# ANTIOXIDATIVE EFFECTS OF CRUDE WATER EXTRACT OF MANGOSTEEN IN HEALTHY VOLUNTEERS

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Thesis
entitled

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## ABSTRACT

Oxidative stress has been implicated in the progression of a number of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Harmful effects of excessive free radicals causing biological damage is in terms of oxidative stress. Reactive oxygen species (ROS) or oxygen free radical are products of normal cellular metabolism and induce apoptosis and damage the protein, DNA, lipid membrane and other organ systems in the body. Biological markers of oxidative stress has many forms such as malondialdehyde (MDA) and hydroxyeicosatetraenoic acid (HETEs). In this study, two biomarkers; i.e., 4-hydroxynonenal (HNE) and dehydroepiandrosterone (DHEA) were of interest. HNE is a byproduct of lipid peroxidation, which is an indicator for oxidative stress. HNE can react with lysine or histidine in protein, and changes the function and structure of the protein. DHEA is a neurosteroid biological marker. Some reports presented that DHEA has also been found to have the ability to protect cells from oxidative damage to the hippocampus part of the brain. Recently, many medicinal plants have been widely used as antioxidants to protect against oxidative stress. Several studies of mangosteen extract have been shown to have several medicinal properties especially as antioxidant and anti-inflammatories in both human cell lines and animal models. Previous studies reported that mangosteen extract could decrease oxidative stress in scopolamine-treated mice and improve the animal's memory.

From the *in vitro* and *in vivo* results, the protective effect of crude water extract of mangosteen (CWM) was obtained as described. Then, the aim of this study was to evaluate the safety of CWM in healthy volunteers and also determine the markers involving oxidative stress during the time of taking CWM over 6 months.

CWM was prepared in a capsule dosage form. The powder of CWM was filled into capsules size No. 1 at 220 mg without filler. For the dose of 280 mg in capsules size No. 0, was adjusted to a weight of 300 mg with corn starch. In this study, we have attempted to determine the properties of CWM after preparation, which consisted of two parts. The first part, we checked the overall quality of CWM capsules on a weight variation test, disintegration test, loss on drying test, DPPH scavenging assay and biological activities on SK-N-SH cells (MTT, ROS). In the second part, the investigation on the safety and effects of CWM in healthy volunteers of clinical tried phase I study were determined from all aspects by the investigators of the whole project. From this study aspect, the antioxidant activity of CWM was assessed in 11 healthy human subjects after taking CWM for 168 days (6 months), whose weight was less either 55 kg or more than 55 kg, had taken a daily dose of CWM at 220 mg and 280 mg respectively. The effectiveness of CWM was evaluated on the changes of ROS in red blood cells (RBCs) by flow cytometry and biological markers; HNE in whole blood (WB) and RBCs by Dot Blot analysis and in the plasma of healthy humans using the ELISA technique.

In the quality control of the extract capsules, weight variation test, disintegration test and loss on drying met the criteria of the British Pharmacopoeia (BP) 2014. The antioxidant activity of CWM by DPPH assay showed the $IC_{50}$ was less than 50 µg/ml and did not show significant change potency between 0 and 6 months of storage. The biological activities of CWM on SK-N-SH cells (MTT, ROS) showed very low cytotoxicity ($IC_{50}$ was more than 100 µg/ml) in on MTT assay, whereas CWM still possessed antioxidant activity in ROS production at 100 and 200 µg/ml respectively. In a clinical trial, volunteers received a daily dose of CME at 220 mg and 280 mg, respectively, corresponding to their weights for 3 months, followed by double dose daily for the last 3 months. The results showed a significant increase in antioxidant capacity of RBCs and led to significantly decrease in HNE modification in whole blood and RBCs after taking CWM at 28, 84 and 168 days respectively and remained until 6 months after ingestion. However, CWM had no significant changes on DHEA levels in the plasma of healthy volunteers.

According to the obtained results, we concluded that the quality of CWM capsules met the standard criteria of BP. It was also safe for cells and in healthy volunteers. CWM capsules possessed antioxidant activity during the 6 months of the study. Therefore, it was supposed that CWM has the potential to reduce oxidative stress in patients with certain diseases in the future study.

KEY WORDS : WATER EXTRACT OF MANGOSTEEN / ANTIOXIDATION / REACTIVE OXYGEN SPECIE / 4-HYDROXYNONENAL / DEHYDROEPIANDROSTERONE

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ฤทธิ์การต้านอนุมูลอิสระของสารสกัดหยาบจากชั้นน้ำของมังคุดในอาสาสมัครสุขภาพดี
ANTIOXIDATIVE EFFECTS OF CRUDE WATER EXTRACT OF MANGOSTEEN IN HEALTHY VOLUNTEERS.

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บทคัดย่อ

ความเครียดออกซิเดชันมีความเกี่ยวข้องในความก้าวหน้าของจำนวนของโรคเกี่ยวกับระบบประสาทเช่นโรคพาร์กินสันและโรคอัลไซเมอร์ ผลกระทบที่เป็นอันตรายจากที่มีอนุมูลอิสระมากเกินไปจะก่อให้เกิดความเสียหายทางชีวภาพ ในช่วงระยะเวลาของการเกิด reactive oxidative stress (ROS) หรือออกซิเจนอนุมูลอิสระซึ่งเป็นผลิตภัณฑ์ของการเผาผลาญของเซลล์ปกติและเหนี่ยวนำให้เกิดการตายและความเสียหายของโปรตีน ดีเอ็นเอ เยื่อไขมันและระบบอวัยวะอื่น ๆ ในร่างกาย ตัวบ่งชี้ทางชีวภาพของความเครียดออกซิเดชัน มีอยู่ด้วยกันหลายตัว อย่างเช่น malondialdehyde (MDA) และ hydroxyeicosatetraenoic acid (HETEs) ในการศึกษานี้ สารบ่งชี้ทางชีวภาพ 2 ตัวอันได้แก่ 4 hydroxynonenal (HNE) และ dehydroepiandrosterone (DHEA) ซึ่งเป็นตัวที่เราสนในการศึกษา HNE เป็นผลพลอยได้จากการเกิด lipid peroxidation ซึ่งเป็นตัวบ่งชี้สำหรับความเครียดออกซิเดชัน HNE สามารถทำปฏิกิริยากับไลซีนหรือฮิสติดีนโปรตีนและเปลี่ยนแปลงการทำงานและโครงสร้างของโปรตีน DHEA เป็นตัวบ่งชี้ทางชีวภาพของ neurosteroid ซึ่งบางรายงานได้นำเสนอว่า DHEA มีความสามารถในการปกป้องเซลล์ในส่วนของ hippocampus ในสมอง จากการทำลายของอนุมูลอิสระ เมื่อเร็วๆ นี้ พืชสมุนไพรจำนวนมากได้ถูกนำมาใช้เป็นสารต้านอนุมูลอิสระอย่างกว้างขวางเพื่อป้องกันความเครียดออกซิเดชัน หลายงานวิจัยของสารสกัดจากมังคุดพบว่า มีคุณสมบัติทางยาหลายอย่างโดยเฉพาะอย่างยิ่งฤทธิ์ต้านอนุมูลอิสระและต้านการอักเสบทั้งในเซลล์ของมนุษย์และสัตว์ การศึกษาก่อนหน้านี้รายงานว่า สารสกัดจากเปลือกมังคุดสามารถลดความเครียดออกซิเดชัน ในหนูที่รับสาร scopolamine และเพิ่มหน่วยความจำของสัตว์

จากการทดลองในหลอดทดลองและสัตว์ทดลอง แสดงประสิทธิภาพในการป้องกันต่างๆของสารสกัดหยาบจากชั้นน้ำของมังคุด (CWM) ตามที่ได้อธิบายไว้ จุดมุ่งหมายของการศึกษาในครั้งนี้เพื่อประเมินความปลอดภัยของ CWM ในอาสาสมัครสุขภาพดีและตรวจสอบตัวบ่งชี้ที่เกี่ยวข้องกับ oxidative stress ในร่างกายของอาสาสมัครภายในช่วงเวลา 24 สัปดาห์ของการรับสารสารสกัด CWM

สารสกัด CWM จะถูกเตรียมในรูปของยาแคปซูล ผงของ CWM จะถูกบรรจุลงในแคปซูลเบอร์หนึ่งปริมาณ 220 และ 280 มก. ในแคปซูลเบอร์ 0 โดยปรับน้ำหนักเป็น 300 มก. ด้วยแป้งข้าวโพด ในการศึกษาครั้งนี้เราได้ตรวจสอบคุณสมบัติของ CWM หลังการผลิต ซึ่งประกอบด้วยสองส่วน ในส่วนแรกเราได้ทำการตรวจสอบคุณภาพโดยรวมของสารสกัด CWM ในแคปซูล โดยทดสอบความแตกต่างของน้ำหนักเฉลี่ย การแตกตัว การวิเคราะห์ปริมาณความชื้น การตรวจการต้านอนุมูลอิสระด้วย DPPH scavenging assay และฤทธิ์ทางชีวภาพต่อเซลล์ SK-N-SH (MTT, ROS) ส่วนที่สองการทดสอบความปลอดภัยและประสิทธิภาพของ CWM ในอาสาสมัครสุขภาพดีทางคลินิค ระยะที่1 ซึ่งเป็นงานในโครงการทั้งหมด ในส่วนของการศึกษานี้ฤทธิ์ต้านอนุมูลอิสระของ CWM ทำการประเมินจากอาสาสมัครสุขภาพดี 11 คนหลังการรับประทาน CWM เป็นเวลา 6 เดือน ซึ่งบุคคนที่มีน้ำหนักน้อยกว่า 55 กก. และมากกว่า 55 กก. จะรับประทานสารสกัด 1 ครั้งต่อวันในขนาด 220 และ 280 มก. ตามลำดับ ประสิทธิภาพของ CWM จะถูกประเมินการเปลี่ยนแปลงของค่า ROS ในเม็ดเลือดแดงโดยวิธี flow cytometry และตัวบ่งชี้ทางชีวภาพ HNE ในเลือด และเม็ดเลือดแดงโดยวิธี Dot Blot และ DHEA ในพลาสมาด้วยเทคนิค ELISA

ในการควบคุมคุณภาพของสารสกัดแคปซูลโดยการทดสอบความแตกต่างของน้ำหนักเฉลี่ย การแตกตัว และ ปริมาณความชื้นซึ่งพบว่าอยู่ในเกณฑ์มาตราฐานของตำรับยาอังกฤษ (BP) 2014 ฤทธิ์ต้านอนุมูลอิสระของ CWM โดยวิธี DPPH scavenging assay ซึ่งแสดงค่า $IC_{50}$ น้อยกว่า 50 ไมโครกรัม/มล. และไม่มีการเปลี่ยนแปลงของฤทธิ์ต้านอนุมูลอิสระระหว่างการเก็บรักษาในเดือนแรกและเดือนที่หก ฤทธิ์ทางชีวภาพของ CWM ต่อ เซลล์ SK-N-SH (MTT, ROS) พบว่า $IC_{50}$ มีค่ามากกว่า 100 ไมโครกรัม/มล. จาก MTT assay แสดงความไม่เป็นพิษต่อเซลล์ และมีฤทธิ์ต้านอนุมูลอิสระต่อการผลิต ROS ในการทดลองทางคลินิก อาสาสมัครรับประทาน CWM ขนาด 220 และ 280 มก. ตามขนาดน้ำหนักที่กำหนด โดยรับประทานวันละครั้งเป็นเวลา 3 เดือน และได้ ปรับปริมาณสารเป็นสองเท่าต่อวันในช่วง 3 เดือนหลัง ผลการศึกษาพบการความสามารถในการต้านอนุมูลอิสระในเม็ดเลือดแดงเพิ่มขึ้นอย่างมีนัยสำคัญและนำไปสู่การลดค่าHNEในเลือดและเม็ดเลือดแดงอย่างมีนัยสำคัญหลังจากการรับประทานสารสกัด CWM ในวันที่ 28, 84 และ 168 วัน ตามลำดับและยังคงอยู่จนถึงเดือนที่ 6 ของการบริโภค แต่ CWM ไม่มีผลต่อการเปลี่ยนแปลงของระดับ DHEA อย่างมีนัยสำคัญ

ตามผลการทดลองทั้งหมดนี้ สรุปได้ว่าคุณภาพของสารสกัด CWM ในแคปซูล อยู่ภายใต้มาตรฐานของ BP ที่มีความปลอดภัยต่อเซลล์และอาสาสมัครสุขภาพดี และยังคงมีประสิทธิภาพต้านอนุมูลอิสระที่ดีตลอดระยะเวลา 6 เดือนของการศึกษา ดังนั้นมีความเป็นไปได้ที่ CWM แคปซูลจะมีศักยภาพในการลดความเครียดออกซิเดชันในผู้ป่วยในโรคที่เกี่ยวข้อง
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# LIST OF ABBREVIATIONS

| | |
|---|---|
| °C | Degree Celsius |
| CWM | Crude water extract of mangosteen |
| DCFH-DA | 2',7'-Dichlorodihydro fluorescein diacetate |
| DHEA | Dehydroepiandrosterone |
| $DPPH^{\bullet}$ | 1,1-Diphenyl-2-picryl-hydrazyl radical |
| EDTA | Ethylene Diamine tetra Acetic Acid |
| ELISA | Enzyme-liked immunosorbent assay |
| et al. | et alli (Latin), and other people |
| etc | et ectera (Latin), and other things |
| EtOH | Ethanol |
| EtOAc | Ethyl acetate |
| $Fe^{3+}$ | Ferric ion |
| $Fe^{2+}$ | Ferrous ion |
| FBS | Fetal bovine serum |
| g | Gram(s) |
| h | hour |
| HNE | 4-hydroxynonenal |
| $^{\bullet}OH$ | Hydroxyl radical |
| $H_2O_2$ | Hydrogen peroxide |
| i.e. | id est (Latin), which is to say, in other words |
| $IC_{50}$ | Inhibitory concentration at 50% |
| LDL | Low Density Lipoprotein |
| $L^{\bullet}$ | Lipid radical |
| MeOH | Methanol |
| µg | Microgram |
| µl | Microliter |
| mg | Milligram |

# LIST OF ABBREVIATIONS (cont.)

| | |
|---|---|
| ml | Milliliter |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| $^{\bullet}NO$ | Nitric oxide radical |
| No. | number |
| ONOO- | Pyroxynitrite |
| $O_2$ | Oxygen |
| PB | Phosphate-buffer |
| PBS | Phosphate-buffered saline |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minute |
| RPMI | Rosewell Park Memorial Institute |
| SEM | Standard error of mean |
| SD | Standard Deviation |
| SOD | Superoxide dismutase |
| $O_2^{\bullet-}$ | Superoxide radical |
| v/v | Volume to Volume |
| $H_2O$ | Water |

# CHAPTER I
# INTRODUCTION

Oxidative stress (OS) has been implicated in various degenerative diseases in aging such as cancer, atherosclerosis, Parkinson's disease, Alzheimer's disease. In order to understand the mechanisms of these diseases that involve OS and to implement treatment or prevention, an analytic method to evaluate OS in living cells is very important.

Reactive oxygen species (ROS) are constantly generated under normal conditions as a consequence of aerobic metabolism. It has been implicated in initiating causing pathogenesis of many diseases. There are some evidences that abnormalities in lipid compounds may cause overproduction of ROS and in turn antioxidant enzyme activities and lipid peroxidation, and these phenomena may be related to the pathophysiology of major depression. ROS include free radical such as hydroxyl radical (OH), hydrogen peroxide ($H_2O_2$), Superoxide anion ($O^{-2}$). They are particularly transient species due to their high chemical reactivity and can react with protein, DNA, carbohydrates and lipid, which lipid peroxidation can lead to cell death. Hydrogen peroxide is thought to be the major precursor of highly reactive free radicals, and it has been reported to induce apoptosis in cells of the central nervous system (1). hydroxynonenal or (HNE) is another one caused by stress event. HNE is an α, β–unsaturated hydroxyalkenal that is produced by lipid peroxidation in cell and consequently will be damage to the cell membrane. Under normal conditions, these chemical events require an accurate balance between ROS production and removal, HNE production and removal, with aging or AD. This balance is markedly disrupted, leading ROS accumulation and oxidative damage (2-4).

Alzheimer's disease has been observed to have lower blood levels of another marker, dehydroepiandrosterone (DHEA) than do non dementia control subjects in cross-sectional studies. DHEA is a major neurosteroids that is presented at high level in brain (5) and it can be detect in tissue, urine, blood and serum. The serum

concentration of DHEA in adult men and women is higher than that of any other steroid except cholesterol (5). It has been clarified that humans are largely depend on peripheral tissues for androgen and estrogen formation from the adrenal precursor DHEA. It has been suggested that DHEA has some protective effect hippocampal neuronal cell against oxidative stress-induced cell damage (5, 6). The hippocampus is a major component of the brains of humans, which belongs to the limbic system and plays important roles in the consolidation of information from short-term memory to long-term memory. In Alzheimer's disease, the hippocampus is one of the first regions of the brain to suffer damage memory loss and disorientation are included among the early symptoms. Some reports present that the level of DHEA is independent on age and inflammation (7). Therefore, the serum concentrations of DHEA are important to the pathophysiology of various diseases DHEA have been applied to the treatment of several disease conditions, such as inflammatory cytokines (7) and cardiovascular disease (8).

As oxidative stress might have an important role in many human diseases, then usage of antioxidants in medical pharmacology is intensively studied, particularly in some diseases such as neurodegenerative and stroke diseases. Antioxidants are also widely used as ingredients in dietary supplements in order to maintain health and prevent diseases such as coronary heart disease, cancer, Parkinson and Alzheimer's disease. Recent studies showed that a number of plant products including polyphenols, flavonoids and various plant extracts exerted the antioxidant action (15-17). Plants, especially medicinal plants, are a great source of the antioxidant; because they have been used as medicine, suggested that they contain some bioactive compounds (15). Natural compounds have been expected to play and important role as antioxidative effect to fight against oxidative damage.

Mangosteen, *Garcinia mangostana* L. is a tropical tree found in Southeast Asia. Different parts of mangosteen, mostly pericarps of fruits, have been used in traditional medicine for the treatments of diarrhea, dysentery, infections and wounds (9, 10). In previous study, has been shown to contain various bioactivities, which include antioxidant, anti-inflammatory, cytotoxic and anticancer activities (9, 11-14). *In vitro*, mangosteen extract showed promising antioxidant activities in SK-N-SH neuroblastoma cells (14), moreover *in vivo*, it could safely protect memory loss in

mice treated with scopolamine (129). Therefore, in this study, the crude water extract part of mangosteen (CWM) was intensively studied in physical control, biological activity on SK-N-SH cells line by MTT and ROS assay and Phase I clinical trial. Phase I clinical trial, especially on the safety daily use for 6 months in healthy volunteers. The plasma and whole blood of the volunteers were collected and evaluated the HNE and DHEA, the interesting markers concerning AD/dementia. Thus the aim of this study is to search for the antioxidative effect of crude water extract of mangosteen observed in the plasma and whole blood of the volunteers after taking CWM. We hope that the outcome of this study would support the antioxidative effect and safety use of our mangosteen preparation and assure the Phase 2 clinical trial study in patients. The scientific and clinical studies of mangosteen would lead to the development of mangosteen as drug and becoming the interesting candidates for the treatment of Alzheimer's disease in the future.

## Objectives

To evaluate the effect of crude water extract of mangosteen in healthy human volunteers on the safety consideration and the changes in ROS and biological markers; HNE and DHEA of Alzheimer's disease after daily taken for 6 months.

# CHAPTER II
# LITERATURE REVIEW

## 2.1 Oxidative stress

The term oxidative stress is a state of unbalanced tissue oxidation refers to a condition in which cells are subjected to excessive levels of molecular oxygen or its chemical derivatives called reactive oxygen species (ROS) (18, 19). The human body is exposed to free radicals both exogenous and endogenous. The exogenous free radicals were radiation, smoke and even sunlight. The endogenous were produced during aerobic respiration of cells when organism consume oxygen molecule to produce energy. Under physiological conditions, the molecular oxygen undergoes a series of reactions that ultimately lead to the generation of superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^\bullet$), lipid peroxides and nitrogen oxides as shown in Figure 2.1 (20, 21). Oxidative stress also plays an important role in the early on set of the disease such as cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, Parkinson disease and Alzheimer's disease (22-25).

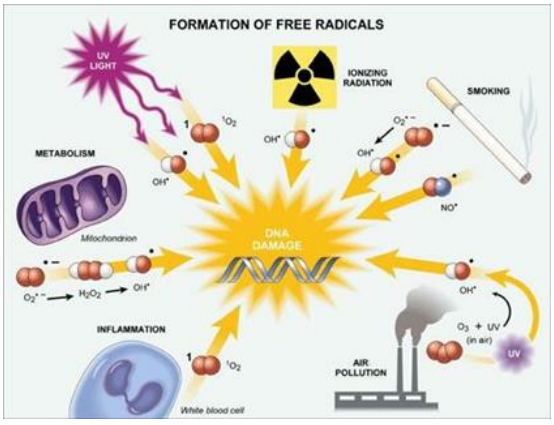



**Figure 2.1** Source of free radical accumulated oxidative damage. (http://radiation-remedies.com/inflammation-and-double-break-dna-rupture)

### 2.1.1 Free radicals

Free radicals are molecules with unpaired electron in their outer orbit. Free radicals have a very important role in the origin of life and biological evolution, leaving beneficial effects on the organisms (26). Oxygen radicals are involved in many biochemical activities of cells such as signal transduction, gene transcription and regulation of soluble guanylatecyclase activity. Humans are constantly exposed to free radicals created by electromagnetic radiation from the manmade environment such as pollutants and cigarette smoke. Natural resources such as radon, cosmic radiation, as well as cellular metabolisms (respiratory burst, enzyme reactions) also add free radicals to the environment. The previous studied presented that the most common reported cellular free radicals are hydroxyl (OH·), superoxide ($O_2^{-}$·) and nitric monoxide (NO·), which generated during aerobic metabolism in cells (29). Even some other molecules like hydrogen peroxide ($H_2O_2$) and peroxynitrite (ONOO–) are not free radicals; they are reported to generate free radicals through various chemical reactions in many cases (29). Superoxide is generated through either incomplete reduction of oxygen in electron transport systems or as a specific product of enzymatic systems, while NO is generated by a series of specific enzymes (the nitric oxide synthases). Both superoxide and NO are reactive and can readily react to form a series of other ROS and RNS (27) while superoxide and NO rapidly react to form peroxynitrite and subsequently other RNS (2-28). Free radicals can cause oxidative damage to biomolecules, (lipids, proteins, DNA) (1), leading to many diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in humans (30, 31).

### 2.1.2 Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) is a phrase used to describe a number of reactive molecules and free radicals derived from molecular oxygen. The production of oxygen based radicals is the bane to all aerobic species. These molecules, produced as byproducts during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalyzed oxidation, have the potential to cause a number of deleterious events. It was originally thought that only phagocytic cells were

responsible for ROS production as their part in host cell defense mechanisms. Recent work has demonstrated that ROS have a role in cell signaling, including; apoptosis; gene expression; and the activation of cell signaling cascades. It should be noted that ROS can serve as both intra- and inter-cellular messengers.

#### 2.1.2.1 Types of Reactive Oxygen Species

Most reactive oxygen species are generated as byproducts during mitochondrial electron transport. In addition ROS are formed as necessary intermediates of metal catalyzed oxidation reactions. Atomic oxygen has two unpaired electrons in separate orbits in its outer electron shell. This electron structure makes oxygen susceptible to radical formation. The sequential reduction of oxygen through the addition of electrons leads to the formation of a number of ROS including: superoxide; hydrogen peroxide; hydroxyl radical; hydroxyl ion; and nitric oxide as shown in Table 2.1 and Figure 2.2.

**Table 2.1** Types of reactive oxygen species.

| Reactive oxygen species | Structure | Properties |
|---|---|---|
| Hydrogen peroxide |  | Can diffuse into cell membranes.<br>Not a free radical, but can generate free radicals by reaction with a transition metal |
| Hydroxyl radical |  | The most reactive species in attacking biologic molecules.<br>Produced from $H_2O_2$ in the Fenton reaction in the presence of $Fe^{2+}$ or $Cu^{+}$ |
| Superoxide anion |  | Produced by the electron transport chain and other sites.<br>Can generate other reactive oxygen species. |

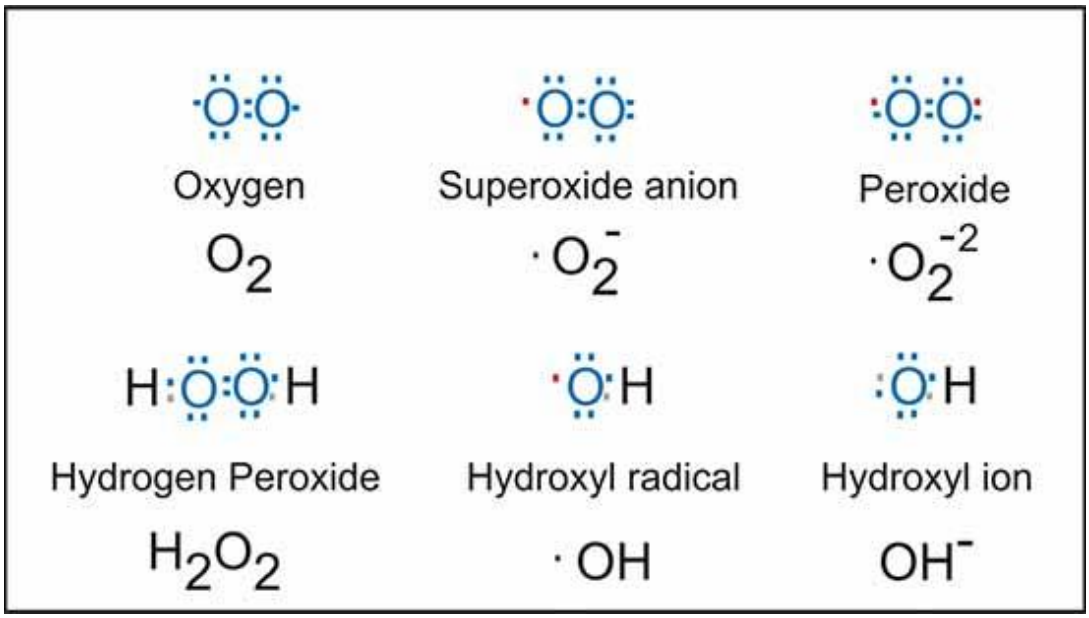



**Figure 2.2** Electron structures of common reactive oxygen species. Each structure is provided with its name and chemical formula. The red • designates an unpaired electron (http://www.biotek.com/resources/articles/reactive-oxygen-species.html).

### 2.1.2.2 Sources of ROS

ROS generation isa prerequisitee of metabolic system in order to interact with organic molecules *in vivo* as interaction of organic molecules with oxygen is energetically unfavorable.

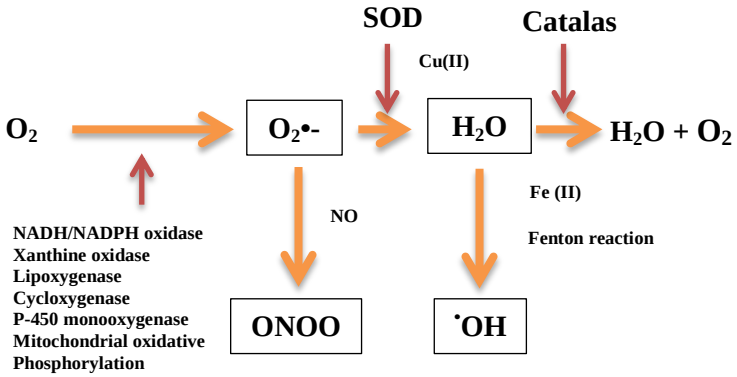



**Figure 2.3** Formation of reactive oxygen species (ROS) from molecular oxygen (32).

ROS can be generated at many different organelles in response to various stimuli. Major sources of ROS production include the mitochondrion, plasma membrane and cytosol. The mitochondria are a major site of generation of

ROS. In all forms of ROS generation, electrons carried by the electron transport chain can leak out of the pathway and pass directly to oxygen, generating $\bullet O_2^-$. Other sources of $\bullet O_2^-$ include enzyme such as P450 in the endoplasmic reticulum, lipoxygenases, xanthine oxidase and NADPH oxidase. The dismutation of superoxide anion by superoxide dismutase (SOD) generates to $H_2O_2$, which can react with $Fe^{2+}$ to form hydroxyl radical, via the Fenton reaction (33) as shown in Figure 2.3:

$$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$$

Hydrogen peroxide can then react with Fe2+ to form hydroxyl radical via the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

Hydroxyl radicals can also be generated via the metal catalyzed Haber-Weiss reaction:

$$\bullet O2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$$

In normal cells, electrons carried by the mitochondria electron tansport chain leak from pathway and form the superoxide free radical. The superoxide anion was generated during respiration, which is converted to $H_2O_2$ by Mn-SOD. In cytosol, superoxide anion is converted to $H_2O_2$ by Zn,Cu-SOD (34). Furthermore, superoxided also has multiple pathways in production such as Complex I, Complex II, NADH ubiquinone oxidoreductase and cytochrome *c* oxidoreductase. The superoxide anion generated by the cycling of ubiquinol in the inner mitochondria matrix (35, 174). High concentration of Mn-SOD ensure that this basal levels of superoxide production is neutralized before it can cause damage to the cell as shown in Figure 2.4.

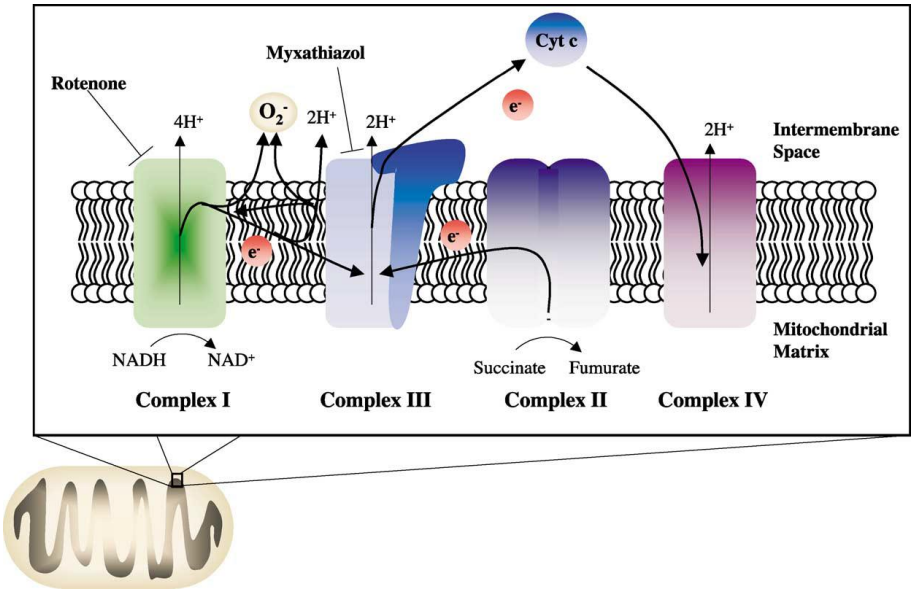



**Figure 2.4** The major subunits of electron transport chain (ETC) and sites of superoxide anion ($\bullet O_2^-$) production. Electron enter the ETC at complex I or complex II following the oxidation of NADH and succinate. Ubiquinone is a lipid soluble electron carrier and carries the electrons from complex I and II to complex III. Superoxide can be produced at both complex I and III (35).

### 2.1.2.3 Effects of ROS in neurodegenerative diseases

ROS comprises hydrogen peroxide ($H_2O_2$), nitric oxide (NO), superoxide anions and the highly reactive hydroxyl and monoxide radicals ($OH^\bullet$, $NO^\bullet$). Damaged mitochondria and activated microglia acts as reservoir of ROS. Initially ROS generation was believed to be an outcome of imbalance between the generation and elimination of ROS but recently many chemistries and molecular biology have been discovered regulating Numerous reports have suggested oxidative stress or ROS plays a key role in neuronal apoptosis. (36-40) For example, Haber Weiss and Fenton reaction initiate the free radical and ROS generation that activates mitogen activated protein (MAP) kinase cascade, excitotoxic calcium mobilization and finally apoptotic cell death (41). ROS are particularly active in the brain and neuronal tissue as the excitatory amino acids and neurotransmitters, whose metabolism is a factory of ROS, which are unique to the brain and serve as sources of oxidative stress. Neuronal biochemical composition is mainly susceptible to ROS since it involves pool of

unsaturated lipids those are labile to peroxidation and oxidative modification. Double bonds of unsaturated fatty acids are sites for attack by free radicals those initiate cascade or chain reaction to damage neighboring unsaturated fatty acids (42). Several studied have shown that Aβ at micromolar concentration showed toxic to neurodegeneration, which they found that it may cause oxidative free radical and cell apoptosis similar to an exposure to $H_2O_2$ or other ROS (41-44). Therefore, cells are particularly sensitive to free radicals, leading to neuronal damage. It has been reported that deleterious effects of ROS on human cells may end in oxidative injury leading to programmed cell death and neuronal loss in cerebral ischemia, seizure disorders, schizophrenia, Parkinson's disease and Alzheimer's disease (45-47).

### 2.1.3 Lipid peroxidation

Lipid peroxidation is a complex process known to occur in both plants and animals (48), which the compound of cholesterol in cell surface membranes influences their susceptibility to peroxidation, probably both by intercepting some of the radicals present and by affecting the internal structure of the membrane by interaction of its large hydrophobic ring structure with fatty acid-side-chains. As lipid peroxidation (LPO) precedes in any membrane, several of the products produced have a detergent-like activity, specially released fatty acids or phospholipids with one of their fatty-acid side-chains removed. (49)

The lipid peroxidation involves three distinct steps: initiation, propagation and termination. The initiation phase of lipid peroxidation may proceed by the reaction of an activated oxygen species such as singlet oxygen ($1O_2$), $O^{2-}$, or $HO^{\bullet}$ with a lipid substrate or by the breakdown of preexisting lipid hydroperoxides by transition metals. In the former case, peroxidation occurs by abstraction of a hydrogen atom from a methylene carbon in the lipid substrate (LH) to generate a highly reactive carbon-centered lipid radical ($L^{\bullet}$). In the propagation phase of lipid peroxidation, molecular oxygen adds rapidly to L at a diffusion controlled rate to produce the lipid peroxyl radical ($LOO^{\bullet}$) (1, 50). The peroxyl radical can abstract a hydrogen atom from a number of in vivo sources, such as DNA and proteins (50), to form the primary oxidation product, a lipid hydroperoxide (LOOH) as shown in Figure 2.5. There are two consequences of lipid peroxidation: structural damage to membranes and

generation of secondary products such as malondialdehyde (MDA, 4-hydroxynonenal (4-HNE). Membrane damage derives from the production of broken fatty acyl chains, lipid-lipid or lipid-protein cross-links, and endocyclization reactions to produce isoprostanes and neuroprostanes (49). This effect is severe for biological systems, produce damage of membrane function, enzymatic inactivation and toxic effects on cellular division and function.

## 2.2 Hydroxynonenal (HNE)

4-hydroxynonenal (HNE), an aldehyde product of membrane lipid peroxidation, can be produced by oxidative stimuli and has been detected in several diseases such as atherosclerosis, Parkinson's disease and Alzheimer's disease (51, 52). The formation of HNE and HNE-protein conjugate has become a biomarker of oxidative stress in tissues or cells. Oxidative stress-induced apoptotic cell death is believed to be involved in the pathological generation of those oxidative stress-related diseases, therefore HNE may be an important mediator of oxidative stress-induced apoptosis. It has been reported that HNE and HNE-protein adduct accumulate in neurons by oxidative insults and that they are associated with the apoptotic events in these cells (53, 54). Exogenously administrated HNE has also been observed to form HNE-protein adduct and to induce apoptotic cell death in macrophages and neurons (53, 55). Much attention has recently been paid to HNE-induced apoptotic cell death in the pathological development of neural and vascular degenerations, particularly because HNE is not only a mediator of amyloid β-peptide-induced damage (56) but also one effective component of oxidized low density lipoproteins. Although studies have shown that HNE-mediated apoptotic cell death involves intracellular calcium uptake (57) and HNE can directly interacts and activates c-Jun amino-terminal kinase (JNKs) (58), the mechanism of HNE induced apoptosis has not been clearly delineated.

### 2.2.1 Mechanism of HNE generation

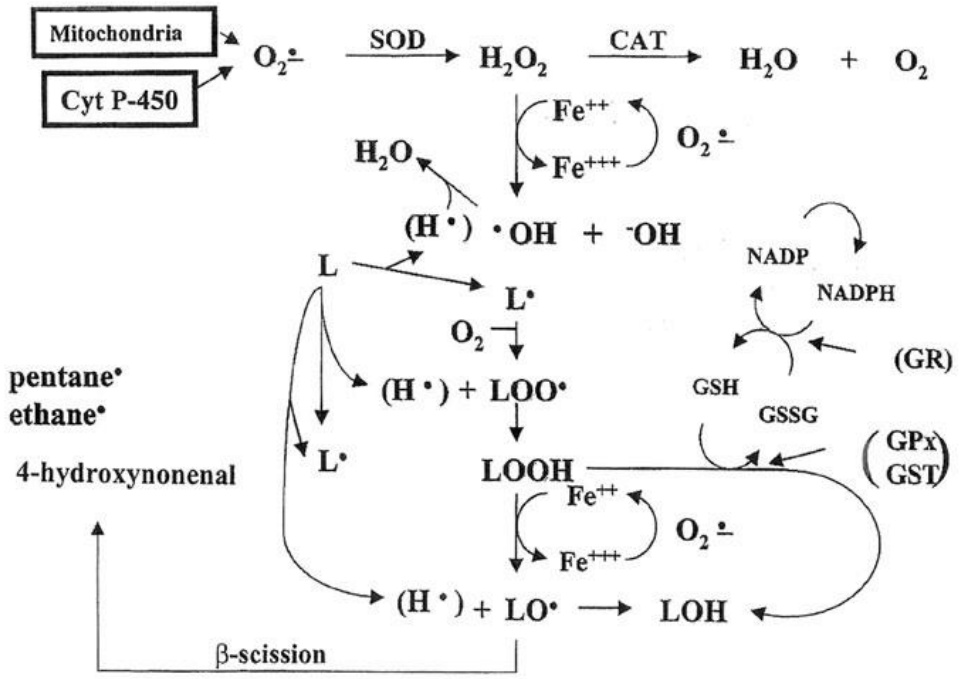



**Figure 2.5** Mechanism of lipid peroxidation and HNE generation (50).

In aerobic organisms, ROS including $O^{2-}$, $H_2O_2$, hydroxyl radical and nitric oxide/ peroxynitrate ($NO/ONOO^-$) are continually generated in cells *via* various endogenous and exogenous sources such as mitochondrial electron transport chain (59). Oxidative stress occurs in the cells as a consequence of an imbalance between the pro-oxidant/antioxidant systems (60). Oxidative stress causes damage to cellular macromolecules such as nucleic acids, proteins, and lipids. Among these targets, the peroxidation of lipids is particularly more damaging because the formation of LPO products leads to propagation of free radical reactions. Abstraction of a hydrogen atom from the polyunsaturated fatty acid (PUFA) moiety of membrane phospholipids initiates the process of lipid peroxidation as shown in Figure 2.5. The resulting alkyl radical may rearrange to a more stable conjugated diene, which enters the autocatalytic lipid peroxidation cascade. Lipid hydroperoxide (LOOH) constitute the major portion of the LPO products and can propagate lipid peroxidation chain reactions (59, 61). The fatty acid carbon chain can be spontaneously cleaved (β-scission) during lipid peroxidation, yielding a variety of highly reactive compounds, including pentane and

ethane radicals, and the α,β-unsaturated aldehydes. In particular 4-HNE, the major α,β-unsaturated aldehyde formed by the degradation of both GO-3 and GO -6 PUFA during LPO is fairly stable and present in relatively higher amounts in biological membranes under the conditions of oxidative stress (62). The biological activities of HNE and other aldehydes include cross-linking with DNA and proteins, which alters the function/activity of these molecules. 4HNE have shown tissue toxicity.

### 2.2.2 Defense against intracellular lipid peroxidation

Uncontrolled generation of ROS can lead to their accumulation causing oxidative stress in the cells. Thus, cells have evolved defense mechanisms to protect against ROS mediated oxidative damage. The antioxidative network as shown in Figure 2.6 (69) acts as a defense mechanism against stress (70). In mammalian cells, there are two tiers of antioxidant defense mechanisms against ROS mediated LPO. Low molecular mass compounds, which act primarily against peroxyl radicals involved in radical propagation provide first-line of defense against LPO. These compounds, referred as "chain-breaking antioxidants" (CBA) can terminate the propagation of free radical mediated reactions and interrupt the autocatalytic chain reaction of LPO (63,68). The main cellular CBAs include tocopherol, ascorbic acid, GSH, uric acid, carotenoids, ubiquinone, polyphenols, etc., (63). Among these, GSH is particularly important because it also serves as the substrate for the two major antioxidant enzyme systems, GPx, and GST. The antioxidant enzymes constitute the second-line of defenses which provide a variety of primary and secondary defenses against oxidative stress. Primary antioxidant enzymes are mainly preventive, and these enzymes such as SOD, CAT, and GPx can decompose ROS and prevent damage to cellular constituents and initiation of LPO. Reduced GSH is an important scavenger of free radicals and a potent endogenous antioxidant, which helps to protect cells from oxidative injury. Besides, its role in the maintaining the redox potential within the cell, it is also a key component of the enzymatic antioxidant system. The reduced GSH concentration was observed to be significantly lower in β-thalassemic and E/β-thalassemic red blood cells (RBCs) compared with the carrier and control subjects, suggesting that the erythrocyte is in a pro-oxidant condition, which may be a partial cause of the increased hemolysis and shortened RBCs survival observed in β-

thalassemic and E/β-thalassemic RBCs (71). Secondary defenses typically involve excision or repair of any lesions caused by ROS. In the event of ROS-induced LPO, secondary defense enzymes are involved in the removal of LOOH to terminate the autocatalytic chain of LPO and protect membranes. GPx and GST, which catalyze GSH-dependent reduction of LOOH through their peroxidase activity, are the major secondary defenses to guard against ROS-induced LPO. In addition to selenium-dependent GPx, the selenium-independent GPx activity of the-α-class GST is also involved in the reduction of LOOH (63-68).

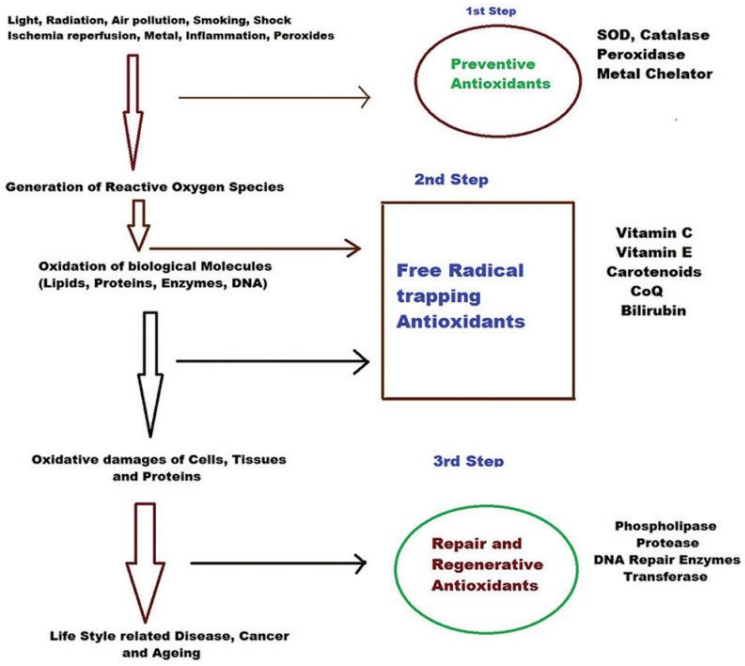



**Figure 2.6** Antioxidative network in biological system (63).

### 2.2.3 Relationship between HNE and diseases

The development of oxidative stress, in which production of highly reactive oxygen species (ROS) overwhelms antioxidant defenses, is a feature of many neurological diseases: ischemic, inflammatory, metabolic and degenerative. Oxidative stress is increasingly implicated in a number of neurodegenerative disorders characterized by abnormal filament accumulation or deposition of abnormal forms of specific proteins in affected neurons, like Alzheimer's disease (AD), Parkinson disease, amyotrophic lateral sclerosis (ALS), and Huntington disease. Causes of neuronal death in neurodegenerative diseases are multifactorial.

In the pathogenesis of AD, lipid peroxidation plays a particular role (72, 42). In previous studies demonstrated to increased levels of lipid peroxidation as indicated by elevated levels of the products of lipid peroxidation such as HNE, neuroprostanes in AD brain (73-75). Further, increased levels of HNE-adducted GSH were found in human postmortem brains from AD patients (76). Normally in cells, HNE-GSH adducts are eliminated by the systems glutathione transferase (GST) and MRP-1. But in AD brain, detoxification system was found to be a target of HNE with consequent decreased efficiency to eliminate HNE, and subsequent accumulation of HNE protein adducts in neuronal cells (77, 78). Even the proteosome, involved in the removal of damaged proteins from cells, has been demonstrated to form conjugates with HNE and neuroprostanes in both MCI and AD (79). In addition, significant increase HNE in cerebrospinal fluid (80), hippocampus, amygdala and parahippocampalgyrus was detected in AD patients compared to control subjects (81, 82). In some studies in blood demonstrated that HNE is significantly higher in AD compared to healthy subjects (82, 83). In addition, some researchers found that increased oxidative damage in neuronal cell bodies is one of the earliest changes, which HNE-adducts to neurofilament has been found, it is the amyloid β-peptide that would induce oxidative stress and the consequent lipoperoxidation. High levels of HNE have been detected in Aβ plaques and in the cerebrospinal fluid in Alzheimer's patients (83). Therefore, these results suggest that HNE can impair key areas in the brain, leading to neuronal cell death and can be identification of markers of neurodegenerative disease including AD.

## 2.3 Dehydroepiandrosterone (DHEA)

Dehydroepiandrosterone (DHEA) is the most abundant circulating hormones in humans. These molecules are synthesized in the adrenal glands and the gonads. The adult adrenal glands have a combined weight of 8-10 grams. The inner medulla stands for 10 % of its weight and the cortex 90 % (84). The adrenal cortex consists of three layers, or zones. DHEA is produced in the inner zona reticularis. However, they can also be produced in the brain and in the peripheral nerves, either by metabolism of circulating hormones or by *de novo* synthesis from cholesterol. It

should also be mentioned that DHEA is also synthesized in the brain independent of supply from the adrenal cortex and the gonads and the concentration of DHEA in CNS is approximately 6-8 times higher than serum concentrations (85-87). Due to the latter fact, they have been named "neurosteroids". In the central nervous system, neurosteroids act via classic intracellular receptor- mediated mechanisms that regulate the transcription of specific genes. In addition to classic genomic steroidal actions, neurosteroids may modulate brain functions, acting as allosteric modulators of ion-gated and other neurotransmitter receptors.

### 2.3.1 Regulation of DHEA synthesis

As mentioned earlier in the introduction, production of DHEA and DHEA-S is regulated by Adrenocorticotropic hormone (ACTH) (88). When ACTH reaches the adrenal cortex through the blood stream, it binds to cell-surface receptors of the adrenal cortex. ACTH then stimulates an increase of the steroidogenic acute regulatory protein (StAR), which carries cholesterol from the outer to the inner mitochondrial membranes (89). Here, the cholesterol is converted to pregnenolone by the cholesterol side chain cleavage enzyme (CYP11A1). Pregnenolone is then further converted in the adrenocortical cells in several steps into different adrenocortical hormones depending on the enzymatic levels within the cells (90, 91). The unique enzymatic phenotype of the zona reticularis cells is a high expression of 17α-hydroxylase/17,20 lyase (CYP17A1), cytochrome b5 (CYB5), DHEA sulfotransferase (SULT2A1), and absence of 3-β-hydroxysteroid dehydrogenase (3β-HSD). This enzyme pattern gives the cells the ability to produce large quantities of DHEA and DHEA-S. CYB5 stimulates the 17,20 lyase activity of CYP17A1and thereby the formation of DHEA and DHEA-S, by leading the precursors into that pathway. The molecular difference between DHEA and DHEA-S is that DHEA-S has a sulphate molecule attached. SULT2A1 converts DHEA to DHEA-S and steroid sulfatase (STS) can convert DHEA-S back to DHEA (92-96). In zona fasciculata cells, there is a high expression of CYP17A1 and abundant 3β-HSD, but absence of CYB5 and SULT2A1, which gives the zona fasciculata cells the ability to produce glucocorticoids. This process of steroid genesis is rapid: within a few minutes from the release of ACTH from the pituitary DHEA, DHEA-S and cortisol are produced. While regulation of cortisol has a

negative feedback function, regulation of DHEA and DHEA-S does not. It should be mentioned that additional hormones have been suggested to function as regulators of DHEA and DHEA-S production (97-99) as shown in Figure 2.7.

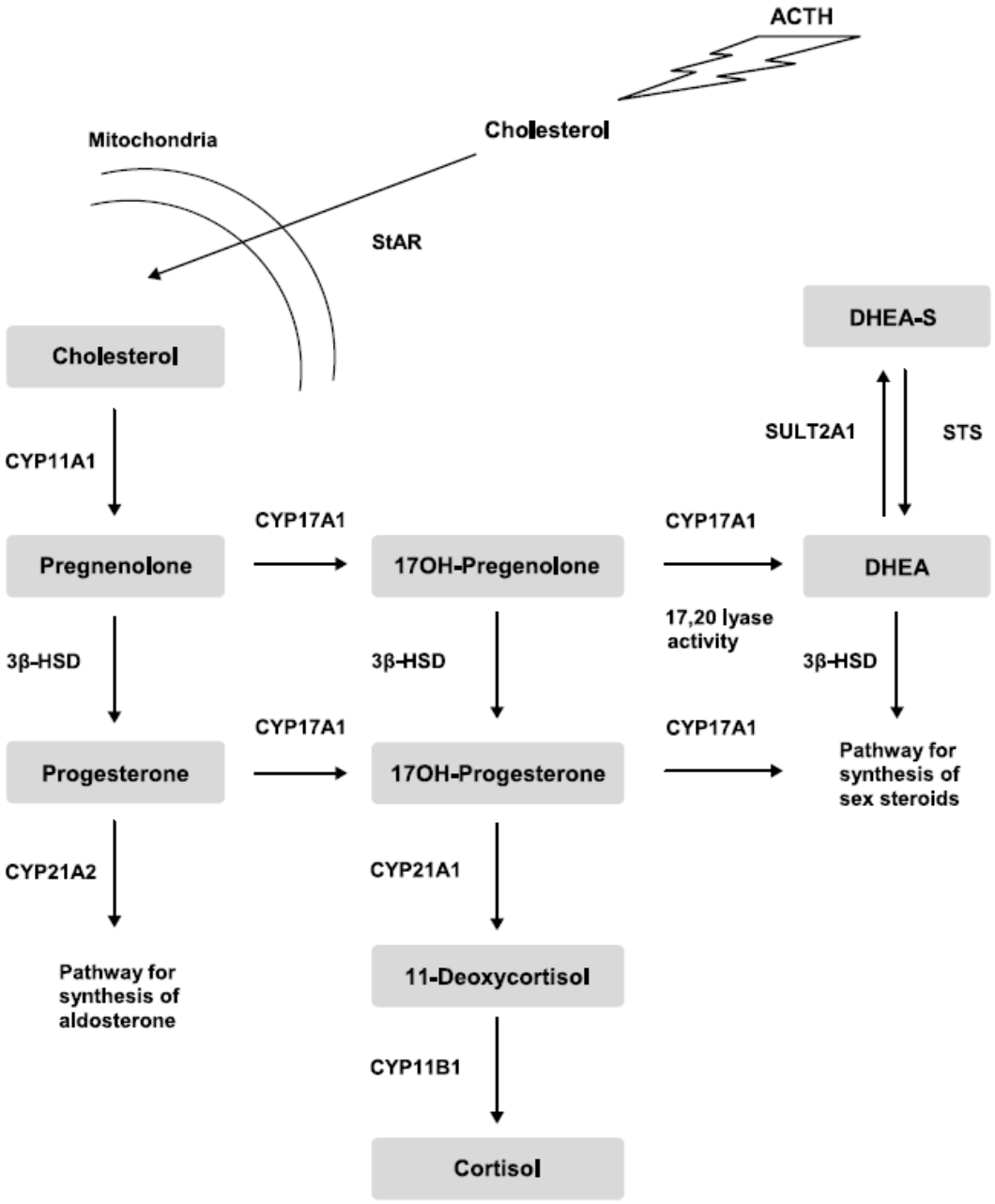



**Figure 2.7** Overview of the different steps in the biosynthesis of DHEA, DHEA-S (89). ACTH stimulates an increase of the steroidogenic acute regulatory protein (StAR), which in turn carries cholesterol from the outer to the inner mitochondrial membranes. Cholesterol is then converted in several steps into different hormones

depending on the enzyme levels within the cells. Enzymes are indicated by above and aside arrows.

However, they can also be synthesized *de novo* in the central or peripheral nervous systems and might accumulate in those structures independently of adrenal and gonadal sources. The term "neurosteroids" has been used to designate steroids directly synthesized from cholesterol in the nervous systems as shown in Figure 2.8.

Neuosteroidogenic enzymes include cytochrome P450 and non-P450 enzymes. The former is found mainly in mitochondria or microsomes. The synthesis of steroids and neurosteroids depends upon the tissue-specific and cell-specific synthesis of a particular array of steroidogenic enzymes. The central nervous system is no exception because steroidogenic enzyme ate produced both in glia and in neurons. In addition. Multiple glial cell types, such as astrobytes and oligodendrocytes, can differentially express some of these enzymes (100).

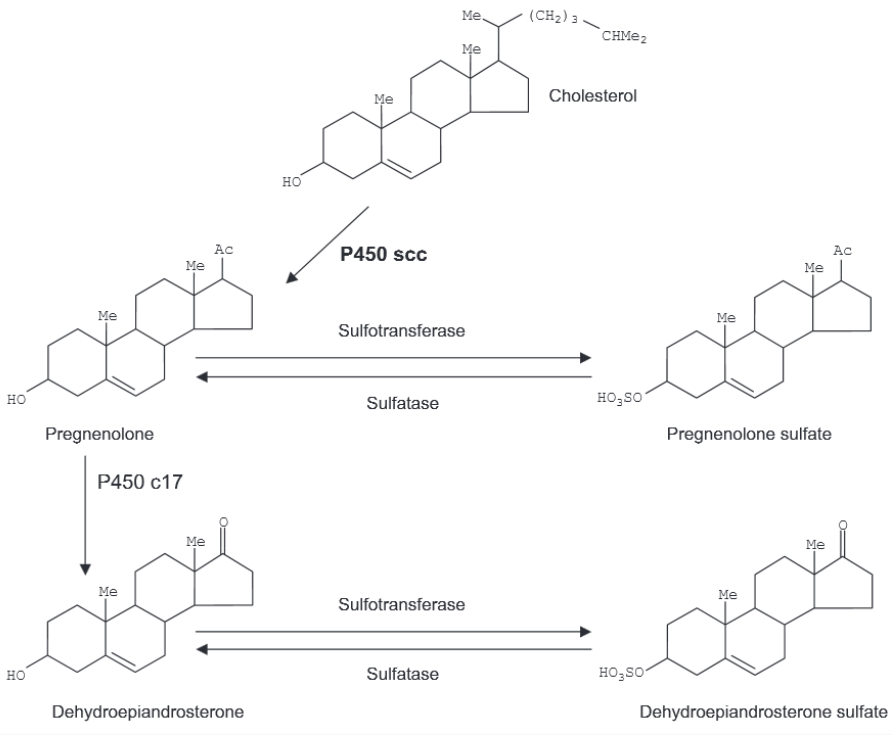



**Figure 2.8** Biosynthetic pathway of DHEA and DHEA-S. The enzymes involved are the cholesterol side chain cleaving cytochrome P450scc (P450scc or CYP11A1), the 17 α-hydroxylase 17,20 lyase cytochrome P450c17 (P450c17 or CYP17A1),

hydroxysteroid sulfotransferase (sulfotransferase) and steroid sulfatase (sulfatase) (100).

### 2.3.2 Functions of DHEA

DHEA serves as a precursor to the sex steroids testosterone and estradiol. Estradiol and testosterone have, besides their functions in reproduction, anabolic functions, and DHEA levels therefore indirectly affect these functions. Since no specific DHEA receptor had been found until quite recently, it was believed that DHEA functioned exclusively as a precursor, and thus, its effects were meditated only through conversion to the sex steroids, which in turn mediate their effects by binding to their specific receptors. Recent research has shown that in addition to mediating their effects via transformation into androgens and estrogens, DHEA are active hormones with effects on their own and that DHEA mediates its action via several nuclear and cell surface receptors (101). DHEA has pleiotropic beneficial effects. DHEA has anti-glucocorticoid effects. For example, DHEA protects against the glucocorticoid-induced involution of the thymus (102) and the neurotoxic effects of corticosterone (1033). This is partly due to the antioxidative properties of DHEA that protect the cells from oxidative stress. In previous studies, DHEA is shown to protect the brain cells against oxidative stress and reduce serum lipid perioxidation (104-106). DHEA are not antioxidants but enhances the activity of antioxidant enzymes. DHEA has been shown to be immune-enhancing and anti-inflammatory properties (107, 108). Some researched, DHEA has been reported to increase the number of monocytes and NK cells and to inhibit the production of pro-inflammatory cytokines such as interleukin-6 (IL-6) and the tumor necrosis factor (TNF-α) (108, 109). DHEA positively modulates endothelial function, partly by stimulating the production of nitric oxide (NO) in the endothelial cells, which in turn have several beneficial effects on the cardiovascular system. DHEA plays an important role in the regeneration of tissues in the body. For example, DHEA accelerates healing of wounds in the skin. DHEA seems to also have beneficial effects on mood (110, 111). However, the functions of DHEA are protective, regenerative and important for the maintenance and restoration of health. Therefore, DHEA levels could be used as markers of regenerative and protective (anabolic) activity.

### 2.3.3 Relationship between DHEA and diseases

Dehydroepiandrosterone (DHEA) is the most common hormone in the body. It is also found in large quantities in the brain (87, 172). DHEA levels decrease in the blood and the brain with age and are thought by many to be associated with many of the symptoms of aging. The precise role of DHEA is unknown other than its role as a source for other steroid hormones in the body. In recent years, some studies have demonstrated an association between decreasing levels of DHEA and the development of age-related conditions such as arthritis, heart disease, diabetes, and obesity. Previous studies suggest DHEA can improve memory and enhance cognitive function in elderly persons with cognitive problems. A case-control study found that a group of 14 persons with Alzheimer's disease had significantly lower levels of DHEA sulfate in the plasma compared to 13 matched healthy controls (112). A study of 52 patients with Alzheimer's found that those with higher plasma DHEA sulfate levels scored higher on a variety of cognitive tests than those with lower DHEA sulfate level (113). DHEA has also been found to have the ability to protect cells from oxidative damage to the hippocampus part of the brain. This is among the regions of the brain most affected by Alzheimer's disease. A randomized, double-blind, placebo-controlled study found that three months of therapy with DHEA improved cognition compared to placebo (114).

## 2.4 Antioxidants

Oxygen is a highly reactive molecule that can damage cells by producing ROS. (115) Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent, which starts chain reactions that damage cells. So, cells need to contain an antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such protein, DNA and lipid (116, 117). Antioxidants are thought to protect the body against the destructive effects of free radicals. Antioxidants neutralize free radicals by donating one of their own electrons, ending the electron- chain reaction. The antioxidant nutrients themselves don't become free radicals by donating an electron because they are stable in either form. They act as scavengers, helping to prevent cell and tissue damage that could lead to

cellular damage and disease. Low levels of antioxidant inhibition of the antioxidant enzymes have been caused oxidative stress and then damage cells.

Antioxidants are classified into two groups, depending on solubility in lipids (hydrophobic) and water (hydrophilic). From studied, water-soluble antioxidants react with oxidants in cell cytosol and blood plasma, while the other group protect cell membranes from lipid peroxidantion (116). These compounds may be obtained from food or synthesized in the body (117).

### 2.4.1 Mechanism of antioxidant

Defense mechanisms against free radicals induced oxidative stress such as repair mechanisms, preventative mechanisms, physical defensed and antioxidant defense had composed of non-enzymatic and enzymatic antioxidant. Enzymatic antioxidants are enzymes which are synthesized in the body such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) (118). Non-enzymatic antioxidants are glutathione (GSH), ascorbic acid, α-tocopherol (vitamin E), carotenoids, polyphenols, flavonoids and other antioxidants. Vitamin C has ability to reduce the reaction of ferric to ferrous, the catalyst of the Fenton reaction. It can remove hypochlorous acid (HOCl) by oxidation and is converted to dehydroascorbic acid, which is an unstable molecule and easily broken down to threonic acid and oxalic. Glutathione has an important role in protection against free radical damage which involves is several enzymatic pathways as shown in Figure 2.9. The reaction of dehydroascorbate reductase enzyme and glutathione can convert dehydroascorbate acid to ascorbic acid (19). Glutahione then has an important role in protection against free radical which involves in several enzymatic pathways.

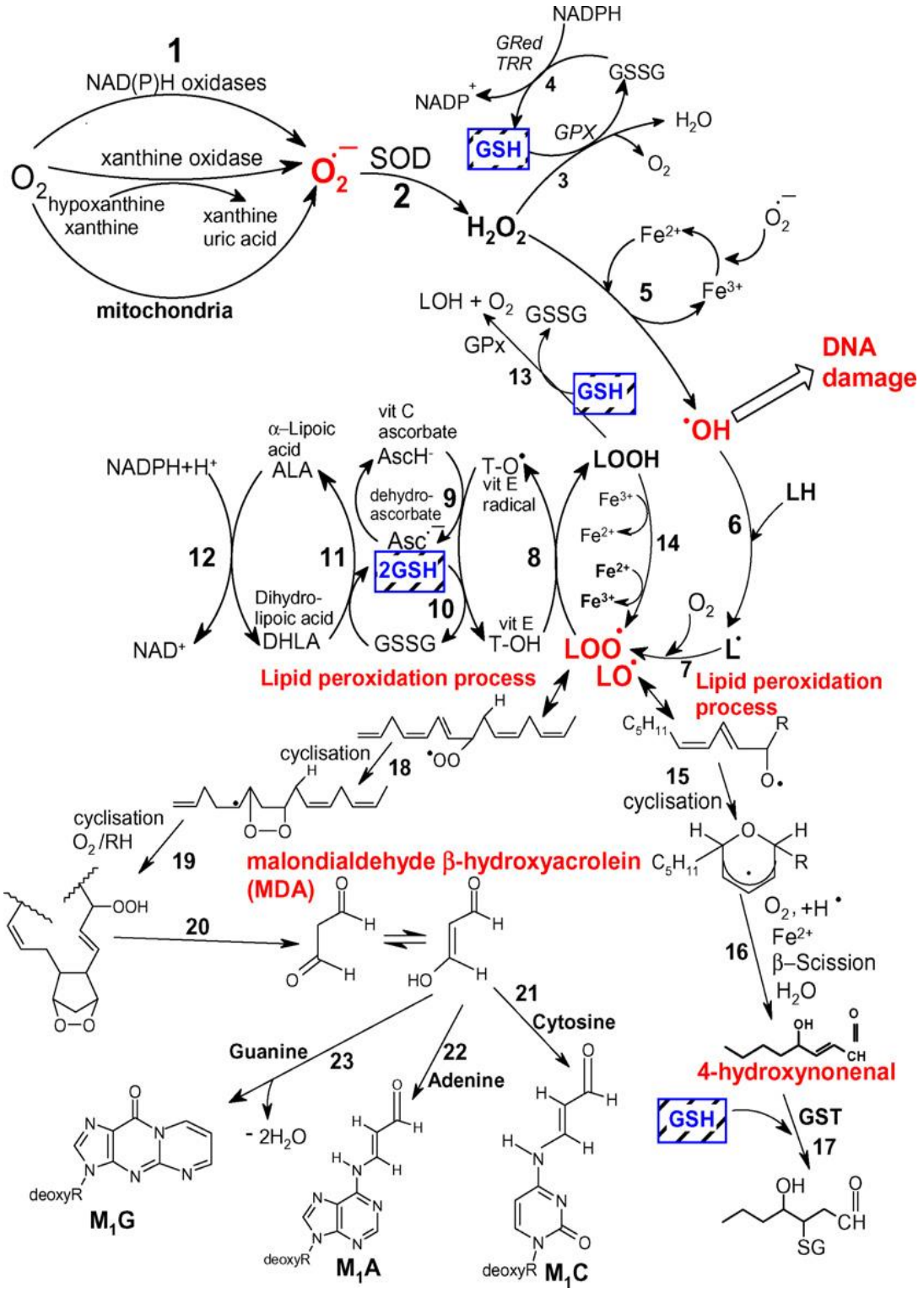



**Figure 2.9** Pathway of ROS formation, lipid peroxidation process and role of glutathione (GSH) including other antioxidants (Vitamin C, Vitamin E, lipoic acid) in prevention of oxidative stress (9).

Reaction 1: The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidase and xanthine oxidase or non-enzymatically by redox-reactive compounds such as semi-ubiquinone compound of the mitochondrial electron transport chain.

Reaction 2: Superoxide radicals are dismutated by superoxide dismutase (SOD), is isoenzyme containing copper iron or manganese (CuSOD, FeSOD or MgSOD) to hydrogen peroxide ($H_2O_2$).

Reaction 3: Hydrogen peroxide is the most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which GSH is the electron donor.

Reaction 4 : The oxidized glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred) which uses NADPH as the electron donor.

Reaction 5: Hydrogen peroxide ($H_2O_2$) are breakdown to the reactive hydroxyl radicals ($^{\bullet}OH$) by some transition metals such as $Cu^{+}$, $Fe^{2+}$ and others (Fenton reaction).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

Reaction 6: The hydroxyl radicals can abstract an electron from polyunsatulated fatty acid (LH) to give rise to a carbon-centred lipid radical ($L^{\bullet}$).

Reaction 7: The Lipid radical ($L^{\bullet}$) can further interact with molecular oxygen to give a lipid peroxyl radical ($LOO^{\bullet}$) If the resulting $LOO^{\bullet}$ is not reduced by antioxidants, the lipid peroxidantion process will occur (reactions 18-23 and 15-17).

Reaction 8: Llipid peroxyl radical ($LOO^{\bullet}$) is reduced within the membrane by the reduced form of vitamin E (T-OH) resulting in the formation of a lipid hydroperoxide and a radical of vitamin E.

Reaction 9: The regeneration of Vitamin E by Vitamin C: the Vitamin E radical ($T\text{-}O^{\bullet}$) is reduced back to Vitamin E (T-OH) by ascorbic acid leaving behind the ascorbyl radical ($Asc^{\bullet -}$).

Reaction 10: The regeneration of Vitamin E by GSH: the oxidized Vitamin E radical ($T\text{-}O^{\bullet}$) is reduced by GSH.

Reaction 11: The oxidized glutathione (GSSG) and the ascorbly radical ($Asc^{\bullet-}$) are reduced back to GSH and ascorbate monoanion, $AscH^{-}$, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to α–lipoic acid (ALA).

Reaction 12: The regeneration of DHLA from ALA using NADPH.

Reaction 13: Lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor (Lipid peroxidantion process).

Reaction 14: Lipid hydroperoxides can react fast with $Fe^{2+}$ to form lipid alkoxyl radical ($LO^{\bullet}$), or much slower with $Fe^{3+}$ to form lipid peroxyl radical ($LOO^{\bullet}$).

Reaction 15: Lipid alkoxyl radical ($LO^{\bullet}$) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring gydroperoxide.

Reaction 16: Six-membered ring gydroperoxide undergoes further reaction (involving β–scission) to form 4-hydroxynonenal.

Reaction 17: 4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST, glutathione *S*-transferase).

Reaction 18: A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce to produce cyclic peroxide adjacent to a carbon-centred radical.

Reaction 19: This radical can either reduce to form a hydroperoxide or it can undergo a second cyclisation to form abicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide.

Reaction 20: Formed compound is an intermediate product for the production of malondialdehyde.

Reaction 21, 22, 23: Malondialdehyde can react with DNA bases cytosine, Adenine and Guanine to form adducts M1A, M1C and M1G respectively.

ROS can be generated as a result of normal metabolic process. It is clear that they are key participants in injury caused to cells, being capable of oxidative damage to all of the major classes of macromolecules, nucleic acid, proteins, carbohydrates and lipid. Under normal conditions, antioxidant systems minimize the adverse effects caused by ROS (Fig. 2.9). However, when ROS overwhelm the biological defenses of the cell, the result is oxidative stress. The function of the antioxidant defense system is defective in case of antioxidant enzyme dysfunctions. This has been associated with several neurodegenerative disorder such as Alzheimer's disease, Parkinson's disease (119, 120).

### 2.4.2 Nutritional therapy with natural antioxidants

From studied, they found that plants and fruits or vegetables are recognized to contain a wide variety of free radical scavenging molecules such as phenolic compounds (phenolic acid, flavonoids, quinons, tannins etc.), vitamin and some other endogenous metabolites, which are rich in antioxidant activity, anti-inflammatory, anti-tumorigenic, anti-allergic, and anti-proliferative (121-124). Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the brain. Many researches have been found that oxidative stress was increased and the other hand total antioxidant capacity of the plasma was decreased in AD patients. Nowadays, a need for identifying alternative and search for natural antioxidants has increased especially herbal traditional medicine. Such as *Curcuma longa* (curcumin) (125, 126), *Ginkgo biloba* (ginkgo) (127), *Camellia sinensis* (green tea) (128) and *Garcinia mangostana* (mangosteen) Previous studies presented that they have important for a traditional medicine and have various activities in preventing disease such as immune functions, anti-inflammation, antioxidant and anti-tumor activity (9, 13, 14). Recently, some studies presented that there active compounds have beneficial effects on the CNS and neurodegeneration, which to prevent cytotoxicity in neuronal of animal study (129).

## 2.5 Mangosteen

Mangosteen (*Garcinia mangostana* L.) known as "The Queen of Fruits". The mangosteen is a tropical evergreen tree in the family Clusiaceae. It can be found in Southeast Asia, Brazil, North America, central America, Hawaii, Southern India and other tropical countries (130), which it is much cultivated in Thailand, which produce fruits from April to June, which fruits are available from June to September. It is one of the most attractive tropical fruits: it has the remarkably pleasant flavor. It also contains many magnificent therapeutic benefits. The tree is slow to grow but can grow from 6-25 m in height. Leaves ate simple opposite, elliptic-oblong or ovate, 4.5-10 cm wide, 15-25 cm long, glabrous leaves with dark green above and dull plate green beneath. Flowers are solitary or dichasium, axillary in uppermost leaf-axil. The color of petals is green with red spots on the outside and yellowish-green. The peel of mangosteen is about 6-10 mm thick. The hard skin of peel of mangosteen is deep reddish purple. The circle of wedge shape arils contains 5 - 8 segments. The segment eatable meat is soft, white, juicy. The taste of aril is slightly acid and mild to distinctly acid in flavor. The mangosteen is usually composed of one to five seeds or sometimes seedless. The seed have ovoid-oblong shape.

**Figure 2.10** Mangosteen Fruits. (http://www.healthyfig.com/purple-mangosteen)

### 2.5.1 Traditional medical use

The pericarp of mangosteen-fruit has been used as a medicinal agent by Southeast Asians for centuries in the treatment of skin infections and wounds, amoebic dysentery in Table 2.2. In Ayurveda medicine the pericarp of mangosteen-fruit has wide use against free radical, inflammation, cancer and diarrhea (9, 131-133).

**Table 2.2** Traditional medicinal properties of mangosteen.

| **Illness** | **References** |
|---|---|
| Acne | Chomnawang et al. J. Ethnopharmacol 2005. |
| Abdominal pain | Moongkarndi et al. J. Ethnopharmacol 2004. |
| Cholera | Sen et al. Indian J. Chem 1980. |
| Dysentry | Yates and Stout J. Am. Chem 1958. |
| Diarrhea | Pedraza-Chaverri et al. Food Chem Toxicol 2008. |
| Fever | Yates and Stout J. Am. Chem 1958. |
| Gonorrhea, cystitis and urethra suppuration | Moongkarndi et al. J. Ethnopharmacol 2004. |
| Inflammation | Chairungsrilerd et al. Br. J.Pharmacol 1996. |
| Leucorrhoea | Moongkarndi et al. J. Ethnopharmacol 2004. |
| Skin infectons | Suksamrarn et al. J. Nat. Prod 2006. |
| Suppuration | Moongkarndi et al. J. Ethnopharmacol 2004. |
| Tuberculosis | Suksamrarn et al. J. Nat. Prod 2006. |
| Wounds | Mahabusarakam et al. J. Nat. Prod 1987. |

### 2.5.2 Chemical constituents

Previous research found that mangosteen contains a variety of phytochemical such as mangostin, tannin, xanthone, isoflavone, α-mangostin, β-mangostin in Table 2.3, which the xanthone derivatives are major compound as shown in Figure 2.10 and 2.11. There are more than 60 derivatives of xanthone, found in different parts of mangosteen including heartwood, bark, seeds, leaves, stem and especially in “pericarp” or “fruit rind”. The pericarp is source of xanthones, anthocyanins, various vitamins and other substances. In previous studies have revealed that xanthone frome fruit hull exhibits antibacterial, antifungal, antitumor, anticancer, cytotoxic activity, anti-inflammatory and antioxidant.

**Table 2.3** List of pure constituents purified from mangosteen.

| **Pure compound** | **Reference** |
|---|---|
| α-Mangostin | Parveen and Ud-Din Khan. Phytochemistry 1988. |
| β-Mangostin | Jefferson et al. J chem 1970 |
| γ-Mangostin | Balasubramanian and Ragagopalan. Phytochemistry 1988. |
| 3-Isomangostin | Gopalakrisnan et al. J Nat Prod 1997. |
| Di-*O*-acetylmangostin | Jefferson et al. J chem 1970 |
| 6-Deoxy-7-demethyl mangostanin | Chin et al . Phytochemistry 2007. |
| Mangostanin | Nilar and Harrison. Phytochemistry 2002. |
| Mangostanol | Chairungsrilerd et al. Phytochemistry 1996. |
| Mangostinone | Matsumoto et al. J Nat Prod 2003. |
| Garcinone A | Sen et al. Indian J Chem 1980 |
| Garcinone B | Suksamrarn et al. Chem Pharm Bull 2003. |
| Garcinone C | Sen et al. Indian J Chem 1980 |
| Garcinone D | Sen et al. Indian J Chem 1986. |
| Garcinone E | Ho et al. Planta Med 2002. |
| Gartanin | Govindachari et al. Tetrahedron 2005. |
| 8-Deoxygartanin | Walker. J Sep Sci 2007. |
| Dulxanthone D | Nguyen et al. Phytochemistry 2005. |
| Mangoxanthone | Nguyen et al. Phytochemistry 2005. |
| 9-Hydroxycalabaxanthone | Ji et al. Pharm Biomed Anal 2007. |
| Coumaric acid | Bunsiri et al. Postharvest Biol Technol 2003. |
| Sinapic acid | Bunsiri et al. Postharvest Biol Technol 2003. |
| 3-Ethyl-3-methyleimide *N*-β-D-glucopyranoside | Krajewski et al. Phytochemistry 1996. |
| Proanthocyanidin | Fu et al. J Agric Food Chem 2007. |
| 3'-6-Dihydroxy-2,4,4'-trimethoxybenzophenone | Nguyen et al. Phytochemistry 2005. |
| D-Galacturonic acid | Chanarat et al. J Med Assoc Thai 1997. |
| L-Rhamnose | Chanarat et al. J Med Assoc Thai 1997. |
| D-Galatose | Chanarat et al. J Med Assoc Thai 1997. |
| Garcimangosone A | Huang et al. J. Nat. Prod 2001. |
| Garcimangosone C | Huang et al. J. Nat. Prod 2001. |
| Garcimangosone D | Huang et al. J. Nat. Prod 2001. |
| Tovophyllin A | Jung et al. J. Agric. Food 2006. |
| Tovophyllin B | Suksamrarn et al. Chem. Pharm. Bull 2003. |

### 2.5.3 Antioxidant properties of mangosteen

Crude extracts and α-, γ-mangostin show antioxidant activity from Reactive Oxygen Species (ROS) production of mangosteen pericarp (9, 159-161). Some researcher studied the antioxidant and neuro protective properties of extract mangosteen pericarp. The antioxidant capacity was evaluated by the DPPH method using showed high antioxidant capacity. The antioxidant capacity was tested on neuroblastoma cell line (NG108-15) induced by hydrogen peroxide ($H_2O_2$); both extracts exhibited neuroprotective activity when they used concentration of 50 µg/mL, showed that mangosteen extract possesses a significant antioxidant activity. α-mangostin decease the human low density lipoproteins (LDL) oxidation induced by copper or peroxyl radicals (162). Various extracts (water, ethanol, ethyl acetate, methanol) showed highest antioxidant capacity and neuroprotective activity in neuroblastoma cell line (14, 129, 162).

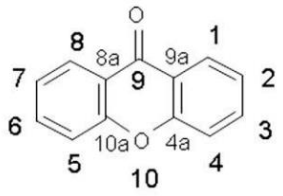
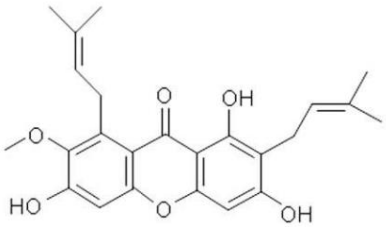
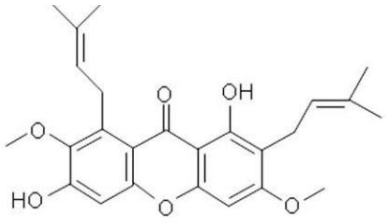


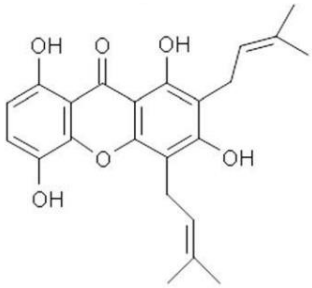

**Figure 2.11** Chemical structure of xanthone nucleus (160).



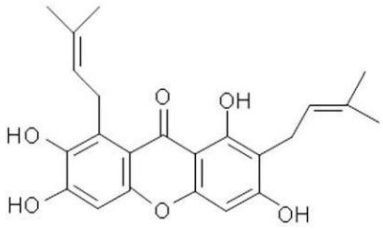
α-mangostin



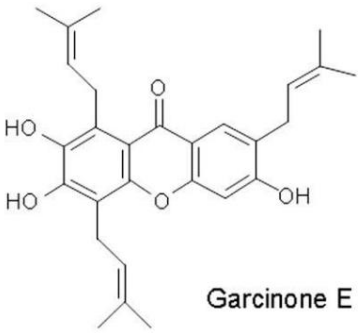
β-mangostin



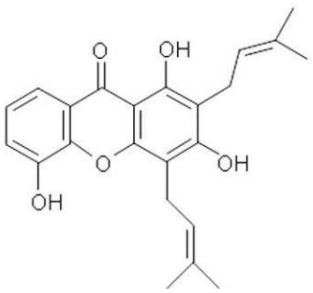
Gartanine



γ-mangostin




Garcinone E



8-deoxygartanine

**Figure 2.12** Chemical structures of xanthone compounds isolated from pericarp of mangosteen (160).

### 2.5.4 Crude Water Extract

Mangosteen pericarp contain various substances. The crude water extract is water-soluble derived from the extraction process of methanol followed by ethyl acetate and water. This crude water extract contains polyphenolic compound and α-mangostin is less than 2%. This extract proposed antioxidant and neuroprotective activities against oxidative stress in NG108-15 cells induce by $H_2O_2$ and against Aβ induced oxidative stress in SK-N-SH cell (14). In previous study, *in vivo*, it could safely protect memory loss in mice treated with scopolamine (129). According to these interesting activity, in this experiment, crude water extract have been selected to test antioxidant activity in healthy volunteers.

# CHAPTER III
# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Cell line

SK-N-SH human neuroblastoma cell line was obtained from American Type Culture Collection (ATCC HTB-11) which was isolated from bone marrow of 4 years old female patient with a neuroblastoma tumor.

### 3.1.2 Culture media

| Name | Catalog No. | Company |
|---|---|---|
| Fetal bovine serum | S0115 | Biochrom |
| L-glutamine | K0282 | Biochrom |
| MEM medium | 61100-053 | Invitrogen |
| Non essential amino acid | K0293 | Biochrom |
| Pennicillin G | M7480 | M&H |
| Sodium pyruvate | P5280 | Sigma |
| Sodium bicarbonate | 6323 | Merck |
| Streptomycin | M7185 | M&H |
| Trypsin | L2133 | Bichrom |

### 3.1.3 Chemicals and solutions

#### 3.1.3.1 Chemical reagents for cell viability by MTT assay

| Name | Catalog No. | Company |
|---|---|---|
| Isopropanal | UN1219 | BDH |
| MTT | M5655 | Sigma |

#### 3.1.3.2 Chemical reagents for DPPH$^{\bullet}$ scavenging activity

| Name | Catalog No. | Company |
|---|---|---|
| DPPH | 300267 | Millipore |
| Ascorbic acid | 100468 | Millipore |

#### 3.1.3.3 Partially purified crude water extract

Partially purified crude water extract were extracted from the dried fruit rind of mangosteen, obtain from Immunology Laboratory, Department of Microbiology, Mahidol University.

#### 3.1.3.4 Reactive oxygen species detection by Flow cytometry

| Name | Catalog No. | Company |
|---|---|---|
| DCFH-DA | D6883 | Sigma-Aldrich |
| Hydrogen peroxide | 107298 | Merck |

#### 3.1.3.5 DHEA determination by ELISA method

| Name | Catalog No. | Company |
|---|---|---|
| DHEA ELISA | IB79120 | IBL AMERICA |

#### 3.1.3.6 Hydroxynonenal detection by Dot Blot analysis

| Name | Catalog No. | Company |
|---|---|---|
| Anti-HNE Fluorophore Rabbit pAb | 393206 | Calbiochem |
| Anti-beta Actin, Rabbit Monoclonal | 04-116 | Millipore |
| Super Signal West Pico Chemiluminescent Substrate | 34079 | Thermo scientific |
| Goat Anti-Rabbit IgG, HRP conjugate | 12-348 | Millipore |
| Bio-Rad Protein Assay | 500-0006 | Bio-Rad |
| Skimmed milk powder | - | Anlene |
| Tween-20 | 20605 | USB |

### 3.1.4 Equipments and instruments

| Name | Model | Company |
|---|---|---|
| **96-well plate** | **167008** | **Nunc** |
| Autoclave | SS-325 | Tomy Seiko |
| Automated pipette | - | Labmate |
| Beaker | 500, 1000 ml | Pyrex |
| Biohazard laminar air flow | CYTAIR | Flufrance |
| Centrifuge | EBA12 | Hettich |
| Centrifuge | Mikro 22R | Hettich |
| Centrifuge tube | 15 ml | Nunc |
| $CO_2$ incubator | BB 15 | Heraeus |
| Flow cytometer | FACSCalibur | Becton, Dickinson |
| Hot air oven | UNE 600 | Memmert |
| Hemocyrometer | 717805 | Brand |
| Inverted microscope | Eclipse TS100 | Nikon |
| Incubator | BD 240 | Binder |
| Microplate reader | Synergy HT | Bio-Tek |
| Microplate reader (UV) | Infinite M200 | Tecan |
| Milli-Q | PF Plus | Millipore |
| Nitrocellulose membrane | 88018 | Thermo scientific |
| pH-meter | Basic | Gibthai |
| Pipette boy | 0301 | High Tech Lab |
| Volumetric flask | 1000 ml | Schott |
| Vortex mixer | Vortex-2 Genie | ScientificIndustries |
| Moisture Analyzer | MA 45 | Sartorius |

## 3.2Methods

### 3.2.1 Recruitment of healthy volunteers

Participants were recruited from healthy volunteers aged 20-60 years old with no history or evidence of cancers and neurological/ cardiac/ psychiatric/ renal/ hepatic diseases.

### 3.2.2 Administration of crude water extract of mangosteen (CWM)

The CWM was prepared in capsules, which were orally taken in the morning. Volunteers who weigh less than 55 kg and more than 55 kg, were given daily dose of CWM at 220 mg and 280 mg, respectively for 3 months consecutively. After that, they were taken the double doses of CWM daily for another 3 months. Blood samples of all subjects were collected at days 0, 7, 21, 84, 112 and 168. Biochemical markers as well as physical examination of each volunteer were determined to evaluate for the safety of CWM.

### 3.2.3 Preparation of crude water extract of mangosteen

Mangosteen pericarp was dried in oven at 60 °C and pulverized grinder. The powder was extracted with EtOH for 4 days. The extract was evaporated at 45 °C by a rotatory evaporator. Crude EtOH extract were partitioned with EtOAc for 2 days. The sediment was collected and dissolved in distilled water at 55-60 °C. The supernatant was collected after centrifuged at 3,790 g for 20 minutes and dried by spray dryer. The powder of CWM was filled in capsules at dose 220 in capsule size number 1 without filler. For the dose of 280 mg, CWE was filled in capsule size number 0 by adjusted with cornstarch as filler to obtain the final weight of 300 mg. The capsules were kept in aluminum foil-packaging for moisture protection**.**

### 3.2.4 Culture conditions of SK-N-SH cells

SK-N-SH cells were continuously cultured and grown in monolayer in the presences of MEM medium containing 10 % fetal bovine serum (v/v), 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, streptomycin (100 µg/ml) and penicillin G (100 units/ml) in humidified atmosphere of

5% $CO_2$ at 37 °C. Typsinization at a concentration of 0.25 % trypsin in EDTA/PBS at pH 7.4 was used to detach cells from surface. After typsinization, cell suspension was washed with MEN medium containing 10% fetal bovine serum and centrifuged at 1,800 rpm for 3 minutes to remove inactivated trypsin and other wastes. Cell pellets were resuspended with 10% MEM medium until they became single cells, then distributed evenly in culture flask. SK-N-SH cells could attach on the surface of culture flask again after an incubation about 3 to 4 h at optimum conditions. Cells were fully growth about 90-100% in 25 cm2 cell culture flask within 27-48 hours and contain about 2-3×$10^6$ cells. In each experiment, suitable number of cells were counted on hemocytometer by inverted microscope then grown in specified conditions.

### 3.2.5 Plasma isolation

Plasma was isolated from the whole blood by density gradient centrifugation. The whole blood was centrifuged at 3,000 rpm for 10 minutes at room temperature. The plasma was collected and aliquoted into microcentrifuge tube and stored at -20 °C.

### 3.2.6 Preparation sample from whole blood

Five hundred microliter of whole blood were transferred into microcentrifuge tube, added 500 µl of PBS. Microcentrifuge tube was centrifuged 800 x g for 5 minutes at 4 °C and supernatant were removed. Cells were washed 3 times with PBS and 40 µl of D.I water were added to suspended cells and stored at -80 °C.

### 3.2.7 Weight variation of content in capsules

Weight accurately 20 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by a suitable means. Weight accurately the emptied shells individually, and calculate for each capsule the net weight of its contents by subtracting the weight of the shell from the respective gross weight.

### 3.2.8 Disintegration of capsules

In each of the 6 tubes, place one capsule was placed into beaker of suitable diameter containing water maintained at 36-37 °C with the level just below the upper perforated disc. Using a pipette, adjust the level with water at 36-37 °C until a uniform film covers the perforations of the disc. Use three vaginal tablets. Place each one on the upper plate of an apparatus and cover the latter with a glass plate to maintain appropriate conditions of humidity. Examine the state of the samples after the period prescribed in the monograph. To pass the test all the samples must have disintegrated.

### 3.2.9 Loss on drying assay

This was carried out using a minimum of 2 – 5 g of material. Drying was effected in a gravity-convention oven maintained at 105-110 °C. The results are expressed as the percentages loss on drying (%LOD).

### 3.2.10 DPPH$^{\bullet}$ scavenging activity

Preliminary antioxidant activity of extract was estimated on the basis of DPPH$^{\bullet}$ scavenging effect. Extract was added to a methanolic DPPH$^{\bullet}$ solution (0.4 mM, 0.1 ml) in a 96 well plate. The reaction mixture was shaken vigorously for 30 min. The degree of purple DPPH$^{\bullet}$ decolorization to yellow DPPH indicated the scavenging capacity of extract. Absorbance of the mixture was determined at 517 nm using microplate reader and ascorbic acid as served as a positive control. Lower absorbance of the reaction mixture indicated higher free radincal-scavenging activity against DPPH$^{\bullet}$ was calculated using the following equation **:**

Scavenging activity (%) = [1 - (A1 - A2) / A0] × 100

Where A0 was the absorbance of control (DPPH• solution without extract), A1 was the absorbance of DPPH$^{\bullet}$ solution in the presence of the extract and A2 was the absorbance without DPPH$^{\bullet}$ solution**.**

### 3.2.11 MTT cell viability assay

The MTT assay is a conventional method to measure the cell viability after metabolic events leading to apoptosis or necrosis. This colorimetric assay is based on

the concept that the survival or metabolically active cells could reduce the yellow tetrazolium MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] to insoluble purple formazan crystal by succinate dehydrogenase enzyme. The absorbance is meausted at 570 nm using microplate reader.

SK-N-SH cells were seeded at a density of $2\times10^5$ cells/100 µl into each well of 96 well plate and incubated at 37 °C in 5% $CO_2$ for 24 h. Medium was removed and replaced with CWM and incubated for indicated time. After exposure, medium was discarded and incubated with MTT solution (5mg/ml, 20 µl/well) for 3 h. Isopropanol was used to dissolve formazan crystal. Survival cells were proportional to the intensity of purple formazan determined by microplate reader at 590 nm (A590 nm = Absorbance at 590 nm.

Survival cells (%) = (A590 nm treated cells / A590 nm untreated cells) × 100

### 3.2.12 Determination of cytosolic ROS

DCFH-DA fluorescent probe was served to estimate the level of cytosolic ROS. Cytoplasmic esterase could cleave DCFH-DA to H2DCF that finally converted to DCF, a fluorescent molecule, by cytosolic peroxide. SK-N-SH cells ($2\times10^5$ cells/ml) were plated in to each well and incubated for 24 h. Cells were treated with CWM for indicated time. After exposure, cells were harvested and washed once with PBS. DCFH-DA (50µM, 5µl) was added into each well and incubated for 30 min in the dark. $H_2O_2$ (600µM, 5µl) was then mixed and incubated for 30 min. The level of cytosolic ROS production was measured by spectrofluorometer with CWM and emission wavelengths at 485 and 530 nm respectively (FI1 = Fluorescence intensity).

Relative amount of cytosolic ROS (%) = (FI1 / FI0) × 100

Where FI0 was the fluorescent intensity of the negative control (cells without CWM) and FI1 was the fluorescent intensity in the presence of CWM.

### 3.2.13 Hydroxynonenal detection by Dot Blot analysis

Samples were dotted on nitrocellulose membrane at 10 µl and dried in air. The membrane was blocked the non-specific sites by soking in 5% skimmed milk for 1 h. The membrane was rinsed two times with PBS. After that, membrane was incubated with primary antibody for overnight, in the dark with flip flop shaking at 4

°C. After incubation with primary antibody, the membrane was washed 3 times with PBST. The membrane was incubated with the secondary antibody for 2 h at room temperature, washed 3 times with PBST and rinsed with PBS. The membrane was placed protein side up on a sheet of Saran Wrap. The mixed detection reagent (Western Bright ECL chemiluminescent HRP substrate) were soaked on the membrane. The membrane was wrapped up and gently smoothened out any air bubbles. Then, the wrapped blots was placed by setting the protein side up in a film cassette. A sheet of CL - exposure film was attached on top of the membrane. The cassette was closed and exposed with film for 3 minutes. Processing of the film ion and fixed solution are performed to detect the results. The observance in each sample was measured with the Image J program.

### 3.2.14 Dehydroepiandrosterone determination by ELISA method

Twenty microliter of each Standard and Samples were dispensed into appropriated wells with new disposable tips. Then, 100 µl of Enzyme Conjugate were dispensed into each well and incubated for 1 h at room temperature. The wells were rinsed 3 times with wash solution. And then, 100 µl of Substrate Solution were added into each well and incubated for 15 minutes at room temperature. After incubated, 100 µl of Stop Solution were added into each well. The absorbance of each well was measured at a wavelength of 450 ± 10 nm.

### 3.2.15 Reactive oxygen species detection by Flow cytometry

Two to three microliter of whole blood were pipetted into 9 ml of PBS, and mixed. Then, 1 ml of solution was pipetted into a 1.6 ml Eppendorf tube and 20 µl of DCFH-DA were added. Tubes were incubated at 37 °C for 15 minutes in the dark, then washed 2 times with PBS and centrifuged at 2,000 g for 5 minutes. The supernatant was removed and 1 ml of PBS was added into each tube following by 50 µl of 2 mM of $H_2O_2$. Data acquisition was performed using a FACS Calibur flow cytometer.

### 3.2.16 Statistical analysis

The data were expressed as mean ± SEM. The normal distribution data were compared by one-way ANOVA with Dunnett's posthoc test or Student's t-test. P-value $< 0.05$ was considered statistically significant. All statistical analysis was performed by SPSS software (SPSS version 17.0, Chicago, IL).

# CHAPTER IV
# RESULTS

## 4.1 Preparation of dried powder from mangosteen hulls

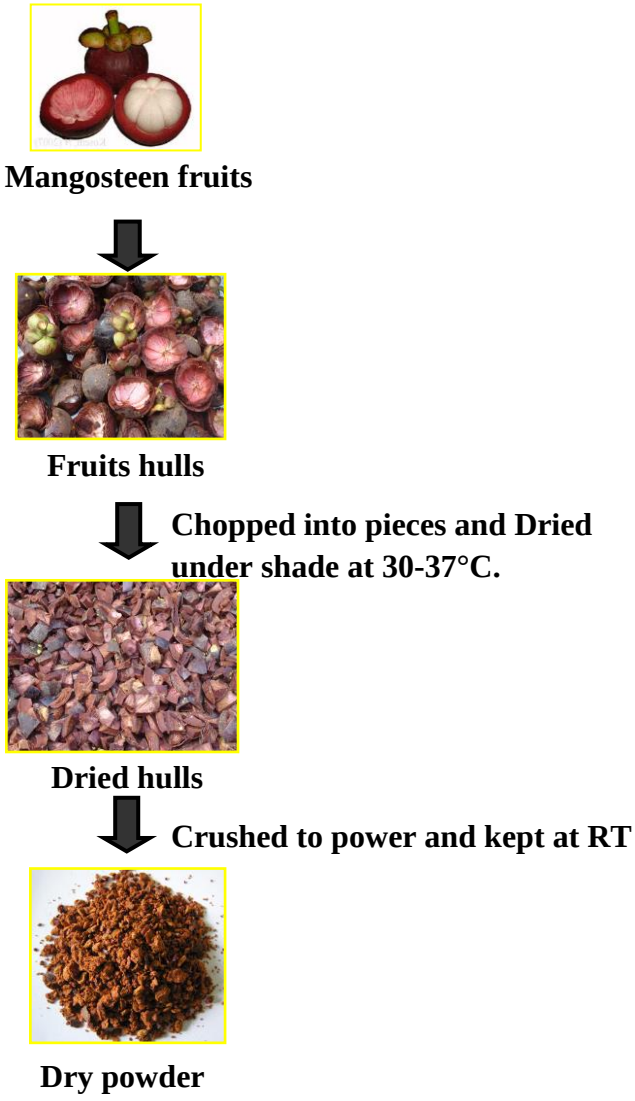



**Figure 4.1** Flow diagram of dry power preparation from mangosteen hulls.

## 4.2 Extraction of crude water extracts of mangosteen

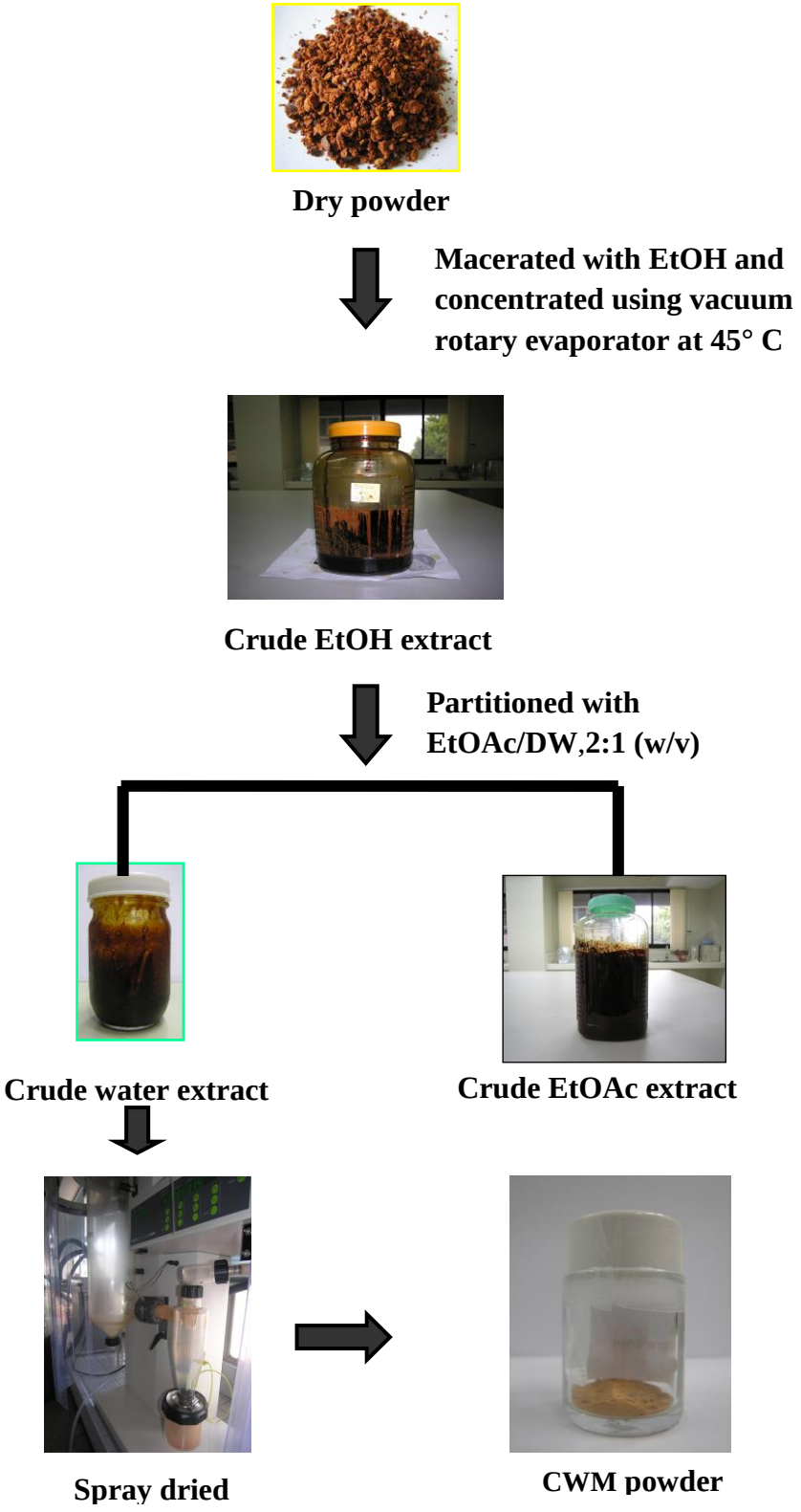



**Figure 4.2** Flow diagram of crude extract preparation.

## 4.3 Capsules preparation

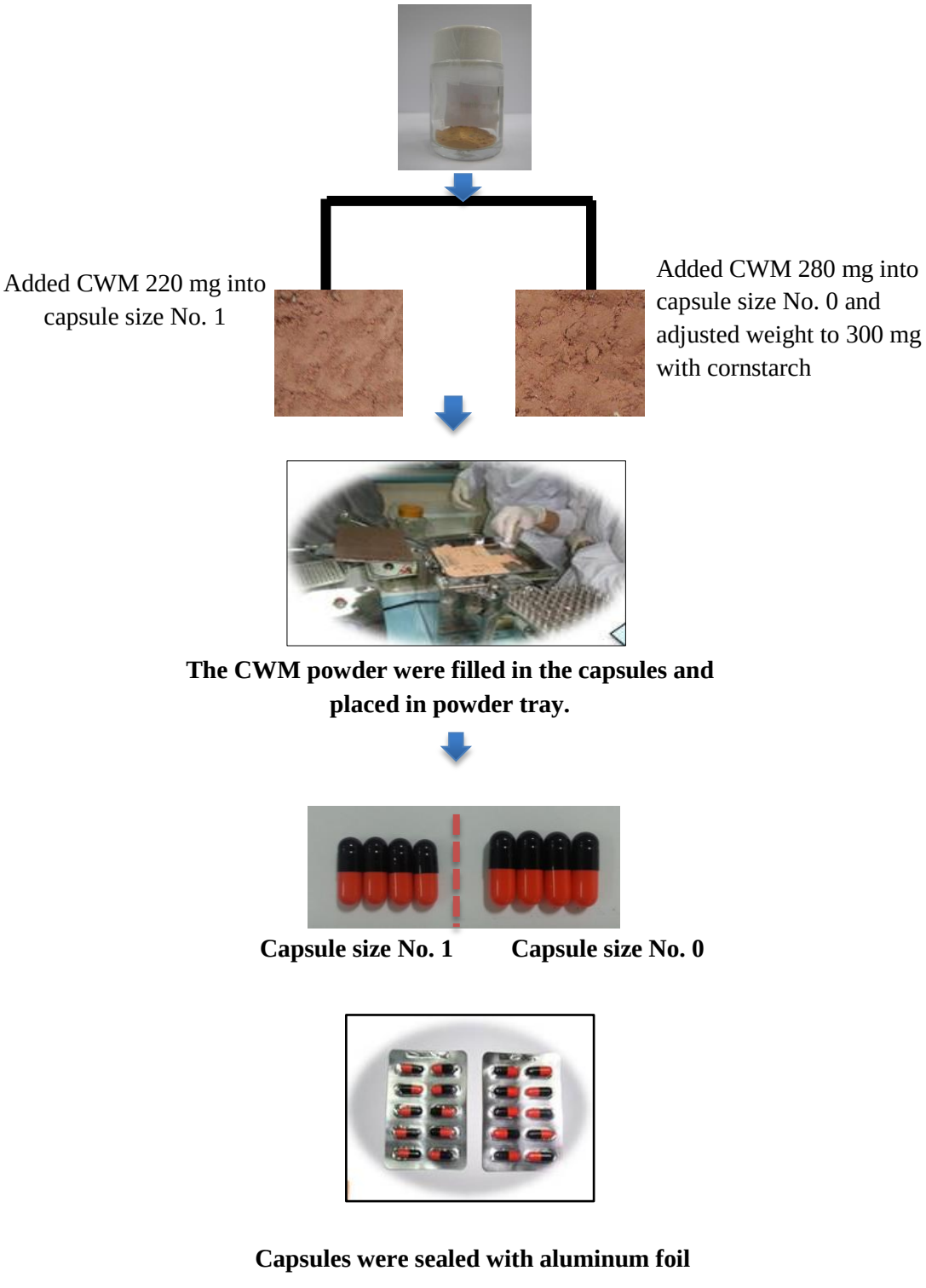



**Figure 4.3** Flow diagram of capsules preparation.

## 4.4 Quality control of capsules after production (weight variation, disintegration and Loss on drying test)

Whether capsules are produced on a small scale or large scale, they are obligatory for quality control according to methods described in British pharmacopoeia 2014, i.e., weight variation test, disintegration test and loss on drying. In brief, twenty capsules were taken at random and weighed. Their average weight was calculated then each capsule was weighed individually and noted. The results showed that the percentages deviation of weights from capsule No 1, capsule No.0 was 6.47 and 7.3 %, respectively (Table 4.1 and 4.2). In the disintegration test, capsules were tested and determined whether capsules disintegrated within the prescribed time when placed in a liquid medium in the experimental conditions. The results in Table 4.2 and Figure 4.4 showed that the capsule began to disintegrate at 2 minute and completely disintegrated at 8 minute. In loss on drying assay, two milligram of CWM was determined using a Moisture Analyzer machine as methods described in Chapter II. The results on the percentages loss on dry (%LOD) of CWM (SP, capsule No 1, capsule No. 0) were 3.4, 3.56 and 3.95 respectively (Table 4.3 and Figure 4.5).

**Table 4.1** Weights of content in each capsules after production.

| **Number** | **220mg/cap (g)** | **Total weight of CWM (g)** Weight of capsule size No. 1 (- 0.0760 g) | **280 mg/cap (g)** | **Total weight of CWM (g)** Weight of capsule size No. 0 (- 0.0980 g) |
|---|---|---|---|---|
| **1** | 0.2689 | 0.1929 | 0.4247 | 0.3267 |
| **2** | 0.3087 | 0.2327 | 0.4051 | 0.3071 |
| **3** | 0.2722 | 0.1962 | 0.4144 | 0.3164 |
| **4** | 0.2973 | 0.2213 | 0.3978 | 0.3448 |
| **5** | 0.2807 | 0.2047 | 0.3882 | 0.3912 |
| **6** | 0.2756 | 0.1996 | 0.4217 | 0.3237 |
| **7** | 0.2798 | 0.2038 | 0.4737 | 0.3757 |
| **8** | 0.2751 | 0.1991 | 0.3897 | 0.3291 |
| **9** | 0.2852 | 0.2092 | 0.4363 | 0.3383 |
| **10** | 0.3054 | 0.2294 | 0.4634 | 0.3654 |
| **11** | 0.2945 | 0.2185 | 0.4018 | 0.3038 |
| **12** | 0.2873 | 0.2113 | 0.4006 | 0.3026 |
| **13** | 0.2990 | 0.223 | 0.4356 | 0.3376 |
| **14** | 0.2729 | 0.1969 | 0.4188 | 0.3208 |
| **15** | 0.2664 | 0.1904 | 0.3652 | 0.355 |
| **16** | 0.3098 | 0.2338 | 0.4333 | 0.3353 |
| **17** | 0.2716 | 0.1956 | 0.4308 | 0.3328 |
| **18** | 0.2954 | 0.2194 | 0.4265 | 0.3285 |
| **19** | 0.2936 | 0.2176 | 0.3936 | 0.3596 |
| **20** | 0.282 | 0.206 | 0.3747 | 0.3719 |
| **Average weight of CWM** | | **0.2101** | | **0.3383** |
| **SD** | | **0.0136** | | **0.0247** |

**Table 4.2** The percentages deviation of capsule**.** The deviation limitation of content in capsule must not exceed 10% (less than 300 mg).

| **Sample** | **Percentage deviation (%)** |
|---|---|
| Capsule size No.1 | 6.47 |
| Capsule size No.0 | 7.3 |


**Figure 4.4 The relation times of disintegration of capsule,** capsule began to disintegrate at 2 minute and completely disintegrated at 8 minute. Which BP standard defined time limit of disintegration of capsule at stomach, must not excess 10 minute.

**Table 4.3** The percentages loss on drying. The percentages **loss on drying of CWM must not excess 5 %**.

| Sample | Percentage loss on drying (%LOD) | |
|---|---|---|
| | 0 month | 6 month |
| SP | 3.4 | 4.12 |
| Capsule size No.1 | 3.56 | 4.38 |
| Capsule size No.0 | 3.95 | 4.92 |

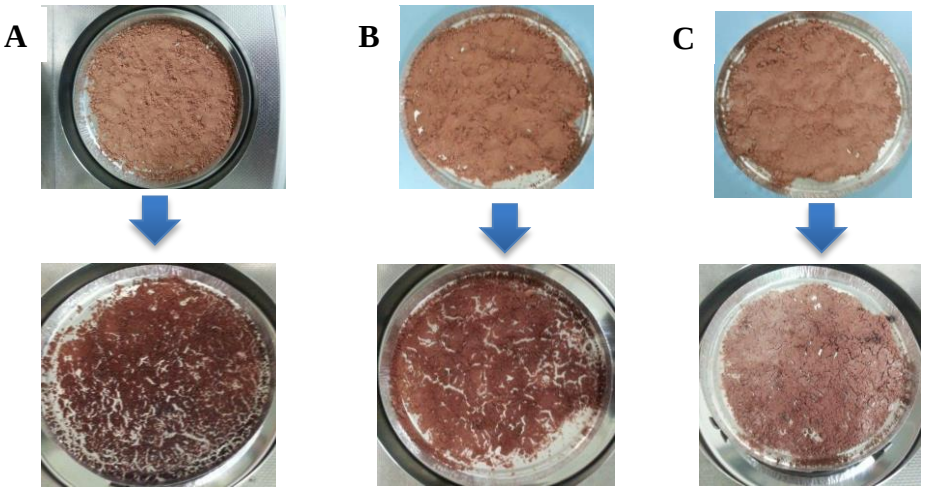



**Figure 4.5** The appearance of CWM before and after measurement loss on drying. (A) Spray dry, (B) Capsule size No. 1, (C) Capsule size No. 0.

## 4.5 The antioxidative effects of CWM against free radical by DPPH radical scavenging assay

Various concentrations of CWM were determined the antioxidation by DPPH scavenging assay. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. The results showed that, $IC_{50}$ values of the CWM, i.e., spray dry, capsule No. 1 and capsule No.0 in the first month of production) were 36.78, 43.65 and 41.49 μg/ml respectively (Table 4.4 and Figure 4.6). In the sixth month, the $IC_{50}$ values were 42.24, 47.13 and 45.72 μg/ml, respectively in Table 4.4 and Figure 4.7. Ascorbic acid, a well known antioxidant was used as positive control and $IC_{50}$ value was obtained at 11.152 and 13.81 μg/ml (Table 4.4, Figure 4.6 D and Figure 4.7 D). The $IC_{50}$ values of the CWM at both month showed that there was different in potency antioxidant activity as showed in Table 4.4.

**Table 4.4** $IC_{50}$ values from antioxidant activities of CWM and Vitamin C against radical species by DPPH assay. * *p* value < 0.05 was considered statistically significant.

| **Samples** | **$IC_{50}$ values** | | |
|---|---|---|---|
| | **0 month** | **6 month** | ***p* value** |
| **Spray dry** | 36.78 | 42.24 | > 0.05 |
| **Capsule size No. 1** | 43.65 | 47.13 | > 0.05 |
| **Capsule size No. 0** | 41.49 | 45.72 | > 0.05 |
| **Control (Vitamin C)** | 11.15 | 13.81 | > 0.05 |

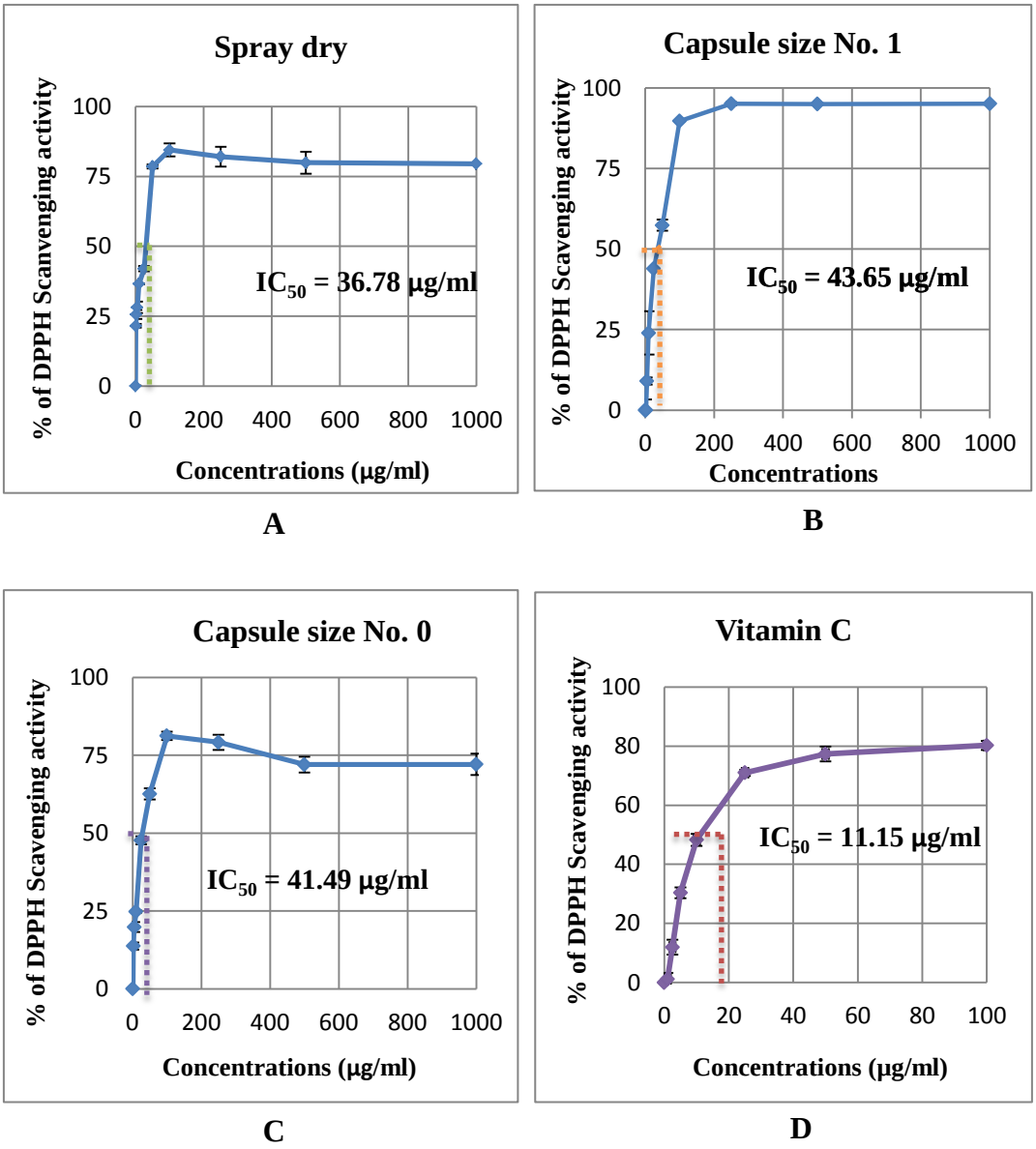



**Figure 4.6** The antioxidant activity of CWM against the radical species of Spray dry (A), Capsule size No. 1 (B), Capsule size No.0 (C) and vitamin C (D) at 0 month. The graph presented as mean ± SD of duplicate measurements.

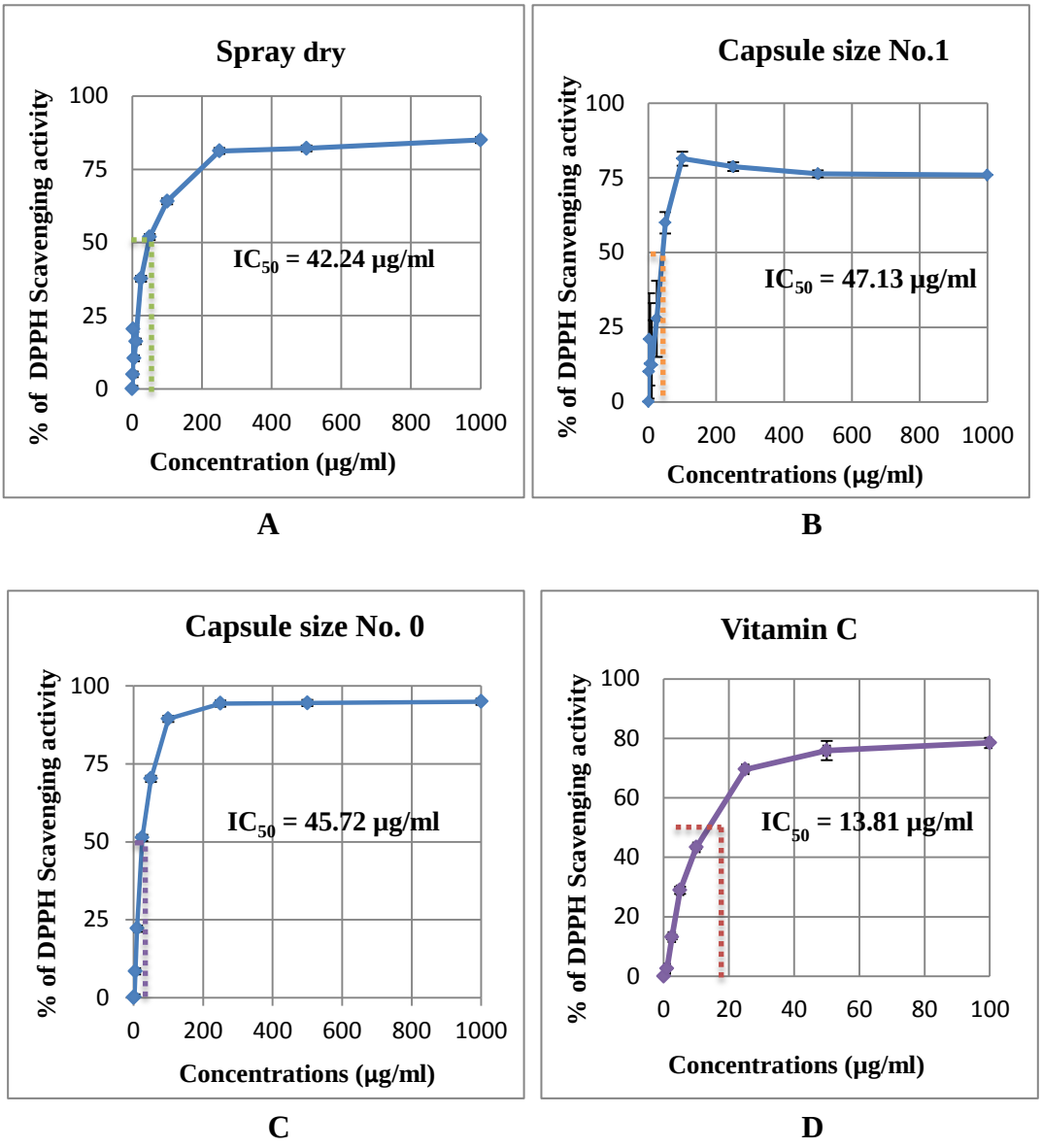



**Figure 4.7** The antioxidant activity of CWM in the DPPH radical scavenging assay of Spray dry (A), Capsule No. 1 (B), Capsule No.0 (C) and vitamin C (D) at 6 month. The graph presented as mean ± SD of duplicate measurements.

## 4.6 Cells viability assay by MTT method

Effects of CWM from preparation on SK-N-SH cells neuroblastoma cells at 24 hours were determined by MTT assay. The SK-N-SH cells were treated with CWM (SP, capsule size No. 1, capsule size No.0 and paclitaxel) at 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 and 1,000 μg/ml in 96-well. The percentages of cell survival compared with untreated cells were presented as mean ± SD. Cells viability after treatment with CWM at 0 month were showed in Table 4.5 A, as after treatment with CWM at 6 month were showed in Table 4.5 B. The 50% of SK-N-SH cells survival in first month resulted from being exposed to 128.73 μg/ml of SP, 168.44 μg/ml of capsule size No. 1 and 156.91 μg/ml of capsule size No. 0 in Table 4.5 A and Figure 4.8, as $IC_{50}$ resulted 157.25 μg/ml of SP, 202.39 μg/ml of capsule size No. 1 and 196.14 μg/ml of capsule size No. 0 in Table 4.5 B and Figure 4.9, as opposed to that of paclitaxel was 0.93 and 0.82 μg/ml as shown in Table 4.6 and Figure 4.8 and 4.9. Paclitaxel was used as positive control.

**Table 4.5** Effect of various concentrations of CWM on the viability of SK-N-SH cells using MTT assay.

A) Effect of various concentrations of CWM on the viability of SK-N-SH cells at first month.

| **Concentration (μg/ml)** | **% relative cell viability** | | |
|---|---|---|---|
| | **Spray dry** | **Capsule size No. 1** | **Capsule size No. 0** |
| **0** | 100 | 100 | 100 |
| **1.95** | 92.84±1.57 | 110.89±2.76 | 121.72±3.20 |
| **3.91** | 97.29±2.58 | 99.94±3.74 | 112.60±5.18 |
| **7.81** | 90.20±2.25 | 88.05±3.04 | 98.05±2.48 |
| **15.63** | 97.19±1.04 | 97.84±0.92 | 117.12±4.48 |
| **31.25** | 89.56±5.62 | 94.84±7.46 | 99.75±1.00 |
| **62.5** | 54.38±2.87 | 72.42±5.72 | 65.44±3.07 |
| **125** | 50.58±3.29 | 53.76±3.98 | 54.47±1.57 |
| **250** | 30.90±1.30 | 42.92±4.21 | 36.93±2.39 |
| **500** | 22.85±0.56 | 16.90±5.58 | 18.23±2.55 |
| **1,000** | 15.48±3.83 | 10.89±3.67 | 16.93±1.55 |
| **$IC_{50}$** | **128.73** | **168.44** | **156.91** |

B) Effect of various concentrations of CWM on the viability of SK-N-SH cells at the sixth month.

| **Concentration (µg/ml)** | **% relative cell viability** | | |
|---|---|---|---|
| | **Spray dry** | **Capsule size No. 1** | **Capsule size No. 0** |
| **0** | 100 | 100 | 100 |
| **1.95** | 130.83±4.71 | 80.22±8.73 | 94.18±4.79 |
| **3.91** | 130.98±11.91 | 80.77±2.56 | 101.80±2.38 |
| **7.81** | 137.32±4.56 | 69.71±2.19 | 106.61±7.60 |
| **15.63** | 125.25±12.71 | 77.78±1.96 | 102.19±12.63 |
| **31.25** | 70.95±2.08 | 64.37±0.91 | 94.43±3.01 |
| **62.5** | 66.73±1.73 | 74.13±6.04 | 73.45±7.86 |
| **125** | 50.57±2.83 | 63.91±1.63 | 63.87±2.45 |
| **250** | 38.70±2.13 | 41.44±4.11 | 39.494±3.19 |
| **500** | 19.59±1.20 | 26.58±4.52 | 15.85±3.52 |
| **1,000** | 20.88±0.95 | 18.75±6.66 | 11.67±4.16 |
| **$IC_{50}$** | **157.25** | **202.39** | **196.14** |

**Table 4.6** Effect of various concentrations of paclitaxel on the viability of SK-N-SH cells using MTT assay.

A) Effect of various concentrations of CWM on the viability of SK-N-SH cell at the 0 month

| **Concentration (µg/ml)** | **% relative cell viability** |
|---|---|
| 0 | 100 |
| 0.78 | 52.94±10.22 |
| 1.56 | 30.50±3.91 |
| 3.13 | -33.73±3.47 |
| 6.25 | -40.04±2.12 |
| 12.5 | -32.29±3.59 |
| 25 | -17.09±2.68 |
| 50 | -62.09±2.96 |
| **% cell viability** | 0.93 |

B) Effect of various concentrations of CWM on the viability of SK-N-SH cell at the 6 month

| **Concentration (µg/ml)** | **% relative cell viability** |
|---|---|
| 0 | 100 |
| 0.78 | 51.94±14.63 |
| 1.56 | 37.18±4.35 |
| 3.13 | 18.70±1.09 |
| 6.25 | -13.72±3.06 |
| 12.5 | -28.01±8.47 |
| 25 | -48.59±3.76 |
| 50 | -57.68±3.31 |
| **% cell viability** | 0.82 |

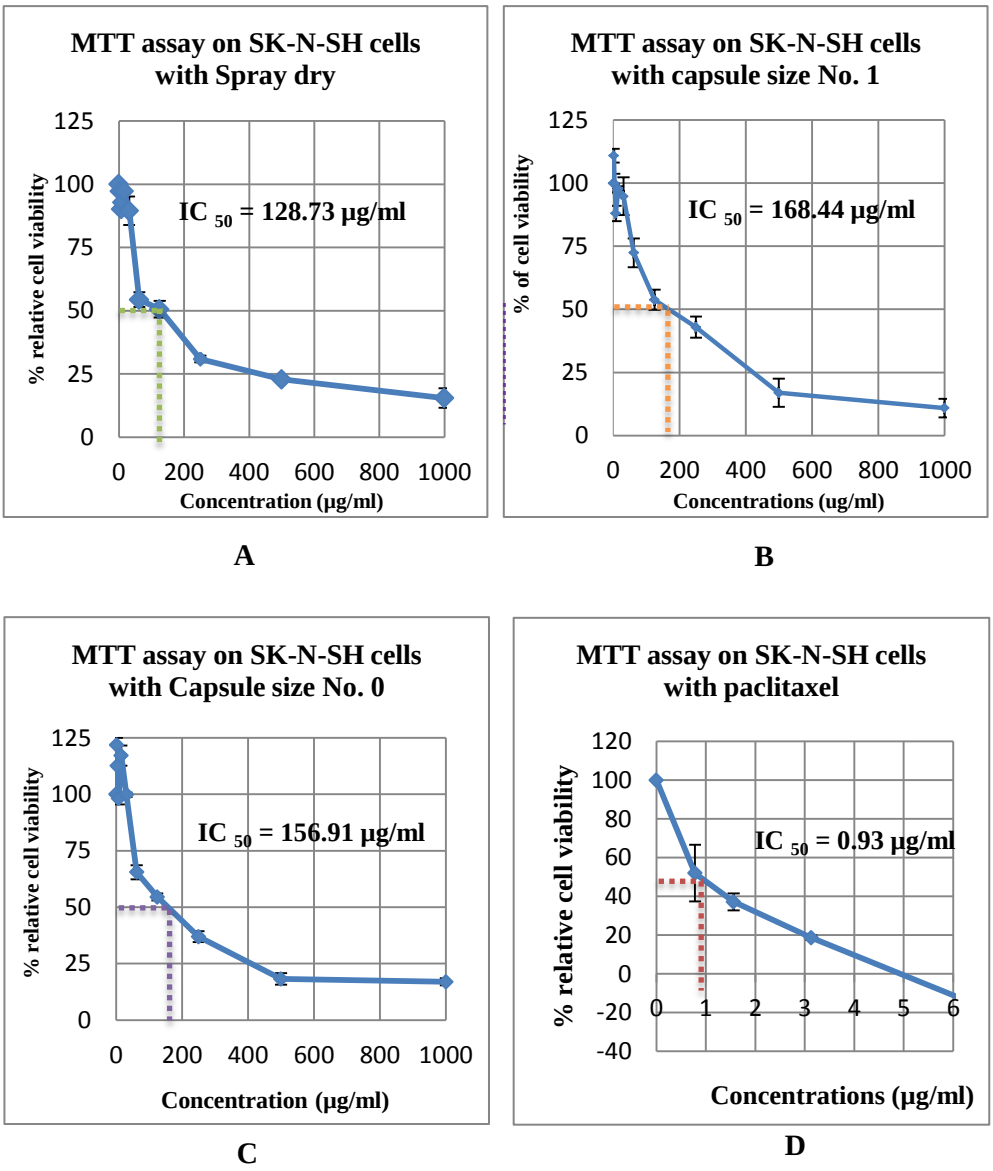



**Figure 4.8** Effect of various concentrations of CWM and paclitaxel on the viability of SK-N-SH cells at 0 month. The $IC_{50}$ values SP, capsule size No. 1, capsule size No.0 and paclitaxel were shown in A, B, C and D. ($IC_{50}$ = the effective dose of a sub stance in inhibit cells at 50% of a population; n = 3 individual samples).

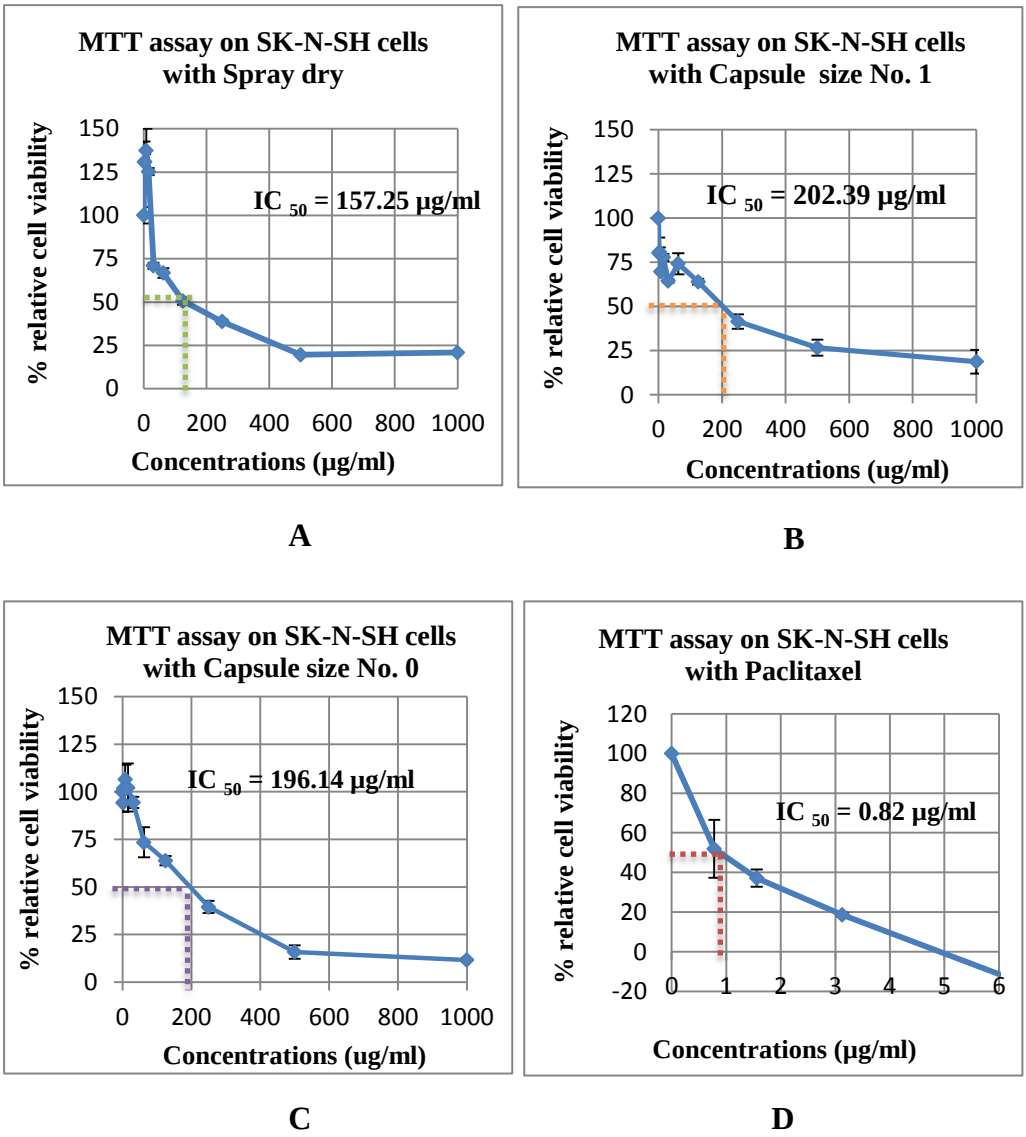



**Figure 4.9** Effect of various concentrations of CWM and paclitaxel on the viability of SK-N-SH cells at 6 month. The $IC_{50}$ values SP, capsule size No. 1, capsule size No.0 and paclitaxel were shown in A, B, C and D. ($IC_{50}$ = the effective dose of a sub stance in inhibit cells at 50% of a population; n = 3 individual samples).

## 4.7 The antioxidative effects of CWM against the $H_2O_2$ induced oxidative stress

The antioxidant effects of the CWM against oxidative stress in cells were obtained. There are many compounds found in the CWM that can act as antioxidants to neutralize the ROS. Therefore, we tested ROS production changes from the protective effect of CWM against $H_2O_2$ induced cell death at 0 and 6 month. ROS production was measured to confirm the protective effect of CWM after SK-N-SH cells were induced by $H_2O_2$. The fluorescence intensity of intracellular ROS after exposing with $H_2O_2$ was compared between untreated cells and treated cells with CWM and vitamin E at 100 and 200 μg/ml. ROS levels were significantly decreased from cells treated with CWM (SP, Capsule No1, 0) at concentrations 100 and 200 μg/ml after exposure to $H_2O_2$ at 50 μM, which showed higher than antioxidant effect of vitamin E (Table 4.7 and 4.8 and Figure 4.10 and 4.11).

**Table 4.7** Protective effect of CWM against $H_2O_2$ induced ROS production at 0 month (shown as fluorescence intensity of DCF). * $p$ value < 0.05 was considered statistically significant.

| **Sample** | | **Fluorescence intensity (unit) (Mean±SEM)** |
|---|---|---|
| **Control ($H_2O_2$)** | 600μM | 1136.50±14.30 |
| **Spray dry** | 100 μg/ml | 487.87±22.45* |
| | 200 μg/ml | 311.90±17.76* |
| **Capsule size No.1** | 100 μg/ml | 470.43±5.70* |
| | 200 μg/ml | 306.03±1.791* |
| **Capsule size No.0** | 100 μg/ml | 459.72±5.56* |
| | 200 μg/ml | 347.92±12.17* |
| **Control (Vitamin E)** | 200 μg/ml | 545.78±11.13* |

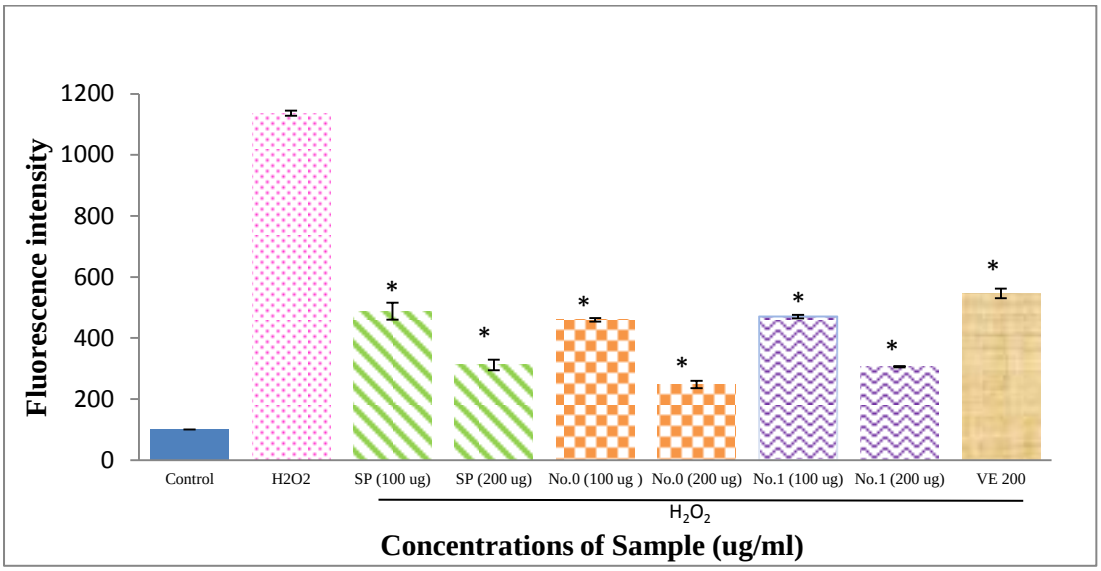



**Figure 4.10** The antioxidative effects of CWM against $H_2O_2$ induced oxidative stress at 0 month. The graph showed the fluorescence intensity of DCF used to measure intracellular ROS after treatment with $H_2O_2$ and with or without CWM. * *p* value < 0.05 was considered statistically significant.

**Table 4.8** Protective effect of CWM against $H_2O_2$ induced ROS production at 6 month (shown as fluorescence intensity of DCF). * *p* value < 0.05 was considered statistically significant.

| Sample | | Fluorescence intensity (unit) (Mean±SEM) |
|---|---|---|
| **Control ($H_2O_2$)** | 600µM | 1241.35±23.52 |
| **Spray dry** | 100 µg/ml | 438.29±16.28* |
| | 200 µg/ml | 295.67±12.88* |
| **Capsule size No.1** | 100 µg/ml | 345.71±7.23* |
| | 200 µg/ml | 237.78±5.13* |
| **Capsule size No.0** | 100 µg/ml | 396.06±5.84* |
| | 200 µg/ml | 262.24±9.84* |
| **Control (Vitamin E)** | 200 µg/ml | 527.32±15.87* |

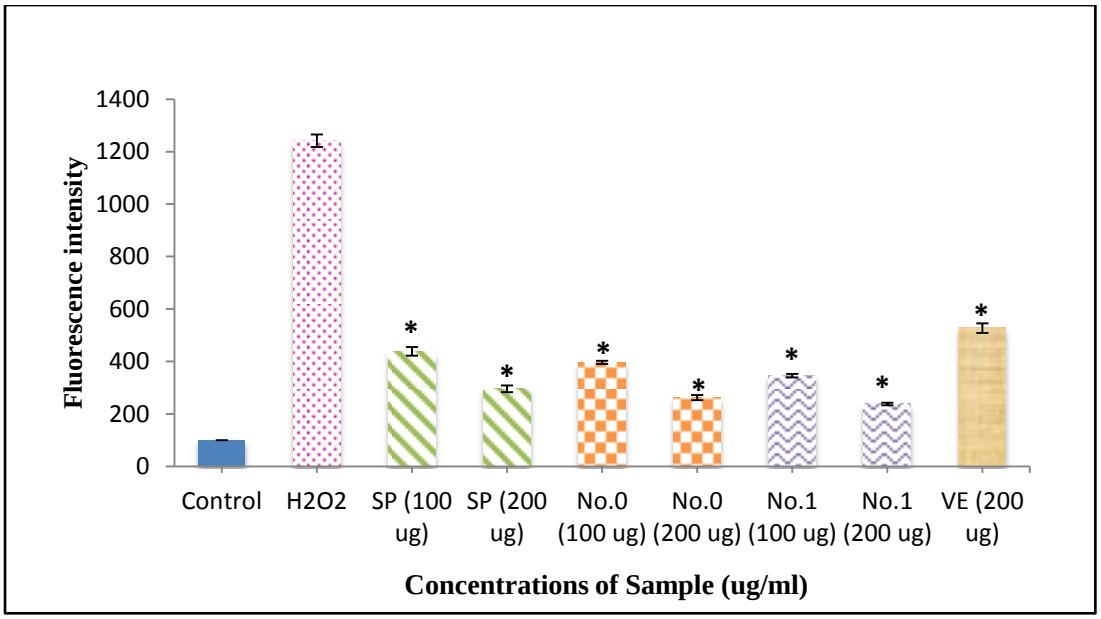



**Figure 4.11** The antioxidative effects of CWM against $H_2O_2$ induced oxidative stress at 6 month. The graph showed the fluorescence intensity of DCF used to measure intracellular ROS after treatment with $H_2O_2$ and with or without CWM. * *p* value < 0.05 was considered statistically significant.

## 4.8 Effects of CWM on reactive oxygen species (ROS) production

This study also investigated that the antioxidant activities of water extracts of mangosteen on red blood cells (RBCs). The volunteers have received a daily dose of CWM at 220 mg and 280 mg, respectively, to their weights for 3 months, then, followed by double dose daily for the further 3 months. Blood samples of all subjects (n = 11) were collected and measured ROS level at days 0, 7, 21, 84 (single doses administration), 112 and 168 (double doses administration). ROS measurement was performed by stimulating isolated RBCs with $H_2O_2$ at 0 and 20 minutes. Mean fluorescent intensity (MFI) of $H_2O_2$ – stimulated RBCs during day 0 – 168 was measured as shown in Table 4.9. The comparisons of MFI between stimulation RBCs by RBCs by $H_2O_2$ at 0 and 20 minutes were determined. Mean values of MFI were calculated by Time at 20 min – Time at 0 min. MFI were significantly reduced starting at 84 days onwards as shown in Table 4.9, Figure 4.12.

**Table 4.9** Mean fluorescent intensity (MFI) of $H_2O_2$-stimulated at days 0 to 168 and Comparisons of MFI between $H_2O_2$-stimulate RBCs at 0 and 20 minutes of a population; n = 11 healthy humans. * *p* value < 0.05 was considered statistically significant.

| | Time (min) | Days of daily administration of CWM of healthy volunteers (n = 11) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 7 | 28 | 84 | 112 | 168 |
| | | ← Single doses → | | | ← Double doses → | | |
| **Mean fluorescent intensity (MFI) of $H_2O_2$– stimulated** | 0 | 9.69±1.30 | 7.79±0.44 | 16.45±3.77 | 12.75±2.06 | 3.06±0.21 | 2.16±0.28 |
| | 20 | 49.14±9.43 | 34.51±3.37 | 38.22±5.79 | 22.77±3.51 | 9.77±2.25 | 4.06±0.65 |
| **Difference of MFI at 0 and 20 minutes** | Mean | 39.45±8.14 | 26.72±2.93 | 21.77±2.02 | 10.03±1.45* | 6.707±2.04 * | 1.90±0.37* |
| | *p*-value | | 0.30 | 0.08 | 0.00 | 0.00 | 0.00 |

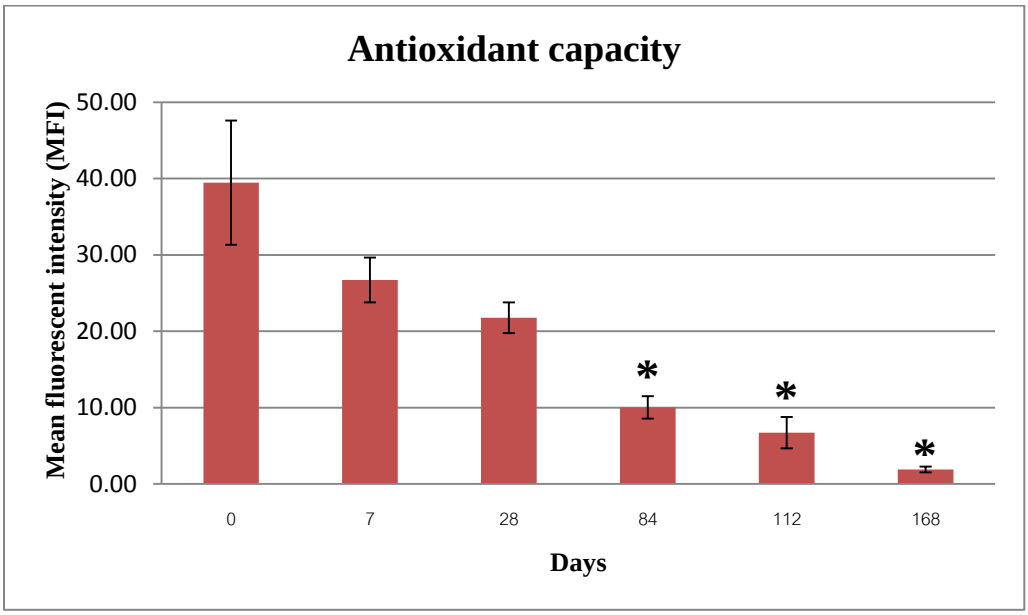



**Figure 4.12** The antioxidant effects of CWM in healthy volunteers. The ROS in RBCs showed the reduction level of MFI from day 0 to 168. * *p* value < 0.05 compared to untreated control (day 0).

## 4.9 Effects of CWM on hydroxynonenal (HNE) production from whole blood and red blood cell

To verify that crude water extract of mangosteen (CWM) had an antioxidant effect on hydroxynonenal (HNE) production, whole blood (WB) and red blood cells (RBCs) of each sample from healthy volunteers after taking CWM were detected by Dot blot analysis. The actin antibody was used as loading control of protein in each sample. The Dot blot analysis showed that the percentage of HNE levels from WB after taking CWM was significantly decreased starting at 28 days onwards (Table 4.10, Figure 4.13A) whereas from RBCs the levels of HNE significantly decreased at 168 days compared with untreated sample (day 0) (Table 4.11, Figure 4.13B).

**Table 4.10** The percentage of ratio between HNE and actin from whole blood healthy volunteers after taking CWM of a population; n = 11 healthy humans. * *p* value < 0.05 was considered statistically significant.

| **Sample No.** | **Days** | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **7** | **28** | **84** | **112** | **168** |
| **1** | 100 | 66.65 | 41.08 | 28.62 | 31.63 | 25.41 |
| **2** | 100 | 136.02 | 63.10 | 79.39 | 88.09 | 74.24 |
| **3** | 100 | 57.61 | 71.99 | 112.86 | 111.78 | 61.87 |
| **4** | 100 | 56.84 | 50.49 | 38.49 | 30.86 | 30.49 |
| **5** | 100 | 96.65 | 91.51 | 117.99 | 101.88 | 78.96 |
| **6** | 100 | 80.19 | 48.79 | 43.11 | 56.98 | 63.41 |
| **7** | 100 | 73.67 | 38.36 | 35.01 | 39.98 | 37.53 |
| **8** | 100 | 94.38 | 73.89 | 75.03 | 72.70 | 39.93 |
| **9** | 100 | 106.92 | 90.80 | 84.96 | 65.69 | 86.80 |
| **10** | 100 | 74.09 | 60.94 | 67.14 | 60.43 | 49.64 |
| **11** | 100 | 114.85 | 103.49 | 75.96 | 63.68 | 47.87 |
| **Average** | **100** | **87.08** | **66.77*** | **68.96*** | **65.79*** | **54.20*** |
| **SEM** | **0** | **7.57** | **6.55** | **9.13** | **8.05** | **6.15** |
| ***P*-value** | | **0.12** | **<0.01** | **0.01** | **<0.01** | **<0.01** |

**Table 4.11** The percentage of ratio between HNE and actin from red blood cells healthy volunteers after taking CWM of a population; n = 11 healthy humans. * *p* value < 0.05 was considered statistically significant.

| **Sample No.** | **Days** | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **7** | **28** | **84** | **112** | **168** |
| **1** | 100 | 123.49 | 252.27 | 81.52 | 130.91 | 93.41 |
| **2** | 100 | 76.70 | 95.41 | 115.53 | 51.82 | 65.64 |
| **3** | 100 | 101.61 | 108.11 | 105.66 | 96.26 | 76.99 |
| **4** | 100 | 94.07 | 75.86 | 81.82 | 81.22 | 52.80 |
| **5** | 100 | 54.12 | 62.54 | 66.19 | 50.87 | 33.68 |
| **6** | 100 | 67.15 | 79.07 | 67.72 | 65.45 | 56.32 |
| **7** | 100 | 95.03 | 126.09 | 117.94 | 97.95 | 86.83 |
| **8** | 100 | 89.27 | 116.81 | 76.91 | 107.40 | 80.86 |
| **9** | 100 | 60.26 | 26.78 | 39.07 | 29.57 | 23.04 |
| **10** | 100 | 123.05 | 107.49 | 116.25 | 140.74 | 147.54 |
| **11** | 100 | 73.24 | 55.27 | 60.27 | 51.67 | 45.14 |
| **Average** | **100** | **87.09** | **100.52** | **88.99** | **82.17** | **69.30*** |
| **SEM** | **0** | **7.04** | **17.60** | **10.59** | **10.77** | **10.29** |
| ***P*-value** | | **0.10** | **0.98** | **0.32** | **0.13** | **0.01** |

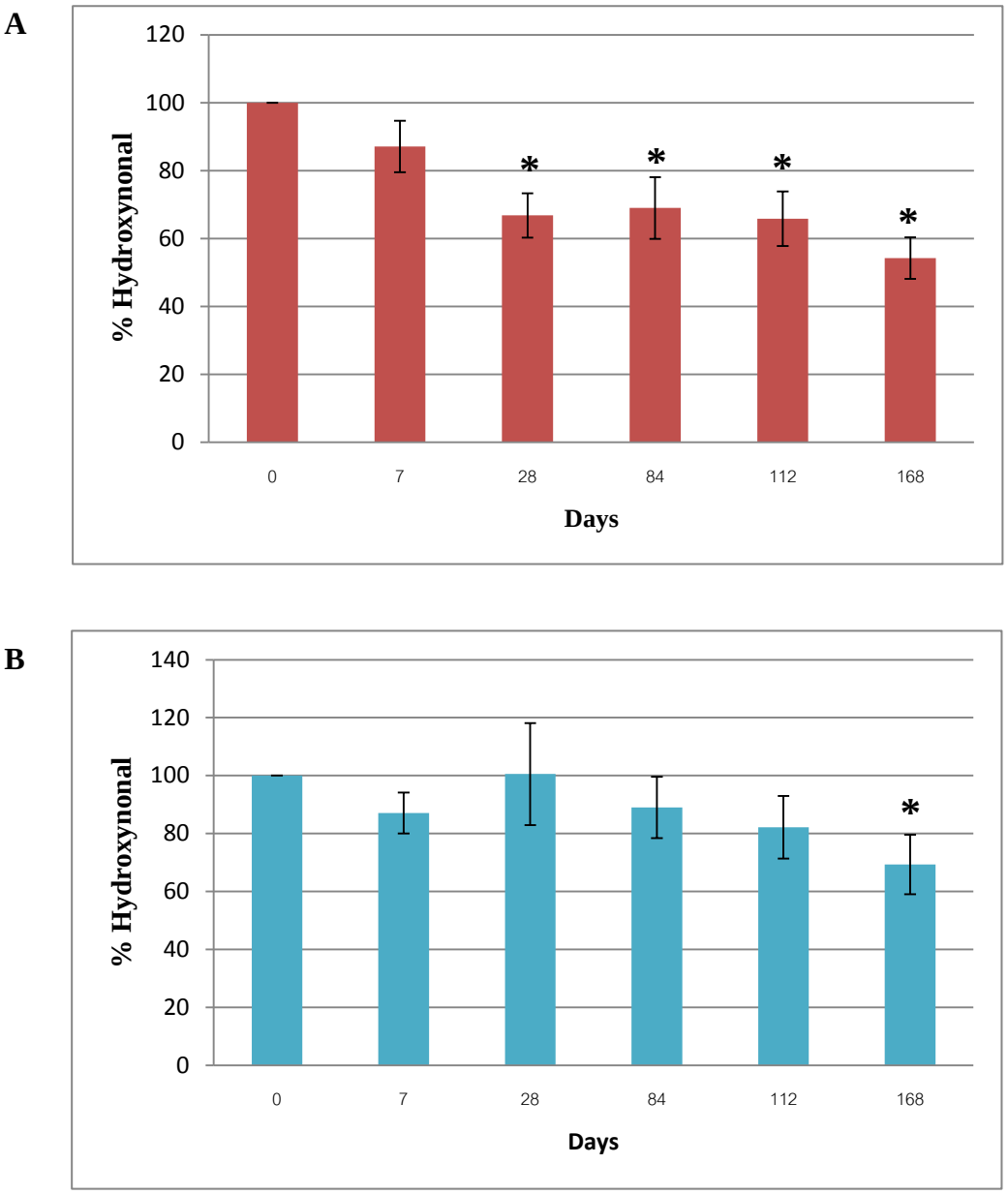



**Figure 4.13** The effects of CWM on HNE production. The percentage of HNE from whole blood (A) and red blood cells (B) of healthy volunteers after taking CWM at days 0, 7, 28, 84, 112, and 168. * *p* value < 0.05 was considered statistically significant.

## 4.10 Effects of CWM on dehydroepiandrosterone (DHEA) in plasma

The contents of DHEA from the plasma of healthy volunteers, which were treated with crude water extract of mangosteen (CWM), were investigated by ELISA technique. The results showed that CWM did not significantly alter the level of DHEA as shown in Table 4.12, Figure 4.14 compared D 84, 168 with Day 0.

**Table 4.12** The percentage of DHEA in plasma of healthy volunteers after taking CWM at 0, 84,168 days of a population; n = 11 healthy humans. * *p* value < 0.05 was considered statistically significant.

| **Sample No.** | **Days** | | |
|---|---|---|---|
| | **0** | **84** | **168** |
| **1** | 100 | 81.98 | 68.56 |
| **2** | 100 | 77.56 | 39.73 |
| **3** | 100 | 34.43 | 52.60 |
| **4** | 100 | 122.79 | 65.45 |
| **5** | 100 | 65.23 | 59.45 |
| **6** | 100 | 107.92 | 214.17 |
| **7** | 100 | 153.92 | 100 |
| **8** | 100 | 155.30 | 68.56 |
| **9** | 100 | 109.53 | 103.53 |
| **10** | 100 | 63.11 | 41.86 |
| **11** | 100 | 80.10 | 66.32 |
| **Average** | **100** | **95.57** | **80.02** |
| **SEM** | **0** | **11.46** | **14.73** |
| ***P*-value** | | **0.78** | **0.23** |

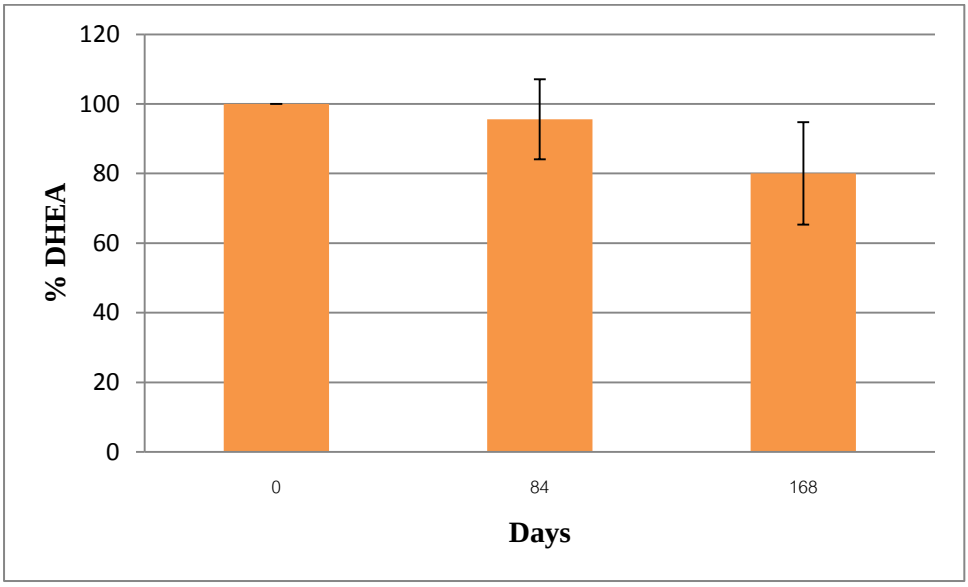



**Figure 4.14** The effects of CWM on DHEA production at 0, 84, 168 days. *$p$ value < 0.05 was considered statistically significant.

# CHAPTER V
# DISCUSSION

Oxidative stress is defined as the imbalance between biochemical process and those responsible for the antioxidant system. Reactive oxygen species (ROS) leads to decrease the activities of antioxidant enzymes and increase lipid peroxidation products and consequently causes damage all components of the cell, including proteins, lipids, and DNA. In humans, oxidative stress is thought to be involved in the development of cancer, atherosclerosis, heart failure, myocardial infarction, fragile X syndrome, sickle cell disease, lichen planus, vitiligo, autism. Infection, chronic fatigue syndrome and neurodegenerative disease such as Parkinson's disease and Alzheimer's disease.

Numerous reports have suggested that oxidative stress or ROS plays a key role in neuronal apoptosis (2-4). Brain has been considered to be abnormally sensitive to oxidative damage (42). Antioxidants are thought to protect the body against the destructive effects of free radicals. Antioxidants neutralize free radicals by donating one of their own electrons, ending the electron- chain reaction. The antioxidant nutrients themselves do not become free radicals by donating an electron because they are stable in either form. They act as scavengers, helping to prevent cell and tissue damage that could lead to cellular damage and disease.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the brain (163). Many researches have been found that in AD patients the oxidative stress was increased and the other hand the total antioxidant capacity of the plasma was decreased (164).

Although novel technologies are applied in health care and medical process, Alzheimer drug discovery is still in need, since the highly effective drug with less toxicity is one of the crucial fields for treatment Alzheimer's disease. Many new studies on Alzheimer's disease drugs are concentrated on the applications of natural products and various biological substances such as Ginkgo biloba, Huperzine A,

Curcumin, Caprylic acid, Amyloid beta (Aβ) Tyrosine receptor kinase B (TrkB) and Brain-derived neurotrophic factor (BDNF) (40, 127, 165-168).

Mangosteen has been used in traditional medicine to treat various ailments, especially diseases of the digestive system and infection. Many previous studies showed that magosteen contains a variety of chemicals such as mangostin, xanthone, tannin, isoflavone, flavone and other bioactive substances with various biological activities (161). Xanthone is a class of polyphenolic compounds which contain high amounts in the pericarp of mangosteen and possesses strong antioxidant activities (9, 14,158,161,162,170). Reports on various isolated chemical constituents from mangosteen have been reported with biological activities (9, 10, 11, 134, 148, 158, 161, 169). In addition, the water soluble extract of mangosteen can demonstrate impressive antioxidant and anti-inflammatory properties (14, 162, 170). However, the water soluble part is of interest, but unfortunately, the isolation of pure and identified chemical constituents with certain biological activities is not so easy to obtain for the Phytochemistry procedure.

The previous study effects of crude water extract of mangosteen (CWM) showed promising antioxidant activities in SK-N-SH neuroblastoma cells (14). Moreover, *in vivo*, it could safely protect memory loss in mice treated with scopolamine (129). According to these attractive properties of CWM, we have an attempt to determine their antioxidant activity in healthy humans. Therefore, this study reported the antioxidant activity of CWM was consisted of two parts. The first part was the *in vitro* study on antioxidant activity of preparative water extract mangosteen in SK-N-SH human neuroblastoma cell. The determination of ROS production by DCFH-DA after treated cells with mangosteen and also the measurement the proliferation of cells by MTT assay. The second part was the evaluation of the effect of CWM in healthy humans. The blood of volunteers was collected and evaluated the changes in ROS after taking CWM daily within 6 months. The biological markers of Alzheimer's disease; HNE and DHEA were also determined.

## 5. 1 Evaluation of preparative mangosteen capsules

Spray dry preparation of CWM was filled in capsules for the clinical trial study. The powder of CWM was filled in capsules at dose 220 mg without filler. For the dose of 280 mg, CWM was filled in capsule size No.0 by adjusting with corn starch as filler to obtain the total weight of 300 mg. We found that CWM in capsules No.1 became aggregated compared to capsule No.0, so we mixed cornstarch to the CWM at dose 220 mg. We found the new problem that we could not fill all the mixtures of CWM dose 220 mg and cornstarch in capsule No.1. We had to change to fill CWM with filler into larger capsule (No. 1). CWM in capsules was evaluated for the antioxidant activity by DPPH scavenging. The preparative capsules were assessed for the quality complied with the standard for capsule dosage form according to British Pharmacopoeia 2014 (BP) (175, 176). In this experiment, capsules after production was tested for the disintegration test, weight variation test and biological activities on SK-N-SH cells (MTT, ROS). After test with weight variation and disintegration, we showed that the percentages deviation of capsule size No 0 and 1 were within the standard follow weight variation limits of BP (less or more than 300 mg was less than 10%) (175). The percentages deviation of capsule size No. 0 and 1 was 7.3 and 6.47 %, respectively as shown in Table 4.2. In the disintegration part, the capsules had begun to disintegrate at 2 minute and completely disintegrated within 8 minute (Figure 4.4). Therefore, the disintegration time of our capsules were in the range within the disintegration time limit of BP standard that a capsule had been dissolved or completely disintegrated within 15 minutes (175). Moreover, the results in the percentages of loss on drying (% LOD) of spray dry CWM (SP), CWM capsule size No. 1 and capsule size No. 0 were 3.4, 3.56 and 3.95 respectively (Table 4.3). Therefore, % LOD of each sample ranged within the loss on the drying limit of BP standard that the moisture of sample should be less than 5 % of LOD (176). With all the data obtained, we concluded that the quality of CWM capsules met the standard criteria.

## 5.2 Effects of CWM by DPPH scavenging assay

DPPH radicals have been used extensively as stable radicals to preliminarily evaluate the antioxidant activity of plant extracts. The DPPH antioxidant assay is based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515 nm and also for a visible deep purple color. In previous study, it was found that xanthones containing in mangosteen composed of dihydroxyl group in B-ring, exhibited strong radical scavenging activity. In this experiment, SP, capsule size No. 1, capsule size No 0 and vitamin C (positive control) were determined antioxidant activity at 0 and 6 months. Therefore, the strong antioxidant could change the color of DPPH from violet to yellow. The results of SP, capsule size No.1, capsule size No.0 and vitamin C at first month showed $IC_{50}$ at 36.78, 43.65, 41.49 and 11.15 μg/ml, respectively (Table 4.4 A and Figure 4.6). In other month, CWM showed $IC_{50}$ at 42.24, 47.13 and 45.72 μg/ml, respectively (Table 4.4 B and Figure 4.7). CWM showed the potent proton-donating ability on DPPH to DPPHH molecule. This activity was an important mechanism of antioxidants. Moreover, we compared the $IC_{50}$ of 0 month CWM and 6 month CWM. We found that there was no difference in potency between 0 and 6 month storage.

## 5.3 Effects of CWM on SK-N-SH cell viability

To evaluate the proliferation of SK-N-SH cells, the extracts were cultured with SK-N-SH cells and assayed with MTT technique. MTT is a tetrazolium salt that can be reduced be living cells into purple color formazan and is used to determine cell viability. The reduction is catalyzed by the enzyme dehydrogenase that localized in mitochondria of cells. The suitable numbers of cells were $2x10^4$ Cells/well in 96 well plate. SK-N-SH cells were cultured with various concentrations of SP, capsule No.1, capsule No.0 at 0 and 6 months. The results in first month showed that 50% of SK-N-SH cells viability resulted from exposed with SP, capsule size No.1, capsule size No.0 was 128.73, 168.44 and 156.91 μg/ml respectively (Table 4.5 A and Figure 4.8). As the $IC_{50}$ at sixth month were 157.25, 202.39 and 196.14 respectively (Table 4.5 B and

Figure 4.9). The $IC_{50}$ showed more than 100 μg/ml (14), which indicated that CWM were safety to cells.

## 5.4 Effects of CWM on reactive oxygen species in SK-N-SH cells

The study of antioxidant activity of water extract of mangosteen (CWM) against oxidative stress was demonstrated on the cytosolic ROS production in cells. The extent of cytosolic cellular oxidative stress was determined by monitoring the amount of ROS by DCFH-DA. The DCFH-DA fluorescence probe was used to estimate for hydrogen peroxide of cytosolic ROS. The $IC_{50}$ in previous test were in the range about 150 μg/ml. So we selected 100 and 200 μg/ml of CWM to evaluate the antioxidant activity on ROS in SK-N-SH cells. The fluorescence intensity of intracellular ROS was compared between untreated cells and treated cells with CWM. ROS levels in cells were significantly decreased from CWM (SP, capsule size No.1, capsule size No.0) at 100 and 200 μg/ml, respectively, compared with $H_2O_2$ as shown in Table 4.7, 4.8 and Figure 4.10, 4.11. Moreover, the study of the protective of CWM compared with some common antioxidant such α-tocopherol (vitamin E) was performed. The ROS production was measured to compare between the protective effect of CWM and vitamin E. Vitamin E at 200 μg/ml showed an antioxidant activity less than CWM at 100 and 200 μg/ml. This result demonstrated that CWM still possessed antioxidant activity after the preparation of spray dry, capsule size No. 1 and capsule size No.0 stronger than antioxidant activity of vitamin E.

## 5.5 Effects of CWM on Reactive Oxygen Species (ROS) in healthy human

ROS are constantly generated under normal conditions as a consequence of aerobic metabolism. ROS are particularly transient species due to their highly chemical reactivity and can react with carbohydrates, DNA, protein and lipid in a destructive manner (1). Several neurodegenrative diseases implicate a deficient natural antioxidant defense as one of their etiological factors. The progression of these

disorders can be delayed by supplementing an antioxidant defense. Several reports showed that ROS are involved in the pathogenesis of AD (10, 14, 16, 18, 20, 24, 42). The previous studies reported the antioxidant activities of CWM of mangosteen pericarp in ROS production in cultured neuronal cells and memory loss improvement of scopolamine treated mice (129). In this study, the healthy volunteers have received CWM for 6 months. It was demonstrated that the CWM significantly decreased ROS levels in RBCs as shown in Table 4.9 and Figure 4.12. Since CWM contains many phenolic compounds that could function as antioxidants as previously reported (14,129,169). The results showed that the level of ROS in RBCs was significantly decreased after daily taking CWM for 84 days and remained until 6 months of ingestion. Our finding demonstrated that CWM could enhance antioxidant activity in healthy volunteers. In addition, other biochemical markers and physical examinations in those subjects were evaluated carefully to approve for the safety of CWM in further clinical trials.

## 5.6 Effects of CWM on hydroxynonenal (HNE)

4-Hydroxynonenal (HNE), an aldehyde product of membrane lipid peroxidation, can be produced by oxidative stimuli and caused by several diseases such as atherosclerosis, Parkinson's disease and Alzheimer's disease (81, 82). There are numerous reports of excess oxidation within the brain of AD patients, but outside the CNS, the evidence is less definite. Blood components have been widely examined for evidence of a systemic oxidative effect, with changes in membrane fluidity, platelet activation and more specifically, reports of leukocyte oxidation. In previous studies, they have found evidence of lipid peroxidation in the blood of patients with AD, with significantly higher levels of HNE (82), an aldehydic product of lipid peroxidation. An important observation was that the concentration of HNE in patients was related to the degree of cognitive impairment. The effects of this compound have been the focus of much recent research. HNE has been shown to accumulate in areas of oxidative stress, with evidence that it inhibits DNA and RNA, and inactivates and modifies enzyme systems (62). Therefore, the presence of HNE can also be a biomarker that reflects oxidative stress in the body. In this study, we determined the

effects of mangosteen extract on the HNE production from whole blood and red blood cells after taking CWM for 6 months by dot blot analysis. The data were analyzed the intensity of HNE divided by the intensity of actin (adhesion molecule as a negative control in Chapter II). CWM showed a significantly antioxidative effect by decreasing HNE level starting at 28 days onwards as shown in Table 4.10 and Figure 4.13 A. Moreover, the results were significantly decreased on the HNE level at 168 days (Table 4.11 and Figure 4.13 B). This data were correlated with the obtained results of ROS determination by Flow cytometry, that the volunteers after taking CWM the level of ROS was reduced. Since HNE is a byproduct of ROS. Therefore, it was confirmed that CWM have antioxidant activity in healthy human.

## 5.7 Effects of CWM on dehydroepiandosterone (DHEA)

Several studies have demonstrated an association between decreasing levels of dehydroepiandosterone (DHEA) and diseases such as arthritis, heart disease, and Alzheimer's disease. DHEA has also been found to have the ability to protect cells from oxidative damage to the hippocampus part of the brain (5, 6). This is among the regions of the brain most affected by Alzheimer's disease. They found that AD patient had significantly lower levels of DHEA compared with control (112). The decreased DHEA in serum, plasma, CSF and brain of AD individuals could have an important bearing on the pathogenesis of this disease in view of widely documented effects of DHEA on neuronal excitability, neuroprotection and memory facilitation (171). Therefore, in this study, we have an attempt to determine the effect of CWM on DHEA in plasma by ELISA kits. From ELISA, CWM showed decreased DHEA level, but non-significantly after taking CWM for 6 months (Table 4.12 and Figure 4.14). In previous study, they tested the presence of this DHEA precursor in human serum using a $Fe^{2+}$- based reaction and determined the amounts of DHEA formed, which $Fe^{2+}$ can induce oxidative stress. $Fe^{2+}$ treatment of the serum resulted in a dramatic increase in DHEA levels in control patients, whereas only a moderate or no increase was observed in AD patients (15). Therefore, DHEA levels were correlated with ROS level in the body. So DHEA and ROS levels of volunteers before taking CWM were shown higher levels when compared with levels in Day 0 in Figure 4.11. After taking

CWM, level of ROS was decreased. As a result, DHEA was decreased to normal level in healthy volunteers (Figure 4.14). Therefore, in this study, DHEA levels of some volunteers showed increasing levels, which were not correlated with previous reports (100, 171). Therefore, it was supposed that some factors had affected to increase DHEA levels. As reported by some studies that DHEA level in the human body could be increased when the volunteers had stress from psychosocial stressor (173). Therefore, this factor may have effect to increase levels in some volunteer as shown in Table 4.12. The result indicated that CWM had an effect on ROS level, thus DHEA was not needed to be produced in the body. With these reasons, the obtained results were opposed our hypothesis that CWM can increase DHEA level. So we aggregated that DHEA seems to associate with oxidative stress condition.

Therefore, our data in this study showed that CWM can enhance antioxidative activity against ROS in cell line and blood of healthy humans. Antioxidant defense categorize into two types: non-enzymatic and enzymatic antioxidant (9, 118). Enzymatic antioxidant defense mechanism is the process that ROS is elimilated by antioxidant enzymes such as catalase and superoxide dismutase. CWM may regulate enzyme activity through gene expression or enhancing antioxidant enzymes directly. The extensive study should be evaluated more on the mechanisms of CWM through the antioxidant activities. The initial results on the effect of CWM in this human study was suggested that CWM should possibly reduce oxidative stress in patients with certain diseases in the future clinical trial.

# CHAPTER VI
# CONCLUSION

Reactive oxygen species (ROS) are products of normal cellular metabolism. The effect of excessive free radicals causing potential biological damage is termed oxidative stress. ROS could induce cell injury has been implicated in the neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease. They are able to initiate some detrimental effects including DNA protein lipid peroxidation, apoptosis and necrosis. The production of ROS occurs mostly in the electron transport chain in the mitochondria where a small number of electrons are interacted with oxygen to form the superoxide free radical. It has been known that antioxidant compounds have the ability to protect oxidative damage. Many compounds from plant have been reported to contain many pharmacological and biological activities. Recent researches on plant derived drugs are receiving high attention in order to produce novel drugs. Traditional medicine using various parts of plants has been reported to enhance cognitive function. However, some drug therapies are limited in their antioxidative effectiveness. Therefore, many antioxidative drugs are on intensive trials to discover a novel potential antioxidative drug.

*Garcinia mangostana* L. is a source of mangostin. xanthone, tannin, flavones and other bioactive substances. It has been reported about the protective effects against oxidative stress, inflammation. Infection and etc. Xantone plentiful in mangosteen have potent antioxidant properties. Therefore, mangosteen should be a good candidate to protect against oxidative damage. Previous studies have demonstrated that the partially purified crude water extract of mangosteen (CWM) can prevent oxidative damage in various cultured neuronal cells and in scopolamine treated mice. In this reason, we chose to evaluate the antioxidative effect of crude water extract of mangosteen (CWM) after filling into the capsules and effect on the changes in reactive oxygen species (ROS) and biological markers; hydroxynonenal (HNE) and dehydroepiandosterone (DHEA) in healthy humans after taking CWM

daily for 6 months. Volunteers whose weight less than 55 kg and more than 55 kg, had taken a daily dose of CWM at 220 mg and 280 mg, respectively. Both dosages of the capsules were checked for quality control. The results showed that capsules of each dosage form after production were within the standard complied with the British Pharmacopoeia 2014 (BP). From preliminary test, they were found that CWM (spray dry, capsule size No 1 and 0) possessed powerful antioxidant activity against DPPH at $IC_{50}$ less than 50 μg/ml and was no different in potency between 6 month storage capsule and freshly prepared capsule. They could significantly decrease ROS levels at 100 and 200 μg/ml. In MTT assay, the 50% of SK-N-SH cell viability resulted from exposed with CWM at dose more than 100 μg/ml, supposed that CWM was safe to cells. From these results demonstrated the interesting efficacy of CWM on antioxidant activity and safety. In this study, we further investigated the antioxidant activity of CWM in healthy volunteers for 6 months.

The crude water extract of mangosteen resulted in a significant increase in antioxidant capacity of RBCs, which was determined by the fluorescent signal from DCF emitted from hydrogen peroxide-incubated RBCs. A significant increase in antioxidant capacity of RBCs could be detected from D84 of oral administration of CWM. Therefore, our results suggested that CWM could augment antioxidant system in human RBCs.

We also investigated the increased antioxidant capacity affected protein related to oxidative damage in the whole blood and RBCs. In our study, we determined the level of 4 hydroxynonenal (HNE) modification for studying the oxidative damage. HNE is a byproduct of lipid peroxidation, which is an indicator for oxidative stress. HNE can react with lysine, histidine or cysteine residue in protein to form adducts, resulting in structural and functional changes of the proteins. In previous reports showed that HNE can be involved in the pathophysiology of many diseases such as cardiovascular disease, Parkinson disease and Alzheimer's disease. We estimated that if the body has an improved antioxidant capacity, lipid peroxidation should be reduced and HNE modification to proteins should then be decreased. The result was shown that CWM could significantly decrease the HNE modification in the whole blood (WB) and red blood cells (RBCs) of healthy volunteers taking CWM

starting on D28 and D168, respectively. This result provided the supporting evidence for antioxidant property of mangosteen extract.

In addition, we investigated the effect of CWM on the change in dehydroxyepiandrosterone (DHEA). In previous studies reported that level of DHEA associated some diseases such as Alzheimer's disease (AD) and found the ability to protect cells from oxidative damage in the hippocampus part of the brain. They found that AD patient had lower level compared with healthy human. In some reports, they reported that oxidative stress had effect to change DHEA level. In this reason, we speculated that if the body have an improved antioxidant capacity DHEA should be increased. Our results show that CWM had an effect on ROS level, thus DHEA was not needed to be produced in the body. From this reason, DHEA level did not significantly alter. Therefore, we aggregated that DHEA seems to associate with oxidative stress condition. The study indicated the antioxidant activity of CWM intake by reducing ROS and HNE levels in healthy volunteers. CWM should possibly reduce oxidative stress in patients with certain diseases in the future after a clinical trial.

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# APPENDICES

# APPENDIX A
# MADIA AND REAGENTS

## 1. MEM medium

| | |
|---|---|
| MEM medium | 9.5 g |
| (with Earle's salts and L-glutamine, without Sodium bicarbonate) | |
| $NaHCO_3$ | 1.5 g |
| Non-essential amino acid | 0.1 mM |
| Sodium pyruvate | 1.0 mM |
| 100 µg/ml Streptomycin – 100 units/ml Penicillin G | 10 ml |
| Sterile water for injection to make | 1,000 ml |

Adjust pH 7.4 with 1.5 N HCL or 1 N NaOH before adjust volume with water and sterilize by cellulose acetate filter (pore size 0.2 µm).

## 2. Freezing medium

| | |
|---|---|
| MEM medium | 63 % |
| Fetal bovine serum | 27 % |
| Dimethysulfoxide (DMSO) | 10 % |

## 3. Phosphate buffer saline

| | |
|---|---|
| NaCl | 8 g |
| KCl | 0.2 g |
| $Na_2HPO_4$ | 1.44 g |
| $KH_2PO_4$ | 0.22 g |

Adjust pH 7.4 with 1.5 N HCL or 1 N NaOH before adjust volume with water and sterilize by autoclave and store at 4 °C.

**4. 0.01% PBST**

| | |
|---|---|
| PBS | 1,000 ml |
| Tween-20 | 1 ml |

**5. 5 mg/ml MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] for MTT assay**

| | |
|---|---|
| MTT | 0.05 g |
| PBS | 10 ml |

**6. 1 mM 2',7'-Dichlorodihydro fluorescein diacetate (DCFH-DA) stock solution**

| | |
|---|---|
| DCFH-DA | 0.0048 g |
| DMSO | 10 ml |

Store in aliquots at -20 °C

# APPENDIX B
# RAW DATA OF ANTIOXIDANT ACTIVITIES

**Intensity of HNE and Actin from red blood cells of individual samples.**

| Sample No. | Day | Intensity of HNE | Intensity of Actin | HNE/Actin |
|---|---|---|---|---|
| **1** | **0** | 11587.63 | 8796.305 | 1.320 |
| | **7** | 8644.497 | 5303.77 | 1.630 |
| | **28** | 5101.719 | 1532.113 | 3.330 |
| | **84** | 10632.55 | 9880.79 | 1.076 |
| | **112** | 10098.72 | 5844.891 | 1.728 |
| | **168** | 13348.79 | 10828.45 | 1.233 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **2** | **0** | 4682.426 | 7687.841 | 0.609 |
| | **7** | 3529.527 | 7555.941 | 0.467 |
| | **28** | 4322.062 | 7438.548 | 0.581 |
| | **84** | 5667.355 | 8055.426 | 0.704 |
| | **112** | 2465.062 | 7811.77 | 0.316 |
| | **168** | 2401.82 | 6008.062 | 0.400 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **3** | **0** | 7453.305 | 6610.113 | 1.128 |
| | **7** | 7268.891 | 6341.82 | 1.146 |
| | **28** | 7406.355 | 6073.648 | 1.219 |
| | **84** | 7162.012 | 6009.012 | 1.192 |
| | **112** | 6437.941 | 5929.184 | 1.086 |
| | **168** | 5058.991 | 5813.941 | 0.868 |

**Intensity of HNE and Actin from red blood cells of individual samples. (continuted)**

| Sample No. | Day | Intensity of HNE | Intensity of Actin | HNE/Actin |
|---|---|---|---|---|
| **4** | **0** | 7201.719 | 3886.355 | 1.853 |
| | **7** | 7206.891 | 4134.305 | 1.743 |
| | **28** | 6994.891 | 4976.355 | 1.406 |
| | **84** | 6334.062 | 4177.820 | 1.516 |
| | **112** | 4938.012 | 3281.234 | 1.505 |
| | **168** | 3867.284 | 3953.113 | 0.978 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **5** | **0** | 9394.912 | 3803.497 | 2.470 |
| | **7** | 6453.134 | 4827.184 | 1.337 |
| | **28** | 7179.426 | 4647.598 | 1.545 |
| | **84** | 7297.012 | 4463.426 | 1.635 |
| | **112** | 5502.062 | 4378.548 | 1.257 |
| | **168** | 4893.891 | 5882.669 | 0.832 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **6** | **0** | 9379.305 | 6096.234 | 1.539 |
| | **7** | 6730.941 | 6513.062 | 1.033 |
| | **28** | 7465.648 | 6135.234 | 1.217 |
| | **84** | 8162.598 | 7832.184 | 1.042 |
| | **112** | 7226.234 | 7174.355 | 1.007 |
| | **168** | 8565.184 | 9881.305 | 0.867 |

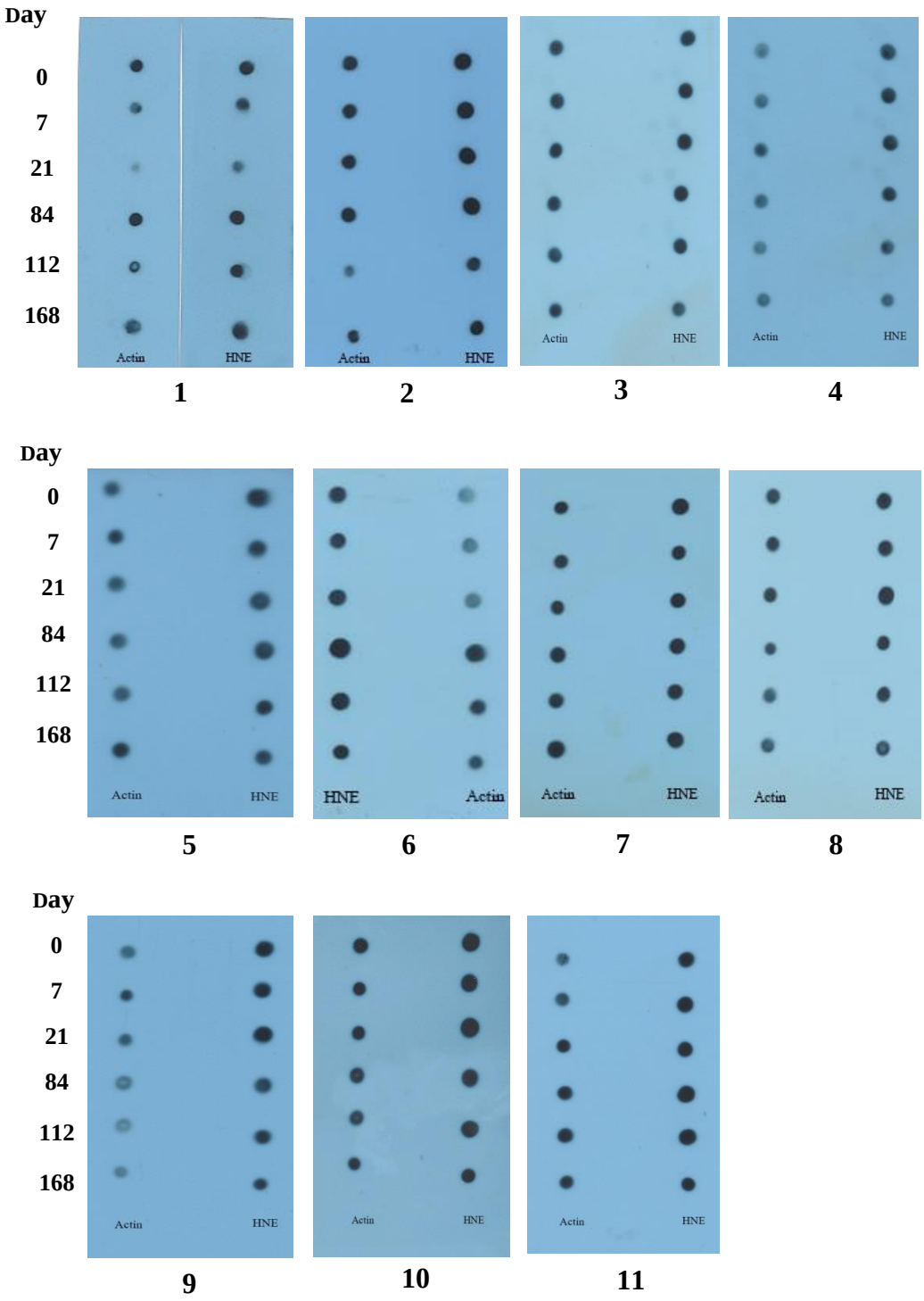
**Intensity of HNE and Actin from red blood cells of individual samples. (continuted)**

| Sample No. | Day | Intensity of HNE | Intensity of Actin | HNE/Actin |
|---|---|---|---|---|
| **7** | **0** | 3134.062 | 6416.527 | 0.488 |
| | **7** | 3363.406 | 5601.062 | 0.600 |
| | **28** | 3504.820 | 6681.477 | 0.525 |
| | **84** | 8200.477 | 10108.134 | 0.811 |
| | **112** | 5271.648 | 7675.77 | 0.687 |
| | **168** | 4194.941 | 5826.477 | 0.720 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **8** | **0** | 9318.719 | 7248.305 | 1.286 |
| | **7** | 8609.891 | 7045.477 | 1.222 |
| | **28** | 10643.891 | 6564.355 | 1.621 |
| | **84** | 6839.355 | 4509.284 | 1.517 |
| | **112** | 7279.77 | 5579.062 | 1.260 |
| | **168** | 6394.527 | 2726.527 | 1.117 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **9** | **0** | 8667.477 | 3694.113 | 2.346 |
| | **7** | 7706.719 | 3680.113 | 2.094 |
| | **28** | 9376.891 | 3421.698 | 2.740 |
| | **84** | 6497.648 | 3601.406 | 1.804 |
| | **112** | 6409.941 | 2543.914 | 2.520 |
| | **168** | 4304.991 | 2269.527 | 1.897 |

**Intensity of HNE and Actin from red blood cells of individual samples. (continuted)**

| Sample No. | Day | Intensity of HNE | Intensity of Actin | HNE/Actin |
|---|---|---|---|---|
| **10** | **0** | 6109.719 | 1365.184 | 4.475 |
| | **7** | 4600.355 | 1705.941 | 2.697 |
| | **28** | 5122.719 | 4274.891 | 1.198 |
| | **84** | 6156.305 | 3521.355 | 1.748 |
| | **112** | 6008.912 | 4540.619 | 1.323 |
| | **168** | 3380.82 | 3279.184 | 1.031 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **11** | **0** | 7969.426 | 3322.284 | 2.399 |
| | **7** | 8228.184 | 4683.234 | 1.757 |
| | **28** | 7359.305 | 5550.770 | 1.326 |
| | **84** | 9195.376 | 6359.770 | 1.446 |
| | **112** | 8533.77 | 6884.305 | 1.240 |
| | **168** | 5766.062 | 5324.770 | 1.083 |

**HNE and Actin from red blood cells of individual samples.**



**Intensity of HNE and Actin from whole blood of individual samples.**

| Sample No. | Day | Intensity of HNE | Intensity of Actin | HNE/Actin |
|---|---|---|---|---|
| **1** | **0** | 3040.548 | 617.698 | 4.922 |
| | **7** | 4110.841 | 1253.698 | 3.279 |
| | **28** | 3888.941 | 1924.406 | 2.021 |
| | **84** | 5709.184 | 4055.477 | 1.408 |
| | **112** | 5420.062 | 3482.719 | 1.556 |
| | **168** | 7861.740 | 6159.841 | 1.250 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **2** | **0** | 6636.184 | 2985.770 | 2.223 |
| | **7** | 8364.891 | 2770.184 | 3.020 |
| | **28** | 8552.255 | 6105.062 | 1.401 |
| | **84** | 8272.083 | 4693.770 | 1.762 |
| | **112** | 8130.891 | 4157.548 | 1.956 |
| | **168** | 8380.962 | 5084.841 | 1.648 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **3** | **0** | 11330.205 | 9633.79 | 1.176 |
| | **7** | 7587.305 | 11160.497 | 0.680 |
| | **28** | 8925.376 | 10507.376 | 0.849 |
| | **84** | 9798.74 | 7357.548 | 1.332 |
| | **112** | 10084.205 | 7645.548 | 1.319 |
| | **168** | 8109.669 | 11108.376 | 0.730 |

**Intensity of HNE and Actin from whole blood of individual samples. (continuted)**

| Sample No. | Day | Intensity of HNE | Intensity of Actin | HNE/Actin |
|---|---|---|---|---|
| **4** | **0** | 10579.255 | 4708.062 | 2.247 |
| | **7** | 10378.012 | 8114.891 | 1.279 |
| | **28** | 10932.719 | 9623.012 | 1.136 |
| | **84** | 7824.134 | 9035.770 | 0.866 |
| | **112** | 5831.648 | 8399.184 | 0.694 |
| | **168** | 6320.598 | 9212.012 | 0.686 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **5** | **0** | 8029.205 | 5917.012 | 1.357 |
| | **7** | 6245.811 | 4751.861 | 1.314 |
| | **28** | 6990.548 | 5616.912 | 1.245 |
| | **84** | 7762.861 | 4837.891 | 1.605 |
| | **112** | 4506.548 | 3252.426 | 1.386 |
| | **168** | 3971.527 | 3698.518 | 1.074 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **6** | **0** | 8414.841 | 8782.619 | 0.958 |
| | **7** | 6603.841 | 8578.782 | 0.770 |
| | **28** | 2967.648 | 6336.619 | 0.468 |
| | **84** | 2870.184 | 6935.296 | 0.414 |
| | **112** | 3326.305 | 6080.841 | 0.547 |
| | **168** | 3018.033 | 4957.598 | 0.609 |

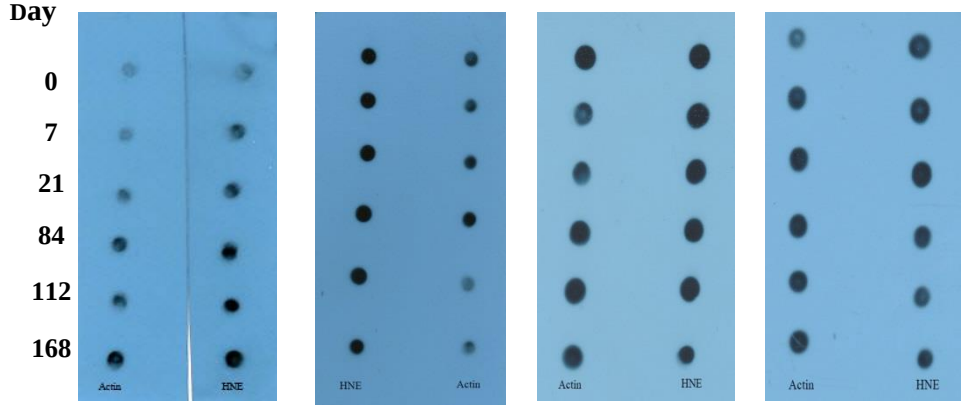
**Intensity of HNE and Actin from whole blood of individual samples. (continuted)**

| Sample No. | Day | Intensity of HNE | Intensity of Actin | HNE/Actin |
|---|---|---|---|---|
| **7** | **0** | 6511.719 | 1321.598 | 4.927 |
| | **7** | 6273.305 | 1720.184 | 3.647 |
| | **28** | 9832.426 | 5178.184 | 1.899 |
| | **84** | 9925.719 | 5727.598 | 1.733 |
| | **112** | 10730.548 | 5421.891 | 1.979 |
| | **168** | 9609.376 | 5172.426 | 1.858 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **8** | **0** | 8147.134 | 7881.426 | 1.034 |
| | **7** | 9052.426 | 9312.548 | 0.972 |
| | **28** | 8940.962 | 11749.861 | 0.761 |
| | **84** | 6464.669 | 8364.790 | 0.773 |
| | **112** | 6460.426 | 8627.426 | 0.749 |
| | **168** | 3675.527 | 8937.841 | 0.411 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **9** | **0** | 10337.497 | 10031.134 | 1.031 |
| | **7** | 9725.426 | 8831.305 | 1.101 |
| | **28** | 7734.891 | 8270.719 | 0.935 |
| | **84** | 7979.841 | 9119.012 | 0.875 |
| | **112** | 6765.598 | 9998.912 | 0.677 |
| | **168** | **6188.719** | **6922.305** | **0.894** |

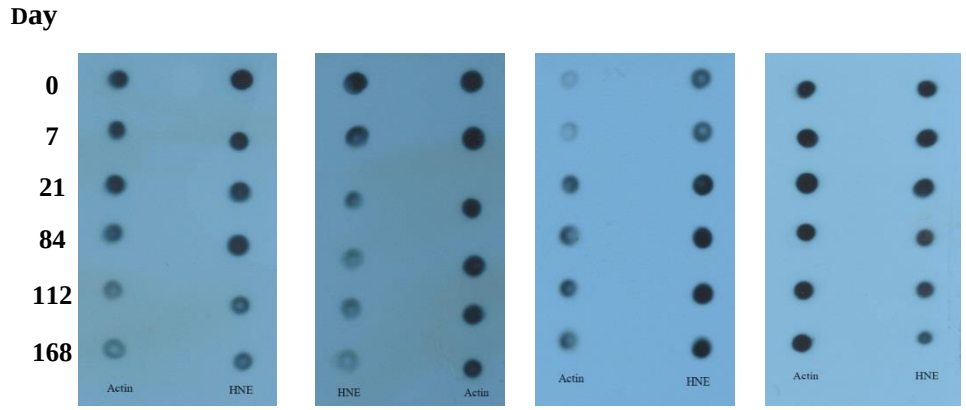
**Intensity of HNE and Actin from whole blood of individual samples. (continuted)**

| Sample No. | Day | Intensity of HNE | Intensity of Actin | HNE/Actin |
|---|---|---|---|---|
| **10** | **0** | 11249.497 | 6788.891 | 1.657 |
| | **7** | 9196.134 | 7477.598 | 1.230 |
| | **28** | 12138.790 | 11999.376 | 1.012 |
| | **84** | 10421.841 | 9351.184 | 1.114 |
| | **112** | 9726.012 | 96953669 | 1.003 |
| | **168** | 8647.012 | 10493.669 | 0.824 |
| **Sample No.** | **Day** | **Intensity of HNE** | **Intensity of Actin** | **HNE/Actin** |
| **11** | **0** | 12372.205 | 6158.477 | 2.009 |
| | **7** | 12032.548 | 5212.154 | 2.309 |
| | **28** | 12294.619 | 5910.598 | 2.080 |
| | **84** | 12548.790 | 8218.598 | 1.527 |
| | **112** | 10913.790 | 8526.184 | 1.280 |
| | **168** | 10140.305 | 10538.086 | 0.962 |

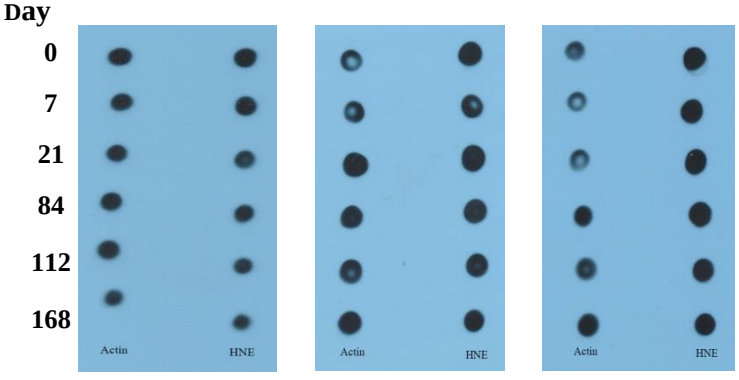
**HNE and Actin from whole blood cells of individual samples.**




1 2 3 4




5 6 7 8

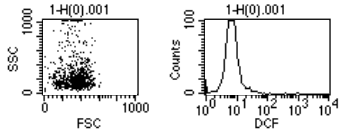



9 10 11

# Mean fluorescent intensity data of individual samples

## Mean fluorescent intensity (MFI) of sample 1

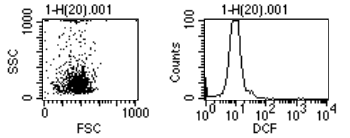
Date: 12122012 Day 0 subject 1(Surachai)




File: 1-H(0).001 Sample ID: 1-H(0)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.73 | 6.90 | 129 |

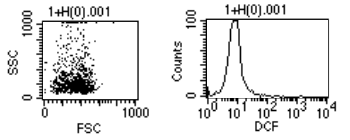
unsti at 0 min




File: 1-H(20).001 Sample ID: 1-H(20)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.95 | 8.86 | 128 |

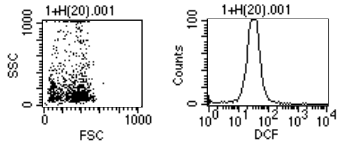
unsti at 20 min




File: 1+H(0).001 Sample ID: 1+H(0)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.00 | 7.72 | 123 |

sti at 0 min

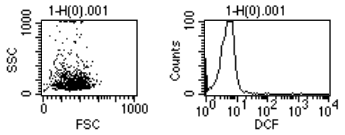



File: 1+H(20).001 Sample ID: 1+H(20)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 33.38 | 29.66 | 115 |

sti at 20 min

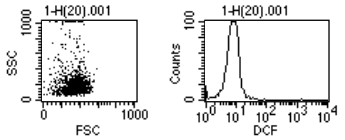
Date: 19122012 Day 7 Subject 1 Surachai




File: 1-H(0).001 Sample ID: 1-H(0)
Patient ID: Surachai Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.76 | 5.00 | 108 |

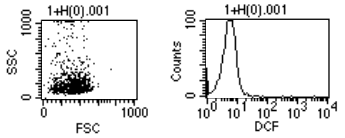
unsti at 0 min




File: 1-H(20).001 Sample ID: 1-H(20)
Patient ID: Surachai Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.92 | 7.33 | 137 |

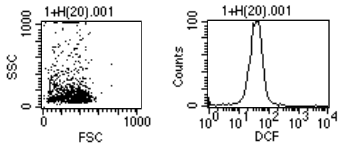
unsti at 20 min




File: 1+H(0).001 Sample ID: 1+H(0)
Patient ID: Surachai Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.30 | 5.39 | 118 |

sti at 0 min



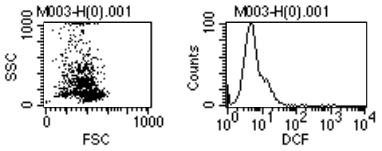

File: 1+H(20).001 Sample ID: 1+H(20)
Patient ID: Surachai Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 39.81 | 35.86 | 115 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 1 (continued)

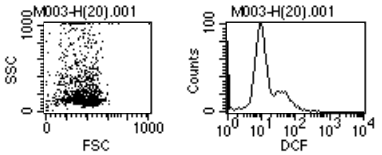
Date: 09012013 Day 28 Subject M003 Surachai




File: M003-H(0).001 Sample ID: M003-H(0)
Patient ID: Surachai Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.98 | 5.53 | 106 |

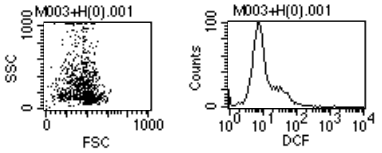
unsti at 0 min




File: M003-H(20).001 Sample ID: M003-H(20)
Patient ID: Surachai Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 16.92 | 12.02 | 110 |

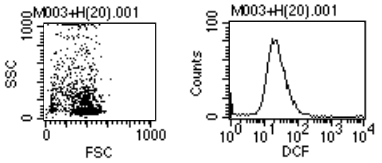
unsti at 20 min




File: M003+H(0).001 Sample ID: M003+H(0)
Patient ID: Surachai Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 13.77 | 9.84 | 102 |

sti at 0 min

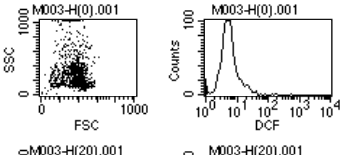



File: M003+H(20).001 Sample ID: M003+H(20)
Patient ID: Surachai Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 29.32 | 22.62 | 80 |

sti at 20 min

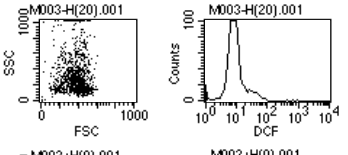
Date: 09012013 Week4 Subject M 003 Surachai




File: M003-H(0).001 Sample ID: M003-H(0)
Patient ID: Surachai Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.19 | 5.37 | 130 |

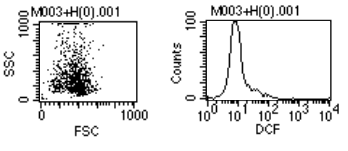
unsti at 0 min




File: M003-H(20).001 Sample ID: M003-H(20)
Patient ID: Surachai Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.01 | 8.49 | 143 |

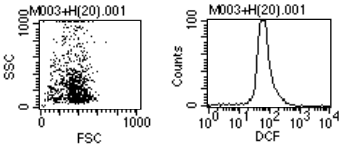
unsti at 20 min




File: M003+H(0).001 Sample ID: M003+H(0)
Patient ID: Surachai Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 11.62 | 8.88 | 121 |

sti at 0 min



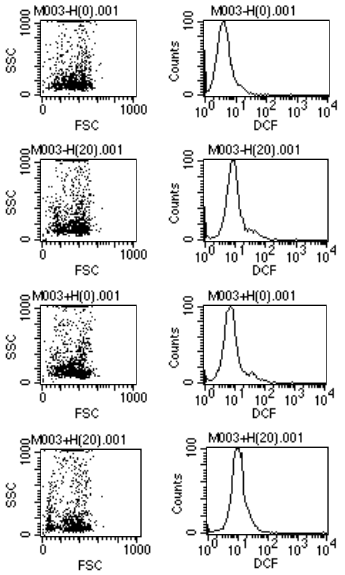

File: M003+H(20).001 Sample ID: M003+H(20)
Patient ID: Surachai Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 71.34 | 62.44 | 115 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 1 (continued)

Date: 06032013 Week 12 Subject M003 Surachi




File: M003-H(0).001 Sample ID: M003-H(0)
Patient ID: Surachai Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 4.78 | 4.12 | 114 |

unsti at 0 min




File: M003-H(20).001 Sample ID: M003-H(20)
Patient ID: Vithoon Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.98 | 8.84 | 105 |

unsti at 20 min




File: M003+H(0).001 Sample ID: M003+H(0)
Patient ID: Surachai Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.52 | 7.50 | 104 |

sti at 0 min

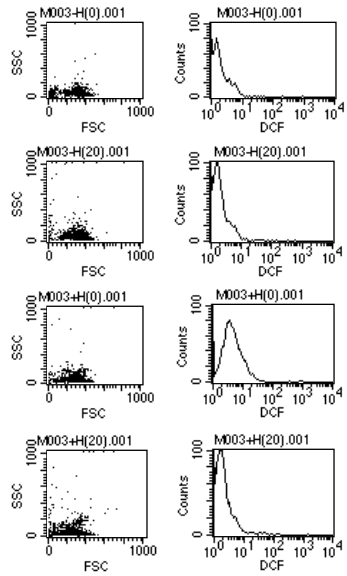



File: M003+H(20).001 Sample ID: M003+H(20)
Patient ID: Vithoon Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 12.18 | 10.19 | 107 |

sti at 20 min

Date: 09042013 Day 112 Subject M003 Surachai




File: M003-H(0).001 Sample ID: M003-H(0)
Patient ID: Surachai Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.93 | 1.54 | 3657 |

unsti at 0 min




File: M003-H(20).001 Sample ID: M003-H(20)
Patient ID: Surachai Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.24 | 1.80 | 1406 |

unsti at 20 min




File: M003+H(0).001 Sample ID: M003+H(0)
Patient ID: Surachai Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.34 | 4.13 | 289 |

sti at 0 min



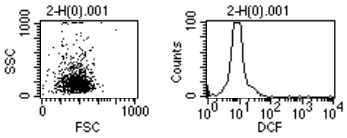

File: M003+H(20).001 Sample ID: M003+H(20)
Patient ID: Surachai Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.56 | 1.94 | 855 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 2

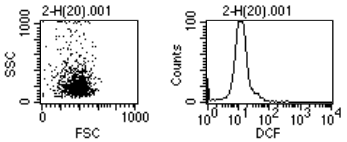
Date: 12122012 Day 0 subject 2 (Vithoon)




File: 2-H(0).001 Sample ID: 2-H(0)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.25 | 8.43 | 128 |

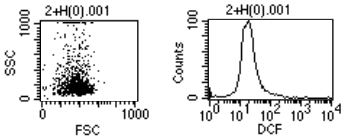
unsti at 0 min




File: 2-H(20).001 Sample ID: 2-H(20)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 12.87 | 11.46 | 126 |

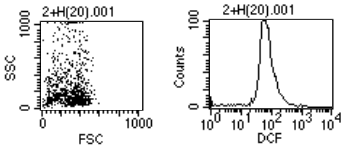
unsti at 20 min




File: 2+H(0).001 Sample ID: 2+H(0)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 22.99 | 18.48 | 98 |

sti at 0 min

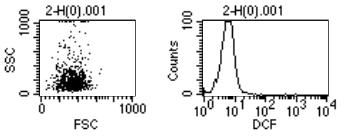



File: 2+H(20).001 Sample ID: 2+H(20)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 75.92 | 65.44 | 112 |

sti at 20 min

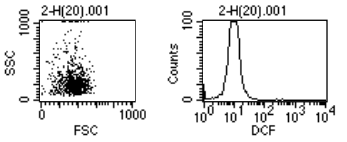
Date: 19122012 Day 7 Subject 2 Vithoon




File: 2-H(0).001 Sample ID: 2-H(0)
Patient ID: Vithoon Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.36 | 5.76 | 111 |

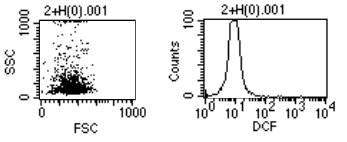
unsti at 0 min




File: 2-H(20).001 Sample ID: 2-H(20)
Patient ID: Vithoon Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.01 | 9.35 | 142 |

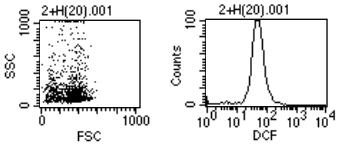
unsti at 20 min




File: 2+H(0).001 Sample ID: 2+H(0)
Patient ID: Vithoon Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.55 | 8.64 | 124 |

sti at 0 min



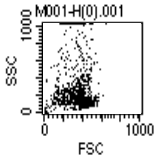

File: 2+H(20).001 Sample ID: 2+H(20)
Patient ID: Vithoon Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 53.68 | 48.62 | 125 |

sti at 20 min

## Mean fluorescent intensity (MFI) of sample 2 (Continued)

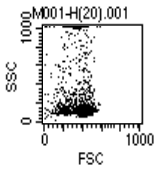
Date: 09012013 Day 28 Subject M001 Witool




File: M001-H(0).001 Sample ID: M001-H(0)
Patient ID: Vithoon Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.78 | 6.29 | 109 |

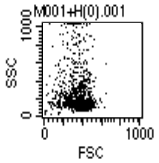
unsti at 0 min




File: M001-H(20).001 Sample ID: M001-H(20)
Patient ID: Witool Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 23.58 | 16.75 | 104 |

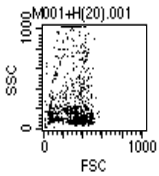
unsti at 20 min




File: M001+H(0).001 Sample ID: M001+H(0)
Patient ID: Vithoon Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 42.64 | 33.52 | 78 |

sti at 0 min

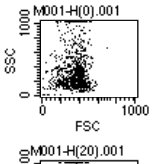



File: M001+H(20).001 Sample ID: M001+H(20)
Patient ID: Witool Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 19.44 | 14.88 | 85 |

sti at 20 min

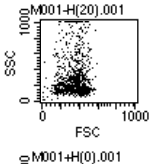
Date: 09012013 Week4 Subject M 001 Vithoon




File: M001-H(0).001 Sample ID: M001-H(0)
Patient ID: Vithoon Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.88 | 6.07 | 125 |

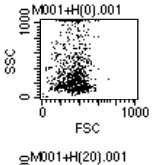
unsti at 0 min




File: M001-H(20).001 Sample ID: M001-H(20)
Patient ID: Yitoon Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.24 | 8.19 | 128 |

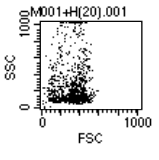
unsti at 20 min




File: M001+H(0).001 Sample ID: M001+H(0)
Patient ID: Vithoon Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 11.94 | 10.01 | 117 |

sti at 0 min



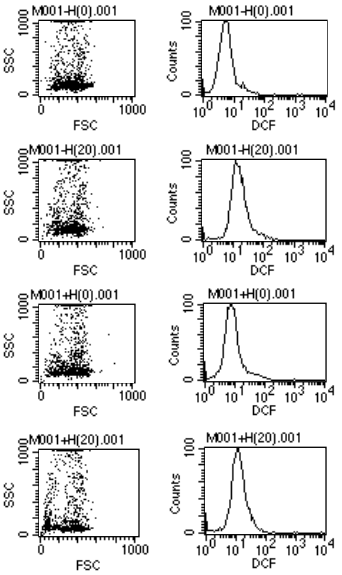

File: M001+H(20).001 Sample ID: M001+H(20)
Patient ID: Yitoon Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 82.90 | 73.70 | 112 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 2 (Continued)

Date: 06032013 Week 12 Subject M001 Vithoon




File: M001-H(0).001 Sample ID: M001-H(0)
Patient ID: Vithoon Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.53 | 5.52 | 111 |

unsti at 0 min




File: M001-H(20).001 Sample ID: M001-H(20)
Patient ID: Vithoon Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 17.51 | 14.74 | 104 |

unsti at 20 min




File: M001+H(0).001 Sample ID: M001+H(0)
Patient ID: Vithoon Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.05 | 8.10 | 103 |

sti at 0 min

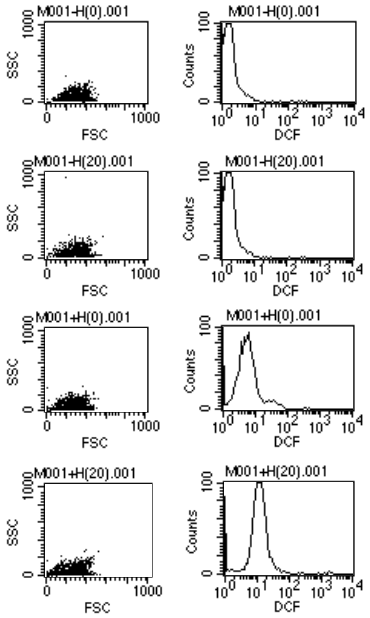



File: M001+H(20).001 Sample ID: M001+H(20)
Patient ID: Vithoon Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 14.94 | 12.28 | 100 |

sti at 20 min

Date: 11042013 Day 112 Subject M001 Vithoon




File: M001-H(0).001 Sample ID: M001-H(0)
Patient ID: Vithoon Acquisition Date: 11-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.92 | 1.66 | 1111 |

unsti at 0 min




File: M001-H(20).001 Sample ID: M001-H(20)
Patient ID: Vithoon Acquisition Date: 11-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.87 | 1.66 | 1068 |

unsti at 20 min




File: M001+H(0).001 Sample ID: M001+H(0)
Patient ID: Vithoon Acquisition Date: 11-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.14 | 5.53 | 137 |

sti at 0 min



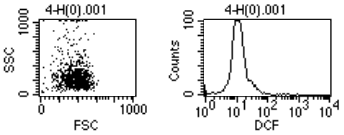

File: M001+H(20).001 Sample ID: M001+H(20)
Patient ID: Vithoon Acquisition Date: 11-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 13.73 | 11.22 | 128 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 3

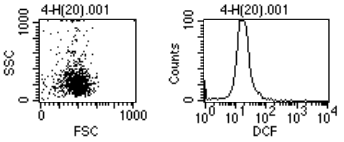
Date: 12122012 Day 0 subject 4 (Pornnapa)




File: 4-H(0).001 Sample ID: 4-H(0)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 13.41 | 11.71 | 119 |

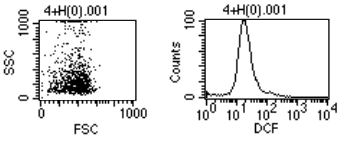
unsti at 0 min




File: 4-H(20).001 Sample ID: 4-H(20)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 19.27 | 16.93 | 116 |

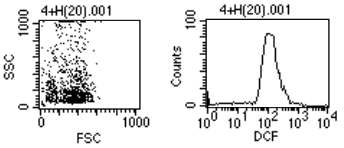
unsti at 20 min




File: 4+H(0).001 Sample ID: 4+H(0)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 21.17 | 17.94 | 110 |

sti at 0 min

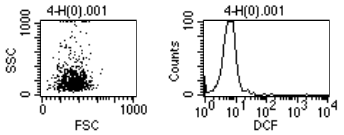



File: 4+H(20).001 Sample ID: 4+H(20)
Patient ID: Acquisition Date: 12-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 130.95 | 107.20 | 83 |

sti at 20 min

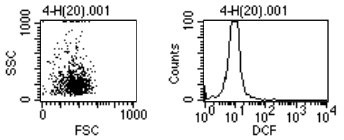
Date: 19122012 Day 7 Subject 4 Pornnapa




File: 4-H(0).001 Sample ID: 4-H(0)
Patient ID: Pornnapa Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 0
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.36 | 5.76 | 111 |

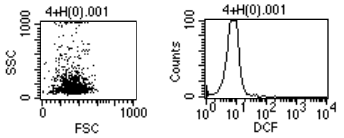
unsti at 0 min




File: 4-H(20).001 Sample ID: 4-H(20)
Patient ID: Pornnapa Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 0
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.01 | 9.35 | 142 |

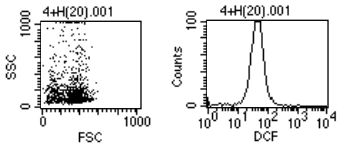
unsti at 20 min




File: 4+H(0).001 Sample ID: 4+H(0)
Patient ID: Pornnapa Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 0
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.55 | 8.64 | 124 |

sti at 0 min



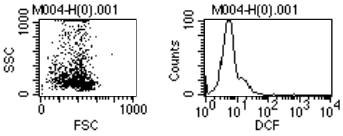

File: 4+H(20).001 Sample ID: 4+H(20)
Patient ID: Pornnapa Acquisition Date: 19-Dec-1
Gate: No Gate Gated Events: 0
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 53.68 | 48.62 | 125 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 3 (Continued)

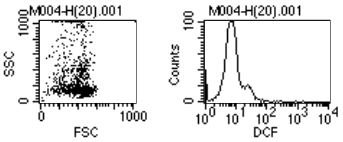
Date: 09012013 Week4 Subject M 004 Pornnapa




File: M004-H(0).001 Sample ID: M004-H(0)
Patient ID: Pornnapa Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.96 | 5.83 | 110 |

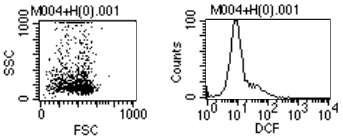
unsti at 0 min




File: M004-H(20).001 Sample ID: M004-H(20)
Patient ID: Pornnapa Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.72 | 7.18 | 119 |

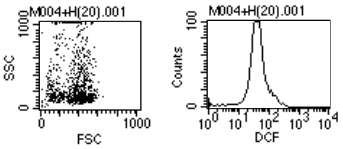
unsti at 20 min




File: M004+H(0).001 Sample ID: M004+H(0)
Patient ID: Pornnapa Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 13.61 | 10.27 | 118 |

sti at 0 min

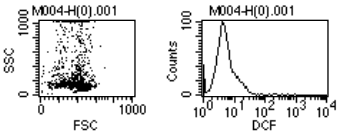



File: M004+H(20).001 Sample ID: M004+H(20)
Patient ID: Pornnapa Acquisition Date: 09-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 45.68 | 39.22 | 110 |

sti at 20 min

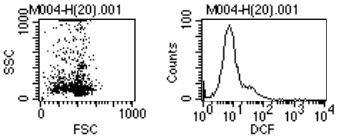
Date: 06032013 Week 12 Subject M004 Pornnapa




File: M004-H(0).001 Sample ID: M004-H(0)
Patient ID: Pornnapa Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.03 | 4.89 | 100 |

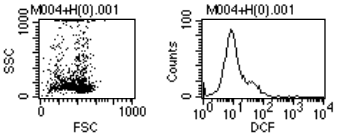
unsti at 0 min




File: M004-H(20).001 Sample ID: M004-H(20)
Patient ID: Vithoon Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 11.32 | 8.12 | 92 |

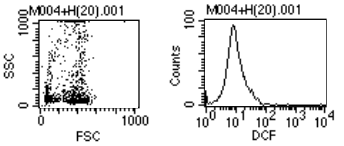
unsti at 20 min




File: M004+H(0).001 Sample ID: M004+H(0)
Patient ID: Pornnapa Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 14.09 | 10.04 | 86 |

sti at 0 min



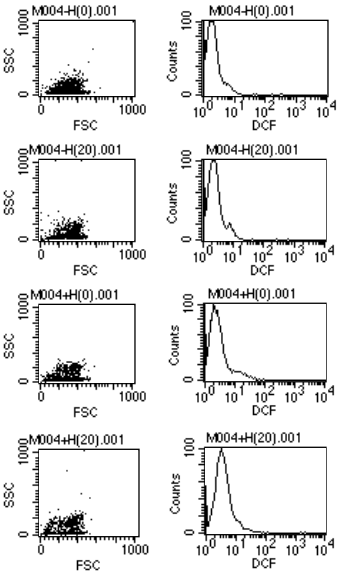

File: M004+H(20).001 Sample ID: M004+H(20)
Patient ID: Vithoon Acquisition Date: 06-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 11.60 | 9.14 | 93 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 3 (Continued)

Date: 09042013 Day 112 Subject M004 Pornnapa




unsti at 0 min

File: M004-H(0).001 Sample ID: M004-H(0)
Patient ID: Pornnapa Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.31 | 1.96 | 665 |

unsti at 20 min

File: M004-H(20).001 Sample ID: M004-H(20)
Patient ID: Pornnapa Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.64 | 2.26 | 419 |

sti at 0 min

File: M004+H(0).001 Sample ID: M004+H(0)
Patient ID: Pornnapa Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 3.49 | 2.67 | 284 |

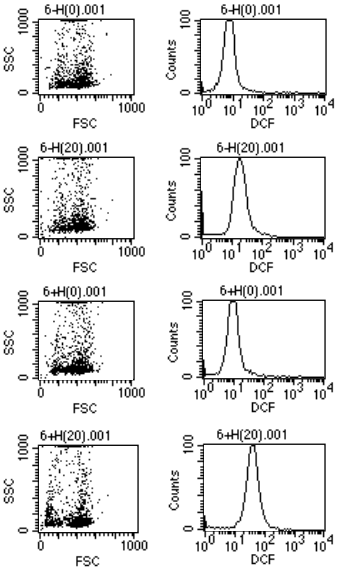
sti at 20 min

File: M004+H(20).001 Sample ID: M004+H(20)
Patient ID: Pornnapa Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 4.63 | 3.67 | 102 |

# Mean fluorescent intensity (MFI) of sample 4

Date: 13122012 Day 0 Subject 6 Sittikarn




File: 6-H(0).001
Patient ID:
Gate: No Gate
Total Events: 10000

Sample ID: 6-H(0)
Acquisition Date: 13-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.23 | 7.60 | 133 |

unsti at 0 min




File: 6-H(20).001
Patient ID:
Gate: No Gate
Total Events: 10000

Sample ID: 6-H(20)
Acquisition Date: 13-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 19.69 | 17.10 | 98 |

unsti at 20 min




File: 6+H(0).001
Patient ID:
Gate: No Gate
Total Events: 10000

Sample ID: 6+H(0)
Acquisition Date: 13-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.82 | 9.31 | 125 |

sti at 0 min



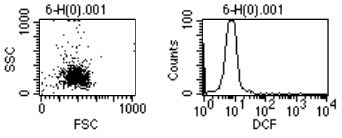

File: 6+H(20).001
Patient ID:
Gate: No Gate
Total Events: 10000

Sample ID: 6+H(20)
Acquisition Date: 13-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 41.55 | 36.61 | 110 |

sti at 20 min

Date: 20122012 Day 7 Subject 6 Sitthikarn

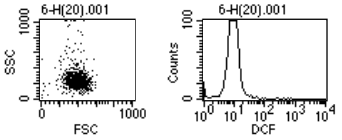



File: 6-H(0).001
Patient ID: Sitthikarn
Gate: No Gate
Total Events: 10000

Sample ID: 6-H(0)
Acquisition Date: 20-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.07 | 7.37 | 148 |

unsti at 0 min

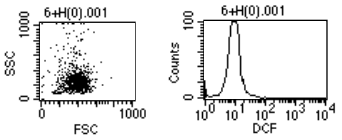



File: 6-H(20).001
Patient ID: Sittikarn
Gate: No Gate
Total Events: 10000

Sample ID: 6-H(20)
Acquisition Date: 20-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.78 | 9.02 | 150 |

unsti at 20 min

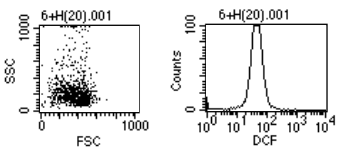



File: 6+H(0).001
Patient ID: Sitthikarn
Gate: No Gate
Total Events: 10000

Sample ID: 6+H(0)
Acquisition Date: 20-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.53 | 8.90 | 141 |

sti at 0 min




File: 6+H(20).001
Patient ID: Sittikarn
Gate: No Gate
Total Events: 10000

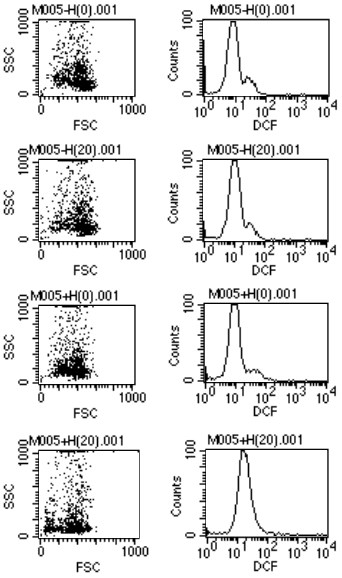
Sample ID: 6+H(20)
Acquisition Date: 20-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 47.56 | 43.61 | 125 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 4 (Continued)

Date: 10012013 Day 28 Subject M005 Sitthikarn




File: M005-H(0).001 Sample ID: M005-H(0)
Patient ID: Sitthikarn Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 11.73 | 9.54 | 124 |

unsti at 0 min




File: M005-H(20).001 Sample ID: M005-H(20)
Patient ID: Sitthikarn Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 13.32 | 10.61 | 119 |

unsti at 20 min




File: M005+H(0).001 Sample ID: M005+H(0)
Patient ID: Sitthikarn Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 12.64 | 9.85 | 112 |

sti at 0 min

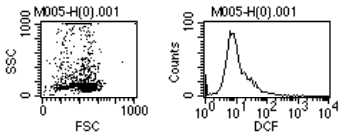



File: M005+H(20).001 Sample ID: M005+H(20)
Patient ID: Sitthikarn Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 21.44 | 17.91 | 99 |

sti at 20 min

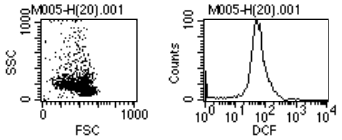
Date: 07032013 Subject M005 Sitthikarn




File: M005-H(0).001 Sample ID: M005-H(0)
Patient ID: Sitthikarn Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 13.41 | 9.50 | 88 |

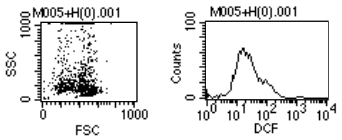
unsti at 0 min




File: M005-H(20).001 Sample ID: M005-H(20)
Patient ID: Sitthikarn Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 55.66 | 46.86 | 104 |

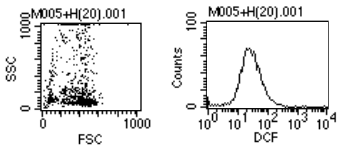
unsti at 20 min




File: M005+H(0).001 Sample ID: M005+H(0)
Patient ID: Sitthikarn Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 33.11 | 22.69 | 64 |

sti at 0 min



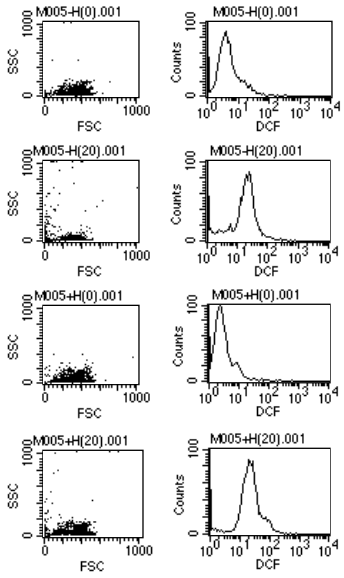

File: M005+H(20).001 Sample ID: M005+H(20)
Patient ID: Sitthikarn Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 34.37 | 25.89 | 68 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 4 (Continued)

Date: 10042013 D 112 Subject M005 Sitthikarn




File: M005-H(0).001 Sample ID: M005-H(0)
Patient ID: Sitthikarn Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.70 | 4.16 | 591 |

unsti at 0 min




File: M005-H(20).001 Sample ID: M005-H(20)
Patient ID: Sitthikarn Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 18.61 | 12.45 | 604 |

unsti at 20 min




File: M005+H(0).001 Sample ID: M005+H(0)
Patient ID: Sitthikarn Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 3.61 | 2.84 | 230 |

sti at 0 min

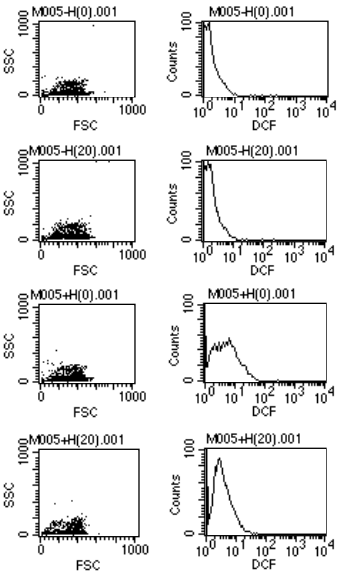



File: M005+H(20).001 Sample ID: M005+H(20)
Patient ID: Sitthikarn Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 26.40 | 19.84 | 244 |

sti at 20 min

Date: 30052013 Subject M005 Sittikarn




File: M005-H(0).001 Sample ID: M005-H(0)
Patient ID: Sittikarn Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.80 | 1.56 | 2189 |

unsti at 0 min




File: M005-H(20).001 Sample ID: M005-H(20)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.82 | 1.59 | 2010 |

unsti at 20 min




File: M005+H(0).001 Sample ID: M005+H(0)
Patient ID: Sittikarn Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.46 | 4.56 | 328 |

sti at 0 min



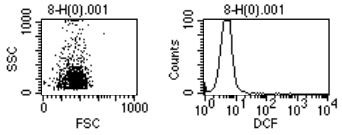

File: M005+H(20).001 Sample ID: M005+H(20)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 3.93 | 3.27 | 171 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 5

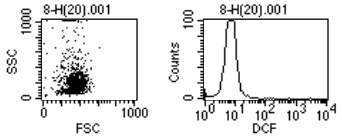
Date: 13122012 Day 0 Subject 8 Chatri




File: 8-H(0).001 Sample ID: 8-H(0)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.32 | 4.88 | 129 |

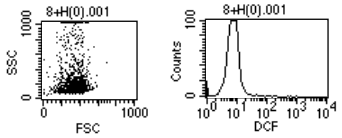
unsti at 0 min




File: 8-H(20).001 Sample ID: 8-H(20)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.63 | 6.86 | 144 |

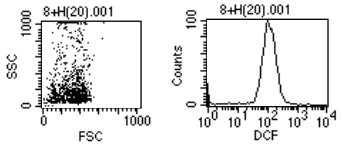
unsti at 20 min




File: 8+H(0).001 Sample ID: 8+H(0)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.10 | 7.31 | 148 |

sti at 0 min

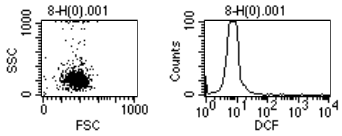



File: 8+H(20).001 Sample ID: 8+H(20)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 118.40 | 103.63 | 101 |

sti at 20 min

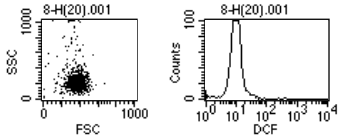
Date: 20122012 Day 7 Subject 8 Chatri




File: 8-H(0).001 Sample ID: 8-H(0)
Patient ID: Chatri Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.39 | 7.12 | 148 |

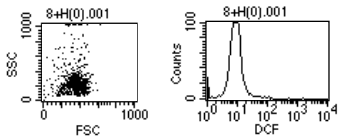
unsti at 0 min




File: 8-H(20).001 Sample ID: 8-H(20)
Patient ID: Chatri Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.11 | 9.45 | 151 |

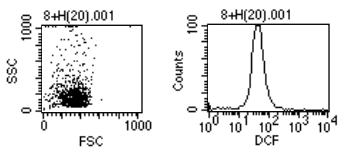
unsti at 20 min




File: 8+H(0).001 Sample ID: 8+H(0)
Patient ID: Chatri Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.24 | 8.31 | 141 |

sti at 0 min



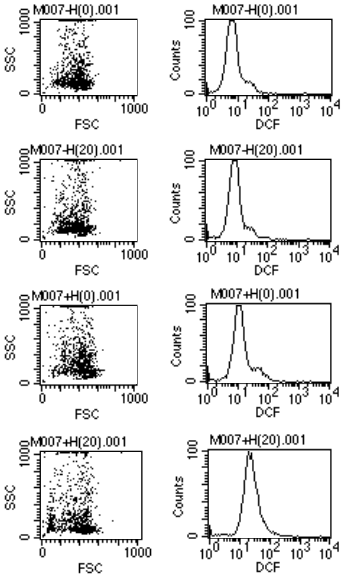

File: 8+H(20).001 Sample ID: 8+H(20)
Patient ID: Chatri Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 47.34 | 42.08 | 124 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 5 (Continued)

Date: 10012013 Day 28 Subject M007 Chatri




File: M007-H(0).001 Sample ID: M007-H(0)
Patient ID: Chatri Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.37 | 6.95 | 133 |

unsti at 0 min

File: M007-H(20).001 Sample ID: M007-H(20)
Patient ID: Chatri Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.04 | 8.33 | 124 |

unsti at 20 min

File: M007+H(0).001 Sample ID: M007+H(0)
Patient ID: Chatri Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 15.36 | 11.97 | 118 |

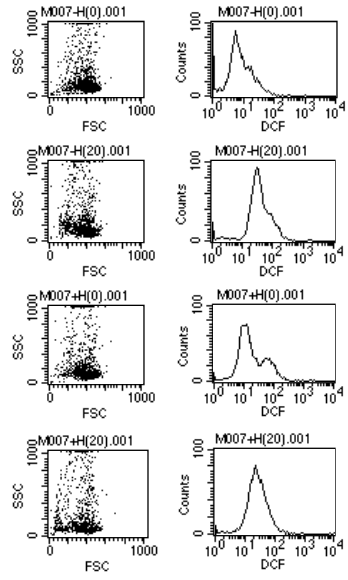
sti at 0 min

File: M007+H(20).001 Sample ID: M007+H(20)
Patient ID: Chatri Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 27.25 | 22.54 | 99 |

sti at 20 min

Date: 07032013 Subject M007 Chatri




File: M007-H(0).001 Sample ID: M007-H(0)
Patient ID: Chatri Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.54 | 7.52 | 164 |

unsti at 0 min

File: M007-H(20).001 Sample ID: M007-H(20)
Patient ID: Chatri Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 41.15 | 32.40 | 90 |

unsti at 20 min

File: M007+H(0).001 Sample ID: M007+H(0)
Patient ID: Chatri Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 25.65 | 16.56 | 73 |

sti at 0 min

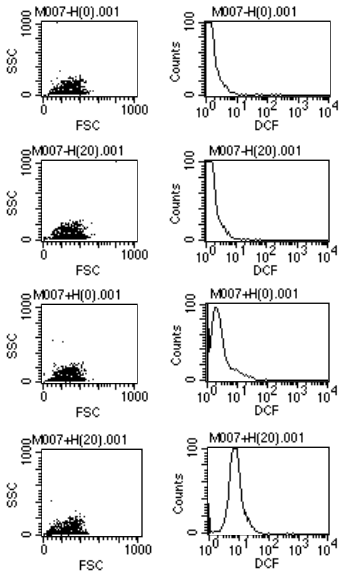
File: M007+H(20).001 Sample ID: M007+H(20)
Patient ID: Chatri Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 30.27 | 23.60 | 79 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 5 (Continued)

Date: 30052013 Subject M007 Chatri




File: M007-H(0).001 Sample ID: M007-H(0)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.62 | 1.43 | 2322 |

unsti at 0 min




File: M007-H(20).001 Sample ID: M007-H(20)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.64 | 1.49 | 1874 |

unsti at 20 min




File: M007+H(0).001 Sample ID: M007+H(0)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.94 | 2.31 | 484 |

sti at 0 min

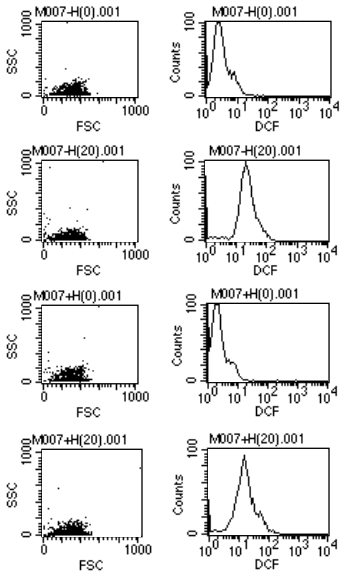



File: M007+H(20).001 Sample ID: M007+H(20)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.76 | 7.71 | 113 |

sti at 20 min

Date: 10042013 D 112 Subject M007 Chatri




File: M007-H(0).001 Sample ID: M007-H(0)
Patient ID: Chatri Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 3.60 | 3.05 | 121 |

unsti at 0 min




File: M007-H(20).001 Sample ID: M007-H(20)
Patient ID: Chatri Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 25.18 | 21.00 | 105 |

unsti at 20 min




File: M007+H(0).001 Sample ID: M007+H(0)
Patient ID: Chatri Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.85 | 2.36 | 247 |

sti at 0 min



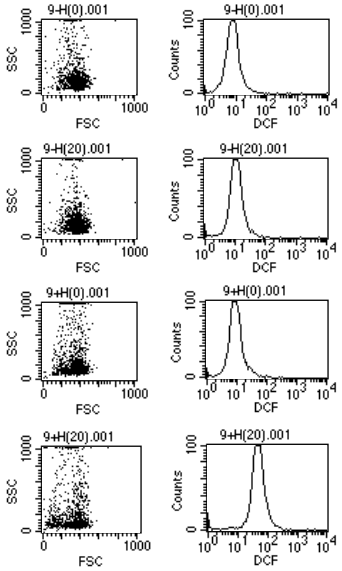

File: M007+H(20).001 Sample ID: M007+H(20)
Patient ID: Chatri Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 20.26 | 16.00 | 91 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 6

Date: 13122012 Day 0 Subject 9 Pornpimon




File: 9-H(0).001 Sample ID: 9-H(0)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.81 | 7.89 | 114 |

unsti at 0 min




File: 9-H(20).001 Sample ID: 9-H(20)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 12.20 | 10.39 | 133 |

unsti at 20 min




File: 9+H(0).001 Sample ID: 9+H(0)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.02 | 8.84 | 121 |

sti at 0 min

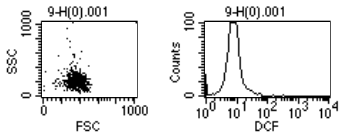



File: 9+H(20).001 Sample ID: 9+H(20)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 54.12 | 46.49 | 111 |

sti at 20 min

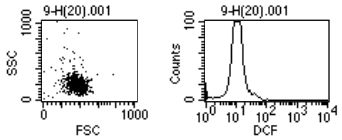
Date: 20122012 Day 7 Subject 9 Pornpimol




File: 9-H(0).001 Sample ID: 9-H(0)
Patient ID: Pornpimol Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.45 | 7.69 | 125 |

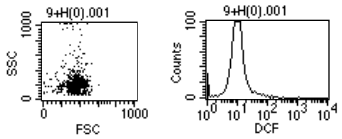
unsti at 0 min




File: 9-H(20).001 Sample ID: 9-H(20)
Patient ID: Pornpimol Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 11.33 | 10.38 | 123 |

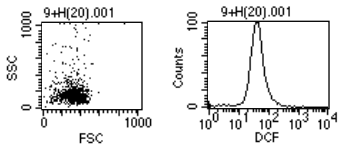
unsti at 20 min




File: 9+H(0).001 Sample ID: 9+H(0)
Patient ID: Pornpimol Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 11.02 | 9.53 | 120 |

sti at 0 min



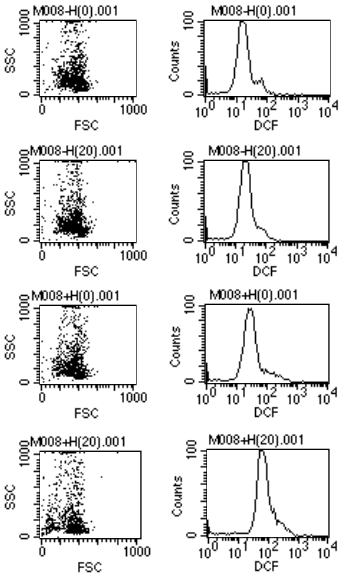

File: 9+H(20).001 Sample ID: 9+H(20)
Patient ID: Pornpimol Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 45.93 | 40.45 | 112 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 6 (Continued)

Date: 10012013 Day 28 Subject M008 Pornpimol




File: M008-H(0).001 Sample ID: M008-H(0)
Patient ID: Pornpimol Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 19.48 | 16.30 | 120 |

unsti at 0 min

File: M008-H(20).001 Sample ID: M008-H(20)
Patient ID: Pornpimol Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 24.26 | 20.66 | 118 |

unsti at 20 min

File: M008+H(0).001 Sample ID: M008+H(0)
Patient ID: Pornpimol Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 38.76 | 29.02 | 94 |

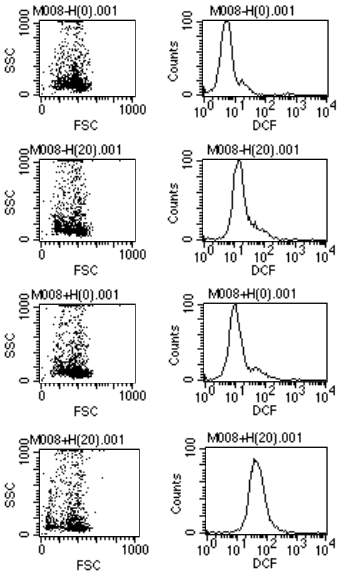
sti at 0 min

File: M008+H(20).001 Sample ID: M008+H(20)
Patient ID: Pornpimol Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 86.44 | 69.30 | 107 |

sti at 20 min

Date: 07032013 Subject M008 Pornpimol




File: M008-H(0).001 Sample ID: M008-H(0)
Patient ID: Pornpimol Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.41 | 5.92 | 113 |

unsti at 0 min

File: M008-H(20).001 Sample ID: M008-H(20)
Patient ID: Pornpimol Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 21.98 | 16.49 | 99 |

unsti at 20 min

File: M008+H(0).001 Sample ID: M008+H(0)
Patient ID: Pornpimol Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 16.33 | 11.91 | 102 |

sti at 0 min

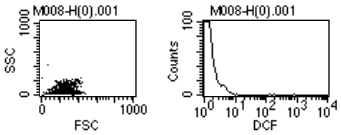
File: M008+H(20).001 Sample ID: M008+H(20)
Patient ID: Pornpimol Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 54.64 | 46.49 | 85 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 6 (Continued)

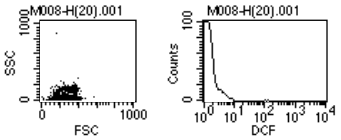
Date: 30052013 Subject M008 Pornpimol




File: M008-H(0).001 Sample ID: M008-H(0)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.63 | 1.41 | 2244 |

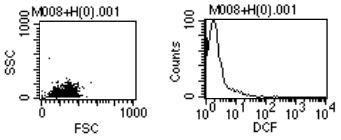
unsti at 0 min




File: M008-H(20).001 Sample ID: M008-H(20)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.52 | 1.39 | 2516 |

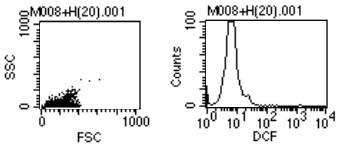
unsti at 20 min




File: M008+H(0).001 Sample ID: M008+H(0)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.47 | 1.98 | 805 |

sti at 0 min

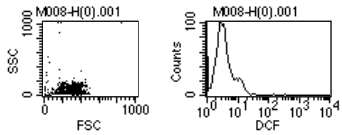



File: M008+H(20).001 Sample ID: M008+H(20)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.94 | 6.76 | 120 |

sti at 20 min

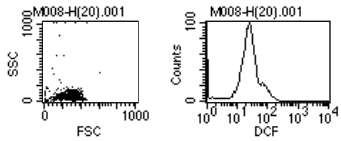
Date: 10042013 D 112 Subject M008 Pornpimol




File: M008-H(0).001 Sample ID: M008-H(0)
Patient ID: Pornpimol Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 4.33 | 3.57 | 194 |

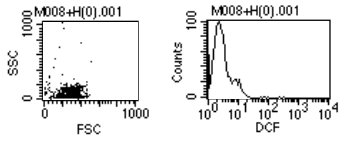
unsti at 0 min




File: M008-H(20).001 Sample ID: M008-H(20)
Patient ID: Pornpimol Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 29.17 | 23.32 | 132 |

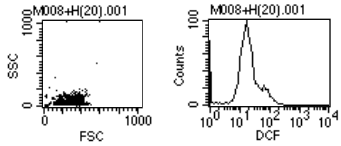
unsti at 20 min




File: M008+H(0).001 Sample ID: M008+H(0)
Patient ID: Pornpimol Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 3.42 | 2.80 | 276 |

sti at 0 min



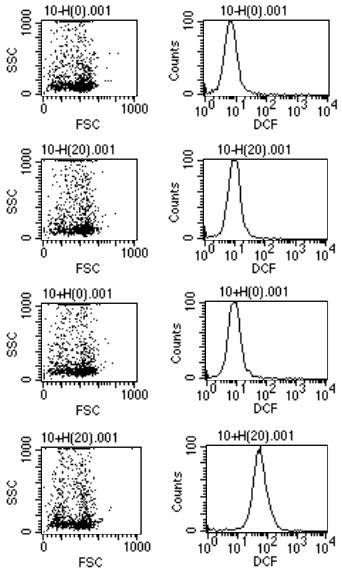

File: M008+H(20).001 Sample ID: M008+H(20)
Patient ID: Pornpimol Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 24.03 | 18.63 | 98 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 7

Date: 13122012 Day 0 subject 10 Varaporn




File: 10-H(0).001 Sample ID: 10-H(0)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.97 | 6.79 | 106 |

unsti at 0 min




File: 10-H(20).001 Sample ID: 10-H(20)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.85 | 8.91 | 111 |

unsti at 20 min




File: 10+H(0).001 Sample ID: 10+H(0)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.40 | 8.02 | 108 |

sti at 0 min

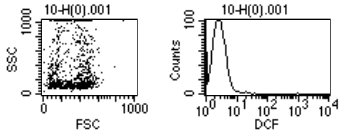



File: 10+H(20).001 Sample ID: 10+H(20)
Patient ID: Acquisition Date: 13-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 59.10 | 51.79 | 105 |

sti at 20 min

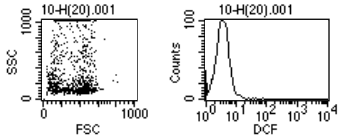
Date: 20122012 Day 7 Subject 10 Varaporn




File: 10-H(0).001 Sample ID: 10-H(0)
Patient ID: Varaporn Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.97 | 2.64 | 143 |

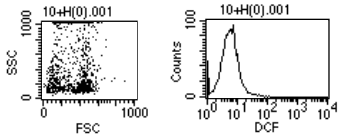
unsti at 0 min




File: 10-H(20).001 Sample ID: 10-H(20)
Patient ID: Varaporn Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 3.79 | 3.48 | 109 |

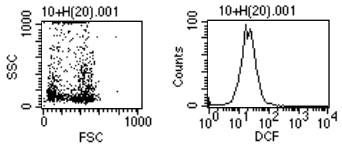
unsti at 20 min




File: 10+H(0).001 Sample ID: 10+H(0)
Patient ID: Varaporn Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.15 | 5.30 | 92 |

sti at 0 min



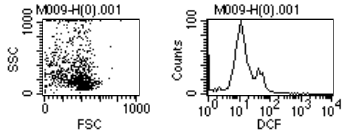

File: 10+H(20).001 Sample ID: 10+H(20)
Patient ID: Varaporn Acquisition Date: 20-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 23.55 | 20.55 | 94 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 7 (Continued)

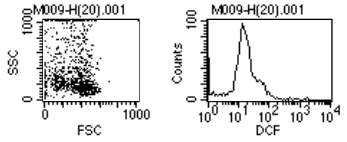
Date: 10012013 Day 28 Subject M009 Varaporn




File: M009-H(0).001 Sample ID: M009-H(0)
Patient ID: Varaporn Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 18.26 | 13.26 | 133 |

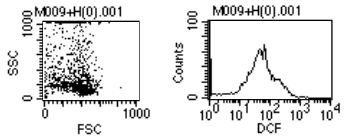
unsti at 0 min




File: M009-H(20).001 Sample ID: M009-H(20)
Patient ID: Varaporn Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 21.71 | 15.36 | 120 |

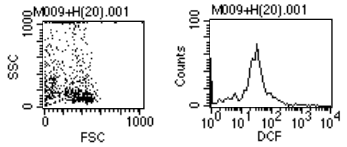
unsti at 20 min




File: M009+H(0).001 Sample ID: M009+H(0)
Patient ID: Varaporn Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 69.64 | 42.97 | 124 |

sti at 0 min

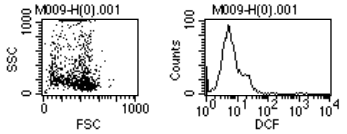



File: M009+H(20).001 Sample ID: M009+H(20)
Patient ID: Varaporn Acquisition Date: 10-Jan-13
Gate: No Gate Gated Events: 7140
Total Events: 7140

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 7140 | 100.00 | 100.00 | 40.83 | 25.95 | 70 |

sti at 20 min

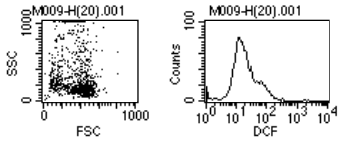
Date: 07032013 Subject M009 Varaporn




File: M009-H(0).001 Sample ID: M009-H(0)
Patient ID: Varaporn Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.45 | 6.17 | 91 |

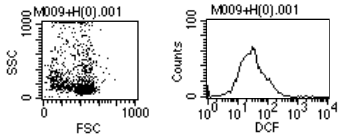
unsti at 0 min




File: M009-H(20).001 Sample ID: M009-H(20)
Patient ID: Varaporn Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 24.19 | 17.09 | 78 |

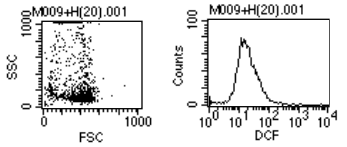
unsti at 20 min




File: M009+H(0).001 Sample ID: M009+H(0)
Patient ID: Varaporn Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 38.06 | 27.18 | 63 |

sti at 0 min



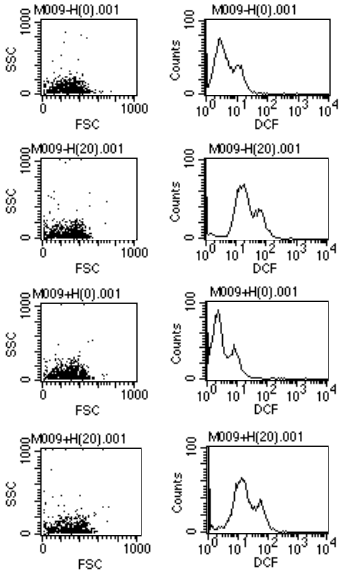

File: M009+H(20).001 Sample ID: M009+H(20)
Patient ID: Varaporn Acquisition Date: 07-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 22.62 | 17.31 | 77 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 7 (Continued)

Date: 10042013 D 112 Subject M009 Varaporn




File: M009-H(0).001 Sample ID: M009-H(0)
Patient ID: Varaporn Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.85 | 4.35 | 179 |

unsti at 0 min




File: M009-H(20).001 Sample ID: M009-H(20)
Patient ID: Varaporn Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 26.16 | 17.63 | 381 |

unsti at 20 min




File: M009+H(0).001 Sample ID: M009+H(0)
Patient ID: Varaporn Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 4.44 | 3.11 | 370 |

sti at 0 min

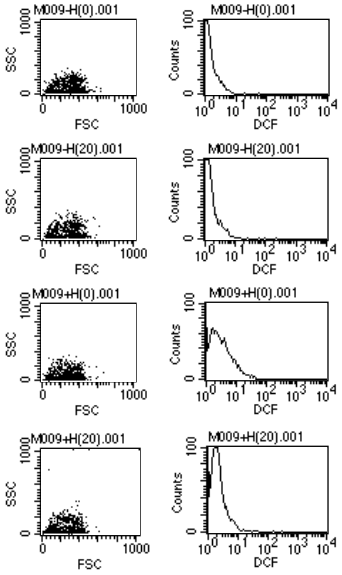



File: M009+H(20).001 Sample ID: M009+H(20)
Patient ID: Varaporn Acquisition Date: 10-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 22.75 | 15.30 | 262 |

sti at 20 min

Date: 30052013 Subject M009 Varaporn




File: M009-H(0).001 Sample ID: M009-H(0)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.44 | 1.32 | 3833 |

unsti at 0 min




File: M009-H(20).001 Sample ID: M009-H(20)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.61 | 1.39 | 3187 |

unsti at 20 min




File: M009+H(0).001 Sample ID: M009+H(0)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 3.68 | 2.74 | 738 |

sti at 0 min



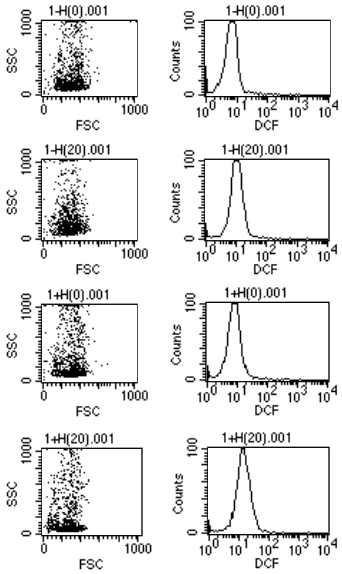

File: M009+H(20).001 Sample ID: M009+H(20)
Patient ID: Acquisition Date: 30-May-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.39 | 2.07 | 460 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 8

Date: 17122012 Day 0 subject 11 Thanapat




File: 1-H(0).001 Sample ID: 1-H(0)
Patient ID: Thanapat Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.31 | 6.46 | 114 |

unsti at 0 min

File: 1-H(20).001 Sample ID: 1-H(20)
Patient ID: Thanapat Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.94 | 9.77 | 129 |

unsti at 20 min

File: 1+H(0).001 Sample ID: 1+H(0)
Patient ID: Thanapat Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.43 | 7.32 | 117 |

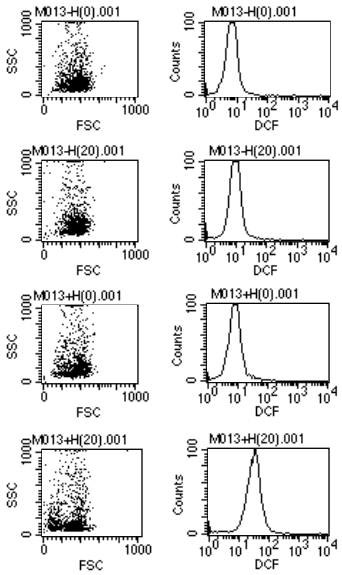
sti at 0 min

File: 1+H(20).001 Sample ID: 1+H(20)
Patient ID: Thanapat Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 15.95 | 14.06 | 107 |

sti at 20 min

Date: 24122012 Day 7 Subject M013 Tanapat




File: M013-H(0).001 Sample ID: M013-H(0)
Patient ID: Tanapat Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.81 | 6.63 | 125 |

unsti at 0 min

File: M013-H(20).001 Sample ID: M013-H(20)
Patient ID: Tanapat Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.87 | 8.92 | 125 |

unsti at 20 min

File: M013+H(0).001 Sample ID: M013+H(0)
Patient ID: Tanapat Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.53 | 7.70 | 122 |

sti at 0 min

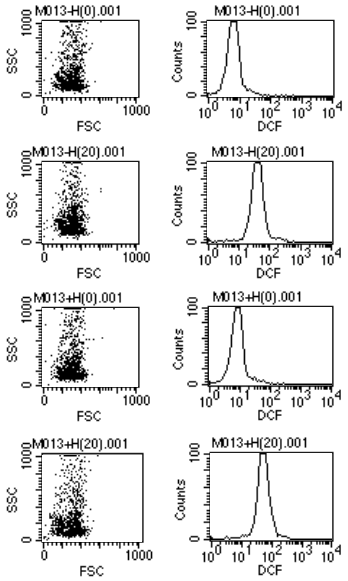
File: M013+H(20).001 Sample ID: M013+H(20)
Patient ID: Tanapat Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 34.44 | 29.60 | 97 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 8 (Continued)

Date: 14012013 Day 28 Subject M013 Thanapat




File: M013-H(0).001 Sample ID: M013-H(0)
Patient ID: Thanapat Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.14 | 6.42 | 135 |

unsti at 0 min




File: M013-H(20).001 Sample ID: M013-H(20)
Patient ID: Thanapat Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 41.69 | 37.87 | 124 |

unsti at 20 min




File: M013+H(0).001 Sample ID: M013+H(0)
Patient ID: Thanapat Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.58 | 7.71 | 129 |

sti at 0 min

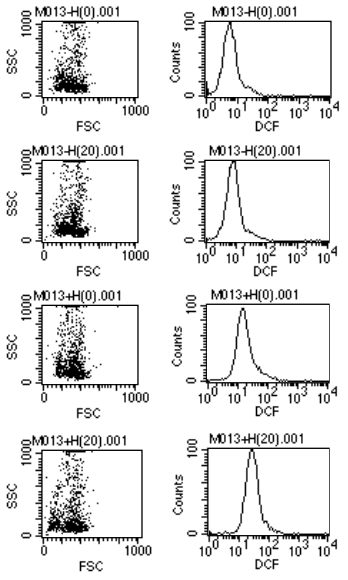



File: M013+H(20).001 Sample ID: M013+H(20)
Patient ID: Thanapat Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 55.83 | 51.40 | 120 |

sti at 20 min

Date: 11032013 Week12 Subject M013 Thanapat




File: M013-H(0).001 Sample ID: M013-H(0)
Patient ID: Thanapat Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.06 | 5.76 | 105 |

unsti at 0 min




File: M013-H(20).001 Sample ID: M013-H(20)
Patient ID: Siriporn Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.08 | 7.49 | 112 |

unsti at 20 min




File: M013+H(0).001 Sample ID: M013+H(0)
Patient ID: Thanapat Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 19.68 | 15.84 | 94 |

sti at 0 min



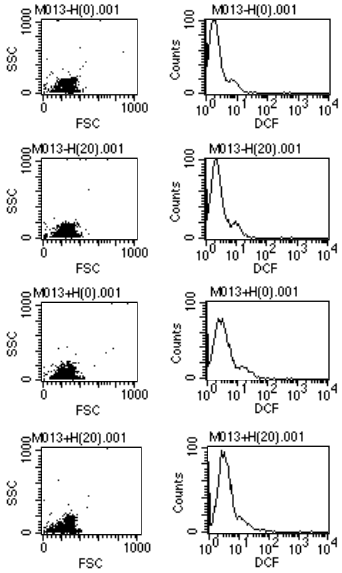

File: M013+H(20).001 Sample ID: M013+H(20)
Patient ID: Siriporn Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 29.44 | 25.56 | 103 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 8 (Continued)

Date: 09042013 Day 112 Subject M013 Thanapat




File: M013-H(0).001 Sample ID: M013-H(0)
Patient ID: Thanapat Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.72 | 2.19 | 530 |

unsti at 0 min




File: M013-H(20).001 Sample ID: M013-H(20)
Patient ID: Thanapat Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 3.25 | 2.56 | 335 |

unsti at 20 min




File: M013+H(0).001 Sample ID: M013+H(0)
Patient ID: Thanapat Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 4.99 | 3.54 | 183 |

sti at 0 min

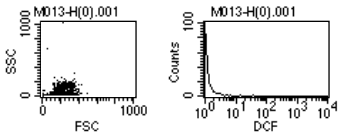



File: M013+H(20).001 Sample ID: M013+H(20)
Patient ID: Thanapat Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.21 | 3.80 | 94 |

sti at 20 min

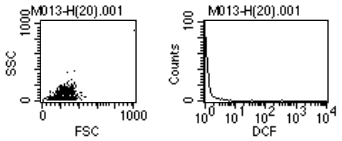
Date: 03062013 Subject M013 Tanapat




File: M013-H(0).001 Sample ID: M013-H(0)
Patient ID: Tanapat Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.05 | 1.03 | 8354 |

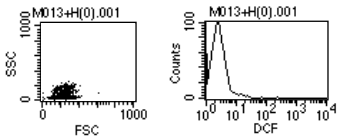
unsti at 0 min




File: M013-H(20).001 Sample ID: M013-H(20)
Patient ID: Tanapat Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.08 | 1.04 | 8198 |

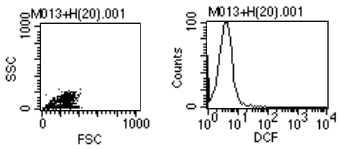
unsti at 20 min




File: M013+H(0).001 Sample ID: M013+H(0)
Patient ID: Tanapat Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.89 | 2.54 | 270 |

sti at 0 min



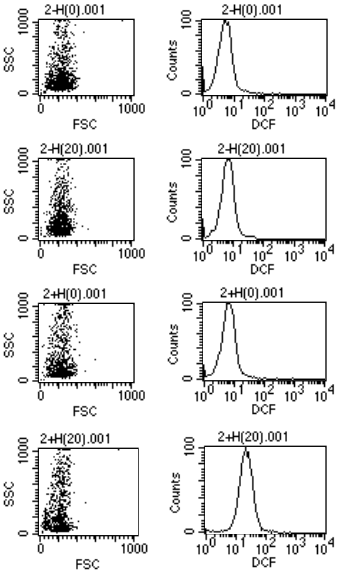

File: M013+H(20).001 Sample ID: M013+H(20)
Patient ID: Tanapat Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 4.20 | 3.82 | 110 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 9

Date: 17122012 Day 0 subject 12 Jumnong




File: 2-H(0).001
Patient ID: jumnong
Gate: No Gate
Total Events: 10000
Sample ID: 2-H(0)
Acquisition Date: 17-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.83 | 5.20 | 104 |

unsti at 0 min




File: 2-H(20).001
Patient ID: Jumnong
Gate: No Gate
Total Events: 10000
Sample ID: 2-H(20)
Acquisition Date: 17-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.97 | 6.33 | 118 |

unsti at 20 min




File: 2+H(0).001
Patient ID: jumnong
Gate: No Gate
Total Events: 10000
Sample ID: 2+H(0)
Acquisition Date: 17-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.13 | 6.30 | 106 |

sti at 0 min

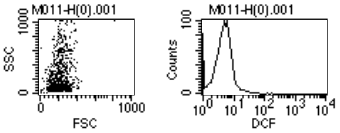



File: 2+H(20).001
Patient ID: Jumnong
Gate: No Gate
Total Events: 10000
Sample ID: 2+H(20)
Acquisition Date: 17-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 23.94 | 21.34 | 97 |

sti at 20 min

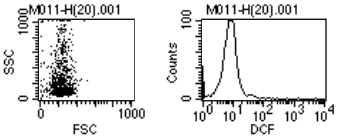
Date: 24122012 Day 7 Subject M011 Jumnong




File: M011-H(0).001
Patient ID: Jumnong
Gate: No Gate
Total Events: 10000
Sample ID: M011-H(0)
Acquisition Date: 24-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.38 | 4.72 | 100 |

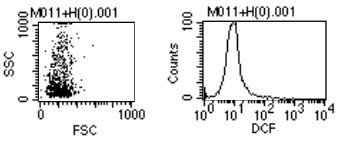
unsti at 0 min




File: M011-H(20).001
Patient ID: Jumnong
Gate: No Gate
Total Events: 10000
Sample ID: M011-H(20)
Acquisition Date: 24-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.09 | 7.55 | 122 |

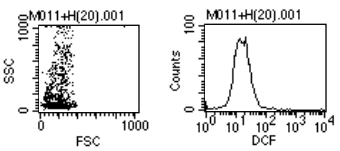
unsti at 20 min




File: M011+H(0).001
Patient ID: Jumnong
Gate: No Gate
Total Events: 10000
Sample ID: M011+H(0)
Acquisition Date: 24-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.26 | 8.63 | 110 |

sti at 0 min



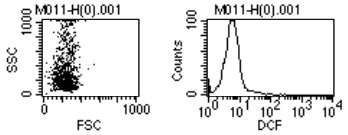

File: M011+H(20).001
Patient ID: Jumnong
Gate: No Gate
Total Events: 10000
Sample ID: M011+H(20)
Acquisition Date: 24-Dec-1
Gated Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 19.14 | 15.96 | 84 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 9 (Continued)

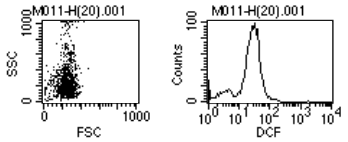
Date: 14012013 Day 28 Subject M011 Jumnong




File: M011-H(0).001 Sample ID: M011-H(0)
Patient ID: Jumnong Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.19 | 5.46 | 104 |

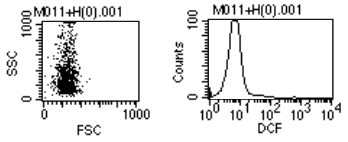
unsti at 0 min




File: M011-H(20).001 Sample ID: M011-H(20)
Patient ID: Jumnong Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 26.04 | 20.72 | 96 |

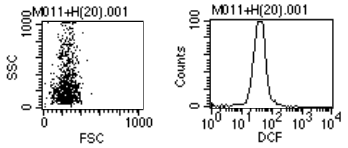
unsti at 20 min




File: M011+H(0).001 Sample ID: M011+H(0)
Patient ID: Jumnong Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.21 | 6.35 | 111 |

sti at 0 min

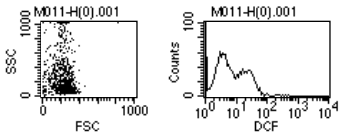



File: M011+H(20).001 Sample ID: M011+H(20)
Patient ID: Jumnong Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 39.99 | 36.69 | 127 |

sti at 20 min

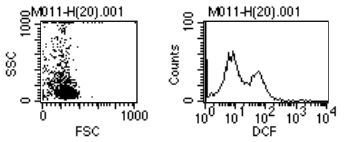
Date: 11032013 Week12 Subject M011 Jumnong




File: M011-H(0).001 Sample ID: M011-H(0)
Patient ID: Jumnong Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.84 | 6.67 | 222 |

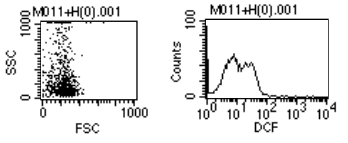
unsti at 0 min




File: M011-H(20).001 Sample ID: M011-H(20)
Patient ID: Jumnong Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 23.50 | 12.80 | 159 |

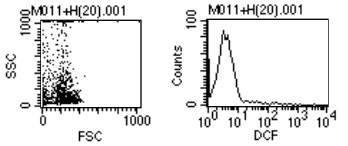
unsti at 20 min




File: M011+H(0).001 Sample ID: M011+H(0)
Patient ID: Jumnong Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 15.73 | 10.60 | 90 |

sti at 0 min



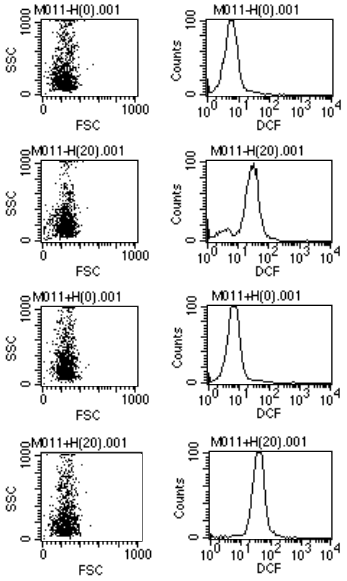

File: M011+H(20).001 Sample ID: M011+H(20)
Patient ID: Jumnong Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.04 | 3.89 | 234 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 9 (Continued)

Date: 14012013 Day 28 Subject M011 Jumnong




File: M011-H(0).001 Sample ID: M011-H(0)
Patient ID: Jumnong Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.19 | 5.46 | 104 |

unsti at 0 min




File: M011-H(20).001 Sample ID: M011-H(20)
Patient ID: Jumnong Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 26.04 | 20.72 | 96 |

unsti at 20 min




File: M011+H(0).001 Sample ID: M011+H(0)
Patient ID: Jumnong Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.21 | 6.35 | 111 |

sti at 0 min

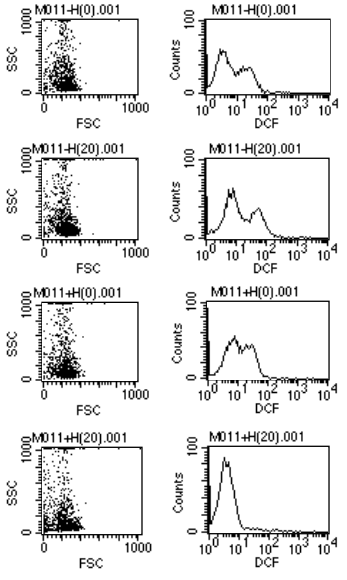



File: M011+H(20).001 Sample ID: M011+H(20)
Patient ID: Jumnong Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 39.99 | 36.69 | 127 |

sti at 20 min

Date: 11032013 Week12 Subject M011 Jumnong




File: M011-H(0).001 Sample ID: M011-H(0)
Patient ID: Jumnong Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.84 | 6.67 | 222 |

unsti at 0 min




File: M011-H(20).001 Sample ID: M011-H(20)
Patient ID: Jumnong Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 23.50 | 12.80 | 159 |

unsti at 20 min




File: M011+H(0).001 Sample ID: M011+H(0)
Patient ID: Jumnong Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 15.73 | 10.60 | 90 |

sti at 0 min



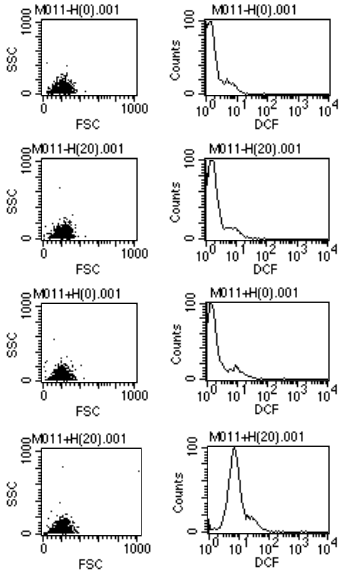

File: M011+H(20).001 Sample ID: M011+H(20)
Patient ID: Jumnong Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.04 | 3.89 | 234 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 9 (Continued)

Date: 09042013 Day 112 Subject M011 Jumnong




File: M011-H(0).001 Sample ID: M011-H(0)
Patient ID: Jumnong Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.01 | 1.65 | 1948 |

unsti at 0 min




File: M011-H(20).001 Sample ID: M011-H(20)
Patient ID: Jomnong Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.39 | 1.87 | 1223 |

unsti at 20 min




File: M011+H(0).001 Sample ID: M011+H(0)
Patient ID: Jumnong Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.53 | 1.82 | 1626 |

sti at 0 min

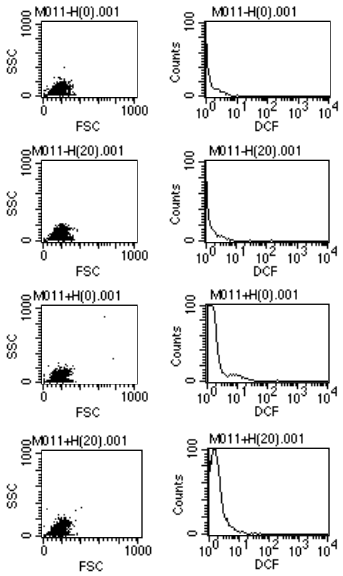



File: M011+H(20).001 Sample ID: M011+H(20)
Patient ID: Jomnong Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.46 | 8.02 | 116 |

sti at 20 min

Date: 03062013 Subject M011 Jumnong




File: M011-H(0).001 Sample ID: M011-H(0)
Patient ID: Jumnong Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.07 | 1.05 | 8505 |

unsti at 0 min




File: M011-H(20).001 Sample ID: M011-H(20)
Patient ID: Jumnong Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.10 | 1.06 | 8370 |

unsti at 20 min




File: M011+H(0).001 Sample ID: M011+H(0)
Patient ID: Jumnong Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.77 | 1.46 | 2883 |

sti at 0 min



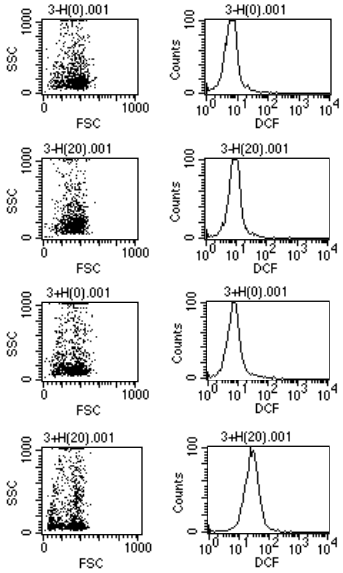

File: M011+H(20).001 Sample ID: M011+H(20)
Patient ID: Jumnong Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.83 | 1.63 | 1577 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 10

Date: 17122012 Day 0 subject 13 Siriporn




File: 3-H(0).001 Sample ID: 3-H(0)
Patient ID: Siriporn Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.74 | 6.05 | 112 |

unsti at 0 min




File: 3-H(20).001 Sample ID: 3-H(20)
Patient ID: Siriporn Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.61 | 7.80 | 124 |

unsti at 20 min




File: 3+H(0).001 Sample ID: 3+H(0)
Patient ID: Siriporn Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.24 | 7.05 | 109 |

sti at 0 min

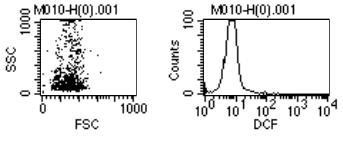



File: 3+H(20).001 Sample ID: 3+H(20)
Patient ID: Siriporn Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 28.92 | 25.54 | 97 |

sti at 20 min

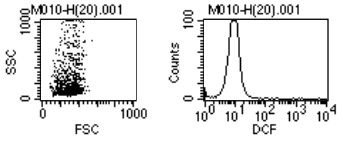
Date: 24122012 Day 7 Subject M010 Siriporn




File: M010-H(0).001 Sample ID: M010-H(0)
Patient ID: Siriporn Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.60 | 7.00 | 130 |

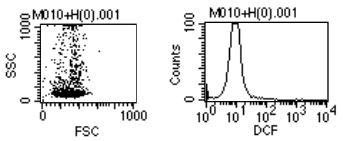
unsti at 0 min




File: M010-H(20).001 Sample ID: M010-H(20)
Patient ID: Siriporn Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.17 | 8.59 | 131 |

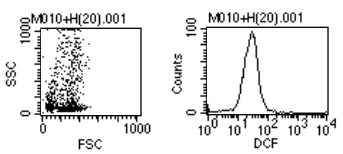
unsti at 20 min




File: M010+H(0).001 Sample ID: M010+H(0)
Patient ID: Siriporn Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.57 | 8.47 | 117 |

sti at 0 min



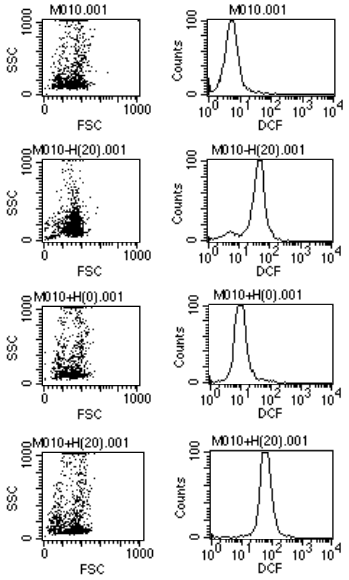

File: M010+H(20).001 Sample ID: M010+H(20)
Patient ID: Siriporn Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 30.49 | 26.29 | 94 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 10 (Continued)

Date: 14012013 Day 28 Subject M010 Siriporn




File: M010.001 Sample ID: M010
Patient ID: Siriporn Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.81 | 5.22 | 108 |

unsti at 0 min




File: M010-H(20).001 Sample ID: M010-H(20)
Patient ID: Siriporn Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 39.94 | 33.28 | 110 |

unsti at 20 min




File: M010+H(0).001 Sample ID: M010+H(0)
Patient ID: Siriporn Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 11.18 | 10.03 | 132 |

sti at 0 min

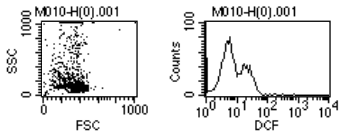



File: M010+H(20).001 Sample ID: M010+H(20)
Patient ID: Siriporn Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 63.40 | 58.48 | 129 |

sti at 20 min

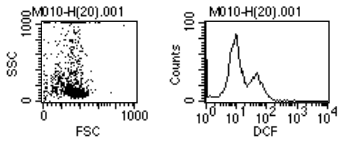
Date: 11032013 Week12 Subject M010 Siriporn




File: M010-H(0).001 Sample ID: M010-H(0)
Patient ID: Siriporn Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.58 | 7.63 | 136 |

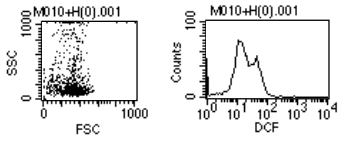
unsti at 0 min




File: M010-H(20).001 Sample ID: M010-H(20)
Patient ID: Siriporn Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 19.79 | 12.83 | 104 |

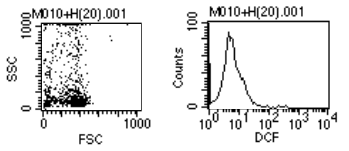
unsti at 20 min




File: M010+H(0).001 Sample ID: M010+H(0)
Patient ID: Siriporn Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 23.57 | 18.16 | 74 |

sti at 0 min



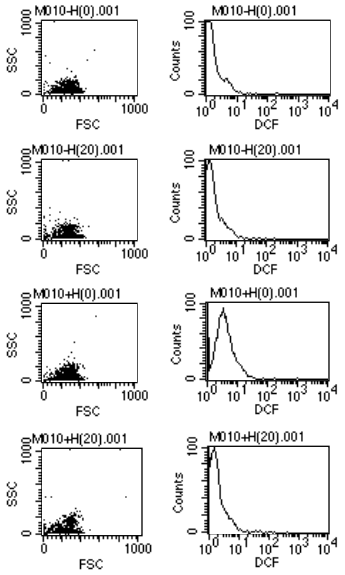

File: M010+H(20).001 Sample ID: M010+H(20)
Patient ID: Siriporn Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.83 | 5.92 | 116 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 10 (Continued)

Date: 09042013 Day 112 Subject M010 Siriporn




File: M010-H(0).001 Sample ID: M010-H(0)
Patient ID: Siriporn Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.81 | 1.53 | 2489 |

unsti at 0 min

File: M010-H(20).001 Sample ID: M010-H(20)
Patient ID: Siriporn Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.88 | 1.57 | 2368 |

unsti at 20 min

File: M010+H(0).001 Sample ID: M010+H(0)
Patient ID: Siriporn Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 4.48 | 3.61 | 174 |

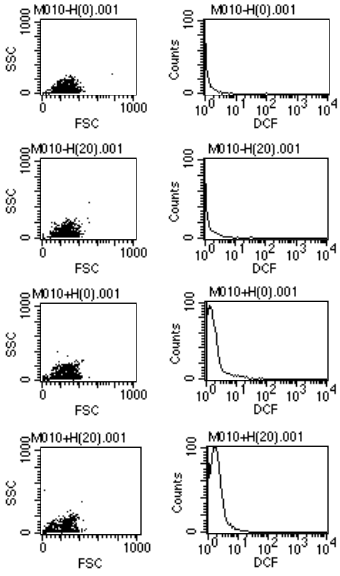
sti at 0 min

File: M010+H(20).001 Sample ID: M010+H(20)
Patient ID: Siriporn Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.17 | 1.75 | 1444 |

sti at 20 min

Date: 03062013 Subject M010 Siriporn




File: M010-H(0).001 Sample ID: M010-H(0)
Patient ID: Siriporn Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.04 | 1.03 | 8941 |

unsti at 0 min

File: M010-H(20).001 Sample ID: M010-H(20)
Patient ID: Siriporn Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.06 | 1.04 | 8741 |

unsti at 20 min

File: M010+H(0).001 Sample ID: M010+H(0)
Patient ID: Siriporn Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.61 | 1.40 | 3423 |

sti at 0 min

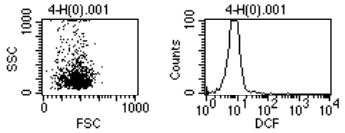
File: M010+H(20).001 Sample ID: M010+H(20)
Patient ID: Siriporn Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.92 | 1.74 | 1197 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 11

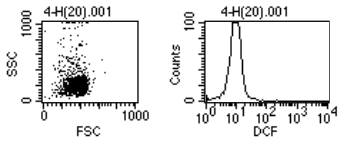
Date: 17122012 Day 0 subject 14 Piyamas




File: 4-H(0).001 Sample ID: 4-H(0)
Patient ID: Piyamas Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.91 | 7.33 | 118 |

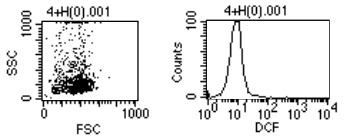
unsti at 0 min




File: 4-H(20).001 Sample ID: 4-H(20)
Patient ID: Piyamas Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.03 | 8.40 | 139 |

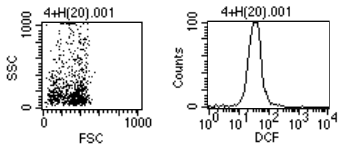
unsti at 20 min




File: 4+H(0).001 Sample ID: 4+H(0)
Patient ID: Piyamas Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.03 | 8.30 | 120 |

sti at 0 min

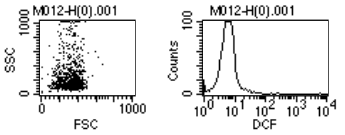



File: 4+H(20).001 Sample ID: 4+H(20)
Patient ID: Piyamas Acquisition Date: 17-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 35.82 | 32.59 | 111 |

sti at 20 min

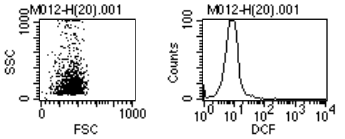
Date: 24122012 Day 7 Subject M012 Piyamas




File: M012-H(0).001 Sample ID: M012-H(0)
Patient ID: PiYamas Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 6.91 | 5.75 | 122 |

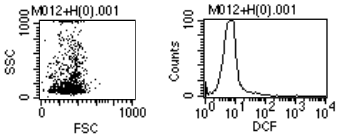
unsti at 0 min




File: M012-H(20).001 Sample ID: M012-H(20)
Patient ID: Piyamas Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.63 | 7.87 | 125 |

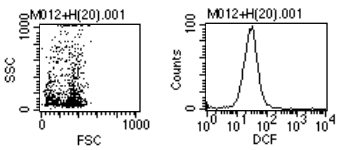
unsti at 20 min




File: M012+H(0).001 Sample ID: M012+H(0)
Patient ID: PiYamas Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 7.23 | 6.22 | 128 |

sti at 0 min



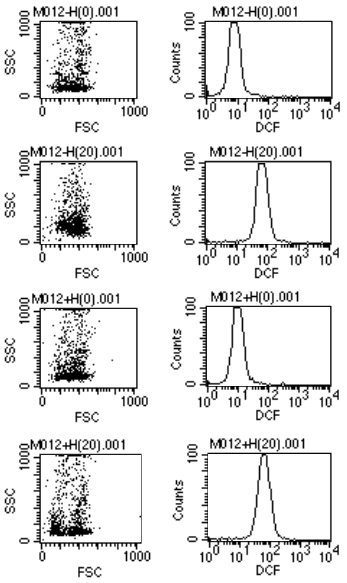

File: M012+H(20).001 Sample ID: M012+H(20)
Patient ID: Piyamas Acquisition Date: 24-Dec-1
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 31.91 | 27.96 | 96 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 11 (Continued)

Date: 14012013 Day 28 Subject M012 Piyamas




File: M012-H(0).001 Sample ID: M012-H(0)
Patient ID: Piyamas Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 8.45 | 7.66 | 129 |

unsti at 0 min




File: M012-H(20).001 Sample ID: M012-H(20)
Patient ID: Piyamas Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 63.41 | 58.06 | 119 |

unsti at 20 min




File: M012+H(0).001 Sample ID: M012+H(0)
Patient ID: Piyamas Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 10.18 | 9.43 | 129 |

sti at 0 min

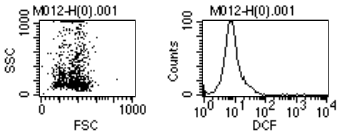



File: M012+H(20).001 Sample ID: M012+H(20)
Patient ID: Piyamas Acquisition Date: 14-Jan-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 66.72 | 61.45 | 113 |

sti at 20 min

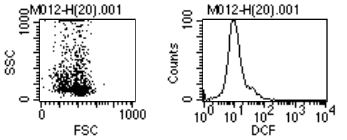
Date: 11032013 Week12 Subject M012 Piyamas




File: M012-H(0).001 Sample ID: M012-H(0)
Patient ID: Piyamas Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 9.56 | 7.99 | 105 |

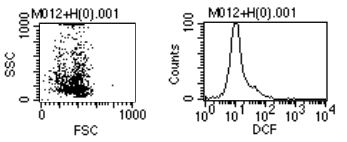
unsti at 0 min




File: M012-H(20).001 Sample ID: M012-H(20)
Patient ID: Piyamas Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 12.67 | 10.62 | 111 |

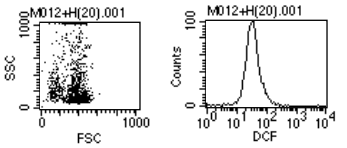
unsti at 20 min




File: M012+H(0).001 Sample ID: M012+H(0)
Patient ID: Piyamas Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 14.25 | 11.42 | 108 |

sti at 0 min



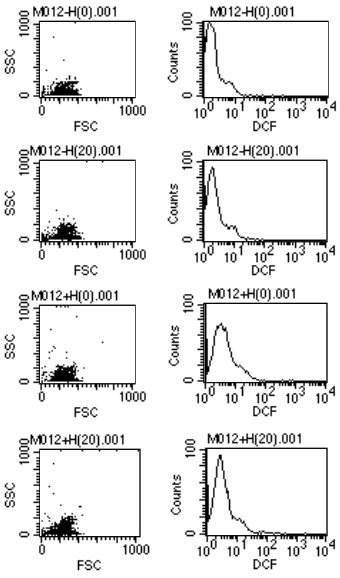

File: M012+H(20).001 Sample ID: M012+H(20)
Patient ID: Piyamas Acquisition Date: 11-Mar-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 37.51 | 32.93 | 105 |

sti at 20 min

# Mean fluorescent intensity (MFI) of sample 11 (Continued)

Date: 09042013 Day 112 Subject M012 Piyamas




File: M012-H(0).001 Sample ID: M012-H(0)
Patient ID: Piyamas Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.40 | 1.95 | 960 |

unsti at 0 min




File: M012-H(20).001 Sample ID: M012-H(20)
Patient ID: Piyamas Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.66 | 2.09 | 1408 |

unsti at 20 min




File: M012+H(0).001 Sample ID: M012+H(0)
Patient ID: Piyamas Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 5.40 | 3.91 | 220 |

sti at 0 min

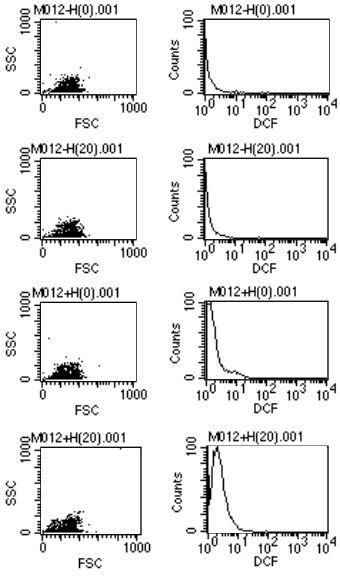



File: M012+H(20).001 Sample ID: M012+H(20)
Patient ID: Piyamas Acquisition Date: 09-Apr-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 4.83 | 3.40 | 189 |

sti at 20 min

Date: 03062013 Subject M012 Piyamat




File: M012-H(0).001 Sample ID: M012-H(0)
Patient ID: Piyamat Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.09 | 1.05 | 8329 |

unsti at 0 min




File: M012-H(20).001 Sample ID: M012-H(20)
Patient ID: Piyamat Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.09 | 1.06 | 7857 |

unsti at 20 min




File: M012+H(0).001 Sample ID: M012+H(0)
Patient ID: Piyamat Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 1.71 | 1.45 | 3117 |

sti at 0 min




File: M012+H(20).001 Sample ID: M012+H(20)
Patient ID: Piyamat Acquisition Date: 03-Jun-13
Gate: No Gate Gated Events: 10000
Total Events: 10000

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | Peak |
|---|---|---|---|---|---|---|---|
| All | 1, 991 | 10000 | 100.00 | 100.00 | 2.57 | 2.25 | 453 |

sti at 20 min

# APPENDIX C

# ETHICAL EXEMPTION DOCUMENT

2 PRANNOK Rd. BANGKOKNOI
BANGKOK 10700

Tel. (662) 4196405-6
FAX (662) 4196405

MAHIDOL UNIVERSITY
*Since 1888*

**Siriraj Institutional Review Board**

**Certificate of Approval**

COA no. Si 333/2012

**Protocol Title** : A study of the safety of mangosteen extract in healthy volunteer and effectiveness on cognitive function and neuropsychiatric symptoms in patients with mild to moderate Alzheimer's disease

**Protocol number** : 110/2555(EC2)

**Principal Investigator/Affiliation** Assist. Prof. Weerasak Muangpaisan, M.D. / Department of Preventive and Social Medicine Faculty of Medicine Siriraj Hospital, Mahidol University

**Research site** : Faculty of Medicine Siriraj Hospital

**Approval includes :**

1. SIRB Submission Form
2. Proposal
3. Informed Consent Form for Phase 1 in healthy volunteer
4. Informed Consent Form for Phase 2 in patients with Alzheimer's disease
5. Case Record Form for Phase 1
6. Case Record Form for Phase 2
7. Principle Investigator's curriculum vitae

**Approval date** : June 21, 2012

**Expired date** : June 20, 2013

This is to certify that Siriraj Institutional Review Board is in full Compliance with international guidelines for human research protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

(Prof. Jarupim Soongswang, M.D.)
Chairperson

June 22, 2012
date

(Clin. Prof. Udom Kachintorn, M.D.)
Dean of Faculty of Medicine Siriraj Hospital

- 4 JUL 2012
date



หน่วยพิมพ์โรงพยาบาลศิริราช 2341 / 3,000 เล่ม / ธ.ค. 51 / Mat. 10023252

# BIOGRAPHY

| | |
|---|---|
| **NAME** | Mr. Vatchara Tunrungruangtavee |
| **DATE OF BIRTH** | 13 October 1987 |
| **PLACE OF BIRTH** | Bangkok, Thailand |
| **INSTITUTION ATTENDED** | King Mongkut's University of Technology North Bangkok, 2006-2010 (Biotechnology)<br>Mahidol University, 2011-2014 Master of Science in Biopharmaceutical (Microbiology) |
| **HOME ADRESS** | 157/1, Phachasongkhro,<br>Din Daeng, Thailand, 10400<br>Tel: 0909180850<br>E-mail: evolution_vb@hotmail.com |
| **PRESENTATION** | Poster presentation:<br>"ANTIOXIDANT EFFECTS OF MANGOSTEEN PERICARP EXTRACT IN HUMAN"<br>The 36$^{th}$ Congress on Pharmacology Of Thailand,<br>Bangkok, Thailand<br>(27-28 March 2014) |