the immunopotentiated candidates. The full adjuvanticity testing must be

done *in vivo* by immunization. The animals will be immunized with a model antigen or other specific antigen and then the antibody titers or antigen specific lymphocytes will be measured. Moreover, the immunized animal may be challenged with the pathogens and the protection will be observed by the survival rate.

One of the *in vitro* models that is usually used for screening the immunopotentiators is the lymphocyte proliferation assay. The lymphocyte proliferation assay is a measurement of the function of lymphocytes. The substances of interest were incubated with the lymphocytes for a period of time, generally 24 - 72 hours. After the incubation, lymphocytes proliferation can be determined by many different methods such as 3H-Thymidine incorporation assay for the detection of cell nucleotide synthesis, MTT assay for the detection of cell viability and CFSE dye dilution assay for the detection of cell divisions.

The other study is about the measurement of antigen presenting cell functions. There are many methods to characterize the function and morphology of antigen presenting cells after incubation with candidate substances.

First is the measurement of the phagocytic activity of dendritic cells. The phagocytic activity of dendritic cell is very high before the antigen encounter. After they phagocytose the antigen, they would differentiate to mature dendritic cells which possess the activity to present the antigen. The mature dendritic cells consist of the antigen carrying protein (MHC Class II) and co-stimulatory molecules (CD80, CD83, CD86) on cell surface. The detection of these markers could be used for the characterization of the maturation of dendritic cells.

The pattern of cytokine released from dendritic cell is one of the important parameters for detection and description the immunopotentiated status. There are many methods used for detection of cytokines such as RT-PCR, gel electrophoresis and ELISA. Each technique gives different aspect of information. ELISA is the most reliable because it has high sensitivity and gives the researcher the existence of intact protein. For RT-PCR couple with gel electrophoresis, the obtained information is the expression of mRNA of the complementary cytokines which is not surely transcribed to the intact cytokines.

IV. Important of dendritic cells as a key model for screening of immunopotentiator

To determine any substance as an immunopotentiator, many methods are employed such as antibody secretion assay, lymphocyte proliferation assay, cytokine secretion assay and antigen presenting cells function assay.

In case of the screening for immunopotentiator for further development to the vaccine adjuvant, the assay should be relevant with the mechanism of adjuvanticity. The immunopotentiators that possess the ability to enhance the function of dendritic cells is the most interesting (Gallucci et al., 1999). Dendritic cells are such a middle man between the innate immunity and adaptive immunity, it is essential in the regulation and the generation of adaptive immune response. Dendritic cells have a talent in communicating with the T lymphocyte in adaptive immune system. They located in several areas that are the portal of the pathogen entry all over the body. Once the dendritic cells meet the antigen, they will phagocytose the antigen and migrate from local area to the lymph node. During the migration, they differentiate into the mature dendritic cells which express a high density of major histocompatibility class II (MHC Class II) and co-stimulatory molecules (CD80, CD83 and CD86). The maturation of Dendritic cell is a key process, without maturation the dendritic cell cannot induce the adaptive immune response. At time they arrive to the lymph node, they would accommodate and find the T lymphocytes clone that suit to the antigen they carry. Finally, the dendritic cells would present the antigen to specific T lymphocytes, and regulate the proliferation of T lymphocytes. The T lymphocytes would proliferate into many subpopulations such as CD4+ T helper cells, CD8+ cytotoxic T cells, regulatory T cells, B lymphocytes and plasma cells depending on the cytokine pattern in the environment. Finally, the specific immune effector, CD8+ T cell and plasma cells, will be obtained and function to eradicate the specific pathogen.

From the description above, the assay of the maturation of dendritic cells was employed in this study. Moreover, in the initial screening of the crude extract, the proliferation assay of peripheral blood mononuclear (PBMCs) was exploited because this assay represents overall outcomes of the adaptive immunity response.

The study of antibody secretion needs to use animal subjects. So the antibody titer assay is not included to this research. The cytokine secretion assay is important to define the cytokine pattern in immune response as a prediction of T helper cell polarization (Th1 or Th2). Thus, this characteristic should be further investigated after the immunopotentiator is confirmed.

V. Immunomodulatory activity from plants

Plants contain various substances that possess the ability to modulate the function of immune system in both stimulatory and suppressive manners. The attempts to discover of the immunomodulators from plants are currently arising due to the demand of immunostimulator for potentiating the function of immune system against cancer and immunosuppressant for graft rejection and autoimmune disease. The immunomodulatory substances can be categorized in two main categories, low molecular weight compounds and high molecular weight compounds (Wagner, 1990). In this review, we interested only low molecular weight compounds. The low molecular weight compounds including alkylamides, phenolic compounds, alkaloids, quinones, saponins and terpenoids and the high molecular weight are polysaccharides, protein, glycolipids peptides and glycoproteins.

Alkylamides are expected to be one of the active substances responsible for boosting immune in Echinacea extract from root of *Echinacea* species (e.g. *Echinacea purpurea*, *Echinacea pallida* and *Echinacea angustifolia*) because they were found in blood circulation of subjects who consumed the Echinacea preparation. The alkylamides showed some activities to the immune components by modulating the expression of TNF-α gene in macrophages (Matthias et al., 2007). However, some alkylamides also exhibited the immuno-inhibitory activity by inhibition of the TNF-α translation. So, the immunostimulatory activity of Echinacea preparation may be a result from the diversity of chemical constituents. In addition, the immune system was multifaceted so that the alkylamides may act at other parts in which the present investigation method cannot reveal.

Phenolic compounds are the chemical substances that possess various biological activities. Within 8,000 known structures of phenolic compounds, 4000 structures are flavonoids, so the phenolic compounds can be divided in two

categories, flavonoid compounds and non-flavonoid compounds (Tsao, 2010). Both categories of phenolic compounds possessed various immunomodulatory activity. The detail of their activities will be described in next topics.

Alkaloids are one of the major secondary phytochemicals in plants. They are the basic compounds that possess the cyclic structure which contains nitrogen atoms inside or outside the cyclic system. Commonly, alkaloids usually possess the anti-inflammatory response. The immunomodulatory activity of alkaloids is the suppressive manner (Barbosa-Filho et al., 2006). The examples of famous alkaloids are colchicine, morphine, melatonin, piperine, quinidine, quinine, caffeine and theophylline. However, some alkaloids play the immunostimulatory activity for example tomatine, imidazoquinoline and punarnavine. Tomatine was studied as an adjuvant for malaria vaccine (Heal et al., 2001). The imidazoquinoline was elucidated the toll-like receptor agonist (Philbin et al., 2012). The punarnavine increased the activity of cell-mediated immunity in metastatic melanoma-bearing mice (Manu and Kuttan, 2007).

Quinones are another compound group that influence the immune components. The unique structure of quinones is the benzene ring with two carbonyl group and this structure could be attached with other ring. Quinones generally suppress the function of immune components and the tumor growth (Kitagawa et al., 2011). However, some quinones such as tetrachlorobenzoquinone and tetrachlorohydroquinone, exerted the ability to increase the immune respones. They acted like a potent hapten that could induce the autoimmune disease by bounding to the endogenous macromolecules (Ezendam et al., 2003).

Saponins are the most famous immunopotentiator which are widely studied as the vaccine adjuvant. Saponins own the special activity that is the ability to induce the cell-mediated immunity. The examples of saponins in adjuvant formulation are Quil A and QS-21 which are isolated from bark of *Quillaja saponaria*. These saponins were examined for the ability to increase the immune response to cancer vaccine and vaccine against intracellular pathogens i.e. HIV, malaria, respiratory syncytial virus, cytomegalovirus, *toxoplasma gondii* and visceral leishmaniasis (Sun et al., 2009). However, the saponins hold many disadvantages which restrain them from

commercial adjuvant. They usually cause heamolysis in mammal, exert high toxicity and are instability in aqueous vehicle.

Sesquiterpene, di- and triterpenoids are the chemical compounds that consist of isoprene units (namely 2-methyl-1,3-butadiene). Sesquiterpenes consists of 3 isoprene unit and may be acyclic or contain rings. Sesquiterpenes which were modified by biochemical reaction such as oxidation and rearrangement would produce any isomers or derivatives called sesquiterpenoids. Dendroside A and dendronobilosides A were the sesquiterpenes isolated from stem of *Dendrobium nobile*. They could stimulate the proliferation of murine T and B lymphocytes *in vitro* but the another sesquiterpene, dendronobilosides B, inhibited the proliferation of T lymphocytes and B lymphocytes (Zhao et al., 2001). Diterpenes compose of four isoprene units. The 14-desoxo-10 and 18-dihydromyrinsol diterpenoids that were isolated from *Euphorbia aellenii* could inhibit T-cell proliferation (Ayatollahi et al., 2010). Triterpenes consist of six isoprene units. Some triterpenes molecules that showed the immunositmulvatory activity is the squalene, which is one of the components in MF59 adjuvant (Tritto et al., 2009).

VI. Phenolic compounds

Phenolic compounds encompass every substance that possesses the structure of benzene ring attached to hydroxyl groups, so phenolic compounds are the large group of phytochemical found in plants. Phenolic compounds can be categorized in two categories that are non-flavonoids and flavonoids.

Non-flavonoid phenolic compounds

There are various compounds in this group. The non-flavonoid compounds that possess the ability to influence immune system are resveratrol, cinnamic acid, caffeic acid and tannin.

Resveratrol is a stilbene derivative which is the chemical substance that composes of two benzene ring separated by two carbon atom. Resveratrol does not widely distribute in plants. It was found in wine, chocolate and grapes. Resveratrol at low concentration (0.6 – 2.5 μg/ml) stimulated the secretion of IL-2, IL-4, IL-6 and IFN-γ from CD4+ and CD8+ T cell (Falchetti et al., 2001; Li et al., 2007). It

stimulated the phagocytosis of phagocytes, caused increase in expression of cell surface molecules of lymphocytes and made the higher restoration of spleen recovery after leucopenia (Vetvicka et al., 2007). The resveratrol enhanced cell-mediated immune response via Toll-like receptor 4 (Yusuf et al., 2009).

Cinnamic acid was expected as an immunostimulatory substances in the propolis that possessed the ability to be vaccine adjuvant (Fischer et al., 2007a). Ivanovska et al. (1995) demonstrated that splenocytes from mice treated with cinnamic acid could enhance the incorporation of thymidine, in the presence of lipopolysaccharide (LPS), phytohemagglutinin (PHA) or concanavalin A (Con A). Moreover, the IL-1 and IL-2 from splenocytes in cinnamic acid treated group were increased .

Caffeic acid is phenolic acid group that was claimed as one of the active components in propolis that stimulate the immune response. Caffeic acid phenethyl ester had the activity to increase T lymphocyte blastogenesis induced by concanavalin A (Con A), CD4+ T cell subpopulation and the IL-2, IL-4 and IFN-γ level (Park et al., 2004). The caffeic acid also increased the function of macrophages in eradiation of *Leishmania donovanii* by increasing the IFN-γ (Kolodziej and Kiderlen, 2005).

Tannin increased the recovery rate from virus infection, enhanced movement of macrophage in early infection (Ivanova et al., 2005). The decoction of *Uncaria tomentosa* which was tannin-rich fraction increase production of inflammatory-related cytokines, namely interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12 (p70), granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-C motif) ligand (CCL) 2, CCL_3, CCL_4, and tumor necrosis factor (TNF)-α in the culture medium of human macrophages (Francisco et al., 2012).

Flavonoids

Flavonoids are one of the most abundant polyphenolic compounds commonly found in every part of the plant. Flavonoids in the petals of the flowers are the pigment of petals that help the plant for mating. Flavonoid in the leaves, stems, roots and other parts, help the plant to maintain physiological status and fighting the insect and other micro-organism. Bees exploit the antimicrobial agent from flavonoids to maintain their hives.

In human, flavonoids were recognized as a foreign body (Stauth, 2007). They were eliminated easily. So, because of the alien characteristic of the flavonoids, it may exert like the PAMPs that activate the response of immune system.

The evidences of using flavonoid containing substances as an immune enhanced remedy are provided below. Their immunostimulatory profiles suggested that the flavonoids have potential to be used as vaccine adjuvants.

Sambucol, a commercial product, was used as an immune enhancing agent for fighting the influenza virus. It strongly activates the immune system by promoting the inflammatory cytokines i.e. IL-1β, TNF-α and IL-6. This product consists of the extract of black elderberry (*Sambuscus nigra*) as a main compound which contained high flavonoid content (Barak et al., 2001).

Silymarin, the mixture of bioactive flavonoids, exhibited the immunostimulatory activity by increasing mRNA expression of IL-1β, IL-6, iNOS, and TNF-α in splenocytes from silymarin treated mice (Johnson et al., 2003). These cellular responses imply the inflammatory process states which are to the mechanism of vaccine adjuvant.

In 2007, Fischer et al. discovered the adjuvant activity of propolis. Cows were immunized with bovine herpes virus type 5 antigen and propolis. The result showed that the group immunized with antigen and propolis showed higher neutralizing antibody than non-propolis group. When the chemical constituents of propolis were analysed, they found that the propolis contain high artepillin C, diprenyl-4-hydroxycinnamic acid compound, and flavonoids. They suggested that the immunostimulatory effect of propolis may come from phenolic compounds including flavonoids.

There was the study demonstrated that the oral treatment of rats with an *Echinacea angustifolia* root extract – containing a rich source of phenolic compounds significantly raised the IgG level to keyhole limpet hemocyanin (KLH) after three boosting doses compared to control rats.

As the other experiment, mice orally given a combination of *Echinacea purpurea* and *Echinacea pallidae* root extracts increased the anti-SRBC PFC and HI titre response almost double compare to control mice. These authors also revealed that this Echinacea treatment could reverse the hydrocortisone-induced

immunosuppression of anti-SRBC PFC response, suggesting a potent immunostimulatory role for Echinacea preparation (Licciardi and Underwood, 2011).

In 2012, Nworu et al. demonstrated the adjuvant potential of high-flavonoid rich fraction from the extract of *Archlorda cardifolia*, a medicinal herb of Nigeria.

However, it should be kept in mind that flavonoids do not always enhance the immune function. Flavnoids is immunomodulatory that can potentiate or suppress the immune response. The direction of activity of flavonoids depends on many factors such as plant species, chemical structure of flavonoids and chemical profiles of flavonoid mixtures (López-Posadas et al., 2008). However, many articles suggested that the flavonoids in crude extract can exert the immunostimulatory activity better than pure flavonoids (Licciardi and Underwood, 2011).

VII. The solubilizing technique for antigen – crude extract solution for injection

There are some considerations related to vehicle for using in pre-clinical study (Maas et al., 2007; Turner et al., 2011). First, the safety of vehicle especially the route of administration that intends to use, must be considered carefully. The unsuitable vehicle could cause the adverse effect to the animal, leads to the confounding results. Second, the vehicle must be compatible with the active substances for the purpose of maintaining the staibility of the substances. Moreover, the properties of vehicle should have the feasibility to be used in human. Finally, the vehicle should be inert.

Solution is a basic pharmaceutical preparation especially for vaccine. The components of solution must not over the approved limits and the ingredients must be approved for the use in that specific route of administration.

For intramuscular injection, the volume of administration is limited. So solubility of active substances must be high enough to reach their effective doses. To increase solubilities of the substances, the use of co-solvent and surfactant is simple and powerful.

Solubilization by cosolvency

Cosolvency is the use of various solvent combinations to modify polarity of solvent systems and consequently affects solubilities of solutes in the solvent systems.

Co-solvents in the parenteral preparation are limited. Common co-solvents used in parenteral preparations are ethanol, propylene glycol (PG) and polyethylene glycol 400 (PEG 400) (ภารุณี ถนอมเกียรติ, 2545). In addition, the obtained candidates would be compatible with protein antigen for *in vivo* adjuvanticity test. The percentages of cosolvents used in formulation containing protein should not cause irredispersible precipitation of protein antigen. The percentages of cosolvents allowed for intramuscular injection and not causing protein precipitation are shown in Table 1.

Table 1 Percentages of cosolvents and tween 80 for intramuscular injection preparation and not precipitating protein.

Excipients	**% U.S. FDA 2011 approved for intramuscular injection**	**% not precipitate protein**	**References**
Ethanol	15 %	10 %	(Elysee-Collen and Lencki, 1996)
Propylene glycol	82 %	30-90%	Gekko and Koga, 1984
PEG 400	36 %	30%	(Atha and Ingham, 1981)
Tween 80	12 %	2 %	Arakawa and Kita, 2000

The crude extracts are poorly soluble in water and mainly are nonpolar or semipolar compounds. The parameter that usually used to indicate the polarity status of the solvents is the dielectric constant (ε). The ε of a solvent system can be calculated using the following equation.

$$\varepsilon_m = \varepsilon_{\text{solvent 1}} f_{\text{solvent 1}} + \varepsilon_{\text{solvent 2}} f_{\text{solvent 2}} + \ldots + \varepsilon_{\text{solvent n}} f_{\text{solvent n}}$$

ε = dielectric constant of each cosolvent

ε_m = dielectric constant of the mixed solvent

f = weight fraction of the cosolvent

By adjusting the proportion of each solvent in mixed solvent system, a wide range of solvent polarity can be prepared.

Solubilization by micellization

Micellization is a method for improving solubility of substances. The surfactants play an important role in solubilizing precesses by forming micelles at the concentration higher than their critical micelle concentration. Nonpolar solutes can be entrapped in the nonpolar core of micelles and semipolar compounds can be located in the palisade layer of micelles. Consequently, solubilities of these compounds are improved in micellar systems. Generally, the concentrations of surfactants that provide the micelles forming are 0.05-0.1 % (Vemula et al., 2010). This implies that using only small amount of surfactant can increase the solubilities.

In the parenteral formulation, the type and concentration of surfactants were limited. Tween 80 is a common surfactant and has been widely used in many parenteral preparations. Thus the tween 80 was selected to increase solubility of crude extract. The percentage of tween 80 that allowed to use in injectable preparation is demonstrated in table 1.

CHAPTER III
MATERIALS AND METHODS

Materials

All chemicals and reagents were analytical or pharmaceutical grade and were used as received.

1. Absolute ethanol (Lot No. K40217883 928, Merck, USA)
2. Advanved RPMI 1640 (Lot No. 1128333, Gibco®, USA)
3. Aluminium chloride anhydrous (Lot No. 2301065, Merck-Schuchardt, USA)
4. Antibiotic Antimycotic solution (100X) (Lot No. 1108200, Sigma® , USA)
5. Bovine serum albumin (Lot No. 2881C295, Amresco®, USA)
6. Carboxyfluorescein succinimidyl ester (CFSE) (Lot. No. 32670W, invitrogen™, USA)
7. Dimethyl sulfoxide (Lot No. 09010221, LAB-SCAN, Thailand)
8. Fetal Bovine serum (Lot No. 41G1780K, Gibco®, USA)
9. Folin ciocalteau reagent (Lot No. 049K0069, Sigma-aldrich, USA)
10. Formaldehyde solution 38% (Lot No. K37735447 736, AnalaR®, England)
11. Gallic acid (Lot No. 046K0131, Sigma®, China)
12. Goat anti-mouse IgG2a FITC conjugate (Lot No. 624801F, invitrogen™, USA)
13. HEPES buffer solution (Lot No. 15630, Gibco®, USA)
14. Hypodermic needle 18G x $1\frac{1}{2}$ (Lot No. 11K18, NIPRO, Thailand)
15. Iscove's modified dulbecco's medium (Lot No. 1380972, Gibco®, USA)
16. Isoprep® (Lot No. 12156620, Robbins scientific corporation, Norway)
17. Lipopolysaccharide from *Escherichia coli* O55:B5 (Lot No. 110M4086V, Sigma®, USA)
18. Lectin from *Phaseolus vulgaris* (red kidney bean), Phytohemagglutinin PHA-P (Lot No. 115K7547, Sigma®, USA)
19. 2-Mercaptoethanol 1000X (Lot No. 1397228 , Gibco®, USA)
20. Ovalbumin (Lot No. 32467, Calbiochem®, Germany)
21. PE mouse anti-human CD80 (Lot No. 11866, BD Pharmingen™, USA)

22. Phytohemagglutinin PHA-P (Lot No. SLBB3948V, Sigma®,
23. Polyethylene glycol 400 (Lot No. E132080208, chemicals of highest quality, England)
24. Potassium acetate (Lot No. 26385, M&B, England)
25. Potassium chloride (Lot No. 4B205275E, Carlo Ebra, Italy)
26. Potassium dihydrogen phosphate (Lot No. A217973 016, Merck, USA)
27. Propylene glycol (Lot No. A135094228, chemicals of highest quality, England)
28. Purified mouse anti-pig SLA-DR (Lot No. 02204, BD Pharmingen™, USA)
29. Quercetin standard (Lot No. 085K0720, Sigma®, USA)
30. Recombinant porcine Interleukine-4 (Lot No. CNM 021152, R&D system®, USA)
31. Recombinan porcine Granulocyte macrophage-colony stimulating factor (Lot No. CNM 015031 R&D system®, USA)
32. Sodium azide (Lot No. 2K K26019806 904, AnalaR®, England)
33. Sodium carbonate (Na_2CO_3) (Lot No. 1006568, UNIVAR, Australia)
34. Sodium chloride (Lot No. 6A105166A, Carlo Ebra, Italy)
35. Sodium heparin (NH) 143 USP Units Plus Blood collection tube (Lot No. 0063871, Becton, Dickinson and Company, USA)
36. di-Sodium hydrogen phosphate (Lot No. F754186 006, Merck, USA)
37. Sterile disposable syringe 10 ml (Lot No. 11A22, NIPRO , Thailand)
38. Thiazolyl blue tetrazolium bromide (Lot No. MKBB9557, Sigma®, USA)
39. Trypan blue (Lot No. 285678, Gibco®, USA)
40. Tween 80 (Lot No. 0000367734, CRODA, Thailand)

Instruments

1. Analytical balance (AX105 DeltaRange®, Mettler Toledo, Switzerland)
2. Flow cytometer (FACS Calibur™, Becton and Dickinson, USA)
3. Hemacytometer (Improve Neubauer, Precicolor Henneberg-Sander GmbH, Germany)
4. Incubator (Heracell, Heraeus kendro products, Germany)
5. Inverted microscope (CK, Olympus, Tokyo)
6. Microplate reader (VICTOR^3, Perkin Elmer, Beaconsfield, UK)
7. Rotary evaporator (BUCHI B169 vacuum-system, Switzerland)
8. Swing rotor bucket centrifuge (Sorvall Legen RT+ centrifuge, Thermo Fisher Scientific, Germany)
9. UV-visible spectrophotometer (UV-1601 UV-Visible, Shimadsu, Japan)
10. Laminar flow hood (VFRS 1206, Clanlaf®, USA)

Methods

1. **Preparation of crude extracts**

Eleven crude extracts were initially included in the study. Four crude methanolic extracts i.e. crude methanolic extracts from root barks of *Artocarpus gomezianus*, heartwood of *Artocarpus heterophyllus*, heartwood of *Dalbergia cultrata*, and root barks of *Morus alba* were obtained from the laboratory of Associate Professor Boonchoo Sritularak. Seven crude ethanolic extracts were prepared in the laboratory. The parts of seven plants including stems of *Artocarpus altilis*, flowers of *Azadirachta indica*, root barks of *Broussonetia papyrifera*, stems of *Derris reticulata*, stems of *Derris scandens*, stem barks of *Erythrina variegata* and stems of *Pithecolobium Dulce* were extracted as described below. Fresh plants were chopped and dry at 45 ˚C. Dry chopped plants were ground by course grinder. The ground plants were soaked in 95% ethanol for 7 days. The ethanolic extracts were filtered through silk to exclude any course particles and filtered again through filter paper (Whatman No.1). The filtrates were evaporated by a rotary evaporator until the solution became concentrated and viscous. The extracts were transferred into evaporating dishes and dried on water bath at 45 ˚C. The dry crude extracts were stored in desiccators and protected from light until used.

The obtained crude extracts were dissolved in DMSO at a concentration of 20 mg/ml and stored at –20°C for further studies. The stock solution was diluted to the concentrations of 0.2, 2, 10 and 20 mg/ml before used. The 0.5 µl of each concentration was incubated in 99.5 µl of cell cultures providing the total volume equal as 100 µl. The final concentrations of each crude extract were 1, 10, 50 and 100 µg/ml.

2. **Total phenolic and total flavonoid content assay**

The total phenolic content of the crude extracts was measured using Folin Ciocalteau colorimetric assay (Javanmardi et al., 2003). Briefly, 50 µl of tested substances were transferred to 10 ml test tube and 2.5 ml of 10% v/v Folin Ciocalteau Reagent was added and mixed thoroughly. After that, 2 ml of 7.5 % w/v sodium carbonate was added and the mixtures were incubated for 15 minutes at 45°C. At the

end of incubation time, the absorbance at 765 nm was read using UV-VIS spectrophotometer. The total phenolic content as mg equivalent to gallic acid was calculated from the standard curve. Gallic acid (0.2-0.8 mg/ml in DMSO) was used as a reference standard. The concentrations of crude extract tested were ranged from 5-8 mg/ml in DMSO. Each experiment was performed in triplicate.

The total flavonoid content was measured using aluminium chloride colorimetric assay (Chang et al., 2002). Briefly, 0.5 ml of tested samples were pipetted into 10 ml test tubes and the reagents were added as following order. The 1.5 ml of 95% ethanol was added and then the 0.1 ml of 10% w/v aluminium chloride in water was added. After that, 0.1 ml of 1M potassium acetate and 2.8 ml of water were added and mixed thoroughly. The mixtures were incubated for 30 minutes at room temperature. When the incubation was completed, the absorbance at 415 nm was read by UV-VIS spectrophotometer. The total flavonoid contents as mg equivalent to quercetin were calculated from the standard curve. Quercetin (15 – 100 μg/ml in 95% ethanol) was used as a reference standard. The concentrations of crude extracts were ranged from 5-8 mg/ml in DMSO. Each experiment was performed in triplicate.

3. Peripheral Blood Mononuclear Cells (PBMCs) isolation

The pig's blood was collected by veterinarian. Pig would be restrained and the head was hold up. The needle with attached syringed was inserted into the jugular vein for collecting of the blood. The needle was withdrawn and injected into the blood collecting vaccutainer. The blood was brought to the laboratory within 2-3 hours. The process of PBMCs isolation was performed according to Yeap et al. 2007. Briefly, 10 ml of porcine blood was gently added into 10 ml of phosphate buffered saline (PBS). Ten ml of the mixture was softly overlaid on the surface of 5 ml isoprep in 15 ml conical tube, centrifuged at 2500 rpm 30 minutes at 20°C in a swing rotor bucket, After that, the middle white band of PBMCs was collected and transferred to a conical tube. The PBMCs were washed three times with PBS. At the second times of washing, the contaminated red blood cells were lysed by adding 2 ml steriled deionized water into the PBMCs pellets and incubating for 1 minute or until the red colour disappeared. After that the 2 ml of 2X PBS was added to stop the cell lysis and further 11 ml PBS was added. After the third times of washing, 900 μl of 10%

advanced complete RPMI 1640 media was added to make a 1000 µl final volume. The PBMCs were stained with trypan blue for the detection of cell viability. PBMCs with the percent cell viability of more than 90 % would be allowed to be used in the studies. The yield of porcine PBMCs isolation was 1 – 2 x 10^6 cells/ml of whole blood.

4. Cytotoxicity test by MTT assay

To find the safe concentration range of each crude extract to PBMCs, the PBMCs viability assay was performed, using the method of MTT assay (Yeap et al., 2007). Briefly, 11 crude extracts at the concentration of 1, 10, 50 and 100 µg/ml were initially tested for their cytotoxicity to PBMCs. After PBMCs isolation, the cells suspension was adjusted to 2 x 10^6 cells / ml of 10% advanced complete RPMI 1640 media, then seeded 50 µl of PBMCs suspension into each well of a 96-well-flatted bottom plate containing 49.5 µl of 10% advanced complete RPMI 1640 media. Then 0.5 µl of crude extract solution of 0.2, 2, 10 and 20 mg/ml were added into each well to make a final concentration of 1, 10, 50 and 100 µg/ml respectively. Every final concentrations - 1, 10, 50 and 100 µg/ml - of each crude extracts in 10% advanced completed RPMI 1640 without PBMCs were used as blank. PBMCs were incubated with crude extracts in 5 % CO_2 incubator at 37 °C, for three days. After three days, 20 µl of 5 mg/ml MTT solution in PBS was added into each well and further incubated for 4 hours then centrifuged at 4,500 rpm 5 minutes and decanted 70 µl of supernatant. After that, the 100 µl DMSO was added and the solution was measured for the absorbance at 570 nm using microplate reader. The absorbance of each well was subtracted from the absorbance of its blank before the % cell viability was calculated using the equation below:

$$\% \text{ viability} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

The crude extracts concentration that did not toxic to PBMCs would be used to screen the effect to lymphocyte proliferation by CFSE dye dilution assay.

5. PBMCs proliferation by CFSE dye dilution assay

The assay was conducted using the method of Parish and Warren, 2002. Briefly, 20 ml of porcine blood was subjected to PBMCs isolation. The allogeneic PBMCs obtained from all tube in the density gradient centrifugation step using isoprep, will be assembled and processed further as described. At last step, the 900 µl of 5% v/v FBS in PBS was added and the total 1000 µl of PBMCs suspension was transferred into new 15 ml conical tube. The 110 µl of 50 µM carboxyfluorescein diacetatesuccinimidyl ester (CFSE) were added to the PBMCs suspension and immediately vortex. The PBMCs were incubated in the dark at room temperature for 5 minutes. The 5% fetal bovine serum in 10 ml PBS was added to stop the staining. CFSE stained PBMCs were washed three times with PBS and counted by hemacytometer. The CFSE stained PBMCs were seeded in 96-well flatted bottom plate at 5 x 10^5 cells per well in 200 µl of 10% advanced completed RPMI 1640 and stimulated with crude extracts. The 5 µg/ml phytohemagglutinin (PHA) and the 10% advanced completed RPMI 1640 were used as a positive control and a negative control, respectively. The unstained PBMCs were seeded and cultured for use as compensation tube. The PBMCs were incubated in a 5% CO_2 incubator at 37°C for three days. After three days, the cultures were washed three times with PBS and were fixed with 400 µl of 2% formaldehyde. The fixed PBMCs were measured for the CFSE fluorescence intensity by flow cytometer. Unstained PBMCs and Non-stimulated CFSE-stained PBMCs were used to adjust the voltage of flow cytometer. The lymphocyte population was selected from forward scatter and side scatter dot plot pattern and the % proliferated lymphocyte were used to calculated the proliferative index as the equation below :

$$\text{proliferative index} = \frac{\text{\% proliferated lymphocyte of sample}}{\text{\% proliferated lymphocyte of control}}$$

The crude extracts that significantly enhance the proliferative index of PBMCs will be selected for studying the effect to MoDCs maturation.

6. Generation of immature monocyte-derived dendritic cells (MoDCs)

The method of Dauer et al, 2003 was used for MoDCs generation. At least, 30 ml of porcine blood was subjected to PBMCs isolation. The allogeneic PBMCs obtained from all tubes in the density gradient centrifugation step using isoprep, would be assembled in a 50 ml conical tube and processed further until counting. The PBMCs were resuspended in IMDM 25 x 10^6 cells/ml and then seeded into six well plate as 1 ml per well. The PBMCs were incubated in 37°C 5% CO_2 for 2 hours to allow the adherence of monocytes. After that, non adherent lymphocytes were removed by gently pipetting. The adherent monocytes were washed with PBS (900 µl/well) for four times. The 50 ng/ml of IL-4 and 50 ng/ml of GM-CSF in 10% advanced completed RPMI 1640 were added into each well in 1000 µl per well and incubated in incubator for 2 days. The immature MoDCs were harvested and resuspended in 10% advanced complete media for 5 x 10^5 cells/ml. The success of derivation was determined by microscopic characteristic and MHC class II and CD80 expression level on immature MoDCs.

7. Maturation of MoDCs by immunophenotypic assay

The crude extracts selected from CFSE dye dilution assay were tested for the effect to the maturation of MoDCs, a major cellular immune system that highly potent in induction of specific immune response by using of major histocompatibility class II (MHC II) and costimulatory molecules (CD80) which would up regulate at mature state.

To detect the expression of MHC Class II and CD 80, the method of Park et al, 2008 were followed. Immature MoDCs in 10% advanced complete media at 5 x 10^5 cells/ml, 200 µl were seed into 96-well flatted bottom plate and added with crude extracts at specific concentration. The 10 µg/ml lipopolysaccharide and 10% advanced complete RPMI 1640 were used as positive control and control, respectively. The MoDCs were incubated in 37°C 5% CO_2 for 24 hours. After incubation the MoDcs were washed with PBS and FACs buffer. The primary antibodies, PE-anti-human CD80 (anti CD80) and mouse IgG2a anti-pig SLA-DR (anti SLA-DR) were diluted 1in FACs buffer at the ratio of 1 : 1 : 98 of anti CD80 :

anti MHC II : FACs buffer. Fifty μl of the primary antibodies solution were added to each well and incubated in 4°C for 30 minutes. After that, the pellets were washed two times with FACs buffer prior to add the 50 μl of secondary antibody, 1:100 of goat anti-mouse IgG2a FITC conjugate in FACs buffer. The pellets were incubated in 4°C for 30 minutes and then were collected and washed two times by FACs buffer. The 400 μl of 2% formaldehyde was added to fix the MoDCs. The MoDCs can be kept in 4°C and protected from light before analysis by flow cytometer. The fluorescent intensity of fluorochromes responding to cell surface molecule will be measured.

8. Preparation of crude extract and antigen solution

The crude extract and a model antigen, ovalbumin, were prepared as a suitable solution for intramuscular injection. Solubilization by cosolvency and micellization were employed to improve solubility of the crude extract. The solvent system should be able to dissolve the extract greater than 20 mg/ml because this concentration will be used for preparing a suitable dosage for injection. The process for determination of proper solvent system is described below.

8.1 Solubilization by cosolvency

The extract was dissolved very well in dimethyl sulfoxide (DMSO). However, DMSO is not permitted in injection dosage form due to its toxicity. Ethanol, propylene glycol (PG) and PEG 400 were used for preparing cosolvent system. Dielectric constant (ε) of the cosolvent system was calculated and the system providing ε close to DMSO was selected as shown in Table 2. Moreover, concentrations of these solvents were not more than concentration limits for injection.

Table 2 Percentage of cosolvents and calculated dielectric constant of solvent system.

Solvent system	Water % w/w	Ethanol % w/w	PG % w/w	PEG 400 % w/w	ε
1	36.5	15	12.5	36	43.65

The crude extract was gradually added in the solvent system and the solution was sonicated for 5 minutes. The solubility was recorded at the concentration before the precipitation observed.

8.2 Solubilization by cosolvency and micellization

As previously reported, tween 80 prevents the aggregation of protein and also improves solubility of non polar compounds (Arakawa and Kita, 2000). Solubilities of the crude extract were determined in the solvent systems as shown in table 3.

Table 3 Percentage of cosolvents and surfactant in solvent systems.

Solvent System	Water % w/w	Ethanol % w/w	PG % w/w	PEG 400 % w/w	Tween 80 % w/w
2	31.5	15	12.5	36	5
3	50	10	8	30	2

The crude extract was added in the solvent systems and solubilities were determined as previously mentioned in 8.1. Then one mg/ml of ovalbumin solution was prepared in the solvent systems by dissolution in the following order. Ovalbumin was dissolved in water and then mixture of other solvents (ethanol, PG, PEG 400) was gradually added. The ovalbumin solution was monitored for any change after each solvent mixture addition. The solvent system providing no ovalbumin precipitation was chosen for further study.

8.3 Evaluation of ovalbumin and crude extract in the solvent system

The crude extract and ovalbumin were incorporated into the same suitable solvent system that was selected from the previous experiment. One mg of ovalbumin was dissolved in water and then the mixture of other solvents (ethanol, PG, PEG400) was gradually added until the total weight was 1000 mg. Finally, the crude extract 20 mg was added and sonicated. The microscopic appearance of the solution was observed.

9. Analysis of Data

The results are expressed as means ± S.D. Difference in mean values between groups were analyzed by a one-way analysis of variance (ANOVA) for randomized block design. The homogeneity of variance were analyzed by Levene's test followed by a post-hoc test using Dunnett test in case of unequal variance or Tukey HSD test in case of equal variance. Statistical significance was assessed as *p value* = 0.05.

CHAPTER IV
RESULTS

1. Preparation of crude extracts

The percent yields of the extraction are shown in Table 4.

Table 4 Percent yields of the ethanolic extraction of plant materials.

Thai name	Scientific name	Part	Crude extract (g)	Dry plant (g)	% yield
สาเก	*Artocarpus altilis*	Stem	13.62	674.80	2.02
สะเดา	*Azadirachta indica*	Flower	8.08	186.77	4.32
ปอกระสา	*Broussonetia papyriferra*	Root bark	5.33	65.28	8.16
ชะเอมเหนือ	*Derris reticulata*	Stem	12.37	218.39	5.66
เถาวัลย์เปรียง	*Derris scandens*	Stem	44.44	1178.39	3.77
ทองหลางด่าง	*Erythrina variegata*	Stem bark	5.06	162.00	3.13
มะขามเทศ	*Pithecolobium dulce*	Stem	3.87	343.22	1.13

From the table, the plant material that providing the highest yield of crude ethanolic extract was the root barks of *Broussonetia papyriferra* (8.16 %), followed by the stems of *Derris reticulata* (5.66 %) and the flowers of *Azadirachta indica* (4.32%). The percent yield of other plant was lower than 4 %. All crude extracts were prepared into 20 mg/ml in DMSO except the extract from the stem of *Derris reticulata*. The extract from the stem of *Derris reticulata* were prepared into 14.85 mg/ml in DMSO.

2. Total phenolic and total flavonoid content

The total phenolic content (TPC) and total flavonoid content (TFC) of crude extracts are summarized in Table 5.

Table 5 The total phenolic and total flavonoid content of crude extracts

Thai name	Scientific name	Part used	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QUE/g)	% of TFC in TPC
สาเก	*Artocarpus altilis*	Stem	48.26 ± 4.53	8.22 ± 0.03	17.03
หาดหนุน	*Artocarpus gomezianus*	Root bark	264.17 ± 16.52	20.95 ± 0.52	7.93
ขนุน	*Artocarpus heterophyllus*	Heartwood	344.59 ± 15.92	189.79 ± 11.18	55.08
สะเดา	*Azadirachta indica*	Flowers	69.8 ± 1.30	21.84 ± 0.20	31.29
ปอกระสา	*Broussonetia papyriferra*	Root bark	92.91 ± 3.06	47.42 ± 5.21	51.04
กระพี้ควาย	*Dalbergia cultrata*	Heartwood	173.19 ± 2.03	22.08 ± 0.45	12.75
ชะเอมเหนือ	*Derris reticulata*	Stem	78.46 ± 5.63	38.04 ± 10.30	48.48
เถาวัลย์เปรียง	*Derris scandens*	Stem	163.54 ± 7.32	22.46 ± 2.56	13.73
ทองหลางด่าง	*Erythrina variegata*	Stem bark	106.62 ± 6.83	16.31 ± 1.69	15.30
หม่อน	*Morus alba*	Root bark	190.65 ± 6.19	23.55 ± 0.94	12.35
มะขามเทศ	*Pithecolobium dulce*	Stem	77.35 ± 2.02	4.46 ± 0.26	5.77

mg GAE/g = mg of gallic acid equivalent to g of crude extract
mg QUE/g = mg of quercetin equivalent to g of crude extract

The highest total phenolic content found in the crude methanolic extract from *Artocarpus heterophyllus* (344.59 ± 15.92 mg GAE/g) followed by the extract from *Artocarpus gomezianus* (264.17±16.52 mg GAE/g). The rest of the crude extracts contained the total phenolic content below 200 mg GAE/g.

For total flavonoid content, the highest content belong to crude methanolic extract from *Artocarpus heterophyllus* (189.79 ± 11.18 mg QUE/g) followed by the extract from *Broussonetia papyriferra* (47.42 ± 5.21 mg QUE/g). The total flavonoid content of the crude extracts from the other plants was lower than 40 mg QUE/g.

3. Cytotoxicity of crude extracts

The effect of crude extracts on the vitality of PBMCs is shown in Figure 1. Cells were exposed to 1, 10, 50 and 100 μg/ml crude extracts for 72 hrs in normal culturing condition. MTT results indicated that crude extracts from *Artocarpus gomezianus*, *Dalbergia cultrata* and *Erythrina variegata* were not toxic to PBMCs in all concentration tested. In contrast, the crude extracts from *Artocarpus gomezianus* and *Dalbergia cultrata* at the concentration of 100 μg/ml significantly enhanced the percentage of cell viability compared with that of cells treated with 1, 10, and 50 μg/ml crude extracts (*p-value* < 0.05). These results suggested that the extracts from *Artocarpus gomezianus* and *Dalbergia cultrata* not only non-toxic to the cells, but also enhanced the vitality of PBMCs at high concentrations. The crude extracts from *Broussonetia papyriferra*, *Artocarpus altilis*, *Artocarpus heterophyllus*, and *Morus alba* caused a decreased in PBMCs viability as increasing concentration. The crude extracts from *Pithecolobium dulce*, *Derris reticulata*, *Derris scandens* and *Azadirachta indica* were toxic to the PBMCs at all concentration tested thus were excluded from the study. The crude extracts concentration of 50 μg/ml (*Artocarpus altilis*, *Artocarpus heterophyllus*, *Broussonetia papyriferra*, and *Morus alba*) and 100 μg/ml (*Artocarpus gomezianus*, *Dalbergia cultrata*, and *Erythrina variegata*), which were the maximum non-toxic concentrations, were chosen for the proliferative study by CFSE dye dilution assay. In addition, all crude extracts at 1 μg/ml, which is the minimum non-toxic concentration, were also included to the CFSE assay.

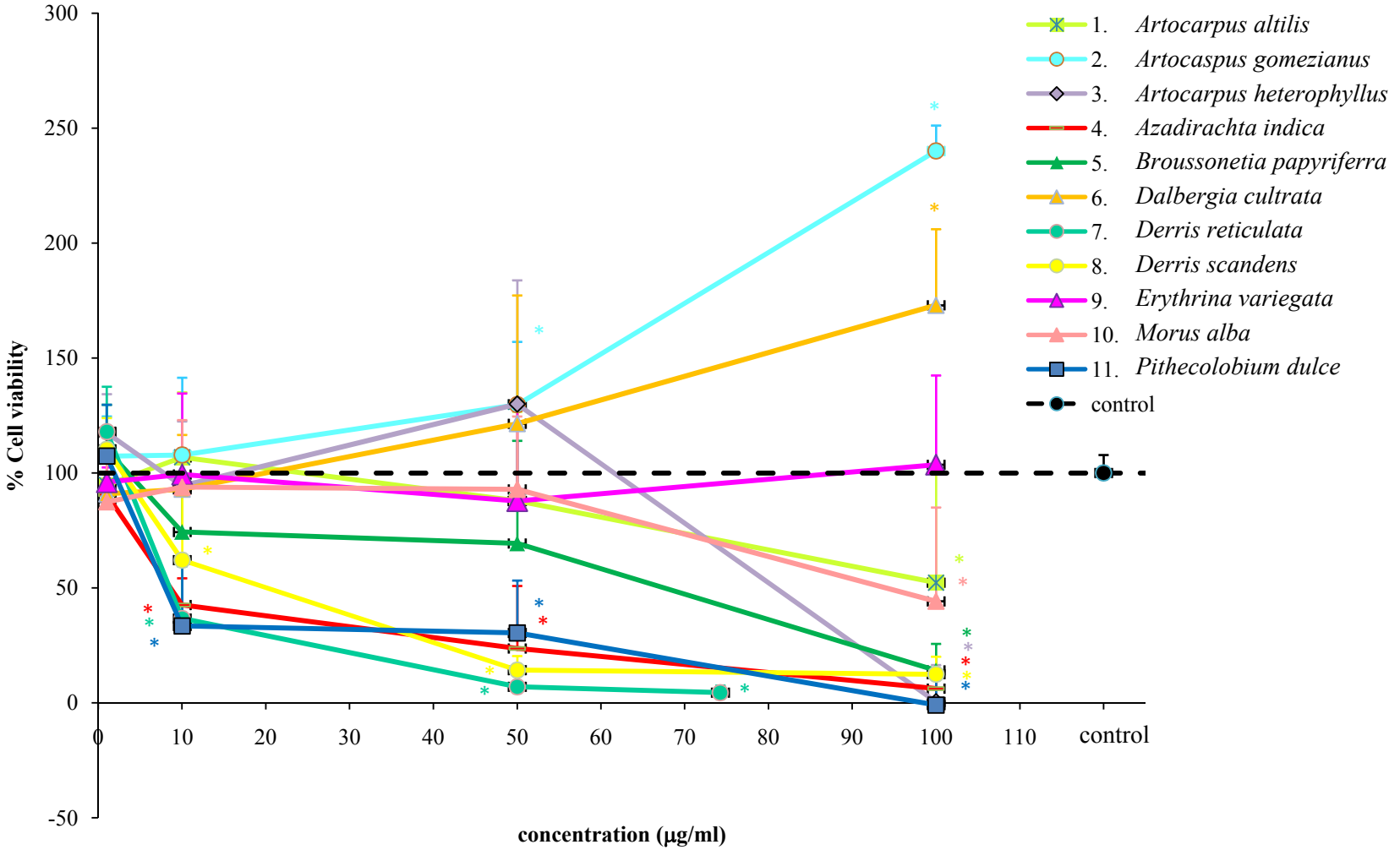


Figure 1 The percent cell viability of PBMCs incubated with 11 crude extracts at 1, 10, 50 and 100 µg/ml for 3 day. * indicate the significantly different from control group at $p < 0.05$, n = 5

4. PBMC proliferation by CFSE dye dilution assay

The effect of crude extracts on the proliferation of PBMCs is shown in Table 6 and figure 2. The crude extracts of concentration 1 and 50 μg/ml (*Artocarpus altilis*, *Artocarpus heterophyllus*, *Broussonetia papyriferra*, and *Morus alba*) and of concentraion 1 and 100 μg/ml (*Artocarpus gomezianus*, *Dalbergia cultrata*, and *Erythrina variegata*) were exposed to the CFSE stained cells for 72 hrs in normal culturing condition. CFSE results indicated that crude extracts from *Artocarpus gomezianus* and *Dalbergia cultrata* had a potential to induce the proliferation of PBMCs at the concentration 100 μg/ml. These proliferation results were in accordance with the percent cell viability in MTT assay. In contrast, the crude extracts from *Erythrina variegata* (100 μg/ml) and *Artocarpus heterophullus* (50 μg/ml) tended to reduce the proliferative index without causing a decrease in viability of PBMCs. The crude extracts from *Broussonetia papyriferra* and *Morus alba* did not increase the proliferation of the PBMCs at both concentration tested. The crude extracts at 100 μg/ml of *Artocarpus gomezianus* and *Dalbergia cultrata* were chosen to study the effect to the maturation of MoDCs. The dendritic cell maturation is the key event in regulating and amplifying the specific immune response.

Table 6 The proliferative indices of PBMCs incubated with crude extract at lowest and highest concentration that is not toxic to the cells.

Types of plant	concentration (μg/ml)	Proliferative indices	*p* value
Artocarpus altilis	1	1.12 ± 0.18	0.80
	50	1.10 ± 0.20	0.95
Artocarpus heterophyllus	1	1.06 ± 0.14	0.84
	50	0.50 ± 0.48	0.55
Broussonetia papyriferra	1	0.84 ± 0.05	0.22
	50	1.02 ± 0.22	1.00
Morus alba	1	1.00 ± 0.02	1.00
	50	0.80 ± 0.40	0.94
Artocarpus gomezianus	1	1.00 ± 0.10	1.00
	100	1.83 ± 0.41	<0.01*
Dalbergia cultrata	1	1.01 ± 0.15	1.00
	100	1.51 ± 0.15	<0.01*
Erythrina variegata	1	1.15 ± 0.19	0.74
	100	0.19 ± 0.32	0.14
PHA	5	2.10 ± 0.73	<0.01*

* indicate the significantly different from control group at $p < 0.05$, n=5

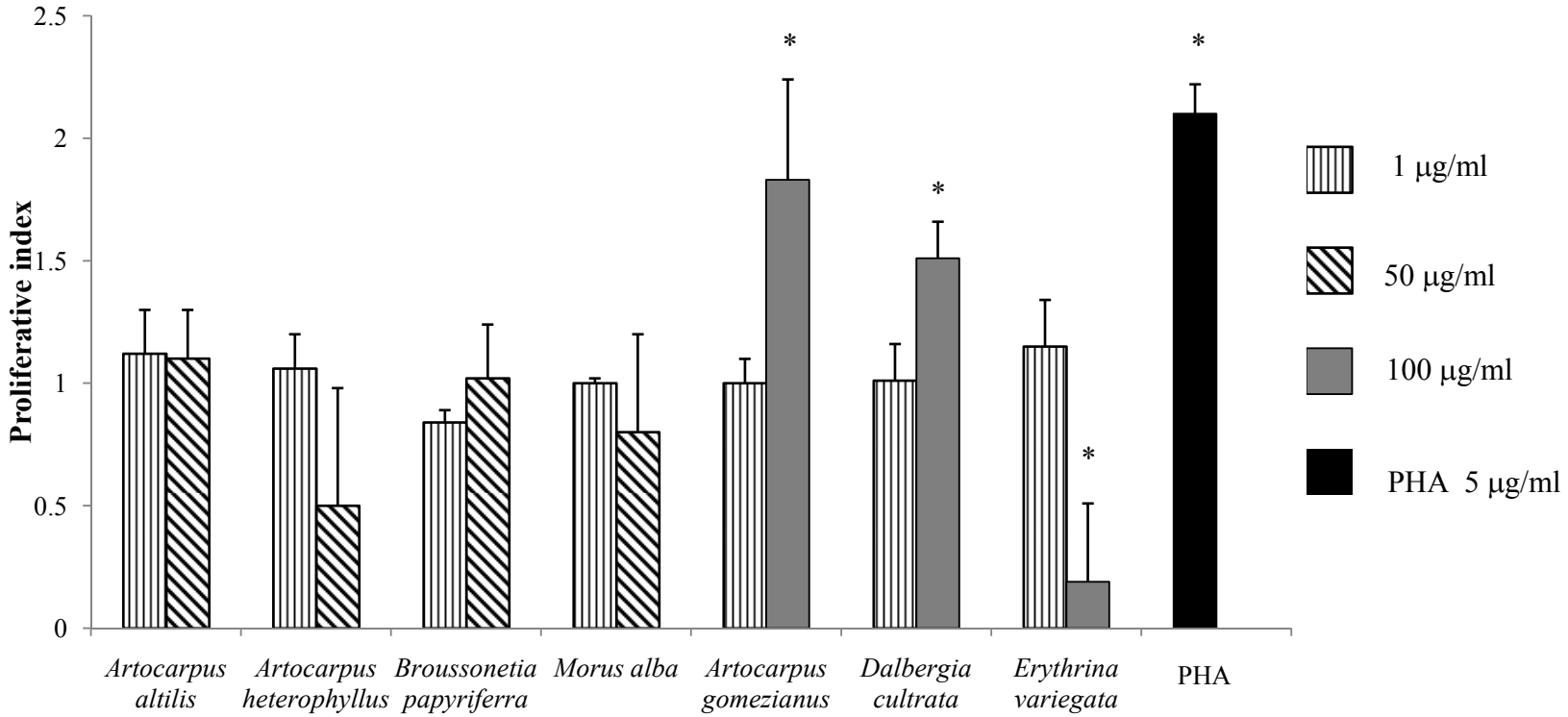


Figure 2 The proliferative indices of the PBMCs incubated with crude extract at the concentration 1 and 50 μg/ml
* indicate the significantly different from control group at $p < 0.05$, n=5

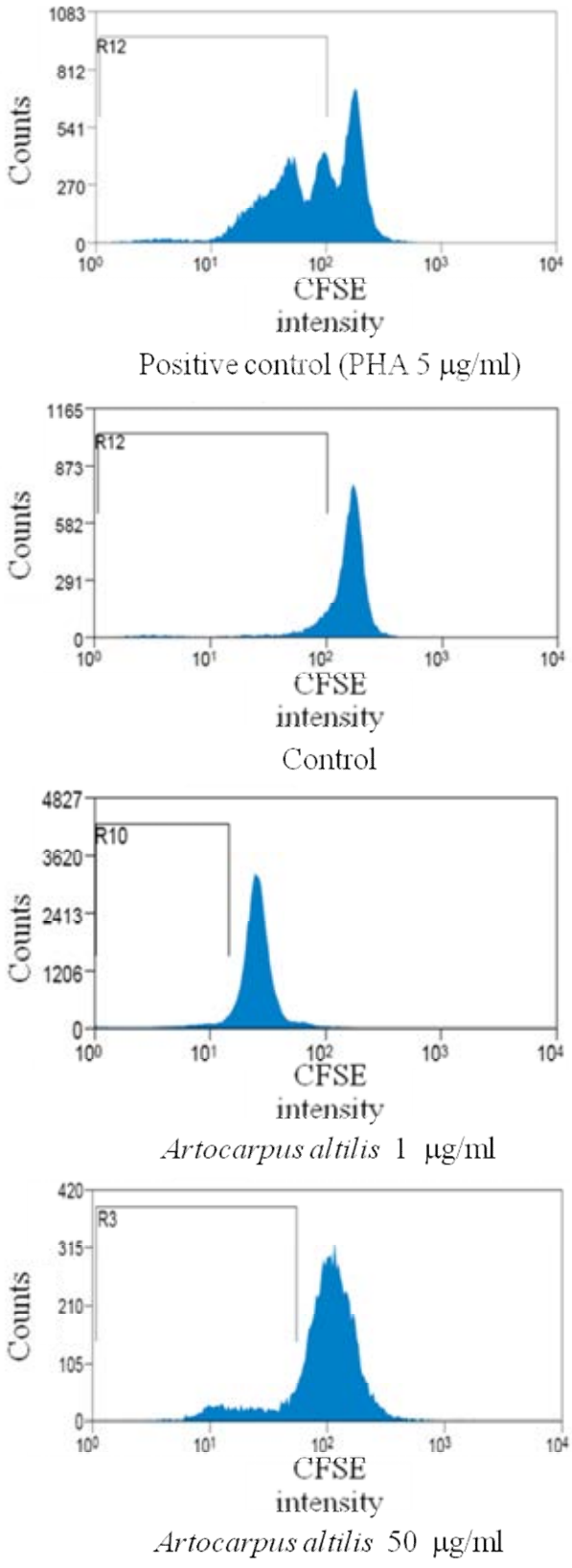
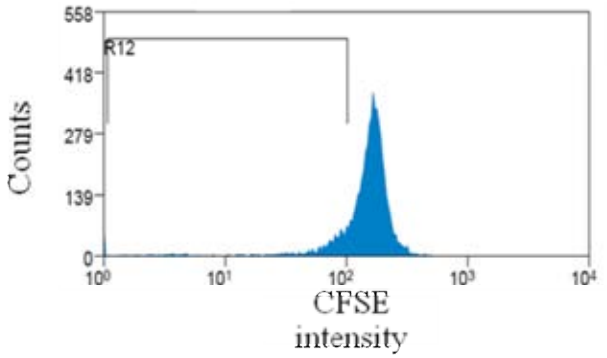
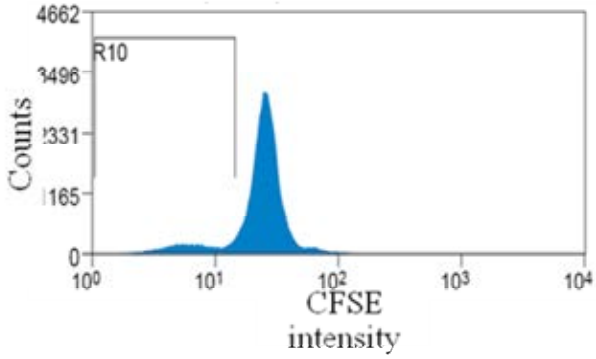


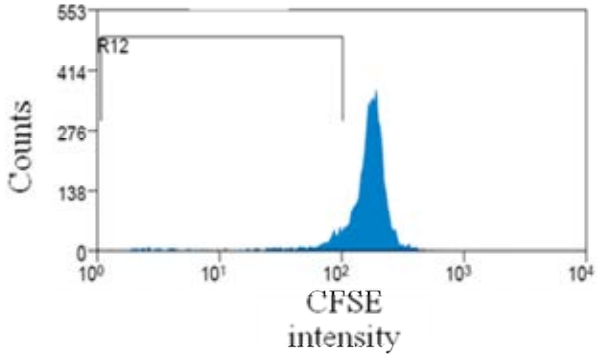
Figure 3 The flow cytometric histogram of CFSE stained PBMCs incubated with each crude extracts, PHA (positive control) and control. The bar markers ⌐¬define the region of proliferated lymphocytes.



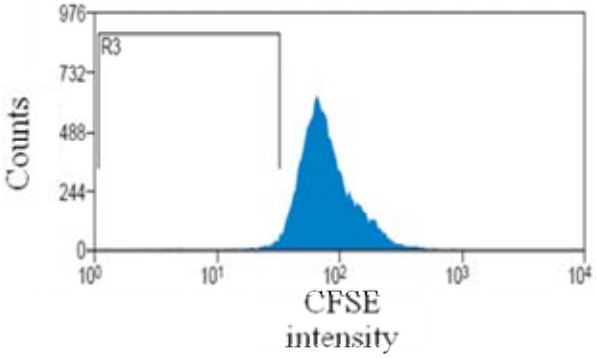
Artocarpus heterophyllus 1 μg/ml



Artocarpus heterophyllus 50 μg/ml

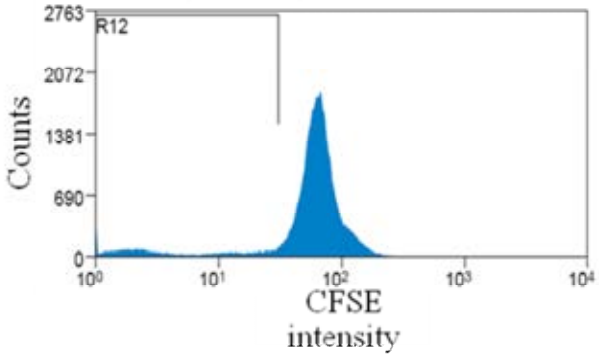


Broussonetia papyriferra 1 μg/ml

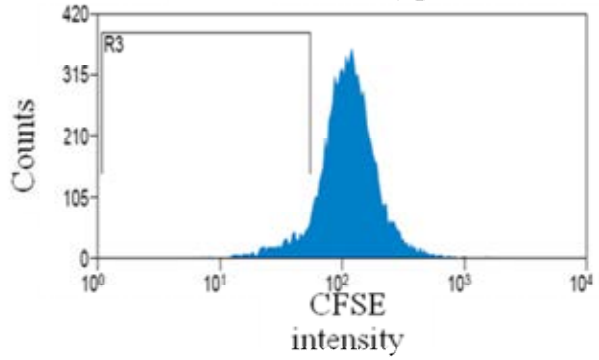


Broussonetia papyriferra 50 μg/ml

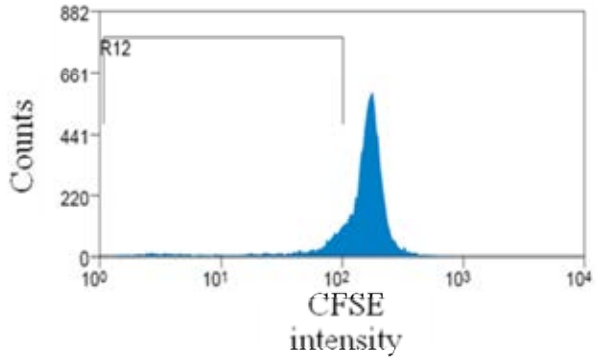
Figure 4 The flow cytometric histogram of CFSE stained PBMCs incubated with each crude extracts. The bar markers ⌐¬ define the region of proliferated lymphocytes.



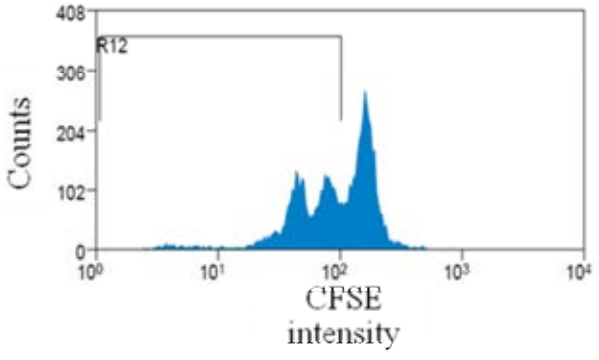
Morus alba 1 μg/ml



Morus alba 50 μg/ml



Artocarpus gomezianus 1 μg/ml



Artocarpus gomezianus 100 μg/ml

Figure 5 The flow cytometric histogram of CFSE stained PBMCs incubated with each crude extracts. The bar markers ⌐¬ define the region of proliferated lymphocytes.

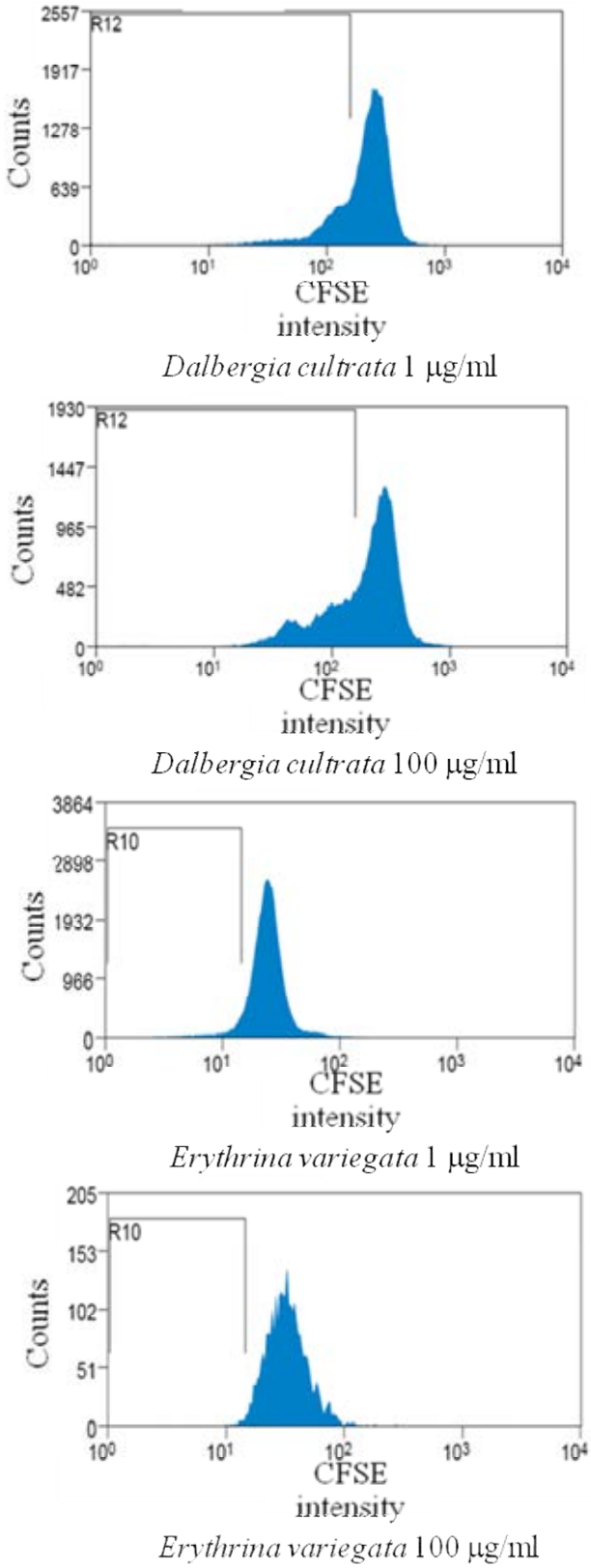


Figure 6 The flow cytometric histogram of CFSE stained PBMCs incubated with each crude extracts. The bar markers ⌐¬ define the region of proliferated lymphocytes.

5. Generation of immature MoDCs

The characteristic of MoDCs were confirmed by the expression of the co-stimulatory molecules and the appearance of dendrite-like cells. After the incubation of monocytes with IL-4 and GM-CSF for 48 hrs, the characteristic of monocytes was changed from round shape to den-drite shape, which is the special characteristic of dendritic cells. Moreover, when measure the expression of MHC class II and CD80 by flow cytometric immunophenotyping, the expression of the molecules were significantly higher than the monocytes ($p < 0.05$), showing the properties of dendritic cells.

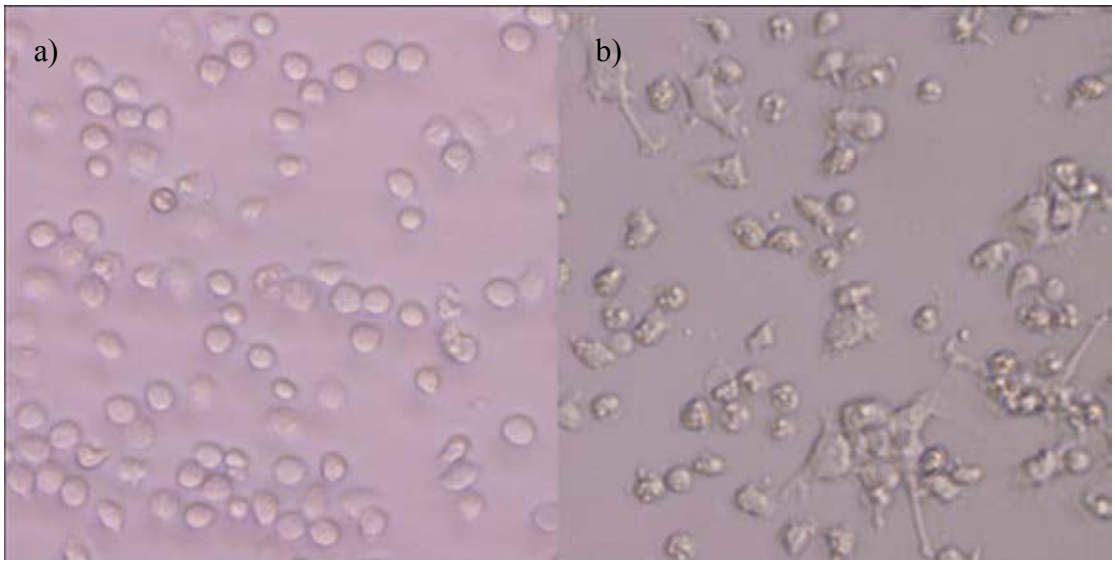


Figure 7 The monocytes (a) and the immature MoDCs (b) microscopic characteristics.

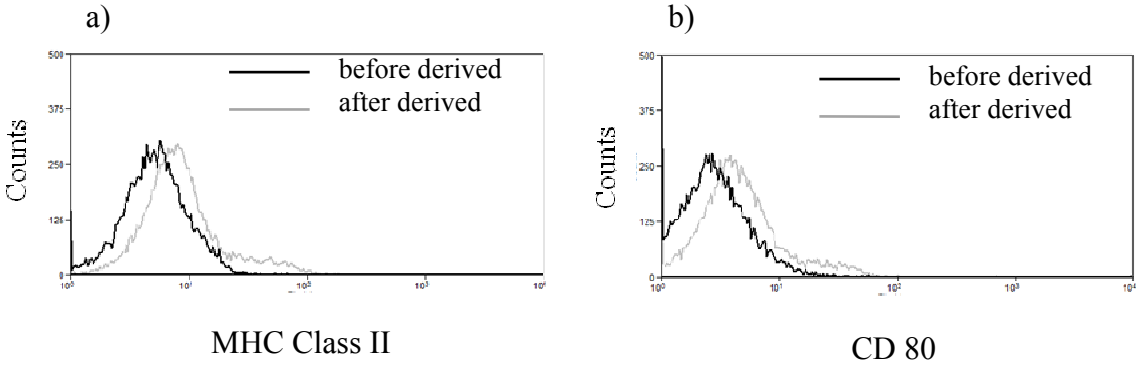


Figure 8 The histogram of MHC Class II (a) and CD80 (b) expression on MoDCs from different state of differentiation.

6. Maturation of MoDCs by immunophenotypic assay

The stimulation effect of the crude extracts from root barks of *Artocarpus gemezianus* and heartwood *Dalbergia cultrata* on the expression of MHC Class II and CD80 on MoDCs surface are shown in figure 9.

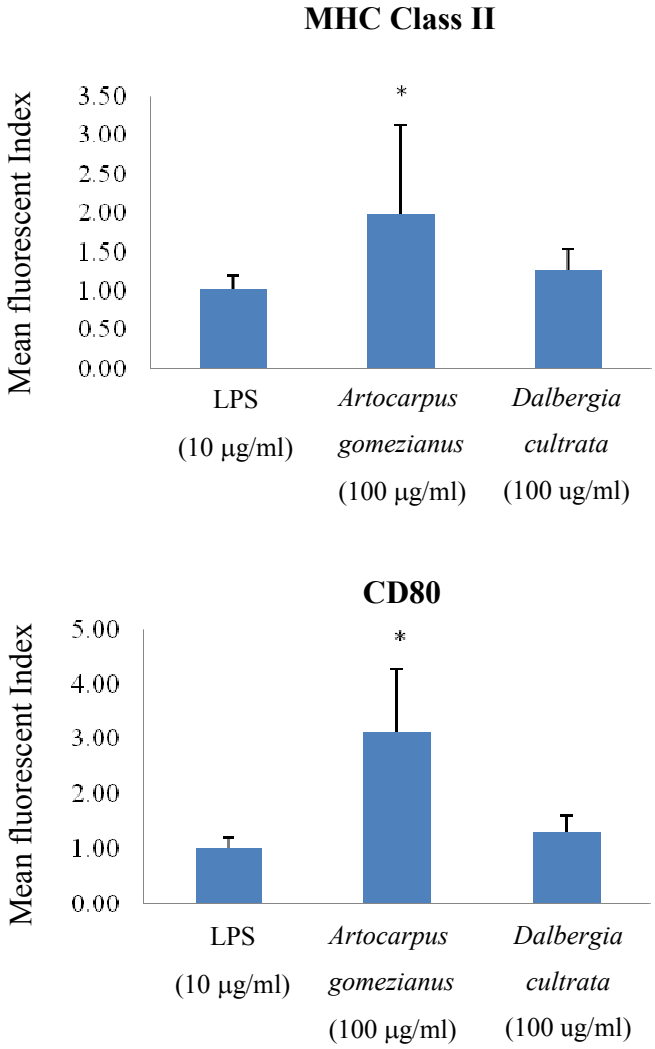


Figure 9 Mean fluorescent intensity of MHC Class II expression and CD80 expression.
* indicate the significantly different from control group at $p < 0.05$, n=5

MoDCs incubated with the crude extract from root barks of *Artocarpus gomezianus* (100 µg/ml) showed higher expression of MHC Class II and CD80 than the positive control (LPS 10 µg/ml), control and the crude extract from heartwood of *Dalbergia cultrata* (100 µg/ml).

7. Preparation of crude extract solution and antigen solution

The crude extract of root barks from *Artocarpus gomezianus* and ovalbumin, were prepared as a solutio. The results are described below.

7.1 Solubilization by cosolvency

The solubility of the crude extract in the cosolvent system 1 is 15.86 mg/ml (Table 7).

Table 7 Solubility of crude extract in solvent system 1

Solvent System	Water % w/w	Ethanol % w/w	PG % w/w	PEG 400 % w/w	Solubility (mg/ml)
1	36.5	15	12.5	36	15.86

7.2 Solubilization by cosolvency and micellization

The solubilities of the crude extract and the ovalbumin antigen are demonstrated in Table 8. The solubilities of crude extract in solvent system 2 and 3 are higher than 20 mg/ml. However, the solutions become turbid when ovalbumin was added in. In solvent system 2, irredispersible precipitation of the antigen was observed. Redispersible coarse dispersion was found in system 3.

Table 8 Solubilities of crude extract and appearance of solvent systems containing 1 mg/ml ovalbumin.

Solvent system	**Water % w/w**	**Ethanol % w/w**	**PG % w/w**	**PEG 400 % w/w**	**Tween80 % w/w**	**Solubility of crude extract (mg/ml)**	**Appearance of 1 mg/ml ovalbumin in solvent system**
2	31.5	15	12.5	36	5	87.48	Precipitate, not redispersible
3	50	10	8	30	2	29.72	Redispersible coarse dispersion

7.3 Evaluation of ovalbumin and crude extract in the solvent system

The microscopic appearance of the crude extract and ovalbumin in the solvent system 3 are shown in figure 10.

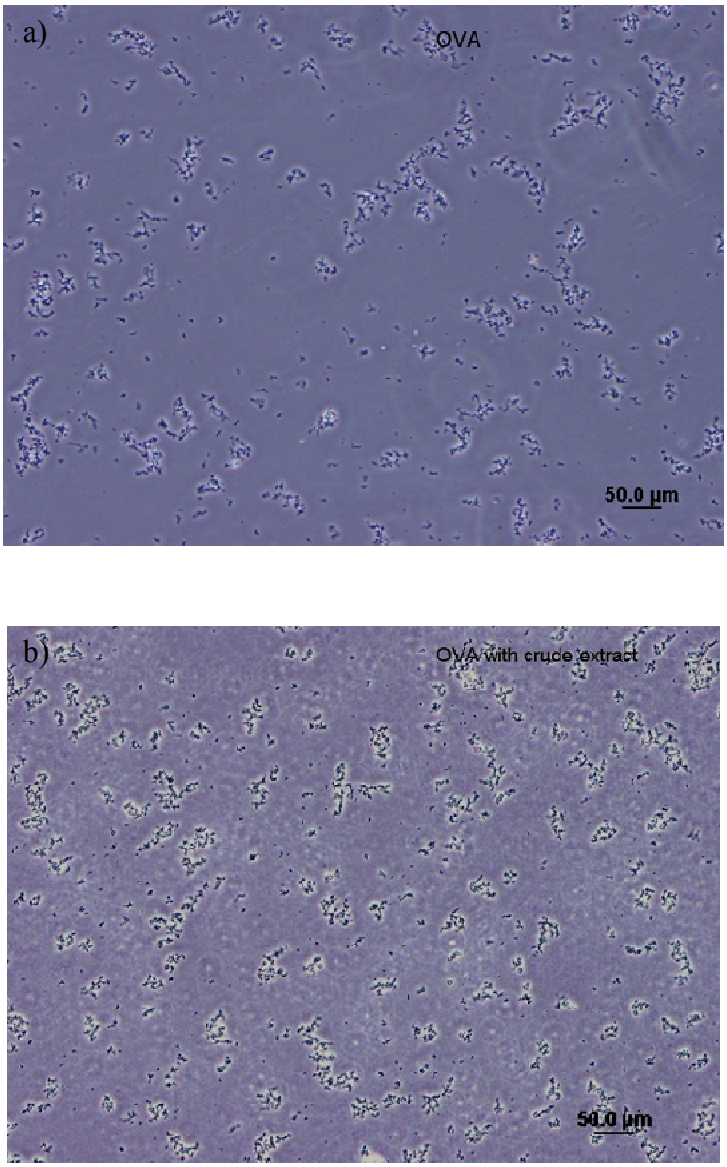


Figure 10 Microscopic characteristic of ovalbumin alone in the solvent system 3 (a) and ovalbumin and crude extract in the solvent system 3 (b)

CHAPTER V
DISCUSSION AND CONCLUSIONS

To screen for the immunopotentiated activity, several methods were reported such as antibody production assay, lymphocyte proliferation assay, phagocytic activity assay, and maturation of antigen presenting cells assay (Chen et al., 2012; Li et al., 2012; Wang et al., 2012). The lymphocyte proliferation assay was the traditional method for screening of the immuopotentiator. The PBMCs are the mixture of immune cells including lymphocytes (85-90%) and monocytes (10-15%) (Netea et al., 2006). The components of PBMCs simulate the immune system that function in the adaptive immune response induction (Pastoret et al., 1997). From the current knowledge, the dendritic cells are a professional presenting cells that are very powerful in inducing adaptive immune response and are the major target of many known immunopotentiators (Millard et al., 2003). One dendritic cell can activate a million of T lymphocytes. Dendritic cells locate in every portal of pathogen entry. The cells are waiting to capture the antigens. After antigen capture, the cells become mature and move to lymph node to present antigen to lymphocytes and regulate the generation of the adaptive immune responses. The mature dendritic cells express high density of several molecules, such as MHC class II, CD80, CD83 and CD86, which are necessary for antigen presentation leading to the generation of adaptive immunity (Figdor et al., 2004). To screen for the appropriate immunopotentiator, the determination of the effect of crude extract on the dendritic cell maturation and lymphocyte proliferation is appropriate (Schijns, 2000; Steinman and Pope, 2002; Schijns, 2003).

Eleven crude extracts from different plants containing phenolic compounds were initially tested for their cytotoxicity by MTT assay. Crude extracts from plants that were not toxic to the cells were further tested for the activity to stimulate the PBMCs proliferation by CFSE dye dilution assay.

The results of cell proliferation by CFSE assay were relevant to the MTT assay. There are two out of eleven crude extracts, which showed the potential to increase the proliferation of PBMCs. Both are crude extract from root barks of

Artocarpus gomezianus (100 μg/ml) and crude extract from heartwood of *Dalbergia cultrata* (100 μg/ml). These crude extracts significantly increased both the viability and the proliferation of PBMCs. The abilities of the crude extract from *Dalbergia cultrata* (100 μg/ml) and crude extract from *Artocarpus gomezianus* (100 μg/ml) to induce PBMCs proliferation by CFSE assay were 50% and 80% higher than the untreated control respectively. However, in the study of the maturation of dendritic cells, only crude extract from root barks of *Artocarpus gomezianus* possessed the ability to induce the maturation of dendritic cells. The *Artocarpus gomezianus* increased the expression of MHC class II and CD80 for 50 % higher than control whereas the *Dalbergia cultrata* did not increase the expression of MHC Class II and CD80. So, this implied that the crude extract from root barks of *Artocarpus gomezianus* have potential to be an immunopotentiator.

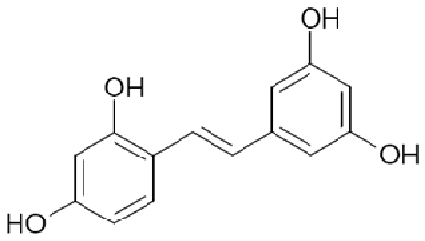
The effect of LPS to porcine MoDCs maturation could not be detected as apparent as human MoDCs (Ardeshna et al., 2000; Sonck et al., 2011). However, there is another parameter on which the LPS could impact. The parameter is the cytokine secretion. Thus, further assay of the maturation of porcine MoDCs by measuring of the level of cytokine by ELISA should be done.

Generally, the plants that were reported as an immunopotentiator usually constituted of polyphenolic compounds in particular flavonoid substances. From the results of total phenolic content, five crude extracts i.e. crude extract from heartwood of *Artocarpus heterophyllus*, root barks of *Artocarpus gomezianus*, root barks of *Morus alba*, heartwood of *Dalbergia cultrata* and stems of *Derris reticulata*, contained high total phenolic content (> 150 mg GAE/g). But only two out of five crude extracts, *Artocarpus gomezianus* and *Dalbergia cultrata*, possessed the immunostimulatory activity i.e. the PBMCs proliferation.

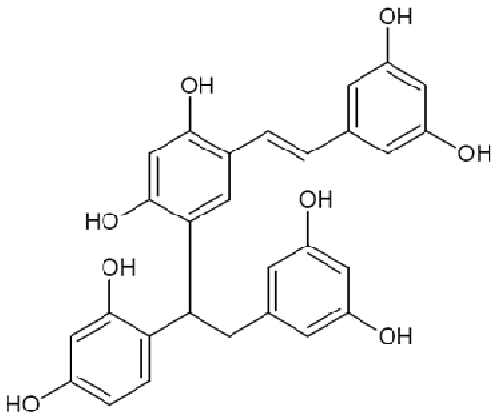
It is of interest that the crude extract from *Artocarpus heterophyllus* contained the highest of total phenolic and flavonoids content but it did not have the immunostimulatory activity. On the other hand, the crude extract from *Artocarpus gomezianus* and *Dalbergia cultrata* contained large amount of total phenolic but small amount of flavonoids showed the immunostimulatory activity. This implied that the major active constituent of the crude extracts involving in the immunostimulating activity may not be the flavonoid compounds. The polyphenolic compound can be

divided into two categories, flavonoids and non-flavonoids compounds, such as benzoic acid, tannin, caffeic acid and stilbene (Tsao, 2010). The immunostimulatory activity from these two crude extracts could be a result of non-flavonoid compounds.

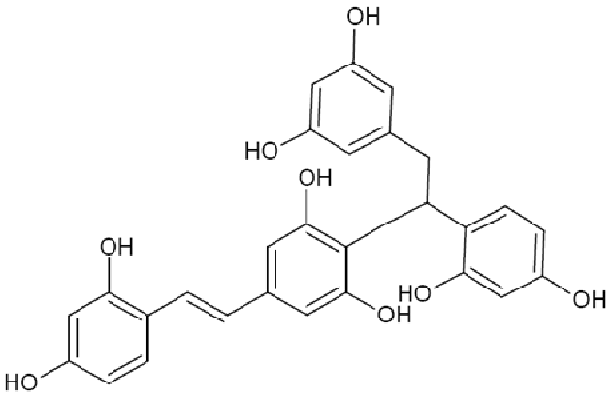
The immunostimulatory activity of crude extract from *Artocarpus gomezianus* is superior to the immunostimulatory activity of crude extract from *Dalbergia cultrata*. The crude extract of *Artocarpus gomezianus* could induce both PBMCs proliferation and maturation of MoDCs, whereas the crude extract from *Dalbergia cultrata* could induce only the PBMCs proliferation. This could be the effect from the difference of crude extract composition. Currently, there are no reports of the phytoconstituents in the root barks of *Artocarpus gomezianus*, so the major immunostimulatory active compounds could not be indicated. However, we suspected that the immunostimulatory activity may be the non-flavonoid phenolic compounds such as resveratrol. There is the report of the resveratrol derivatives in the root, which is another part of this plant (figure 11). One of the resveratrol derivatives is oxyresveratrol, which has structure similar to the resveratrol that was reported as a ligand for Toll-like receptor 4 (Yusuf et al., 2009).



oxyresveratrol

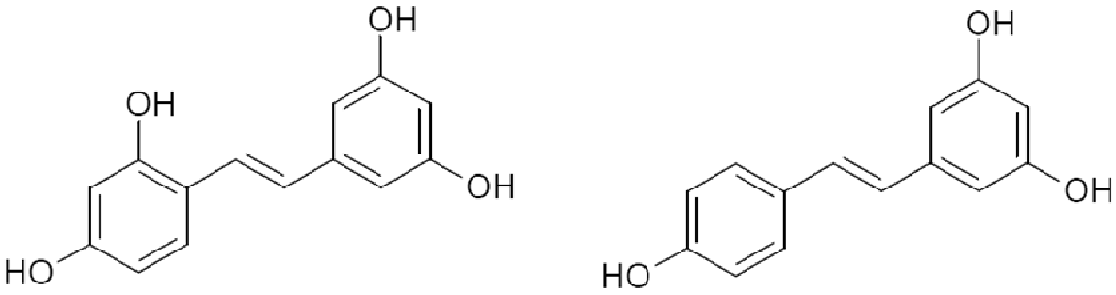


artogomezianol



andalasin

Figure 11 Chemical structures of oxyresveratrol, artogomezianol and andalasin found in root of *Artocarpus gomezianus* (Likhitwitayawuid and Sritularak, 2001)



oxyresveratrol resveratrol

Figure 12 Chemical structures of oxyresveratrol and resveratrol

The activation of Toll-like receptor 4 would lead to the maturation of dendritic cell (Thomas et al., 2003). Moreover the structure of oxyresveratrol seems similar to the tucaresol which has the ability to increase the lymphocytes proliferation (Marciani, 2003).

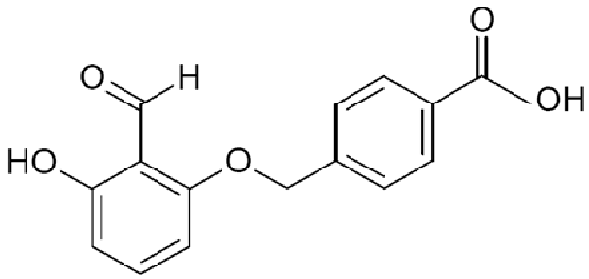
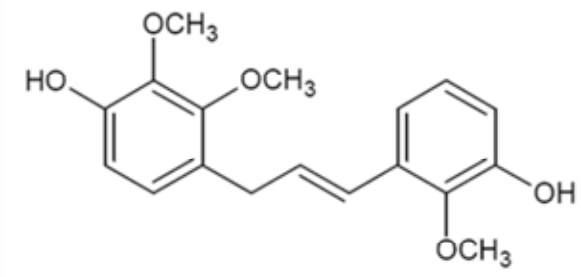


Figure 13 Chemical structure of tucaresol

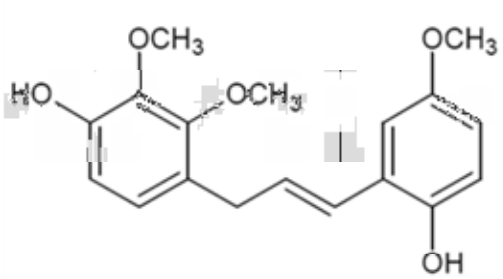
However, the results from our pre-liminary identification of the oxyresveratrol constituent in the crude extract from root barks of *Artocarpus gomezianus* by thin-layer chromatography showed that there are no oxyresveratrol in the crude extract. Hence, the immunostimulatory substance in the crude extracts was not oxyresveratrol. The phytoconstituent in root barks of *Artocarpus gomezianus* have not been investigated, but from the chemical profile in the root part, the major types of reported chemicals were stilbene derivatives (Figure 11). So, we guess that the phytochemicals in the root barks of *Artocarpus gomezianus* may also be the stilbene derivatives and these compounds may give the immunostimulatory activities that were observed in

this study. However, the evidence supporting which constituents in crude extract are responsible for the immunostimulatory activity is ambiguous, so the further studies of phytoconstituent of crude extract from root barks of *Artocarpus gomezianus* are required.

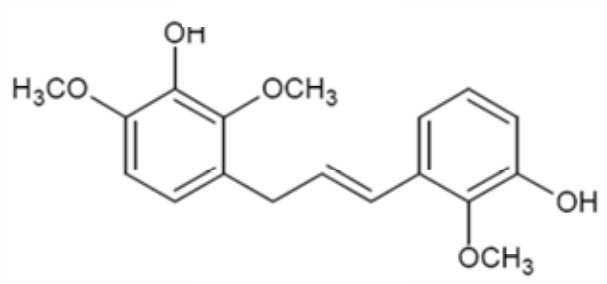
For the non-flavonoid phenolic compounds of *Dalbergia cultrata* were dalberatins. There are five dalberatins, dalberatin A, B, C, D and E (Figure 14). The dalberatin are also stilbene derivatives. In addition, with the low total flavonoid content and the previous report of non-flavonoid constituent, the lymphocyte proliferation stimulatory activity of crude extract from heartwood of *Dalbergia cultrata* may also be the result of some non-flavonoid phenolic compounds.



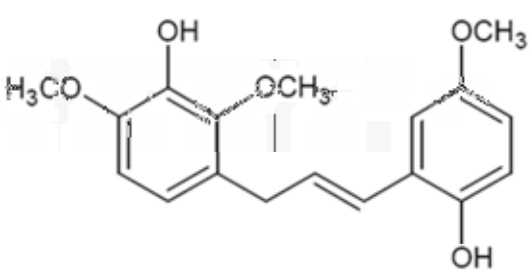
Dalberatin A



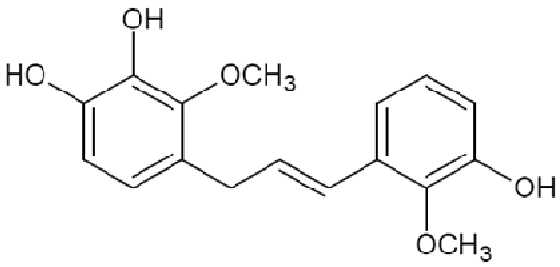
Dalberatin B



Dalberatin C



Dalberatin D



Dalberatin E

Figure 14 Chemical structures of dalberatin A, B, C, D and E found in heartwood of *Dalbergia cultrata*

Interestingly, the effect of *Artocarpus heterophyllus* (50 μg/ml) and *Erythrina variegata* (100 μg/ml) to viability of PBMCs in the MTT cytotoxicity assay was not in accordance with the effect of such compounds to the PBMCs proliferation in the CFSE cell proliferation assay. In cytotoxicity assay, the % cell viabilities of PBMCs incubated with crude extracts from *Artocarpus heterophyllus* (50 μg/ml) and *Erythrina variegata* (100 μg/ml) were comparable to the control, but in CFSE cell proliferation assay, the proliferative index of PBMCs incubated with both crude extracts tended to decline. The PI of *Artocarpus heterophyllus* (50 μg/ml) and *Erythrina variegata* (100 μg/ml) are 0.5 and 0.19 respectively, this means that the proliferative index of the two crude extracts were 50 % and 80 % lower than the control (Proliferative index = 1), respectively. The mechanism of this irrelevant cytotoxicity and proliferation could not be explained and need further studies. Interestingly, the properties of the crude extract from heartwood of *Artocarpus heterophyllus* and crude extract from stem barks of *Erythrina variegata* that tended to inhibit the PBMCs proliferation but did not affect the cell viability may be beneficial to be developed to an immunosuppressant.

The development of some candidate substances showing immunosuppressive activities could be a new choice for immunosuppressive drugs. Now, the problem from immunosuppressant is their side effects such as infection and malignancy (Gummert et al., 1999). These problems could be ameliorated by the combination of various immunosuppressive drugs that act at different steps of immune system suppression. The leflunomide is one of the immunosuppressant that has recently passed through the drug development process. Initially, it was tested for the activity to suppress the two enzymes that are protein tyrosine kinase and DHODH enzymes (Gummert et al., 1999). The protein tyrosine kinase plays many important roles in cell signal transduction that required for many cell functions including cell proliferation. The DHODH enzyme is essential in the *de novo* pathway of pyrimidine nucleotide synthesis, so the inhibition of this enzyme would result to the shortage of nucleotide for DNA synthesis. Thus, the inhibition of these enzymes would give rise to the inhibition of lymphocyte proliferation. So, the two crude extracts, the crude extract from heartwood of *Artocarpus heterophyllus* and the crude extract from stem barks of

Erythrina variegata, may be studied more in dept in the mechanism of proliferation inhibition by testing the suppressive activity to any enzymes that important for cell proliferation. After the *in vitro* immunosuppresive, the following process would be the *in vivo* immunosuppressive testing. The models for immunosuppresive activity testing are the heterotopic heart transplant and concordant and discordant xenotransplantation model in rat or other animal. In these models, a graft from other animal would be engraft to another animal model before treat with the sample substances and the graft survival duration would be monitor.

The crude extract from root barks of *Artocarpus gomezianus* was prepared as the injectable preparation. At least, the concentration of crude extract should be 20 mg/ml, because this concentration can provide a sufficient dose for immunostimulatory activities (Fischer et al., 2007a; Fischer et al., 2007b; Fischer et al., 2010). Moreover, the crude extract was intended to be mixed with a water soluble model antigen. Since the crude extract is insoluble in water, the solubility of the crude extract should be improved.

The solubility of the crude extract in the cosolvent system 1 (15.86 mg/ml) was much greater than that in water. Even though, the dielectric constant of this cosolvent was comparable to that of DMSO (ε_{DMSO} = 47), solubility of the crude extract in the cosolvent was less than that in DMSO (20 mg/ml). It implied that the solubility did not depend only on dielectric constant of the solute and the solvents. It could be also due to the difference of interaction between the solute and the solvents.

Although, the solubility of crude extracts did not reach the target (20 mg/ml), the addition of any cosolvents was discontinued due to the limited concentration of cosolvents for intramuscular injection (US FDA, 2007). The percentages of ethanol and PEG400 reached the maximum permitted concentrations. For propylene glycol, the percentage allowed in the injection preparation is as high as 80 % but the ε of propylene glycol ($\varepsilon_{propylene\ glycol}$ = 35.1) is not low enough to decrease ε of the system. The use of cosolvency quite reach the maximum of their potency. The micellization is worth a try because of different mechanism of solubilization. Moreover, some surfactants have the ability to prevent protein aggregation (Arakawa and Kita, 2000).

As expected, addition of a surfactant, tween 80, improved solubility of the crude extract. The critical micelle concentration (CMC) of tween 80 is 0.24 % w/v

(Patist et al., 2000). Therefore, the tween 80 formed micelles at the concentration used. The percentage of tween 80 permitted for the intramuscular injection formulation is not more than 12% since surfactants can cause irritation at the injected area. It was reported that 5% of tween 80 improved homogeneity of BCG vaccine preparation (Obayashi et al., 1955). From the results, at 5% of tween 80, the solubility of the crude extract exceeded 20 mg/ml, but this system also induced ovalbumin precipitation. It could be that high percentage of cosolvents (ethanol, PG and PEG400) captured the water molecules from the protein environment and displaced the water molecules on protein surface known as dehydration. Dehydration causes the protein aggregation and results in precipitation (Ghosh, 2006). A big lump of protein precipitation was observed in the solvent system.

So, the percentage of cosolvents and tween 80 in solvent system 2 were adjusted to be the solvent system 3. The percentage of ethanol and PEG400 were reduced to the percentages that were previously reported as the protein-unprecipitated concentration (Table 1). Tween 80 at 2 % was reported to prevent protein aggregation (Arakawa and Kita, 2000). The adjusted concentrations of solvents were shown in solvent system 3. However, partially dehydrated protein molecules were observed. The dehydrated protein molecules were easily redispersible in the solvent system 3. Then the solvent system 3 was further evaluated.

From the microscopic appearance (Figure 10), the degree of ovalbumin aggregation in the solvent system 3 containing the crude extract was more than the ovalbumin in the solvent system without the crude extract. This could be a result from the polyphenolic compounds containing in the crude extract. The polyphenolic compounds could interact with protein molecules leading to the protein precipitation (Hagerman et al., 1998; Handique and Baruah, 2002). The size range of the particles is below 50 μm. This size can be used in intramuscular injection (Buckwalter and Dickison, 1958). Although ovalbumin antigens used for immunization are commonly found in the soluble form, the insoluble form was also reported of the ability to induce immunity (Rosenberg, 2006). Therefore, the ovalbumin-crude extract coarse suspension in solvent system 3 was accepted for using in further *in vivo* experiments. However, there is a suggestion that the potential to induce the immunogenicity of the ovalbumin-crude extract preparation should be confirmed in small laboratory animals

such as mice, rat and guinea pigs etc., prior to be studied in large animal models. Moreover, the loss of soluble crude extract in solution due to the precipitation should be investigated. The soluble molecules of crude extract in the preparation may precipitate because the phenolic constituent in the crude extract is able to interact with protein antigen and then form the aggregates. Therefore, other determination of this phenomenon might be required. Some suggested methods are the determination of total phenolic content before and after the addition of ovalbumin antigen and the characterization of aggregated protein by SDS page.

These crude extracts may be developed for using in other field beside the vaccine adjuvant. There are many diseases that required immunostimulant for curing or relieving. Cancer has been the most deadly distress of the human race. In our body, the immune system is the surveillant system that functions as surveillance for seeking and destroying the abnormal cells and preventing the host from cancer. In patients, the immunostimulator can increase their life span and supports the activity of the main treatment. In immunocompormised patients such as HIV patients and diabetes, the immunostimulant may help to decrease the risk of opportunistic infection. So, the crude extract from root barks of *Artocarpus gomezianus* and the crude extract from *Dalbergia cultrata* can be evaluated in other assays that are useful for describing the activity related to all diseases above.

In Summary, the crude methanolic extract from root barks of *Artocarpus gomezianus* showed the activities to increase porcine PBMCs proliferation and induce the maturation of porcine MODCs, at the concentration 100 μg/ml. The crude extracts could be prepared as a solution in the solvent system that consisted of 10 % w/w ethanol, 8 % w/w propylene glycol, 30 % w/w PEG 400 and 2 % w/w tween 80 in water at the concentration of 29.72 mg/ml. The ovalbumin could be incorporated into the crude extract solution at the concentration of 1 mg/ml as a redispersible coarse dispersion.

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APPENDICES

Appendix A

Reagent Formula in cell cultures

1. Phosphate buffer saline (1X PBS)

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.2	g
DI water qs.ad to	1000	ml

Preparation

Each solid substances was weighed accuratedly and mixed together. The mixture was dissolved in 1000 ml DI water. The solution was steriled by autoclaving.

2. FACs buffer

NaN_3	0.5	g
Bovine serum albumin	2.5	g
PBS qs.ad. to	500	ml

Preparatoin

The 0.5 g NaN3 was weighed accurately and dissolved in 500 ml PBS. The 2.5 g ovalbumin was addded into the NaN3 solution. After that, the solution was filtered through 0.2 μm filter in laminar flow hood.

3. 10% Advanced complete RPMI 1640

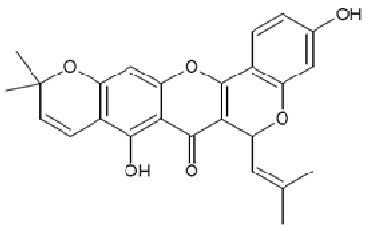
Fetal bovine serum	50	ml
100X L-glutamine	5	ml
100X Antibiotic/antimycotic	5	ml
1M HEPES	12.5	ml
50 mM 2-mercaptoethanol	0.5	ml
RPMI 1640 qs.ad. to	500	ml

Preparation

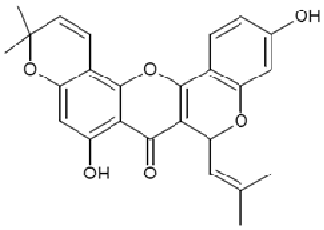
Mix all reagent in laminar flow hood by aseptic techniques

Appendix B
Structure of phenolic compounds in studied plants

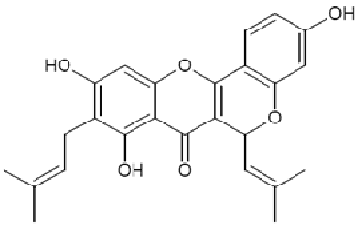
1. ***Artocarpus altilis***



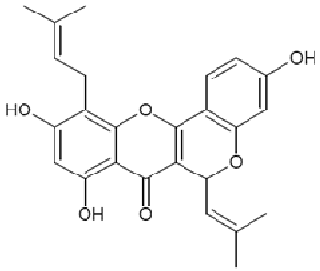
isocyclomorusin



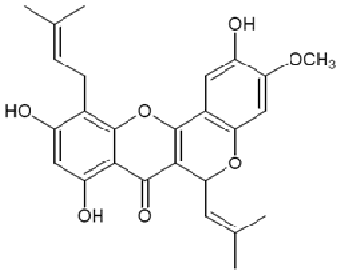
cyclomorusin



isocyclomulberrin



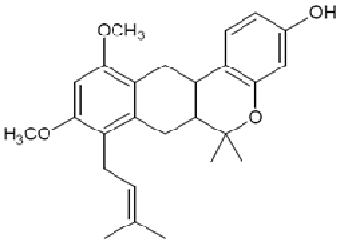
cyclomulberrin



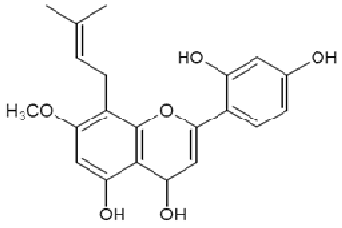
cycloaltilisin

Figure D1 Chemical structures of substances found in stem bark of *Artocarpus altilis* (Chen et al., 1993)

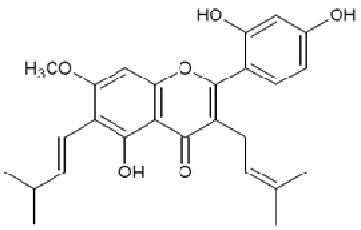
2. *Artocarpus heterophyllus*



heterophylol



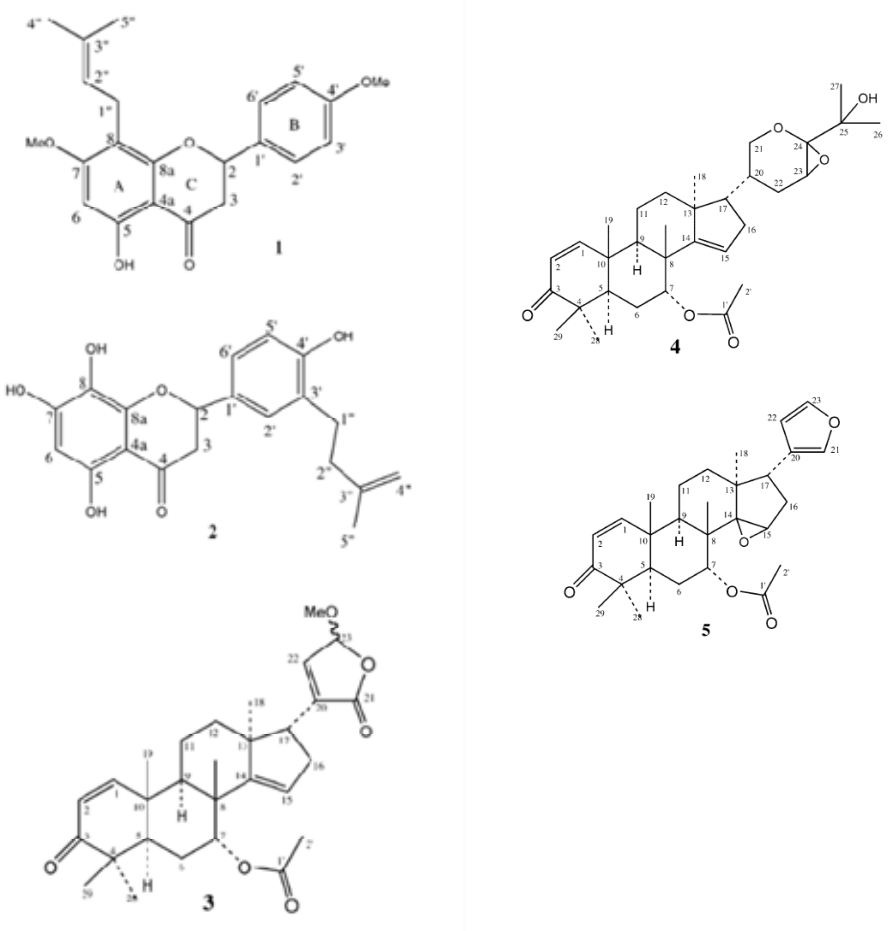
artocarpetin A



artocarpin

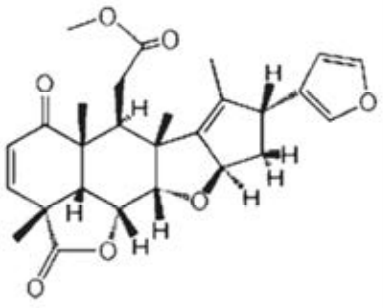
Figure D2 Chemical structures of substances found in heartwood of *Artocarpus heterophyllus* (Lin et al., 1995)

3. ***Azadirachta indica***

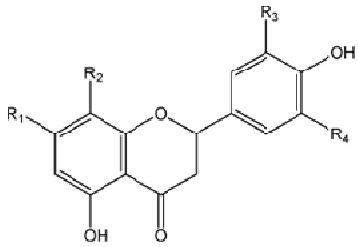


flowerine (1), flowerone (2) , O-ethylazadironolide (3), diepoxyazadirol (4) and trichilenone acetate (5) (Siddiqui et al., 2003)

Figure D3 Chemical structures of substances found in flowers of *Azadirachta indica*



nimbolide (Roy et al., 2007)



	R_1	R_2	R_3	R_4
1	OH	CH_2-CH=C(CH_3)$_2$	H	H
2	OCH_3	CH_2-CH=C(CH_3)$_2$	H	H
3	OH	CH_2-CH=C(CH_3)$_2$	CH_2-CH=C(CH_3)$_2$	H
4	OH	H	CH_2-CH=C(CH_3)$_2$	CH_2-CH=C(CH_3)$_2$

sophoraflavanone B (**1**), munduleaflavanone B (**2**), euchrestaflavanone A (**3**) and abyssinone V (**4**) (Nakahara et al., 2003)

Figure D4 Chemical structures of substances found in flowers of *Azadirachta indica*

4. *Broussonetia papyriferra*

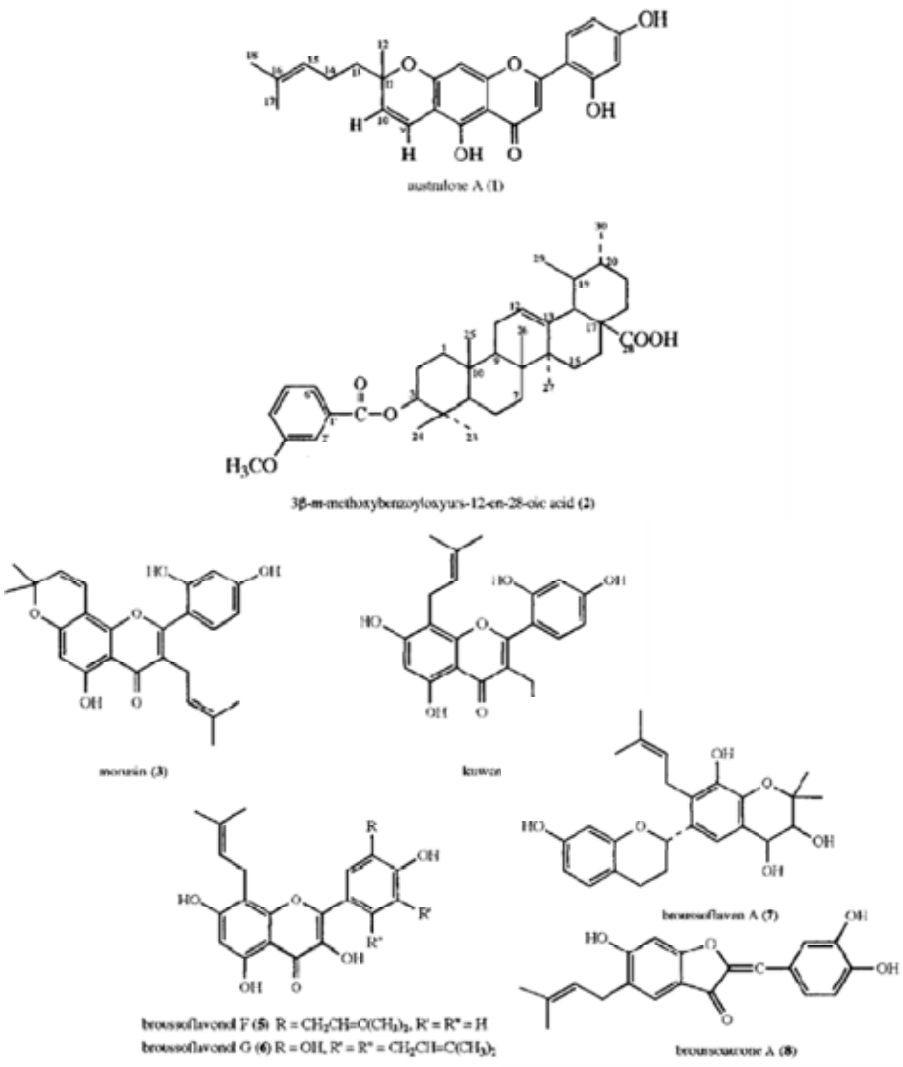
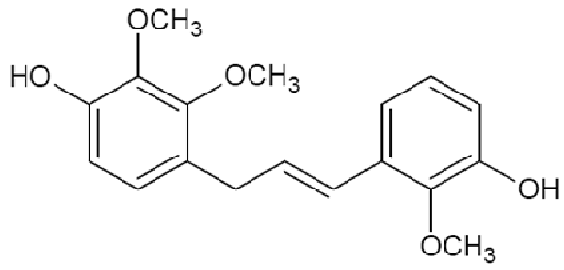
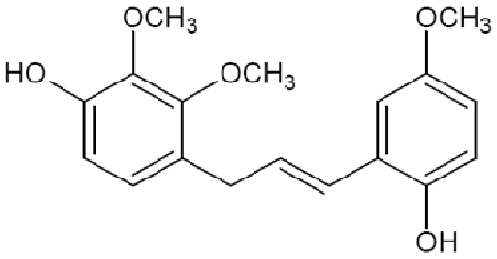


Figure D5 Chemical structures of substances found in root bark of *Broussonetia papyriferra* (Ko et al., 1997)

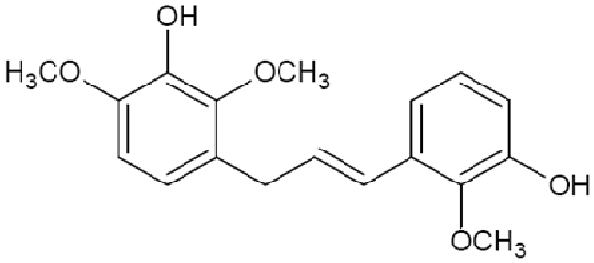
5. *Dalbergia cultrata*



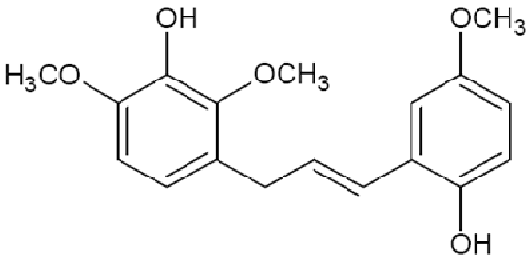
Dalberatin A



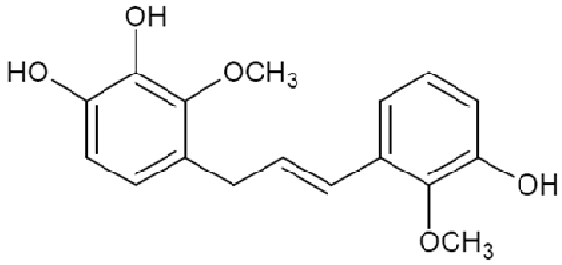
Dalberatin B



Dalberatin C

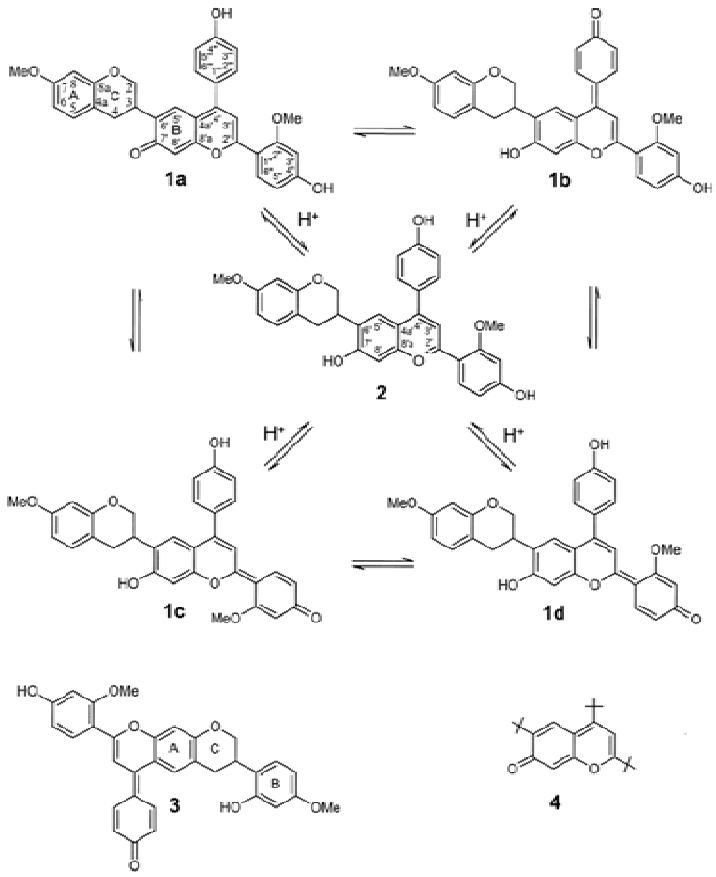


Dalberatin D



Dalberatin E

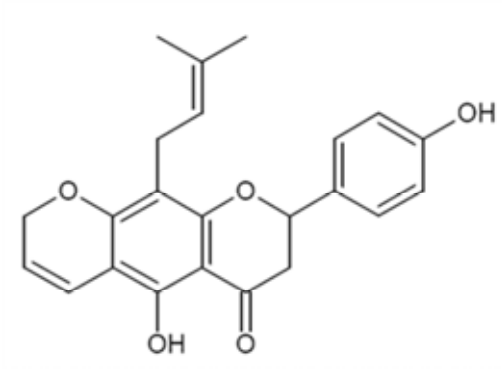
Figure D6 Chemical structures of substances found in heartwood of *Dalbergia cultrata* (Ito et al., 2003)



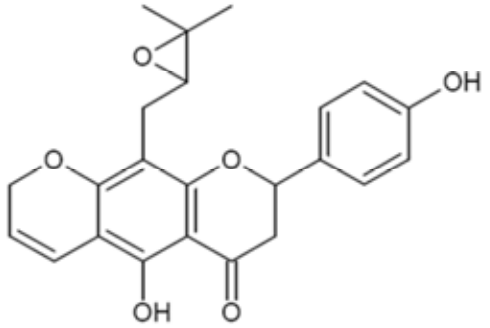
Retusapurpurin (1a), three of its possible tautomers (1b-1d), their protonated form (2), the partial structure (4), and candenatone (3) (Czakó and Márton, 2001)

Figure D7 Chemical structures of substances found in heartwood of *Dalbergia cultrata*

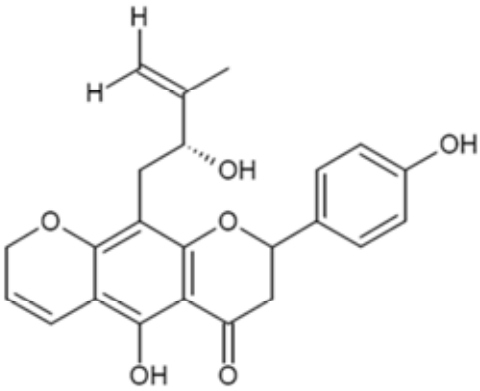
6. *Derris reticulata*



lupinifolin



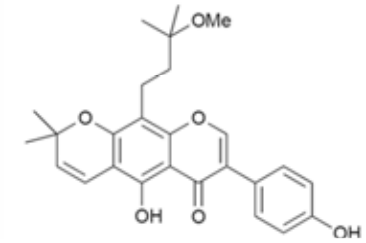
2‴,3‴-epoxylupinifolin



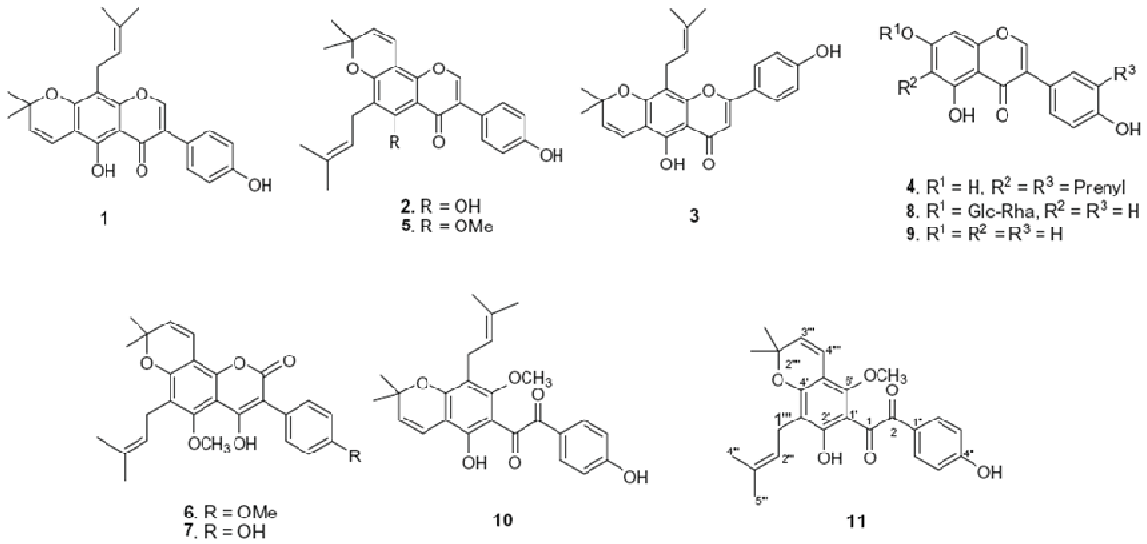
dereticulatin

Figure D8 Chemical structures of substances found in stem of *Derris reticulata* (Mahidol et al., 1997)

7. *Derris scandens*



Eturunagarone (Boland and Donnelly, 1998)



scandenone (1), osajin (2), laxifolin (3), lupalbigenin (4), scandinone (5),scandenin A (6), scandenin (7), sphaerobioside (8), genistein (9), (10) and derrisdione A (11) (Rao et al., 2007)

Figure D9 Chemical structures of substances found in stem of *Derris scandens*

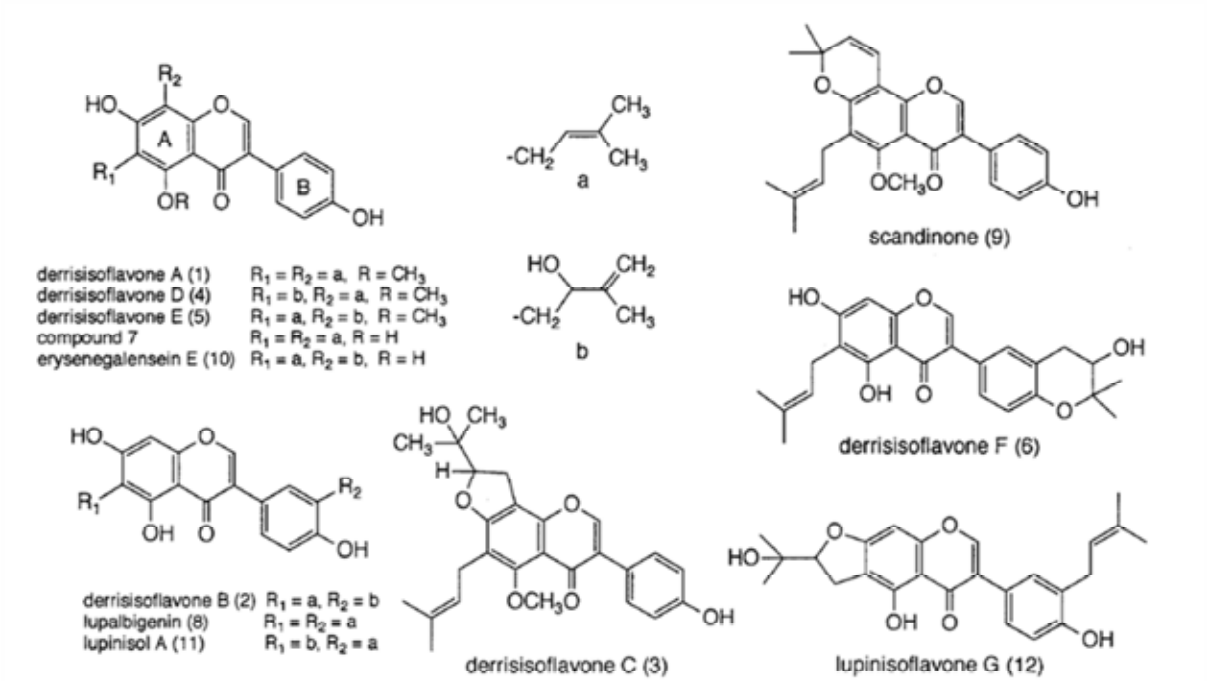
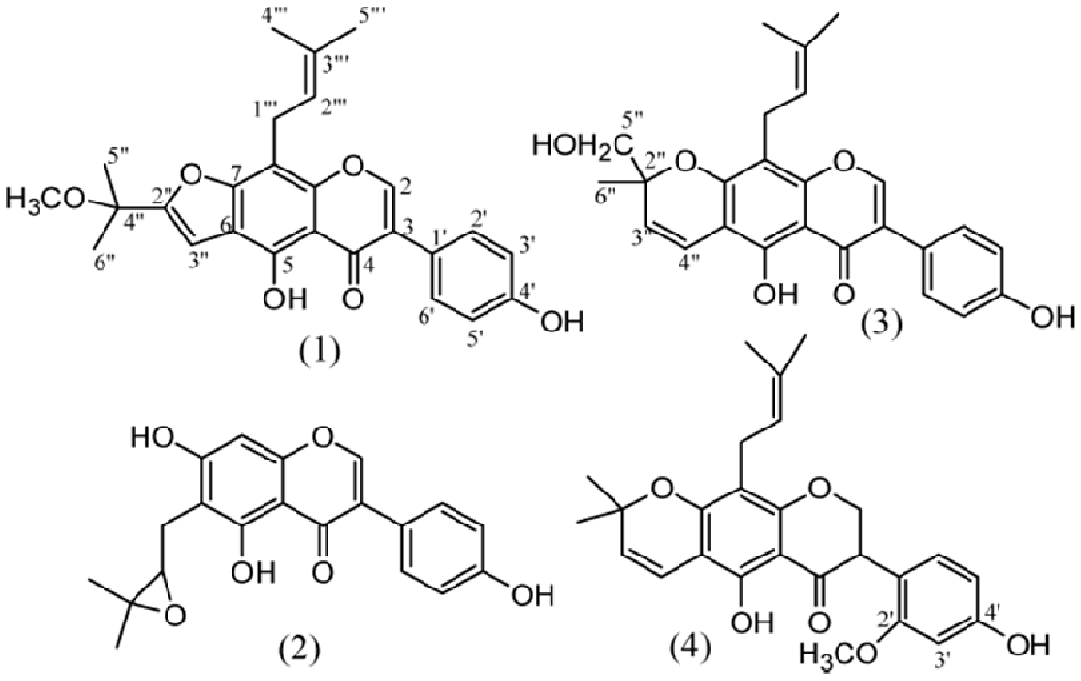


Figure D10 Chemical structures of substances found in stem of *Derris scandens* (Sekine et al., 1999)

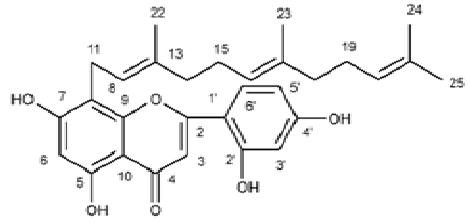
8. ***Erythrina variegata***



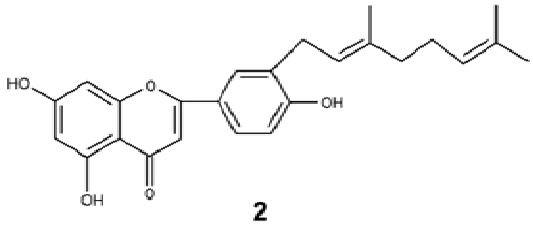
Compounds 1-4 (Xiaoli et al., 2006)

Figure D11 Chemical structures of substances found in stem bark of *Erythrina variegata*

9. *Morus alba*



1



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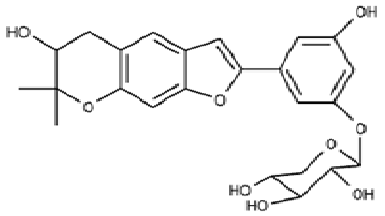

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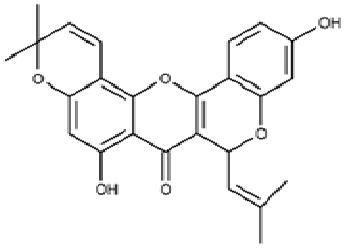

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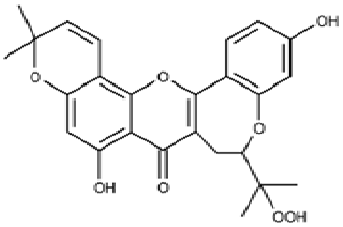

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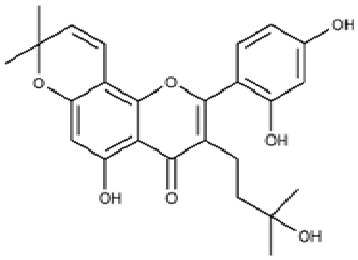

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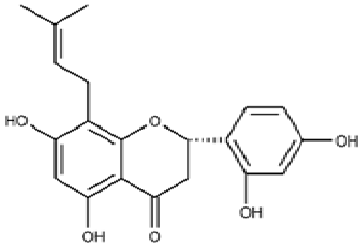

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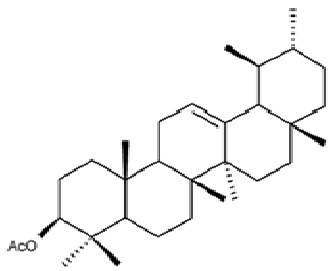
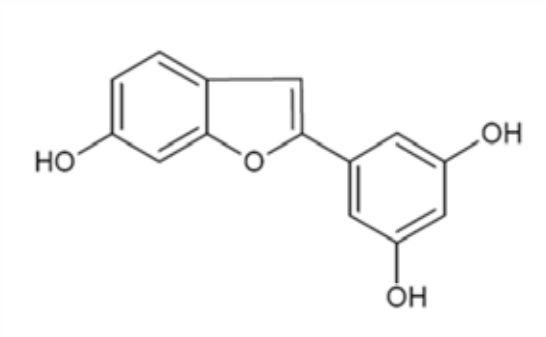


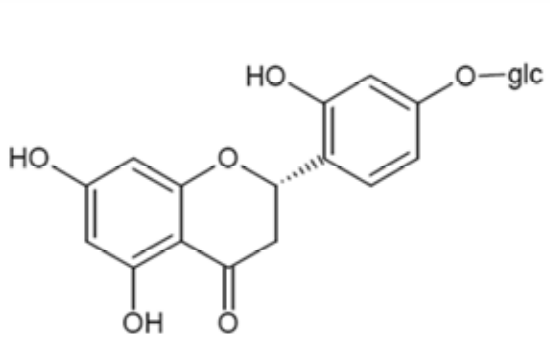
8

moralbanone (1), kuwanon S (2), mulberroside C (3), cyclomorusin A (4), eudraflavone B hydroperoxide (5), oxydihydromorusin (6), leachianone G (7) and α-acetyl-amyrin (8) (Du et al., 2003)

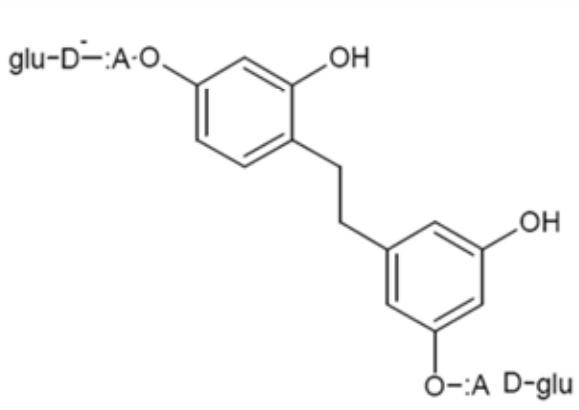
Figure D12 Chemical structures of substances found in root bark of *Morus alba*



Moracin M



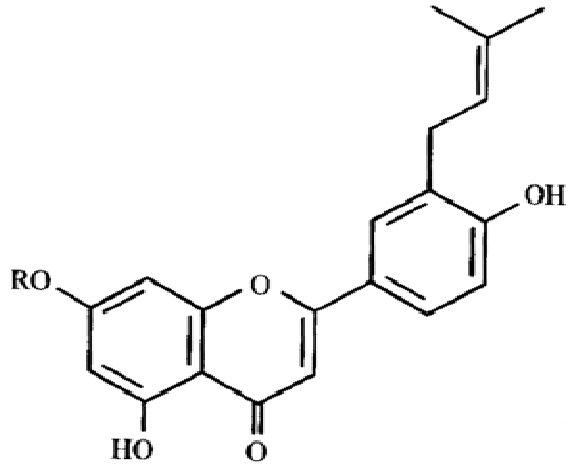
Steppogenin-4'-O-β-D-glucosiade



Mullberroside

Figure D13 Chemical structures of substances found in root bark of *Morus alba* (Zhang et al., 2009)

10. ***Pithecolobium dulce***



1. R = α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl
2. R = H
3. R = β-D-glucopyranosyl

3'-Prenylapigenine 7-O-rutinoside (1), 3'-prenylapigenine (2) and pyranoside (3) (Saxena and Singhal, 1999)

Figure D14 Chemical structures of substances found in stem of *Pithecolobium dulce*

Appendix C

The bar graph showing cytotoxicity results of 11 crude plant extracts

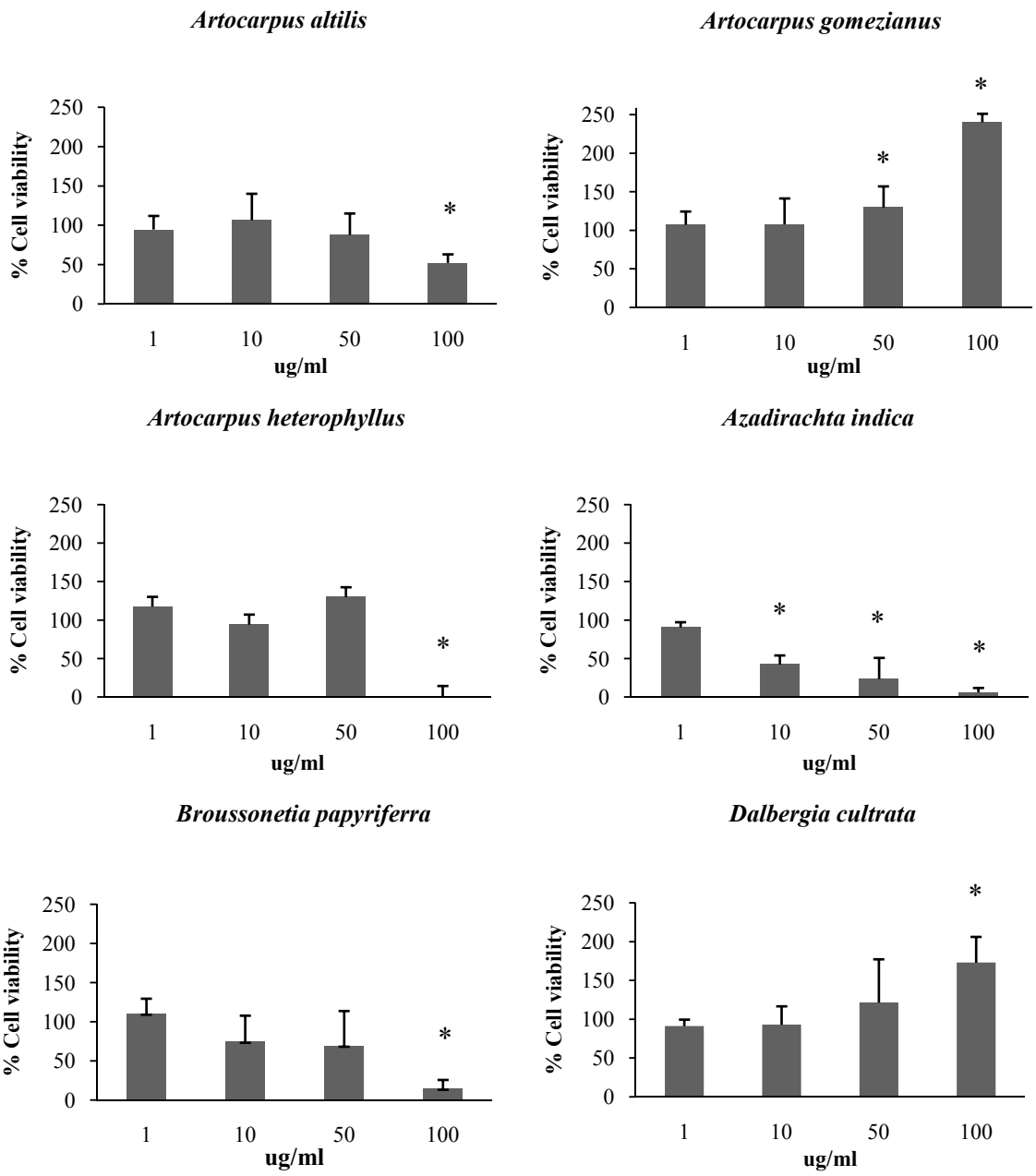


Figure D15 The percent cell viability of PBMCs incubated with 11 crude extracts at 1, 10, 50 and 100 μg/ml for 3 days.
* indicate the significantly different from control group at $p < 0.05$, n = 5

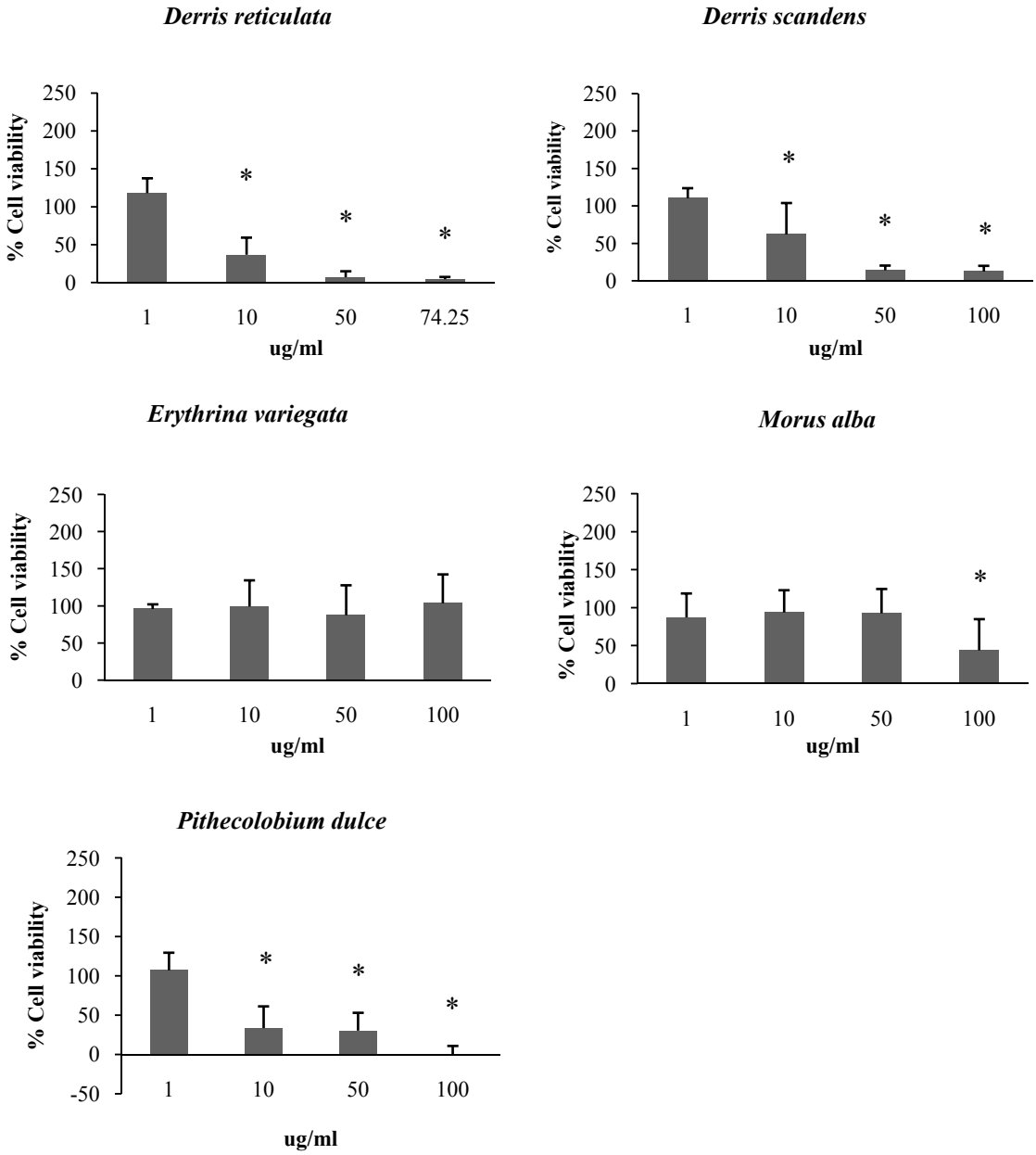


Figure D16 The percent cell viability of PBMCs incubated with 11 crude extracts at 1, 10, 50 and 100 μg/ml for 3 days.
* indicate the significantly different from control group at $p < 0.05$, n = 5

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 12-33-017	**Approval No. 12-33-017**

Protocol Title
Screening of medicinal plants containing flavonoids as a vaccine adjuvant and preparation of crude extract solution

Principal Investigator
ANGKANA TANTITUVANONT, Ph.D.

Certification of Institutional Animal Care and Use Committee (IACUC)
This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	Date of Expiration
August 21, 2012	August 21, 2015

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan BKK-THAILAND. 10330

Signature of Chairperson	Signature of Authorized Official
Thongchai Lt.	Parkpoom Tengamnuay
Name and Title THONGCHAI SOOKSAWATE, Ph.D. Chairman	**Name and Title** PARKPOOM TENGAMNUAY, Ph.D. Associate Dean (Research and Academic Service)

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

VITA

Miss Tullaya Pungchaipat was born on October 26, 1985 in Saraburi, Thailand. She received her Bachelor Degree with second honor from the Faculty of Pharmaceutical Sciences, Chulalongkorn University in 2009. She continued the enrollment to the Master degree program in Pharmacy at Chulalongkorn University in the same year.