ฤทธิ์ยับยั้งการสร้างไนตริกออกไซด์ในเซลล์แมโครฟาจ RAW 264.7 ของโปรตีนจากเหง้าของพืชวงศ์ขิง

นางสาวเชิญขวัญ จันทรโณทัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

INHIBITORY ACTIVITY AGAINST NITRIC OXIDE PRODUCTION IN MACROPHAGE RAW 264.7 OF PROTEINS FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS

Miss Chernkhwan Chantaranothai

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

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Thesis Title	INHIBITORY ACTIVITY AGAINST NITRIC OXIDE PRODUCTION IN MACROPHAGE RAW 264.7 OF PROTEINS FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS.
By	Miss Chernkhwan Chantaranothai
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Polkit Sangvanich, Ph.D.
Thesis Co-advisor	Associate Professor Tanapat Palaga, Ph.D.
Thesis Co-advisor	Aphichart Karnchanatat, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr. rer. nat.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Sirirat Rengpipat, Ph.D.)

…….…… Thesis Advisor
(Associate Professor Polkit Sangvanich, Ph.D.)

…….. Thesis Co-advisor
(Associate Professor Tanapat Palaga, Ph.D.)

…….. Thesis Co-advisor
(Aphichart Karnchanatat, Ph.D.)

…….. Examiner
(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

…….. External Examiner
(Prapas Khorphueng, Ph.D.)

เชิญขวัญ จันทรโณทัย : ฤทธิ์ยับยั้งการสร้างไนตริกออกไซด์ในเซลล์แมโครฟาจ RAW 264.7 ของโปรตีนจากเหง้าของพืชวงศ์ขิง (INHIBITORY ACTIVITY AGAINST NITRIC OXIDE PRODUCTION IN MACROPHAGE RAW 264.7 OF PROTEINS FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.พลกฤษณ์ แสงวณิช, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.ธนาภัทร ปาลกะ, อ. ดร.อภิชาติ กาญจนทัต, 57 หน้า

การอักเสบเป็นหนึ่งปัจจัยที่สำคัญอันเป็นสาเหตุของการเกิดโรคมะเร็งและโรคความเสื่อมของอวัยวะอื่นๆ โดยการศึกษานี้จะเน้นที่กระบวนการผลิตไนตริกออกไซด์ซึ่งเป็นโมเลกุลที่มีบทบาทสำคัญในการเกิดการอักเสบและได้รับสารเอนโดทอกซินในการเหนี่ยวนำการบาดเจ็บของเนื้อเยื่อ พืชวงศ์ขิงเป็นพืชที่เจริญได้ในเขตร้อน เช่น ประเทศไทย โดยมีการใช้เป็นยาต้านการอักเสบมาตั้งแต่โบราณอย่างกว้างขวาง ในงานวิจัยนี้ได้ตรวจสอบฤทธิ์ยับยั้งการสร้างไนตริกออกไซด์ของโปรตีนที่สกัดจากเหง้าของพืชวงศ์ขิง 15 ชนิดในเซลล์แมโครฟาจ RAW 264.7 โดยให้เซลล์แมโคร-ฟาจถูกกระตุ้นด้วยไลโพโพลีแซคคาไรด์ และอินเตอร์เฟียรอน-แกมมา เพื่อกระตุ้นการสังเคราะห์ไนตริกออกไซด์จากเซลล์นี้ ผลการทดลองพบว่าโปรตีนจากเหง้าของไพลดำมีแอคติวิตียับยั้งการสังเคราะห์ไนตริกออกไซด์สูงสุด โดยมีค่า IC_{50} ของโปรตีนที่ 38.6 ± 0.34 ไมโครกรัมโปรตีนต่อมิลลิลิตร นอกจากนี้ได้ทำการศึกษาการลดการแสดงออกของ mRNA ของ *iNOS, IL-6* และ *TNF-*α ผลการทดลองแสดงให้เห็นว่าโปรตีนจากเหง้าของไพลดำลดระดับการถอดรหัสของ mRNA ของ *iNOS, IL-6* และ *TNF-*α อีกด้วย จากผลที่กล่าวมาโปรตีนของไพลดำมีฤทธิ์ต้านการอักเสบได้ดี และสามารถที่จะพัฒนาเป็นสารบำบัดได้ นอกจากนี้เมื่อทำการวิเคราะห์โปรตีนด้วยเทคนิคพอลิอะคริลา-ไมด์เจลอิเล็กโตรฟอเรซิสแบบเสียสภาพและแมสสเปกโทรเมทรี แสดงแถบโปรตีนที่เด่นชัดคือ เลคติน ซิสเตอีนโปรติเอส และเอนไซม์ต้านออกซิเดชันซูเปอร์ออกไซด์ดิสมิวเทส ซึ่งมีฤทธิ์ในการต้านอนุมูลอิสระ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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CHERNKHWAN CHANTARANOTHAI: INHIBITORY ACTIVITY AGAINST NITRIC OXIDE PRODUCTION IN MACROPHAGE RAW 264.7 OF PROTEINS FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS. ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., CO-ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., APHICHART KARNCHANATAT, Ph.D., 57 pp.

Inflammation is one of the most critical factors implicated in carcinogenesis and other degenerative disorders. This study focused on nitric oxide (NO), a molecule that plays a key role in the pathogenesis of inflammation and has been implicated in endotoxin-induced tissue injury. *Zingiberaceae* is an indigenous plant of tropical region country such as Thailand, which has been traditionally and widely used as an anti-inflammatory agent. In the present study, we investigated the inhibitory activity against nitric oxide production of the crude protein from fifteen of rhizomes of *Zingiberaceae* plants in RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS) and interferon-gramma (IFN-γ) for induced NO release in cells. The results suggested that the crude protein of *Zingiber ottensii* Valeton exhibited the highest activity against NO release among fifteen *Zingiberaceae* plants with an IC_{50} of 38.6±0.34 µg protein/ml. Moreover, we demonstrated the attenuation in *iNOS, IL-6* and *TNF-α* mRNA expression that was also observed suggesting interference at transcriptional level. The result showed that the crude protein of *Z. ottensii* decreased cellular *iNOS, IL-6* and *TNF-α* mRNA level. These results suggest that the crude protein possesses a strong anti-inflammatory activity and has a potential to be developed as a therapeutic compound. Furthermore, the analytical SDS-PAGE and mass spectrometry showed a high abundance of protein bands, including lectin, cysteine protease, superoxide dismutase (SOD) that are related with antioxidant activity.

Field of Study : Biotechnology Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

Co-advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

Abs	Absorbance
ATCC	American Type Culture Collection
bp	Base pair
cDNA	Complementary DNA
CO_2	Carbon dioxide
DMSO	Dimethylsulfoxide
DPPH•	2,2-diphenyl-1-picrylhydrazyl radical
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
g (centrifugation speed)	Gravity
h	Hour
HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
IC50	Median inhibitory concentration, 50% maximum inhibition
IFN-γ	Interferon-gamma
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRF-1	Interferon regulatory factor 1
kDa	Kilodalton
LPS	Lipopolysaccharide
mA	Milliampere

mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NFκB	Nuclear factor-kappa B
ng	Nanogram
nm	Nanometer
nNOS	Neuron nitric oxide synthase
NO•	Nitric oxide
O2•-	Superoxide anion
O.D.	Optical Density
OH•	Hydroxyl radical
OONO-	Peroxynitrite
PBS	Phosphate buffered saline
PCR	Polymerase chain raction
PGE2	Prostaglandin E2
psi	Pound per square inch
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Round per minute
RT	Reverse transcription
s	second

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel Electrophoresis
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet
α	Alpha
β	Beta
°C	Degree Celsius
γ	Gamma
µg	Microgram
µl	Microliter
µM	Micromolar
/	Per
%	Percentage
:	Ratio

CHAPTER l

INTRODUCTION

Nitric oxide (NO) is a molecule that plays a key role in the pathogenesis of inflammation and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues (Sharma *et al*., 2006). Nitric oxide is also an important component of the antineoplastic and antimicrobial armament of macrophages (Coleman, 2001). This highly labile and noxious gas is produced in large and sustained quantities by macrophages following exposure to a variety of immunologic and inflammatory mediators (Fang, 1997). In mammals, nitric oxide synthesized by the oxidation of L-arginine catalyzed by inducible nitric oxide synthase (iNOS) in phagocytes such as macrophages, induced apoptosis in neighboring cell. In addition, apoptosis can also be induced by $ONOO^-$ generated as O_2^- produced by phagocytes reacts with nitric oxide. The macrophages are induced NO release by lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). LPS is the major component of cell wall found mainly in Gram-negative bacteria functioning in protecting the chemical attack. It is prototypical endotoxin which could induce innate immune response in animals (Lydyard *et al*., 2004). In addition, Reactive oxygen species (ROS) propagate inflammation by stimulating release of these mediators, which stimulate recruitment of additional macrophages at the inflammatory site. It is believed that persistent inflammatory cells recruitment, repeated generation of ROS and pro-inflammatory mediators, and continued proliferation of genomically unstable cells contribute to neoplasic transformation and ultimately result in tumor invasion and metastasis (Khan *et al*., 2008).

The family Zingiberaceae is a world-wide family comprising more than 1500 species, distributed throughout tropical Africa, Asia, and the America. It is now estimated that Thailand has about 25 genera which some 250 species (Larsen *et al*., 1996). Zingiberaceae is well known for its medicinal and economic significance, furnishing as it does a number of species and condiments. The various plant tissues and parts of this species of ginger are rich in volatile aromatic compounds. The majority of the essential oils extracted from the leaves, stems and rhizomes. Currently, other species of Zingiberaceae are the subjects of extensive research into

the various antioxidant compounds present in their leaves and rhizomes. Their ethnomedical use in order to conserve the native knowledge about folk medicines and to search for new potential candidates for modern drug investigations and development (Chuakul and Boonpleng, 2003).

Proteins are made up of amino acids. These amino acids bond together into long chains to form proteins. The amino acids must be present in the proper ratios in order for protein synthesis to occur. These natural products may exert their effect on bacterial cell membranes and/or specific protein targets (Stone and Williams, 1992; Butler, 2004; and Koehn and Carter, 2005). Nowadays, there are many studies about bioactive such as proteins, peptide. They were developed to be drugs due to the proteins have specific to target, highly efficacy and less side effect. In this study, the anti-inflammatory properties of the crude protein extracts were dialyzed from fifteen *Zingiberaceae* plants on the responses in macrophages RAW 264.7 cell line that were stimulated with lipopolysaccharide (LPS) and interferon-gramma (IFN-γ) as an *in vitro* model.

CHAPTER ll

LITERATURE REVIEWS

2.1 Biological significance of nitric oxide

Elevated production of reactive oxygen and nitrogen species (ROS and RNS respectively) are increasingly recognized as contributing to cell dysfunction in a variety of disease states by inducing oxidative damage to cell macromolecules such as lipids, DNA and proteins (Alvarez and Radi, 2003). Peroxynitrite is a damaging reactive nitrogen species produced in vivo when nitric oxide (NO) and superoxide react (Pacher *et al*., 2007). Nitric oxide (NO, formula •N=O) is a simple, inorganic, gaseous free radical whose predominant functions are that of a messenger and effector molecules (Furchgott and Zawadzki, 1980). NO in an uncharged lipophilic molecule that contains a single unpaired electron (NO•), which causes it to be reactive with other molecules, such as oxygen, glutathione, and superoxide radicals (Smutzer, 2001). NO undergoes a number of chemical reactions, many of which occur under biological conditions. Some of the more important chemical reactions involving NO are discussed below (Blough and Zafiriou, 1985):

1. Reactivity with oxygen to yield NO_2 gas or NO_2^- in solution:

$$2NO + O_2 \longrightarrow 2NO_2^-$$

2. Reactivity with superoxide anion (O_2^-) to yield the unstable intermediate peroxonitrite anion ^-OONO), which rearranges to form NO_3^-

$$NO + O_2^- \longrightarrow {}^-OONO \longrightarrow NO_3^-$$

NO is chemically unstable, with a half-life of 3-5 s in aqueous solution under physiological conditions of concentration, temperature, pH, and oxygen tension. The lability of NO, however, may be much greater in the actual tissues in vivo. In aqueous solution NO spontaneously oxidizes primarily to NO^-_2, which is 5 or 6 orders of magnitude less potent than NO as a vasodilator (Ignarro and Gruetter, 1980; Gruetter *et al*., 1979). NO is one of the inflammatory mediators that have been implicated in a variety of pathophysiological conditions including inflammation,

carcinogenesis and atherosclerosis (Mordan *et al*., 1993; Ohshima and Bartsch, 1994; Kranche *et al*., 1998). The excessive production of this free radical is pathogenic to the host tissue, since NO can bind with other superoxide radicals which directly damages the function of normal cells (Moncada *et al*., 1991). NO acts as a host defense by damaging pathogenic DNA, and as a regulatory molecule with homeostatic activities (Kou and Schroder, 1995). Moreover, the potentially lethal substances released from microglia, overproduction of NO has been reported to cause oxidative damages and cell death (Minghetti and Levi, 1998). In animal cells, NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). Three NOS isoforms have been identified neuronal NOS (nNOS), inducible NOS in macrophages (iNOS), and endothelial NOS (eNOS) (Table 1) (Nathan and Xie, 1994). The nNOS and the eNOS are referred to as constitutive NOSs, whereas *iNOS* gene expression is induced in macrophages and many other cell types in response to inflammatory agents and cytokines (Mayer and Hemmens, 1997). The isoforms of nitric oxide synthase and their major physiological functions and implications in various diseases are summarized in Figures 1. It is this low level of production that is biological significance, while overproduction may lead to circulatory shock, chronic inflammation and carcinogenesis (Hidaka *et al*., 1997).

Table 2.1 Classification of mammalian nitric oxide synthase

Isoform[a]	Typical localization	Cellular localization	Dependence on intracellular free Ca2+ concentration	Monomer molecular mass (kDa)
nNOS	Neuron	Soluble and particulate	Yes	-160
eNOS	Endothelial cells	Particulate	Yes	-135
iNOS	Macrophages	Soluble	No	-130

[a]nNOS, neuronal nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS

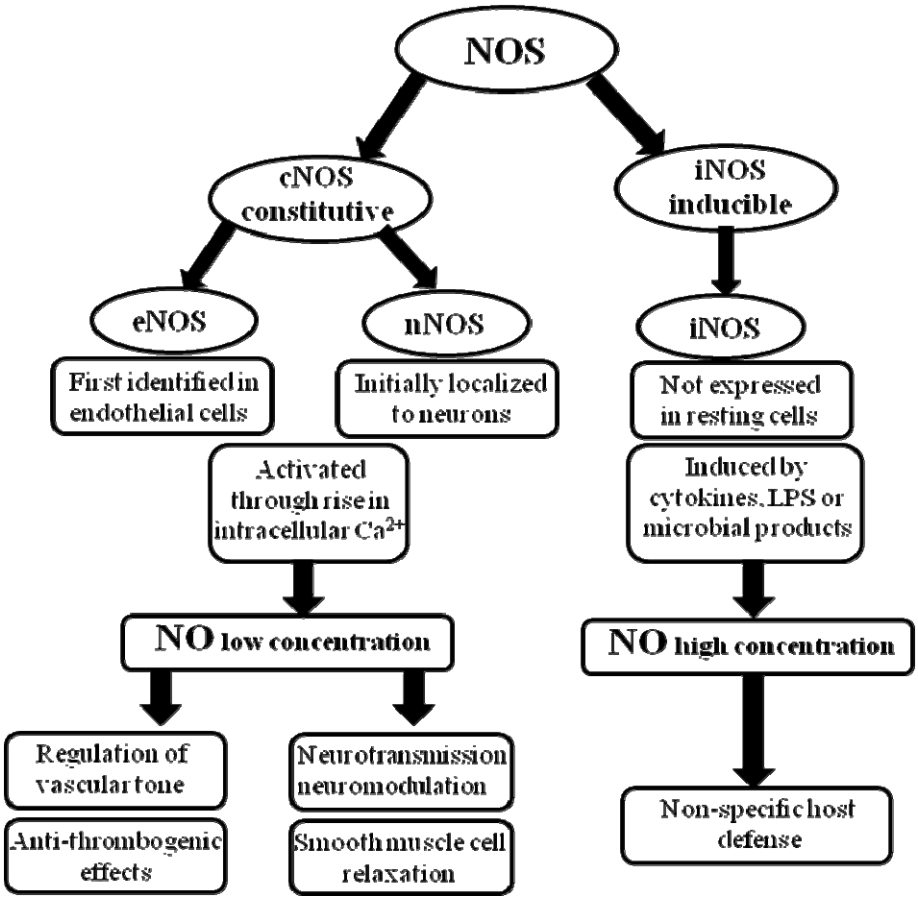
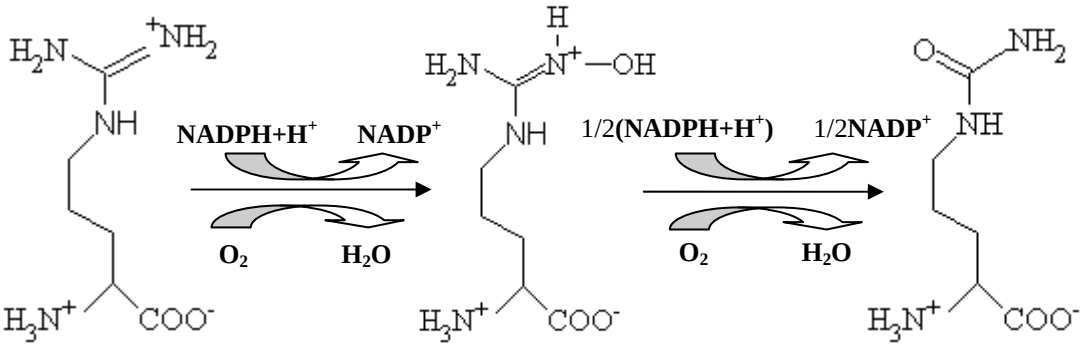


Figure 2.1 The isoforms of nitric oxide synthases. Two cNOS enzymes (eNOS, nNOS) are contrasted by a third, inducible NOS (iNOS) (Hemmrich *et al*., 2003).

All NOSs are active as homodimers. In their active form, NOSs oxidize L-arginine to L-citrulline and NO (Figure 2). Under conditions of reduced L-arginine availability, NOSs also produce both superoxide anion ($O_2^{\bullet -}$) and NO, leading to the production of peroxynitrite ($ONOO^-$), which is a cytotoxic species implicated in several pathophysiological processes (Xia *et al*., 1998). Activity of constitutive NOSs is strictly dependent on the elevation of intracellular free Ca_2^+ and resultant binding of the Ca_2^+-CaM complex. Thus, both nNOS and eNOS show fast and transient activation (over a matter of minutes) (Nathan and Xie, 1994; Mayer and Hemmens, 1997).



L-arginine N^G-hydroxy-L-arginine L-citrulline

Figure 2.2 The reaction catalyzed by mammalian nitric oxide synthases (NOSs). NOSs catalyze the conversion of L-arginine to L-citrulline and NO in a two-step reaction: oxygenation of L-arginine to NG-hydroxy-L-arginine, followed by oxygenation of NG-hydroxy-L-arginine to L-citrulline and NO. The reaction requires O_2 and NADPH. The catalytic mechanism involves flavin-mediated electron transport from C-terminal-bound NADPH to the heme iron, where O_2 is reduced and incorporated into the guanido group of L-arginine. Bound calmodulin functions as a molecular switch between the two domains of NOS, allowing the electron to pass from the flavins to the heme group.

The small quantities of NO generated by the constitutive NOSs help to maintain normal homeostasis of the vasculature and the central nervous systems (Schmidt and Walter, 1994; Nathan and Xie, 1994). By contrast, activity of iNOS is independent of the intracellular free Ca_2^+ concentration (Nathan and Xie, 1994; Mayer and Hemmens, 1997) with CaM remaining tightly bound to the enzyme even in the absence of elevated intracellular $Ca_2^{+\cdot}$ Consequently, iNOS activity is sustained for periods ranging from hours to days. This stability, in addition to its higher activity, results in the generation of two to three orders of magnitude more NO than with the constitutive NOS enzymes (Beck *et al*., 1999). The high amounts of NO produced by iNOS exert cytotoxic and antimicrobial effects on the immune system.

2.1.1 iNOS expression

More recently, a new isoform of NOS was found in mitochondria isolated from rat liver (Tatoyan and Giulivi, 1998). This isoform, named mtNOS, is similar to iNOS. All NOS isoforms show ~50–60% identity in their amino acid sequences. Each NOS is a bi-domain enzyme consisting of an N-terminal oxygenase and a C-terminal reductase (Mayer and Hemmens, 1997). The oxygenase domain contains a cytochrome P-450 type heme center and a binding site for the cofactor tetrahydrobiopterin. The reductase domain contains NADPH, FAD and FMN binding sites and exhibits significant homology with NADPH cytochrome P-450 reductase. Both domains are connected by a calmodulin (CaM) binding site in the middle of the enzyme. In addition, each NOS has a different N-terminal extension determining the intracellular localization of the enzyme (Table1) (Mayer and Hemmens, 1997).

Inducible NOS (iNOS) is induced in response to various pro-inflammatory cytokines, including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), and mediates several inflammatory responses. NO synthesized by iNOS has also been considered as an important mediator of carcinogenesis. Over-expression of iNOS that has been detected in human cancer and tumor associated with the production of NO by iNOS may promote cancer progression (Jung *et al.*, 2007). Overproduction of NO has been reported to cause oxidative damage cell death. Inhibition of NO overproduction has been proved to increase cell survival in several models. For example, addition of iNOS antagonist prevented rat amoeboid microglia from lysing oligodendrocytes *in vitro* (Merrill *et al.*, 1993). NO has been correlated

with the inflammatory process, in which multiple cytotoxic effects are related to the ability to increase vascular permeability and edema. This involves changes in local blood flow and increases in pro-inflammatory prostaglandins (Lantz *et al.*, 2005). The macrophages can be activated by lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). LPS is one of the major constituents of the outer membrane of Gram-negative bacteria, and the immune system is constantly exposed to low levels of LPS through infections. LPS recognition and signal transmission are the key events aimed at eliminating an invading pathogen. The LPS-induced activation of macro phages results in the production of bioactive lipids, reactive oxygen species, and in particular, inflammatory cytokines to fight and clear the bacterial infection. However, LPS mediates both the beneficial and deleterious reaction to the host. The excessive and uncontrolled production of inflammatory mediators triggered by LPS is harmful, and can lead to potentially lethal systemic disorders such as septic shock (Cho *et al.*, 2008). The antigen can activate macrophages to release some inflammatory mediators such as NO, TNF-α, PGE_2 and others. Therefore, the inhibition of NO, PGE_2 and TNF-α production is an important therapeutic consideration in the development of anti-inflammatory agents.

2.1.2 Nitric oxide activity

Measurement of NO generation can be performed by different experimental setups. The rate of conversion of ^{3}H- or ^{14}C- labeled L-arginine into the respective labeled L-citrulline and subsequent spectrophotometry is a standard procedure for indirect confirmation of NO production. L-citrulline can be readily detected using ion-exchange procedures to separate substrate and product (Chan *et al.*, 1997). In addition, there are many spectrophotometric assays available for the determination of NO metabolites. In oxygenated solution, NO reacts with O_2 to form nitrite and nitrate which can be measured using the Griess reaction (Kelm *et al.*, 1997). The Griess Reagent System is based on the chemical reaction shown in Figure 3, which uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects NO_2 in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium (Griess, 1879).

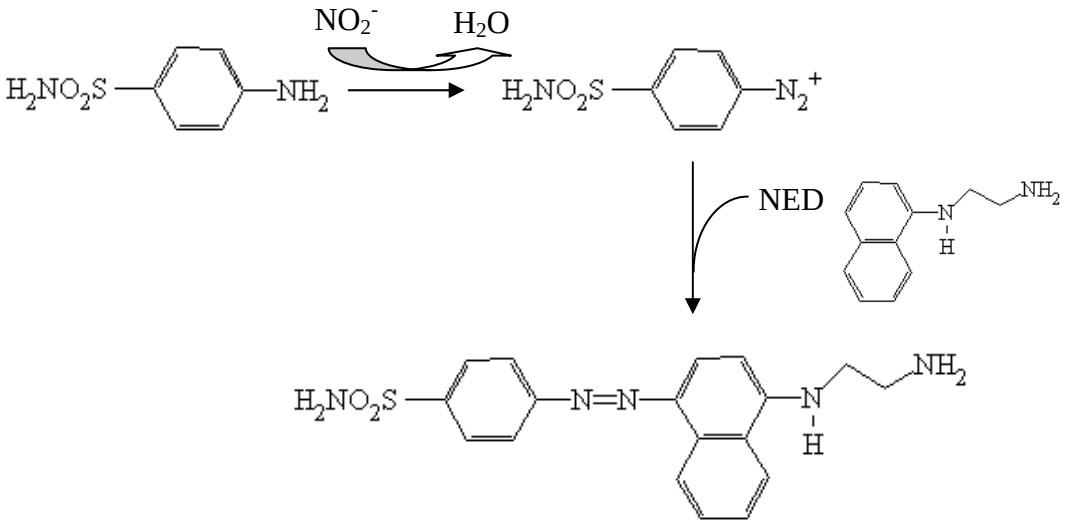


Figure 2.3 Chemical reactions involved in the measurement of NO_2 using the Griess Reagent System.

2.1.3 Activation of macrophage

iNOS modulation has been extensively studied in *in vitro* and *in vivo* murine macrophage models (Bottley and Fernandez, 2000). Macrophages are known to play an important role in host defense mechanism (Krol *et a*l., 1995). They are ubiquitous cells that are involved in various physiological processes such as pathogen destruction, inflammation, tissue repair, and remodeling (Nordin and Abastado, 2008). In addition, chemical mediators such as ROS, NO propagate inflammation by stimulating release of these mediators, which stimulate recruitment of additional macrophages at the inflammatory site. It is believed that persistent inflammatory cells recruitment, repeated generation of chemical mediators and pro-inflammatory mediators, and continued proliferation of genomically unstable cells contribute to neoplasic transformation and ultimately result in tumor invasion and metastasis (Khan *et al.*, 2008). The macrophages can be activated by LPS and IFN-γ. Additionally, inflammation and sepsis caused by gram-negative bacteria are frequently primarily due to LPS) released from bacterial outer membranes, LPS can activate several signal transduction pathways through induction of G-proteins, cAMP dependent kinase, protein kinase C, tyrosine kinase, PI3 kinase, and the ERK, p38, and JNK mitogen activated kinase (MAPK) families (Furukawa *et al.*, 1987). The cascade of LPS-induced events in macrophages is initiated by LPS binding to the LPS-binding protein

(LPSBP), then LPS is transferred to CD14 on the surface of macrophages for further interactions with Toll-like receptors (TLRs). Through the C- Jun N-terminal kinase and p38 mitogen-activated protein kinases (MAPKs), TLRs activate nuclear factor (NF)-κB, which mediates the production of inflammatory cytokines (Scott and Hancock, 2000). The combination of LPS (20 ng/mL) with different concentrations of IFN-γ resulted in a synergistic effect, which enhanced the amounts of NO released by macrophages compared to the endotoxin alone (Scuro *et al*., 2004). Macrophages-activated produce a variety of pro-inflammatory mediators, including interleukin, cytokines, nitric oxide and prostaglandin E_2 (PGE_2) (Chien *et al*., 2008).

Restoration/activation of improperly working or repressed antioxidant machinery or suppression of abnormally amplified inflammatory signaling can provide important strategies for chemoprevention. Consequently, determination of anti-inflammatory and/or antioxidant properties has been proposed as a good indicator for screening anti-cancer agents. To prevent the LPS-induced cascade in macrophages, several approaches were explored such as antibodies (Abs), anti-TNF-α Abs, endotoxinbinding molecules, and antimicrobial peptides (AMPs) (Dankesreiter *et al*., 2000). Thus, agents that down-regulate these pro-inflammatory mediators would be beneficial in the treatment of inflammation, which are mediators or can serve as an indicator of the presence of septic shock (Waage and Espevik, 1988; Chen *et al*., 2001; Wang and Mazza, 2002; Chien *et al*., 2008). To find a substance that can effectively neutralize or suppress LPS-induced production of inflammatory molecules is a critical step in the therapy against gram-negative bacterial infections. Therapeutic approaches based on molecules that bind and neutralize LPS, thereby directly blocking the primary stimulus for the proinflammatory cytokine cascade, are an attractive concept. However, macrophages are in direct contact with the ambient environment, and these cells recognize bacteria and their products (Waage and Espevik, 1988). In addition, most AMPs cloned from natural organisms have the potential to be applied as therapeutics by a peptide-synthesis method to suppress inflammatory responses caused by LPS or may be promising candidates for use as agents against septic shock (Nagaoka *et al*., 2001). For example, application of the peptide of Limulus anti-LPS factor (LALF) and black tiger shrimp (Penaeus monodon) anti-LPS factor (SALF) in an experimental animal model of gram-negative sepsis showed a pattern of cytokine gene expression in the spleen and liver in peptide- and LPS-treated mice including an abrogation of the systemic TNF-α response,

reduced organ damage, and increased survival of infected mice (Pan *et al*., 2007; Vallespi *et al*., 2000). Thus, ALFs may be useful peptides for preventing sepsis.

2.2 Cytotoxic activity

The predictive value of *in vitro* cytotoxicity tests is based on the idea of "basal" cytotoxicity – that toxic chemicals affect basic functions of cells which are common to all cells, and that the toxicity can be measured by assessing cellular damage. The development of *in vitro* cytotoxicity assays has been driven by the need to rapidly evaluate the potential toxicity of large numbers of compounds, to limit animal experimentation whenever possible, and to carry out tests with small quantities of compound. Evidence for the utility of *in vitro* cytotoxicity tests (Barile *et al*., 1994; Davila *et al*., 1990; Todd *et al*., 1999). "Cytotoxic" or "Cytotoxicity" meant in medical reports were usually get into mainly two denotations; the certain substance which contains ability to kill cancer or tumor cells, the certain substance which can kill certain animals or certain animal cells (including human) (Chang, 1983; Mizutani and Yoshida, 1990; Clark *et al*., 1976; Kaewdoungdee, 2006; Galvez *et al*., 2003). Further studies are needed to clarify the risk of these materials as well as their application for human use. Recently, *in vitro* methods have shown a significant potential for assessing the toxicity of environmental and occupational health risks (Bakand *et al*., 2006; Lestari *et al*., 2006).

The general principle for the detection of cell growth or cell kill via the MTT cytotoxicity assay (Figure 4). This method has been proven to be user friendly, rapid and highly sensitive (Promega, 2005; Bakand *et al*., 2006; Potera, 2007). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed (Mosmann, 1983).

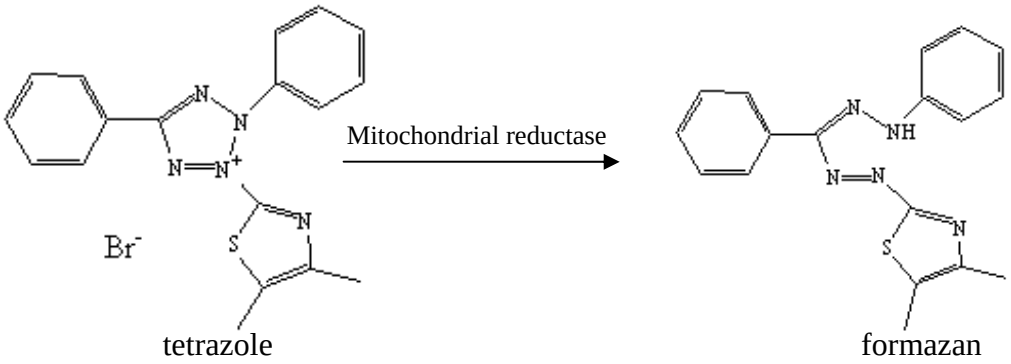


Figure 2.4 MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide a yellow tetrazole), is reduced to purple formazan in living cells.

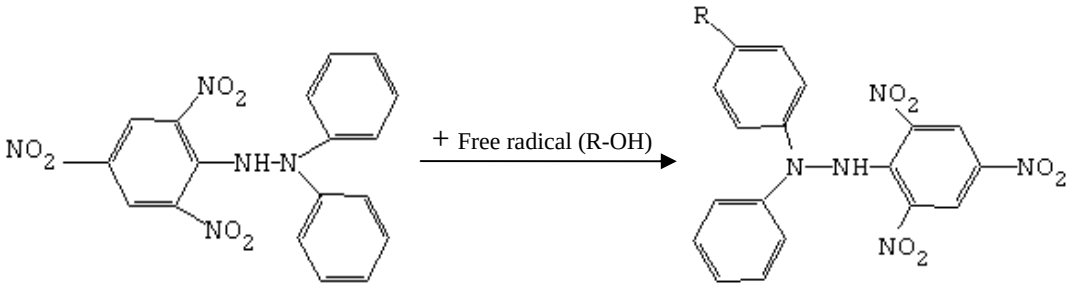
2.3 Antioxidant

Oxygen may be partially reduced during normal metabolism to yield reactive molecules termed reactive oxygen species (ROS). Oxidative stress occurs when ROS overload the body's antioxidant defenses or when the antioxidant defense system loses its capacity for response, and can lead to damage of vital cellular components. Large amounts of ROS are detrimental and have been shown to participate in the etiology of several human degenerative diseases, including inflammation, cardiovascular and neurodegenerative disorders, and cancer (Ames *et al*., 1993). Antioxidant is an agent that prevents or inhibits oxidation. They are naturally occurring or synthetic substances that help protect cells from the damaging effects of oxygen free radicals (Davies *et al*., 1984). Anti-oxidation substances in nature can be divided into two groups (Huang *et al*., 2005); (i) the enzymatic group, for example superoxide dismutase (SOD), glutathione peroxidase and catalase, and (ii) the non-enzymatic antioxidants, for example carotenoids, ascorbic acid, α-tocopherol, glutathione and phenolics. With the growing medical interest in natural antioxidants, it is of no surprise that antioxidant proteins in plants have gained attention and reports of their existence and bioactivities have correspondingly increased as well (Arcan and Yemenicioglu, 2007; Ningappa and Srinivas, 2008; Rios-Gonzalez *et al*., 2002; Sarkar *et al*., 2009; Sivapriya and Leela, 2007). Biological antioxidants have a further broad definition, which include systems such as metal transport proteins (e.g. transferrin, albumin, ferritin and ceruloplasmin) to prevent the redox properties

expression of metal, antioxidant enzymes and factors involved in vascular homeostasis, signal transduction and gene expression (Frankel and Meyer, 2000). Thus, the cellular antioxidant mechanisms involve suppressing of ROS formation, reducing oxygen free radicals ($O^{\cdot}{}_2^-$, $HO^{\cdot}$, $ROO^{\cdot}$) and H_2O_2, sequestering metal ions, scavenging active free radicals, repairing and/or clearing the oxidative damage on biomolecules. Moreover, some antioxidants also induce the biosynthesis of other antioxidants or defence's enzymes. It is necessary to note, that the bioactivity of an antioxidant also depends on factors like its physico-chemical characteristics and in vivo radical generating conditions (Tiwari, 2001). Research in the recent past has accumulated enormous evidences revealing that natural antioxidants can prevent the onset as well as to treat diseases caused and/or fostered due to oxygen free radicals (Hsu, 2006; Bruckdorfer, 1996; Tiwari, 1999; Pietta, 2000).

2.3.1 DPPH free radical scavenging assay

Antioxidative activities are evaluated by various methods. Diphenyl-picrylhydrazyl radical (DPPH) bleaching is one of the strategies used to evaluate the antioxidant properties of herbal extracts; this method has shown to be rapid and simple and it measures the capacity of herbal extract to bleach the DPPH radical, a nitrogen-centred free radical (Antolovich *et al*., 2002). The structural changes that this radical provokes on herbal principles as well as the involved mechanism however, are not clear yet (Wang and Zhang, 2003). The DPPH is a stable radical, as the radical is heavily delocalized. As a stable radical is can be used to trap other radicals and thus reacting as an inhibitor in radical reactions. The DPPH radical is strongly colored violet in alcoholic media, while the molecule is pale yellow after capturing a hydrogen donors or free radicals (Figure 5) (Ebada *et al.*, 2008). The DPPH assay can be used to evaluate the antioxidant capacity of antioxidants to donate hydrogen during a free radical attack (Liangli, 2002). Recently, Curcuma longa L., or turmeric, was found to have a potential antioxidant protein, b-turmerine (Smitha *et al*., 2009). Although an antioxidant activity was also been reported in the related Curcuma comosa (Niumsakul *et al*., 2007) this was found to be due to a small nonprotein molecule, 4,6-dihydroxy-2-O-(b-D-glucopyranosyl) acetophenone, and showed not only a powerful antioxidant activity but was also cytotoxic to HeLa cells in tissue culture.



DPPH: 2,2-diphenyl-1-picryl hydrazyl

R= -OH or $-NO_2$

1(4-Hydroxyphenyl)-1-phenyl-2-picryl hydrazine

1(4-Nitrophenyl)-1- phenyl-2-picryl hydrazine

$/\lambda_{max}$ 517 nm

Figure 2.5. The DPPH free radical scavenging assay. The DPPH radical is strongly colored violet in alcoholic media, while the molecule is pale yellow after capturing a hydrogen donors or free radicals (Ebada *et al.*, 2008).

2.4 Bioactive protein

In general, protein is a nutrient needed by the human body for growth and maintenance. Aside from water, protein is the most abundant molecule in the body. Protein is found in all cells of the body and is the major structural component of all cells in the body, especially muscle. This also includes body organs, hair and skin. Proteins also are utilized in membranes, such as glycoproteins. When broken down into amino acids, they are used as precursors to nucleic acid and vitamins (Hermann and Janice, 1998). Hormones and enzymes are also formed from amino acids in which they help regulate metabolism, support the immune system and other body functions (Jeong *et al.*, 2003). In addition to simple functions such as being structural components, enzymes, hormones, receptors, transporters, immune species, storage proteins, and moving organelles (for examples; flagella, cilia, and spindle fibers), the protein can also involve in cell to cell or cell to environment signal transduction element (Voet, 2007). Finally, protein is needed to form blood cells (Hermann and Janice, 1998). The heart of protein function is amino acid sequence which can be called “peptide”. The diversity of protein functions come from difference of peptide sequences, both in amino acid type ordered and chain length. Moreover, nascent synthesized peptides are possibly managed by many post-translation modification

procedures presented in different kinds of cells that make different in overall structures of final proteins. These mechanisms all cause changes in molecular structures and properties of proteins towards different three dimension compatibilities suitable for various functions (Elliott and Elliott, 2009). With the growing medical interest in natural antioxidants, it is of no surprise that antioxidant proteins in plants have gained attention and reports of their existence and bioactivities have correspondingly increased as well (Arcan and Yemenicioglu, 2007; Ningappa and Srinivas, 2008; Rios-Gonzalez *et al*., 2002; Sarkar *et al*., 2009; Sivapriya and Leela, 2007). Recently, *Curcuma longa* L., or turmeric, was found to have a potential antioxidant protein, β-turmerine (Smitha *et al*., 2009). In addition, biologically active peptides are either naturally occurring or can be derived from soy protein hydrolysates by various methods, such as enzyme digestion and fermentation. Some peptides have been found to be bioactive and to exert anticancer, antihypertensive, hypocholesterolaemic, antiobesity, antioxidant and immunomodulatory properties (Wang and de Mejia, 2005). Lunasin is a chemopreventive peptide which has been isolated from soybean, barley, wheat and other plant sources (Galvez *et al*., 2001; Jeong, jeong, Kim *et al*., 2007; Jeong, jeong, Park *et al*., 2007; Jeong *et al*., 2002; Silva-Sanchezet *et al*., 2008). It is composed of 43 amino acid residues and contains nine aspartic acid residues on its carboxyl end, a cell adhesion motif composed of arginine-glycine-aspartic acid residues and a predicted helix with structural homology to a conserved region of chromatin binding proteins (Galvez *et al*., 2001; Jeong *et al*., 2002). Lunasin has been reported to have chemopreventive properties, including suppression of colony formation induced by ras-oncogene and inhibition of core H3-histone acetylation (Jeong *et al*., 2003). Earlier studies in animals showed that lunasin can be absorbed intact and can enter target tissues (de Lumen, 2005). Recent studies have shown that the anticancer potential of lunasin can be attributed to its capability to selectively kill cells that are being transformed or are newly transformed by disrupting the dynamic of histone acetylation– deacetylation (Galvez *et al*., 2001; Jeong *et al*., 2007). In order to better understand the role of lunasin in disease prevention, further in vivo studies are needed. However, the high cost involved in obtaining synthetic lunasin limits its application in chemopreventive studies.

2.5 Zingiberaceae plants

Zingiberaceae is one of the largest families of the plant kingdom. It is important natural resources that provide many useful products for food, spices, medicines, dyes, perfume and aesthetics to man (Larsen, 1964). Zingiberaceae are distributed mostly in tropical and subtropical areas. It is usually found as ginger, and exists in about 50 genera and 1,300 species worldwide, distributed mainly in South and Southeast Asia (Wu and Larsen, 2000). Species of the Zingiberaceae are the ground plants of the tropical forests. They mostly grow in damp and humid shady places. They are also found infrequently in secondary forest. Some species can fully expose to the sun, and grow on high elevation (Larsen, 1980). Perennial rhizomatous herbs. Leaves simple, distichous. Inflorescence terminal on the leafy shoot or on the lateral shoot. Flower delicate, ephemeral and highly modified. All parts of the plant aromatic (Larsen, 1964). Recently, scientific study has sought to reveal the bioactive compounds of the rhizome. It has been found to be effective in the treatment of thrombosis, sea sickness, migraine and rheumatism (Farnsworth and Bunyapraphatsara, 1992). Moreover, Zingiberaceous plants have been reported for their biological activities in antifungal, antioxidant, insecticidal, and anti-inflammatory activities (Sirat 1994; Sirat *et al*., 1996; Sirat and Liamen, 1995).

2.5.1 Anti-inflammatory effect from Zingiberaceae plants

Kaempferia parviflora Wall. ex Baker, is one of the plants in the Zingiberaceae family, locally known in Thai as kra-chai-dam. The rhizome of this plant has been used for treatment of gout, apthous ulcer, abscesses, allergy and gastrointestinal disorders, as well as an aphrodisiac (Pengcharoen, 2002). *Kaempferia parviflora* has recently been reported to possess antimycobacterial, antiplasmodial (Yenjai *et al*., 2004), anti-peptic ulcer (Rujjanawate *et al*., 2005) and anti-viral protease effects (Sookkongwaree *et al*., 2006). Moreover, it has been reported that the ethanolic extract of this plant promoted NO production in human umbilical vein endothelial cells (Wattanapitayakul *et al*., 2007). Previously, Tewtrakul and Subhadhirasakul (2008) reported the effects of 5-hydroxy-3,7,3',4'-tetramethoxyflavone from *Kaempferia parviflora* on nitric oxide (NO), PGE_2 and tumor necrosis factor-alpha (TNF-α) productions in RAW264.7 macrophage cells. Since *Kaempferia parviflora* rhizomes have long been used for treatment of inflammation and possessed marked anti-NO activity.

Curcuma comosa Roxb. (Zingiberaceae), commonly known as Waan chak mod look in Thai, is an indigenous plant of Thailand which has been generally used in folk medicine as an anti-inflammatory agent for the treatment of postpartum uterine bleeding, peri-menopausal bleeding, and uterine inflammation. Despite its long-term and wide use, there is scant scientific evidence on the anti-inflammatory activity of the plant. The result from previous study demonstrated the estrogenic-like activity of the plant and they suggested the presence of phytoestrogens in the hexane extract of this plant (Piyachaturawat *et al*., 1995). It has been reported that phytoestrogens possess anti-inflammatory and neuroprotective activities in the CNS (Dixon, 2004; Wang *et al*., 2001). Since the anti-inflammatory effect of estrogen in LPS-activated microglia (Vegeto *et al*., 2000) and of phytoestrogens in reducing NO production were observed (Kim *et al*., 1999), attempts have been made in this study to investigate whether *C. comosa* could exhibit a similar inhibitory activity in microglia.

Curcuma mangga Val. and Zijp. is a perennial herb and a member of the Zingiberaceae family, commonly grown in Thailand, Peninsular Malaysia, and Java. It is locally known as “mango tumeric” because of its mango-like smell when the fresh rhizomes are cut (Thai name is Kha-Min-Khao). Due to this characteristic and its palatable taste, *C. mangga* has become a popular vegetable, of which the tips of young rhizomes and shoots are consumed raw with rice. Medicinally, the rhizomes are used as a stomachic and for chest pains, fever, and general debility. It has been reported that compounds from *C. mangga* showed high cytotoxic activity against a panel of human tumor cell lines, such as human leukemia (HL-60), breast cancer (MCF-7) and liver cancer (HepG2) (Abas *et al.*, 2005). Moreover, the ethanol and water extracts from *C. mangga* also possessed anti-allergic activity (Tewtrakul and Subhadhirasakul, 2007). Since the EtOH extract of *C. mangga* rhizomes possessed potent NO inhibitory effect (Kaewkroek *et al*., 2009).

CHAPTER llI

EXPERIMENTAL

3. Material and methods

3.1. Plant materials

The fresh rhizomes of 15 plants in Zingiberaceae family were periodically (April 2010 - May 2010) purchased from Chatuchak park market in Bangkok, Thailand.

3.2. Chemical and biological materials

100 bp DNA ladder	Fermentas,Canada
100mM dNTP Mix	Fermentas,Canada
AccQ-Flour reagent	
Acetic acid	Merck Ag Darmstadt, Germany
Acetonitrile	
Acrylamind	Plusone Pharmacia Biotech, Sweden
Agar	
Ammonium persulfate	Plusone Pharmacia Biotech,Sweden
Ammonium sulfate	Merck Ag Darmstadt, Germany
Bis-acrylamide	Promega, USA
Bovine serum albumin (BSA)	Sigma, USA
Bromophenol Blue	USB, USA
Chloroform	Lab-Scan, Ireland
Coomassie Brilliant Blue G-250	USB, USA
Diethylpyrocarbonatev(DEPC)	Sigma, USA
Dimethylsulfoxide (DMSO)	Sigma, USA
Di- Potassiumhydrogen phosphate	Merck Ag Darmstadt, Germany
Disodium hydrogen phosphate (Na_2HPO_4)	Merck Ag Darmstadt, Germany
Dulbecco's modified minimum essential medium (DMEM)	Hyclone, England

Endotoxin-free water	Sigma, USA
Ethylenediaminetetraacetic acid, EDTA	Sigma, USA
Ethanol	Merck Ag Darmstadt, Germany
Ethidium Bromide	Sigma, USA
Ethyl acetate	Ajax Finechem, New Zealand
Fetal Bovine Serum (FBS)	Hyclone, England
Glycerol	Fluka, Germany
Glycine	Sigma, USA
HEPES (N-2-hydroxyethylpiperazine-	Hyclone, England
N, N'-methylene-bis-acrylaminde	Plusone Pharmacia Biotech, Sweden
Hydrochloric acid (HCl)	J.T. Baker, USA
Isopropanol	Merck, Germany
L-α-amino-*n*-butyric acid	
Lipopolysaccharide from *E.coli* serotype O26:B6	Sigma,USA
Methanol	Merck Ag Darmstadt, Germany
M-MuLVReverse Transcriptase	Fermentas, Canada
NED (N-1-naphthylethylenediamine dihydrochloride)	Sigma, USA
PCR reagent	Takara, Japan
Penicillin	General Drugs House, Thailand
Potassium chloride (KCl)	Merck Ag Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck Ag Darmstadt, Germany
Quant-iT^{TM} RNA Assay Kit	Invitrogen, England
Random Hexamer Primer	Qiagen, Germany
Recombinant mouse interferon-γ	R&D systems, Inc., USA
Ribolock^{TM} Ribonuclease Inhibitor	Fermantas, Canada
Sodium acetate	
Sodium azide (NaN_3)	Merck Ag Darmstadt, Germa
Sodium chloride (NaCl)	Merck Ag Darmstadt, Germany
Sodium hydroxide (NaOH)	Merck Ag Darmstadt, Germany

Sodium nitrite ($NaNO_2$)	Carlo Erba, Italy
Sodium pyruvate	Hyclone, England
Standard Molecular Weight Marker	Sigma, U.S.A
Streptomycin	M&H Manufacturing, Thailand
Sulfanilamide ($C_6H_8N_2O_2S$)	BDH Chemicals Ltd., England
Tetramethylethylenediamine, TEMED	Plusone Pharmacia Biotech, Sweden
Trisma Base (hydroxymethyl-aminomethane, $CH_4H_{11}NO_3$)	Sigma, USA
TRI®zol reagent	Invitrogen, England
Trypan Blue solution	Hyclone, Englan

3.3 Equipment

-20°C Freezer model MDF-U332	Sanyo, Japan
-80°C Deep Freezer model ULT1780	Forma Scientific, USA
4°C Refrigerator	Mitsubishi Electric, Japan
5% CO_2 Incubator model 311	Thermo Electron Corporation, USA
Autoclave model MLS 3020	Sanyo, Japan
Centrifuge tube 15 and 50 ml	Corning Incorporation, USA
CX2 Inverted Microscope	Olympus, USA
Dialysis bags	Snake Skin Dialysis Tubing, Pierce, USA
Electrophoresis unit	Cosmo Bio, Japan
Gel Documentation and Quantity One 4.4.1	Bio-Rad, USA
Haemocytometer	Boeco, Germany
High performance liquid chromatography.	
High Speed Refrigerated Centrifuge	Kubota 6500, Japan
Hot Air Oven	Memmert, Germany
Hot plate stirrer	HL instrument, Thailand
Hypersil Gold column C18	
Laminar Flow Cabinet model H1	Lab Service., Ltd., Thailand
Microcentrifuge	Axygen Scientific, USA
Micropipette P2, 20, 100 and 1000	Gilson, France

Microplate Reader Elx 800	Bio-Tek Instrument, Canada
PCR tube	Corning Incorporation,USA
Petri Dish	Hycon, Germany
pH meter	Mettler Toledo, USA
Pipette tips	Bioline, U.S.A
Power Supply	Bio-rad, USA
Spectrophotometer	Synergy HT Biotek, USA
Speed vacuum centrifuge (Heto-Holten, Denmark)	
Tissue Culture Plate 24 and 96 well	Corning Incorporation, USA
Ultrasonic	leaner D200, D.S.C.,
Vortex mixer	Scientific Industries, U.S.A
Water Bath NTT-1200	Tokyo kikakikai, Japan

3.4 Preparation of the crude protein from Zingiberaceae rhizomes

The rhizomes (1.5 kg wet weight) of the 15 selected plant species from within the Zingiberaceae family were peeled, cut into small pieces (~ 10 × 10 × 10 mm) and then homogenized in 5 L of PBS (20 mM phosphate buffer (pH 7.2) containing 0.15 M NaCl) using a blender and then left with stirring overnight at 4 ^{o}C. The suspension was then clarified by filtration through double-layered cheesecloth followed by centrifugation at 15,000 × g for 30 min. The clarified supernatant (crude homogenate) was harvested and ammonium sulfate added, with stirring, to 80% saturation and then left with stirring overnight at 4 ^{o}C. The precipitate was collected from the suspension by centrifugation at 15,000 × g for 30 min with discarding of the supernatant. The pelleted materials were then dissolved in PBS, dialyzed (3,500 MWCO) against 3 changes of 5 L of water at 4 ^{o}C and then freeze dried. This is referred to as the 'ammonium sulphate cut fraction'.

3.5 Cell culture

Mouse macrophage cell line RAW 264.7 (ATCC TIB-71) was purchased from American Type Culture Collection (USA) and maintained in RAW 264.7 growth medium (DMEM) at 37 °C in a humidified atmosphere with 5% CO_2 incubator.

DMEM media contained 10%v/v fetal bovine serum, 100U/ml penicillin-G, 0.4 mg/ml streptomycin, 1% sodium pyruvate and 1% HEPES.

3.5.1 Harvesting RAW 264.7 cells

Old medium of RAW 264.7 grown in non-tissue culture treated dish were aspirated old medium from the cells. RAW 264.7 cells were gently rinsed with repetitive pipetting in 1X PBS and gently scraped the cells until all are dislodged from dish. RAW 264.7 cells were harvested in PBS by centrifugation at 1,000 rpm for 5 min. After that, PBS was removed and cell pellets were resuspended in DMEM complete media with pipette, up and down. Count the cells using Trypan blue dye exclusion assay. The following equation was used to calculate viable cell numbers.

Total cell count (cells/ml) = The number of cell counted in 16-large squares$\times 2 \times 10^4$

3.5.2 Maintaining RAW 264.7 cell line

RAW 264.7 cells were maintained in DMEM complete media at 37 °C in a humidified atmosphere with 5% CO_2. For routine maintenance in culture (passage), cells are seeded at a confluence of approximately 10% and grown to a confluence of approximately 80%. This procedure requires the cells to be split every two days. For passage over the weekend to ensure no more than 80% confluence when harvested after three days. Cultures are not maintained beyond three months.

3.5.3 Cell storage

Cells were harvested and cell pellet was resuspended in cold freezing media containing DMEM complete media and 10% DMSO. Cell pellet was aliquoted in the cryogenic tubes. The cryogenic tubes were stored at -80°C immediately for at least overnight.

3.5.4 Thawing cells

RAW 264.7 cells were thawed by gently rubbing the vial between your hands and followed by in a 37° C water bath. RAW 264.7 cells were transferred into 10 ml

of serum free DMEM media and centrifuged at 1,000 rpm for 5 min that harvested RAW 264.7 cells. Removed supernatant and the cell pellets were resuspended in warmed fresh DMEM complete media. Cells were maintained at 37°, in a humidified atmosphere, with 5% CO_2. After the cells have adhered, approximately 6 h, aspirated the old medium and added 7 ml of fresh medium. When the cells are 80% confluent, passed them into a new vessel with fresh media.

3.6 Pretreatment of RAW 264.7 cells

The RAW 264.7 cells were seeded in 96-well plates with density of 1×10^4 cells/well in 100 µl in 96-well plate and incubated overnight in 5% CO_2 incubator. After that the medium was replaced with a fresh medium containing the crude proteins at various concentrations of each *Zingiberaceae* plants and was incubated for 1 h. After 1 h incubation, stimulated NO production with 100 ng/ml lipopolysaccharide (LPS) and 10 ng/ml interferon-gamma (IFN-γ), incubated for 18-24 h.

3.7 Determination of nitric oxide (NO) production from RAW 264.7 cell

NO production was determined by measuring the nitrite in culture supernatants. Sodium nitrite was used as standard ranging from 0-100 µM. A volume of 50 µl of the culture supernatant was added in 96-well plate and added 50 µl of sulfanilamide, incubated at room temperature 10 min and dark condition. After added 50 µl of NED solution and incubated 10 min at room temperature and dark condition (Griess reagent). The absorbance at 540 nm was measured using a microplate reader.

3.8 MTT assay for the measuring of cell proliferation

RAW 264.7 cell proliferation was measured using the MTT assay. Cells were plated at density of 1×10^4 in 96-well plate and added 100 µl of 5 mg/ml MTT solution (in PBS) per well. After incubated at 37°C, 5% CO_2 incubator for 4 h and 100 µl of isopropanol containing 0.04N HCl was added to dissolve the formazan production in the cells. Pipette up and down until purple crystals are completely dissolved. The absorbance at 540 nm was measured using a microplate reader.

$$\% \text{ viability} = \frac{(\text{O.D.test average}) - (\text{O.D.blank average})}{(\text{O.D.control average}) - (\text{O.D.blank average})} \times 100$$

3.9 Total RNA isolation from RAW 264.7 cells

RAW 264.7 cells (4×10^5 cell/well) were added to 6-well culture plate and allowed to adhere for overnight at 37°C in incubator containing 5% CO_2. After that, cells were treated with crude protein and 2.5 µg/ml parthenolide (positive control) (Wangthong *et al*., 2010) for 1 h. Then, cells were stimulated with 100 ng/ml lipopolysaccharide (LPS) and 10 ng/ml interferon-gamma (IFN-γ). After 6 h incubation, added 1 ml of TRI®zol reagent to 6-well culture plate for harvested the total RNA and incubated at room temperature for 5 min. After that, added 200µl of chloroform and mixed for 15 sec, incubated at room temperature about 2-3 min. After incubation, RNA was centrifuged with 12000×g at 2-8°C for 15 min. The RNA in upper supernatant was removed to new microcentrifuge tube then added 500µl of isopropanol and inversion mixed, incubated at room temperature for 10 min. Then, centrifuged with 12000×g at 2-8°C for 10 min. RNA was washed with 1 ml of 75% ethanol, mixed by vortex and centrifuged with 7500×g at 2-8°C for 5-10 min. After that, dried RNA about 15-20 min and redissolved 20µl of RNase free water. Then, incubated at 55-60°C for 10 min. The isolated RNA was stored at -80 ◦C until use. To measure amount of RNA in sample, the using Qubit® fluorometer and Quant-iT™ RNA Assay Kit (Invitrogen) were used according to manufacturer's recommendation.

3.10 Detection *iNOS, IL-6* and *TNF-α* mRNA by Reverse transcription (RT-PCR)

A content of 0.2 µg total RNA from each condition was subjected to reverse transcription became single-stranded complementary DNA (cDNA) consisting of RNA solution 12 µl, random hexamer primer 0.5 µl, 5X RT buffer 4 µl, 10mM dNTP mixture 2 µl, RNase inhibitor (10U/ml) 1 µl and reverse transcriptase 1 µl for 20 µl reaction. Reverse transcription was performed at 25°C for 10 min, 42°C for 60 min and 70°C for 10 min. The resulting cDNA was used as a template for subsequent PCR. The *iNOS, IL-6, TNF-α* and *β-actin* genes were amplified by PCR. The *β-actin*,

a constitutively expressed gene, was analyzed as an internal standard. The oligonucleotide primers for each gene were as follows Table 3.1.

Table 3.1: Oligonucleotide primers used in experiments

Primer	Gene Bank Association Number	Sequence (5' to 3')	References
β-actin forward	NM_001101	ACCAACTGGGACGACATGGAGAA	(Palaga *et al*., 2008)
β-actin reverse	NM_001101	GTGGTGGTGAAGCTGTAGCC	(Palaga *et al*., 2008)
iNOS forward	NM_010927	CCCTTCCGAAGTTTCTGGCAGCAGC	(Lee *et al*., 2007)
iNOS reverse	NM_010927	GGCTGTCAGAGCCTCGTGGTCTTGG	(Lee *et al*., 2007)
IL-6 forward	NM_010548	CATGTTCTCTGGGAAATCGTGG	(Palaga *et al*., 2008)
IL-6 reverse	NM_010548	AACGCACTAGGTTTGCCGAGTA	(Palaga *et al*., 2008)
TNF-α forward	NM_013693	CCTGTAGCCCACGTCGTAGC	(Lee *et al*., 2007)
TNF-α reverse	NM_013693	TTGACCTCAGCGCTGAGTTG	(Lee *et al*., 2007)

The PCR mixture was consisted of cDNA 2 µl, highpure water (RNase free water) 9.5 µl, PCR reagent (Takara, Japan) 12.5 µl, 10mM forward primer 0.5 µl and 10mM reverse primer 0.5 µl, to give a final volume of 25 µl. Amplification was performed for 30 cycles, except *β-actin* was amplified for 25 cycles using Takara PCR Thermal Cycler Dice TP600 (Takara, Japan) with the following programme: denaturation at 98 °C for 30 s, annealing at 60 °C for 30 s, and extension at 74 °C for 1min. The 580 base pairs (bp) of *iNOS*, 475 bp of *IL-6*, 239 bp of *TNF-α* and 514 bp of *β-actin* DNA fragments were obtained and separated on 1.5% (w/v) agarose gel electrophoresis. The bands of DNA were detected by ethidium bromide staining for 10 min and were observed under a UV light using gel documentation system (Bio-Rad; Hercules, CA).

3.11 Total amino acid analysis

3.11.1 Acid hydrolysis

Five milliliters of HCl 6N was added (5.06 mg protein/ml HCl) and mixed. The tube was flushed with nitrogen for 1 min to remove air. Hydrolysis was then carried out at 110 °C for 22 h. The internal standard (10 ml of 2.5mM L-α-amino-*n*-butyric acid in HCl 0.1 M) was added and diluted with water to 250 ml. The solution was filtered with 0.20 µm filter and was then derivatized with 6 aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Flour reagent). It was then heated in a heating block at 55 °C, for 10 min. Heating converts a minor side product of tyrosine to a major mono-derivatized compound. Total amino acid content was determined by high performance liquid chromatography.

3.11.2 Chromatographic conditions

Chromatographic separation was carried out in a Waters Alliance 2695 with heater amino acid analysis Hypersil Gold column C18. The column was thermostatted at 35±1 °C and the flow rate was 1.0 ml/min. The injection volume was 5 µl. Mobile phase A consisted of sodium acetate buffer pH 4.90 and 60% acetonitrile.

3.12 Measurement of free radical scavenging capacity using DPPH assay

The experiment was carried out according to the method slight modification. Briefly, the crude protein extract and vitamin C (positive control) are pipetted into each tube that covered tube with aluminum foil. The appropriate concentration of sample were added in vary concentration viz 200, 100, 50, 25, 20, 10, 5, 0 µg/ml. After that added 800 µl of DPPH solution at 200µM in absolute ethanol into each tube. The mixture was shaken vigorously and incubated at 37°C for 30 min. After incubation, The volume 200 µl of mixture solution was added to 96-well plate. The absorbance was measured at 517 nm using micro-plate reader. The percentage of DPPH radical scavenging activity was calculated as follows;

$$\% \text{ DPPH radical activity} = [(A_{DPPH}-A_{sample})/A_{DPPH}] \times 100$$

3.13 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The sodium dodecyl sulfate–polyacrylamide gel was prepared with 0.1% (w/v) SDS in 15% separating gels and 5% stacking gels. Tris-glycine buffer pH 8.3

containing 0.1% SDS was used as the electrode buffer. Discontinuous SDS-PAGE in reducing conditions was performed according to the procedure of Laemmli. (Laemmli, 1970) Samples to be analyzed were treated with reducing sample buffer and boiled for five min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standards were co-resolved in adjacent lanes and used to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were visualized by staining with Coomassie Brillion blue R-250 in 20% ethanol in deionized water for overnight. Then, the gels were washed with destain solution (20% v/v acetic acid and 20% v/v methanol in deionized water) and the solution would be always changed until blue color absence from gel textures. Relative molecular weights were achieved by comparison with co-resolved sample bands from molecular.

3.14 Internal amino acid sequencing by liquid chromatography / mass spectrometry / mass spectrometry (LC/MS/MS)

3.14.1 *In situ* (in gel) trypsinization

The sample preparation process followed the published method of Mortz *et al*. (1994). The single band from the SDS-PAGE resolution of the crude protein was excised from the gel, cut into small pieces (ca. 1 mm^3) and washed with 100 µl deionized water. The gel pieces were destained by adding 200 µl of a 2:1 (v/v) ratio of acetonitrile: 25 mM NH_4HCO_3 for 15 min, and this step was performed several times until the gel pieces were completely destained. The supernatant was removed and gels were then dehydrated by adding 200 µl acetonitrile for 15 min prior to drying in a vacuum centrifuge. Then, 50 µl of a 10 mM DTT solution in 100 mM NH_4HCO_3 was added, and the proteins were reduced for 1 h at 56 $^{\circ}$C. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and gels were incubated for 45 min at room temperature in the dark. The solution was then removed, the gel pieces were dehydrated in acetonitrile and the solvent evaporated off before adding 10 µl of a trypsin solution (proteomics grade, Sigma) (10 ng / µl in 50 mM NH_4HCO_3). After allowing the gel plug to swell for 15 min at 4 $^{\circ}$C, 30 µl of 50 mM NH_4HCO_3 was added and the digestion allowed to proceed at 37 $^{\circ}$C overnight. The supernatant was

then harvested following centrifugation at 10000×rpm for 1 min. The remaining peptides in the gel were extracted with a solution of 50% (v/v) acetonitrile containing 5% (v/v) formic acid for 10 min with shaking, and subsequently pooled with the previous supernatants and taken to dryness.

3.14.2 LC-MS/MS and peptide blasting

The likely amino acid sequence of each internal fragment of the trypsinized protein was analyzed by LC/MS/MS. The extracted tryptic peptides were then subjected to LC-nano ESI/MS/MS. All collected LC/MS/MS data were processed and submitted to a MASCOT (http://www.matrixscience.com) search of the NCBI database (http://blast.ncbi.nlm.nih.gov). The following criteria were used in the Mascot search: trypsin cleavage specificity with up to three missed cleavage sites, cysteine carbamidomethyl fixed modification, methionine oxidation variable modifications, ± 0.2 Da peptide tolerance and MS/MS tolerance, and ESI-TRAP fragmentation scoring (Mortz *et al*., 1994).

3.15 Statistical analysis

All determinations, except for NO activity, were done in triplicate, and the results are reported as the mean $\pm$ 1 standard error of the mean (SEM). Regression analyses and calculation of IC_{50} values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc.).

CHAPTER lV

RESULT AND DISCUSSION

4.1. Screening of Zingiberaceae plants protein for inhibitory activity against nitric oxide production in RAW264.7 cells and antioxidant activity

The crude protein of fifteen Zingiberaceae rhizomes were prepared as described in materials and methods. The crude extracts were subjected to test their inhibitory activity against nitric oxide production in macrophages RAW 264.7 which were stimulated by LPS and IFN-γ (See in Table 4.1). From the result in table 4.1 suggested that, the crude protein of *Z. ottensii* exhibited the highest activity against NO production with an IC_{50} value of 38.6 μg/ml (Table 4.1). This concentration of crude protein of four Zingiberaceae had no effect on the viability of RAW 264.7 macrophages (data not shown). It has been reported that dried rhizomes ethanol extract of *Z. officinale* had the analgesic, anti-inflammatory and hypoglycaemic effects in mice and rats (Ojewole, 2006). In addition to *C. comosa*, other plants in curcuma species, *C. xanthorrhiza* and *C. longa* have long been used for anti-inflammatory, anti-cancer, anti-microbial, anti-oxidation effects, etc. (Claeson *et al.*, 1993; Lechtenberg *et al.*, 2004). Moreover, there is the first report of the anti-inflammatory properties of soy high in this type of protein (Hernandez-Ledesma *et al.*, 2009).

The antioxidant properties of crude protein from Zingiberaceae plants were determined using DPPH assays. The results of the IC_{50} value of DPPH radical scavenging activities were also showed (in Table 4.1). The result suggested that *H. coronarium* extract exhibited the highest activity, followed by *C. comosa*, *C. aeruginosa*, *Z. officinale*, *Z. ottensii*, *C. amarissima*, *Z. zerumbet*, *Z. cassumunar*, *C. longa*, *Curcuma sp.*, *C. zedoaria*, and *C. aromatic*, respectively. These results were comparable to vitamin C as the standard for inhibiting this reaction. No significant correlation was found DPPH at $P < 0.005$ (data not shown) except *C. zedoaria*, and *C. aromatic*. Therefore, the crude protein had DPPH radical scavenging activities equate with vitamin C. It has previously been reported that the seed coat extract of *T. indica* contains a polyphenolic flavonoid that displays antioxidant

properties. An inhibitory effect of the seed coat extract of *T. indica* on nitric oxide production *in vivo* and *in vitro* has also been reported (Komutarin *et al*., 2004). Correlation between DPPH bleaching activity of herbal extracts and some antioxidant properties of natural product have been observed (Wright, 2003; Hurd *et al.*, 2005). Taken together, DPPH and oxygen free radicals interact with phospholipids and protein thiol groups. The herbal extract considered as a mixture of antioxidant principles prevented the oxidative changes induced by oxygen free radicals.

Table 4.1 Inhibition on NO production in RAW 264.7 cells and DPPH radical scavenging activity of crude protein from fifteen selected plants in the Zingiberceae family

Plants	IC_{50} value (μg protein/ml)	
	Inhibition on NO production	DPPH radical scavenging activity
Alpinia galanga (L.) Swartz	ND	ND
Boesenbergia pandurata (Roxb.)	ND	ND
Curcuma aeruginosa Roxb.	54.6±0.31	1.020±0.069
C.amarissima Roscoe	ND	1.419±0.005
C. aromatic	52.7±0.96	9.849±4.698
C. longa L.	ND	2.404±0.021
Curcuma sp. (Kuntamara)	ND	2.554±0.528
C. comosa	ND	0.953±1.051
C. zedoaria (Berg) Roscoe	ND	3.136±0.210
Hedychium coronarium Roem.	41.8±0.27	0.254±0.003
Kaempferia galanga L.	ND	ND
Zingiber cassumunar	ND	2.040±0.066
Z. officinale Roscoe	ND	1.064±0.020
Z. ottensii Valeton.	38.6±0.34	1.101±0.056
Z. zerumbet (L.) Smith	ND	2.036±0.328
Vitamin C	-	0.186±0.009

*ND = not detected

4.2 Effect of crude protein of *Z. ottensii* on *iNOS IL-6* and *TNF-α* mRNA expression in LPS and IFN-γ-stimulated RAW 264.7 cells

From bioassay-guided screening of the crude protein from fifteen Zingiberaceae rhizomes (in Table 4.1), the crude protein of *Z. ottensii* exhibited the highest activity against NO production. In order to determine the mechanism of crude protein of *Z. ottensii* on LPS and IFN-γ-induced NO production, we therefore examined the effect of the crude protein, compared with parthenolide (positive control) on LPS-induced expression of *iNOS*, *IL-6* and *TNF-α* mRNA. LPS which is known to stimulate macrophage RAW 264.7 to produce NO and enhance *iNOS*, *IL-6* and *TNF-α* expression was used in this study. The result showed that the crude protein and parthenolide decreased *iNOS*, *IL-6* and *TNF-α* mRNA level (Figure 4.1B, C, and D). When the crude protein was compared with parthenolide as the positive control, it was found that both showed similar effect on *iNOS*, *IL-6* and *TNF-α* mRNA expression. However, the concentration of parthenolide for treated cells was less than the concentration of crude protein. Inducible NOS (iNOS) is induced in response to various pro-inflammatory cytokines, including interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), and mediates several inflammatory responses (Jung *et al.*, 2007). Therefore, these results correlated well with the results of inhibitory effect on nitric oxide production. Furthermore, the modulation either at the transcriptional and/or post-transcriptional levels could also involve the inhibition of gene expression. Previously reported have identified the presence of the regulation of iNOS activity occurs mainly at the transcriptional level of which *IRF-1* is an essential transcription factor that can bind *iNOS* promoter (Martin *et al.*, 1994). In mouse macrophage cell line, disruption of *IRF-1* gene resulted in loss of NO production and barely detectable *iNOS* expression in response to stimulation (Kamijo *et al*., 1994). Moreover, it has been reported that the inhibition on *iNOS* mRNA expression might involve in the blockade of NFκB, an essential transcription factor for *iNOS* gene transcription (Diaz-Guerra *et al*., 1996; and Jung *et al*., 2007).

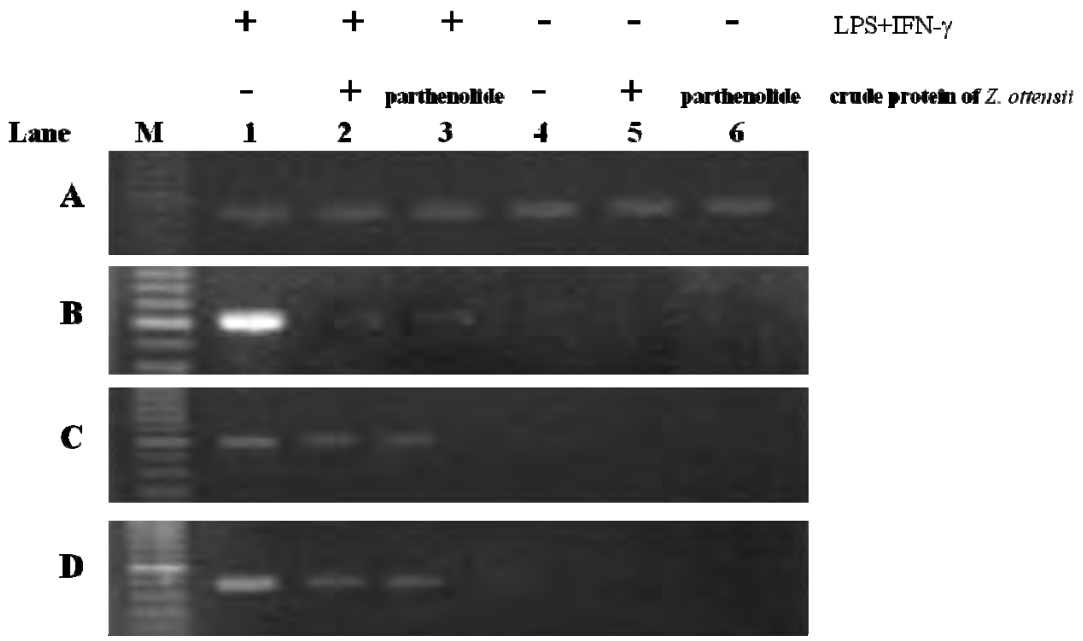


Figure 4.1. Effect of crude protein of *Z. ottensii* on *iNOS*, *IL-6* and *TNF-α* expression in LPS and IFN-γ-stimulated RAW 264.7 cells. (A) *β-actin* mRNA expression. (B) *iNOS* mRNA expression. (C) *IL-6* mRNA expression. (D) *TNF-α* mRNA expression; Lane1: (+) LPS+IFN-γ; Lane2: (+) LPS+IFN-γ and crude protein; Lane3: (+) LPS+IFN-γ and parthenolide (positive control); Lane 4: (-) LPS+IFN-γ; Lane5: (-) LPS+IFN-γ and crude protein; Lane6: (-) LPS+IFN-γ and parthenolide.

4.3 Total amino acid contents of the crude protein of *Z. ottensii*

The total amino acid contents of the crude protein of *Z. ottensii* are shown in Table 4.2 (on a dry weight basis). The aspartic acid had the highest contents of the total essential amino acid (2.42 mg/100mg), followed by glutamic acid, proline, leucine, arginine, lysine, phenylalanine, valine, threonine, isoleucine, glycine, tyrosine, serine, alanine and histidine, respectively. It has previously been reported that the anti-inflammatory properties of soy high in this type of protein (Hernandez-Ledesma *et al.*, 2009). These results are similar to those reported by Song *et.al*., 2008.

Table 4.2 Amino acid contents of the *Z. ottensii* (mg/100mg dry weight)

Amino acids	Contents (mg/100mg)
Aspartic acid	2.42
Serine	0.65
Glutamic acid	1.82
Glycine	0.72
Histidine	0.31
Arginine	1.25
Threonine	0.77
Alanine	0.42
Proline	1.29
Tyrosine	0.71
Valine	0.79
Lysine	1.17
Isoleucine	0.73
Leucine	1.29
Phenylalanine	0.88

4.4 Protein identification

The crude proteins from *Z. ottensii* was separated by SDS-PAGE (Figure 4.2) and the three dominant bands, marked Z1, Z2 and Z3, were excised and subjected to in-gel trypsin digestion. The resultant mixture of tryptic peptides was evaluated with tandem mass spectrometry and the tandem mass spectra so obtained were used to deduce the amino acid sequences of each principal peptide using the Masslyxn software. The sequences of peptides were compared against existing known proteins using the MS-Blast algorithm to search the NCBI database.

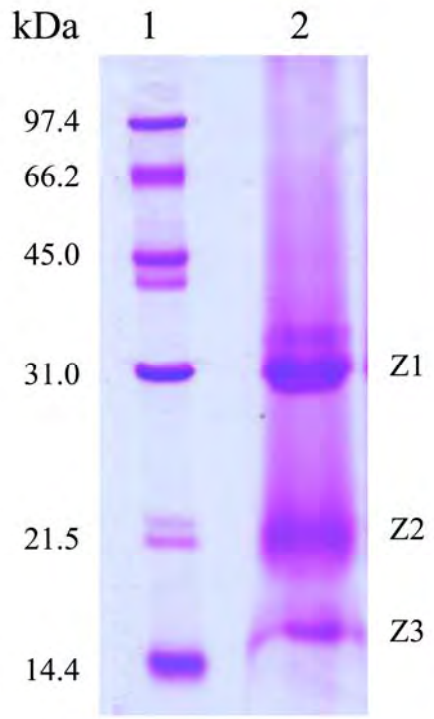


Figure 4.2. Reducing SDS-PAGE resolution of the crude protein from the rhizomes of *Z. ottensii*. Lane 1 molecular weight standards; lane 2, crude lane was loaded with 30 μg of protein in total.

For the Z1 protein, the MS-Blast result revealed that five cysteine proteases proteins (Table 4.3) from various plants with a total HSP score of more than the threshold score (HSP =170). Cysteine proteases were identified as band Z1 at molecular weigh about ~31.0 kDa. This enzyme family plays a role in plant growth, development and senescence. Most plant cysteine proteases belong to the papain and

legumain families. Recently this enzyme family was reported from three members of the ginger family, in *C. longa* (Nagarathnam *et al*., 2010), *C. aromatica* (Tipthara *et al*., 2008) and *Z. offinale* (Choi, and Laursen, 2000), and this ginger protease is used as a food improver and anti-inflammatory agent. Founding cysteine protease in four members of Zingiberaceae plant, *C. comosa, C. longa, C. aromatica* and *Z. offinale* at difference molecular weigh, the ginger cysteine protease might be a protein marker to classify specific species in this family in the future.

Interestingly, an antioxidant protein was found in the gel. Superoxide dismutase (SOD), a class of enzymes that convert the reactive superoxide radical into oxygen and hydrogen peroxide, was identified in band Z2. This result is in accord with the recent report of an antioxidant activity and the isolation of a SOD homologue from *C. comosa* (Boonmee *et al*., 2011). Indeed, SOD homologues have also been reported in other Zingiberaceae plant species, such as *C. longa* (Kochhar and Kochhar, 2008) and *C. zedoaria* (Loc *et al*., 2008). Their current biotechnological application has mainly been in cosmetic products to reduce free radical levels that otherwise cause skin damage (Christian Diehl and Danijela LediÄ-Drvar, 2009). The discovery of antioxidant enzyme may suggest some benefit for *Z. ottensii* for the natural product based cosmetic industry, but this will depend upon their relative specific activity or ease of enrichment.

Moreover, Comparisons amino acid sequences of the three tryptic peptides from the Z3, with ~15 kDa, using BLASTP searching identified this fragment as a likely homolog of parts of a lectin precursor from the common bean, *Phaseolus vulgaris* L. (Fabales: Fabaceae) (Hoffman and Donaldson, 1985). The high degree of internal amino acid sequence identity between the peptide fragments of this Z3 band, from *Z. ottensii* rhizomes, with those of other members of the leucoagglutinating phytohemagglutinin precursor family suggests that this protein could be a member of this lectin family as well. A mannose binding lectin with a molecular mass of 13.4 kDa was also isolated from *C. zedoary* (Tipthara *et al*., 2007). In addition, six homologous lectin proteins of various molecular masses (8.84-32.8 kDa) were found in *C. aromatica* (Tipthara *et al*., 2008). Most of them are mannose binding lectins. With respect to high throughput protein identification, agglutinin was also found to be present in the *C. longa* 2-D IEF-SDS-PAGE protein profile (Chokchaichamnankit *et al*., 2009).

Organism		Sequence	
Zingiber ottensii (Z1)		AVANQPVSVTMDAAGR NRNH GCEGG WPYR NWGESGYIR	
Zingiber officinale (Cysteine proteinase GP-I)	126	AVANQPVSVTMDAAGRDFQLYRNGIFTGSCNISANHYRTVGGRETENDK-DYWTVKNSWGKNWGESGYIR	194
Zingiber officinale (Cysteine protease gp2a)	267	AAANQPISVGIDASGRNFQLYHSGIFTGSCNTSLNHGVTVVGYGTENGN-DYWIVKNSWGENWGNSGYIL	335
Zingiber officinale (Cysteine protease gp3a)	267	AAANQPISVGIDASGRNFQLYHSGIFTGSCNTSLNHGVTVVGYGTENGN-DYWIVKNSWGENWGNSGYIL	335
Oryza sativa (Cysteine protease precursor)	268	AVANQPVAVAVEASGQDFQFYSEGVFTGECGTDLDHGVAAVGYGITRDGTKYWIVKNSWGEDWGERGYIR	337
Jacaratia mexicana (Mexicain)	214	AIANQPVSVVTDSRGRGFQFYKGGIYEGPCGTNTDHAVTAVGYGKT-----YLLLKNSWGPNWGEKGYIR	188
Zingiber ottensii (Z2)		AVVVHADPDDLGKGGHELSK VACGIIGLQ	
Ananas comosus (Cu/Zn-superoxide dismutase)	101	SQIPLSGSNSIIGRAVVVHADPDDLGKGGHELSK-TTGNAGGRVACGIIGLQG	152
Zea mays (Cu/Zn-superoxide dismutase)	101	SQIPLTGPNSIIGRAVVVHADPDDLGKGGHELSK-STGNAGGRVACGIIGLQG	152
Malus xiaojinensis (Cu/Zn-superoxide dismutase)	101	KQIPLAGPHSIIGRAVVVHADPDDLGKGGHELSK-STGNAGGRVACGIIGLQG	152
Citrus limon (Cu/Zn-superoxide dismutase)	101	NQIPLSGPNSIIGRAVVVHADPDDLGKGGHELSK-TTGNAGGRVACGIIGLQG	152
Populus tremula (Cu/Zn-superoxide dismutase)	101	NQIPLTGPNSIVGRAVVVHADPDDLGKGGHELSK-STGNAGGRVACGVIGLQG	152
Zingiber ottensii (Z3)		DNLSWSFASK	
Phaseolus vulgaris (Phytohemagglutinin)	245	DILSWSFASK	254
Vigna unguiculata (Seed lectin subunit I)	247	LSWSFASK	254
Medicago truncatula (Truncated lectin)	251	DILSWSFDSK	260
Phaseolus vulgaris (Arcelin-5A)	240	LSWSFSSK	247

Figure 4.3 Amino acid sequence from the tryptic fragments of the *Z. ottensii* rhizome crude protein. Comparisons are made with other cysteine proteinases, Cu/Zn-superoxide dismutase, and lectin from the others family that showed the highest sequence homology in BLASTP searches of the NCBI and SwissProt databases.

CHAPTER V

CONCLUSION

In conclusion, the data presented in this study demonstrates inhibitory activity against nitric oxide of the crude protein of fifteen Zingiberaceae rhizomes in macrophages RAW 264.7 that were stimulated by LPS and IFN-γ. The crude protein of *Z. ottensii* exhibited the highest activity against NO production from the crude protein of fifteen Zingiberaceae rhizomes with an IC_{50} value of 38.6 µg protein/ml and it had ability for free radical scavenging activity with an IC_{50} value of 1.101 µg protein/ml. In addition, the result of part gene expression showed that the crude protein of *Z. ottensii* decreased cellular *iNOS*, *IL-6* and *TNF-α* mRNA level which a potent pro-inflammatory cytokine. The protein patterns showed a high abundance of protein bands, including lectin proteins, metabolic and defense enzyme, superoxide dismutase (SOD) that are related with antioxidant activity. Furthermore, cysteine protease was found in this plant. These results suggest that the crude protein of *Z. ottensii* possesses a strong anti-inflammatory activity and has a potential to be developed as a therapeutic compound.

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APPENDICE

APPENDIX A

Media: Complete DMEM

DMEM	100%
Fetal Bovine Serum (FBS)	10%
Penicillin G	100 U/ml
Streptomycin	0.4 mg/ml
HEPES	1%
Sodium pyruvate	1%

Freezing media

Complete DMEM	10%
DMSO	90%

1X PBS 1 L

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Adjusted pH to 7.4 with 1M HCl and adjusted volume to 1 L with deionized water. After that, sterile by autoclave at 121°C and pressure 15 psi for 15 min.

50X Tris-acetic acid EDTA (TAE) buffer solution 200 ml

Trisma base	48.4 g
Glacial acetic acid	11.42 ml
0.5M EDTA	20 ml

Adjusted pH to 8.0 and volume to 200 ml with deionized water. After that, sterile by autoclave at 121°C and pressure 15 psi for 15 min.

1X TAE for agar gel preparation

Ratio = 50X TAE : deionized water = 1 : 49

0.5X TAE for running buffer

Ratio = 50X TAE : deionized water = 1 : 99

1.5% agar gel preparation

Agar	0.3 g
1X TAE	20 ml

Griess reaction preparation

- Sulfanilamide solution 50 ml (stored at 4°C and protected from light)
  Sulfanilamide 1% (w/v) was dissolved in 5% phosphoric acid, and adjusted total volume to 50 ml by deionized water.

- NED solution 50 ml (stored at 4°C)

NED 0.1% (w/v) was dissolved in 50 ml deionized water. NED Solution may change color if it is not stored protected from light.

- Preparation of a Nitrite Standard Curve

Prepare 1ml of a 100μM nitrite solution by diluting the provided 0.1M Nitrite Standard 1:1,000 in DMEM complete media. Add 100μl of the 100μM nitrite solution to the remaining 3 wells in row A of 96-well plate. Immediately perform 6 serial two-fold dilutions (50μl/well) in triplicate down the plate to generate the Nitrite Standard curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56μM), discarding 50μl from the 1.56μM set of wells. Do not add any nitrite solution to the last set of wells (0μM).

200 μM DPPH solution 25 ml

DPPH 0.2 mg was dissolved in 25 ml ethanol, stored protected from light.

APPENDIX B

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solution

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volumn to 100 ml with distilled water.

1M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjustes pH to 6.8 with 1M HCl and adjusted volumn to 100 ml with distilled water.

10% SDS (w/v)

Sodium dodecyl sulfate (SDS) 10 g

Adjusted volumn to 100 ml with distilled water.

50% Glycerol (w/v)

100% Glycerol 50 ml

Added 50 ml of distilled water

1% Bromopenol blue (w/v)

Bromophenol blue 100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylaminde, 0.8% (w/v) bis-acrylaminde)

Acrylaminde	29.2 g
N, N-methylene-bis-acrylaminde	0.8 g
Adjust volumn to 100 ml with distilled water	

Solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS)

2M Tris-HCl (pH 8.8)	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (0.5M Tris-HCl pH 6.8, 0.4% SDS)

1 M Tris-HCl (pH 6.8)	50 ml
10% SDS	4 ml
Distilled water	46 ml

10% Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)

Tris (hydroxymethyl-aminomethane	3 g
Glycine	14.4 g
SDS	1 g
Dissolved in distilled water to 1 liter without pH adjustment	
(Final pH should be 8.3)	

5x sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue)

1M Tris-HCl (pH6.8)	0.6 ml
50% Glycerol	5 ml
10% SDS	2 ml
2-mercaptoethanol	0.5 ml

1% Bromophenol blue	0.5 ml
Distilled water	0.9 ml

3. SDS-PAGE

15% Seperating gel

Solution A	5 ml
Solution B	2.5 ml
Distilled water	2.5 ml
10% Ammonium persulfate	50 µl
TEMED	5 µl

5.0% Stacking gel

Solution A	0.67 ml
Solution C	1 ml
Distilled water	2.3 ml
10% Ammonium persulfate	30 µl
TEMED	5 µl

Coomassie Gel Stain

Coomassie Blue R-250	1 g
Methanol	450 ml
Distilled water	450 ml
Glacial Acetic Acid	100 ml

Coomassie Gel Destain

Methanol	100 ml
Glacial Acetic Acid	100 ml
Distilled water	800 ml

APPENDIX C

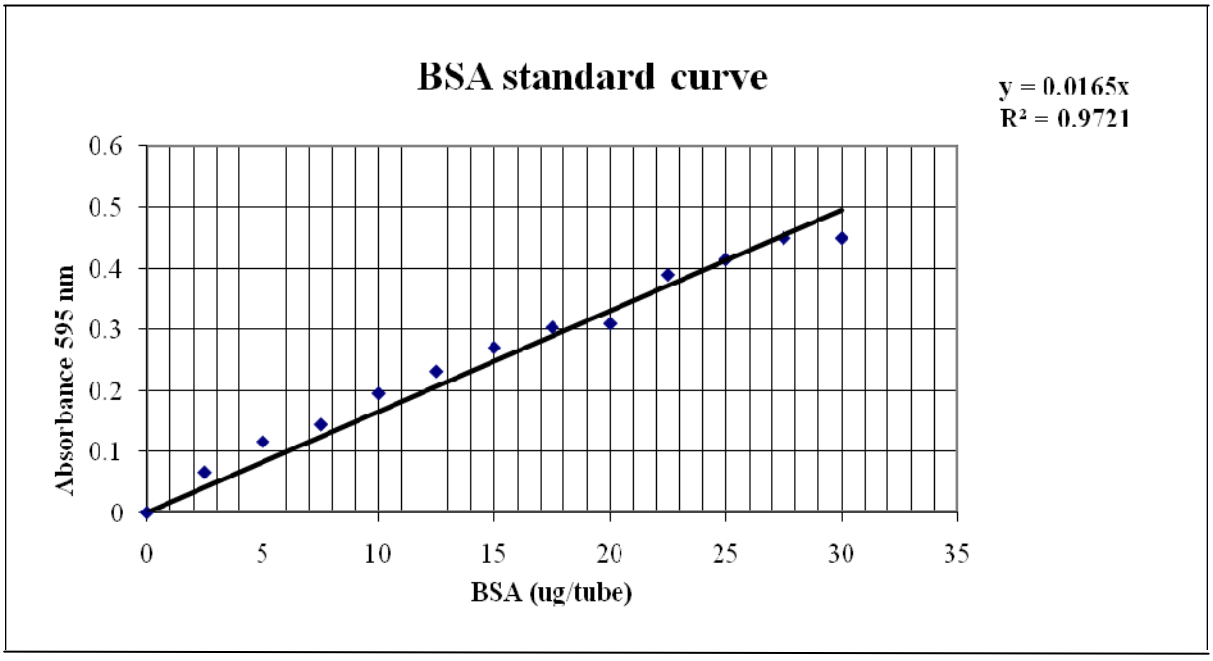
Bradford Stock Solution 300 ml

95% ethanol	100 ml
88% phosphoric acid	200 ml
Serva Blue G	350 mg

Bradford Working buffer 500 ml

95% ethanol	15 ml
88% phosphoric acid	30 ml
Bradford stock solution	30 ml
Distilled water	425 ml

Calibration curve for protein determination by Bradford method



APPENDIX D

Amino acid abbreviations

Amino acid	Three-letter	One-letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic-acid	Asp	D
(Asn + Asp)	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
(Gln + Glu)	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX E

Scientific name and Thai name of fifteen selected plants in the Zingiberceae family

Scientific name	Thai name
Alpinia galanga (L.) Swartz	ข่า
Boesenbergia pandurata (Roxb.)	กระชายดำ
Curcuma aeruginosa Roxb.	ว่านมหาเมฆ
C.amarissima Roscoe	ขมิ้นดำ
C. aromatic	ว่านนางคำ
C. longa L.	ขมิ้นชัน
Curcuma sp.	คัณฑมาลา
C. comosa	ว่านชักมดลูก
C. zedoaria (Berg) Roscoe	ขมิ้นอ้อย
Hedychium coronarium Roem.	ว่านมหาหงส์
Kaempferia galanga L.	เปราะหอม
Zingiber cassumunar	ไพลเหลือง
Z. officinale Roscoe	ขิง
Z. ottensii Valeton.	ไพลดำ
Z. zerumbet (L.) Smith	กระทือ

BIOGRAPHY

Miss Chernkhwan Chantaranothai was born on December 3, 1987 in Nakhonratchasima, Thailand. She graduated with Bachelor Degree of Science from Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2008. She was admitted to the Master degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2008.

Academic presentation;

1.) Chantaranothai, C., Sangvanich, P., Palaga, T. and Karnchanatat, A. Inhibitory Activity against Nitric Oxide Production in Macrophage RAW 264.7 from the Protein of the Rhizomes of Zingiberaceae Plants. The 12th Graduate Research Conference Khon Kaen University 2011, 28 January 2011, Khon Kaen, Thailand.