EVALUATION ON IMMUNOMODULATORY ACTIVITIES OF MANGOSTEEN (*GARCINIA MANGOSTANA* L.)

PIMOLKAN KASEMWATTANAROJ

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……
Miss Pimolkan Kasemwattanaroj
Candidate

……
Assoc. Prof. Primchanien Moongkarndi,
Dr. rer. nat. (Immunology)
Major advisor

……
Asst. Prof. Supachoke Mangmool,
Ph.D. (Pharmaceutical Sciences)
Co-advisor

……
Prof. Kovit Pattanapanyasat,
Ph.D. (Iron Metabolism)
Co-advisor

……
Prof. Banchong Mahaisavariya,
M.D., Dip. Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

……
Assoc. Prof. M.L. Sumarn Saraya,
Ph.D. (Pharmacokinetics)
Program Director
Master of Science in Pharmacy
Program in Microbiology
Faculty of Pharmacy
Mahidol University

Thesis
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on
June 24, 2013

……
Miss Pimolkan Kasemwattanaroj
Candidate

……
Assoc. Prof. Wanla Kulwichit,
M.D. (Hons), Dip. Thai Board of Internal Medicine and Infectious Diseases
Chair

……
Assoc. Prof. Primchanien Moongkarndi,
Dr. rer. nat. (Immunology)
Member

……
Asst. Prof. Supachoke Mangmool,
Ph.D. (Pharmaceutical Sciences)
Member

……
Prof. Kovit Pattanapanyasat,
Ph.D. (Iron Metabolism)
Member

……
Prof. Banchong Mahaisavariya,
M.D., Dip. Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

……
Assoc. Prof. Chuthamanee Suthisisang,
Ph.D. (Pharmacology)
Dean
Faculty of Pharmacy
Mahidol University

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EVALUATION ON IMMUNOMODULATORY ACTIVITIES OF MANGOSTEEN (*GARCINIA MANGOSTANA* L.)

PIMOLKAN KASEMWATTANAROJ 5336321 PYMI/M

M.Sc. in Pharm. (MICROBIOLOGY)

THESIS ADVISORY COMMITTEE: PRIMCHANIEN MOONGKARNDI, Dr.rer.nat. (IMMUNOLOGY), KOVIT PATTANAPANYASAT, Ph.D. (IRON METABOLISM), SUPACHOKE MANGMOOL, Ph.D. (PHARMACEUTICAL SCIENCES)

ABSTRACT

Mangosteen, *Garcinia mangostana* L., has been used as a traditional medicine for the treatment of many diseases. Several reports show that crude extract and active constituents of mangosteen exhibit miscellaneous activities including antimicrobial, antioxidant, anti-inflammatory, cytotoxic and anticancer activities. Mangosteen contains a variety of phytochemicals in which xanthones are the principal constituents exhibiting these activities. Based on these reported activities, a large amount of products derived from mangosteen has been widely used to improve health status as well as treat many diseases. However, the immunomodulatory evidence of mangosteen is limited. This study, therefore, aimed to investigate the immunomodulatory activity of two mangosteen extracts, purified α-mangostin and partially purified crude water extract, on human immune cells and cytokines *in vitro*.

Peripheral blood mononuclear cells (PBMCs) were cultured with the selected concentrations of α-mangostin (1, 2 and 4 µg/ml), crude water extract (50, 100 and 200 µg/ml), T cell mitogen concanavalin A (ConA; 10 µg/ml), and α-mangostin (or crude water extract) plus ConA. After 24 h of incubation, the treated cells were enumerated in the percentages of $CD3^+$ (T cells), $CD19^+$ (B cells) and $CD3^-CD16^+/CD56^+$ (NK cells) by immunophenotypic method, while the cell cultured supernatants were evaluated the primary cytokine (TNF-α, IL-1β and IL-2) production by ELISA technique. The results demonstrated that three concentrations of α-mangostin or crude water extract could not change the expression level of three lymphocyte markers compared with unstimulated cells; moreover, the combinations of α-mangostin (or crude water extract) plus ConA could not change the expression level of these three lymphocyte markers compared with ConA-stimulated cells. On the contrary, α-mangostin could show an obvious tendency to inhibit IL-2 secretion; whereas, crude water extract could significantly induce TNF-α and IL-1β secretion as much as ConA-stimulated cells produced. The crude water extract was further examined the stimulatory activity on proinflammatory cytokine expression by measuring intracellular TNF-α of monocytes. The whole blood was incubated with 50 and 100 µg/ml of crude water extract, lipopolysaccharide (LPS), dexamethasone (Dex), and the combined substances between crude water extract and LPS (or Dex) for 4 h. The results showed that crude water extract could not induce intracellular TNF-α expression. This possibly suggested that crude water extract could exert its effect on other cells, but not monocytes, in whole blood to produce TNF-α.

These results indicated that α-mangostin could be developed into an immunosuppressant, suppressing IL-2 secretion, and crude water extract could be employed as an immunostimulant triggering proinflammatory cytokine production without activating via monocytes. Nevertheless, further studies of both extracts on other immune cells and cytokines with an increase sample size are necessary to deeply understand their mechanisms of actions.

KEY WORDS: *GARCINIA MANGOSTANA* L. / IMMUNOMODULATORY ACTIVITY / HUMAN IMMUNE CELLS / CYTOKINES

88 pages

การประเมินฤทธิ์การปรับภูมิคุ้มกันของมังคุด (*GARCINIA MANGOSTANA* L.)
EVALUATION ON IMMUNOMODULATORY ACTIVITIES OF MANGOSTEEN (*GARCINIA MANGOSTANA* L.)

พิมลกานท์ เกษมวัฒนาโรจน์ 5336321 PYMI/M

ภ.ม. (จุลชีววิทยา)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: ปริ่มเฉนียน มุ่งการดี, Dr.rer.nat. (IMMUNOLOGY), โกวิท พัฒนาปัญญาสัตย์, Ph.D. (IRON METABOLISM), ศุภโชค มั่งมูล Ph.D. (PHARMACEUTICAL SCIENCES)

บทคัดย่อ

มังคุด , *Garcinia mangostana* L, ถูกใช้เป็นยาพื้นบ้านในการรักษาโรคมากมาย หลายรายงานแสดงให้เห็นว่าสารสกัดหยาบและสารสำคัญจากมังคุดแสดงฤทธิ์ที่หลากหลาย ได้แก่ ฤทธิ์ต้านจุลชีพ, ต้านออกซิเดชั่น, ต้านการอักเสบ, เป็นพิษต่อเซลล์ และต้านมะเร็ง จากรายงานมังคุดประกอบไปด้วยสารเคมีจากพืชหลากหลายชนิดโดยมีสารกลุ่มแซนโทนเป็นสารหลักที่ออกฤทธิ์เหล่านี้ ทำให้ผลิตภัณฑ์จำนวนมากที่มาจากมังคุดถูกใช้อย่างกว้างขวางเพื่อส่งเสริมสุขภาพและรักษาหลายโรค อย่างไรก็ตามหลักฐานด้านการปรับภูมิคุ้มกันจากมังคุดยังมีจำกัด ดังนั้นการศึกษานี้มุ่งหมายที่จะค้นหาฤทธิ์การปรับภูมิคุ้มกันของสารสกัดมังคุดสองชนิด: สารแอลฟา-แมงโกสตินบริสุทธิ์ และสารสกัดหยาบชั้นน้ำที่ถูกทำให้บริสุทธิ์บางส่วน ที่มีต่อเซลล์ระบบภูมิคุ้มกันและไซโตไคน์ของมนุษย์ในหลอดทดลอง

เพอริเฟอรัลโมโนนิวเคลียสเซลล์ถูกเลี้ยงร่วมกับสารที่เลือกความเข้มข้นแล้วของแอลฟา-แมงโกสติน (1, 2 และ 4 ไมโครกรัม/มล.), สารสกัดหยาบชั้นน้ำ (50, 100 และ 200 μg/ml), สารคอนคานาวาลิน เอ ที่กระตุ้นทีเซลล์ (คอนเอ; 10 ไมโครกรัม/มล.), และสารแอลฟา-แมงโกสติน (หรือสารสกัดหยาบชั้นน้ำ) ร่วมกับคอนเอ หลังจากบ่มนาน 24 ชั่วโมง เซลล์ที่ถูกทดสอบถูกนับจำนวนร้อยละของ $CD3^+$ (ทีเซลล์), $CD19^+$ (บีเซลล์) และ $CD3^-CD16^+/CD56^+$ (เอ็นเคเซลล์) โดยใช้วิธี immunophenotype ในขณะที่ส่วนสารละลายเหนือตะกอนของเซลล์ที่เลี้ยงถูกนำมาประเมินหาปริมาณสารไซโตไคน์หลักที่ถูกสร้างหลั่งออกมา (ทีเอ็นเอฟ-แอลฟา, ไอแอล-1เบต้า และ ไอแอล-2) โดยใช้เทคนิค ELISA ผลการทดลองแสดงให้เห็นว่าความเข้มข้นทั้งสามของสารแอลฟา-แมงโกสตินหรือสารสกัดหยาบชั้นน้ำไม่สามารถเปลี่ยนแปลงระดับการแสดงออกของโมเลกุลเครื่องหมายของลิมโฟไซต์ทั้งสามได้เมื่อเทียบกับเซลล์ที่ไม่ได้รับการกระตุ้น นอกจากนี้การใส่สารแอลฟา-แมงโกสติน (หรือสารสกัดหยาบชั้นน้ำ) ร่วมกับคอนเอไม่สามารถเปลี่ยนแปลงระดับการแสดงออกของโมเลกุลเครื่องหมายของลิมโฟไซต์ทั้งสามได้เมื่อเทียบกับเซลล์ที่ถูกกระตุ้นด้วยคอนเอ ในทางตรงกันข้าม สารแอลฟา-แมงโกสตินสามารถแสดงแนวโน้มที่ชัดเจนในการยับยั้งการหลั่งไอแอล-2 ขณะที่สารสกัดหยาบชั้นน้ำสามารถเหนี่ยวนำการหลั่งทีเอ็นเอฟ-แอลฟาและไอแอล-1เบต้าได้อย่างมีนัยสำคัญซึ่งมากเท่ากับเซลล์ที่ถูกกระตุ้นด้วยคอนเอสร้างได้ สารสกัดหยาบชั้นน้ำถูกตรวจสอบฤทธิ์กระตุ้นการแสดงออกของไซโตไคน์หลักที่เกี่ยวกับการอักเสบเพิ่มเติมโดยการวัดทีเอ็นเอฟ-แอลฟาภายในโมโนไซต์ เลือดถูกบ่มร่วมกับ 50 และ 100 ไมโครกรัม/มล. ของสารสกัดหยาบชั้นน้ำ, ไลโปโพลีแซคคาไรด์ (แอลพีเอส), เดกซาเมทาโซน (เดกซ์), และสารร่วมระหว่างสารสกัดหยาบชั้นน้ำและแอลพีเอส (หรือเดกซ์) นาน 4 ชั่วโมง ผลการทดลองแสดงให้เห็นว่าสารสกัดหยาบชั้นน้ำไม่สามารถเหนี่ยวนำการแสดงออกของทีเอ็นเอฟ-แอลฟาภายในเซลล์ได้ ผลที่ได้นี้แนะให้เห็นว่าสารสกัดหยาบชั้นน้ำสามารถส่งผลต่อเซลล์อื่นแต่ไม่มีผลต่อโมโนไซต์ในเลือด ให้สร้างทีเอ็นเอฟ-แอลฟา

ผลการศึกษาเหล่านี้ชี้ให้เห็นว่าสารแอลฟา-แมงโกสตินสามารถถูกพัฒนามาเป็นสารกดภูมิคุ้มกันโดยยับยั้งการหลั่งไอแอล-2 และสารสกัดหยาบชั้นน้ำสามารถถูกใช้เป็นสารกระตุ้นภูมิคุ้มกันที่กระตุ้นการสร้างไซโตไคน์ที่เกี่ยวข้องกับการอักเสบโดยปราศจากการกระตุ้นผ่านโมโนไซต์ อย่างไรก็ตามการศึกษาเพิ่มเติมของสารสกัดทั้งสองที่มีต่อเซลล์ภูมิคุ้มกันและไซโตไคน์อื่นๆ พร้อมกับการเพิ่มขนาดตัวอย่าง มีความจำเป็นอย่างมากต่อการเข้าใจอย่างลึกซึ้งถึงกลไกการออกฤทธิ์ของสาร

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LIST OF ABBREVIATIONS

APC	allophycocyanin
APC	antigen-presenting cell
BCR	B-cell receptor
CD	cluster of differentiation
ConA	concanavalin A
Crude	crude water extract
CTLs	cytotoxic T lymphocytes
Dex	dexamethasone
E. coli	*Escherichia coli*
ED_{50}	effective dose at 50%
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
IC_{50}	inhibitory concentration at 50%
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MBC	minimum bactericidal concentration
MFC	minimum fungicidal concentration
MHC	major histocompatibility complex
MIC	minimum inhibitory concentration
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa B
NK	natural killer
NKT	natural killer T
no.	number
O.D.	optical density

LIST OF ABBREVIATIONS (cont.)

PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll
PMNs	polymorphonuclear neutrophils
SEM	standard error of mean
Tc	cytotoxic T cells
TCR	T-cell receptor
TGF	transforming growth factor
Th	T helper cells
TNF	tumor necrosis factor

CHAPTER I
INTRODUCTION

The immune system is a complex network of cells, tissues, and organs that work together to protect the body against non-self invaders. It can be divided into two systems: innate and adaptive immune systems. The innate immune system is the first line defense against infection mediated by cell components (including neutrophils, monocytes and complements) and host soluble products such as cytokines. This system rapidly mediates nonspecific response to pathogens. Unlike the innate response, the adaptive immune system is slower in its response to pathogens but provides long-term protection. The adaptive immune of host needs to be confronted with a pathogen or antigen earlier to develop antigen-specific T cell receptors (cell-mediated immunity) and antibody productions (humoral immunity), which depend on T and B lymphocytes, respectively (1-3). T lymphocytes function in eradication of intracellular pathogens, whereas B lymphocytes destroy extracellular microbes and their toxins. Furthermore, natural killer cells (NK cells), which bridge innate and adaptive immunities, can destroy the target cells without prior sensitization, and can also recognize tumor cells followed by kill tumor cells (4-7). Numerous evidences have pointed out that various human diseases stem from the defectiveness of the immune system. Immunomodulators, substances that are capable to normalize immune state, consequently becomes a wide use as a therapeutic option. Nevertheless, immunomodulator therapies regularly require lifelong use and cause the adverse events. For example, immunosuppresants nonspecifically suppress the entire immune system, which leads the patients to have significantly higher risks of infection and cancer; moreover, drug resistance is one of the most important obstacles of immunosuppresants to cure the disease (8, 9). While the complications of immunostimulants depend on individual response; one immunostimulant may generate systemic effects at one patient but low efficacy at different patients (3, 9, 10). According to these problems, searching for new immunomodulators is in the field of

interest. Plants, especially medicinal plants, are a great source of the immunomodulator; because they have been used as a medicine, which suggests that they contain some bioactive compounds.

Mangosteen, *Garcinia mangostana* L., is a tropical tree found in Southeast Asia. It is named as "the queen of fruits" because of the pleasant tasty flavor of the edible fruit. Moreover, the non-edible parts of mangosteen, for example, roots, bark, stem and especially pericarp, have been used as a traditional medicine for the treatments of diarrhea, dysentery, infections and wounds (11, 12). Mangosteen contains a variety of phytochemicals, which the majority group is xanthones. Bioactive secondary metabolite xanthones exhibit a powerful antioxidant activity. There are more than 60 derivatives of xanthones found in mangosteen (13-16), and α-mangostin, one of xanthone derivatives, is present in the highest content (13, 17, 18). Several studies have reported the versatile activities of crude extract and active constituents from mangosteen including antibacterial, antifungal, antioxidant, anti-inflammatory, cytotoxic activities and anticancer (14, 16, 17). Based on these evidences, a large amount of products derived from mangosteen has been widely used for improving health status or treating a variety of the diseases. However, the activity of mangosteen on immune system is still equivocal. This study, therefore, attempted to clarify the immunomodulatory activity of two interesting mangosteen extracts: α-mangostin and crude water extract (a promising antioxidant activity, presented in a water-soluble part of the extraction process). We focused their activities on human T cells, B cells, NK cells (the important cells associated with adaptive and innate immunity), and monocytes (the main antigen presenting cells involved in inflammatory cytokine secretion), as well as on the primary cytokine expression of TNF-α, IL-1β (the initiators of inflammation process) (19) and IL-2 (the activator of T, B and NK cells proliferation and other cytokine syntheses (20).

Objective

The present study aims to determine immunomodulatory activities of the purified α-mangostin and partially purified crude water extract on normal human immune cells and cytokines *in vitro.*

CHAPTER II
LITERATURE REVIEW

2.1 Immune systems

The immune system is a complex network of cells, tissues, and organs that work together to protect the body against non-self invaders and cancer. The immune system can be divided into two systems: innate (or non-specific) and adaptive (or acquired) immune systems. The innate immune system is the first line defense against infection mediated by the presence of anatomical barriers such as skin, cell components (including neutrophils, macrophages and complements) and host soluble products such as cytokines and antimicrobial compounds. This system rapidly mediates nonspecific response to pathogens. Unlike the innate response, the adaptive immune system is slower in its response but provides long-term protection. The adaptive immune of the host has to be firstly confronted with a pathogen or antigen to develop antigen-specific T cell receptors (cell-mediated immunity) and antibody productions (humoral immunity), which depend on T and B lymphocytes, respectively (1-3). The second exposure of the same challenger results in a memory response: the immune response is stronger and more potent to eradicate the pathogens. Even if both arms of immunities have their individual functions, there is a relationship between two immunities, which one of them can exert influence on each other (21). In addition, type of encounter antigen is one of the factors that influences on the duration and potency of immunity expression (22).

2.1.1 Innate (non-specific) immune system

The components of innate immunity include anatomical/physiologic barriers, cell components and soluble factors of the host.

2.1.1.1 Anatomical/physiologic barriers

Epithelial cells of the skin and the surface of the mucous membranes are the first line defines that prevent infectious agent penetrate into the

body. Secretion such as saliva and tears are a great washing agent for invaders and also contain antibacterial or antiviral substances. The mucus of respiratory and gastrointestinal tract is a trap to protect the lungs and digestive systems from infection (22). Moreover, the conditions of physiologic barriers (e.g., temperature, pH, and various soluble and cell-associated molecules) can inhibit the growth of pathogen (21).

2.1.1.2 Cells of innate immune system

Inflammatory response is one of the important defense mechanisms of innate immunity. The blood monocytes, neutrophils and tissue macrophages play an essential role in this phenomenon. The invasion of microorganism stimulates the recruitment of these cells to the site of infection, followed by phagocytosis: the cells extend their plasma membranes to engulf the organism and digest the captured organism by lysozyme (21-23).

Monocytes/Macrophages

Monocytes are mononuclear leukocytes containing granules, found in the blood stream. The normal range of monocyte count is 2-8% of white blood cells. The classical marker expression of monocytes is cluster of differentiation (CD) 14. Once the cells settle and differentiate in the tissue, they become "macrophages". Macrophages reside throughout the connective tissue and around the basement membrane of small blood vessels, lung and spleen sinusoid. Monocytes and macrophage can clear pathogens and foreign materials by phagocytosis and they can stimulate adaptive immunity by initiating specific immune mechanism (e.g., cytokine production, antigen presentation to stimulate lymphocytes) to respond against pathogens. The results of destroying pathogens by these cells can cause the pathogenesis of inflammatory and degenerative diseases (24, 25).

Neutrophils

Neutrophils or polymorphonuclear neutrophils (PMNs) are granular short-lived cells with multilobulated nucleus. They are the most abundant white blood cells (50-70% of white blood cells) (26). Neutrophils migrate to the sites of infection and inflammation by chemotaxis process. The methods for directly attacking pathogen are phagocytosis and the production of anti-microbial enzymes (27).

Eosinophils

For the protection from large pathogens such as parasites, the responsibility belongs to eosinophils. They are bone marrow-derived granulocytes which have granule enzymes for cell lysis. In addition, granule proteins of them can function as a transmembrane plug in the target membrane. They also have surface receptors to react with complement proteins (25, 28).

Natural killer cells

The infectious agents can reside in the host cells, which results in an escape from phagocytosis by macrophages and neutrophils. This situation can be managed by the natural killer (NK) cells. NK cells are cytotoxic lymphocytes. The phenotype of NK cells is characterized by $CD3^-$ $CD56^+/CD16^+$. Amount of NK cells is approximately 5 to 15% of peripheral blood lymphocytes (29). They can recognize and directly kill infected cells, abnormal cells as well as tumor cells by detecting unusual protein expression or aberrance of major histocompatibility complex (MHC) expression on the cells. NK cells can cause apoptosis in abnormal cells by directly releasing cytotoxic granules (including pore-forming granules and protease) or by depending on caspase activity via receptor complex activation (25).

Many evidences currently indicate that NK cells are also closely related to adaptive immunity by many reasons: NK cells are differentiated from the common lymphoid progenitor; they require the certain cytokines (IL-2 common–γ-chain family, particularly IL-15) for their development, homeostasis, and survival; which is similar to T and B cells; NK cells are functionally similar to T cells to produce IFN-γ and TNF-α; and NK cells mediate cytotoxic activity which is the same ability as cytotoxic T lymphocytes (CTL). Furthermore, NK cells are able to develop themselves to recognize the rechallenging specific antigens, which indicate that NK cells are a type of memory cells. However, NK cells do not rearrange the genes that encode their receptors, which is the same characteristic as innate cells (30, 31). As described, NK cells, therefore, are the cells that bridge and contain activities of both innate and adaptive immunities together.

2.1.1.3 Soluble factors

The soluble factors are secretory proteins, for example, lysozyme, interferons, and complements. They play important roles in inflammation.

Lysozyme, a hydrolytic enzyme found in secretions, can break down the peptidoglycan cell wall of the bacteria. Interferons are cytokines produced by infected cells from viruses, bacteria, parasites or by the presence of tumor cells. They perform the ability to trigger nearby cells to produce large amount of enzymes to eradicate pathogens or tumor cells. Interferons also exert their effects on NK cells and macrophages and increase antigen presentation to specific T cells (32). Complements are composed of a large group of plasma proteins that circulate in an inactive state. The complements are activated by the presence of cell-surface components of pathogens (which is related to a trigger of innate immune system) or by the presence of antigen-antibody complex (which is related to a trigger of adaptive immune system). The reactions of destroying pathogen include lysis of cells, opsonization, binding to complement receptors of immune cells and phagocytosis (21, 33).

2.1.2 Adaptive (acquired) immune system

Adaptive immune system provides a meticulous detection on non-self antigens, which tightly rely on interaction between antigen-presenting cells and lymphocytes. These interactions further modulate pathogen-specific immunologic effector pathways, generate immunologic memory cells, and regulation of host immune homeostasis.

2.1.2.1 Cells of adaptive immune system

The cells of adaptive immunity are typically present in the blood, lymphoid organ and tissues. These followings are crucial cells of adaptive immune responses.

Lymphocytes

Lymphocytes are special cells that can recognize and respond to a variety of antigens. From the origin of cells (i.e., lymphoid progenitor), lymphocytes are classified into three outstanding subsets: T lymphocytes, B lymphocytes and NK cells

B lymphocytes

B lymphocytes are crucial cells of the humoral immune response. B lymphocytes originate from bone marrow and migrate to secondary lymphoid tissues. The surface marker of human B lymphocytes at all stages

of maturation is CD19, which is present on approximately 7% to 23% of human peripheral blood lymphocytes (34). The major function of B cells is to secrete antibodies against foreign antigens. B cell expresses miscellaneous cell surface immunoglobulin (Ig) receptors to recognize specific antigenic determinants, once the cell encounter the cognate antigen and receive co-stimulatory signal from a T helper cell, the cell further differentiate into either plasma B cell (the effector B cell producing large amount of antibodies) or memory B cell (the cell that has a longer lifetime and respond to the same antigen quickly) (25, 28). To terminate pathogens, the secretory antibodies may either directly neutralize pathogens or collaborate with other immune materials (i.e., complement proteins or phagocytic cells) to trigger an elimination process (25).

T lymphocytes

T-lymphocytes are the most abundant lymphocytes (60% to 80% of the lymphocytes), which play a central role in immunoregulation and immunostimulation via cell-mediated immunity. T lymphocytes are differentiated from other lymphocytes by the presence of T-cell receptor (TCR) on the cell surface. CD3 is an antigen presented on the surface of mature T lymphocytes. The $CD3^+$ antigen is present on 61% to 85% of normal peripheral blood lymphocytes (35). When this antigen forms complex with TCR, they play an essential part in the maturation of thymocytes from immature precursors and they are also associated with signal transduction during antigen recognition (36, 37). The activated T lymphocytes may differentiate into T-helper cells (Th cells), cytotoxic T-lymphocytes (Tc cells, or CTLs) or become memory T cells (38). According to the difference in cytokine production and function, Th cells expressing $CD4^+$ antigen are further subdivided into Th1, Th2, Th17, and regulatory T (Treg) cells (39, 40). The characteristics of each Th lineage are shown in Table 2.1. While cytotoxic T-lymphocytes expressing $CD8^+$ are crucial effector cells for killing tumor cells and cells infected with intracellular pathogen (25, 41).

Table 2.1 T-helper cell subsets (40, 42)

Th cell subsets	Cytokine secretion	Function
Th1	IFN-γ	Initiate and maintain functions of $CD8^+$ memory T cells Induce inflammation Protective against intracellular pathogens
Th2	IL-4, IL-5, IL-13	Modulate B-cell proliferation and immunoglobulin class switching Involved in allergy Protective against extracellular parasites
Th17	IL-17, IL-17F, IL-21, IL-22	Protective against extracellular pathogens Induce autoimmunity
Treg	TGF-β	Suppress the excessive immune and inflammatory response

2.1.3 Cytokines

Cytokines are regulatory polypeptides or glycoproteins produced by various cells, especially cells of the immune system, to respond to an immune stimulus or to communicate between cells. Cytokines may be secreted or on the membrane. Cytokines function via binding to a specific cell surface receptor, resulting in transducing a signal to the recipient cell and initiating a cascade of cell activating events in the innate and adaptive immune systems (28, 43). Many individual cytokines are sometimes produced by the same cells, involved in both the innate and adaptive immune response. The effects of cytokines are pleiotropic and redundant: pleiotropic effects, individual cytokines have the ability to act on many cell types; and redundant effects, many cytokines have similar actions, which is due to the nature of the cytokine receptors. Cytokines are currently concerned that they play the crucial role in the

development of various disorders; therefore, they are used clinically as biological response modifiers in the treatment of diseases.

2.1.3.1 Cytokine Classification

Cytokines can be classified based on various criteria, for example, structural characteristics (Table 2.2) or functional characteristics (Table 2.3).

Table 2.2 Cytokine classification based on structural homology (44-46)

Protein Family	Cytokine	Producing Cells
TNF	TNF-α	Macrophages, NK cells, T-cells
	TNF-β	T-cells, B-cells
Interferons	IFN-α	Dendritic cells
	IFN-β	Fibroblasts
	IFN-γ	T-cells, NK cells
IL-10 related	IL-10	T-cells, Macrophages
	IL-29	T-cells, NK cells
IL-12 related	IL-12	Macrophages, dendritic cells
	IL-23	Dendritic cells
Hematopoietins	IL-2	T-cells
	IL-3	T-cells, thymic epithelial cells
	IL-4	T-cells, mast cells
	IL-6	T-cells, macrophages, endothelial cells
	IL-17	Th-17 cell, NKT cell
Miscellanous	TGF-β	Monocytes, T-cells
	IL-1	Macrophages, endothelial cells
	IL-25	T-cells, mast cell

Table 2.3 Cytokine classification based on functional mechanisms (45)

Protein Family	Cytokine
Inflammation, acute phase response and fever	TNF-α
	IL-1
	IL-6
Anti-viral protection	IFN-α
	IFN-β
	IL-29
B-cell proliferation	IL-7
T-cell proliferation	IL-2
	IL-7
Th2 responses	IL-4
	IL-25
Th1 responses	IFN-γ
	IL-12
	IL-23
Anti-inflammatory	IL-10
	TGF-β

From the functional mechanisms of cytokines, they can also be divided into 3 groups with different activities and mechanisms.

2.1.3.1.1 Cytokines of innate immunity

Cytokines of innate immunity have a direct action against the foreign invader during the early infected stages. In other words, these cytokines are able to initiate other immune-modulatory mechanisms (e.g., produce inflammatory process, provoke NK cells and monocytes-macrophages, which induce the other cytokines to release later). Cytokines that play a major role in the innate immune system include TNF-α, IL-1, IL-6, IL-10, IL-12, type I interferons (IFN-α and IFN-β) and chemokines (21, 43, 47).

2.1.3.1.2 Cytokines of adaptive immunity

Cytokines of adaptive immunity are mainly secreted by T-cells to respond to a specific antigen (Ag). Cytokines that play a major role in the adaptive immune system include IL-2, IL-4, IL-5, IFN-γ, TGF-β, IL-13 and IL-17 (21).

2.1.3.1.3 Cytokines of hematopoiesis

To protect the body from infectious agent, the immature immune cells have to be prepared earlier and differentiated into specific mature cells in favor of hematopoietic cytokines. Some of discovered hematopoietic cytokines are granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage-stimulating factor (M-CSF), erythropoietin (EPO), thrombopoietin, IL 3, IL 7, IL-9 and IL-11 (18-19).

The cytokine features of innate immunity, adaptive immunity and hematopoiesis are summarized in Table 2.4.

Table 2.4 A summary of cytokine features (21, 43, 48, 49)

Cytokines	Secreted by	Cell target	Effects
Some cytokines of innate immunity			
TNF-α	Macrophages T cells	T cells; B cells Endothelial cells Hypothalamus Liver	- Activation of inflammation - Vasculature - Fever - Lipid metabolism (cachexia) - Induction of apoptosis in every cell type
IL-1	Monocytes and macrophages (main) Fibroblasts Epithelial cells Endothelial cells Astrocytes	T cells; B cells Endothelial cells Hypothalamus Liver NK cells	- Modulation of inflammatory response (e.g., fever, acute phase protein) - T-cell activation - Chemokine secretion by endothelial cells - Stimulation of IFN-γ secretion by NK cells

Table 2.4 A summary of cytokine features (continued).

Cytokines	Secreted by	Cell target	Effects
Some cytokines of innate immunity (continued)			
IL-6	T cells; Macrophages; Fibroblasts	T cells; B cells Mature B cells Liver	- Influences adaptive immunity (proliferation and antibody secretion of B cell lineage) - Acute phase reactants
IL-10	T cells (T_H2)	T cells Macrophages	- Inhibits production of IFN-γ by Th1 cells - Inhibits APC activity - Inbits cytokine production by macrophages
IL-12	Macrophages Dendritic cells	Naive T cells Tc cells NK cells	- Differentiation of naïve T cells into a Th1 cell - Enhances the cytolytic functions of Tc and NK cells - Stimulation of the IFN-γ synthesis by T-cells and NK cells
IFN-α	Macrophages	Nucleated cells T cells NK cells	- Inhibit viral replication in most nucleated cells - Activation of MHC class I expression - NK-cell activation
IFN-β	Fibroblast	Nucleated cells T cells NK cells	- Induction of an antiviral state in most nucleated cells - Activation of MHC class I expression - NK-cell activation
Some cytokines of adaptive immunity			
IL-2	Antigen-activated T cells	T cells B cells NK cells Monocyte	- T-cell proliferation - B-cell proliferation - NK cell activation/proliferation - Monocyte proliferation

Table 2.4 A summary of cytokine features (continued).

Cytokines	Secreted by	Cell target	Effects
Some cytokines of adaptive immunity (continued)			
IL-4	Th2 (main) Mast cells Basophils NK cells	Naive T cells B cells Monocytes/macrophages	- Th2 differentiation and proliferation - Isotype switching to IgE - Inhibitor of IFN-γ activation
IL-5	Th2 Eosinophil Mast cells NK cells	B cells Eosinophils Mast cells	- B-cell differentiation, IgA production - Eosinophil differentiation/ activation - Th/Tc maturation
IFN-γ	Th1 cells $CD8^+$ cells NK cells	Monocytes Endothelial cells Many tissue cells - especially macrophages	- Monocyte activation - Endothelial cell activation - Increased class I and II MHC
TGF-β	T cells; Macrophages	T cells Macrophages B cells	- Inhibitor of growth of T cells , B cells and macrophages - Promotes isotype switch to IgE
IL-17	Th17 cells	Neutrophils Stromal cells Fibroblasts	- Recruitment of neutrophils - Activate the expression of proinflammatory cytokines, chemokines, and cell adhesion molecules
Some cytokines of hematopoiesis			
IL-3	Antigen-activated T cells	Bone marrow progenitors	- Growth and differentiation
GM-CSF	T cells; Macrophages; Endothelial cells, Fibroblasts	Bone marrow progenitors	- Granulocyte and monocyte maturation - Hemopoietic effects

2.2 Immunomodulators

The disorders of immune system, resulting from either excessive or insufficient immune response, have enormous impacts on human diseases (10); therefore, immunomodulators, nowadays, are used and become a drug of choice for many diseases.

Immunomodulators or biologic response modifiers are natural or synthetic substances that are able to interact with immune system in order to upregulate or downregulate specific aspects of the host response. They are generally classified into two types based on their effects: immunosuppressants and immunostimulants (50, 51).

2.2.1 Immunosuppressants

Immunosuppressants are used to weaken the immune response in organ transplantation and autoimmune disease (10). The classification of immunosuppressants is shown in Table 2.5.

2.2.2 Immunostimulants

In contrast to immunosuppressants, immunostimulants are used to stimulate or amplify immune responses against infectious pathogens and tumor cells as well as used in immunodeficiency patients (10, 52). Immunostimulants can be classified into two types: specific immunostimulants (which perform antigenic specificity in immune response, for example, vaccines or any antigen); and non-specific immunostimulants (which can increase immune responses by magnifying immune response of other antigen or activate components of immune system without antigenic specificity, for example, adjuvants) (53).

Table 2.5 Classification of immunosuppressants (10, 50)

Mechanism of action	**Drugs**
Inhibit lymphocyte gene transcription	Glucocorticoids
Inhibit lymphocyte signaling a) Calcineurin inhibitors b) Mammalian target of rapamycin (mTOR) inhibitors	Cyclosporine, Tacrolimus Sirolimus, Everolimus
Cytotoxic agents a) Antimetabolites b) Alkylating agents	 Azathiprine, Methotrexate Cyclophosphamide
Cytokine inhibitors (anticytokine-antibodies) a) TNF-α inhibitors b) IL-1 inhibitors c) IL-2 inhibitors	 Etanercept, Infliximab, Anakinra Daclizumab, Basiliximab
Antibodies against specific immune cell molecules a) Polyclonal antibodies b) Monoclonal antibodies	 Antithymocyte globulin (ATG) Muromunab (anti CD3 antibodies)
Inhibitors of immune cell adhesion	Efalizumab (LFA-1 Inhibitor)

2.3 Mangosteen overviews

2.3.1 Botanical information

Mangosteen, *Garcinia mangostana* L., is an evergreen fruit tree in the family Clusiaceae. The original place of mangosteen is in Southeast Asia and it is much cultivated in Thailand. It is one of the most attractive tropical fruits: it has the remarkably pleasant flavor, leading to the name “the queen of fruits”; it also contains many magnificent therapeutic benefits (11, 14). The tree is slow to grow but can reach 6–25 m in height. Leaves are simple opposite, ovate or elliptic-oblong, 4.5-10 cm

wide, 15-25 cm long, leathery, glabrous leaves with dark green above and dull pale green beneath. Flowers are solitary or dichasium, axillary in uppermost leaf-axil. There are four sepals. The color of petals is yellowish-green inside and green with red spots on the outside. The fruit is capped by the prominent calyx at the stem end with 4–8 triangular remnants of the stigma in a shape of a rosette at the apex. The external of the fruit is smooth and round with dark-purple to red-purple color. The fruit rind is 6-10 mm thick, red in cross-section, purplish-white on the inside, contains bitter yellow latex and a purple. The edible part of the fruit is aril, which is white, juicy, soft flesh and divided into 4-8 triangular segments. The taste of aril is slightly acid and mild to distinctly acid in flavor. The seeds have ovoid-oblong shape. The number of seeds per one fruit is 1 to 5 fully developed seeds or sometimes seedless (13, 54).

2.3.2 Traditional medical use

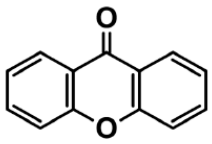
Different parts of mangosteen, mostly fruit rind, have been used in traditional medicine for the treatments of diarrhea, dysentery, infections and wounds (11, 55) according to the following formulations (Table 2.5).

Table 2.6 Formulations of the mangosteen fruit rind for treatment of diseases (56, 57).

Conditions	Formulations
● Diarrhea	- The well-dried fruit rind is rubbed with special stone using water as a solvent. - A quarter of the dried fruit rind is macerated with lime water (a saturated calcium hydroxide solution) or drinking water. - The dried fruit rind is boiled with water. Doses for children are 1-2 teaspoonfuls every 4 hours. The adult dose is one tablespoonful every 4 hours.
● Dysentery	- A quarter of the dried fruit rind is grilled until burnt and macerated with about half a glass of clear lime water or ground to a powder and dissolved in rice water or boiled water. Drink every two hours.
● Skin infections	- The dried fruit peel is rubbed with stone using water as a solvent. The suspension is applied over the wound areas.

2.3.3 Chemical constituents from *G. mangostana*

Mangosteen contains a variety of phytochemicals, which the major compounds are xanthone derivatives (Figure 2.1). There are more than 60 derivatives of xanthones, for example, α-mangostin, β-mangostin, γ-mangostin, 1-isomangostin, 3-isomangostin, 9-hydroxycalabaxanthone, 8-deoxygartanin, demethylcalabaxanthone, garcinone B, garcinone D, garcinone E, gartanin, mangostanol, mangostanin and mangostinone, found in different parts of magosteen including heartwood, stem, bark, leaves, seeds and especially in “fruit rind” or “pericarp” (13-16). The pericarp is a source of various vitamins (B1, B2 and C), anthocyanins, xanthones and other substances (11, 58) as therapeutic agents (described in Table 2.6). Among of them, α-mangostin is present in the highest concentration of all xanthones (59) in the dried fruit rind.



xanthone

Figure 2.1 Chemical structure of xanthone. The structure was redrawn from Walker EB (59).

2.3.4 Pharmacological activities and clinical trials

From the evidence that mangosteen has been used as traditional medicine for a long time and it contains the high content of xanthones, theses arouse the scientists to discover more information about mangosteen activities. The following information is some of main medicinal properties of mangosteen extracts.

2.3.4.1 Antioxidant property

Alpha- and γ- mangostins show antioxidant activity using the ferric thiocyanate method and α-mangostins decreased the human low density lipoproteins (LDL) oxidation induce by copper or peroxyl radical (14). Various extracts of mangosteen (water, ethanol, ethyl acetate, methanol) possess antioxidant (11) and neuroprotective activity in cell culture (60, 61).

2.3.4.2 Antiproliferative and anticancer properties

Crude methanolic extract could inhibit proliferation of breast cancer cell line (median effective dose (ED_{50}) is equal to 9.25 ± 0.64 μg/ml) (11). The combination of α- and γ- mangostins exhibit *in vivo* anti-colon cancer activity (62).

2.3.4.3 Antitumoral property

Crude methanolic extract from mangosteen pericarp can reduce tumor size at 50-70% in the footpad of tumor-bearing mice (63).

2.3.4.4 Anti-inflammatory and antiallergy properties

Alpha- and γ- mangostins reduce inflammation in human macrophage cell-line (64). Gamma-mangostin can inhibit inhibitor-κB kinase and reduce LPS-induced COX-2 gene expression in cell culture (65). An ethanol extract of mangosteen pericarp can inhibit IgE-mediated histamine secretion and prostaglandin E2 synthesis (66).

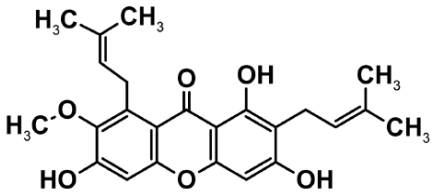
2.3.4.5 Antibacterial, antifungal and antiviral properties

Alpha-mangostin can inhibit *Propionibacterium acnes* and *Staphylococcus epidermidis* (MIC values are 3.91 µg/ml but minimum bactericidal concentration (MBC) values are 3.91 and 15.63 µg/ml against *P. acnes* and *S. epidermidis* , respectively) (67) and *Candida albicans* (MIC and minimum fungicidal concentration (MFC) value are 1,000 and 2000 µg/ml, respectively). γ-Mangostin exhibits activity as a noncompetitive HIV-1 protease inhibitor (median inhibitory concentration (IC_{50}) = 4.81 ± 0.32 µM) (68).

2.3.4.6 Antimalarial property

Prenylated xanthone has *in vitro* antiplasmodial activity against *Plasmodium falciparum* (69).

2.3.5 α-Mangostin



α-mangostin

Figure 2.2 Chemical structure of α-mangostin. The molecular weight of α-mangostin is equal 410.47. The structure was redrawn from Walker EB(59).

α-Mangostin (Figure 2.2) is a phenolic compound. It is present at the highest concentration than other xanthones (59) in the dried pericarp. α-Mangostin exhibits remarkable pharmacological effects: it can induce apoptosis and cell cycle arrest in several cancer cells, possesses antibacterial activity and has cytoprotective effects (Table 2.7).

Table 2.7 Pharmacological effects of α-mangostin

Effects	Actions
● Induction of apoptosis in cancer cells	-Activates caspase-3 and -9 in mitochondria on human leukemia HL60 cell line (70). -Inhibits sarcoplasmic/endoplasmic Ca^{2+} ATPase (SAECA) and activate autophagy in mouse colon carcinoma and Her-2/CT26 tumor-bearing mice (71). -Modulates bcl-2, bax and p53 level in HNSCC cell lines (72).
● Induction of cell cycle arrest in cancer cells	-Induces G1-phase arrest and suppresses S-phase by altering the expression of cell-cycle-related molecules (i.e., CDKs) in breast cancer cell line (17).
● Bactericidal property	-Disrupts cytoplasmic membrane integrity of gram positive pathogens and methicillin-resistant *Staphylococcus aureus* MRSA (MIC = 0.78–1.56 μg/ml).
● Cytoprotection	-Protects cardiac ischemia by reducing oxidative stress in a mouse model (73). -Protects renal by reducing reactive oxygen species in a rat model (74).

2.3.6 Crude water extract

Mangosteen pericarp contains various substances, not only α-mangostin that possesses remarkable clinical activities, but also other substances such as crude water extract. The crude water extract is water-soluble substances derived from the left extraction process of methanol followed by ethylacetate and water. This partially purified crude water extract contains phenolic compounds which α-mangostin concentration is less than 2% (60). The crude water extract has promising antioxidant and neuroprotective activities against H_2O_2-induced oxidative stress in NG108-15 cells (61) and against *β*-amyloid -induced oxidative stress in SK-N-SH neuronal cells (60). Moreover, it acts as an anti-inflammatory agent by inhibiting prostaglandin E2 synthesis in C6 rat glioma cells (66). According to these interesting activities, in this

experiment, α-mangostin and crude water extract have been selected to test for their immunomodulatory activities.

2.4 Properties of control substances

In this study, we have four control substances routinely used to measure the responses of immune cells from different donors. The positive controls were composed of paclitaxel, concanavalin A and lipopolysaccharide; while, the negative control was dexamethasone.

2.4.1 Paclitaxel

Paclitaxel is an antitumor drug, used to treat a variety of malignant tumors. Paclitaxel is able to bind β-tubulin to enhance the polymerization of microtubules and stabilize microtubules by preventing depolymerization. This effect diminishes the amount of microtubules, leading to the disruption to cell mitosis and other normal cellular activities where microtubules are involved, and ultimately inhibits the proliferation of cells (75-77). Because paclitaxel is derived from natural product, firstly isolated from Pacific yew tree, *Taxus brevifolia* and has antiproliferative activity; therefore, it was used as a control to compare cytotoxicity between mangosteen extracts in MTT assay.

2.4.2 Concanavalin A (ConA)

Concanavalin A (ConA) is a plant lectin, a protein first isolated from jack bean and characterized by specifically binding to α-D-mannose and α-D-glucose moieties in which appear on a variety of cell surfaces. ConA has the ability to induce T-cell blastogenesis and mitosis as well as to increase other syntheses of cellular products such as cytokines. ConA is useful for studying carbohydrate, glycoprotein purification, enzyme tagging, cell membrane, cell agglutination, and cell typing. (78-80). This was the reason why ConA was used as a positive control in immunophenotypic assay and cytokine determination by ELISA method.

2.4.3 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) or endotoxin is a characteristic component existing on outer membrane of the Gram-negative bacteria. LPS consists of three parts: lipid A, core oligosaccharide and O-specific polysaccharide. The three parts of LPS are immunogenic, which can stimulate the formation antibodies (81). To protect the body from bacterial infection, monocytes and macrophages were the major cells that respond to the presence of LPS by secreting primary mediator TNF-α, IL-1, IL-6 and arachidonic acid metabolites via NF-κB activation in order to stimulate other immune cells to fight against bacteria (81-83). LPS was used a positive control in intracellular cytokine determination.

2.4.4 Dexamethasone

Dexamethasone, a potent synthetic glucocorticoid, exhibits anti-inflammatoy and immunosuppressant properties by inhibiting NF-κB expression in a dose-dependent manner (84, 85). This action is mediated through on lymphocytes, endothelial cells and monocyte/macrophages, at least (86). Dexamethasone, therefore, was used in this study as an inhibitor to suppress NF-κB-mediated effects on TNF-α expression.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and solutions

3.1.1.1 Purified α-mangostin and partially purified crude water extract

Purified α-mangostin and partially purified crude water extract were extracted from the dried fruit rind of mangosteen, obtained from Immunology Laboratory, Department of Microbiology, Mahidol University.

3.1.1.2 PBMCs isolation and culture

Name	Catalog No.	Company
Lymphoprep™	1114547	Axis-Shield
Penicillin G/Streptomycin	A2210	Biochrom AG
RPMI 1640 medium	T121-01	Biochrom AG
Sodium bicarbonate	478537	Carlo Erba
FBS superior	S0615	Biochrom AG
Concanavalin A (ConA)	234567	Calbiochem
Trypan blue	T6146	Sigma

3.1.1.3 Cell viability assay by MTT method

Name	Catalog No.	Company
MTT	19265	USB corporation
Paclitaxel	-	Boryung Pharm

3.1.1.4 Imunophenotyping by flow cytometry

Name	Catalog No.	Company
Fluorochrome-labeled antibodies	340503	Becton, Dickinson

3.1.1.5 Intracellular cytokine detection by flow cytometry

Name	Catalog No.	Company
Brefeldin A	B7651	Sigma-Aldrich
E. Coli LPS (O111:B4)	437627	Calbiochem
EDTA	15701	USB corporation
FACS lysing solution	349202	Becton, Dickinson
Permeabilization buffer	554723	Becton, Dickinson
Mouse IgG1 Isotype reagents	554679	Becton, Dickinson
FITC mouse anti-human TNF	562082	Becton, Dickinson
PerCP mouse anti-human CD14	340585	Becton, Dickinson

3.1.1.6 Cytokine detection by ELISA method

Name	Catalog No.	Company
Human TNF-α ELISA kit	DTA00C	R&D Systems
Human IL-1β ELISA kit	DLB50	R&D Systems
Human IL-2 ELISA kit	D2050	R&D Systems

3.1.2 Equipments and instruments

Name	Model	Company
6-well plate	3516	Costar
96-well plate	167008	Nunc
Analytical balance	SPB 33	Scaltec
Autoclave	SS-325	Tomy Seiko
Automated pipette	-	Labmate
Biohazard laminar air flow	CYTAIR	Flufrance
Centrifuge	EBA12	Hettich
Centrifuge	Mikro22R	Hettich
Centrifuge tube	15, 50 ml	Nunc
Cellulose acetate filter	11107--47------N	Sartorius Stedim
CO_2 incubator	BB 15	Heraeus
Flow cytometer	FACSCalibur	Becton, Dickinson
Hemocytometer	717805	Brand

3.1.2 Equipments and instruments (continued)

Name	Model	Company
Hot air oven	UNE 600	Memmert
Inverted microscope	Eclipse TS100	Nikon
Incubator	BD 240	Binder
Microplate reader (UV scan)	InfiniteM200	Tecan
Needle	21-G x 1 1/2	Nipro
Pasteur pipette	747720	Brand
pH- meter	Basic	Gibthai
Pipette boy	0301	High Tech Lab
Round bottom test tubes	352054	Becton, Dickinson
Syringe	10 ml	Nipro
Syringe filter	PN 4612	Pall
Venous Blood Collection Tubes	454051	Greiner Bio-One
Vortex mixer	Vortex-2 Genie	ScientificIndustries

3.2 Methods

The procedures of testing immunomodulatory activity of *G. mangostana* extracts are illustrated in Figure 3.1.

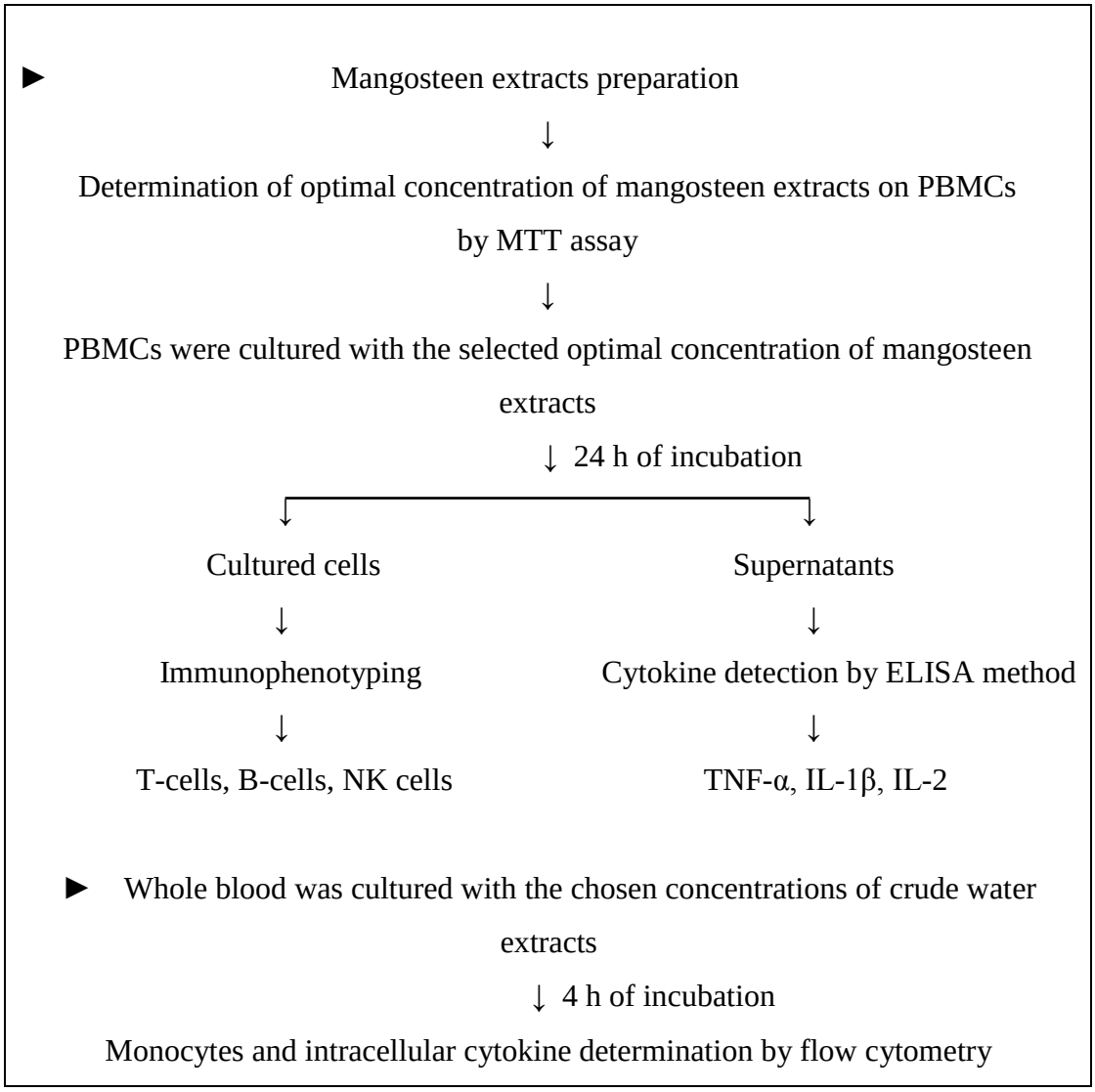


Figure 3.1 The experimental diagram of evaluating effects of mangosteen extracts on immune cells.

3.2.1 Ethical Consideration

The study protocol was approved by Faculty of Pharmacy, Mahidol University, Thailand. The Certification of Exemption number was MU-DT/PY-IRB 2013/003.0402 (Appendix E).

3.2.2 Preparation of stock solutions of test compounds

Purified α-mangostin and partially purified spray-dried crude water extract were kindly obtained from Immunology Laboratory, Department of Microbiology, Mahidol University. The stock solutions of test compounds were freshly prepared for an individual experiment. Purified α-mangostin was dissolved with 10% (v/v) dimethyl sulfoxide (DMSO; Labscan Asia, Bangkok, Thailand) in RPMI 1640 medium containing 10% (v/v) fetal bovine serum as a 1,000 µg/ml stock solution and further diluted with the complete medium to achieve the desired concentrations before treating cells in each experiment. The final DMSO concentration in the working solution would not exceed 0.1% v/v. While partially purified crude water extract was dissolved with RPMI 1640 medium supplemented with 10% fetal bovine serum. The stock solution was filtered through a cellulose acetate membrane (pore size 0.2 µm).

3.2.3 PBMCs isolation

PBMCs contain essential components of human immune system, which mainly are lymphocytes, monocytes and a small number of NK cells (87). Therefore, in this study, PBMCs were selected to be the experimental cells to analyze the mangosteen extracts in immune monitoring.

PBMCs were isolated by density gradient centrifugation method. Buffy coats of healthy blood donors (obtained from Ramathibodi Hospital) were diluted 1:1 with PBS. Six ml of the diluted buffy coat was carefully layered over 3 ml of Lymphoprep™ in a 15-ml centrifuge tube. The tube was centrifuged at 800 x g for 30 min at room temperature with the brake off. The opaque interface (mononuclear cells) was collected into a new conical tube by using a Pasteur pipette. The harvested fraction was washed with PBS by centrifugation at 250 x g at room temperature for 10 min and the supernatant was aspirated. The lymphocyte pellet was resuspended in RPMI 1640 medium with 10% heat-inactivated FBS. The number of viable cells were counted by using a trypan blue dye exclusion method and used for the further experiments.

3.2.4 MTT cell viability assay

MTT assay is a simple method to evaluate the number of viable cells by detecting the conversion of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) color. The only mitochondria of living cells are able to produce intracellular succinate dehydrogenase to reduce yellow MTT into purple formazan crystal. The formazan is then solubilized by detergent reagent and spectrophotometrically measured (88). The change of formazan color is directly proportional to the number of viable cells.

3.2.4.1 MTT assay for determination of optimal cell number

The optimal cell count and incubation period were determined by making serial dilutions of PBMCs per well (in a 96-well plate), ranging from 4 x 10^6, 2 x 10^6, 1 x 10^6, 5 x 10^5, 2.5 x 10^5, 1.25 x 10^5, 6.25 x 10^4, 3.13 x 10^4 and 1.6 x 10^4 cells/well, respectively. The cells were periodically observed under an inverted microscope for the presence of intracellular punctate purple precipitate. The absorbance of each well was measured at a wavelength of 570 nm with background subtraction at 690 nm. The cell number selected should typically be within the linear portion of the plot and yield an absorbance of 0.75 - 1.25 (89).

3.2.4.2 MTT assay for determination of optimal test compound concentrations

To determine the optimal concentrations of the test compounds for PBMCs culture, each test compound was dissolved in RPMI 1640 supplemented with 10% FBS and added into a 96-well plate. Then, 50 µl of the selected PBMCs count was seeded on the plate. Therefore, the final concentrations of α-mangostin were 0.5, 1, 2, 4, 6, 8 and 10 µg/ml; the final concentrations of crude water extract were 25, 50, 100, 150, 200, 250 and 300 µg/ml; and the final concentrations of a positive control paclitaxel, were 0.63, 1.25, 2.5, 5 and 10 µg/ml in the total volume of 100 µl/well. For the untreated control wells of α-mangostin treatment were added with the DMSO-containing RPMI medium as the final concentrations of DMSO at 0.005, 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1 µg/ml; meanwhile, the wells of untreated cell control of crude water extract treatment were added with only RPMI medium. Plates were incubated in a 37 °C, 5% CO_2 and 90% humidity incubator for 24 hours. Freshly

prepared 10 µl of MTT reagent at 5 mg/ml in PBS was added into each well and incubated for 4 hours in a 37 °C, 5% CO_2 and 90% humidity incubator. Finally, 100 µl of detergent reagent was added to each well and incubated for overnight at 37 °C to completely solubilize the formazan crystal (90). The observance in each well was measured at 570 nm in a microplate reader with the reference wavelength 690 nm. The treated cells, untreated cells and blank were assayed in triplicate for three times. The relative cell survival was calculated by the following formula:

$$\text{Relative cell survival (\%)} = \frac{\text{(A treated-A blank)}}{\text{(A untreated-A blank)}} \times 100$$

$A_{treated}$ = Absorbance of wells containing the test compound + PBMCs

$A_{untreated}$ = Absorbance of wells containing the solvent + PBMCs

A_{blank} = Absorbance of wells containing the test compound

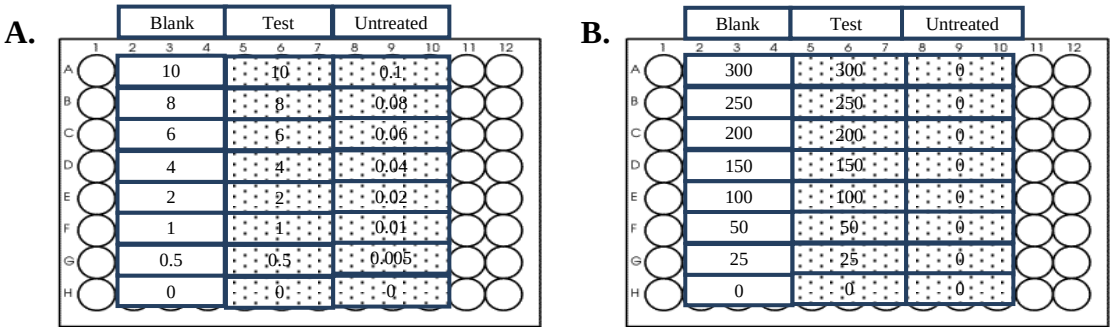


Figure 3.2 The schematic layout of the MTT plates. The MTT plates of α-mangostin (A) and crude water extract (B) treatment. The dotted wells were shown as containing PBMCs.

3.2.5 PBMCs culture and stimulation

PBMCs (6 x 10^6 cells/well) were seeded in the 6-well plates and treated with α-mangostin (final concentration 1, 2 and 4 µg/ml); crude water extract (final concentration 50, 100 and 200 µg/ml); ConA (final concentration 10 µg/ml); a combination of α-mangostin and ConA (1, 2 and 4 µg/ml of α-mangostin plus 10

µg/ml of ConA); a combination of crude water extract and ConA (50, 100 and 200 µg/ml of crude water extract plus 10 µg/ml of ConA); and RPMI 1640 medium supplemented with 10% FBS. Unstimulated cells were served as a negative control and ConA-treated cells were served as a positive control. The plate was incubated in a 37 °C, 5% CO_2 and 90% humidity incubator. After 24 h of incubation, the cells were collected by centrifugation for further analysis of immunophenotyping and the supernatants were kept at -70 °C for cytokine detection.

3.2.6 Imunophenotyping by flow cytometry

The effects of mangoteen extracts on lymphocyte subsets were analyzed by immunostaining method. The expression of cell surface markers on mature human T lymphocytes ($CD3^+$), B lymphocytes ($CD19^+$) and NK lymphocytes ($CD3^-CD16^+$ and/or $CD56^+$) were enumerated by staining with a four-color immunofluorescence reagent kit (FITC-labeled CD3, PE-labeled CD16, CD56, PerCP-labeled CD45, and APC-labeled CD19) (35). The stained cells were passed through the laser beam in the flow cytometer and scattered the laser light; therefore, the instrument was able to provide information about the size, internal feature and relative fluorescence intensity of each cells.

After 24 h of incubation, the cultured cells were harvested by centrifugation at 500 x g for 5 min at 4 °C, washed three times with 1 ml of staining buffer, distributed in tubes (2×10^5cells/tubes) and stained with 10 µl of a cocktail of antibodies on ice for 30 min in the dark. After staining, the cells were washed twice with 1 ml of staining buffer and fixed with 500 µl of 1% paraformaldehyde in PBS. The fixed cells were washed, resuspended with staining buffer and kept at 4 °C in the dark and analyzed within 3 days. This experiment was performed in three individual samples. Data acquisition was performed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowJo software version 10.0.5 for Microsoft (TreeStar, San Carlos, CA). The gating strategy was based on $CD45^+$ to identify the lymphocyte populations. The total percentages of $CD3^+ + CD19^+ + CD56^+$ and/or $CD16^+$ were between 100 ± 5% to control flow result accuracy.

3.2.7 Cytokine detection by ELISA method

Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive and specific method to detect a targeted antigen in a complex mixture by using a specific antibody. Sandwich ELISA is one of the ELISA techniques. The antigen of determined cytokine is bound to the pre-coated antibody on the microplate well and then the second specific antibody linking to an enzyme is added to bind the capture antigen. The reaction was complete by incubating this enzyme-complex with a substrate that produces a detectable product (91).

To examine the effects of mangosteen extracts on immune cell-secreted cytokines (i.e., TNF-α, IL-1β and IL-2), the sandwich ELISA technique was performed. The frozen supernatants of treated PBMCs were thawed to room temperature and then added into the ELISA wells. The plates were processed and measured according to the manufacturer's protocol. The sensitivities of TNF-α, IL-1β and IL-2 detection kits were 5.5, 1 and 7 pg/ml, respectively (91).

3.2.8 Intracellular cytokine detection by flow cytometry

Monocytes are the major cells that secrete inflammatory cytokine such as TNF-α. Therefore, to validate the effect of crude water extract activating inflammatory cytokine secretion, the intracellular TNF-α of monocytes cultured with crude water extract were determined by flow cytometry.

The test substances in this experiment including crude water extract, LPS and dexamethasone were prepared by dissolving in PBS. Fresh whole blood sample from one healthy volunteer was collected in heparinized blood collection tubes. Five hundred µl of whole blood was added into 15-ml centrifuge tubes and followed by adding 10 µl of the prepared substances: crude water extract (final concentration 50 and 100 µg/ml); combinations of crude water extract and LPS (crude water extract final concentration 50 and 100 µg/ml plus LPS final concentration 10 µg/ml); combinations of crude water extract and dexamethasone (crude water extract final concentration 50 and 100 µg/ml plus dexamethasone final concentration 0.02 nM); LPS (as a positive control, final concentration 10 µg/ml); a combination of LPS and dexamethasone (final concentration 10 µg/ml and 0.02 nM, respectively); and PBS (as a negative control). The tubes were then added 10 µl of 0.5 mg/ml Brefeldin A to

inhibit a protein transport of Golgi (92) and incubated for 4 h in a 37 °C, 5% CO_2 and 90% humidity incubator. After incubation, 50 µl of 20 mM EDTA was added into the tubes for 15 min at room temperature. To lyse red blood cells, 4.5 ml of FACS lysing solution was added and the tubes were incubated for 10 min at room temperature. After centrifugation at 500 x g for 5 min and washing with 8 ml of staining buffer, 2.5 ml of FACSPerm was added into the tubes and incubated for 10 min. The cells were washed with 8 ml of staining buffer at 500 x g for 5 min and resuspended with 0.5 ml of staining buffer. Fifty µl of each cell suspension was brought into a 12 x 75 mm polystyrene tube containing fluorochrome-conjugated antibodies: 5 µl of PerCP-labeled CD14 and 5 µl of FITC-labeled TNF-α (with an additional tube for 5 µl of the FITC-labeled isotype control) for 30 min at room temperature in the dark. The stained cells were washed with 2 ml of staining buffer, centrifuged at 500 x g for 5 min to discard supernatant and resuspended with 500 µl of 1% paraformaldehyde. The fixed cells were kept at 4 °C in the dark and analyzed within 24 h on FACSCalibur flow cytometer with FlowJo software**.** Gates were drawn based on forward scatter versus side scatter plot, and bright $CD14^+$ fluorescence versus side scatter plot. A minimum of 15,000 $CD14^+$ monocyte events were collected for TNF-α analysis.

3.2.9 Statistical analysis

The data were expressed as mean ± SEM. The comparison was performed by using one-way ANOVA with Tukey's post hoc test or Student's *t*-test. The nonparametric data were analyzed by Kruskal-Wallis test with Mann-Withney U test. The difference was considered statistically significant with P-value < 0.05. All statistics were performed by using SPSS software (SPSS, Chicago, IL, USA).

CHAPTER IV
RESULTS

4.1 Peripheral blood mononuclear cells (PBMCs) preparation

PBMCs were isolated from diluted buffy coat by using Lymphoprep™. After centrifugation, a white band of mononuclear cells was present at the interface between Lymphoprep™ and plasma, while the red blood cells (RBC) and polymorphonuclear leukocytes (PMNs) were sedimented (Figure 4.1A). The morphology of PBMCs was shown in Figure 4.1.

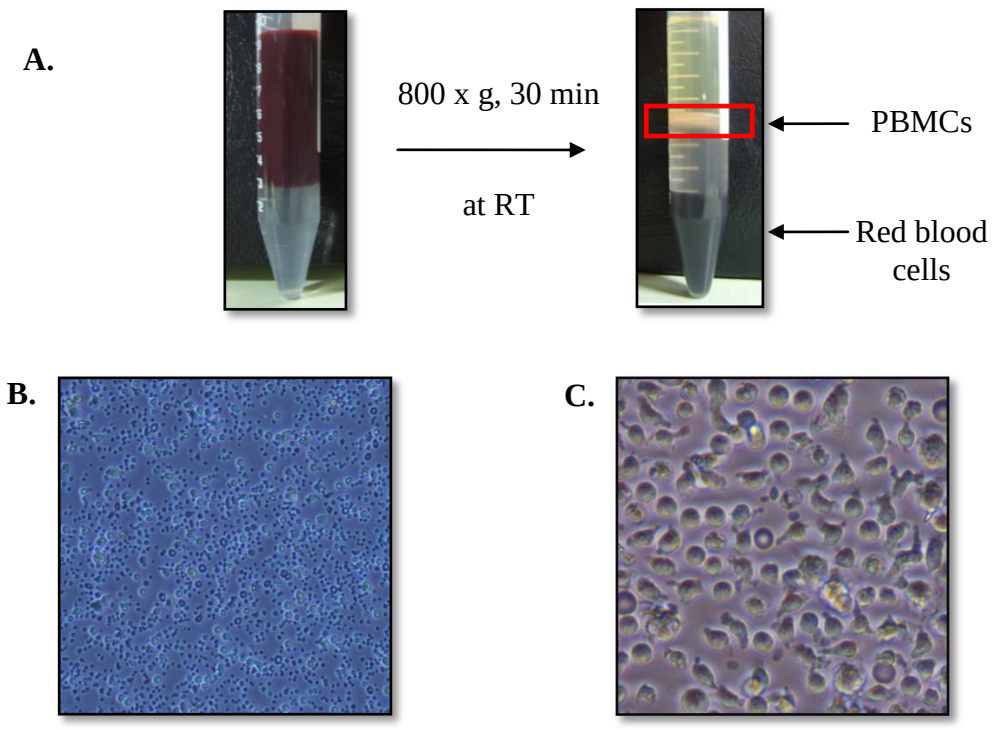


Figure 4.1 PBMCs isolation by density gradient method. Mononuclear cells were present at the interface between Lymphoprep™ and plasma (A). The morphology of PBMCs was observed under 100X (B) and 400X (C) magnification of phase contrast microscope.

4.2 Cell viability assay by MTT method

The optimal PBMCs count in a 96-well plate for 24-houred culture was determined by MTT method. The suitable amount of cells in the proper optical density (O.D.) range from 0.75 - 1.25 was between 2 x 10^5 and 4.6 x 10^5 cells/well (Figure 4.2A). The results of microscopic observation showed that the approximate 80% of cell confluency was at 2 x 10^5 cells/well (Figure 4.2B). In addition, at 2 x 10^5 cells/well, the purple formazan crystals were completely dissolved by solubilizing detergent. Therefore, 2 x 10^5 cells/well was selected as the optimal PBMCs count.

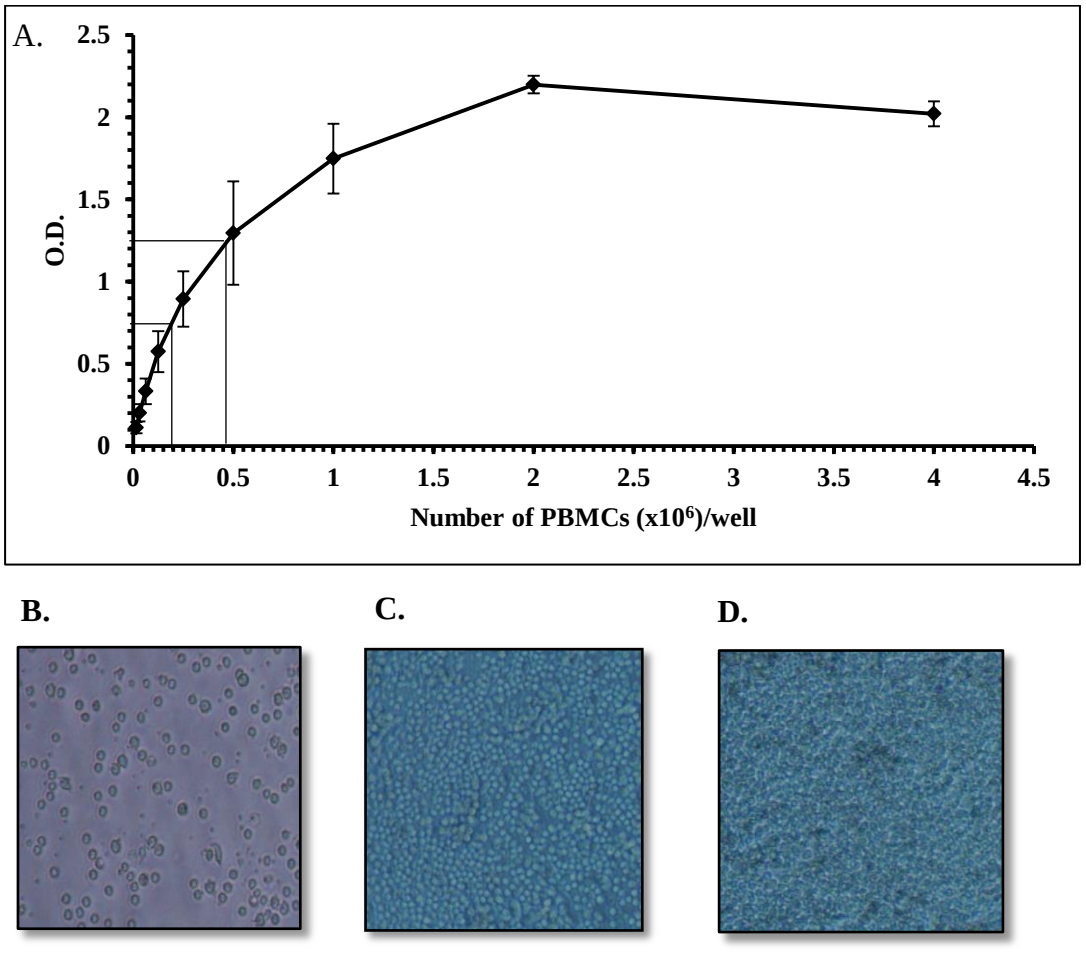


Figure 4.2 The optical density curve of PBMCs by MTT method. The varied amounts of PBMCs/well were ploted against optical density (A). The microscopic pictures of cells at 3 x10^4 (B), 2 x 10^5 (C) and 2 x 10^6 cells/well (D) (*n* = 3 individual samples).

The optimal concentrations of mangosteen extracts on immune cells were evaluated by MTT assay. Two hundred thousand PBMCs/well were treated with α-mangostin, crude water extract and paclitaxel (a positive control) in 96-well plates for 24 h. Comparing the percentage of PBMCs survival with untreated cells, the 50% of PBMCs survival resulted from being exposed to 5.55 µg/ml of α-mangostin, 150 µg/ml of crude water extract, or 5.85 µg/ml of paclitaxel. For more than 95% of cell survival, the concentrations of α-mangostin, crude water extract or paclitaxel had to be lower than 4.5, 55 and 2.35 µg/ml, respectively, compared with untreated cells (Figure 4.3).

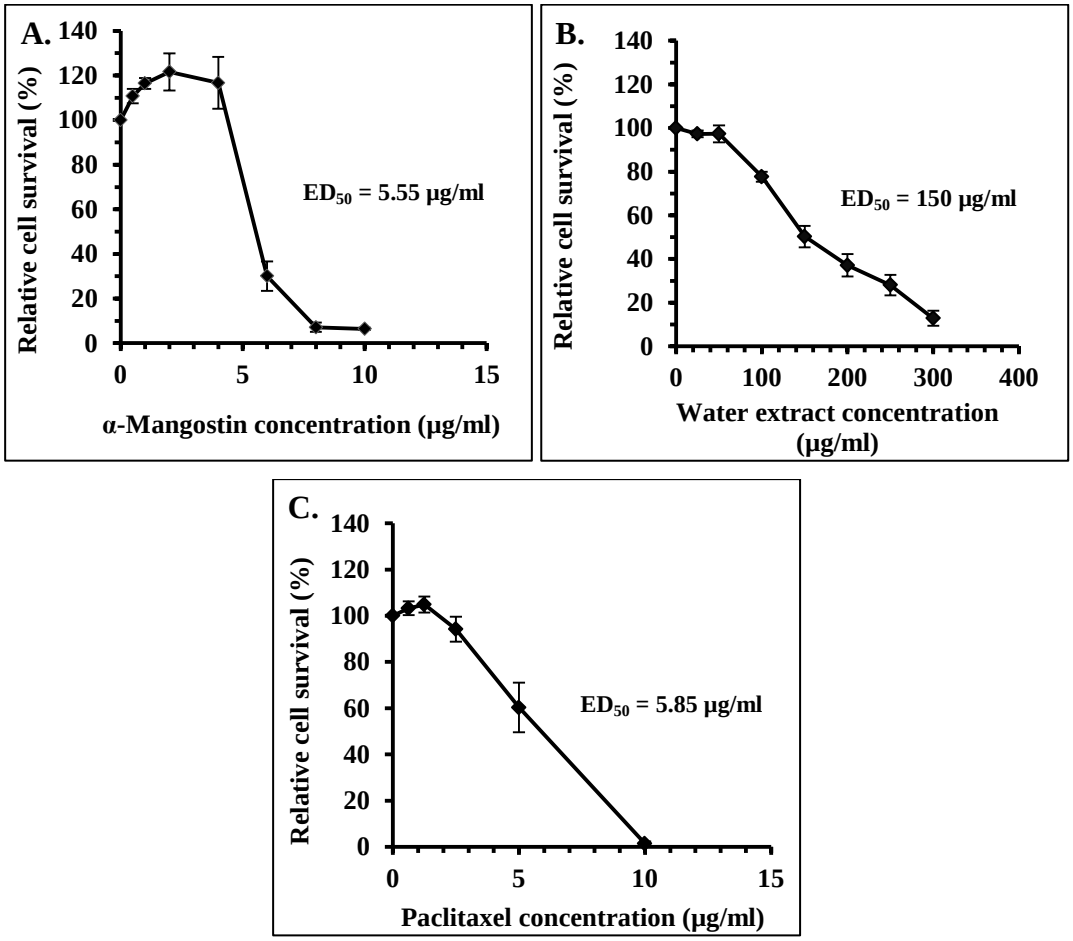


Figure 4.3 The relative cell survival of PBMCs after treating with the test compounds. The ED_{50} values of α-mangostin, crude water extract and paclitaxel were shown in A, B and C, respectively. (ED_{50} = the effective dose of a substance that produced cell survival at 50% of a population; *n* = 3 individual samples).

4.3 Effects of α-mangostin and crude water extract on lymphocyte lineages

To investigate the immunomodulatory activities of mangosteen extracts on immune cells (i.e., T lymphocytes, B lymphocytes and NK cells), PBMCs from three different samples were cultured with α-mangostin, crude water extract, mitogen ConA, and the combinations of α-mangostin/crude water extract and ConA for 24 h. The percentages of $CD3^+$ (T cells), $CD19^+$ (B cells) and $CD16^+/CD56^+$ (NK cells) were not significantly changed by the treatment with different concentrations of α-mangostin or crude water extract compared with untreated PBMCs (Figure 4.4A, 4.5A, and 4.6A). Even though, α-mangostin and crude water extract were combined with ConA, the combinations did not significantly change the percentages of three lymphocytes compared with ConA-treated PBMCs (Figure 4.4B, 4.5B and 4.6B). The total values of each treatment were shown in Table 4.1.

Table 4.1 The percentages of $CD3^+$, $CD19^+$ and $CD16^+/CD56^+$ after treating PBMCs with α-mangostin, crude water extract and the combinations with ConA.

Culture conditions	$CD3^+$ (%)	$CD19^+$(%)	$CD16^+/CD56^+$(%)
Untreated	72.0 ± 4.6	8.6 ± 2.5	18.9 ± 3.4
α-Mangostin 1 µg/ml	76.0 ± 4.3	7.6 ± 2.4	15.9 ± 3.5
α-Mangostin 2 µg/ml	75.5 ± 5	8.2 ± 2.4	15.8 ± 4.2
α-Mangostin 4 µg/ml	75.2 ± 3.9	7.8 ± 2.3	16.3 ± 3.6
Crude water extract 50 µg/ml	74.0 ± 4.0	8.8 ± 2.9	16.4 ± 2.1
Crude water extract 100 µg/ml	73.5 ± 3.2	6.7 ± 2.2	17.6 ± 1.4
Crude water extract 200 µg/ml	73.2 ± 2.4	7.4 ± 2.1	16.6 ± 2.1
ConA 10 µg/ml	77.5 ± 4.9	5.5 ± 1.6	15.4 ± 3.6
ConA 10 µg/ml + α-mangostin 1 µg/ml	77.8 ± 4.7	4.3 ± 1.0	16.5 ± 4.1
ConA 10 µg/ml + α-mangostin 2 µg/ml	77.7 ± 4.3	4.7 ± 1.6	16 ± 3.8
ConA 10 µg/ml + α-mangostin 4 µg/ml	77.8 ± 3.6	3.8 ± 1.7	15.5 ± 3.5
ConA 10 µg/ml + crude water extract 50 µg/ml	77.2 ± 4.0	4.1 ± 1.1	16.6 ± 3.7
ConA 10 µg/ml + crude water extract 100 µg/ml	74.8 ± 3.2	3.3 ± 1.1	17.9 ± 3.6
ConA 10 µg/ml + crude water extract 200 µg/ml	77.7 ± 2.4	5.3 ± 1.7	14.7 ± 3.3

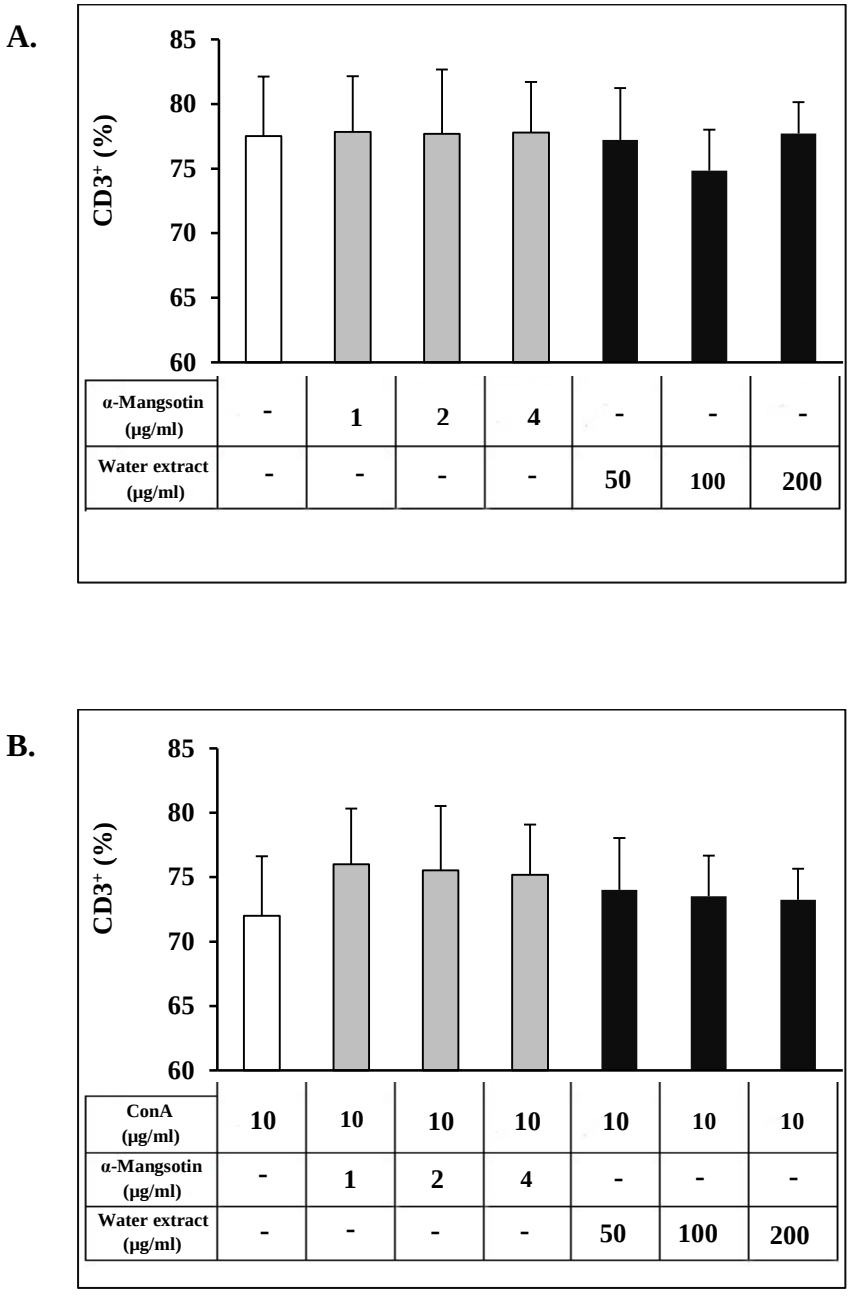


Figure 4.4 The effects of α-mangostin and crude water extract on $CD3^+$ expression. The percentage of $CD3^+$ after treating with α-mangostin and crude water extract were compared with untreated PBMCs (A). The percentage of $CD3^+$ after treating with α-mangostin/crude water extract plus ConA were compared with ConA-treated PBMC (B) (n = 3 individual samples, * = $P < 0.05$).

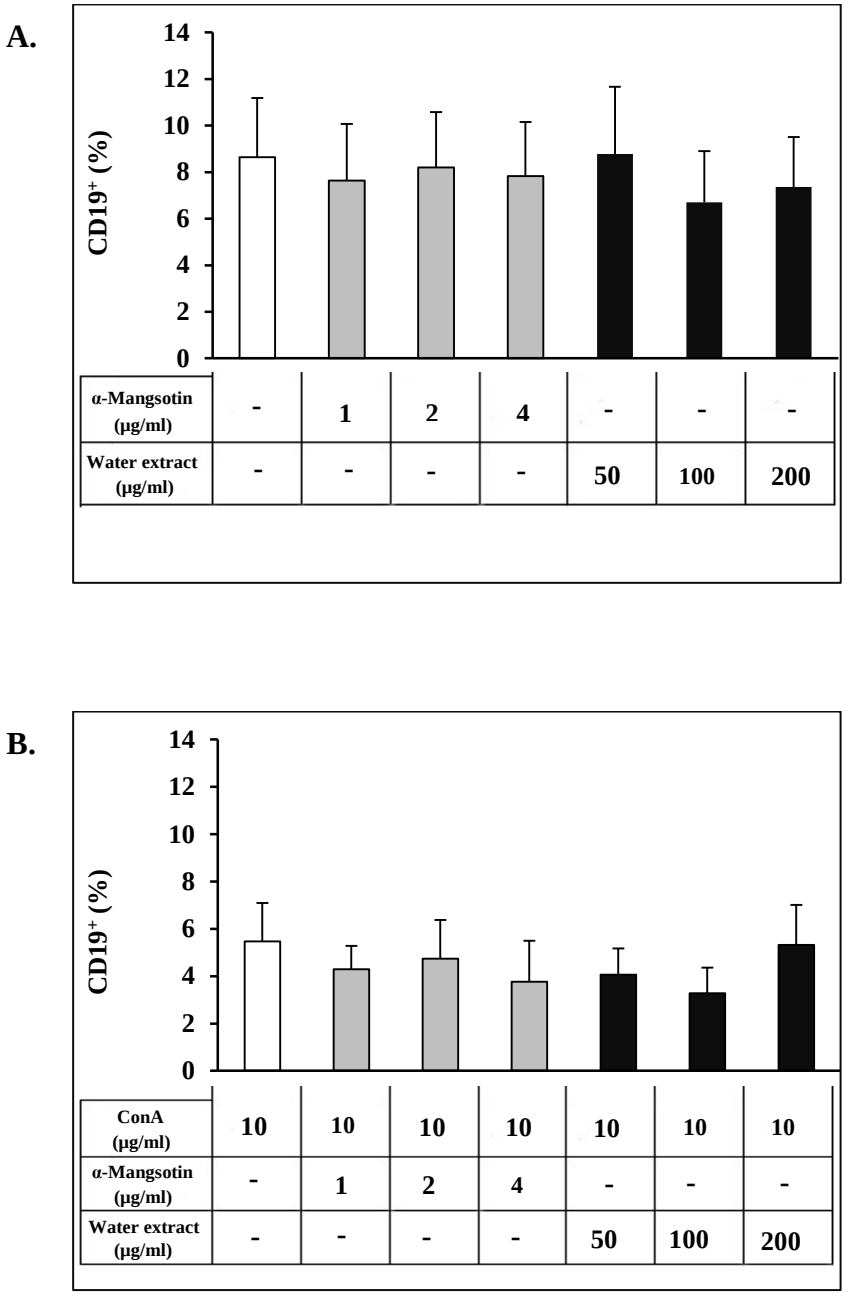


Figure 4.5 The effects of α-mangostin and crude water extract on $CD19^+$ expression. The percentage of $CD19^+$ after treating with α-mangostin and crude water extract were compared with untreated PBMCs (A). The percentage of $CD19^+$ after treating with α-mangostin/crude water extract plus ConA were compared with ConA-treated PBMC (B) (n = 3 individual samples, * = $P < 0.05$).

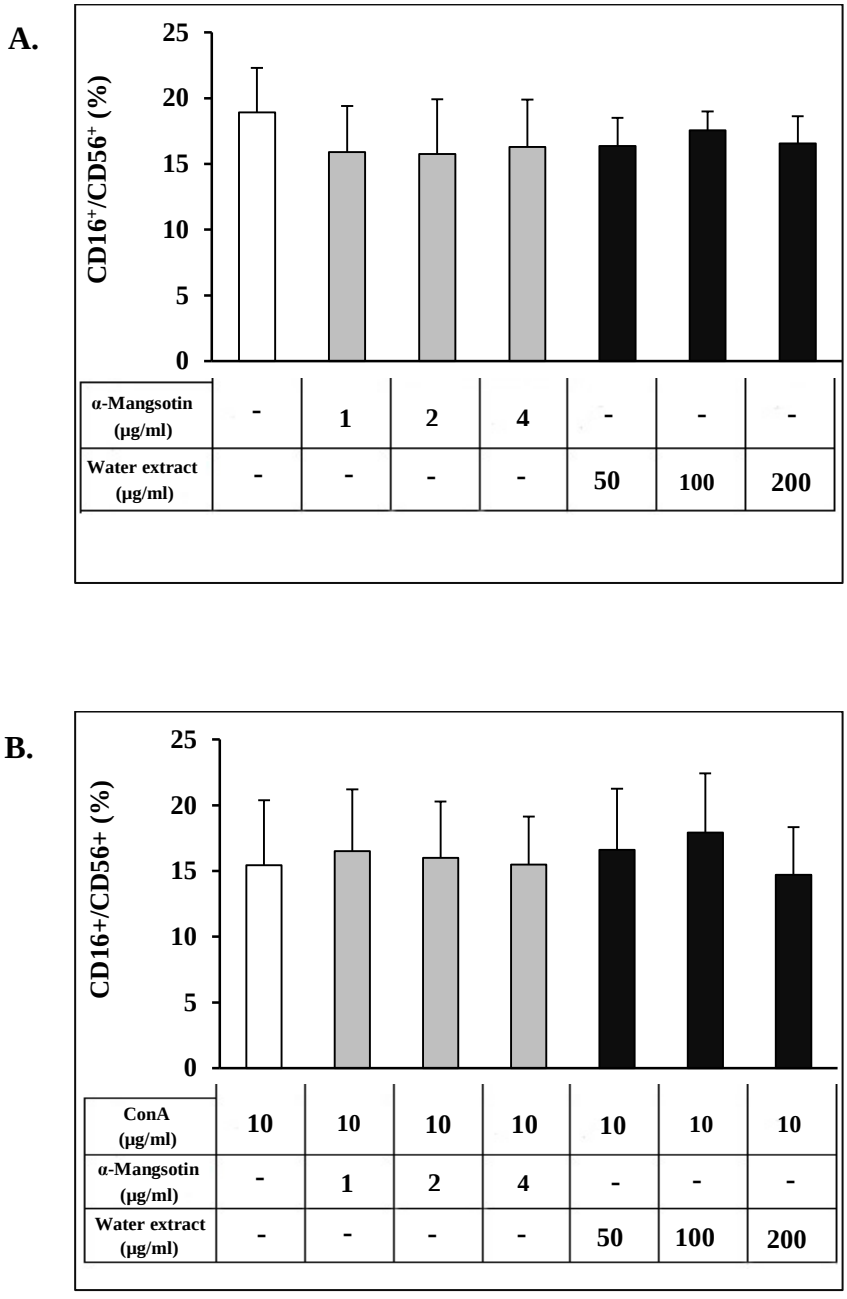


Figure 4.6 The effects of α-mangostin and crude water extract on $CD16^+/CD56^+$ expression. The percentage of $CD16^+/CD56^+$ after treating with α-mangostin and crude water extract were compared with untreated PBMCs (A). The percentage of $CD16^+/CD56^+$ after treating with α-mangostin/crude water extract plus ConA were compared with ConA-treated PBMC (B) (n = 3 individual samples, * = $P < 0.05$).

4.4 Effects of α-mangostin and crude water extract on cytokine secretion by PBMCs

The contents of secreted cytokines from PBMCs, which were treated by α-mangostin and crude water extract, were investigated by ELISA technique. After 24 h of incubation, three concentrations of α-mangostin did not significantly alter the TNF-α (Figure 4.7A), IL-1β (Figure 4.8A) and IL-2 levels (Figure 4.9A) compared with untreated PBMCs. In contrast, three concentrations of crude water extract significantly increased the levels of TNF-α (Figure 4.7A) and IL-1β (Figure 4.8A) but did not significantly change the IL-2 level (Figure 4.9A) compared with untreated PBMCs.

For the combined effect, the combination of α-mangostin and ConA could not show the significant effect on TNF-α (Figure 4.7B) and IL-1β secretion (Figure 4.8B) but they could obviously reduce IL-2 secretion compared with ConA-treated PBMCs (Figure 4.9B). For the treatment with ConA-combined crude water extract, the TNF-α levels were not significantly changed (Figure 4.7B); nevertheless, the lowest concentration of crude water extract (50 μg/ml) plus ConA had a noticeable effect to reduce IL-2 production compared with ConA-treated PBMCs (Figure 4.9B) and the highest concentration of crude water extract (200 μg/ml) plus ConA significantly increased the IL-1β levels compared with ConA-treated PBMCs (Figure 4.8B).

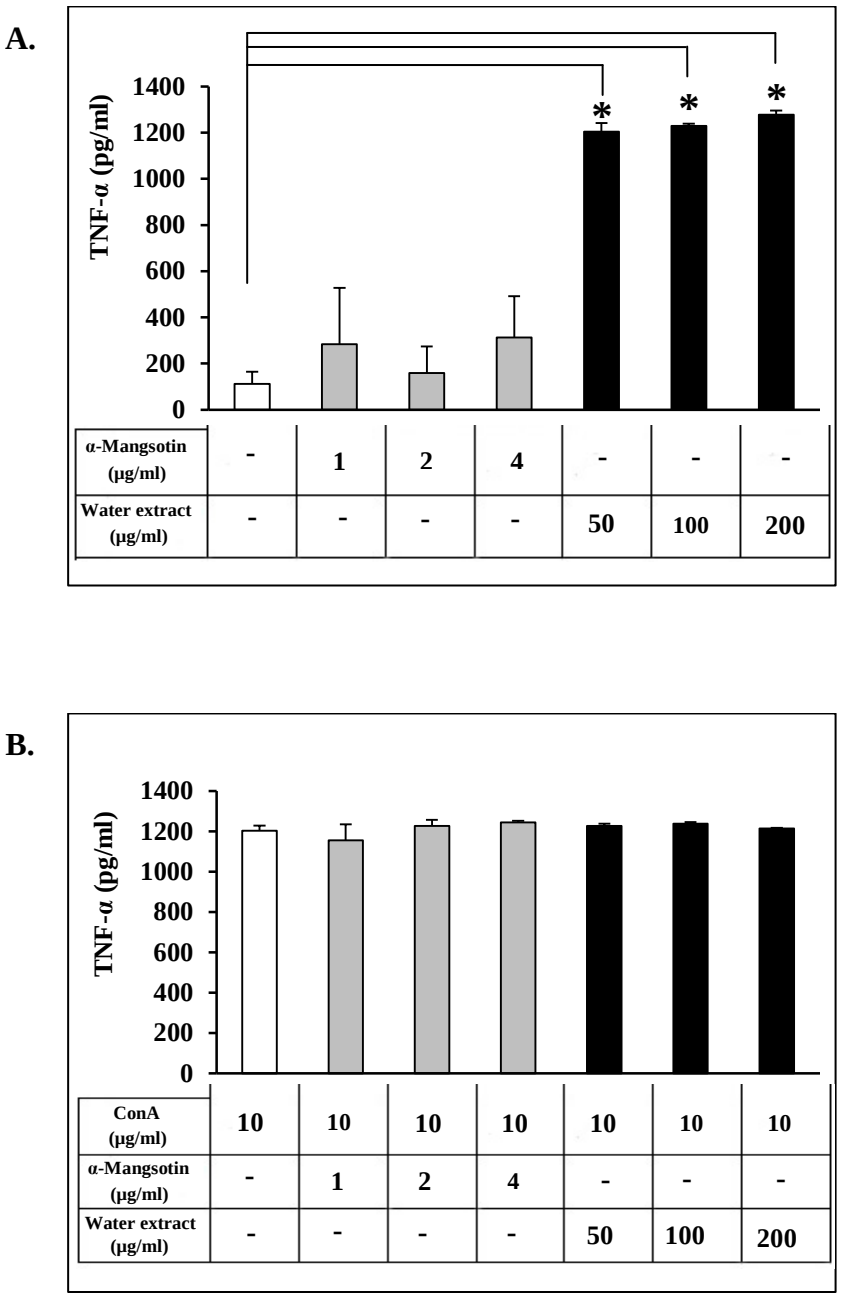


Figure 4.7 The effects of α-mangostin and crude water extract on TNF-α secretion. TNF-α levels of α-mangostin/crude water extract treated PBMCs were compared with untreated PBMCs (A). TNF-α levels of α-mangostin/crude water extract plus ConA treated PBMCs were compared with ConA-treated PBMC (B) (n = 3 individual samples, * = $P < 0.05$).

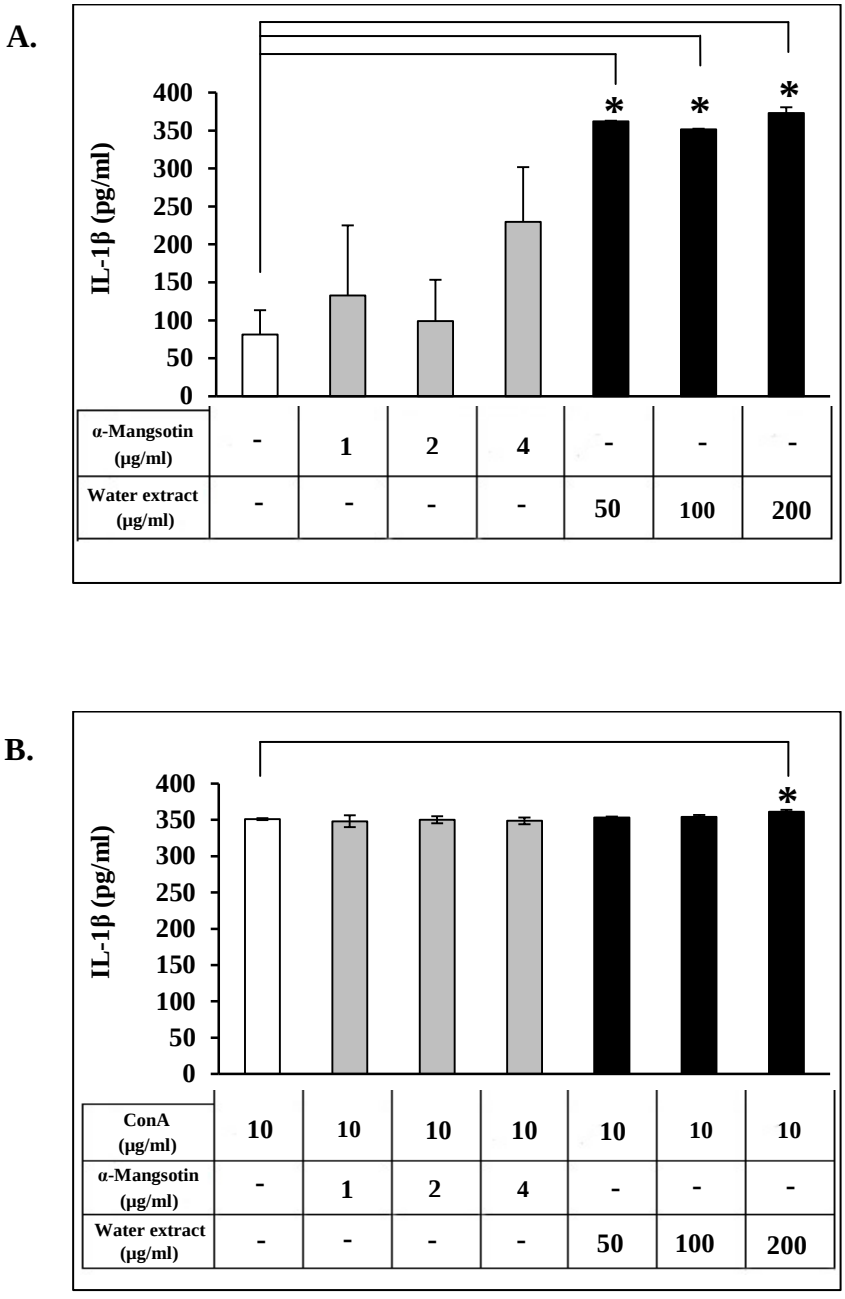


Figure 4.8 The effects of α-mangostin and crude water extract on IL-1β secretion. IL-1β levels of α-mangostin/crude water extract treated PBMCs were compared with untreated PBMCs (A). IL-1β levels of α-mangostin/crude water extract plus ConA treated PBMCs were compared with ConA-treated PBMC (B) (n = 3 individual samples, * = $P < 0.05$).

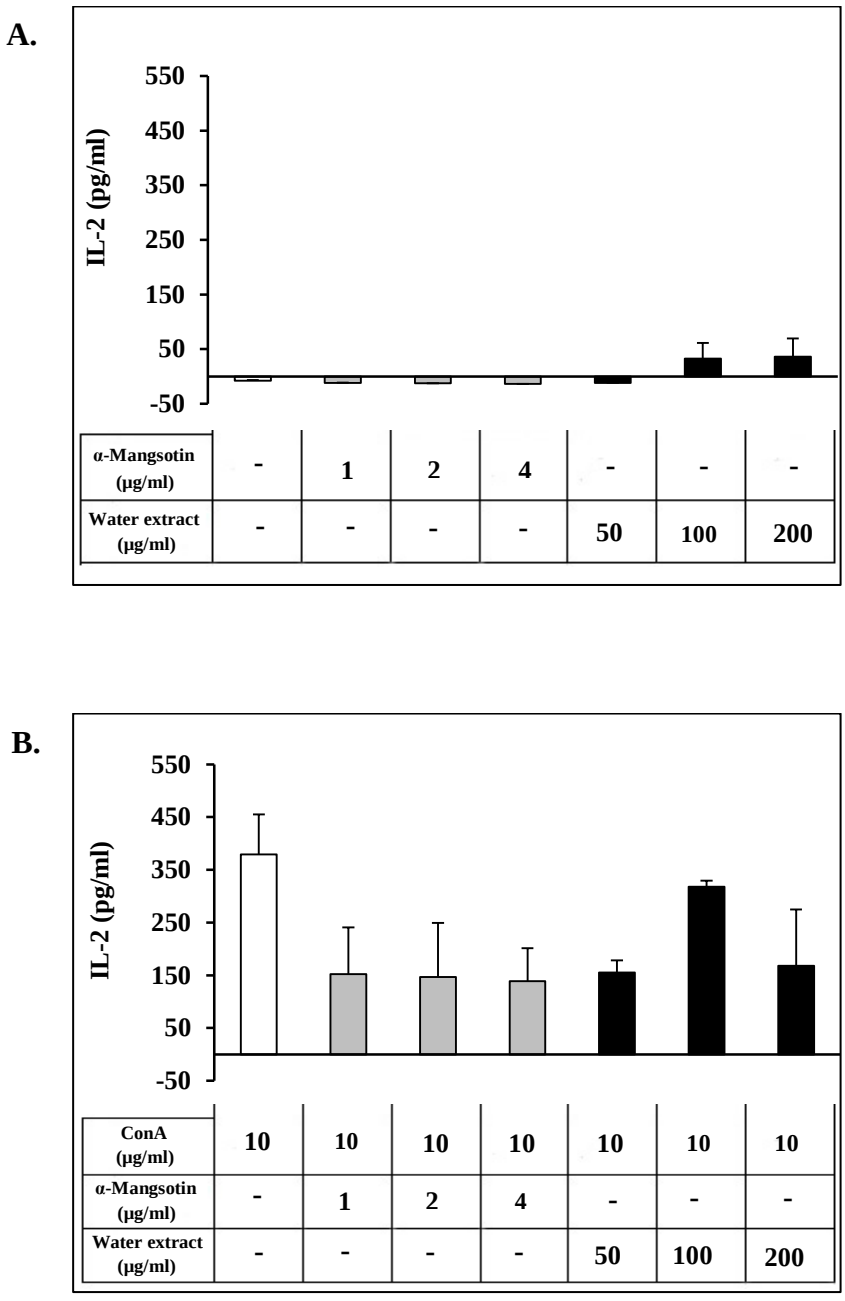


Figure 4.9 The effects of α-mangostin and crude water extract on IL-2 secretion. IL-2 levels of α-mangostin/crude water extract treated PBMCs were compared with untreated PBMCs (A). IL-2 levels of α-mangostin/crude water extract plus ConA treated PBMCs were compared with ConA-treated PBMC (B) (n = 3 individual samples, * = $P < 0.05$).

4.5 Effects of crude water extracts on intracellular cytokine secretion

To verify that crude water extract whether had a stimulating effect on monocytes to secrete TNF-α, whole blood was cultured with crude water extract, LPS, dexamethasone or combined substances of crude water extract and LPS/dexamethasone for 4 h. The FITC isotype control was used to distinguish positivity of TNF-α in monocytes. The flow cytometric analysis showed that the percentage of TNF-α positive events of LPS-treated cells increased to higher level at 73.2% compared with untreated cells (0.204%; Figure 4.10A); while the percentages of 50 and 100 µg/ml of crude water extract-treated cells (0.375 and 0.378%, respectively) did not change from the untreated cells (0.204%; Figure 4.10B). For the combined effect, the percentages of TNF-α positive events of 50 or 100 µg/ml of crude water extract plus LPS were 81.4 and 81.8%, respectively, which lightly increased compared with LPS-treated cells (73.2%; Figure 4.11A). When cells were treated with LPS plus dexamethasone, the percentages of TNF-α positive events of this combination was 76.8%, which was not lower than LPS-treated cells (73.2%; Figure 4.11A). In addition, the combined substances, which were 50 or 100 µg/ml of crude water extract and dexamethasone, could not alter the percentage of TNF-α positive events compared with the cells that were treated with crude water extract alone (Figure 4.11B). The values of intracellular TNF-α of monocytes in each treatment were shown in Table 4.2.

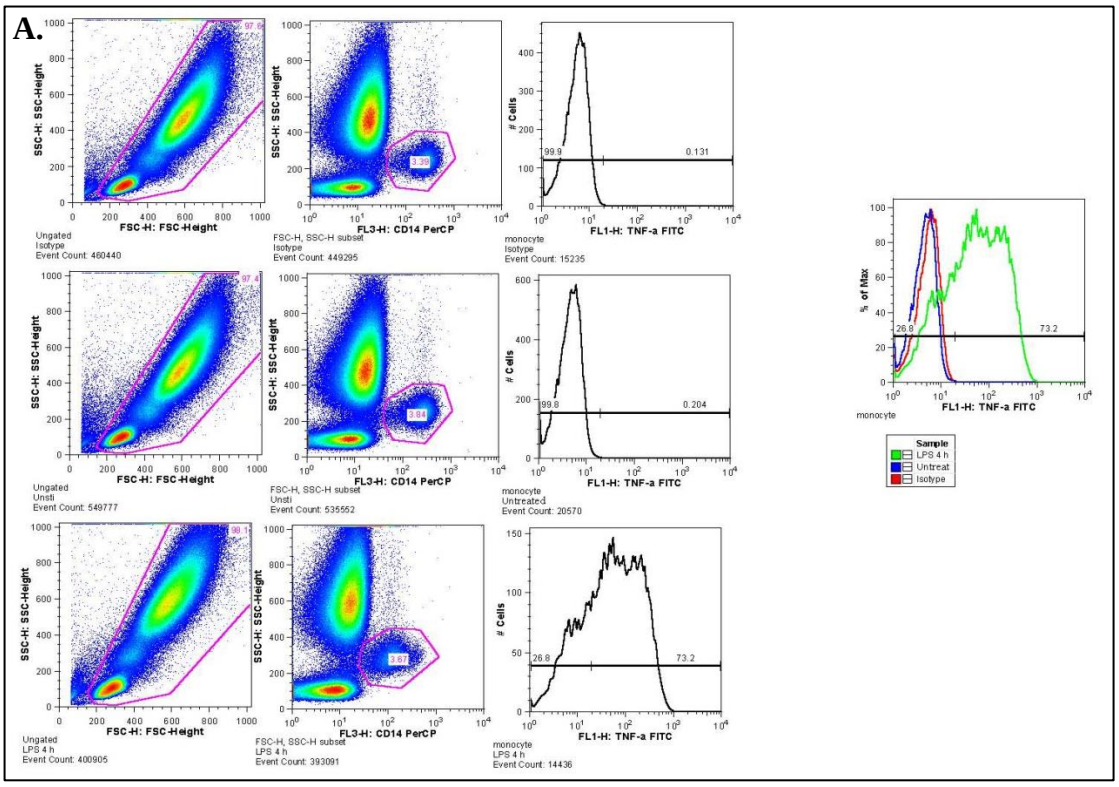


Figure 4.10 Effect of crude water extract and LPS on intracellular TNF-α expression in monocytes. The percentages of intracellular TNF-α^+ of LPS-treated cells and untreated cells were shown in the histograms with the background of mouse IgG1 isotype control (A); and the percentages of intracellular TNF-α^+ crude water extract treated-cells was compared with unstimulated cells (B).

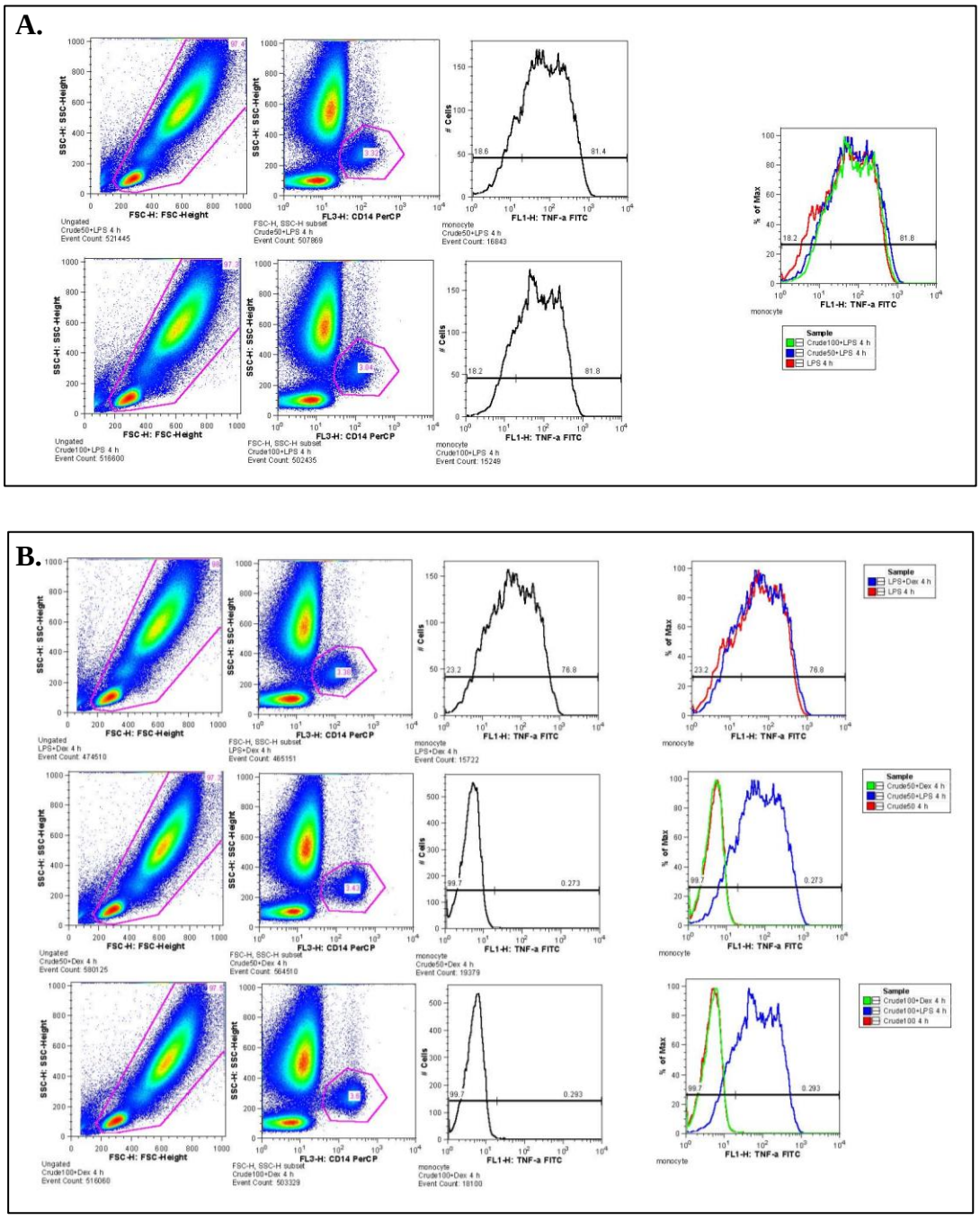


Figure 4.11 Effect of combined substances between crude water extract plus LPS/Dex on intracellular TNF-α expression in monocytes. The percentages of TNF-α^+ events of crude water extract plus LPS-treated cells were compared with LPS-treated cells (A); the percentage of TNF-α^+ of LPS plus Dex treated cells was compared with LPS-treated cells (B); and the percentages of TNF-α^+ crude water extract plus LPS/Dex treated cells were compared with crude water extract-treated cells (B).

Table 4.2 The flow cytometric analysis results of intracellular TNF-α

Sample	FSC-H,, SSC-H subset Freq of parent (%)	monocyte Freq of parent (%)	FITC+ Freq of parent (%)
Isotype	97.6	3.39	0.131
Untreated	97.4	3.84	0.204
LPS	98.1	3.67	73.2
Crude50	96.6	3.79	0.375
Crude50+Dex	97.3	3.43	0.273
Crude50+LPS	97.4	3.32	81.4
Crude100	97.1	3.79	0.378
Crude100+Dex	97.5	3.6	0.293
Crude100+LPS	97.3	3.04	81.8
LPS+Dex	98	3.38	76.8

Crude = crude water extract; Dex = dexamethasone; Freq. = frequency; and LPS = lipopolysaccharide.

CHAPTER V
DISCUSSION

The immune system is a complex network of cells, tissues, and organs that work together to protect the body against the foreign invader and control health status. The disorder of immunity can cause a variety of diseases. Therefore, immunomodulators have been intensely used to cope with the failure of immunity. However, the problems of immunnomodulator treatment are longterm use, drug resistance and adverse events (8-10). The discovery of novel immunomodulators, hence, is in a field of interest.

Mangosteen has been used as a traditional medicine. Many previous studies have obviously shown that mangosteen contains a variety of chemical constituents which the major compound is xanthone derivatives, α-mangostin (59). α-Mangostin exhibits a diversity of its actions, for example, anti-inflammatory (64), antioxidant (14), antiallergy, antiproliferative (62), antitumor (11), anticancer (17) and antimicrobial properties (67, 68). In addition, the water soluble extract of mangosteen can demonstrate impressive antioxidant (60, 61) and anti-inflammatory properties (66). According to these attractive properties of them, the plentiful sources of mangosteen in Thailand, and the scarce evidence of their immunomodulatory activities, we have an attempt to determine their immunomodulatory activities that act on the major immune cells (i.e., T, B and NK lymphocytes) and the primary cytokines (i.e., TNF-α, IL-1β and IL-2) in the immune system by using the PBMCs model.

5.1 Effect of α-mangostin and crude water extract on PBMCs viability

To evaluate the optimal concentrations of purified α-mangostin and partially purified crude water extract for PBMCs culture, both extracts were cultured with normal human PBMCs and assayed with MTT technique. The suitable number of

PBMCs for 24-houred culture was 2 x 10^5 PBMCs/well in a 96-well plate. This number of PBMCs was enough to cover 80% of one well and brought the absorbance value lined within the range 0.75 - 1.25 (89) with completely dissolved formazan crystals. PBMCs (2 x 10^5 cells/well) were cultured with various concentrations of both extracts. The results showed that 50% of PBMCs viability resulted from being exposed to 5.55 µg/ml of α-mangostin (Figure 4.3A), which was nearly the same concentration as paclitaxel expressed (5.58 µg/ml; Figure 4.3C). This data indicated that α-mangostin could exhibit a potent cytotoxic effect on PBMCs, which correleated to the previous studies on cancer cell lines *in vitro* (62) and tumor cells *in vivo* (63). On the other hand, the concentration of crude water extract had to be highly used to 150 µg/ml in order to destroy 50% of cells (Figure 4.3B). For non-cytotoxic concentrations, PBMCs would survive more than 95%, when they were treated with α-mangostin, crude water extract, or paclitaxel at the concentration lower than 4.5, 55 and 2.35 µg/ml, respectively (Figure 4.3A, 4.3B, 4,3C).

Therefore, to prevent cell damage, we selected the concentrations of α-mangostin that would not be toxic to the cells (< 4.5 µg/ml) at 1, 2 and 4 µg/ml to conduct further experiments. For crude water extract concentration, according to the previous study of Moongkarndi P. et. al. (60), they showed the protective effect of crude water extract against β-amyloid-induced cytotoxicity at the highest concentration 400 µg/ml. For this reason, the selected concentrations of crude water extract in this study were broader to cover all possible activity ranges, which were 50, 100 and 200 µg/ml.

5.2 Effects of α-mangostin and crude water extract on lymphocyte lineages

Lymphocytes are special cells that can recognize and respond to a variety of antigens. They play crucial roles in cell-mediated and humoral immunity to response to the stimuli to protect the body. In this study, to determine the immunomodulatory activity of mangosteen extracts on lymphocytes, we used immunophenotypic analysis to identify and enumerate the affected lymphocytes. Due to the requirement for mitogen-induced clonal expansion, PBMCs have to be cultured

with ConA approximately 3 - 10 days to induce CD3 expression (93). However, we were concerned about the short lifespan of PBMCs and the unfavorable environment with mangosteen extracts, leading the reason to culture cells for only 24 h. PBMCs were cultured with α-mangostin (1, 2 and 4 μg/ml), crude water extract (50, 100 and 200 μg/ml), T-cell mitogen concanavalin A (ConA, a positive control; 10 μg/ml), the combination of α-mangostin/crude water extract plus ConA, and RPMI supplemented with 10% FBS (a negative control). After 24 h of incubation, the flow cytometric results showed that the percentages of $CD3^+$ (T cells), $CD19^+$ (B cells) and $CD16^+/CD56^+$ (NK cells) were not significantly changed by the treatment with 1, 2 and 4 μg/ml of α-mangostin or by the treatment with 50, 100 and 200 μg/ml crude of water extract compared with untreated PBMCs (Figure 4.4A, 4.5A, and 4.6A). Moreover, the combination of α-mangostin/crude water extract plus ConA did not significantly change the percentages of three lymphocytes compared with ConA-treated PBMCs (Figure 4.4B, 4.5B and 4.6B). The data indicated that these selected concentrations of both mangosteen extracts did not have any significant effect on the clonal expression of T, B or NK lymphocytes and did not have antagonist or synergistic effect when combined with ConA to increase T lymphocytes.

5.3 Effects of α-mangostin and crude water extract on cytokine secretion by PBMCs

Different cell types of immune system have a distinct role and communicate with each other using secreted glycoprotein cytokines. Cytokines function as regulators of the growth and differentiation of immune cells as well as maintain homeostasis of the immune system. The presence of each cytokine can also be a biomarker that reflects physiological and pathological process in the body (94). In this experiment, we would like to determine the immunomodulatory activity of mangosteen extracts on primary cytokine secretion. We focused only on three important cytokines: TNF-α, IL-1β and IL-2. TNF-α is mainly secreted from activated antigen presenting cells (e.g., monocytes, macrophages and dendritic cells) and $CD4^+$ T lymphocytes. It exhibits an interesting activity that can destroy tumor cells, inhibit viral gene expression and replication as well as activates B cells (95). IL-1β is mainly

secreted by monocytes and macrophages. It can activate a variety of cell types via NF-κB pathway and induces T cell to secrete IL-2 (21, 96). Both TNF-α and IL-1β are proinflammatory mediators of the innate immune system (95) that can independently trigger other inflammatory cytokine cascades and involved in the pathophysiology of many diseases (97). On the contrary, IL-2 is a predominant cytokine of the adaptive immunity. IL-2 is produced by activated T lymphocytes: mainly by $CD4^+$ T helper cells in secondary lymphoid organs and, to a lower extent, by $CD8^+$ T cells, NK cells and natural killer T (NKT) cells (98). IL-2 is responsible for promoting T cell proliferation, differentiation and apoptosis; NK cell proliferation; and being a cofactor for B-cell proliferation (43, 48, 96).

The concentration of TNF-α, IL-1β and IL-2 were detected from cultured supernatants of mangosteen extract-treated PBMCs by ELISA method. T cell mitogen ConA was used as a positive control to stimulate cytokine secretion, because a cascade of intracellular signals and cytokine production also occur during cell proliferation process (93). The concentration of ConA, 10 μg/ml, was selected from the preliminary test in cytokine secretion detected by ELISA method: PBMCs could give the highest response at this concentration (data not shown). The results showed that three concentrations of α-mangostin did not significantly alter the TNF-α (Figure 4.7A) and IL-1β (Figure 4.8A) levels compared with untreated cells; additionally, the combinations of α-mangostin and ConA could not show the significant effect on TNF-α (Figure 4.7B) and IL-1β secretion (Figure 4.8B) as well. This data implicated that α-mangostin did not have an activity on proinflammatory cytokine secretion of normal immune cells; it did not significantly activate or inhibit TNF-α or IL-1β cytokine secretion by itself and did not have any interaction with ConA to affect the proinflammatory cytokine production.

The previous studies (64, 99, 100) showed that α-mangostin has anti-inflammatory activity, but in our study α-mangostin could not express anti-inflammatory activity. This was possibly due to the condition of cultured cells: we examined anti-inflammatory activity of α-mangostin on normal immune cells but the previous studies used cell lines or animal models that had been induced into inflammatory state before treatment (64, 99, 100). From the collected data and our experimental results ensure that α-mangostin do not affect on normal immune cells

and it do not exhibit anti-inflammatory activity unless pathophysiological condition has been occurred. For IL-2 secretion, the IL-2 levels of α-mangostin-treated cells and untreated control cells could not be measured; the calculated concentration of them were the negative values, which indicated that α-mangostin could not stimulate IL-2 secretion compared with untreated cells (Figure 4.9A). However, the tendency of IL-2 levels obviously declined when PBMCs were exposed to α-mangostin and ConA compared with ConA-treated PBMCs (Figure 4.9B), which demonstrated that α-mangostin had an inhibitory activity towards IL-2 secretion either by directly interacting with T cells or by interacting with ConA. According to the knowledge that inhibition of IL-2 results in suppression of T cell proliferation; thereby, α-mangostin could be developed into an immunosuppressant using either alone or combined with other immunosuppressant to treat or reduce severity of autoimmune diseases and rejection of organ transplantation (10).

In contrast, three concentrations of crude water extract significantly increased the levels of TNF-α (Figure 4.7A) and IL-1β (Figure 4.8A) compared with untreated cells. Nevertheless, the combinations of crude water extract and ConA did not significantly increase the TNF-α and IL-1β levels, except the highest concentration 200 µg/ml of crude water extract plus ConA that could increase IL-1β levels compared with ConA-treated PBMCs (Figure 4.8B). These no significant effects of combination treatments were due to the effect of ConA: ConA at this concentration (10 µg/ml) extremely stimulated PBMCs to produce the maximal level of proinflammatory cytokines; thereby, crude water extract could not show the additive or synergist effect with ConA to increase more TNF-α and IL-1β levels than the ConA-treated group. These results indicated that crude water extract was able to stimulate TNF-α and IL-1β secretion from normal immune cells; in addition, the high dose (200 µg/ml) of crude water extract could exert an effect of ConA to stimulate IL-1β production. For IL-2 production, crude water extract could not significantly induced IL-2 secretion compared with untreated cells (Figure 4.9A), but the combinations of ConA and crude water extract, especially the lowest concentration at 50 µg/ml of crude water extract, had an effect to diminish the IL-2 level compared with ConA-treated cells (Figure 4.9B). The results indicated that crude water extract did not have a stimulant activity

to increase IL-2 secretion but it had an antagonist effect with ConA resulting in lesser IL-2 secretion.

5.4 Effects of crude water extracts on intracellular TNF-α secretion

Monocytes are the major cells that produce proinflammatory cytokines. The expression of proinflammatory cytokines by monocytes is efficiently activated by bacterial products such as LPS (101). From ELISA test, crude water extract showed substantial effect to induce proinflammatory cytokine secretion, which reasonably suggested that crude water extract probably composed of some constituents that were bacterial-like products and associated with monocyte activation. Therefore, to investigate this hypothesis, whole blood was cultured with 50 and 100 µg/ml of crude water extract to determine proinflammatory cytokine expression within the monocytes by flow cytometry, and we detected only TNF-α which was a representative of the proinflammatory cytokines. Whole blood culture assay requires a minimal step to process the sample. It is useful to evaluating an elementary response in a complex system, in which a variety of proteins and cells are present and interact with each other in a conserved physiological environment. Before testing this experiment, in our test system, we preliminarily cultured whole blood with crude water extract for 24 h, but nearly most monocyte population was not able to collect for further analysis (data not shown), resulting in shorten incubation time only at 4 h. After 4 h of incubation, the flow cytometric analysis showed that the percentages of TNF-α positive of crude water extract group did not change from untreated group (Figure 4.10B) and the percentages of TNF-α positive in the presence of the combinations of LPS and crude water extract were not different from LPS-stimulated group (Figure 4.11A). These results proved that crude water extract could not induce intracellular TNF-α expression by activating monocytes. For negative control, dexamethasone typically has potent anti-inflammatory effects on innate immune cells, but in this experiment dexamethasone could not reduce intracellular TNF-α expression in both LPS-stimulated whole blood and crude water extract-treated whole blood (Figure 4.11B). This might be due to the effects of concentration and incubation time: in our study, the concentration of dexamethasone, which was applied from Hodge et. al. method (102),

was too low to inhibit TNF-α expression (85, 103); and the duration of incubation was not long enough for dexamethasone to exhibit its action in time (104).

In inflammatory process, many cells participate in inflammatory reactions, including circulating cells (lymphocytes, neutrophils, eosinophils, basophils and platelet) and tissue-resident cells (mast cells and macrophages). Most acute inflammatory reactions rely on three major inflammatory cells: neutrophils, macrophages and lymphocytes (105). Even though, proinflammatory cytokines are mainly secreted from monocytes/macrophages, a variety of cells can produce them as well. The results of crude water extract stimulating TNF-α expression in PBMCs cultured supernatants and whole blood culture were different, which implied that crude water extract did not contain LPS-like substance to stimulate TNF-α expression on monocytes. On the other hand, crude water extract was capable to stimulate proinflammatory cytokine secretion via other cells. Hence, crude water extract, could possibly be a proinflammatory-activating agent that could be employed as an immunostimulant to trigger other specific cells, such as T cells, and other cytokine secretion to destroy tumor cells (106). However, crude water extract is composed of many constituents. The clarification of its active constituents is helpful to clearly understand its activity and we are now trying to characterize its active constituents by HPLC method. Moreover, the activity of proinflammatory cytokines is still controversial: prolonged or dramatically production of proinflammatory cytokines can cause a variety of the diseases (107). Meanwhile, the precise role of individual cytokine in immune responses is intricate to determine: one type of cell can produce many cytokines and one cytokine can exert many effects on diverse targets (96). Therefore, further studies of the active constituents and the activity of crude water extract on other immune cells and cytokines are necessary to be investigated.

CHAPTER VI
CONCLUSION

Magosteen has been used as a traditional medicine for the treatment of many diseases. It contains a variety of substances, for instance, xanthones consisting more than 60 derivatives, which some exhibit as a potent antioxidant. There are abundant reports of the mangosteen activities, especially of the major xanthone derivative, α-mangostin; low polar constituent, whereas water extract; high polar constituents. Since mangosteen shows the diversity of its activities, a large amount of products derived from mangosteen has been widely used for improving health status or treating a variety of the diseases. However, the immunomodulatory evidence of mangosteen is scarce. This study, therefore, aimed to investigate the immunomodulatory activity of two mangosteen extracts which have been previously studied and contain the promising activities: α-mangostin and crude water extract.

Based on the culture of normal human immune cells with mangosteen extracts *in vitro*, we concluded that α-mangostin did not have an activity on clonal expression of human lymphocytes and did not affect proinflammatory cytokine (TNF-α and IL-1β) secretion; nevertheless, it showed the noticeable tendency to suppress IL-2 production and had an effective antiproliferative activity that could destroy the normal cell at very low concentration. With previous reports of other studies and our results indicated that α-mangostin could be developed into an antitumor drug with immunosuppressive activity, which was possibly used alone or combined with the other drug to treat or reduce severity of the diseases.

On the contrary, crude water extract did not have an activity on clonal expression of human lymphocytes and did not have a stimulant activity to increase IL-2 production. However, it had a potential activity to induce proinflammatory cytokine secretion significantly without activating monocytes. These results suggested that crude water extracts could be employed as an immunostimulant that was able to

trigger other specific cells, such as T cells, and other cytokine secretion to destroy tumor cells.

Nevertheless, this study was performed only in an *in vitro* model. The real immune system of the human body contains a variety of cells that works together as a network. Besides, one type of immune cell or cytokine can orchestrate the cascade of immune responses, and these responses can be varied between individual samples. Hence, the further studies of α-mangostin and crude water extract on other cells and cytokines with an increase sample size are necessary to deeply understand their mechanisms of actions.

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APPENDICES

APPENDIX A
MEDIA AND REAGENTS

1. Phosphate buffered saline (PBS)

0.2 M phosphate buffer (PB)

0.2 M NaH_2PO_4 pH 4.5	27.998 g/l
0.2 M Na_2HPO_4 pH 9.4	35.598 g/l

Add NaH_2PO_4 to Na_2HPO_4 dropwise to adjust the pH to 7.4

0.01 M PBS

0.2 M PB pH 7.4	50 ml
NaCl	8.76 g
Distilled water to make	1000 ml

Sterilized by cellulose acetate filter (pore size 0.2 µm).

2. RPMI 1640 medium

RPMI 1640 medium (BIOCHOM AG Cat.No. T121-01)	10.43 g
(with L-glutamine and 25 mM HEPES buffer, without $NaHCO_3$)	
$NaHCO_3$	2.00 g
100 units/ml Penicillin G - 100 µg/ml Streptomycin	10 ml
Sterile water for injection to make	1000 ml

Adjust pH to 7.0-7.2 with 1 N HCl or 1 N NaOH before adjust volume with water and sterilize by cellulose acetate filter (pore size 0.2 µm).

3. Detergent reagent for solubilizing MTT crystal

Sodium dodecyl sulfate (SDS)	11 g
0.02 M HCl	50 ml
Isopropanol	50 ml

Filter through cellulose acetate filter (pore size 0.45 µm).

4. 0.25% w/v Trypan blue

Trypan blue	0.25 g
Distilled water to make	100 ml

5. 1% w/v Paraformaldehyde fix solution

Paraformaldehyde	0.5 g
1 N NaOH	100 µl
Diluted HCl for adjusting pH	
0.01 M PBS to make	50 ml

For 50 ml of 1% Formaldehyde, add 40 mL of PBS to a glass beaker on a stir plate in a fume hood. Heat while stirring to approximately 60 °C. Add 0.5 g of paraformaldehyde powder to the heated PBS solution. Add 100 µl of 1 N NaOH to obtain the clear solution. Adjust pH to7.0-7.2 with diluted HCl before adjust volume of the solution to 50 ml with PBS and sterilize by cellulose acetate filter (pore size 0.2 µm). Store at 2-8 °C for up to one month.

6. Staining buffer for flow cytometry

NaN_3	0.45 g
Fetal bovine serum	10 ml
0.01 M PBS to make	500 ml

Adjust pH to 7.0-7.2 with 1 N HCl or 1 N NaOH before adjust volume with PBS and sterilize by cellulose acetate filter (pore size 0.2 µm).

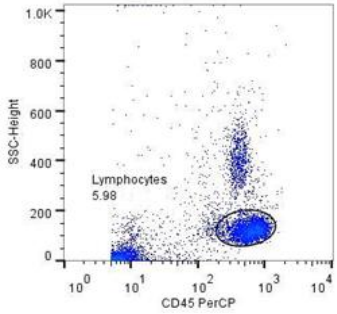
7. 20 mM Ethylenediamine Tetraacetic Acid (EDTA)

$EDTA.Na_2.2H_2O$	372.24 mg
0.01 M PBS to make	50 ml

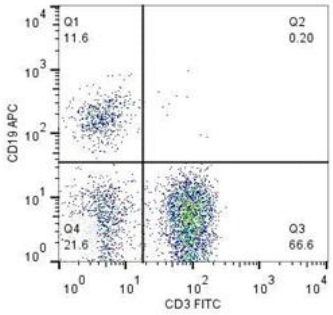
Adjust pH to 7.0-7.2 with 1 N NaOH before adjust volume with PBS and sterilize by cellulose acetate filter (pore size 0.2 µm).

APPENDIX B
IMMUNOPHENOTYPIC DATA OF INDIVIDUAL SAMPLES

Density plots of the PBMCs donor no. 1



Untreat 0 h
Gate: Ungated
Count: 84304



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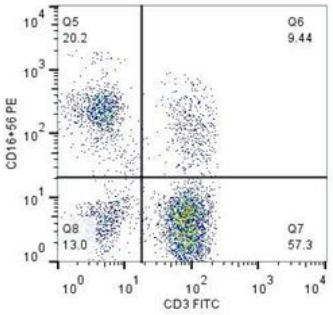
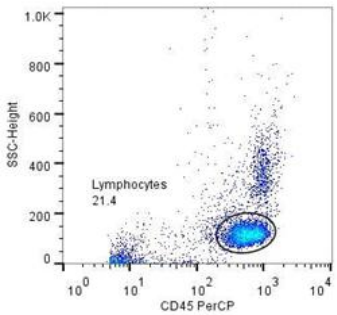
Gate: Lymphocytes
Count: 5043



Untreat 0 h
Gate: Lymphocytes
Count: 5043



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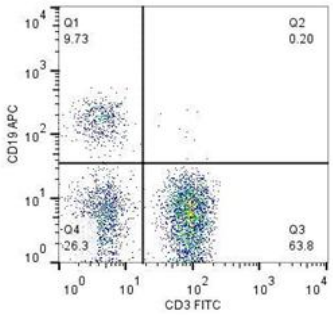
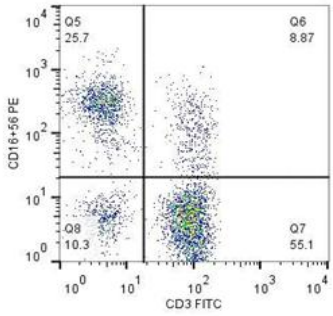
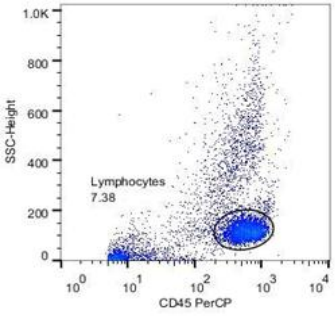


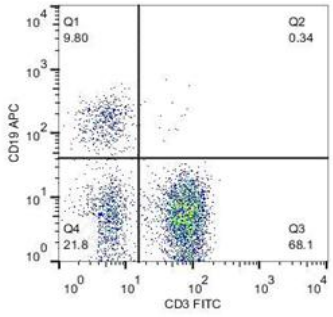
Untreat 24 h
Gate: Lymphocytes
Count: 5004



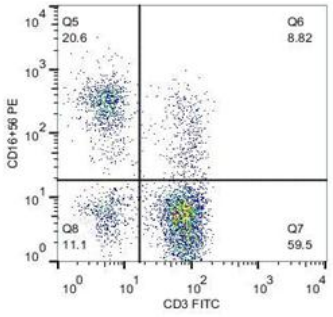
Untreat 24 h
Gate: Lymphocytes
Count: 5004



Water Extract 50 µg/ml
Gate: Ungated
Count: 68032

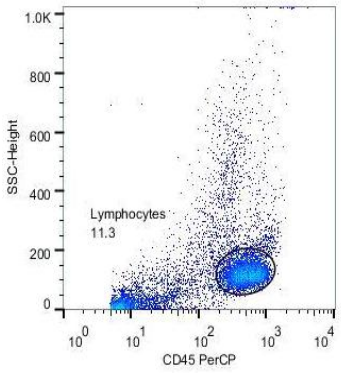


Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 5020

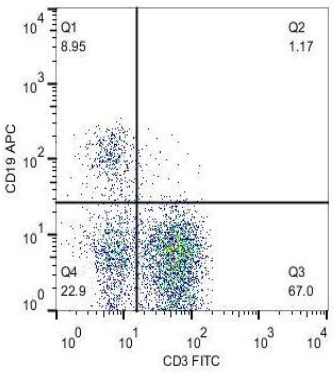


Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 5020

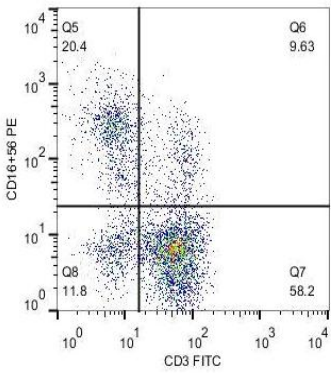
Density plots of the PBMCs donor no. 1 (continued)



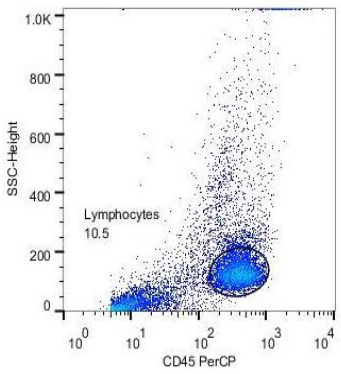
Water Extract 100 µg/ml
Gate: Ungated
Count: 45466



Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 5117



Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 5117



Water Extract 200 µg/ml
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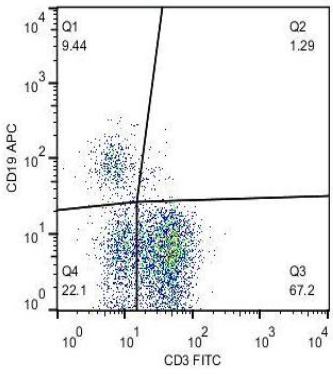
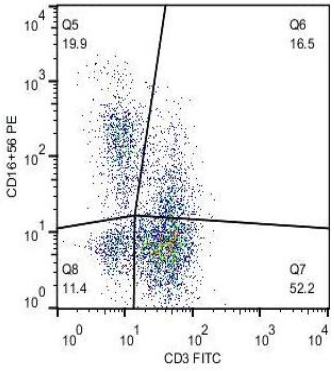
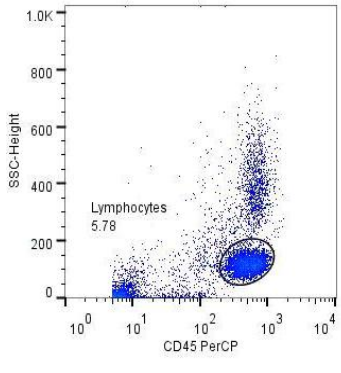


Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5190



Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5190



α-Mangostin 1 µg/ml
Gate: Ungated
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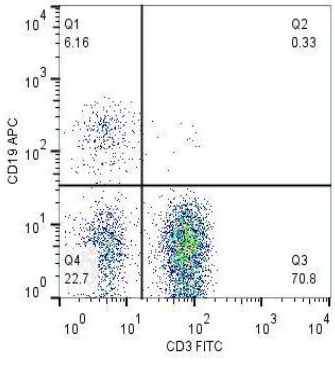
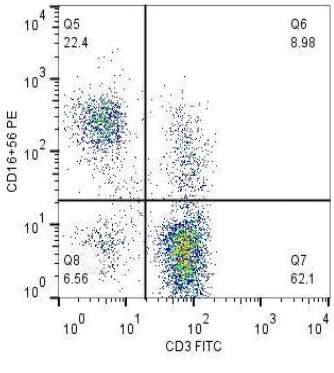
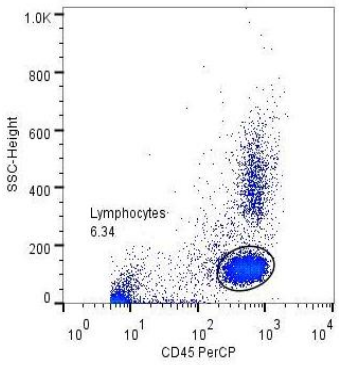


α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 5110



α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 5110

Density plots of the PBMCs donor no. 1 (continued)



α-Mangostin 2 µg/ml
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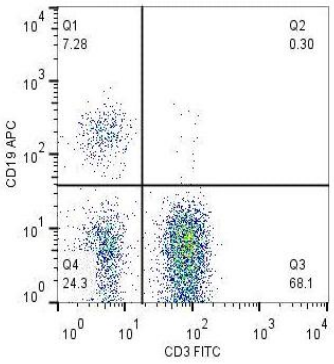

α-Mangostin 2 µg/ml
Gate: Lymphocytes
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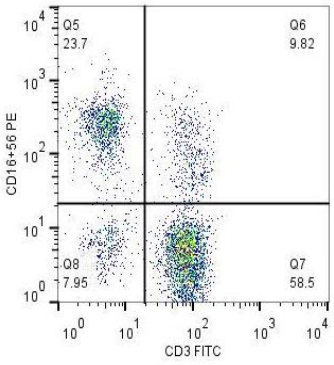
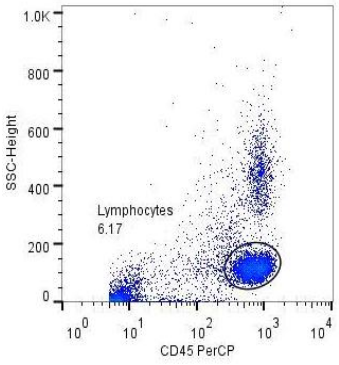


α-Mangostin 2 µg/ml
Gate: Lymphocytes
Count: 5083



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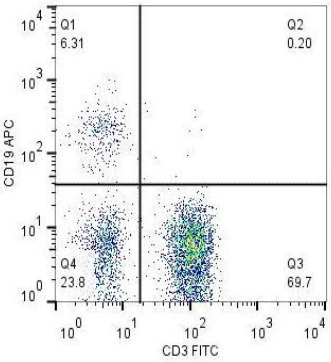
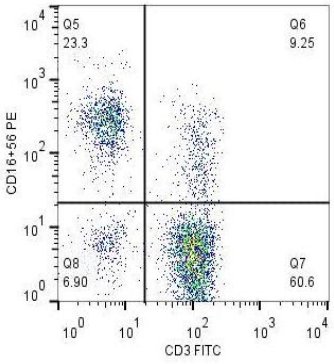
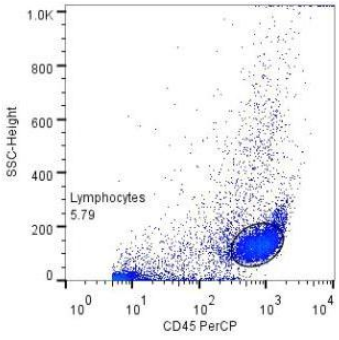


α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4974



α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4974



ConcanavalinA 10 µg/ml
Gate: Ungated
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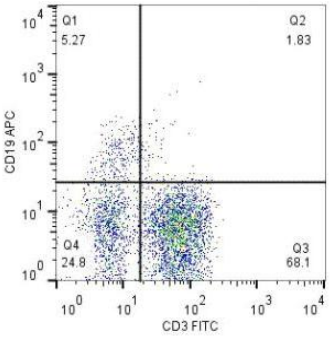

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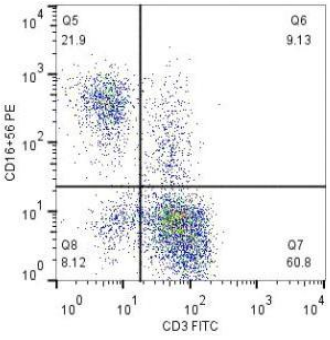
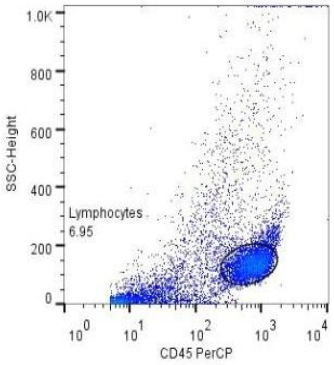
Gate: Lymphocytes
Count: 5026

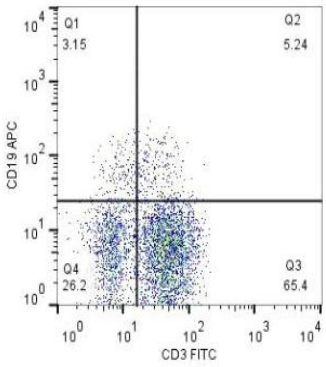


ConcanavalinA 10 µg/ml
Gate: Lymphocytes
Count: 5026

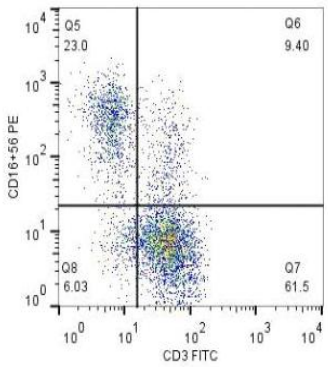
Density plots of the PBMCs donor no. 1 (continued)



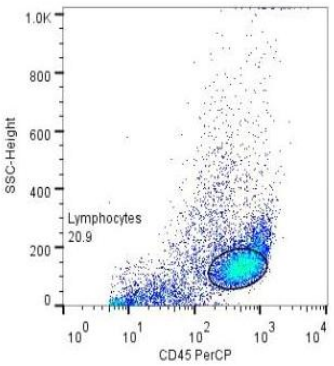
ConcanavalinA 10 µg/ml + Water Extract 50 µg/ml
Gate: Ungated
Count: 71201



ConcanavalinA 10 µg/ml + Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 4946



ConcanavalinA 10 µg/ml + Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 4946



ConcanavalinA 10 µg/ml + Water Extract 100 µg/ml
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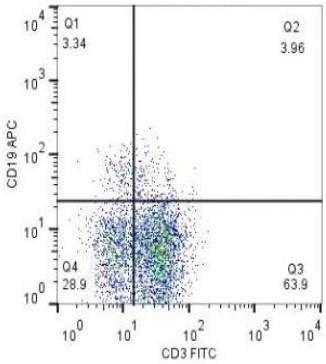
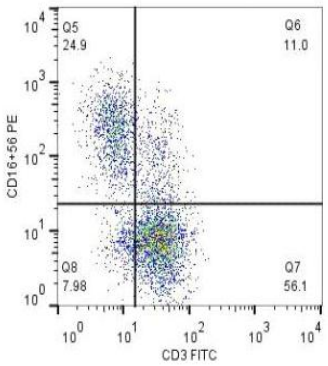
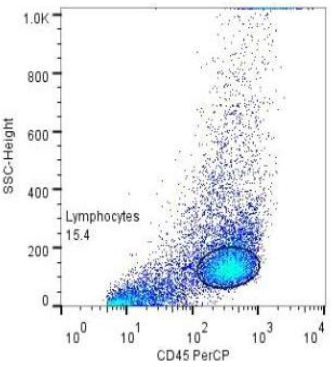
Count: 23000



ConcanavalinA 10 µg/ml + Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 4797



ConcanavalinA 10 µg/ml + Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 4797



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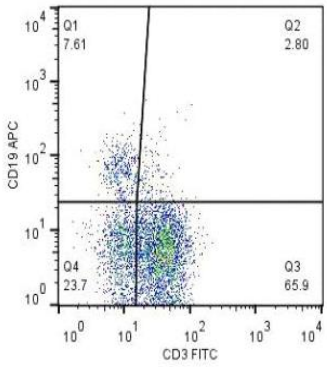
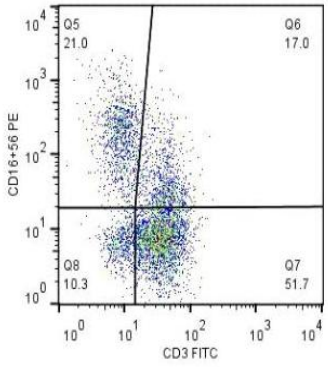
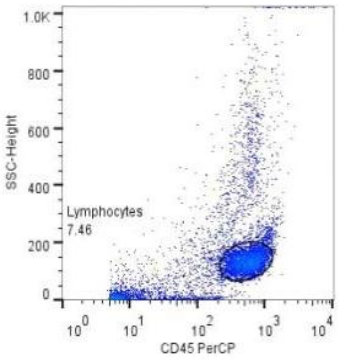


ConcanavalinA 10 µg/ml + Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5364

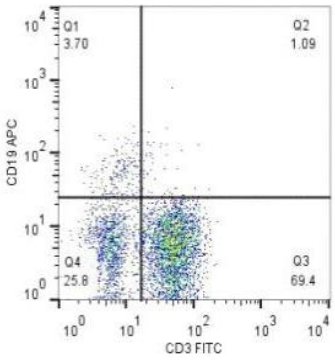


ConcanavalinA 10 µg/ml + Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5364

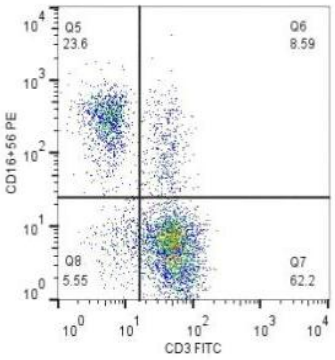
Density plots of the PBMCs donor no. 1 (continued)



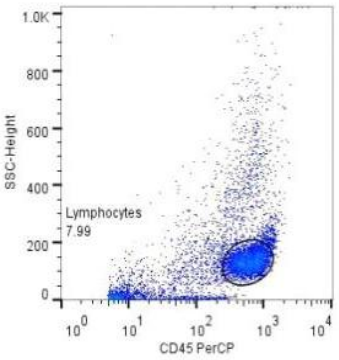
ConcanavalinA 10 µg/ml + α-Mangostin 1 µg/ml
Gate: Ungated
Count: 68780



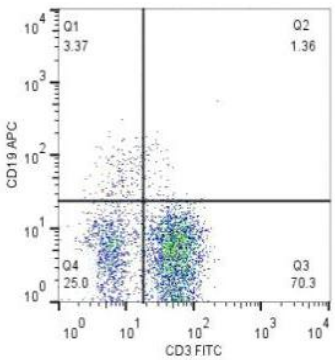
ConcanavalinA 10 µg/ml + α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 5132



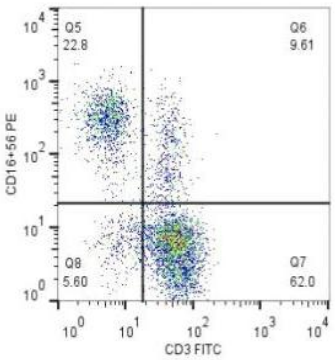
ConcanavalinA 10 µg/ml + α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 5132



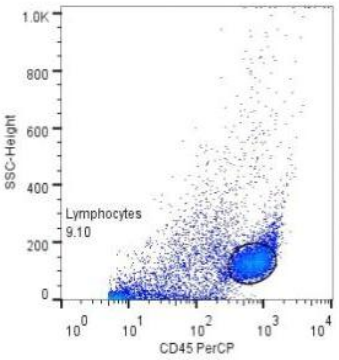
ConcanavalinA 10 µg/ml + α-Mangostin 2 µg/ml
Gate: Ungated
Count: 62783



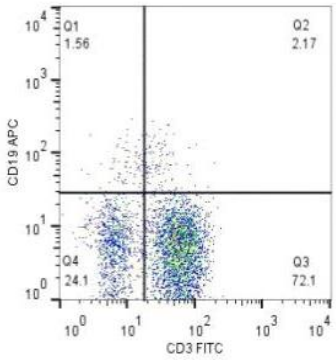
ConcanavalinA 10 µg/ml + α-Mangostin 2 µg/ml
Gate: Lymphocytes
Count: 5016



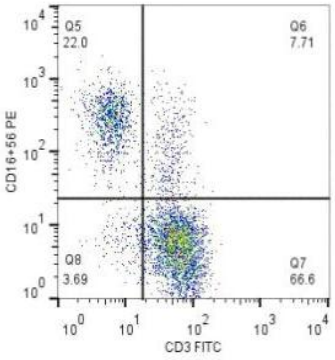
ConcanavalinA 10 µg/ml + α-Mangostin 2 µg/ml
Gate: Lymphocytes
Count: 5016



ConcanavalinA 10 µg/ml + α-Mangostin 4 µg/ml
Gate: Ungated
Count: 54110

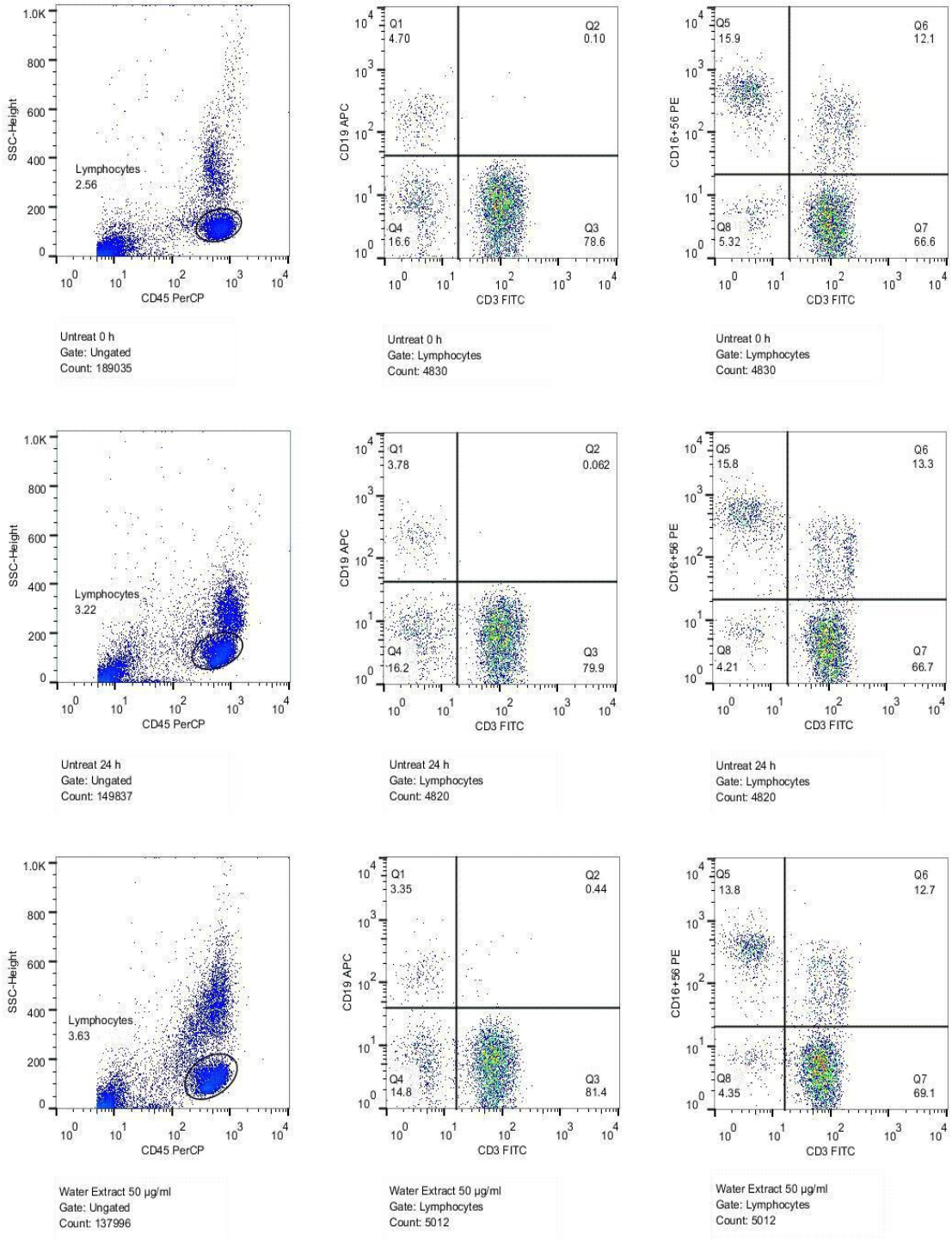


ConcanavalinA 10 µg/ml + α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4926

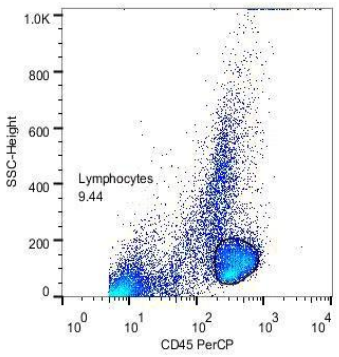


ConcanavalinA 10 µg/ml + α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4926

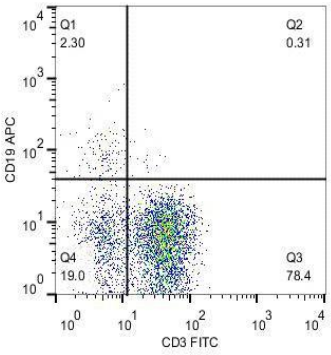
Density plots of the PBMCs donor no. 2



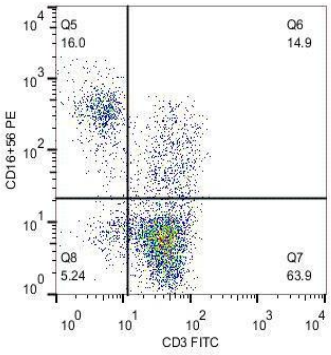
Density plots of the PBMCs donor no. 2 (continued)



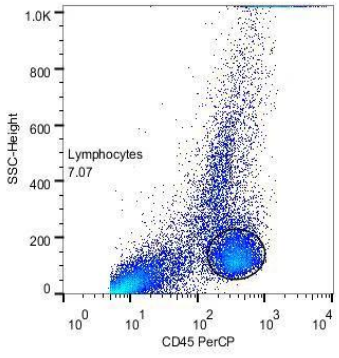
Water Extract 100 µg/ml
Gate: Ungated
Count: 50719



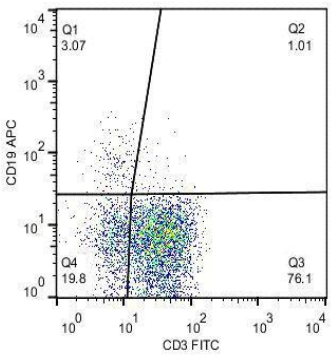
Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 4787



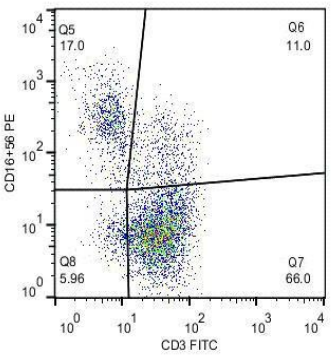
Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 4787



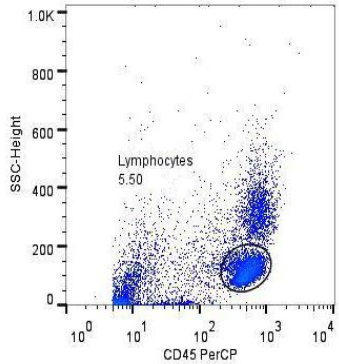
Water Extract 200 µg/ml
Gate: Ungated
Count: 75535



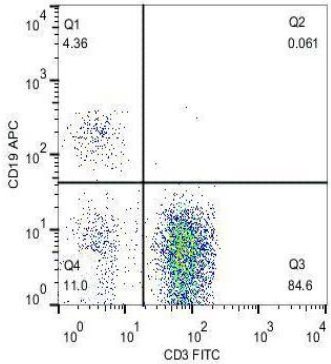
Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5340



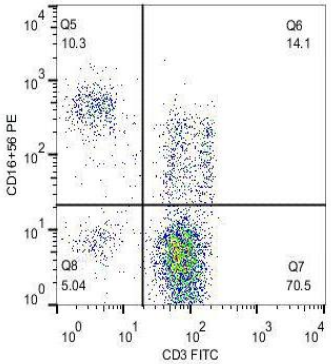
Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5340



α-Mangostin 1 µg/ml
Gate: Ungated
Count: 88762

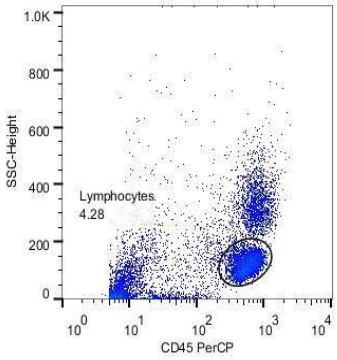


α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 4880

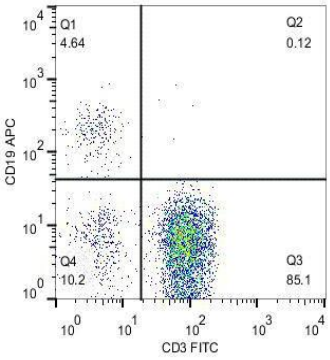


α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 4880

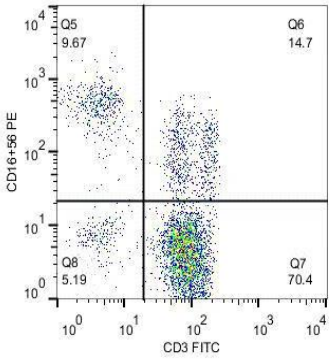
Density plots of the PBMCs donor no. 2 (continued)



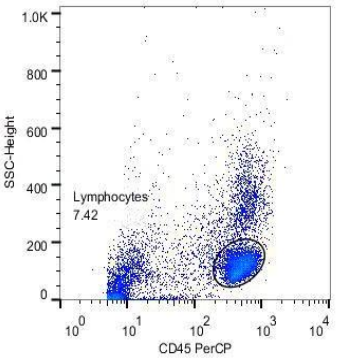
α-Mangostin 2 µg/ml
Gate: Ungated
Count: 1148172



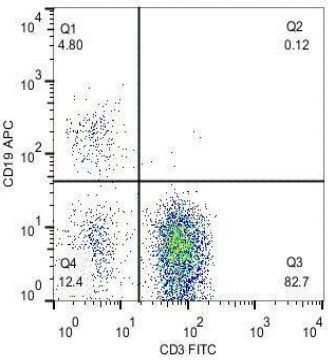
α-Mangostin 2 µg/ml
Gate: Lymphocytes
Count: 4912



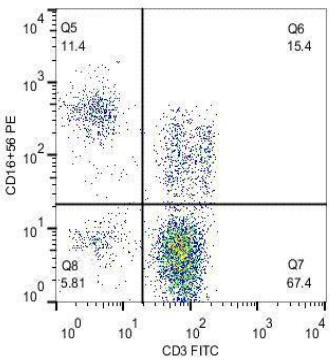
α-Mangostin 2 µg/ml
Gate: Lymphocytes
Count: 4912



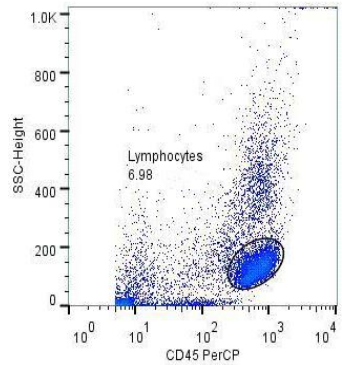
α-Mangostin 4 µg/ml
Gate: Ungated
Count: 66558



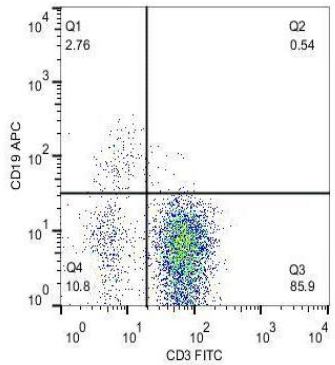
α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4941



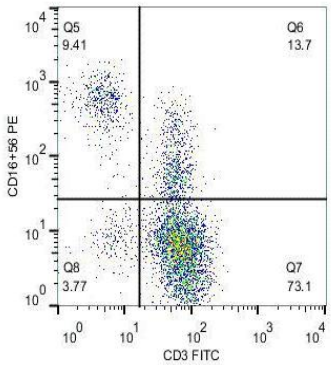
α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4941



ConcanavalinA 10 µg/ml
Gate: Ungated
Count: 71084

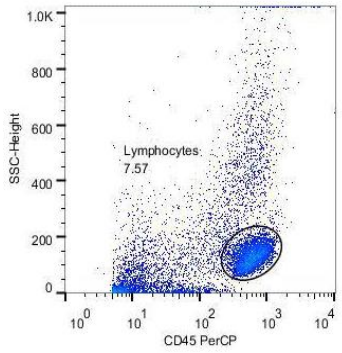


ConcanavalinA 10 µg/ml
Gate: Lymphocytes
Count: 4962

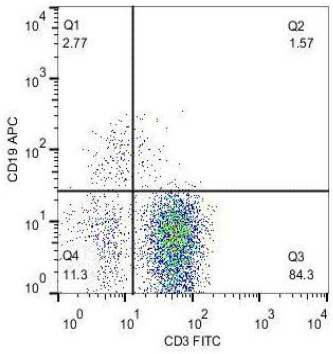


ConcanavalinA 10 µg/ml
Gate: Lymphocytes
Count: 4962

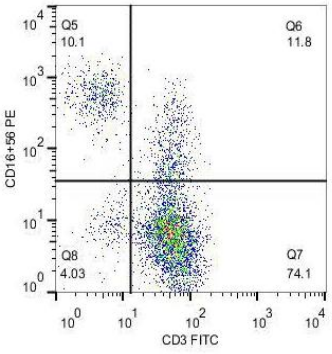
Density plots of the PBMCs donor no. 2 (continued)



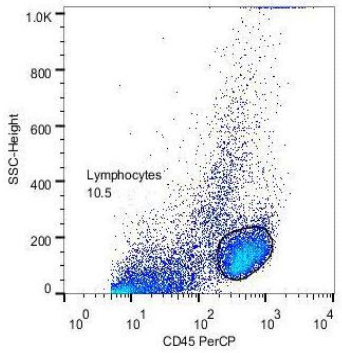
ConcanavalinA 10 µg/ml + Water Extract 50 µg/ml
Gate: Ungated
Count: 66288



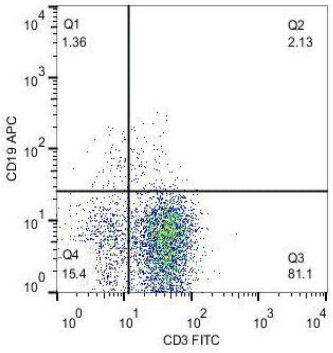
ConcanavalinA 10 µg/ml + Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 5017



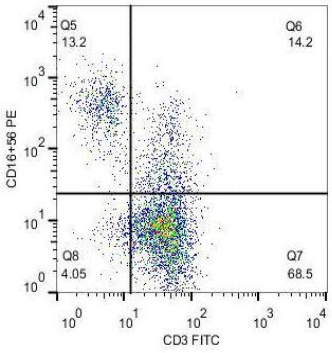
ConcanavalinA 10 µg/ml + Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 5017



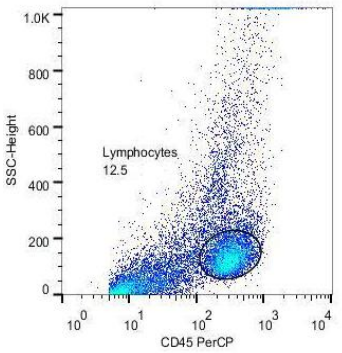
ConcanavalinA 10 µg/ml + Water Extract 100 µg/ml
Gate: Ungated
Count: 47657



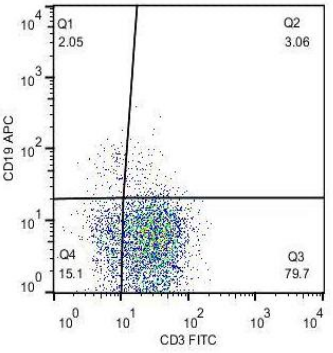
ConcanavalinA 10 µg/ml + Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 5016



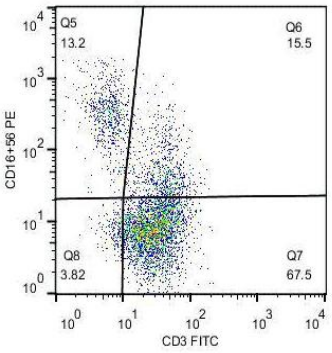
ConcanavalinA 10 µg/ml + Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 5016



ConcanavalinA 10 µg/ml + Water Extract 200 µg/ml
Gate: Ungated
Count: 40225

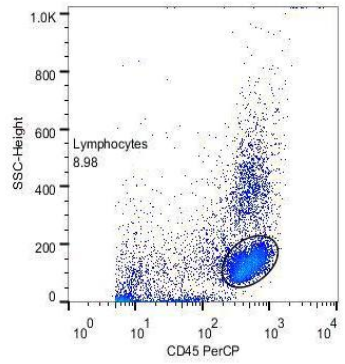


ConcanavalinA 10 µg/ml + Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5025

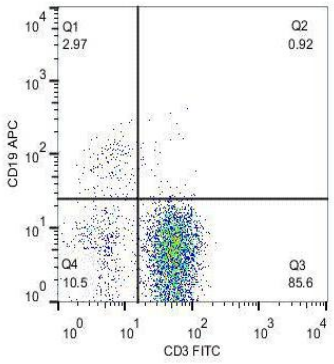


ConcanavalinA 10 µg/ml + Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5025

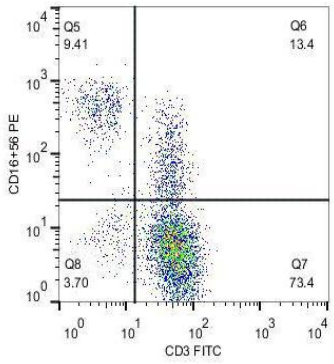
Density plots of the PBMCs donor no. 2 (continued)



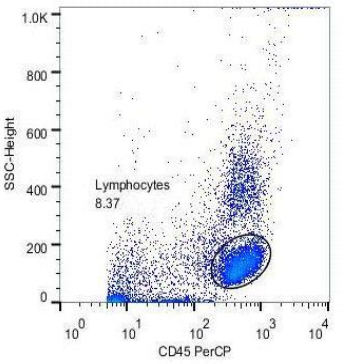
ConcanavalinA 10 µg/ml + α-Mangostin 1 µg/ml
Gate: Ungated
Count: 55380



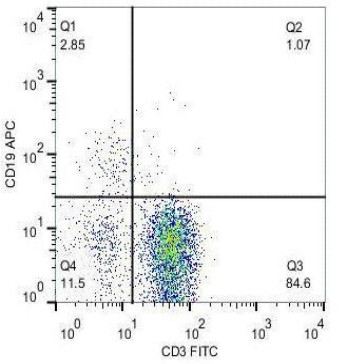
ConcanavalinA 10 µg/ml + α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 4975



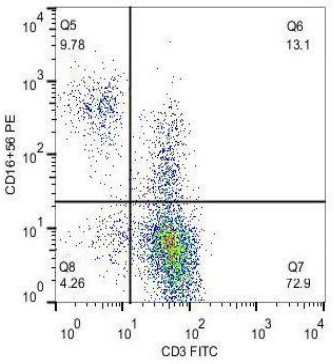
ConcanavalinA 10 µg/ml + α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 4775



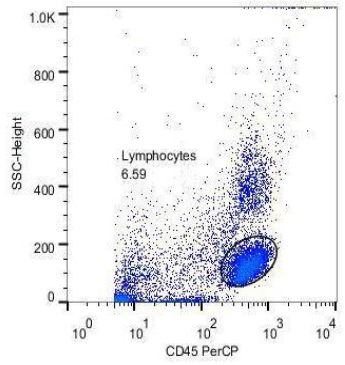
ConcanavalinA 10 µg/ml + α-Mangostin 2 µg/ml
Gate: Ungated
Count: 60335



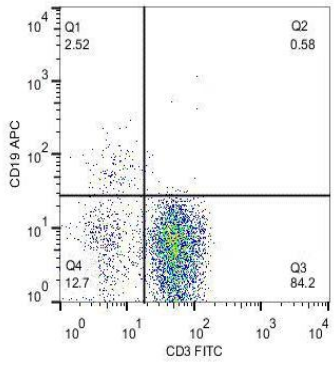
ConcanavalinA 10 µg/ml + α-Mangostin 2 µg/ml
Gate: Lymphocytes
Count: 5051



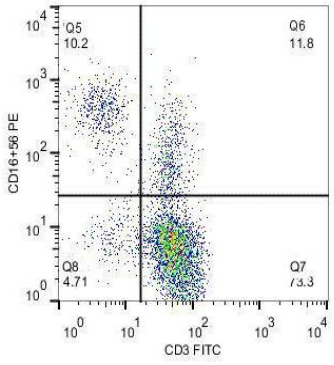
ConcanavalinA 10 µg/ml + α-Mangostin 2 µg/ml
Gate: Lymphocytes
Count: 5051



ConcanavalinA 10 µg/ml + α-Mangostin 4 µg/ml
Gate: Ungated
Count: 75371

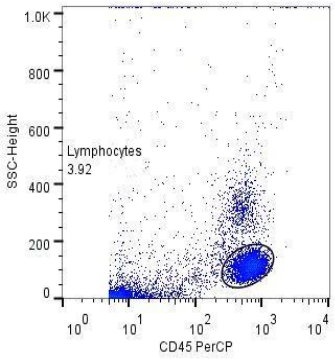


ConcanavalinA 10 µg/ml + α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4964

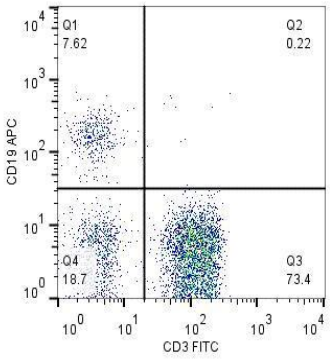


ConcanavalinA 10 µg/ml + α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4964

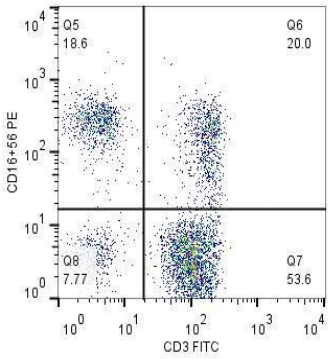
Density plots of the PBMCs donor no. 3



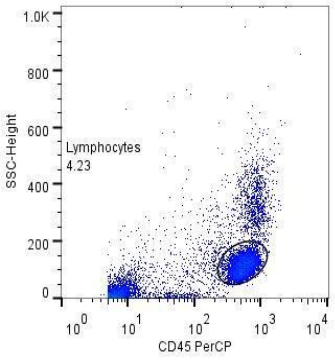
Untreat 0 h
Gate: Ungated
Count: 124896



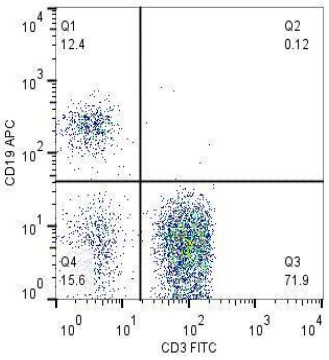
Untreat 0 h
Gate: Lymphocytes
Count: 4893



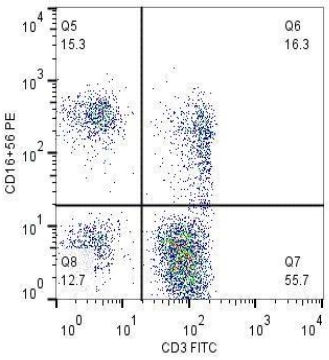
Untreat 0 h
Gate: Lymphocytes
Count: 4893



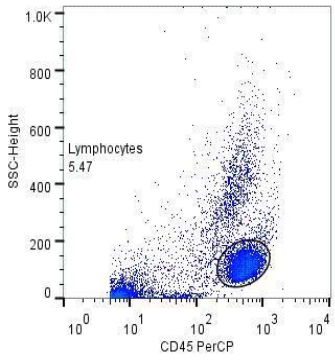
Untreat 24 h
Gate: Ungated
Count: 116321



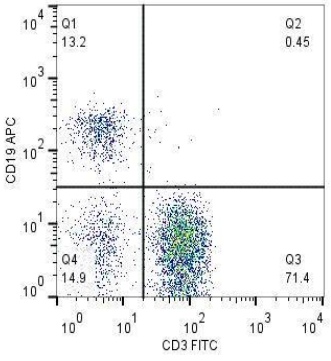
Untreat 24 h
Gate: Lymphocytes
Count: 4915



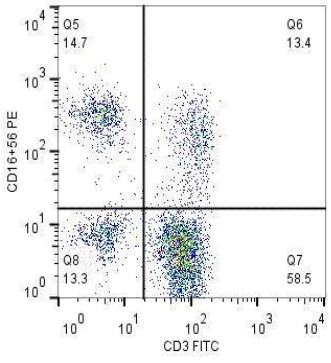
Untreat 24 h
Gate: Lymphocytes
Count: 4915



Water Extract 50 µg/ml
Gate: Ungated
Count: 89802

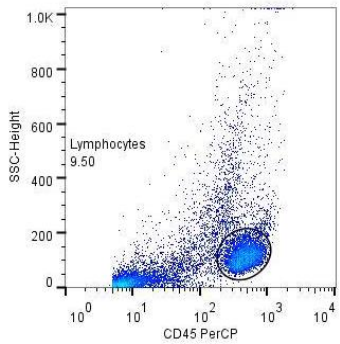


Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 4914

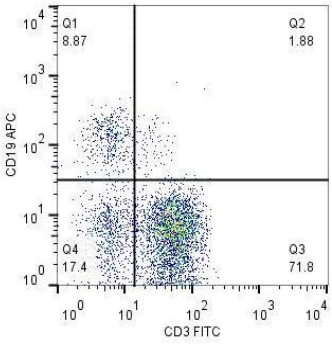


Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 4914

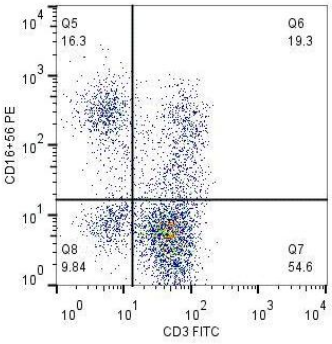
Density plots of the PBMCs donor no. 3 (continued)



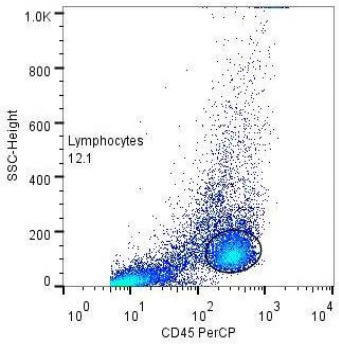
Water Extract 100 µg/ml
Gate: Ungated
Count: 52190



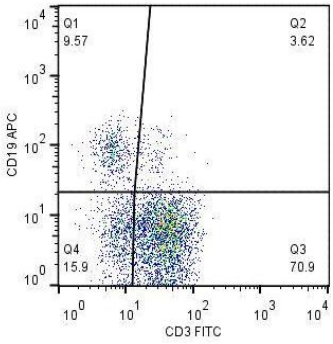
Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 4958



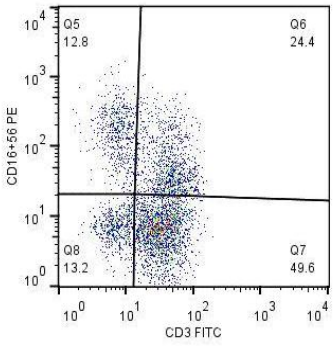
Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 4958



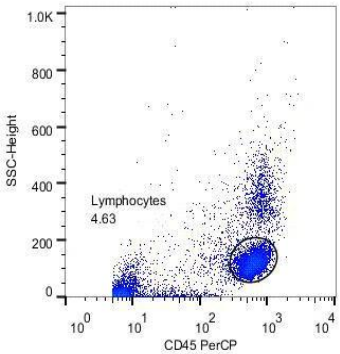
Water Extract 200 µg/ml
Gate: Ungated
Count: 39361



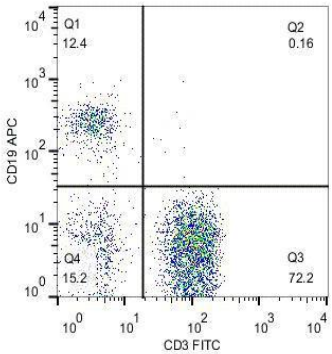
Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 4776



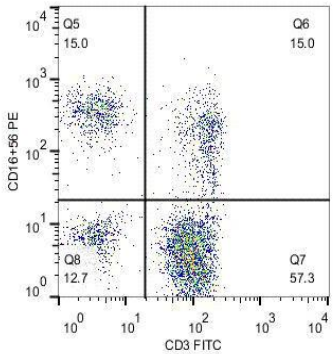
Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 4776



α-Mangostin 1 µg/ml
Gate: Ungated
Count: 105016

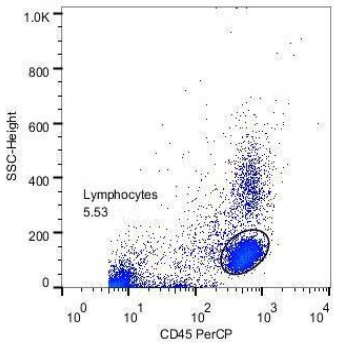


α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 4860

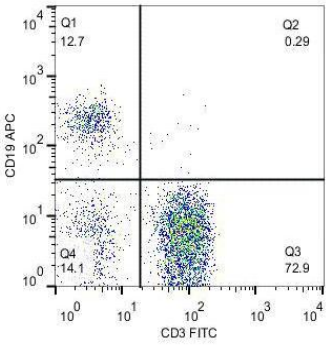


α-Mangostin 1 µg/ml
Gate: Lymphocytes
Count: 4860

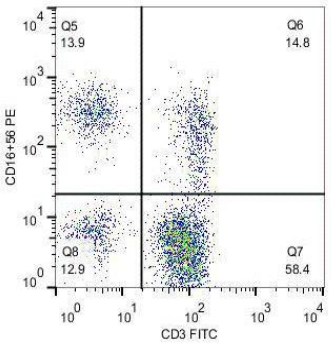
Density plots of the PBMCs donor no. 3 (continued)



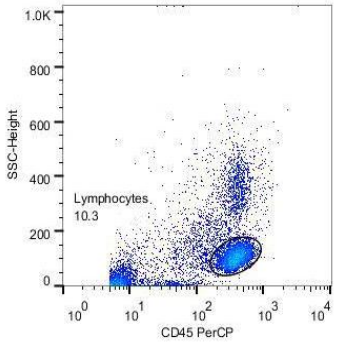
α-Mangostin 2 µg/ml
Gate: Ungated
Count: 88698



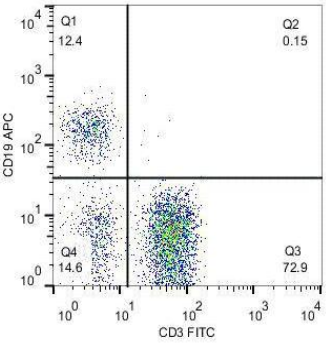
α-Mangostin 2 µg/ml
Gate: Lymphocytes
Count: 4909



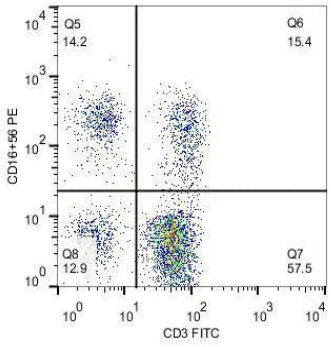
α-Mangostin 2 µg/ml
Gate: Lymphocytes
Count: 4909



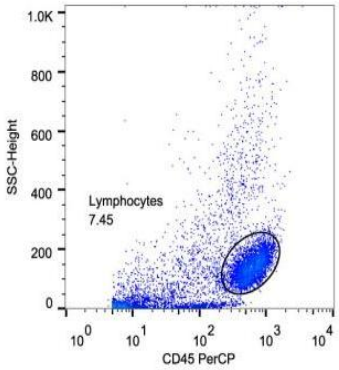
α-Mangostin 4 µg/ml
Gate: Ungated
Count: 46476



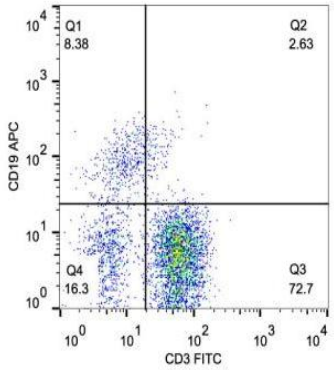
α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4793



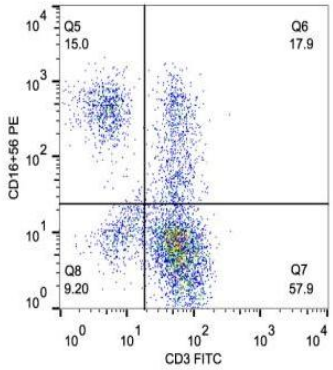
α-Mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4793



ConcanavalinA 10 µg/ml
Gate: Ungated
Count: 69885

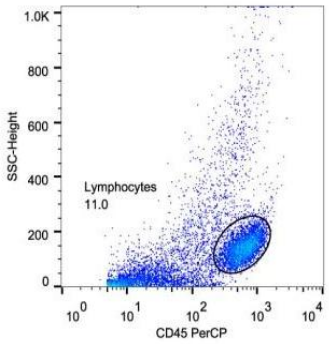


ConcanavalinA 10 µg/ml
Gate: Lymphocytes
Count: 5205

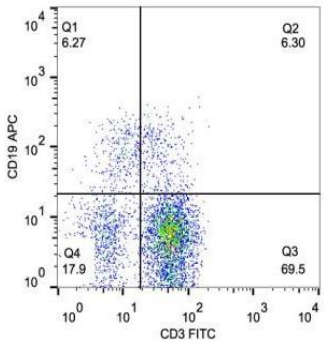


ConcanavalinA 10 µg/ml
Gate: Lymphocytes
Count: 5205

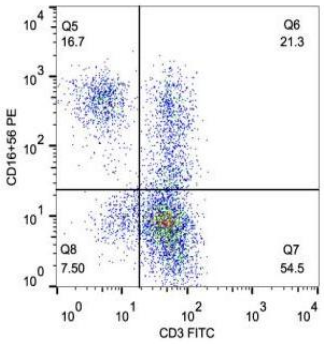
Density plots of the PBMCs donor no. 3 (continued)



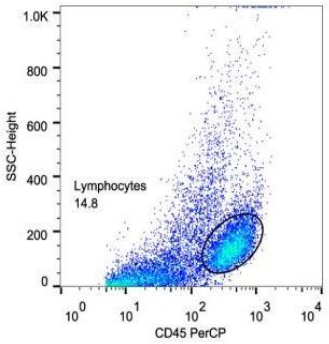
ConcanavalinA 10 µg/ml + Water Extract 50 µg/ml
Gate: Ungated
Count: 46842



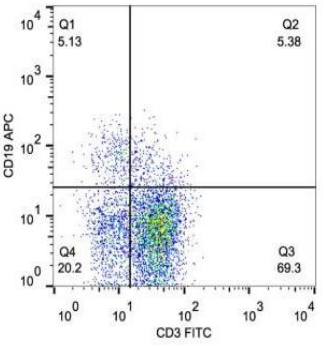
ConcanavalinA 10 µg/ml + Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 5171



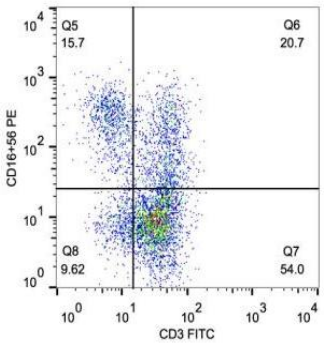
ConcanavalinA 10 µg/ml + Water Extract 50 µg/ml
Gate: Lymphocytes
Count: 5171



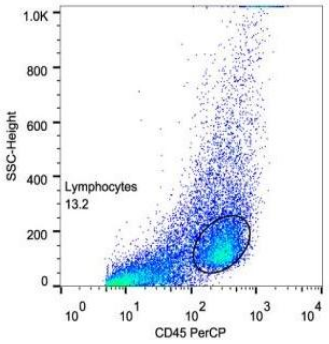
ConcanavalinA 10 µg/ml + Water Extract 100 µg/ml
Gate: Ungated
Count: 36621



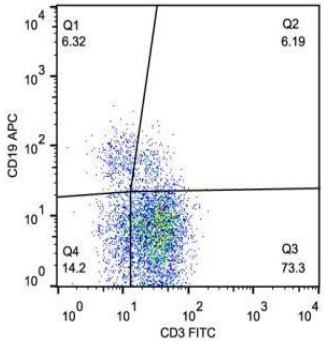
ConcanavalinA 10 µg/ml + Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 5424



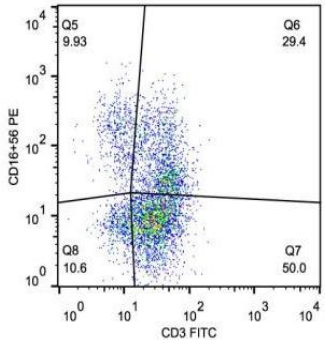
ConcanavalinA 10 µg/ml + Water Extract 100 µg/ml
Gate: Lymphocytes
Count: 5424



ConcanavalinA 10 µg/ml + Water Extract 200 µg/ml
Gate: Ungated
Count: 40787

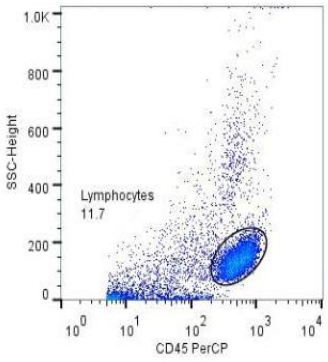


ConcanavalinA 10 µg/ml + Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5398

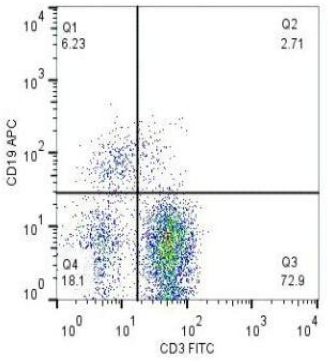


ConcanavalinA 10 µg/ml + Water Extract 200 µg/ml
Gate: Lymphocytes
Count: 5398

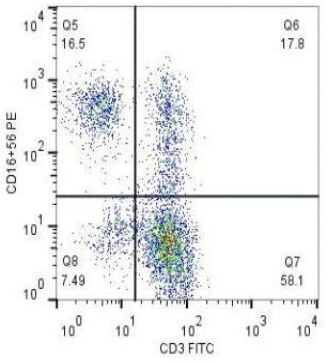
Density plots of the PBMCs donor no. 3 (continued)



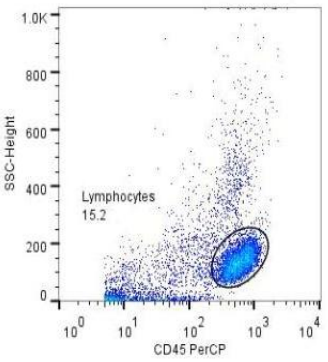
ConcanavalinA 10 µg/ml + α-mangostin 1 µg/ml
Gate: Ungated
Count: 43665



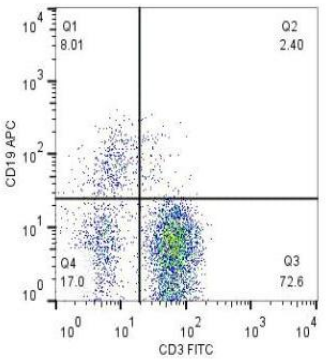
ConcanavalinA 10 µg/ml + α-mangostin 1 µg/ml
Gate: Lymphocytes
Count: 5088



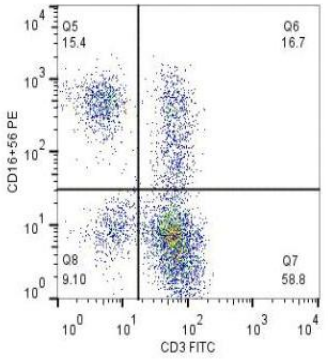
ConcanavalinA 10 µg/ml + α-mangostin 1 µg/ml
Gate: Lymphocytes
Count: 5088



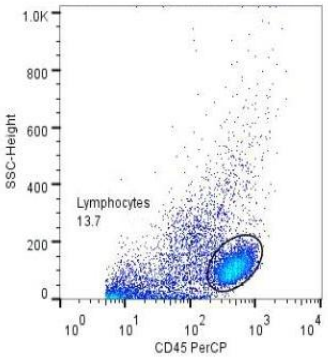
ConcanavalinA 10 µg/ml + α-mangostin 2 µg/ml
Gate: Ungated
Count: 33368



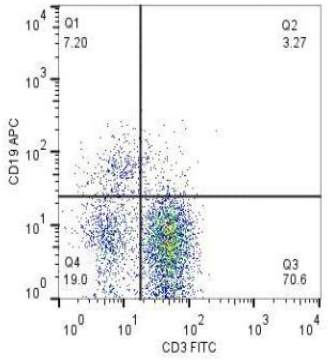
ConcanavalinA 10 µg/ml + α-mangostin 2 µg/ml
Gate: Lymphocytes
Count: 5078



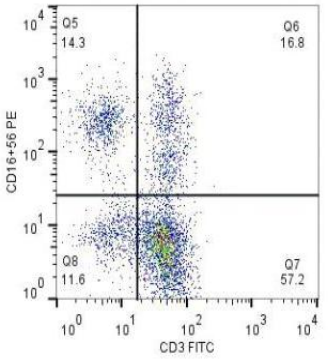
ConcanavalinA 10 µg/ml + α-mangostin 2 µg/ml
Gate: Lymphocytes
Count: 5078



ConcanavalinA 10 µg/ml + α-mangostin 4 µg/ml
Gate: Ungated
Count: 36409



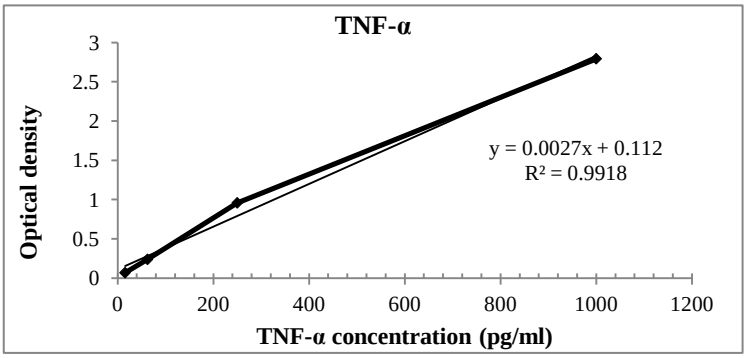
ConcanavalinA 10 µg/ml + α-mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4986



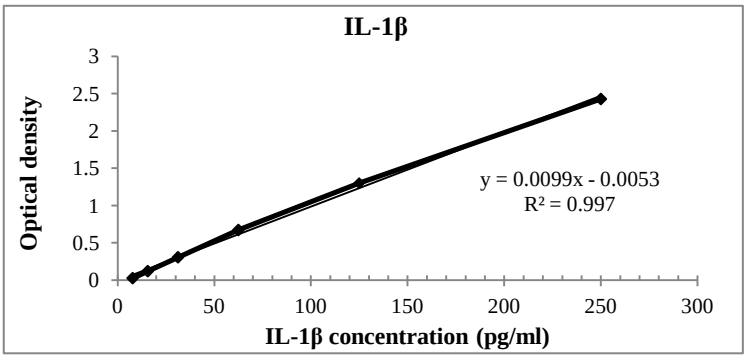
ConcanavalinA 10 µg/ml + α-mangostin 4 µg/ml
Gate: Lymphocytes
Count: 4986

APPENDIX C
ELISA STANDARD CURVES OF THREE CYTOKINES

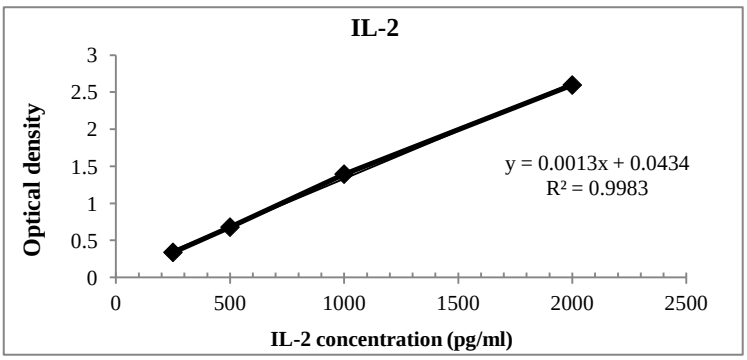
1. Standard curve of TNF-α



2. Standard curve of IL-1β



3. Standard curve of IL-2



APPENDIX D

ANALYSIS RESULTS OF INTRACELLULAR TNF-α

Sample	FSC-H,, SSC-H subset Freq of parent	monocyte Freq of parent	monocyte Geo mean FITC	monocyte Mean FITC	FITC+ Freq of parent	FITC+ Geo mean FITC	FITC+ Mean FITC	FITC- Freq of parent	FITC- Geo mean FITC	FITC- Mean FITC
Isotype	97.6	3.39	5.09	6.7	0.131	56.3	704	99.9	5.07	5.78
Untreated	97.4	3.84	4.27	4.94	0.204	36.4	49.5	99.8	4.25	4.84
LPS	98.1	3.67	47.4	104	73.2	93.9	139	26.8	7.29	8.81
Crude50	96.6	3.79	4.6	5.48	0.375	50.8	89.5	99.6	4.56	5.16
Crude50+ Dex	97.3	3.43	4.4	5.11	0.273	40.4	60.1	99.7	4.37	4.96
Crude50+ LPS	97.4	3.32	66.7	139	81.4	105	169	18.6	8.97	10.3
Crude100	97.1	3.79	4.51	5.26	0.378	34.1	49.4	99.6	4.47	5.09
Crude100 +Dex	97.5	3.6	4.69	5.44	0.293	38.5	62.9	99.7	4.66	5.27
Crude100 +LPS	97.3	3.04	65.2	126	81.8	99	151	18.2	9.93	11.1
LPS+Dex	98	3.38	56.2	121	76.8	98.8	155	23.2	8.65	10

APPENDIX E

HUMAN SUBJECTS APPROVAL DOCUMENT

Certificate of Exemption

COE. No. MU-DT/PY-IRB 2013/003.0402

Documentary Proof of Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Institutional Review Board

Title of Project: Evaluation on Immunomodulatory Activities of Mangosteen

Project Number: MU-DT/PY-IRB 2013/007.0402

Principle Investigator: Miss Pimolkan Kasemwattanaroj

Name of Institution: Faculty of Pharmacy

Date of Recommendation: February 4, 2013

Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Institutional Review Board is in full compliance with International Guidelines for Human Research Protection such as Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Signature of Chair:

(Associate Professor Dr.Choltacha Harnirattisai)

Chair

Office of Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Institutional Review, Board Building 4, Fifth Floor, Faculty of Dentistry, Mahidol University, 6 Yothi Street, Rajthevi, Bangkok 10400, THAILAND Tel: (662)-200-7622

BIOGRAPHY

NAME	Miss Pimolkan Kasemwattanaroj
DATE OF BIRTH	5 January 1986
PLACE OF BIRTH	Nakhon Pathom, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 2005-2009 Bachelor of Science in Pharmacy (First class honors) Mahidol University, 2010-2013 Master of Science in Pharmacy (Microbiology)
HOME ADDRESS	9/1 Moo 7, Tumbon Nongpak-long, Amphur Muang, Nakhon Pathom, Thailand, 73000 Tel: 089 202 2252 E-mail: pimolkan.k@gmail.com
PRESENTATION	Poster presentation: "EFFECT OF MANGOSTEEN EXTRACT ON NK CELL POPULATIONS" The 35th Pharmacological and Therapeutic Society of Thailand Meeting, Phitsanulok, Thailand (20 – 22 March 2013)