ฤทธิ์ต้านอนุมูลอิสระ และไซโตไคน์ต้านการอักเสบโดยสารสกัดจากฮว่านง็อก

(*Pseuderanthemum palatiferum* (Nees) Radlk.)

นางสาวพัชรวรรณ สิทธิศาสตร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาชีววิทยาสิ่งแวดล้อม

มหาวิทยาลัยเทคโนโลยีสุรนารี

ปีการศึกษา 2556

ANTIOXIDANT AND ANTI-INFLAMMATORY CYTOKINE ACTIVITY BY EXTRACTS FROM HOAN-NGOC (*PSEUDERANTHEMUM PALATIFERUM* (NEES) RADLK.)

Patcharawan Sittisart

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

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ANTIOXIDANT AND ANTI-INFLAMMATORY CYTOKINE ACTIVITY BY EXTRACTS FROM HOAN-NGOC (*PSEUDERANTHEMUM PALATIFERUM* (NEES) RADLK.)

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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พัชรวรรณ สิทธิศาสตร์ : ฤทธิ์ต้านอนุมูลอิสระ และไซโตไคน์ต้านการอักเสบโดยสารสกัดจากฮว่านง็อก (*Pseuderanthemum palatiferum* (Nees) Radlk.) (ANTIOXIDANT AND ANTI-INFLAMMATORY CYTOKINE ACTIVITY BY EXTRACTS FROM HOAN-NGOC (*PSEUDERANTHEMUM PALATIFERUM* (NEES) RADLK.) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เบ็ญจมาศ จิตรสมบูรณ์, 169 หน้า.

สารสกัดจากใบฮว่านง็อกมีฤทธิ์ต้านอนุมูลอิสระ แต่ในปัจจุบันยังไม่มีรายงานฤทธิ์ต้านการอักเสบ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาสารพฤกษเคมี ตรวจสอบฤทธิ์ต้านอนุมูลอิสระและค้นหาฤทธิ์ต้านการอักเสบของ [1] สารสกัดจากใบสดของฮว่านง็อกด้วยเอทานอล 95% (95EE-FLP) [2] สารสกัดจากใบสดของฮว่านง็อกด้วยเอทานอล 80% (80EE-FLP) [3] สารสกัดจากใบตากแห้งของฮว่านง็อกด้วยเอทานอล 80% (80EE-DLP) [4] สารสกัดจากใบสดของฮว่านง็อกสกัดซ้ำด้วยน้ำ (WE-FLP) และ [5] สารสกัดจากใบตากแห้งของฮว่านง็อกสกัดซ้ำด้วยน้ำ (WE-DLP) ผลการทดลองพบว่า ทุกสารสกัดจากใบสดของฮว่านง็อกมีปริมาณสารฟินอลิกและฟลาโวนอยด์สูงกว่าสารสกัดจากใบตากแห้ง ในทำนองเดียวกัน ฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากใบสดเมื่อประเมินโดยวิธี DPPH และ FRAP มีค่าสูงกว่าสารสกัดจากใบตากแห้งเช่นกัน ทั้ง 95EE-FLP และ WE-FLP สามารถลดการสร้างอนุมูลอิสระ (Reactive oxygen species, ROSs) ภายในเซลล์แมคโครฟาจ RAW264.7 เมื่อถูกชักนำให้เกิดสภาวะเครียดออกซิเดชั่นโดย *tert*-Butyl hydroperoxide (tBuOOH) เมื่อติดตามโดยใช้ DCFH-DA probe ที่ให้สารเรืองแสง ทั้ง 95EE-FLP และ WE-FLP ยังสามารถแสดงคุณสมบัติต่อต้านการอักเสบ ในระดับความเข้มข้นที่ไม่เกิดพิษต่อเซลล์ (50-250 μg/mL) 95EE-FLP และ WE-FLP มีประสิทธิภาพในการยับยั้งการเพิ่มการผลิตไนตริกออกไซด์ และการสังเคราะห์โปรตีนของเอนไซม์ iNOS และ COX-2 ที่เกิดจากการกระตุ้นเซลล์ RAW264.7 ด้วย 1 μg/mL LPS และ 25 U/mL IFN-γ สารสกัด 95EE-FLP ที่มีศักยภาพสูงสุดถูกคัดเลือกเพื่อนำมาศึกษาผลต่อระบบภูมิคุ้มกันที่เกิดผ่านสารสื่อกลางที่มีความสำคัญยิ่งในกระบวนการอักเสบได้แก่ TNF-α และ IL-6 การศึกษาครั้งนี้พบว่าสารสกัด 95EE-FLP ยับยั้งทั้ง TNF-α และ IL-6 ที่หลั่งจากเซลล์แมคโครฟาจในช่องท้องของหนูเม้าส์ C57BL/6 เมื่อกระตุ้นด้วย LPS (100 ng/mL) โดยฤทธิ์การยับยั้งเป็นปฏิภาคโดยตรงกับความเข้มข้นของสารสกัด นอกจากนั้น 95EE-FLP ยังยับยั้งทั้งการสังเคราะห์โปรตีนและการแสดงออกของ mRNA ของ TNF-α และ IL-6 ในเซลล์ RAW264.7 ที่กระตุ้นด้วย LPS การศึกษาในระดับอณูชีววิทยาพบว่า กลไกบางส่วนของการลดการสังเคราะห์โปรตีนและการแสดงออกของ mRNA ของ TNF-α และ IL-6 โดย 95EE-FLP เกิดผ่านการยับยั้งการจับกันระหว่าง ทรานสคริบชั่นแฟคเตอร์ NF-κB กับ รหัสจำเพาะบน DNA ในโปรโมเตอร์ TNF-α ของหนู และการจับกันระหว่างทรานสคริบชั่นแฟคเตอร์ NF-κB C/EBP และ AP-1 กับรหัสจำเพาะบน DNA ของโปรโมเตอร์ IL-6 ของหนู นอกจากนั้น 95EE-FLP สามารถยับยั้งการกระตุ้นของ LPS ต่อโปรโมเตอร์ของ mIL-6 ที่ติดฉลากด้วยรีพอร์ตเตอร์ยีน ลูซิเฟอร์เรส (IL-6 promoter-reporter construct, pmIL-6.Luc(-231) luciferase

reporter) ในเซลล์ RAW264.7 ที่ได้รับการปลูกถ่ายยีนดังกล่าว ทั้งนี้ฤทธิ์การยับยั้งของ 95EE-FLP เป็นปฏิภาคโดยตรงกับความเข้มข้นที่ใช้ โดยภาพรวมสารสกัดจากใบฮว่านง็อกมีศักยภาพควรค่าต่อการพัฒนาในอนาคตเพื่อใช้ประโยชน์ในเชิงป้องกัน หรือรักษาโรคต่าง ๆ อันมีสาเหตุจากอนุมูลอิสระ และกระบวนการอักเสบ

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

PATCHARAWAN SITTISART : ANTIOXIDANT AND ANTI-INFLAMMATORY CYTOKINE ACTIVITY BY EXTRACTS FROM HOAN-NGOC (*PSEUDERANTHEMUM PALATIFERUM* (NEES) RADLK.). THESIS ADVISOR : ASST. PROF. BENJAMART CHITSOMBOON, Ph.D. 169 PP.

PHENOLIC/FLAVONOID/ANTIOXIDANT/CELL VIABILITY/ ANTI-INFLAMMATORY

Extracts of *Pseuderanthemum palatiferum* (PP) leaves have been demonstrated to possess antioxidant activities, but their anti-inflammatory effects have not yet been reported. The present study aimed to further investigate the phytochemicals and explore the antioxidant and anti-inflammatory activities of [1] 95% ethanol extract of fresh leaves of PP (95EE-FLP), [2] 80% ethanol extract of fresh leaves of PP (80EE-FLP), [3] 80% ethanol extract of dried leaves of PP (80EE-DLP), [4] water extract of fresh leaves of PP (WE-FLP), or [5] water extract of dried leaves of PP (WE-DLP). The results suggested that all fresh leaf extracts significantly exhibited higher phenolic and flavonoid contents than those of dried leaf extracts (p<0.001). Concordantly, the antioxidant activity, assessed by DPPH and FRAP assays, was also more prominent in fresh leaf extracts than that of dried leaf extracts. Both 95EE-FLP and WE-FLP could also effectively attenuate intracellular reactive oxygen species (ROSs) generation in tBuOOH-induced oxidative stress in macrophage RAW264.7 cells as monitored by using DCFH-DA fluorescent probe. Both 95EE-FLP and WE-FLP also possess anti-inflammatory properties. At non-cytotoxic concentrations (50-250 µg/mL), 95EE-FLP and WE-FLP effectively diminished the up regulation of nitric oxide and protein levels of inducible NO synthase (iNOS) and cyclooxygenase (COX-2) enzymes in 1 µg/mL LPS plus 25 U/mL IFN-γ-stimulated RAW264.7 cells. 95EE-FLP, the most potent extract, was selected for further investigation of its immune modulation on critical

proinflammatory cytokines, TNF-α and IL-6. The results showed that 95EE-FLP induced a dose-dependent suppression of LPS (100 ng/mL)-induced TNF-α and IL-6 secretion in mouse (C57BL/6) peritoneal macrophages. In addition, 95EE-FLP also suppressed LPS-induced TNF-α and IL-6 protein synthesis and mRNA expression in RAW264.7 cells. The study of the molecular mechanism of 95EE-FLP-mediated immune modulation of TNF-α and IL-6 revealed that the suppression in RAW264.7 cells was mediated, at least in part, through decreasing the binding activity of NF-κB to the response element in mTNF-α promoter and the binding activities of NF-κB, C/EBP and AP-1 in mIL-6 promoters. Furthermore, the LPS activation of pmIL-6.Luc(-231) promoter/luciferase reporter gene in transfected RAW264.7 cells was also suppressed by 95EE-FLP in a dose-dependent manner. Collectively, the extracts from PP leaves exhibit a high potential for future development to be exploited in prevention or treatment of diseases caused by over production of ROSs and inflammatory disorders.

School of Biology Student's Signature_____

Academic Year 2013 Advisor's Signature _____

Co-advisor's Signature_____

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
AP-1	=	Activator protein 1
BCA	=	Bicinchoninic acid
BSA	=	Bovine serum albumin
CAA	=	Cellular antioxidant activity
CE	=	Catechin equivalent
C/EBPs	=	CCAAT-enhancer-binding proteins
cNOS	=	Constitutive nitric oxide synthase
COX-1	=	Cyclooxygenase-1
COX-2	=	Cyclooxygenase-2
DCFH-DA	=	2',7'-dichlorofluorescin-diacetate
DI water	=	Distilled water
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	Dimethyl sulfoxide
DPPH	=	2,2-Diphenyl-1-picryl-hydrazyl
DTT	=	Dithiotrietol
ECL	=	Enhanced chemiluminescence system
EDTA	=	Ethylenediaminetetraacetic acid
80EE-DLP	=	80% ethanol extract of dried leaves of PP
80EE-FLP	=	80% ethanol extract of fresh leaves of PP
95EE-FLP	=	95% ethanol extract of fresh leaves of PP
ELISA	=	Enzyme-linked immunosorbent assay

LIST OF ABBREVIATIONS (Continued)

EMSA	=	Electrophoretic mobility shift assay
FBS	=	Fetal bovine serum
FRAP	=	Ferric reducing antioxidant power
g	=	Gram
GAE	=	Gallic acid equivalent
h	=	Hour
HBSS	=	Hank’s balanced salt solution
HEPES	=	N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
IC_{50}	=	50% inhibitory concentration
IFN-γ	=	Interferon-γ
IL-6	=	Interleukin-6
iNOS	=	Inducible nitric oxide synthase
kg	=	Kilogram
L	=	Liter
LB	=	Luria broth
LPS	=	Lipopolysaccharide
M	=	Molar
mAb	=	Monoclonal antibody
$MaCl_2$	=	Magnesium chloride
min	=	Minute
mg	=	Milligram
mg/mL	=	Milligram per milliliter
mL	=	Milliliter

LIST OF ABBREVIATIONS (Continued)

mM	=	Millimolar
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	=	Naïve
NaCl	=	Sodium chloride
$NaHCO_3$	=	Sodium bicarbonate
NaOH	=	Sodium hydroxide
NED	=	Naphthylethylenediamine dihydrochloride
NF-κB	=	Nuclear factor-kappaB
nm	=	Nanometer
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
OD	=	Optical density
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffered saline
PGs	=	Prostaglandins
PI	=	Propidium iodide
PMSF	=	Phenylmethanesulfonyl fluoride
PP	=	*Pseuderanthemum palatiferum*
qRT-PCR	=	Quantitative reverse transcription PCR
RLU	=	Relative light units
RNSs	=	Reactive nitrogen species
ROSs	=	Reactive oxygen species
rpm	=	Revolution per minute
RPMI 1640	=	Roswell Park Memorial Institute

LIST OF ABBREVIATIONS (Continued)

RT	=	Room temperature
SA	=	Sulfanilamide
SDS	=	Sodium dodecyl sulfate
SEM	=	Standard error of the mean
tBuOOH	=	*tert*-butyl hydroperoxide
TEMED	=	N, N, N′, N′-tetramethylethylenediamine
TFC	=	Total flavonoid content
TLR4	=	Toll-like receptor 4
TNF-α	=	Tumor necrosis factor alpha
TPC	=	Total phenolic content
Trolox	=	6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid
VCEAC	=	Vitamin C equivalent antioxidant capacity
VH	=	Vehicle
v/v	=	Volume : Volume
WE-DLP	=	Water extract of dried leaves of PP
WE-FLP	=	Water extract of fresh leaves of PP
%	=	Percent
°C	=	Degree celsius
µg	=	Microgram
µg/mL	=	Microgram per milliliter
µL	=	Microliter
µM	=	Micromolar
x *g*	=	x gravitational acceleration

CHAPTER I

INTRODUCTION

1.1 Introduction

Reactive oxygen species (ROSs) are produced by exposure to environmental factors and by activated neutrophils and macrophages during mitochondria catalyzed electron transport reactions, and other mechanisms (Gülcin, Bursal, Şehitoğlu, Bilsel, and Gören, 2010). Excessive release of ROSs has been implicated in the pathogenesis of tissue injury and development of several degenerative and inflammatory diseases, including cancers, cardiovascular diseases, diabetes, neural disorders, and other chronic inflammations (Gutteridge, 1995; Valko, Rhodes, Moncol, Izakovic, and Mazur, 2006). ROS propagate inflammation by stimulating the release of several proinflammatory mediators such as nitric oxide (NO) and proinflammatory cytokines, which in turn mediated the recruitment of additional neutrophils and macrophages. Therefore, ROSs are prime mediators provoking or sustaining inflammatory processes, and, consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation (Geronikaki and Gavalas, 2006). Consumption of foods rich in antioxidants has been shown to play an essential role in the prevention of cancers (Ames, Gold, and Willett, 1995), neurodegenerative diseases (Christen, 2000), and inflammations (Kaur and Kapoor, 2001). Therefore, it is possible to reduce the risk of chronic diseases and prevent disease progression by using natural antioxidants.

Inflammation is a complex biological response to the harmful stimuli and is associated with many pathological conditions. Macrophages play a key role in

inflammatory and immune reactions by releasing a variety of inflammatory mediators such as proinflammatory cytokines (IL-1, TNF-α, and IL-6), ROSs, NO, chemokines, growth factors, and inducible enzymes (iNOS and COX-2) (Adams and Hamilton, 1984; Ramana et al., 2006). These inflammatory mediators can be stimulated by a variety of agents such as TNF, IFN-γ, and microbial products including lipopolysaccharide (LPS) (Lowenstein et al., 1993; Xie, Whisnant, and Nathan, 1993). After LPS stimulation of macrophage, inducible isoforms of NOS and COX-2 are responsible for the production of large amounts of NO and prostaglandin E_2 (PGE_2), respectively (Senthil Kumar and Wang, 2009; Yoon et al., 2009). There are indications that activation of nuclear factor kappaB (NF-κB) is involved in iNOS and COX-2 expression as suggested by the presence of NF-κB consensus site in the upstream promoter region of both the iNOS and COX-2 genes (Park et al., 2006; Chung et al., 2008). Hence, suppression of NF-κB leading to decreased activity of NO, iNOS, and COX-2 have been used as screening tools for substances with anti-inflammatory activity.

Cytokines are important in recruitment of immune cells and in regulation of inflammatory responses (Gonzalo et al., 1996). NF-κB, CCAAT/enhancer binding protein (C/EBP), and activator protein-1 (AP-1) are common transcription factors involved in the regulation of many proinflammatory cytokines. The transcriptional promoters of proinflammatory genes TNF-α and IL-6 contain typical binding sites for NF-κB, AP-1, and C/EBP (Zagariya et al., 1998; Kuprash et al., 1999; Baccam, Woo, Vinson, and Bishop, 2003). NF-κB plays a critical role in the expression of many genes (TNF-α, IL-1β, IL-6, IL-8, and COX-2) involved in immune and inflammatory responses. It is regarded as one of the most important transcription factors that regulate inflammation and play a key role in several inflammatory diseases, such as rheumatoid arthritis (RA) synovia fibroblasts, asthma, inflammatory bowel disease, sepsis, neurodegeneration, and ischemia/reperfusion injury (Tak and Firestein, 2001; Prajapati, singhal, Yashwant, and Gupta, 2010). Among many transcription factors, AP-1 is one of the most important transcription factors that has

been implicated as a critical regulator of gene expression in the setting of inflammation. Like NF-κB, activation of AP-1 complexes, composed of Jun and Fos family members, contributes to RA-associated inflammation (Firestein and Manning, 1999). C/EBP is a transcription factor that is involved in multiple biological functions including a role in inflammation (Ramji and Foka, 2002). Therefore, modulation of NF-κB, AP-1, and/or C/EBP activations might serve as an effective strategy for suppressing the levels of proinflammatory mediators implicated in inflammatory diseases.

There has been increasing interest in the discovery and development of novel pharmaceuticals from medicinal plants possessing antioxidant and anti-inflammatory activity with minimal side effects. Natural products and herbal remedies used in traditional folk medicine have been the sources of many medicinally beneficial drugs. In some countries, government encourages the use of indigenous forms of medicine rather than expensive imported drugs. In Thailand, a large percentage of the population is dependent on herbal medicines because the international commercial medicines are becoming increasingly expensive and out of reach. Among several medicinal plants in Thailand, *Pseuderanthemum palatiferum* Nees Radlk. (PP) is widely used. PP leaves have been long used among the native population of Thailand and Vietnam as remedies against different diseases such as hypertension, diabetes, cancers, and inflammatory diseases (Dieu, Loc, Yamasaki, and Hirata, 2005; Padee, Nualkaew, Talubmook, and Sakuljaitrong, 2010). Recently, this plant has been reported to possess phenolics and flavonoids as well as antioxidant properties (Nguyen and Eun, 2011). Phenolic compounds in plants are known to exhibit a wide range of biological activities, including anticancer, antibacterial, antioxidant, and anti-inflammatory properties (Lu and Foo, 2002). Therefore, the aim of this study was to investigate the effect of the extracts of PP leaves on antioxidant activity, RAW264.7 macrophage cell viability, anti-inflammatory activity as well as *in vitro* production and transcriptional regulation of inflammatory mediators in order to gain

a further insight into the molecular mechanisms whereby this plant leaf extract may mediate its immunosuppressive action *in vitro.*

1.2 Research objectives

The main objectives of this study are as following :

1.2.1 To investigate the phytochemicals and antioxidant properties of PP leaf extracts.

1.2.2 To investigate the effect of PP leaf extracts on RAW264.7 cell viability.

1.2.3 To investigate the cellular antioxidant activity (CAA) of PP leaf extracts in RAW264.7 cells.

1.2.4 To investigate anti-inflammatory effect of PP leaf extracts by modulation of NO, iNOS, and COX-2 protein expressions in LPS plus IFN-γ-stimulated RAW264.7 cells.

1.2.5 To investigate immunomodulation of PP leaf extract on protein synthesis and mRNA expressions of proinflammatory cytokines TNF-α and IL-6.

1.2.6 To explore whether the anti-inflammatory mechanism of PP leaf extract was mediated by suppression of NF-κB, C/EBP, or AP-1 activation.

1.3. Research hypothesis

1.3.1 PP leaf extracts contain high contents of total phenolic and flavonoid.

1.3.2 PP leaf extracts have high antioxidant activities as compared to standard antioxidants.

1.3.3 PP leaf extracts are not toxic on RAW264.7 macrophage cells, but appear to have biological activity.

1.3.4 PP leaf extracts suppress COX-2 protein expression and NO levels by modulation of iNOS protein.

1.3.5 PP leaf extracts suppress TNF-α and IL-6 proinflammatory cytokines by regulation of NF-κB, AP-1, or C/EBP transcription factors.

1.4 Scope and limitations of the study

Phytochemicals of various extracts of PP leaves were evaluated for total phenolic and flavonoid contents. Antioxidant properties of the extracts were determined *in vitro* by both chemical and cell-based assays. RAW264.7 macrophage cells were used to investigate the effects of extracts of PP leaves on *in vitro* cytotoxic studies. The anti-inflammatory properties of extracts of PP leaves were investigated *in vitro* using LPS and/or IFN-γ-activated RAW264.7 macrophage cell line. The inflammatory parameters measured were NO production, iNOS and COX-2 protein expressions, TNF-α and IL-6 productions and mRNA expressions. Anti-inflammatory-mediated modulation was examined by determination of activation of NF-κB located in mouse TNF-α promoter, and NF-κB, C/EBP, and AP-1 located in mouse IL-6 promoters using the electrophoretic mobility shift assay. The effect of extract from PP leaves on activation of pmIL-6.Luc(-231) promoter/luciferase reporter gene in transfected RAW264.7 cells activated with LPS was also examined using transient transfection followed by luciferase assays. The limitation of this study was antioxidant and anti-inflammatory cytokine activity of PP. It was only investigated *in vitro* and mainly focused on RAW264.7 cells. The overviews of the studies were summarized in Figure 1.1.

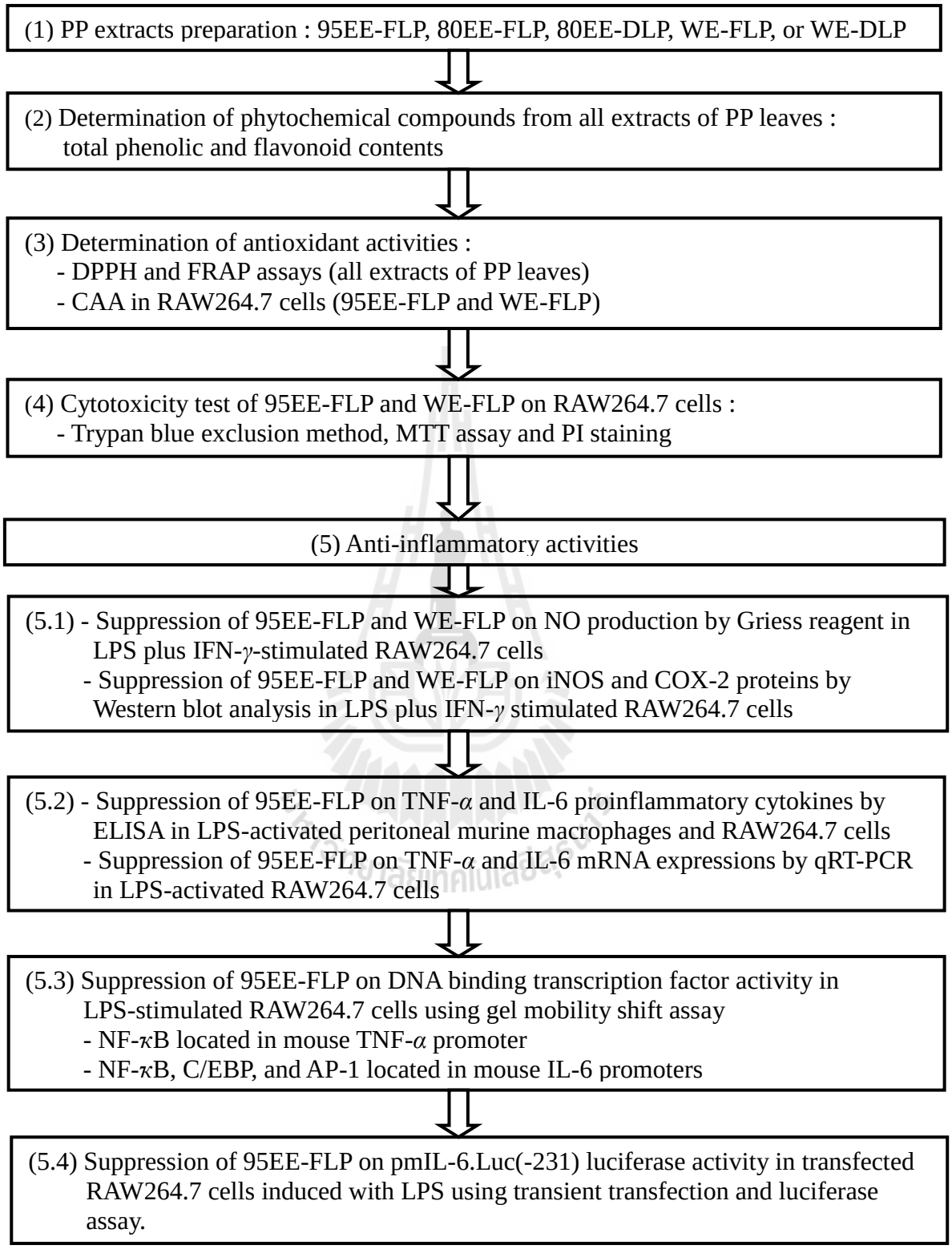

Figure 1.1 Scope and limitation of the studies.

95EE-FLP, 95% ethanol extract of fresh leaves of PP; 80EE-FLP, 80% ethanol extract of fresh leaves of PP; 80EE-DLP, 80% ethanol extract of dried leaves of PP; WE-FLP, water extract of fresh leaves of PP; WE-DLP, water extract of dried leaves of PP; CAA, cellular antioxidant activity.

1.5 Expected results

1.5.1 The outcomes from the study of phytochemical and antioxidative properties of PP leaf extracts can be used as basic pharmacological data for future research.

1.5.2 Scientific data obtained from PP leaf extracts will help to support and strengthen the knowledges and practices of using leaves of PP which has already gained popularity in Thailand.

1.5.3 Information on anti-inflammatory activity of PP leaf extracts can be used to develop anti-inflammatory drugs in the future.

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CHAPTER II

LITERATURE REVIEW

2.1 *Pseuderanthemum palatiferum*

Pseuderanthemum palatiferum (Nees) Radlk. (PP) or *Eranthemum palatiferun* Nees, known as Hoan-Ngoc, is a native plant of Vietnam belonging to the Acanthaceae family. During the latter half of the 1990's, the plant was found in Cuc Phuong forest in Northern Vietnam. After the discovery, the plant has been cultivated throughout the country (Figure 2.1) as a medicinal and ornamental plant including the Mekong Delta region, the most southern region in the country. The Vietnamese people believe that PP is a miracle-medicinal plant. Chewing its fresh leaves, or drinking its juice prepared fresh or boiled is supposed to cure many diseases such as wounds, trauma, stomachache, colitis, blood pressure, nephritis, and diarrhea (Dieu, Loc, Yamasaki, and Hirata, 2005, 2006). In addition, PP leaves have also been used for prevention and treatment of piglet diarrhea (Dieu et al., 2006). PP was taken through the northeast of Thailand, Surin, Buriram, and Sisaket provinces, about 25-30 years ago by a Vietnam Era veteran. Its Thai name is "Wan Ling" or "Payawanorn". PP can be widely grow in Thailand and Thai people have used PP leaves for prevention and treatment of many diseases, such as chronic pain in older adults, trauma, nerve disease, influenza, allergy, high or low blood pressure, digestive disorder, diarrhea, bone fracture, stomachache, nephritis, hepatitis, diabetes, and cancer. Therefore, commercial products made from PP leaves including powder decoctions, herbal tea bags and capsules have been developed (Figure 2.2). These products are available in markets both in Thailand and Vietnam, but without quality control.

Figure 2.1 PP (Hoan-Ngoc) has been cultivated in various regions throughout Vietnam country (Sinh, 2010; Trần, 2013).

Figure 2.2 Commercial products from PP leaves (Thaiza, www, 2013).

At present, there are very limited published studies of PP and most of them are written in Vietnamese. The showed that PP leaves contain n-pentacosan-1-ol, *β*-sitosterol, stigmasterol, *β*-sitosterol 3-O-*β*-glucoside, stigmasterol 3-O-*β*-glucoside, kaempferol 3-methyl ether 7-O-*β*-glucoside, apigenin 7-O-*β*-glucoside, 1-triacontanol, and salicylic acid (Phan, Ha, and Phan, 2003; Dieu et al., 2005, 2006). The leaves also contain pseuderantin,

a proteinase with high thermal stability and proteolytic activity. Moreover, the leaves of PP contain crude proteins, minerals such as Ca, Mg, Fe and Cu, and amino acids such as lysine, methionine, and threonine (Dieu et al., 2005) as demonstrated in Table 2.1. Flavonoids, phenol carboxylic acids, and terpenoids were also detected in dried leaf extract of PP by 80% ethanol maceration with thin-layer chromatography fingerprinting (Chayarop et al., 2011). Dried leaf extracts of PP macerated with different solvents (methanol, acetone, ethanol, and water) showed phenolic content determined by the Folin-Cioculteu method, flavonoid content determined by aluminium trichloride colorimetric assay, and antioxidant activities with reducing power (FRAP), metal chelating activity, and 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging activity (Nguyen and Eun, 2011). The ethyl acetate and n-butanol fractions of PP leaves contained high flavonoid contents and have antioxidant activity in human blood peroxidase model. Moreover, PP leaf extract had antibacterial activity against *Salmonella typhi* 158, *Shigella flexneri*, and *Escherichia coli*, and antifungal activity against *Candida albicans* and *Candida stellatoides* (Phan, Ha, and Phan, 2005). Dieu et al. (2006) reported that the dried and fresh-leaves had comparable effect in the treatment of diarrhea in piglets, and the efficacy was almost equivalent to Coli-norgent and Co-trimoxazold which are the best prevalent drugs in treating diarrhea. Nam, Kim, Bae, and Ahn (2003) reported that methanol extract from PP leaves was toxic to B16 melanoma and could inhibit new blood vessel growth in human umbilical venous. Aqueous and ethanol crude extracts from PP leaves had anti-mutagenic effect with Ames test. In addition, two extracts from PP had anti-proliferative activity against 3 types of colon cancer cell lines; HCT15, SW48, and SW480 with 3'-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay (Pamok, Saenphet, Vinitketkumnuen, and Saenphet, 2012). Moreover, crude extract of PP with 80% ethanol showed hypoglycemic effect on streptozotozin-induced diabetic rats (Padee, Nualkaew, Talubmook, and Sakuljaitrong, 2010).

Table 2.1 Chemical composition, dry matter yield and crude protein, and amino acid contents of PP leaves.

Isolation and identification of compounds from PP leaves	
n-Pentacosan-1-ol	
β-Sitosterol	
Stigmasterol	
β-Sitosterol 3-O-*β*-glucoside	
Stigmasterol 3-O-*β*-glucoside	
Kaempferol 3-methyl ether 7-O-*β*-glucoside	
Apigenin 7-O-*β*-glucoside	
1-Triacontanol	
Salicylic acid	
Minerals (mg/100 g fresh leaves)	
Ca	875.5
Mg	837.6
Fe	38.8
Cu	0.43
Amino acids (mg/100 g fresh leaves)	
Lysine	30.6
Methionine	29.7
Threonine	61.0
Dry matter (%)	13.4
Crude protein (% dry matter)	30.8

Source: Phan et al., 2003; Dieu et al., 2005, 2006.

2.2 Free radicals (ROSs and RNSs) and antioxidants

In recent years, there has been considerable interest in free radicals and related species. They are mainly derived from oxygen (reactive oxygen species/ROSs), including not only the oxygen radicals (superoxide anion $O_2^{\cdot -}$, hydroxyl radicals $^{\cdot}OH$ etc.) but also some non-radical derivatives. In addition, they are also derived from nitrogen (reactive nitrogen species/RNSs) such as nitric oxide (NO) and RNSs, which play also important roles in biology similar to ROS (Hallwell, 2006; Fang, Seki, and Maeda, 2009). Free radicals are very small highly reactive molecules due to the presence of unpaired valence shell electrons. They are very short lived, with half-lifes in milli-, micro- or nanoseconds. The well-regulated levels of ROS and RNS are produced to maintain cellular homeostasis in the normal healthy tissue and they play important roles as signaling molecules that regulate cell growth and reduction-oxidation (redox) status (Devasagagam et al., 2004).

Free radicals are normally generated in our body by various endogenous systems. They are generated from the autoxidation and consequent inactivation of small molecules such as reduced thiols and flavins, and from the activity of certain oxidases, lipoxygenases, cyclooxygenases, peroxidases, and dehydrogenases. (Machlin and Bendich, 1987; Nicholis and Budd, 2000). Other sources of free radicals also include redox cycling of xenobiotics, exposure to environmental (atmospheric pollutants and cigarette smoking etc.) or physicochemical agents (ultraviolet rays and radiation etc.), or toxic chemicals, overnutrition, or pathophysiological state (Machlin and Bendich, 1987; Cadenas and Davies, 2000; Devasagayam and Kamat, 2002) (Figure 2.3). However, during time of environmental stress, free radicals can adversely alter lipids, proteins, and DNA, resulting in significant damage to cell structure and functions. Membrane lipids present in subcellular organelles are highly prone to free radical damage resulting in lipid peroxidation that can lead to adverse alterations (Devasagayam, Boloor, and Ramasarma, 2003). Oxidation of proteins by ROSs/RNSs can result in loss of enzyme activity (Stadtman, 1992,

2001). Free radical damage to DNA can result in mutagenesis and carcinogenesis. The hydroxyl radical is known to react with all components of the DNA molecule. Hydroxyl radicals can cause damage to purine and pyrimidine bases and also the deoxyribose backbone (Halliwell and Gutteridge, 1999).

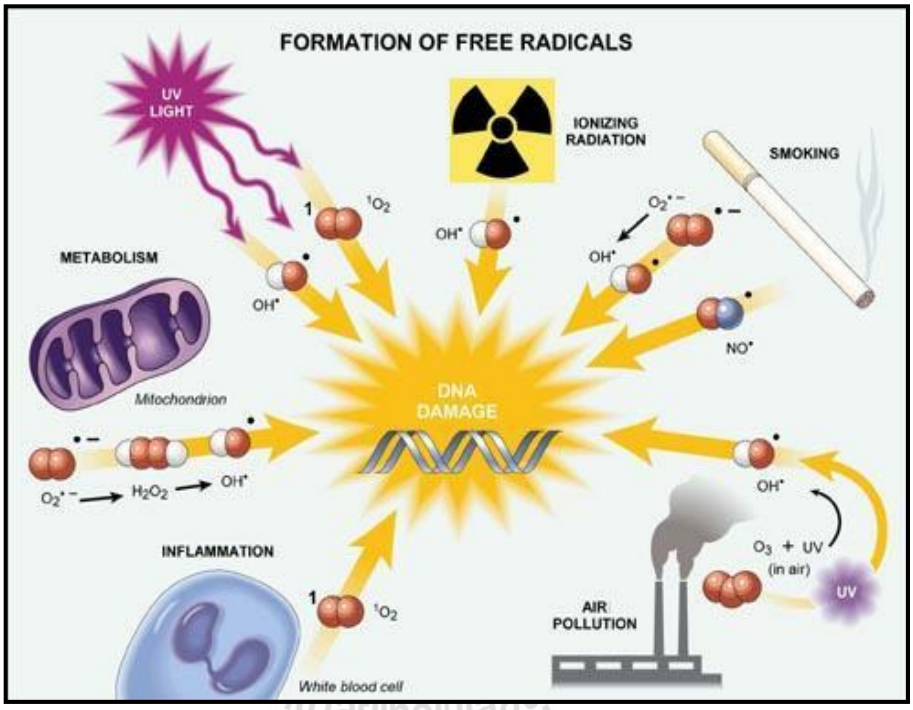


Figure 2.3 Sources of free radicals (Symmetry, www, 2013).

Oxidative stress or overproduction of ROSs and RNSs have been implicated in aging and a number of human diseases such as atherosclerosis, malaria, rheumatoid arthritis, neurodegenerative diseases, ischemia-reperfusion (I/R) injury, cardiovascular diseases, viral pathogenesis, hypertension, formation of drug resistant mutant, drug-induced tissue injury, and inflammation (Aroma, 1998; Fang et al., 2009). Antioxidants are known as substances or nutrients that neutralize free radicals in various stages (Sies, 1997). Nature has endowed each cell with adequate protective mechanisms against any harmful effects of free radicals. As shown in Figure 2.4, these defense systems include intracellular superoxide dismutase

(SOD), glutathione peroxidase, and glutathione reductase. Other non-enzymatic antioxidants include essential nutrient such as vitamin E and C. Vitamin E acts as a chain-breaking antioxidant which prevents the propagation of free radical reactions in all cell membranes in our body. Vitamin E is also considered as the standard antioxidant to which other compounds with antioxidant activities are compared. Ascorbic acid or vitamin C is also part of the normal protecting mechanism (Buettner, 1993; Mahadik and Mukherjee, 1996; Kagan et al., 2002). Other non-enzymatic antioxidants also include carotenoids, flavonoids, and related polyphenols such as α-lipoic acid, glutathione. Carotenoids, such as beta-carotene, lycopene, lutein and other carotenoids, function as important antioxidants to quench O_2 and $ROO^{\cdot}$ (Stahl and Sies, 2003). Flavonoids, mainly present as coloring pigments in plants also function as potent antioxidants at various levels. Antioxidant can act at the levels of prevention, interception, and repair. Preventive antioxidants attempt to stop the formation of ROS like intracellular SOD. Interception of free radicals is mainly mediated by radical scavengers such as vitamin C and E, glutathaione, other thiol compounds, carotenoids, flavonoids etc. Repair enzymes are involved at the repair and reconstitution level of antioxidants (Devasagagam et al., 2004).

Natural products from dietary components such as Indian spices, Thai spices, traditional Chinese spices as well as medicinal plants are known to possess antioxidant activity. They have important roles in bioprospecting of new medicines from medicinal plants, which are also rich sources of antioxidants. Current estimate indicates that about 80% of people in developing countries still rely on traditional medicine from medicinal plants and animals for various diseases prevention and treatment (Devasagagam et al., 2004).

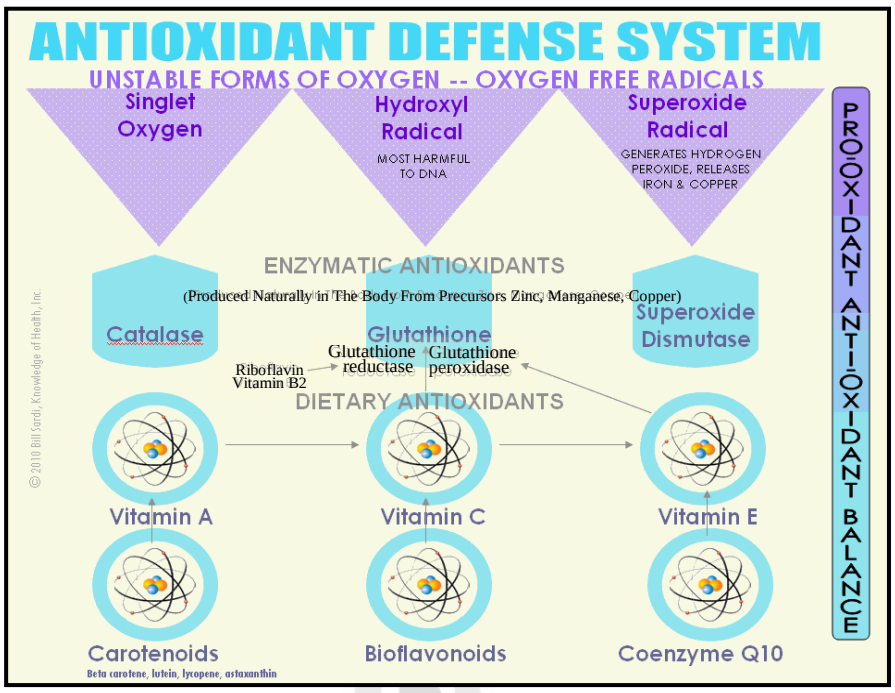


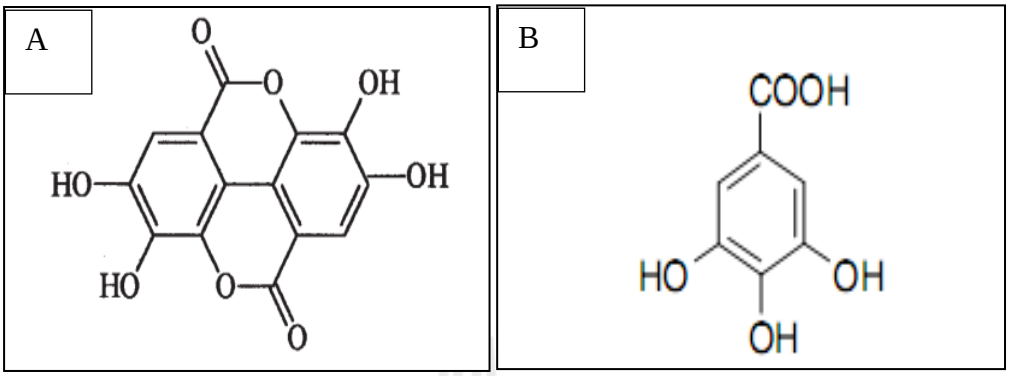
Figure 2.4 Antioxidant defense system (Lewrockwell, www, 2010).

2.3 Phenolic compounds and flavonoids

Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits, vegetables, and medicinal plants (Hertog, Hollman, Katan, and Kromhout, 1993; Parr and Bolwell, 2000; Barros, Dueñas, Carvalho, Ferreira, and Santos-Buelga, 2012). The beneficial effects derived from plant phenols have been attributed to their antioxidant properties (Heim, Tagliaferro, and Bobilya, 2002). They are also considered to possess anti-atherogenic, anti-allergenic, anti-inflammatory, anti-microbial, anti-thrombotic, vasodilatory, and cardioprotective effects. Basic structures of phenolic compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer (Balasundram, Sundram, and Samman, 2006). Phenolic phytochemicals are the largest category of phytochemicals and the most widely distributed in the plant kingdom. More than 8,000 phenolic phytochemicals have been identified (Croft, 1998). Phenolic compounds are plant secondary metabolites that are frequently

attached to sugars (glycosides). Occasionally, phenolic compounds also occur in plants as aglycones. Thus, these phenolics can be classified into non-soluble compounds (condensed tannin, lignins, and cell-wall bound hydroxycinnamic acids) and soluble compounds (phenolic acids, phenylpropanoids, flavonoid, and quinones) (Bravo, 1998). Phenolic compounds can be divided into several classes based upon their chemical structures. Though such structural diversity results in the range of phenolic compounds that occur in nature, the three most important groups of dietary phenolics are phenolic acid, tannins, and flavonoids (King and Young, 1999). Phenolic acids consist of two subgroups: the hydroxybenzoic and hydroxycinnamic acids. The two major dietary hydroxybenzoic acids are ellagic (Figure 2.5A) and gallic acid (Figure 2.5B), which usually occur as hydrolyzable tannins and are found mainly in berries and nuts (Maas and Galletta, 1991). Tannins, the relatively high molecular weight compounds, are divided into 2 groups. Condensed tannins are the first group, which are polymers of catechins or epicatechins. And the second group is hydrolyzable tannins which are polymers of gallic acid **(**Chang, Collins, Bailey, and Coffey, 1994; King and Young, 1999). Flavonoids are the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Baxter, Harborne, and Moss, eds., 1999). Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms which are arranged in a C6-C3-C6 configuration. Essentially, the structure consists of two aromatic rings A and B, joined by a 3-carbon bridge usually in the form of heterocyclic ring, C (Bohm, 1998; Marken and Beecher, 2000) (Figure 2.6). Flavonoids can be further subdivided into 6 major subclasses, based on the connection of an aromatic ring to the heterocyclic ring, as well as the oxidation state and functional groups of the heterocyclic ring. In addition, within each subclass, individual compounds are characterized by specific hydroxylation and conjugation patterns (Beecher, 2003). The six major subclasses of flavonoids include flavones, flavonols, flavanones, catechins or flavanols, isoflavones, and anthocyanidins (Ross and Kasum,

2002). Chemical structures and major food sources of flavonoid subclasses are shown in Figure 2.7.



Ellagic acid Gallic acid

Figure 2.5 Structure of (A) ellagic acid (King and Young, 1999), and (B) gallic acid (Balasundram et al., 2006).

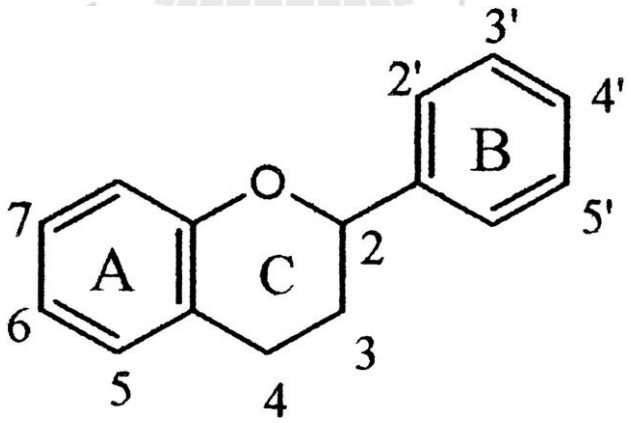


Figure 2.6 Generic structure of a flavonoid molecule (Balasundram et al., 2006).

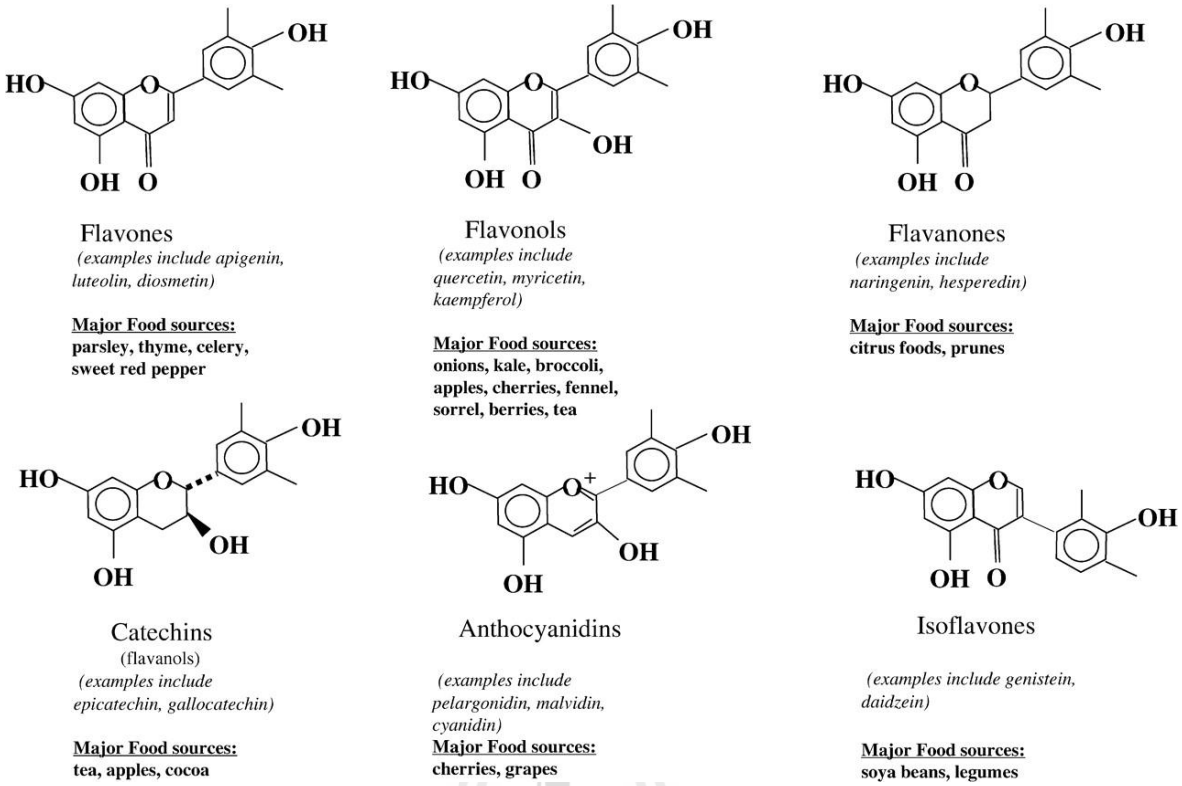


Figure 2.7 Chemical structures and major food sources of flavonoid subclasses (Ross and Kasum, 2002).

2.4 Inflammation

Inflammation is the reaction of vascularized tissue to local injury. The causes of inflammation are many and varied but most commonly result from an immune response to infectious microorganisms (Sommer, 2005) as shown in Figure 2.8. Inflammation can be classified into acute and chronic. Though the temporal profiles differ, both acute and chronic inflammation share common mechanisms with different timescales and amplitudes of the biological response (Cheng et al., 2010). Acute inflammation is the early or immediate response to vascular changes where the widespread effects of inflammatory mediators produce pain, heat, and swelling, usually of short duration (Sommer, 2005). Subacute or chronic phase is characterized by the development of specific humoral and

cellular immune responses to the pathogen present at the site of tissue injury (Faghali and Wright, 1997). Chronic inflammation is self-perpetuating and may be present for weeks, months, or even years. It may develop as the result of a recurrent or progressive acute inflammatory process or from low-grade, smoldering responses that fail to evoke an acute attack (Sommer, 2005). During both the acute and chronic inflammatory state, a variety of soluble factors are involved in leukocyte recruitment through increased expression of cellular adhesion molecules and chemoattraction. In addition, a variety of these soluble mediators regulate resident cells and the newly recruited inflammatory cells and some of these mediators contribute to the systemic responses associated with the inflammatory state. The soluble factors that mediate these responses are divided into four main categories: (1) inflammatory lipid metabolites and the numerous derivatives from arachidonic acid such as prostaglandins and leukotrienes, (2) three cascades of soluble proteinase/substrates such as clotting and complement which generate numerous proinflammatory peptides, (3) NO, a potent endogenous vasodilator, (4) a group of cell-derived polypeptides, known as cytokines, which to a large extent orchestrate the inflammatory response (Faghali and Wright, 1997). Thus, many inflammatory conditions are associated with many and varied factors including production of large amounts of NO (Liu and Hotchkiss, 1995), proinflammatory cytokines (TNF-α and IL-6) (Faghali and Wright, 1997), inducible enzymes (iNOS and COX-2) (Dannhardt and Kiefer, 2001; Calixto, Otuki, and Santos, 2003), and numerous of genes are expressed in association with the inflammatory process by activating several types of transcription factors (NF-κB, C/EBP, and AP-1) (Koj, 1996).

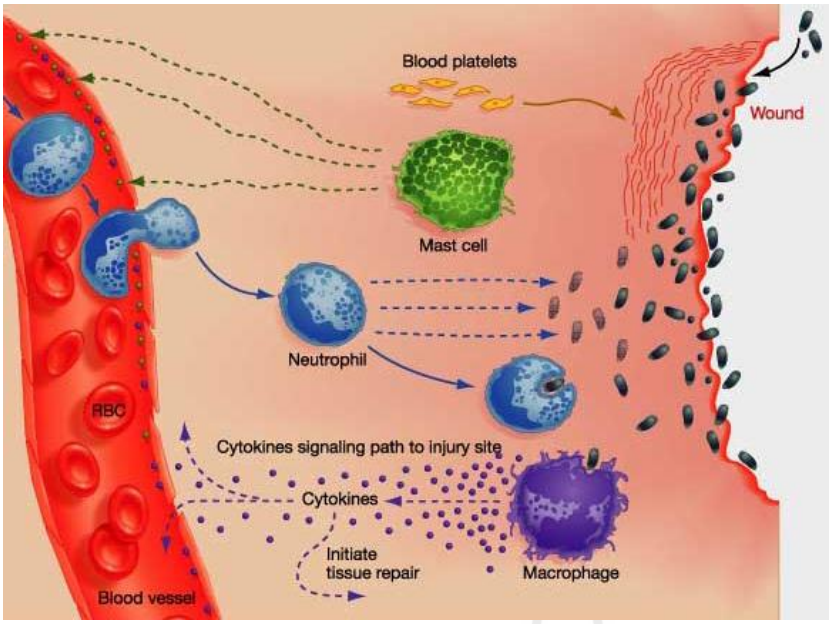


Figure 2.8 Inflammatory response against pathogens (Inflammation relief guide, www, 2011).

2.4.1 Role of nitric oxide in physiological function and inflammation

NO is an important bioregulatory molecule regulating numerous physiological functions such as blood pressure, neural signal transmission, and platelet functions, as well as playing critical roles in the anti-pathogen and tumoricidal response of the immune system. Low concentrations of NO are sufficient, in most cases, to affect these functions (Liu and Hotchkiss, 1995; Wink et al., 1998). However, during infection and inflammation, *in vivo* formation of NO is increased suggesting that NO concentrations are well above those found in normal physiological functions in infection of tissues. Increased NO levels may bring about some adverse effects of NO such as formation of carcinogenic N-nitroso compounds, deamination of DNA bases and mutagenesis, oxidation of DNA bases and tissue injury caused by potent oxidative agents (Liu and Hotchkiss, 1995). It is likely that the release of NO via iNOS has also been implicated as a deleterious agent in various pathophysiological conditions and tissue damages (Moncada, Palmer, and Higgs, 1991; Wink et al., 1998). Figure 2.9 shows physiological and pathological roles of NO.

2.4.2 Inducible nitric oxide synthase (iNOS)

The molecular NO is synthesized from the guanidino group of L-arginine by three isoforms of the enzyme NO synthase (NOS). Each isoform has been described and cloned: endothelial cell NOS (ecNOS or type 3), brain NOS (bNOS, nNOS, or type 1), and inducible macrophage-type NOS (iNOS or type 2) (Cuzzocrea, Riley, Caputi, and Salvemini, 2001). Historically, NOS have been classified into two distinct categories, constitutive (nNOS and ecNOS) and inducible (iNOS). Each isoform is the product of a distinct gene (Wink et al., 1998). As shown in Figure 2.10, biosynthesis of NO, generally, nNOS and ecNOS are present continuously in neurons and endothelial cells, respectively, and require elevation in intracellular Ca^{2+} and attendant activation of calmodulin (Ca^{2+}/calmodulin dependent) to produce NO. These isoforms are regulated primarily by calcium influx and generate low levels of NO for brief periods of time in response to receptor or physiological stimulation. Therefore, when NO is formed by vascular endothelial, it diffuses into the vascular smooth muscle cells adjacent to the endothelium where it binds to and activates guanylyl cyclase. This enzyme catalyzes the dephosphorylation of GTP to cGMP, which serves as a second messenger for many important cellular functions, particularly for signaling smooth muscle relaxation (Moncada et al., 1991; Wink et al., 1998). Another enzyme, iNOS needs to be induced by cytokines primarily by cells of the myeloid lineage, macrophages, enthothelial cells, and a number of other cells and can generate locally high concentration of NO for prolonged periods of time (Wink et al., 1998). iNOS is expressed typically in response to immunological stimuli, bacteria lipopolysaccharide (LPS), and a variety of proinflammatory cytokines, and produces nanomoles, rather than picomoles of NO (Cuzzocrea et al., 2001). iNOS is a cytosolic Ca^{2+}-independent enzyme. It requires tetrahydrobiopterin as well as other cofactors, and its induction is suppressed by glucocorticoids (Moncada et al., 1991). Induction of iNOS can also be suppressed by numerous agents, including thrombin,

macrophage deactivation factor, transforming growth factor-β, platelet-derived growth factor, interleukin (IL)-4, IL-8, IL-10, and IL-13 (Cuzzocrea et al., 2001). Macrophages are capable of producing wide ranges of NO concentrations depending on the source of stimulus such as LPS, proinflammatory cytokines, or LPS plus proinflammatory cytokines. Interestingly, macrophages are stimulated by LPS plus IFN-γ. The amount of NO generation is higher when compared to stimulation with LPS alone or LPS plus TNF-α or IL-1β (Cheng et al., 2010).

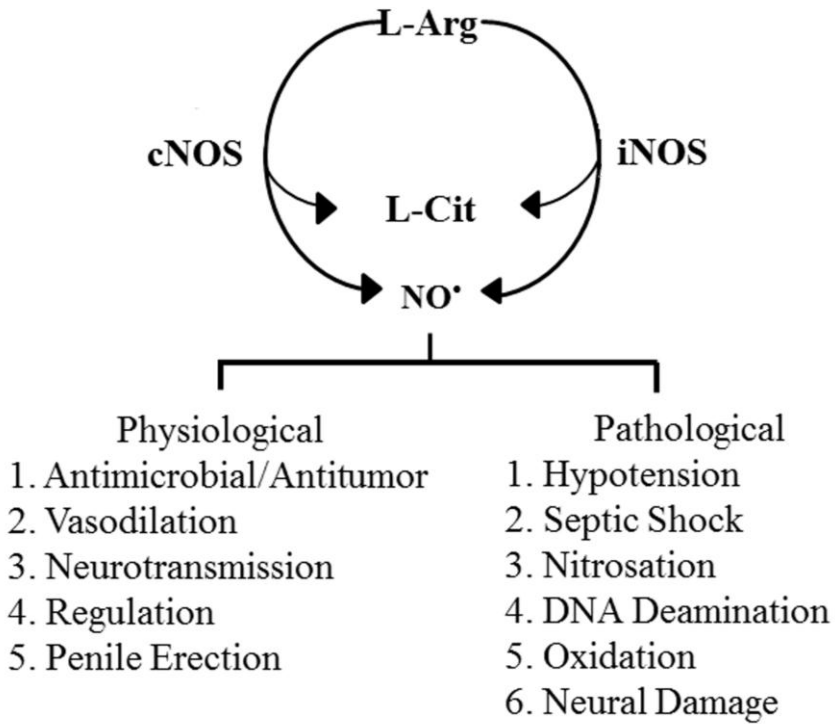


Figure 2.9 Physiological and pathological roles of NO (Liu and Hotchkiss, 1995).

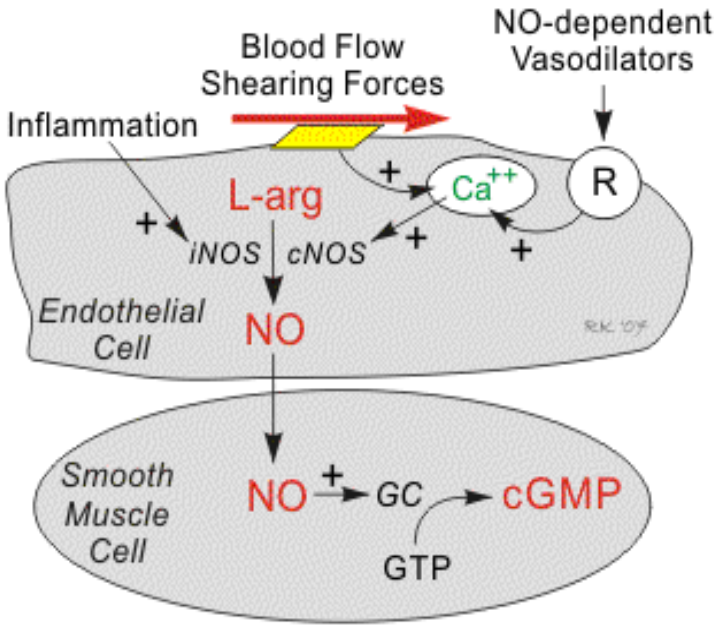


Figure 2.10 Biosynthesis of NO (Cardiovascular physiology concepts, www, 2008).

2.4.3 Direct and indirect effects of NO

The biological effects of NO can be divided into two types, direct and indirect. The direct effects of NO generally occur at low concentrations, whereas indirect effects occur at much higher concentration. Direct effects are those chemical reactions in which NO reacts directly with given biological targets. Low levels of NO can react directly with heme-containing proteins such as guanylate cyclase, oxyhemoglobin, and cytochrome P450, and thereby may account for the neuromodulatory effects of nNOS and the vasodilatory effects of ecNOS. In contrast, the indirect effects require the reaction of NO with oxygen (O_2) or superoxide (O_2^-) to generate RNS which subsequently react with the biological targets and change their downstream functions. These reactions require high local concentrations of NO of which iNOS may be the sole biological source (Wink et al., 1998; Thomas et al., 2008; Cheng et al., 2010). Figure 2.11 shows the overview of the chemistry of direct and indirect reactions of NO.

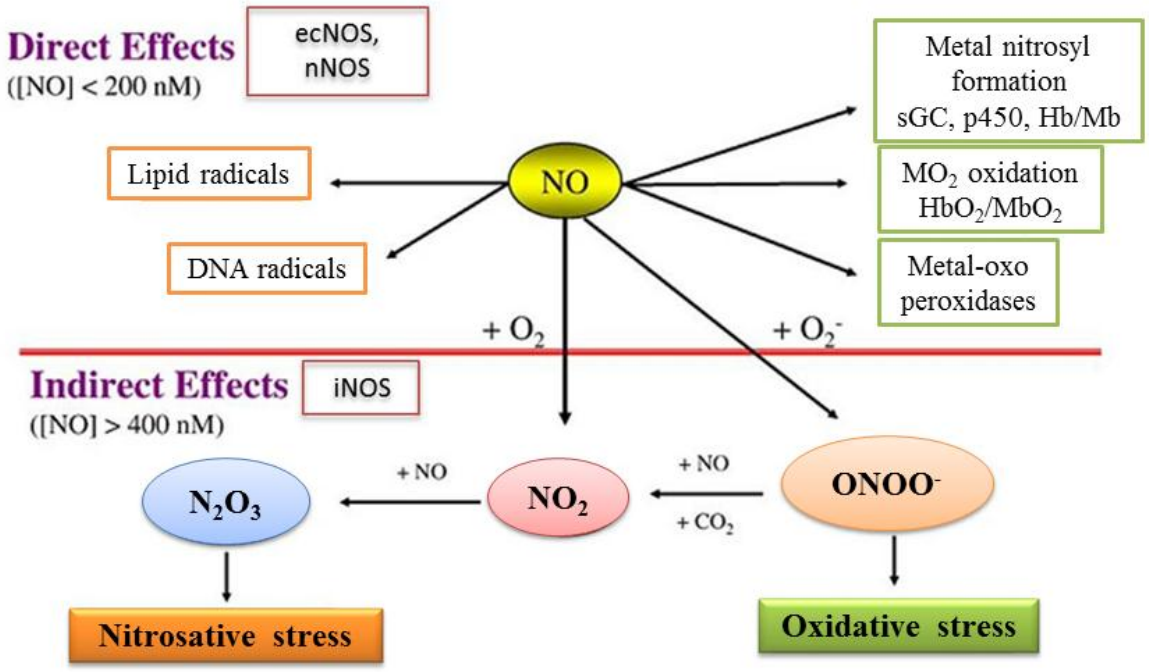


Figure 2.11 The chemistry of direct and indirect reactions of NO (adapted from Thomas et al., 2008).

2.4.4 Inducible cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) or prostaglandin H_2 synthase (PGHS), first purified in 1976 and cloned in 1988, is the key enzyme in the synthesis of prostaglandins (PGs) from arachidonic acid (AA) (DeWitt and Smith, 1988; Merlie, Fagan, Mudd, and Needleman, 1988; Yokoyama, Takai, and Tanabe, 1988). Two closely related forms of COX known as COX-1 and COX-2 have been identified. Both isoforms are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations. Both isoenzymes transform AA to PGs, but they differ in their distribution and their physiological roles. Thus, rather than classifying PG biosynthesis into physiological and pathological, it may be better to classify based on COX isoforms: either constitutive or induced. COX-1 activity is constitutive and is present in nearly all cell types at a constant level, whereas COX-2 activity is normally absent from cells, and when induced, the protein

level can increase or decrease in a matter of hours after a single stimulus (Vane, Bakhle, and Botting, 1998).

It is now known that under basal conditions the constitutive enzyme COX-1 is expressed in nearly all tissues including the kidney, colon, stomach, liver, spleen, heart, lung, and brain (Vane et al., 1998; Dannhardt and Kiefer, 2001). In contrast, COX-2 expression is largely undetectable unless it is induced by inflammatory stimuli in cells such as macrophages, endothelial cells, and synoviocytes. Such stimuli are proinflammatory cytokines (IL-1α/β, IL-2, IFN-γ, and TNF-α), endotoxin, mitogens, oncogenes (phorbolic ester), growth factors (fibroblast growth factor, FGF; platelet derived growth factor, PDGF; epidermal growth factor, EGF), hormones (luteinizing hormone, LH) and disorders of water-electrolyte homeostasis. The anti-inflammatory cytokines, IL-4, IL-10, and IL-13, and glucocorticoids can decrease induction of COX-2 (Dannhardt and Kiefer, 2001; Wang and Dubois, 2010). In addition, COX-2 expression is regulated at both transcriptional and post-transcriptional levels. It is well established that the COX-2 transcription can be regulated by various transcription factors such as NF-κB, C/EBP, CREB, NFAT, AP-1, and PPAR (Wang and Dubois, 2010).

Induction of COX-2 by several stimuli associated with cell activation and inflammation assured the relationship of this particular isoform to inflammatory disease in general. Apart from its involvement in inflammatory process, COX-2 seems to play a role in angiogenesis, colon cancer, and Alzheimer's disease, based on the fact that it is expressed during these diseases. The discovery of the COX-2 has made possible the design of new drugs that can reduce inflammation without removing the protective PGs in the stomach and kidney made by COX-1 (Vane et al., 1998). Therfore, the selective COX-2 inhibitors have offered a new perspective for anti-inflammatory treatment.

2.4.5 LPS and toll-like receptor

LPS is a principal outer membrane component of Gram-negative bacteria, which potently activates the innate immune system. The known components of LPS recognition complex in macrophages is summarized in Figure 2.12.

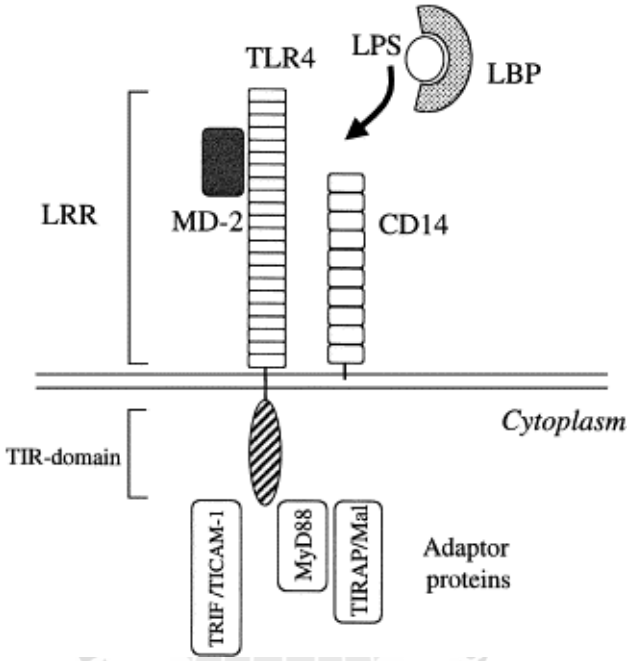


Figure 2.12 Schematic illustration of the LPS receptor complex on macrophages. LPS is recognized by a complex of three proteins: CD14, TLR4, and MD-2. A serum LBP transfers LPS to CD14. CD14 concentrates LPS and presents it to TLR4-MD-2. MD-2 plays a role in LPS recognition and regulates the cellular distribution of TLR4. TLR4 is a transmembrane protein characterized by an extracellular domain containing multiple LRR, a transmembrane domain, and an intracellular TIR domain. TLR4 also plays a role in LPS recognition and functions as the signal-transducing receptor for LPS. Three adaptor proteins including MyD88, TIRAP (also known as Mal), and TRIF (also known as TICAM-1) are responsible for the TLR4-mediated signaling (Fujihara et al., 2003).

CD14 serves as a ligand-binding component of the LPS sensing complex, having a role in concentration and presentation of LPS to the downstream

recognition/signaling components. It is Toll-like receptor 4 (TLR4) that works downstream of CD14 and is responsible for delivering an LPS signal. Although TLR4 is indispensable for LPS signaling, MD-2 is required for recognition of LPS that involves physical association of MD-2 with TLR4. Therefore, in macrophages and dendritic cells, TLR4-MD-2 and CD-14 are important for LPS recognition (Miyake, 2003).

2.4.6 Role of cytokines in inflammation

Inflammation is mediated by many and a variety of soluble factors including a group of secreted polypeptides known as cytokines. They are major determinants leading to cellular infiltrate, the state of cellular activation, and the systemic responses to inflammation. Cytokines are multifunctional and pleiotropic molecules that elicit their effects locally or systemically in an autocrine or paracrine manner. They are associated in extensive networks that involve synergistic as well as antagonistic interactions. In addition, these cytokines exhibit both negative and positive regulatory effects on various target cells. Cytokines can be divided into two major groups that mediate acute and chronic inflammation (Faghali and Wright, 1997). Figure 2.13 shows the types of multiple cytokines that play key roles in mediating acute and chronic inflammatory reactions.

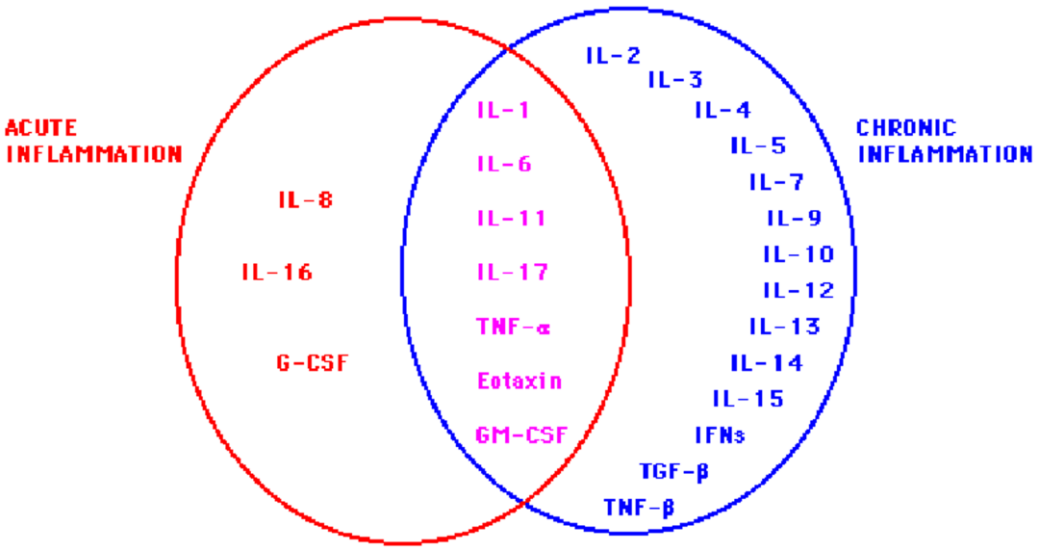


Figure 2.13 Cytokines involved in acute and chronic inflammatory responses (Faghali and Wright, 1997).

2.4.6.1 Tumor necrosis factor alpha

Tumor necrosis factor (TNF, also known as TNF-α) was identified in 1975. TNF has since been implicated in common biological properties, and in a diverse range of inflammatory infectious and malignant conditions. The importance of TNF in inflammation has been highlighted by the efficacy of anti-TNF antibodies or administration of soluble TNF receptor (TNFRs) in controlling disease activity in rheumatoid arthritis and other inflammatory conditions. TNF is one of the products which is produced predominantly by activated monocyte/macrophage. Although these cells are the main source of TNF in inflammatory disease, a wide range of cells types can produce TNF, including mast cells, T and B lymphocytes, natural killer (NK) cells, neutrophils, endothelial cells, smooth and cardiac muscle cells, fibroblasts, and osteoclasts (Bradley, 2008).

Regulation of the transcription factor NF-κB is a key component of TNF signal transduction. All known responses to TNF are triggered by binding to one of two distinct receptors as shown in Figure 2.14. Designated TNFR1 (also known as TNFRSF1A, CD120a, p55) and TNFR2 (also known as TNFRSF1B, CD120b, p75) are differentially regulated on various cell types in normal and diseased tissue. Both the proinflammatory and the programmed cell death pathways activated by TNF, and associated with tissue injury, are largely mediated through TNFR1. In addition to mediating cell survival and proinflammatory signals through NF-κB and AP-1, TNFR1 can also initiate cell death signaling pathways. Moreover, both TRAF1 and TRAF2 can contribute to NF-κB activation, through binding of the IKK complex and through recruitment of inhibitor of cellular apoptosis associated proteins. However, the utilization of different signaling mechanisms by TNFR1 and TNFR2 is consistent with the ability of each receptor to signal distinct biological responses in cultured cells (Bradley, 2008).

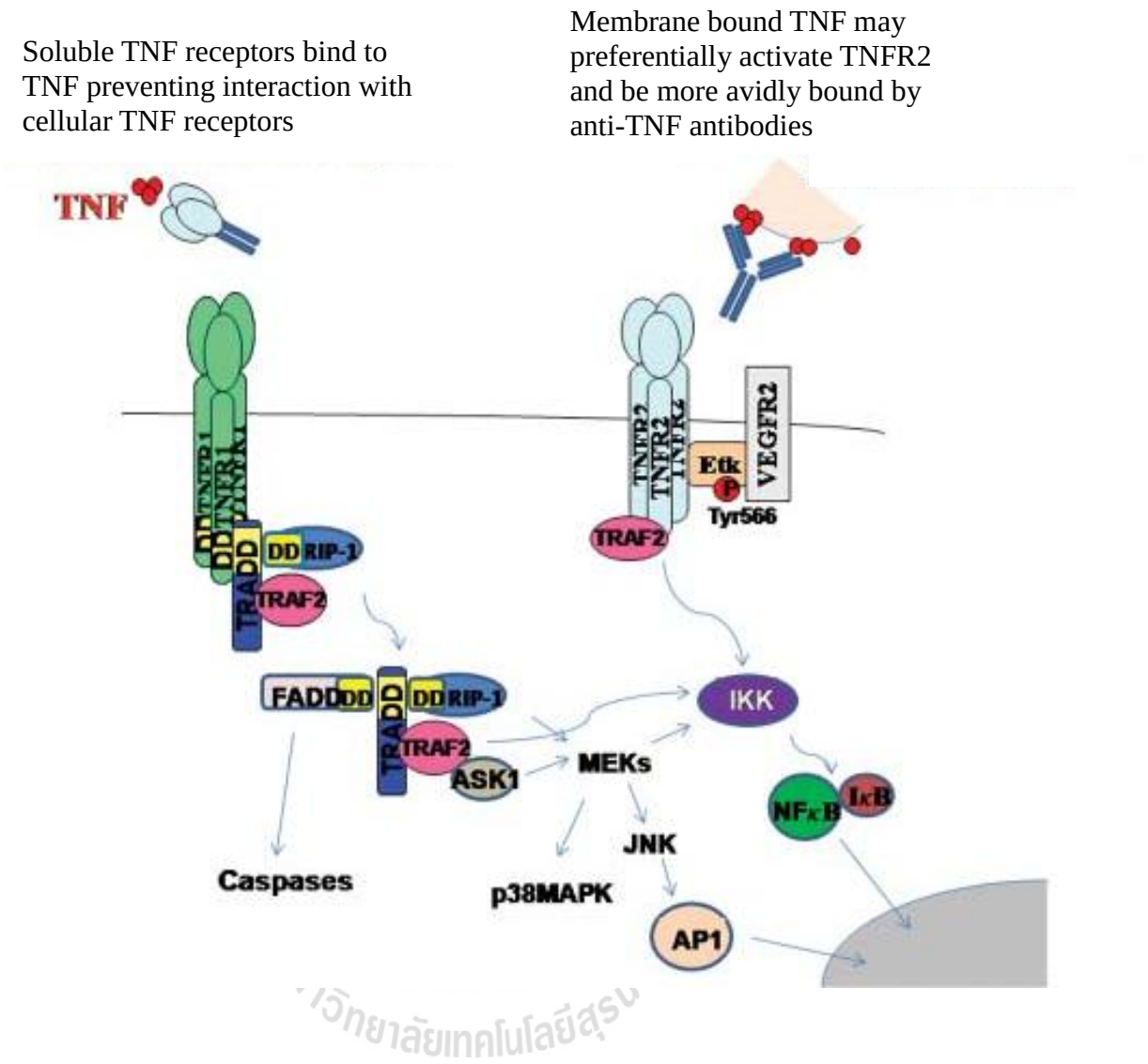


Figure 2.14 Signaling pathways leading to the main cellular responses of TNF. Soluble TNF receptors or monoclonal anti-TNF antibodies, which prevent TNF interacting with its receptors to activate the pathways, can be used to treat inflammatory disease (Bradley, 2008).

In response to TNF, endothelial cells promote inflammation by displaying in distinct temporal, spatial, and anatomical patterns including different combinations of adhesion molecules (Bradley, 2008). Many of the classical features of inflammation can be produced by local effects of TNF on endothelial cells. In addition, TNF can induce the expression of COX-2 leading to vasodilation, thus causing ‘rubor’ and

'color' through increased local blood flow (Mark, Trickler, and Miller, 2001). TNF-α exhibits several proinflammatory properties and can share these properties with IL-1. TNF-α and IL-1 can induce fever directly via stimulation of PGE_2 production by hypothalamic vascular endothelium. For indirect effects, TNF-α and IL-1 induce release of IL-1 (Faghali and Wright, 1997). Furthermore, TNF-α shares similar inflammatory activity with IL-6 and IL-1. Both TNF-α and IL-6 proinflammatory cytokines exhibit secondary inflammatory effects by stimulating IL-6 synthesis in many cell types (Warren, 1990).

2.4.6.2 Interleukin-6

IL-6 has many synonyms with some of its biological activities such as interferon-β_2 (IFN-β_2), hybridoma/plasmacytoma growth factor, hepatocyte-stimulating factor, B cell stimulatory factor (BSF-2), and B cell differentiation factor (BCDF) (Van Snick, 1990). IL-6, a cytokine featuring redundancy and pleiotropic activity, contributes to host defense against acute environmental stress. In addition, dysregulated persistent IL-6 production has been demonstrated to play a pathological role in various autoimmune and chronic inflammatory diseases. The proinflammatory cytokine, IL-6 is produced by a variety of cell types, including mononuclear phagocytes, T cells, and fibroblasts (Hirano et al., 1990; Van Snick, 1990; Hirano, 1992a). In the early phase of infectious inflammation, IL-6 is produced by monocytes/macrophages immediately after the stimulation of toll-like receptors (TLRs) with distinct pathogen-associated molecular patterns (PAMPs). This acute IL-6 expression plays a central role in host defense by stimulating various cell populations. When acting on hepatocytes, IL-6 strongly induces a broad spectrum of acute-phase proteins such as C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, hepcidin, haptoglobin, and antichymotrypsin, whereas it reduces albumin, cytochrome P450, fibronectin, and transferrin (Figure 2.15). CRP is the best biomarker of inflammation and its expression mainly depends on IL-6. IL-6 combined with TGF-β preferentially induces the differentiation of naïve CD4 positive T cells into Th17 cells, whereas IL-6

inhibits TGF-β induced regulatory T cell (Treg) development. Consequently, Th17/Treg imbalance may cause the onset and progression of autoimmune and chronic inflammatory diseases. In bone marrow, IL-6 induces maturation of megakaryocytes into platelets and activation of hematopoietic stem cells. IL-6 also promotes the differentiation of osteoclasts and angiogenesis, the proliferation of keratinocytes and mesangial cells, and the growth of myeloma and plasmacytoma cells (Tanaka and Kishimoto, 2012).

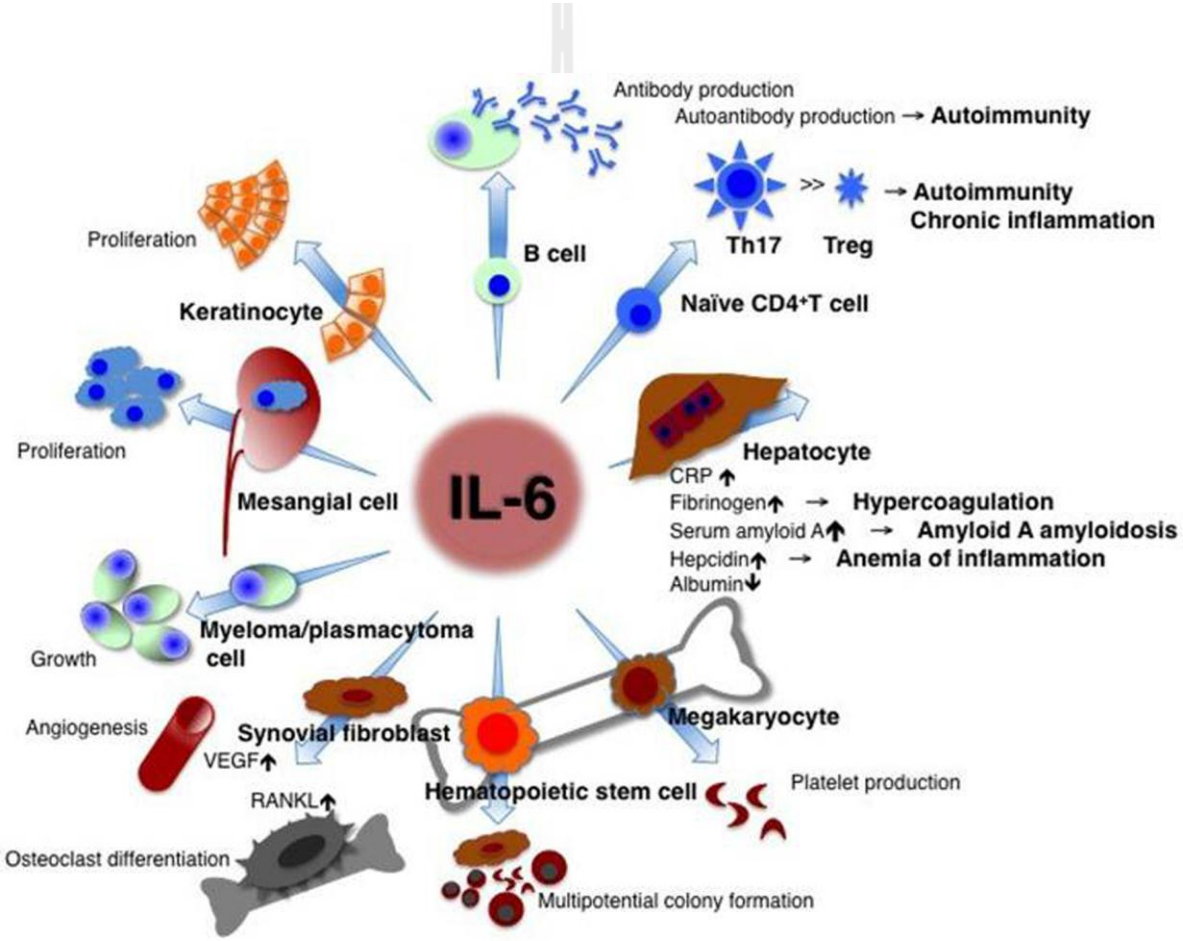


Figure 2.15 IL-6 has a pleiotropic effect but its dysregulated persistent production causes the onset and development of various autoimmune and chronic inflammatory diseases (Tanaka and Kishimoto, 2012).

IL-6 acts as a growth factor for mature B cells and induces their final maturation into antibody-producing plasma cells. IL-6 is also involved in T cell activation

and differentiation, and participates in the induction of IL-2 and IL-2 receptor expression. Some regulatory effects of IL-6 involve inhibition of TNF production and provide negative feedback for limiting the acute inflammatory response (Feghali, Bost, Boulware, and Levy, 1992). An increase in IL-6 production has been observed in a variety of chronic inflammatory and autoimmune disorders such as type I diabetes, thyroiditis, RA (Tan, Farmiloe, Yeoman, and Watson, 1990; Hirano, 1992b), systemic sclerosis (Feghali et al., 1992), mesangial proliferative glomerulonephritis and psoriasis, and neoplasms (Hirano, 1992b).

2.4.7 Major transcription factors involved in cytokine gene expression

NF-κB, C/EBP, and AP-1 are a major family of transcription factors which participate in the basal and induced expression of proinflammatory cytokines, especially acute phase cytokines.

2.4.7.1 C/EBPs transcription factor

CCAAT/enhancer-binding proteins (C/EBPs) are a family of leucine zipper transcription factors involved in regulation of various aspects of normal cellular differentiation and function in multiple tissues. In addition, C/EBPs act as pivotal regulators of response to inflammatory insults (Lekstrom-Himes and Xanthopoulos, 1998). Six different members of the family C/EBP, basic leucine zipper (bZIP) transcription factors, includes C/EBPα, -β, -γ, -δ, -ε, and –ζ, which share related sequences and functions (Figure 2.16A). Except for CEBP ζ which lacks a canonical basic region, each protein contains similar basic region and leucine zipper sequences at its C-terminus, which mediate DNA binding and dimerization, respectively. In addition, Figure 2.16B showed C/EBP bind to palindromic DNA sites as homo-or heterodimers. The N-terminal portion of each protein contains effector domains that mediate transcriptional activation, repression, and autoregulatory functions (Johnson, 2005).

A B

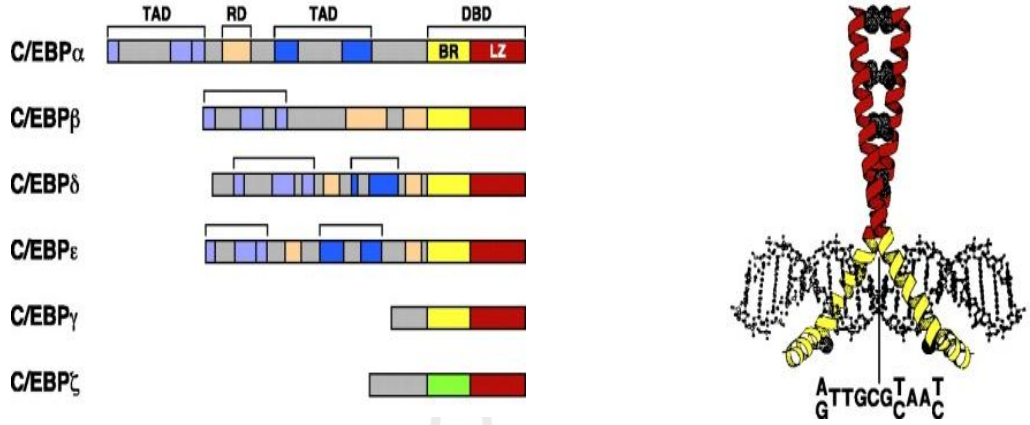


Figure 2.16 (A) Domain organization of C/EBP-family members. Each C/EBP protein contains a functionally related leucine zipper dimerization domain (LZ) at its C-terminus. All family members, with the exception of C/EBP*ζ*, share an adjacent highly conserved basic region (BR) that mediates sequence-specific DNA binding. C/EBP*α*, C/EBP*β*, C/EBP*δ* and C/EBP*ϵ* each contain transactivation domains (TADs) and a regulatory domain (RD) located in their N-terminal regions. A tripartite TAD at the N-terminus displays homology among several of the family members. (B) The predicted structure of a C/EBP bZIP dimer bound to its cognate DNA site (Johnson, 2005).

Many target genes in a variety of tissues have been reported for C/EBP proteins. In liver parenchymal cells, C/EBP proteins have been exhibited to form constitutive and inducible complexes on IL-6- and IL-1-responsive elements of acute phase protein genes. Among C/EBP isoforms, C/EBP*α* and -*β* may be responsible for constitutive transcription of these genes. C/EBP*β* and –*δ* isoforms appear to predominate in the IL-6-induced expression of such proteins as complement component 3 (C3) or mouse serum amyloid A3 in response to inflammation (Koj, 1996). Furthermore, both C/EBP*β* and –*δ* are strongly up-regulated at the transcription level by inflammatory stimuli such as LPS and

turpentine oil, and by recombinant cytokines such as IL-6, IL-1, and TNF-α (Poli, 1998). Both C/EBPβ and –δ isoforms participate in the induced expression of TNF-α, IL-6, IL-1β, IL-8, and other cytokines. Interestingly, C/EBPβ and especially C/EBPδ are highly inducible or activated via several signaling pathways in multiple tissues including monocytes/macrophages during the acute phase response elicited by LPS (Koj, 1996). Constitutive expression of C/EBPβ was found in liver, intestine, lung, and adipose. Moreover, it is also detectable in kidney, heart, and spleen of mice. Stimulation with LPS strongly induces C/EBPβ expression, suggesting a role in the mediation of the inflammatory response. Constitutive expression of C/EBPδ is detected in intestines, adipose, and lung, with high levels of expression in all tissues following LPS stimulation (Lekstrom-Himes and Xanthopoulos, 1998).

2.4.7.2 AP-1 transcription factor

Activator protein 1 (AP-1) transcription factor complex which has a function in cell proliferation, differentiation, and cell transformation during development and in adult tissues, is also a key player in regulating inflammatory processes. AP-1 is composed of homodimeric and heterodimeric complexes consisting of members of the Jun, Fos, or activating transcription factor (ATF) that bind to a common DNA site, the AP-1 binding site (Karin, Liu, and Zandi, 1997; Schonthaler, Guinea-Viniegra, and Wangner, 2011) (Figure 2.17). It can be activated by bacterial endotoxin (LPS), growth factors, cytokines, chemokines, hormones, and multiple environmental stresses (Pocock et al., 2003; Schonthaler et al., 2011). The leucine zipper is also a prominent feature of AP-1 transcription factor like C/EBP, which was first identified in the human metallothionein gene. AP-1 protein consists of either Jun homodimers or Fos/Jun heterodimeric complexes, which binds to palindromic sequence TRE (phorbol ester - TPA-responsive element) TGA(C/G)TCA. TRE sequences are presented in numerous mammalian promoters, including genes that code for cellular growth factors, cytokines, and acute phase protein

(Koj, 1996). AP-1 is usually composed of c-jun/c-fos subunits. After stress-induced phosphorylation, AP-1 enters the nucleus and binds to a specific sequence in the promoter of numerous target genes (Karin et al., 1997). The regulation of AP-1 complexes can occur at several levels, including transcription, mRNA translation, protein stability, or by interactions with other transcription factors (Schonthaler et al., 2011). Two almost identical AP-1 sequences have been identified in the human and mouse promoters of IL-6 gene. AP-1 and NF-κB transcription factors differ in their structure and bind to distinct enhancer motifs. AP-1, the heterodimer of c-Fos and c-Jun, are capable of physically reacting with NF-κB p65 subunit and the resulting heterodimer exhibits functional synergy (Koj, 1996). Current research has shown that AP-1 factors possess important roles in inflammatory state as well as in inflammatory diseases (e.g., psoriasis, fibrosis) (Schonthaler et al., 2011).

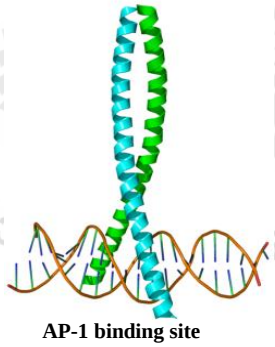


Figure 2.17 AP-1/DNA complex. Crystallographic structure of the AP-1 heterodimer is comprised of c-FOS (cyan) and c-Jun (green) complexed with DNA (Wikipedia, www, 2013).

2.4.7.3 NF-κB transcription factor

Nuclear factor kappaB (NF-κB) is a ubiquitous and well-characterized protein involved in physiological and pathological conditions, with a pivotal role in controlling cell signaling in the intact organism. NF-κB controls the expression of encoding adhesion molecules (e.g., ICAM, VCAM, E-selectin), chemokines (e.g., IL-8,

MIP-1α, MCP1, RANTES, eotaxin, etc.), proinflammatory cytokines (e.g., IL-1, IL-2, IL-6, TNF-α, etc.), inducible enzymes (COX-2 and iNOS), growth factors, some of the acute phase proteins, and immune receptors, and all of which play a critical role in controlling inflammatory processes (Calixto et al., 2003). The NF-κB family of transcription factors is composed of homo- and heterodimers of Rel proteins; NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (Rel) (Hayden and Ghosh, 2012). The most prevalent activated form of NF-κB is p50/p65 heterodimer. In unstimulated cells, NF-κB is present in the cytoplasm as a heterodimer consisting of p50, p65, and IκBα subunits. In response to an activation signal with inflammatory agents such as LPS, IκBα is degraded, leading to nuclear translocation and binding to a specific sequence in the promoter of numerous target genes (Calixto et al., 2003) (Figure 2.18). The highest DNA binding affinity is exhibited by heterodimer recognizing sequent motif GGGACTTTCC; however, numerous variations exist (Koj, 1996). Since NF-κB plays a central role in various inflammatory diseases, much research has been focused on the identification of compounds that selectively interfere with this pathway. Currently, a great number of plant-derived substances has been evaluated as possible inhibitors of the NF-κB pathway (Calixto et al., 2003).

Regulatory promoter regions with NF-κB bindings are common in genes primarily involved in inflammation, immune reactions, and the acute phase response to external stimuli. Activation of cytokine gene expression by NF-κB is probably the most important pathway, but additional factors are also required for the maximum response. Interaction of C/EBPα, C/EBPβ, NF-κB1 (p50), and NF-κB RelA (p65) was required for full IL-6 induction by LPS in differentiating bovine monocytes (Koj, 1996).

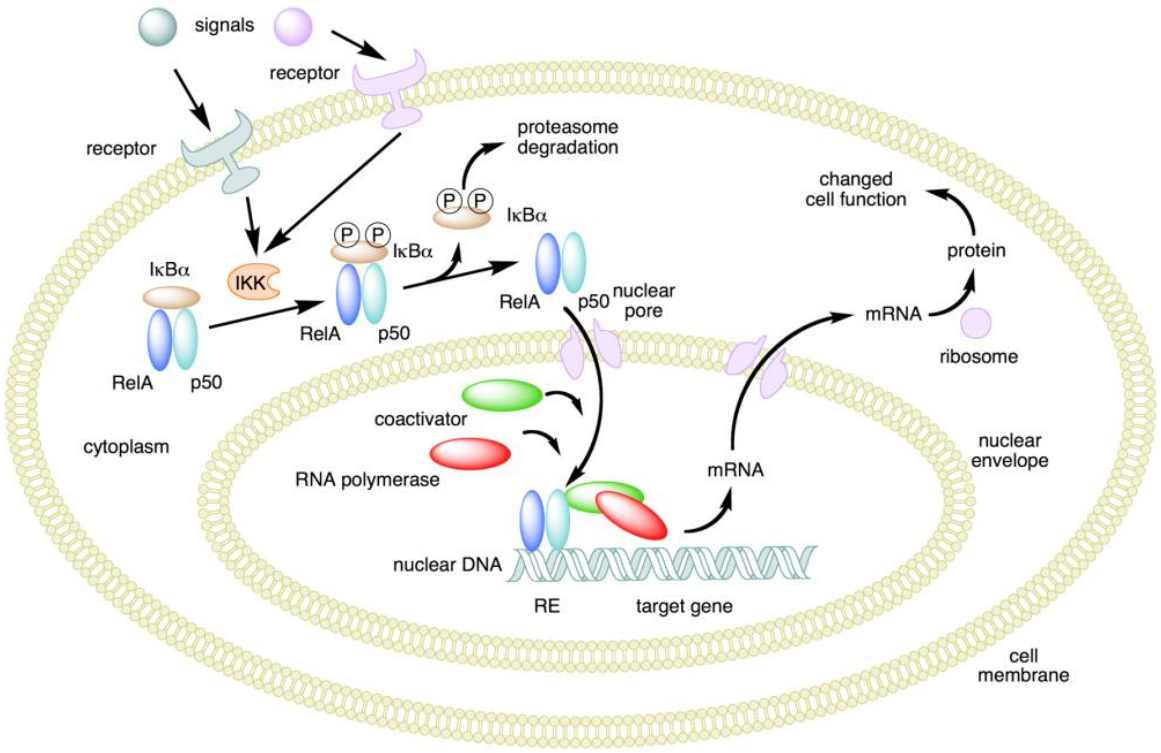


Figure 2.18 Mechanism of NF-κB activation. In unstimulated cells, NF-κB is present in the cytoplasm as an inactive heterodimer composed of two sub-units, p50 and p65 (RelA). The heterodimer is complexed with an inhibitory protein IκB*α*, preventing it from moving into the nucleus. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IκB kinase (IKK). IKK, inturn, phosphorylates the IκB*α* protein, which results in ubiquitination, dissociation of IκB*α* from NF-κB. Phoshorylated IκB*α* is then rapidly degraded by the proteasome, leading to the translocation of NF-κB to the nucleus, binding to its specific responsive element of DNA. The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, inturn, is translated into protein, resulting in alteration of cellular functions (Prajapati, singhal, Yashwant, and Gupta, 2010).

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CHAPTER III

PHYTOCHEMICAL AND ANTIOXIDANT PROPERTIES OF EXTRACTS FROM *PSEUDERANTHEMUM PALATIFERUM* LEAVES

3.1 Abstract

Beneficial antioxidant phytochemicals are found in many medicinal plants including the extracts from leaves of *Pseuderanthemum palatiferum* (PP). However, reports on the phytochemical constituents and antioxidant activities of these extracts are still very limited. Therefore, the present study aimed to evaluate and compare phytochemical contents and antioxidant activities of [1] 95% ethanol extract of fresh leaves of PP (95EE-FLP), [2] 80% ethanol extract of fresh leaves of PP (80EE-FLP), [3] 80% ethanol extract of dried leaves of PP (80EE-DLP), [4] water extract of fresh leaves of PP (WE-FLP), or [5] water extract of dried leaves of PP (WE-DLP). All extracts from PP were evaluated for total phenolic content (TPC) by Folin-Ciocalteu method, total flavonoid content (TFC) by aluminium trichloride colorimetric assay, free radical scavenging capacity by DPPH assay, and reducing capability by ferric reducing-antioxidant power (FRAP) method. The present study showed that WE-FLP had significantly higher levels of TPC and TFC than 95EE-FLP and 80EE-FLP, followed by WE-DLP, and 80EE-DLP, respectively, at $p<0.001$. The highest scavenging activity against DPPH radicals was found in WE-FLP, followed by 95EE-ELP, 80EE-FLP, WE-DLP, and 80EE-DLP, respectively, at $p<0.01$. The highest FRAP value was found in 95EE-FLP and 80EE-FLP, followed by WE-FLP, WE-DLP, and 80EE-DLP, respectively, at $p<0.001$. As a consequence, high levels of TPC and TFC, and

antioxidant activities were observed in extracts of fresh leaves of PP, whereas low levels of them were found in extracts of dried leaves of PP. This study also indicated that there were no significant difference in the levels of phenolic and flavonoid as well as ferric reducing antioxidant ability between 95EE-FLP and 80EE-FLP. Therefore, both 95EE-FLP and WE-FLP were employed to evaluate anti-oxidative stress in RAW264.7 cells using a fluorogenic dye marker, DCFH-DA. The result showed that both 95EE-FLP and WE-FLP suppressed reactive oxygen species (ROSs) generation in RAW264.7 cells. This study also indicated that 95EE-FLP more potently suppressed ROSs generation from RAW264.7 cells than did WE-FLP. Collectively, the present study suggested that all extracts of PP had levels of TPC and TFC, and possess antioxidant properties in an *in vitro* assay, and both 95EE-FLP and WE-FLP also displayed strong antioxidant properties when analyzed using a cell-based assay. Therefore, increased medicinal plant consumption is an effective strategy to increase antioxidant intake and decrease oxidative stress, and may lead to reduced risk of many chronic diseases.

3.2 Introduction

The balance between oxidants and antioxidants in humans is important for sustaining optimal physiological conditions. Reactive oxygen species (ROSs) are continuously produced in the human body, being essential for energy supply, detoxification, chemical signaling, and immune function (Dimitrios, 2006). However, during oxidative stress, large amounts of ROSs can adversely alter lipids, proteins, and DNA, resulting in significant damage to cell structure and function (Devasagayam et al., 2004). These types of damage have been implicated in increased risks of cancer, atherosclerosis, rheumatoid arthritis, and neurodegenerative diseases including inflammation (Fang, Seki, and Maeda, 2009). Although, the body can produce endogenous superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase that help prevent the damaging effects of ROSs, these

endogenous systems are often insufficient for fully scavenging ROSs (Dimitrios, 2006). Thus, finding exogenous sources of antioxidants is an important reason for discovery and synthesis of novel antioxidant compounds. Potential sources of natural antioxidants have been found in fresh fruits and vegetables, leaves, oilseeds, grains, bark and roots, spices, and herbs (Ramarathnam, Ochi, and Takeuchi, 1997). Natural antioxidants like flavonoids and other phenolics are free radical scavengers, and exhibit many biological activities (Rice-Evans, Miller, and Paganga, 1996). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelators (Rice-Evans, Miller, and Paganga, 1997; Parr and Bolwell, 2000).

Pseuderanthemum palatiferum (PP) is one of the most popular medicinal plants in both Thailand and Vietnam. It is referred to as a miracle plant in folk medicine because it anecdotally cures or prevents various conditions such as wounds, trauma, stomachache, colitis, hypertension, nephritis, diarrhea, diabetes, and cancer (Dieu, Loc, Yamasaki, and Hirata, 2005; Padee, Nualkaew, Talubmook, and Sakuljaitrong, 2010). Oxidative stress can contribute to the pathology of these diseases. However, reports on the phytochemical constituents and antioxidant activity of extracts of PP leaves are still very limited. Therefore, the present study aimed to investigate and compare the phytochemical contents and the antioxidant activities of extracts of PP leaves.

3.3 Materials and methods

3.3.1 Chemicals and reagents

(+)- Catechin hydrate and vitamin C were purchased from Fluka Chemine GmbH (Buchs, Switzerland). 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), penicillin G, streptomycin sulfate, resveratrol, 2',7'-dichlorofluorescin-diacetate (DCFH-DA), and *tert*-butyl hydroperoxide (tBuOOH), were ordered from Sigma-Aldrich (St. Louis, MO). RPMI

medium 1640 and Hank's balanced salt solution (HBSS) were obtained from Gibco Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, Utah). Quercetin dihydrate was obtained from INDOFINE Chemical Company, Inc. (Hillsborough, NJ). Finally, 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (trolox) was purchased from Sigma-Aldrich Chemine Gmbh (Steinheim, Germany).

3.3.2 Cell lines

The mouse macrophage RAW264.7 cell line (CLS, Cell Lines Service, Eppelheim, Germany) was used to investigate effects of extracts of PP leaves on suppression of ROS generation in cells.

3.3.2.1 Maintenances of RAW264.7 cells

Cells were cultured at 37 °C, 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 15 mM HEPES. Exponentially growing cells were used for the experiments when they reached approximately 80% confluence.

3.3.3 Plant materials

Fresh leaves of PP were purchased from producers in Yasothon province, Thailand (Figure 3.1). The plant was identified and authenticated by Dr. Kongkanda Chayamarit, the Forest Herbarium, Royal Forest Department, Bangkok, Thailand. A voucher specimen (BKF 174009) was deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Figure 3.1 *Pseuderanthenum palatiferum* (PP) in Yasothon province, Thailand.

3.3.4 Plant extract preparation

Extracts of PP leaves were prepared by two different methods. In the first method, fresh leaves of PP were extracted by blending. In the second method, air-dried leaf powder of PP was extracted by maceration.

Concerning the first method, fresh leaves (1.5 and 2.1 kg) were cut into small pieces and blended in 6 L 95% and 8.4 L 80% ethanol (100 g : 400 mL), respectively (Figure 3.2). This suspension was then filtered through several layers of gauze. The pooled extracts were centrifuged at 3,500 x *g* for 10 min at 4 °C and the supernatant was filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). Next, the ethanolic filtrate was concentrated using a vacuum rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) and dried by lyophilization (Freeze-Zone 12 plus, Labconco Corporation, Missouri) (Figure 3.3) to give crude dried extracts of 95% ethanol extract of fresh leaves of PP (95EE-FLP) and 80% ethanol extract of fresh leaves of PP (80EE-FLP), respectively. Forty grams of 95EE-FLP was further partitioned between hexane and water (1:1, v/v) using a separatory funnel (Figure 3.4). The water fraction was collected, centrifuged at 14,000 x *g* for 10 min at 4 °C, evaporated and lyophilized to obtain water extract of fresh leaves of PP (WE-FLP).

Figure 3.2 Fresh leaves of PP were blended in ethanol.

Figure 3.3 The extract was concentrated using a vacuum rotary evaporator and dried using a lyophilizer.

Figure 3.4 Crude dried extract was partitioned between hexane and water (1:1, v/v) using a separatory funnel.

For another method, air-dried leaves were ground into powder with an electric grinder (Figure 3.5). Powder air-dried leaves (420 g and 300 g) were then extracted with 12.6 L 95% and 9 L 80% ethanol, respectively (60 g : 1.8 L) by maceration for 72 h at room temperature (RT), with the used solvent being replaced with fresh solvent after every 24 h. The pooled extracts were centrifuged at 3,500 x *g* for 10 min at 4 °C, and the supernatant was filtered through Whatman No. 1 filter paper. Then, the ethanolic filtrate was concentrated using a vacuum rotary evaporator and dried by lyophilization to give crude

dried extracts of 95% ethanol extract of dried leaves of PP (95EE-DLP) and 80% ethanol extract of dried leaves of PP (80EE-DLP), respectively. Twenty grams of 95EE-DLP was further partitioned between hexane and water (1:1, v/v). The water fraction was later collected, centrifuged at 14,000 x *g* for 10 min at 4 °C, evaporated and lyophilized to obtain water extract of dried leaves of PP (WE-DLP). The summary of all extracts of PP leaves is shown in Figure 3.6.

Figure 3.5 Air-dried leaves and their ground powder prepared from PP.

All crude dried extracts were stored at -20 °C until use. When used in experiments, the ethanol and water crude dried extracts were dissolved in dimethyl sulfoxide (DMSO) and water, respectively. For cell culture, WE-FLP was dissolved in phosphate buffered saline (PBS). 95EE-FLP was dissolved in DMSO and then diluted to 0.25% (v/v) DMSO in cell culture medium when preparing the designated concentrations.

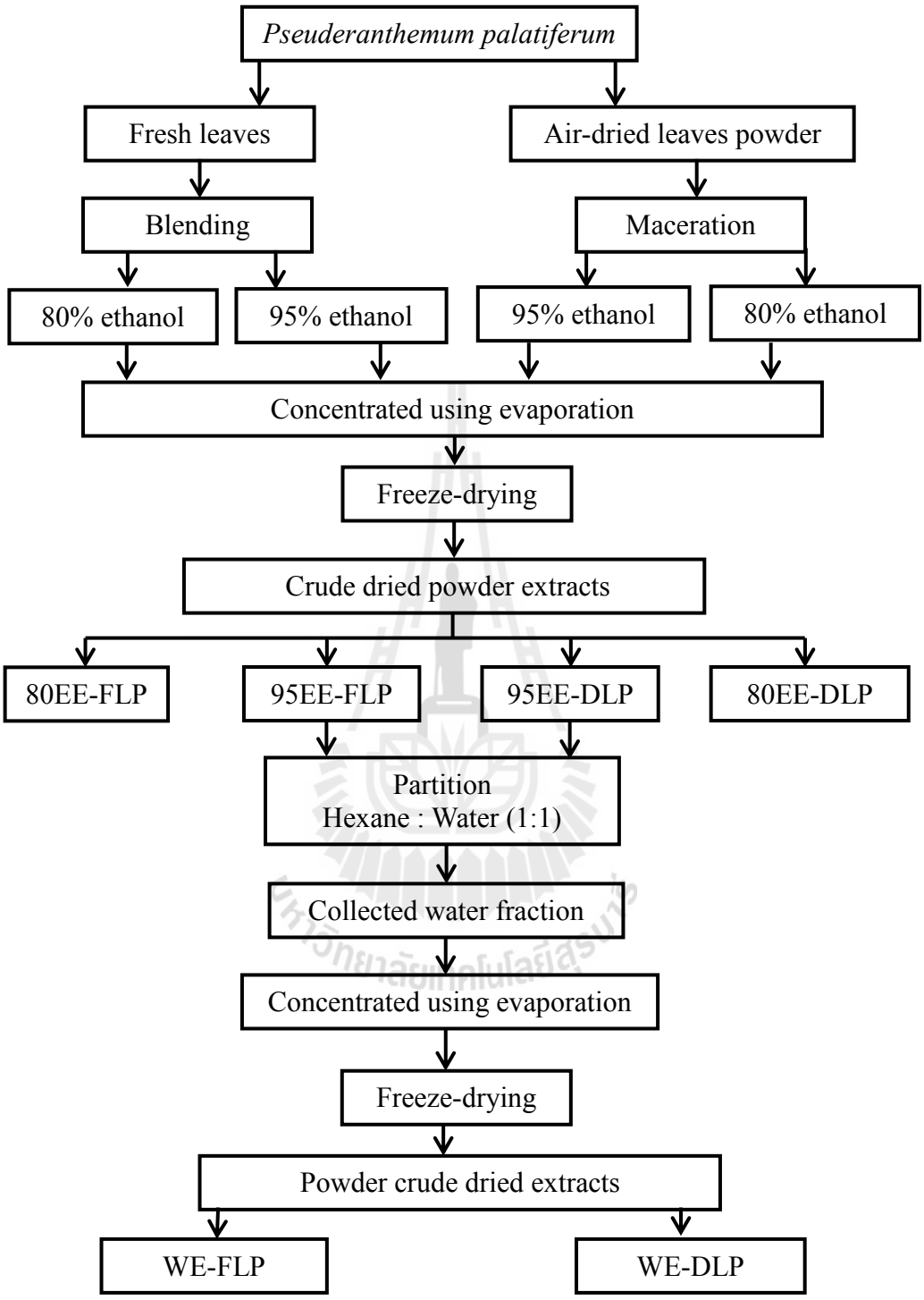

Figure 3.6 The flow chart of the process of PP leaf extracts.

3.3.5 Determination of phytochemical compounds

3.3.5.1 Total phenolic content (TPC)

The total phenolic content of individual extract was determined by the method of Folin-Ciocalteu (Mariod, Matthäus, Eichner, and Hussein, 2006). This method is based on the reduction of phosphotungstic acid in alkaline solution to phosphotungstic blue. The absorbance of formed phosphotungstig blue is proportional to the number of aromatic phenolic groups (Singleton, Orthofer, and Lamuela-Raventos, 1999). Briefly, 100 µL of test solutions were added to 2.0 mL of 2% Na_2CO_3 and mixed thoroughly. After 2 min, 100 µL of 50% Folin-Ciocalteu reagent were added, mixed, and allowed to stand at RT for 30 min. The absorbance of extracts was measured at 750 nm by a Cecil 1000 series spectrophotometer (Cecil Instruments, Cambridge, UK) against a blank consisting of all reagents and solvents without the extract. A gallic acid solution ranging from 0.05-0.3 mg/mL was used to prepare a standard curve. The concentration of phenolic compounds in the extracts was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract.

3.3.5.2 Total flavonoid content (TFC)

The total flavonoid content was determined using the aluminium trichloride colorimetric assay (Liu et al., 2002). The reaction is based on aluminium trichloride forming acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Moreover, it also forms acid labile complexes with the ortho-dihydroxyl groups in the A or B ring of flavonoids (Kiranmai, Kumar, and Ibrahim, 2011). Briefly, 250 µL of samples were diluted with 1.25 mL of distilled water (DI water). Then 75 µL of 5% $NaNO_2$ solution were added to the mixture. After 6 min, 150 µL of a 10% $AlCl_3 \cdot 6H_2O$ solution were added and the mixture was allowed to stand for another 5 min. One half mL of 1 M NaOH were added, and the total was brought up to 2.5 mL with DI water. The solution was thoroughly mixed, and the absorbance was

measured immediately against the prepared blank at 510 nm using a spectrophotometer. A catechin standard solution (0.05-0.4 mg/mL) was used to prepare a standard curve. The concentration of flavonoids in the extracts was expressed as milligrams of catechin equivalent (CE) per gram of dry extract.

3.3.6 Determination of antioxidant properties

3.3.6.1 DPPH assay

One of the most frequently used techniques for antiradical capacity measurements is DPPH free radical scavenging assay. DPPH assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 515 nm. When the stable proton free radical DPPH encounters proton radical scavengers, its purple color fades rapidly (Figure 3.7) (Ndhlala, Moyo, and Staden, 2010).

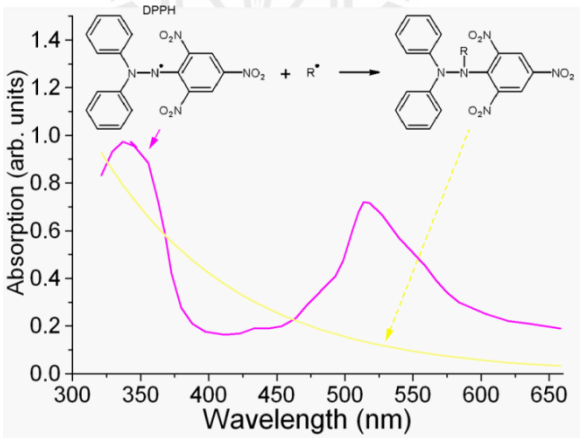

Figure 3.7 The mechanism of DPPH radical interact with antioxidant compound. R= antioxidant compound, DPPH= 2,2-diphenyl-1-picrylhydrazyl (Wikipedia, www, 2012).

This method was determined as described by Sánchez-Moreno, Larrauri, and Saura-Calixto (1999). Briefly, 100 µL of the extracts at different concentrations were added to 3.9 mL of DPPH methanolic solution (63 mM). The mixture was shaken vigorously and left to stand at RT for 45 min in the dark. The mixture was

measured spectrophotometrically at 515 nm. The free radical scavenging activity was calculated as shown below. The IC_{50} of $DPPH^{\cdot}$ was determined from a dose response curve using linear regression analysis. Decreasing DPPH solution absorption indicates an increase of DPPH radical scavenging activity.

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{control}-A_{sample}}{A_{control}} \times 100$$

Where A_{sample} = absorbance of different concentrations of sample extracts

$A_{control}$ = The absorbance of control

3.3.6.2 FRAP assay

The ferric reducing ability of the extracts was measured colorimetrically according to the method developed by Benzie and Strain (1996). The principle of this method is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the present of antioxidants. Briefly, the FRAP reagent included 0.1 M acetate buffer (pH 3.6), 10 mM 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing acetate buffer, TPTZ solution, and $FeCl_3 \cdot 6H_2O$ solution (10:1:1, v/v/v). The FRAP reagent (3 mL) was added to 0.1 mL of the extract and mixed. Readings were recorded on the spectrophotometer at 593 nm, and the reaction was monitored for 10 min. A vitamin C standard solution (10-90 µg/mL) was used to perform the calibration curve. The ferric reducing ability of the extracts was expressed as milligrams of vitamin C equivalent antioxidant capacity (VCEAC) per gram of dry extract.

3.3.6.3 Cellular antioxidant activity (CAA)

Though currently, there are many *in vitro* chemistry models for antioxidant evaluation; however, most of these assays have certain limitations. ABTS and

FRAP assays are not conducted under physiological condition, DPPH radical does not exist in human body (Pérez-Jiménez et al., 2008; Alamed, Chaiyasit, McClements, and Decker, 2009). For these reasons, the results from *in vitro* chemical antioxidant assays might not necessarily correlate with biological responses. Therefore, antioxidant activity of a compound should also be tested in a cellular system for a step closer to *in vivo* result. The popular cell-based model use 2',7'-dichlorofluorescin-diacetate (DCFH-DA) as the probe to reflex ROSs and oxidative stress in cells. When the nonfluorescent DCFH-DA is uptaken into cells, its diacetate moiety will be hydrolyzed by cellular esterases to generate the more polar DCFH which is trapped inside the cells. In the presence of ROSs, intracellular DCFH is oxidized to form the fluorescence DCF product (Wolfe and Liu, 2007) as illustrated in Figure 3.8.

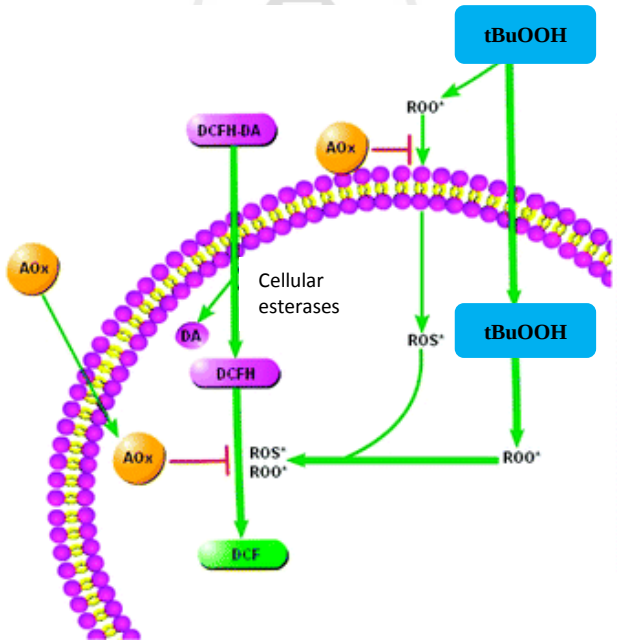

Figure 3.8 The proposed principle of the cellular antioxidant activity (CAA) assay. Cells were pretreated with antioxidant compounds or plant extract (AOx) prior exposed to DCFH-DA. The oxidant initiator (tBuOOH) can generate peroxyl radicals (ROO˙) which can oxidize the intracellular DCFH to the fluorescent DCF. AOx antioxidant prevent oxidation of DCFH, of membrane lipids and reduce the formation of DCF (adapted from Wolfe and Liu, 2007).

Intracellular oxidative stress was detected using the 2',7'-dichlorofluorescin-diacetate as described by Kim, Kwon, and Jang (2011) with slight modification. Briefly, RAW264.7 cells ($4x10^4$ cells/well) were plated in a Costa^{TM} 96-well black clear bottom plate (Corning Inc., Corning, NY) and incubated for 16-18 h at 37 °C and 5% CO_2. After incubation, cells were washed with PBS twice. To assess antioxidant activity, cells were pre-exposed to different concentrations of 95EE-FLP or WE-FLP (50, 150, or 250 μg/mL), or the antioxidant positive controls, catechin (250 μM), resveratrol (20 μM), or quercetin (10 μM), for 24 h. After washing twice with PBS, cells were exposed to 20 μM DCFH-DA in HBSS and further incubated in the dark for another 30 min. The DCFH-DA was removed by washing the cells with PBS twice, 500 μM tBuOOH were added. The intensity of the fluorescence signal was detected time-dependently with an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

3.3.7 Statistical analysis

All statistical significances (GraphPad Prism 5, USA) were determined by performing a one-way analysis of variance (ANOVA) with a *post-hoc* Turkey' s analysis to determine differences between treatments. Values were considered statistically significant when $p<0.05$. Data were presented as mean ± SEM (n=3). The data from CAA were statistically analyzed by two-way analysis of variance followed by Bonferonni's *post-hoc test* with $p<0.05$ as statistical significant. Data of CAA experiment were presented as mean ± SEM (n=4).

3.4 Results

3.4.1 Percent yield of extracts from PP leaves

The percentage yields of crude extracts from fresh and dried leaves of PP are shown in Table 3.1. It shows that the percentage yield of different extracts from PP leaves

ranged widely from 3.17 to 84.80%. In addition, high percentage yield was found in the water extracts (WE-FLP and WE-DLP).

Table 3.1 The percentage yields of crude extracts from fresh and dried leaves of PP.

Extracts	Amount and source of preparation	Yield (g)	% yield
95EE-FLP	1500 g of fresh leaves	60.41	4.03
80EE-FLP	2100 g of fresh leaves	66.65	3.17
80EE-DLP	300 g of dried leaves	46.99	15.66
WE-FLP	40 g of 95EE-FLP	32.71	81.77
WE-DLP	20 g of 95EE-DLP	16.96	84.80

95EE-FLP, 95% ethanol extract of fresh leaves of PP
80EE-FLP, 80% ethanol extract of fresh leaves of PP
80EE-DLP, 80% ethanol extract of dried leaves of PP
WE-FLP, Water extract of fresh leaves of PP
WE-DLP, Water extract of dried leaves of PP

3.4.2 Phytochemical compounds of extracts from PP leaves

3.4.2.1 Total phenolic contents (TPC)

Various extracts of PP leaves were used to investigate the best sources of antioxidant properties. Initial studies were conducted to investigate and determine the total phenolic contents (TPC) of extracts of PP leaves. The content of total phenolic content was expressed as mg gallic acid equivalent per gram of dry extract (mg GAE/g dry extract). TPC of 95EE-FLP, 80EE-FLP, 80EE-DLP, WE-FLP, and WE-DLP are shown in Figure 3.9. WE-FLP had a significantly higher level (p<0.001) of TPC (212.47 ± 0.52 mg GAE/g dry extract) than 95EE-FLP (200.14 ± 0.77 mg GAE/g dry extract) and 80EE-FLP (201.32 ± 0.72 mg GAE/g dry extract), followed by WE-DLP (177.56 ± 0.43 mg GAE/g dry extract), and 80EE-DLP (98.63 ± 0.26 mg GAE/g dry extract), respectively. There was no significant difference in the level of total phenolic

content between 95EE-FLP and 80EE-FLP. This study also demonstrated that WE-FLP had the highest total phenolic content, while 80EE-DLP had the lowest value. These results suggested that fresh leaves, rather than dried leaves, provide more extractable phenolic compounds.

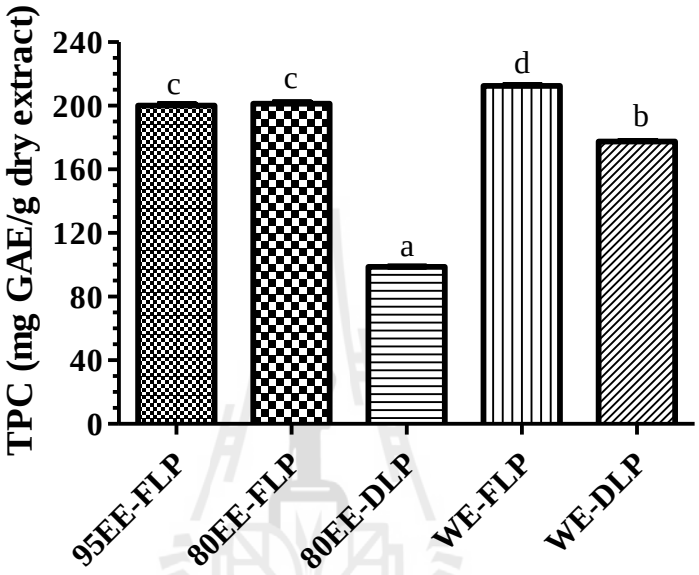

Figure 3.9 Total phenolic contents of extracts of PP leaves. The contents were expressed as mg of GAE/g dry extract. Values are mean ± SEM (n=3) of representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $p<0.001$.

3.4.2.2 Total flavonoid content (TFC)

The content of flavonoid was expressed as mg catechin equivalent per gram of dry extract (mg CE/g dry extract). Total flavonoid contents of 95EE-FLP, 80EE-FLP, 80EE-DLP, WE-FLP, and WE-DLP are shown in Figure 3.10. WE-FLP had a significantly higher level ($p<0.05$) of TFC (133.43 ± 11.27 mg CE/g dry extract) than 95EE-FLP (100.19 ± 0.68 mg CE/g dry extract) and 80EE-FLP (99.80 ± 1.02 mg CE/g dry extract), followed by WE-DLP (68.95 ± 0.42 mg CE/g dry extract), and 80EE-DLP

(30.73 ± 0.30 mg CE/g dry extract), respectively. Both 95EE-FLP and 80EE-FLP did not have significantly different level of total flavonoid. Collectively, high levels of flavonoid were found in extracts of fresh leaves of PP (95EE-FLP, 80EE-FLP, and WE-FLP), while low levels of flavonoid were found in extracts of dried leaves of PP (80EE-DLP and WE-DLP).

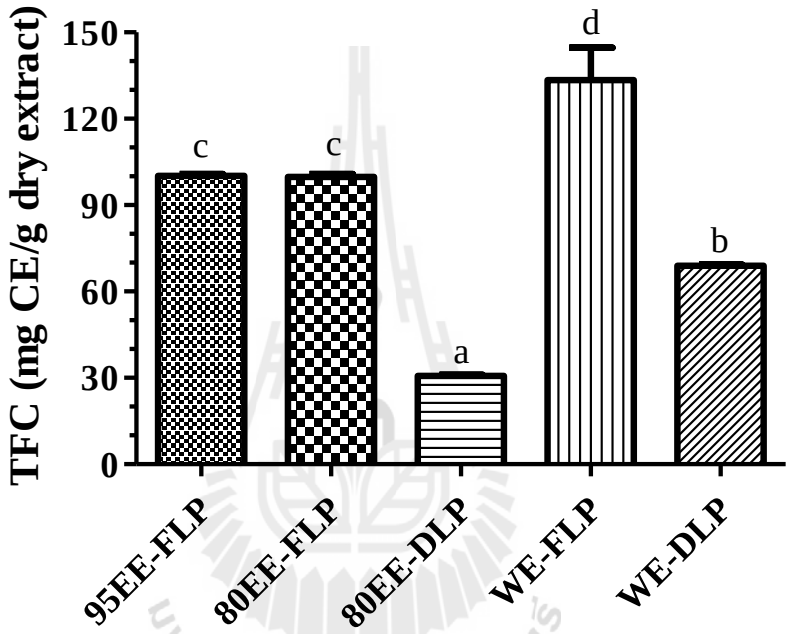

Figure 3.10 Total flavonoid contents of extracts of PP leaves. The contents were expressed as mg of CE/g dry extract. Values are mean ± SEM (n=3) of representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $p<0.05$.

3.4.3 Antioxidant properties of extracts from PP leaves

3.4.3.1 DPPH free radical scavenging activity

DPPH assay is one of the most extensively used methods to evaluate *in vitro* antioxidant activity. The free radical scavenging capacity of extracts of PP leaves are shown in Figure 3.11. The result suggested that all extracts exhibited the capability to scavenge DPPH free radical. The highest scavenging activity against DPPH radicals was

found in WE-FLP (IC_{50}= 21.55 ± 0.06 µg/mL), followed by 95EE-FLP (IC_{50}= 23.45 ± 0.12 µg/mL), 80EE-FLP (IC_{50}= 25.35 ± 0.03 µg/mL), WE-DLP (IC_{50}= 31.08 ± 0.52 µg/mL), and 80EE-DLP (IC_{50}= 64.38 ± 0.52 µg/mL), respectively, at p<0.01. This study also exhibited that high level of scavenging activities against DPPH radicals was found in extracts of fresh leaves of PP, while low level of them were found in extracts of dried leaves of PP. However, the scavenging capacity of all extracts of PP leaves was not as effective as that of other positive antioxidant controls. In the present study, the scavenging ability of vitamin C (IC_{50}= 3.94 ± 0.01 µg/mL) and catechin (IC_{50}= 3.55 ± 0.01 µg/mL) was comparable, and was significantly higher than trolox (IC_{50}= 5.90 ± 0.27 µg/mL), at p<0.001.

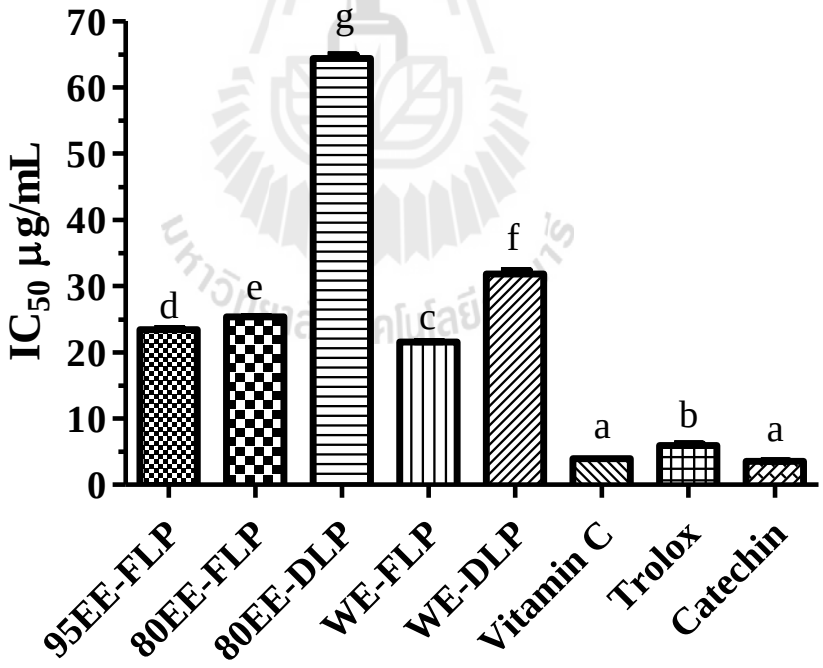

Figure 3.11 Comparison of DPPH radical scavenging activity of extracts of PP leaves with various antioxidants controls. Values are mean ± SEM (n=3) of representative of three independent experiments with similar results. Bars marked with different letters are significantly different at p<0.01.

3.4.3.2 Ferric reducing antioxidant power

The reducing power of extracts of PP leaves, expressed as milligram vitamin C equivalent antioxidant capacity per gram dry extract (mg VCEAC/g of dry extract), are shown in Figure 3.12. The highest FRAP value was found in 95EE-FLP (213.23 ± 1.09 mg VCEAC/g dry extract) and 80EE-FLP (216.73 ± 0.44 mg VCEAC/g dry extract), followed by WE-FLP (193.40 ± 2.65 mg VCEAC/g dry extract), WE-DLP (168.40 ± 2.63 mg VCEAC/g dry extract), and 80EE-DLP (91.53 ± 0.44 mg VCEAC/g dry extract), respectively, at $p<0.001$. Degrees of electron donating capacity of 95EE-FLP and 80EE-FLP were comparable. This study also showed that high level of FRAP values were found in extracts of fresh leaves of PP, while low level of them were found in extracts of dried leaves of PP.

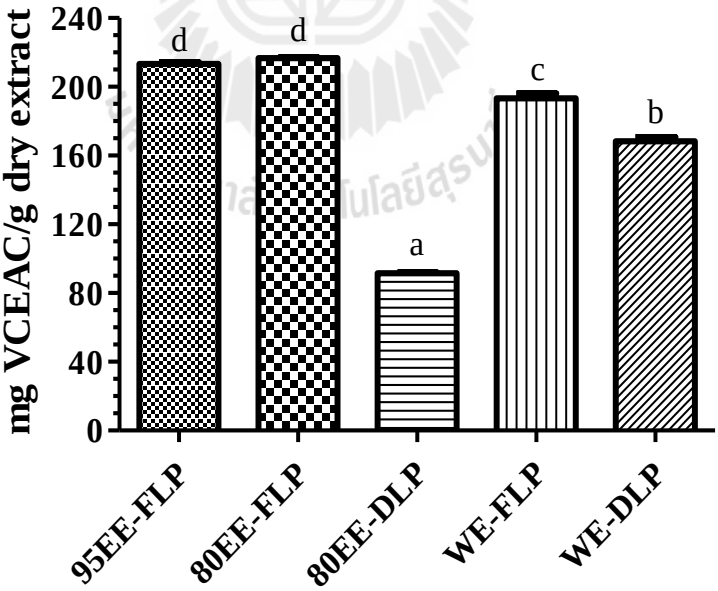

Figure 3.12 Total antioxidant (FRAP) activity of extracts of PP leaves. The activity was expressed as mg of VCEAC/g dry extract. Values are mean ± SEM (n=3) of representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $p<0.001$.

3.4.3.3 Cellular antioxidant activity (CAA) of extracts from PP leaves

The direct scavenging effect of extracts of PP leaves on intracellular free radical stress was investigated in RAW264.7 cells using the DCFH-DA assay. The increment of DCF fluorescence emission following ROSs-mediated oxidation of DCFH was following for 240 min. As shown in Figure 3.13A, standard antioxidants positive controls, catechin (250 μM), resveratrol (20 μM), and quercetin (10 μM), could scavenge ROSs significantly throughout the incubation time when compared to the vehicle control (VH) (p<0.05). As early as 30 min of incubation, catechin, resveratrol, and quercetin showed a considerable radical scavenging activity. 95EE-FLP (Figure 3.13B) and WE-FLP (Figure 3.13C) decreased the DCF fluorescent emission in a dose-and time-dependent manner. As early as 30 min of incubation, both 95EE-FLP and WE-FLP at lower concentrations (50 μg/mL) showed a comparable radical scavenging activity to the antioxidant controls. Increasing concentrations of 95EE-FLP significantly decreased the DCF fluorescent emission throughout the incubation time when compared to the VH control (p<0.05). At high concentration (150 μg/mL), 95EE-FLP exhibited a strong scavenging activity as suggested by the capability to reduce the fluorescent intensity to approximately basal level of unstimulated control or naïve (NA). In addition, the highest concentration of 95EE-FLP (250 μg/mL) significantly decreased DCF fluorescent intensity to the level of less than basal unstimulated condition at all time points (p<0.05). Similarly, 150 and 250 μg/mL of WE-FLP significantly decreased the DCF fluorescent emission throughout the incubation time when compared to tBuOOH control (p<0.05). At the lowest concentration (50 μg/mL), WE-FLP significantly decreased the DCF fluorescent emission at time points from 30 to 210 min (p<0.05).

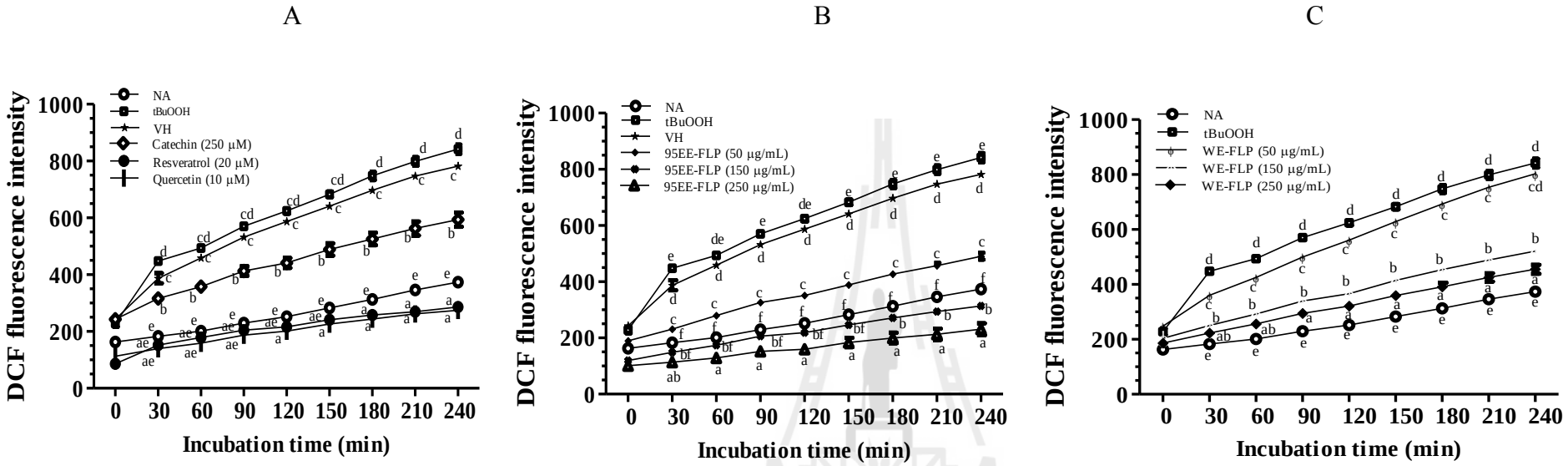

Figure 3.13 Time course of cellular radical scavenging activity in tBuOOH-activated RAW264.7 cells. Intracellular ROSs level generated in cells was measured by the 2',7'-dichlorofluorescin-diacetate (DCFH-DA). RAW264.7 cells were pretreated with indicated concentrations of antioxidants (A), 95EE-FLP (B) or WE-FLP (C) for 24 h prior allowed to take up 20 µM DCFH-DA for 30 min. Results are mean ± SEM (n=4) of representative of three independent experiments with similar results. Points marked with different letters are significantly different at $p<0.05$ when compared at the same time point as determined by two-way ANOVA.

3.5 Discussion and conclusion

It is well known that the major components of plant leaf extracts that act as antioxidants are phytochemicals such as phenolic compounds and flavonoids. Phan, Ha, and Phan (2003) found flavonoids in ethyl acetate, chloroform and *n*-butanol-soluble fractions of PP leaves. Moreover, flavonoids from PP leaves displayed antioxidant activity (Phan, Ha, and Phan, 2005). In addition, Nguyen and Eun (2011) reported that phenolics and flavonoids were found in PP leaf extracts when assessed with Folin-Ciocalteu and aluminum trichloride. PP leaf extracts also had antioxidant activities when evaluated with DPPH and FRAP assays. Similarly, the present study also suggested that flavonoids and phenolics could exhibit antioxidant activities in all extracts from PP leaves. Several methods have been performed and developed to measure the total antioxidant activity of natural medicinal plants *in vitro*. The antioxidant capacity of medicinal plant extracts cannot be investigated by using only one single method, due to the complex composition of phytochemicals and oxidative process (Böhm, Schlesier, Harwat, and Bitsch, 2001). The use of at least two methods should be employed in order to evaluate the total antioxidant activity. The secondary metabolites in plants, vitamin C, trolox, catechin, resveratrol, and quercetin were used as positive antioxidant controls in the present study as they are the most frequently used antioxidant standards for food samples. DPPH assay is one of the most extensively used methods to evaluate antioxidant activity. The present study revealed that DPPH radical scavenging capacity of WE-FLP was greater than that of 95EE-FLP, 80EE-FLP, WE-DLP, and 80EE-DLP, respectively as shown in Figure 3.11. In contrast, 95EE-FLP and 80EE-FLP had higher ferric reducing power than that of WE-FLP, WE-DLP, and 80EE-DLP, respectively as shown in Figure 3.12. These contradictory results between DPPH and FRAP assays are not unusual. Though both assays are based on a single electron transfer reaction (Bunea et al., 2011), their characteristics, sensitivities, mechanisms of the reaction, and endpoints are totally different. For instance, DPPH

method is based on the free radical scavenging activity while FRAP measures the reduction ability of Fe^{3+} to Fe^{2+}. Depending on what specific phytochemical constituents present in the extract are providing the antioxidant activity, their discrete chemical structures, positions, numbers, and types of substitutions of features can influence their redox properties and hence their antioxidant potentials. The structure antioxidant activity relationships of flavonoids have been studied, for example, multiple hydroxyl groups of flavan nucleus of flavonoid favor antioxidant and chelating activity, methoxy group has unfavorable steric effects and increase lipophilicity, a double bond and carbonyl group in the herterocycle of the nuclear structure offer a more stable radical and hence increase the activity (Heim, Tagliaferro, and Bobilya, 2002). Phenolics may have hydroxyl or methoxyl groups; while flavones possess hydroxyl, keto, and free carboxylic groups; and flavonoids have flavan nucleus with different types of substitutions. Typically, all of these compounds can provide different reducing properties.

The present study indicated that WE-DLP had higher levels of phenolic and flavonoid contents including free radical scavenging capacity and ferric reducing ability than those of 80EE-DLP. Notably, both WE-DLP and 80EE-DLP had lower levels of phenolic and flavonoid contents, including antioxidant activities by DPPH and FRAP assay, than those of 95EE-FLP, 80EE-FLP, and WE-FLP. This study also suggested that high levels of TPC and TFC as well as antioxidant properties of various extracts of PP leaves were found in fresh leaves (95EE-FLP, 80EE-FLP, and WE-FLP), while low level of them were found in dried leaves (80EE-DLP and WE-DLP). The profile of phytochemicals, both TPC and TFC, and antioxidant properties of extracts of PP leaves between fresh leaves and dried leaves was remarkable. It is possible that extracts of dried leaves of PP exhibited a decrease in TPC and TFC resulting in less antioxidant capacity. Nguyen and Eun (2011) found high levels of phenolic and flavonoid contents as well as antioxidant activities of extracts of dried leaves of PP by DPPH and FRAP assay when macerated with different solvents

(methanol, acetone, ethanol, and water). Using similar investigation methods, Nguyen and Eun (2011) also found comparable levels of TPC, TFC, free radical scavenging, and ferric reducing capability of 80% ethanol extract of PP dried leaves. Therefore, regardless of their geographic differences, leaves of PP collected from Thailand and Vietnam share similar antioxidant properties.

Though both DPPH and FRAP assays are frequently used methods, they have some drawbacks for antioxidant activity measurement. In DPPH assay, other compounds may have high absorbance or interference at the same wavelength at 515 nm. In addition, DPPH radical is absent in living organisms. The drawbacks of the FRAP method are that the compounds with low redox potential (lower than 0.77) still can reduce the Fe^{3+} even though they do not behave as antioxidants *in vivo*. Interfering compounds may also absorb at the same wavelength, and this assay is also performed at a non-physiological pH (Pérez-Jiménez et al., 2008). Therefore, the present study also determined the scavenging activity in the cell-based assay using intracellular fluorescent probe DCFH-DA.

Among various extracts of PP leaves, WE-FLP had the highest levels of TPC and TFC, and DPPH free radical scavenging activity, while 95EE-FLP and 80EE-FLP had the highest FRAP value. In addition, there seemed to be no differences in TPC and TFC levels as well as antioxidant activity of 95EE-FLP and 80EE-FLP. Therefore, both 95EE-FLP and WE-FLP were selected for subsequent evaluation of cellular antioxidant activity. The macrophage is usually the cell of choice in studying ROSs-mediated cellular events since they could generate high amount of ROSs following stimulation. The present study demonstrated that all antioxidant standards, 250 μM catechin, 20 μM resveratrol, and 10 μM quercetin, exerted a strong inhibition of ROSs generation induced by tBuOOH over a period of 30 to 240 min. In addition to extracellular antioxidant capacity, 95EE-FLP and WE-FLP also possessed intracellular antioxidant activity in scavenging ROSs and decreasing the oxidation of DCFH (Figure 3.13B and C). Both 95EE-FLP and WE-FLP

exhibited comparable efficiency of scavenging ROSs formation to the antioxidants standards. Notably, the highest concentrations (250 µg/mL) of 95EE-FLP reduced DCFH oxidation better than those of WE-FLP. Several studies showed that crude water and crude ethanol extracts of plant leaves contained phenolic compounds that act as antioxidants (Aiyegoro and Okoh, 2009, 2010; Shi, Xu, Hu, Na, and Wang, 2011; Shukla, Mehta, Mehta, and Bajpai, 2011). In addition, polyphenols are known to support bioactive activities in medicinal plants. Therefore, the antioxidant activity of PP might be attributed to the presence of high contents of phenolic and flavonoid in both ethanol and water crude extracts of PP.

In summary, all extracts from PP leaves had phenolic and flavonoid compounds and displayed antioxidant properties by chemical antioxidant assays (DPPH and FRAP). High levels of TPC and TFC and antioxidant activities were found in extracts from fresh leaves of PP. However, there were no significantly different levels of phenolic and flavonoid as well as ferric reducing antioxidant ability between 95EE-FLP and 80EE-FLP. The antioxidant activity in cell-based study revealed that both 95EE-FLP and WE-FLP exhibited high potential to prevent ROS-induced oxidative stress in RAW264.7 cells, and 95EE-FLP exerted more pronounced effects than that of WE-FLP.

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CHAPTER IV

CYTOTOXICITY AND SUPPRESSION OF NO, iNOS AND COX-2 EXPRESSION BY EXTRACTS FROM LEAVES OF *PSEUDERANTHEMUM PALATIFERUM*

4.1 Abstract

Pseuderanthemum palatiferum (PP), a well-known Vietnamese traditional medicinal plant in Thailand, has long been used in folk medicine for curing inflammatory diseases. This study aimed to determine cytotoxicity and anti-inflammatory activities of 95% ethanol extract of fresh leaves of PP (95EE-FLP), and water extract of fresh leaves of PP (WE-FLP). In an initial study, the cell viability of RAW264.7 cells exposed to various concentrations of 95EE-FLP (0.05, 0.25, 0.50, 1.00, or 1.50 mg/mL) or WE-FLP (0.10, 0.50, 1.00, 1.50, or 4.50 mg/mL) for 24 h were assessed by MTT, trypan blue methods, and propidium iodide staining. Both 95EE-FLP and WE-FLP displayed low toxicity towards RAW264.7 cells as evidenced by no apparent effect on cell viability up to the concentration of 1.5 mg/mL of each extract was used as assessed by MTT and trypan blue methods. However, cytotoxicity of RAW264.7 cells as assessed by PI staining showed that 95EE-FLP at concentrations ranging from 0.05-0.25 mg/mL do not alter the viability of RAW264.7 cells. Similarly, WE-FLP at a concentration range from 0.1-0.5 mg/mL did not alter the viability of RAW264.7 cells. Therefore, both 95EE-FLP and WE-FLP at the concentration range of 0-0.25 mg/mL were selected for RAW264.7 cell treatment in anti-

inflammatory test. Anti-inflammatory effects of 95EE-FLP and WE-FLP were evaluated on LPS plus IFN-γ-stimulated RAW264.7 macrophage cells. Various concentrations (50, 100, 150, 200, or 250 µg/mL) of 95EE-FLP and WE-FLP significantly suppressed LPS plus IFN-γ-induced nitric oxide (NO) production. However, 95EE-FLP more potently suppressed LPS plus IFN-γ-induced NO production when compared to WE-FLP. Both 95EE-FLP and WE-FLP also suppressed LPS plus IFN-γ-induced protein production of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Collectively, these results suggested that both 95EE-FLP and WE-FLP at the concentration range of 0-0.25 mg/mL did not cause cell death in RAW264.7 cells and their certain concentrations also displayed the anti-inflammatory properties by suppressing the production of NO, iNOS, and COX-2 proteins. Therefore, the findings of this study could provide the rational for future studies on the mechanism of anti-inflammatory properties of extracts from PP leaves.

4.2 Introduction

Macrophages play an important role in inflammatory diseases through the release of proinflammatory cytokines and other proinflammatory molecules involved in the immune response such as nitric oxide (NO) and prostaglandins (PGE_2) (MacMicking, Xie, and Nathan, 1997; Harris, Padilla, Koumas, Ray, and Phipps, 2002). NO is a short lived free radical gas produced by the conversion of L-arginine to citrulline, catalyzed by nitric oxide synthase (NOS). Under normal physiological functions, NO production is regulated by the constitutive isoform of NOS (cNOS). At nanomolecular levels, NO plays important roles in vasodilation, neuron communication, and host defense. However, under pathological conditions, NO level is increased by the inducible isoform of nitric oxide synthase (iNOS) leading to regulation of almost all stages of inflammation development, in particular, the early stages of inflammatory cell transmigration to the sites of inflammation (MacMicking et al., 1997; Bernstein, Bogerts, and Keilhoff, 2005; Korde (Choudhari), Sridharan,

Gadbail, and Poornima, 2012). Among a variety of inflammatory mediators, PGE_2 is also of central importance in the regulation of inflammation. PGE_2 is produced from arachidonic acid by prostaglandin synthase or cyclooxygenase (COX) enzymes. COX exists in two isoforms: COX-1 and COX-2. COX-1 is constitutively expressed, and is a housekeeping enzyme required for normal physiological functions, whereas COX-2 is only induced during inflammatory processes (Donnelly and Hawkey, 1997; Harris et al., 2002). Many reports have demonstrated that when NO was up-regulated in inflammatory cells, iNOS and COX-2 expression increased in parallel (Lin, Juan, Shen, Hsu, and Chen, 2003; Park et al., 2005; Kim et al., 2006). Therefore, reduction of the levels of NO, iNOS, and COX-2 expression could be an effective strategy for suppressing inflammation.

Plants have been used medicinally all over the world for many centuries. In Thailand, as well as in other parts of the world, traditional healers have prescribed herbal preparations for the treatment of different types of diseases. *Pseuderanthemum palatiferum* (PP) leaves have long been used for health improvement and treatment for a variety of diseases especially inflammatory diseases associated with wound healing, general trauma, colitis, and nephritis (Dieu, Loc, Yamasaki, and Hirata, 2005). Therefore, the objective of the present study was to assess the anti-inflammatory effect of extracts from PP leaves, a common herbal medicine used in Thailand, on LPS and IFN-γ-activated RAW264.7 macrophage cells. The effect of extracts from PP leaves on cell viability of RAW264.7 cells was predetermined in order to identify the optimum concentration range of the extract for RAW264.7 cell treatment.

4.3 Materials and methods

4.3.1 Chemicals and reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Invitrogen Molecular Probes (Eugene, OR).

Vitamin C was purchased from Fluka Chemine GmbH (Buchs, Switzerland). Penicillin G, streptomycin sulfate, N-(1-Naphthyl) ethylenediamine dihydrochloride, sodium nitrite, and LPS (*Escherichia coli* O111:B4), were purchased from Sigma-Aldrich (St. Louis, MO). RPMI medium 1640 was obtained from Gibco Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, Utah). 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (trolox) was purchased from Sigma-Aldrich Chemine Gmbh (Steinheim, Germany). Mouse interferon gamma (mIFN-γ) was purchased from Piearce Protein Research Products (Rockford, IL).

4.3.2 Cell lines

The mouse macrophage RAW264.7 cell line was used to investigate the effects of 95% ethanol extract of fresh leaves of PP (95EE-FLP), and water extract of fresh leaves of PP (WE-FLP) on *in vitro* cytotoxic and anti-inflammatory studies. Cells were cultured as previously described in Chapter III: Cell lines.

4.3.3 Plant materials

Fresh leaves of PP were purchased from producers in Yasothon province, Thailand. The plant was identified and authenticated by Dr. Kongkanda Chayamarit, the Forest Herbarium, Royal Forest Department, Bangkok, Thailand. A voucher specimen (BKF 174009) was deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

4.3.4 Plant extract preparation

95EE-FLP and WE-FLP were prepared as previously described in Chapter III: Plant extract preparation. 95EE-FLP was dissolved in DMSO and then diluted to 0.25% (v/v) DMSO in cell culture medium when prepared at the indicated concentrations. Another extract, WE-FLP, was dissolved in PBS when used in experiments.

4.3.5 Cytotoxicity

The effect of 95EE-FLP and WE-FLP on RAW264.7 cell viability was evaluated by using different methods. For MTT and trypan blue exclusion methods, cells were plated at a density of $5x10^4$ cells/well in a 96-well plate and incubated overnight. For the other methods, PI staining, cells were plated at a density of $2x10^6$ cells/well in a 6-well plate and incubated overnight. Cells were then exposed to various concentrations of 95EE-FLP or WE-FLP at 37 ℃ under 5% CO_2 for 24 h.

4.3.5.1 MTT method

MTT is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tratrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria. Then, mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring yielding purple MTT formazan crystals (dark purple colure) which are insoluble in aqueous solutions (Figure 4.1). The crystal formazan product can be dissolved in acidified isopropanol or DMSO and measured spectrophotometrically. An increase in cell number results in increasing amount of formazan product which in turn, is proportional to the absorbance at 540 nm. As reduction of MTT can only occur in metabolically active cells, the level of MTT formazan formed could be a measurement of cell viability (Byun et al., 2008).

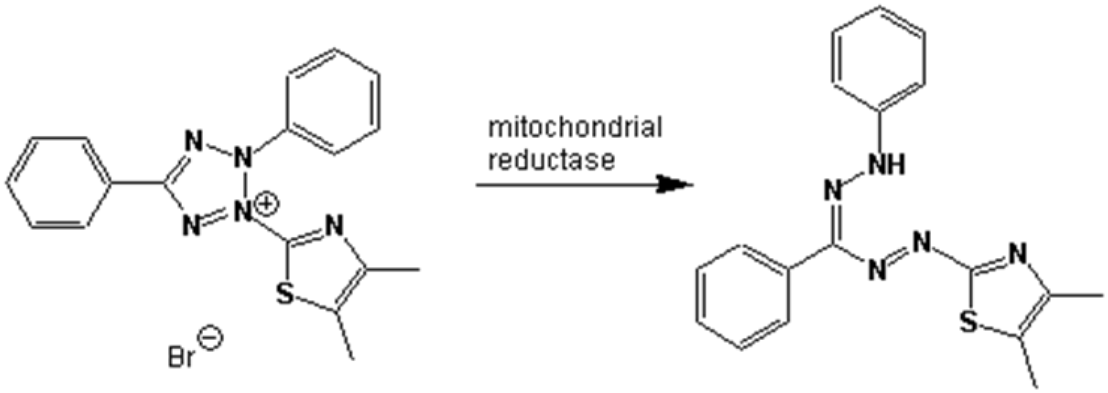

Figure 4.1 A yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tratrazolium bromide (MTT) is reduced to purple formazan product by mitochondrial reductase in living cells (Wikipedia, www, 2012).

Cell viability was determined with MTT assay as described by Chun et al. (2007). Briefly, MTT (0.5 mg/mL) dye solution was added in each well and incubated at 37 °C, 5% CO_2 for 4 h. The medium was removed and DMSO was added into each well to dissolve insoluble formazan crystals giving a uniform dark purple color before reading OD at 540 nm by Benchmark Plus Microplate Spectrophotometer System (Bio-Rad Laboratories, Inc., Hercules, CA). The percentage of cell viability was calculated by the following equation :

$$\text{Percent cell viability} = \frac{\text{Average OD for test group}}{\text{Average OD for control group}} \times 100$$

4.3.5.2 Trypan blue exclusion method

The trypan blue exclusion test is a rapid, simple, and inexpensive method to assess cell viability. Trypan blue is a diazo dye that has been widely used to color dead tissues or cells selectively. The reactivity of trypan blue is based on it being negatively charged and not interacting with cells unless the membrane is damaged. Undamaged cells are very selective concerning the compounds that pass through their

membrane, and thus should not take up trypan blue. Therefore, all the cells that exclude the dye are considered viable. In contrast, cells with damaged membranes are stained in a distinctive blue color readily observed under a microscope (Longo-Sorbello, Saydam, Banerjee, and Berniti, 2006).

Cell viability was determined with trypan blue exclusion method. Briefly, after incubation with 95EE-FLP and WE-FLP, cells were washed three times with PBS. Then cells were harvested by trypsinization and stained with 0.4% (w/v) trypan blue. A small amount of this diluted sample was transferred to one or both chambers of the haemocytometer and these chambers were filled by capillary action. The 10X objective lens was used to focus on the grid lines of the chamber. The viable cells (exclude trypan blue) and non-viable cells (stained blue) were counted in the four 1 mm^2 corner squares in one chamber. Cells that lie on the lines should only be counted if they are touching the top and left hand lines of each corner squares. The percentage of cell viability was calculated by the following equation:

$$\text{Percent cell viability} = \frac{\text{Total cells - Dead cells}}{\text{Total cells}} \times 100$$

4.3.5.3 Propidium iodide staining

One method to assess cell viability is propidium iodide staining. Propidium iodide (PI) is a membrane impermeant dye that is generally excluded from viable cells. PI cannot pass through intact cell membranes, but may freely enter cells with compromised cell membranes. Upon entering a non-viable cell, these dyes bind to the double stranded DNA by intercalating between base pairs.

Cell viability was determined with PI staining as described by Düchler and Stepnik (2008) with slight modifications. Briefly, after incubation with 95EE-FLP or WE-FLP, cells were washed three times with PBS, trypsinized, and transferred to tube. Then, PBS was removed, and cells were stained with 100 μL of PI

solution (1 mg/mL stock solution diluted to 1:200) for 10 min in the dark. After staining, cells were washed in PBS and analyzed by flow cytometry (BD FACSCalibur flow cytometer, BD, San Diego, CA).

4.3.6 Nitrite assay

The Griess test is a chemical analysis test which detects the presence of organic nitrite compounds. The Griess reaction was first described in 1879 and this assay has also been used extensively in analysis of numerous biological samples including cell culture media. In this assay, nitrite is first treated with a diazotizing reagent, sulfanilamide (SA) in acidic media to form a transient diazonium salt. This intermediate is then allowed to treat with a coupling reagent, naphthylethylenediamine dihydrochloride (NED) to form a stable azo compound. This overall reaction is described in the Figure 4.2. The absorbance of purple color of the product at 540 nm is in linear proportion to the nitrite concentration in the sample (Sun, Zhang, Broderick, and Fein, 2003).

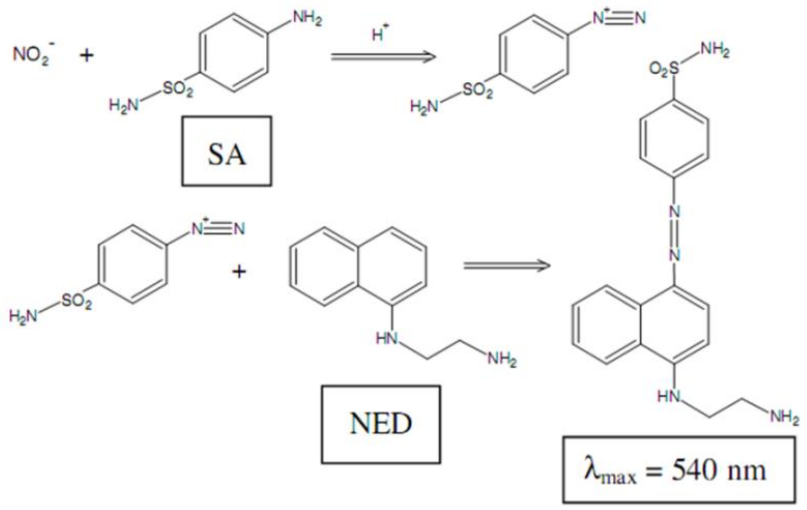

Figure 4.2 The overall of Griess reaction. Nitrite is detected by formation of purple color upon treatment of NO_2-containing sample with Greiss reagent. When SA in acidic media is added, the nitrites form a diazodium salt. When azo dye agent (NED) is added, a purple color of the product develops (Sun et al., 2003).

Therefore, the level of NO in the culture media was detected as nitrite, a major stable product of NO, and was determined using Griess reagent (de Oliveira et al., 2012). Briefly, RAW264.7 cells were seeded at a density of $2x10^5$ cells/well in a 96-well plate. Cells were grown for 3 h to allow plate attachment prior treating with antioxidant positive control vitamin C (500 µM) or various concentrations (50, 100, 150, 200, or 250 µg/mL) of 95EE-FLP or WE-FLP. After 1 h incubation, RAW264.7 cells were stimulated with 1 µg/mL plus 25 U/mL IFN-γ. The activated cells were further incubated for 24 h. Then 100 µL of supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 3% phosphoric acid). After 10 min of dark incubation, the absorbance of samples was measured at 540 nm using a microplate spectrophotometer. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was derived from a standard curve of sodium nitrite.

4.3.7 Western blot analysis

4.3.7.1 Preparation for protein lysate

RAW264.7 cells were plated at a density of $2x10^6$ cells/well in a 6-well plate. After an attachment period of approximately 3 h, cells were treated with various concentrations (50, 100, 150, 200, or 250 µg/mL) of 95EE-FLP or WE-FLP for 1 h. 50 µg/mL trolox and 500 µM vitamin C were used as antioxidant positive controls. Cells were then stimulated with 1 µg/mL LPS plus 25 U/mL IFN-γ for 18 h. After incubation, cells were washed three times with PBS and placed in 150 µL of ice-cold lysis buffer (1 mL RIPA buffer supplemented with 2 mM PMSF, 2 µM leupeptin, and 1 µM E-64) for 20 min. Then the disrupted cells were transferred to microcentrifuge tubes and centrifuged at 14,000 x *g* at 4 °C for 30 min. The supernatant was collected and kept in -80 °C for further analysis. The aliquot part was used for protein determination.

4.3.7.2 Determination of protein concentration

The protein concentration was measured according to the Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951). The protein lysate was diluted in DI water (1:10). Twenty microliters of diluted protein lysate was mixed with 200 μL of reagent D in a 96-well plate. After 10 min, 20 μL of reagent C were added to 96-well plates. The reaction was incubated at RT for 30 min and the absorbance at 750 nm was measured. Bovine serum albumin (BSA) was used to prepare a standard curve for protein determination.

4.3.7.3 SDS-PAGE gel electrophoresis and Western blot

Denaturing polyacrylamide gel was performed according to the method of Laemmli (1970) with some modification. Cell lysate was boiled for 5 min in 6X sample buffer (50 mM Tris-base, pH 7.4, 4% SDS, 10% glycerol, 4% 2-mercaptoethanol, and 0.05 mg/mL of bromophenol blue). Thirty micrograms of cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% and 10% polyacrylamide gels for determination the expression of iNOS and COX-2, respectively (125 volts, 120 min). The proteins in the gel were transferred onto nitrocellulose membrane (AMersham, Pittsburgh, PA) at 80 volts for 1 h. The membrane was blocked overnight at 4 °C with 5% nonfat milk in 0.1% Tween-20 in PBS-buffer (TPBS). Membranes were then incubated with 1:1,000 dilution of primary antibody anti-iNOS mouse monoclonal (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or 1:2,000 dilution of primary antibody anti-COX-2 mouse polyclonal (Cayman Chemical, Ann Arbor, MI) at RT for 2 h. After extensive washing with TPBS, the membranes were incubated with a 1:10,000 dilution of the secondary antibody goat-anti-mouse-HRP conjugate (Santa Cruz) for iNOS and goat-anti-rabbit IgG-HRP conjugate (Cayman) for COX-2 at RT for 1 h. To control the equal loading of total protein in all lanes, the same blots were also stained with primary antibody anti-tubulin mouse monoclonal (Santa Cruz) at a dilution of

1:2000 at RT for 2 h. After washing, the membranes were incubated with a 1:10,000 dilution of the secondary antibody goat-anti-mouse-HRP conjugate (Santa Cruz). Membranes were washed three times with TPBS, 10 min per wash. Blots were incubated for 3 min in ECL Western Blotting Substrate (Piearce Protein Research Products) and exposed to film. The relative expression of proteins was quantified densitometrically using the ImageJ software and calculated according the reference band of tubulin.

4.3.8 Statistical analysis

All parameters statistical significance (GraphPad Prism 5, USA) were determined by performing a one-way analysis of variance (ANOVA) with a *post-hoc* Turkey' s analysis to determine differences between treatment and control groups. Values were considered statistically significant when $p<0.05$. Data were presented as mean ± SEM (n=3). PI staining and western blotting analysis experiments were repeated at least twice.

4.4 Results

4.4.1 Effect of extracts from PP leaves on RAW264.7 cell viability

The cell viability of RAW264.7 cells exposed to 95EE-FLP and WE-FLP was determined by MTT (Figure 4.3A and B), trypan blue exclusion assay (Figure 4.3C and D), and PI staining (Figure 4.4A and B). Cells were incubated for 24 h with various concentrations of 95EE-FLP (0.05, 0.25, 0.50, 1.00, or 1.50 mg/mL) or WE-FLP (0.10, 0.50, 1.50, or 4.50 mg/mL). As shown in Figure 4.3, both 95EE-FLP (Figure 4.3A and C) and WE-FLP (Figure 4.3B and D) displayed low toxicity towards RAW264.7 cells as evidenced by no apparent effect of each extract on cell viability only observed when the treatment concentration reached 1.5 mg/mL, as assessed by MTT (Figure 4.3A and B) and trypan blue exclusion methods (Figure 4.3C and D). At 1.5 mg/mL, 95EE-FLP (Figure 4.3A) and WE-FLP (Figure 4.3B) decreased the viability of RAW264.7 cell by 34.14 ± 9.69% ($p<0.05$) and 21.58 ± 1.66% ($p<0.05$), respectively. However, the cytotoxic effect

was more pronounced at higher concentration, WE-FLP at 4.50 mg/mL decreased the cell viability to 54.21 ± 1.74% (p<0.05). The suppression of cell viability by 37.56 ± 14.37%, as assessed by trypan blue, (Figure 4.3C) was only observed in the highest treatment group of 1.50 mg/mL 95EE-FLP (p<0.05). The cell viability of RAW264.7 (Figure 4.3D) decreased by 8.16 ± 1.79% (p<0.05) and 65.32 ± 0.38% (p<0.05) at 1.50 and 4.50 mg/mL WE-FLP, respectively.

Increasing concentrations of 95EE-FLP and WE-FLP also exhibited low toxicity towards RAW264.7 cell as evidenced by PI staining. As shown in Figure 4.4A, 95EE-FLP at concentrations ranging from 0.05 to 0.25 mg/mL did not alter the viability of RAW264.7 cells. However, at higher concentrations, 95EE-FLP at 0.50, 1.00, and 1.50 mg/mL decreased the viability of RAW264.7 cell by 8.57%, 20.57% and 32.89%, respectively. As shown in Figure 4.4B, WE-FLP at concentration ranging 0.1-0.5 mg/mL did not alter the viability of RAW264.7 cells. At 1.50 mg/mL, WE-FLP decreased the viability of RAW264.7 cells by 21.64%. However, the cytotoxic effect was more pronounced at higher concentrations, WE-FLP at 4.50 mg/mL decreased the cell viability to 60.6%. Therefore, a non-toxic concentration range of 0-0.25 mg/mL of both 95EE-FLP and WE-FLP was selected for RAW264.7 cell treatment in the subsequent studies.

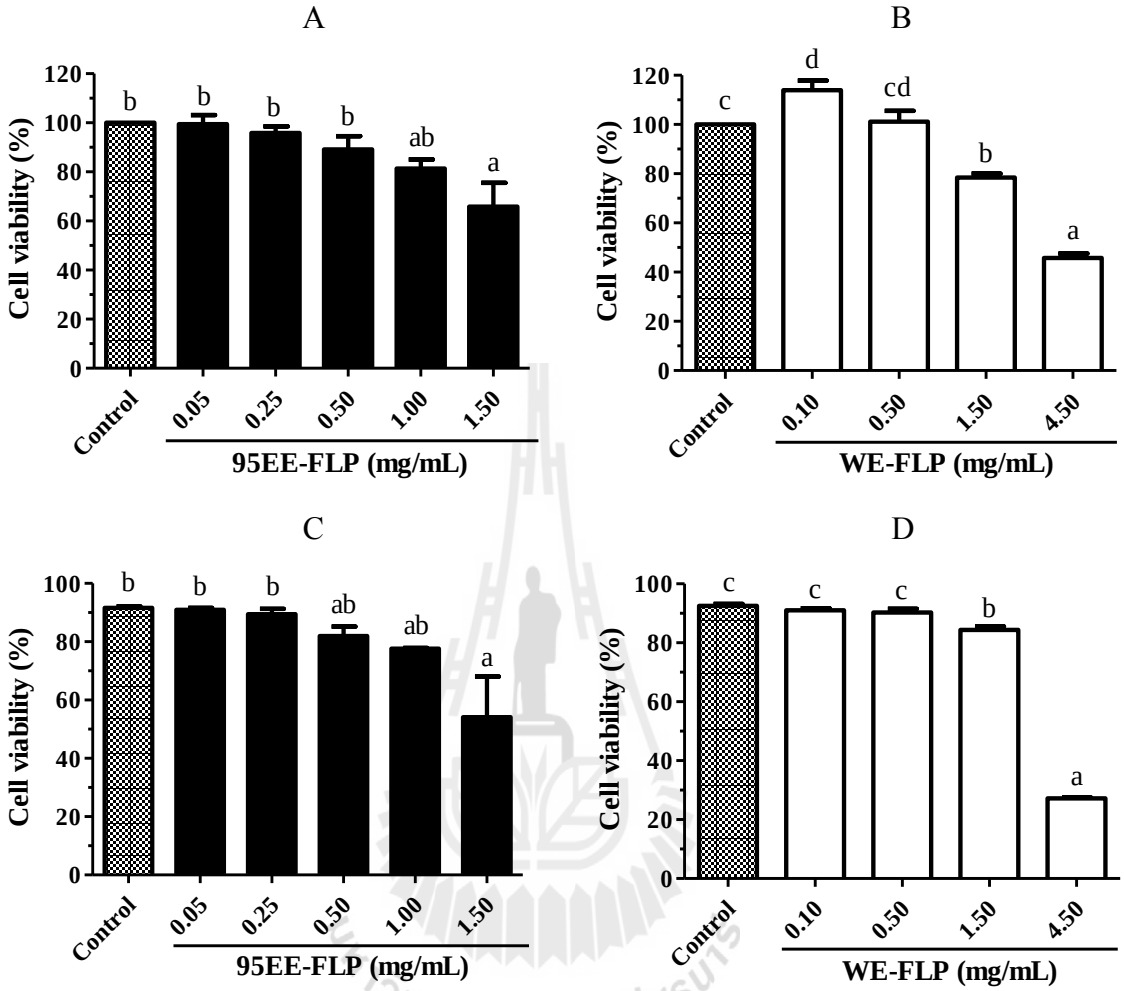

Figure 4.3 Effects of 95EE-FLP (A and C) and WE-FLP (B and D) on cell viability of RAW264.7 cells were assessed by MTT (A and B) and trypan blue (C and D). Values were expressed as mean ± SEM of representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $p<0.05$ as determined by one-way ANOVA.

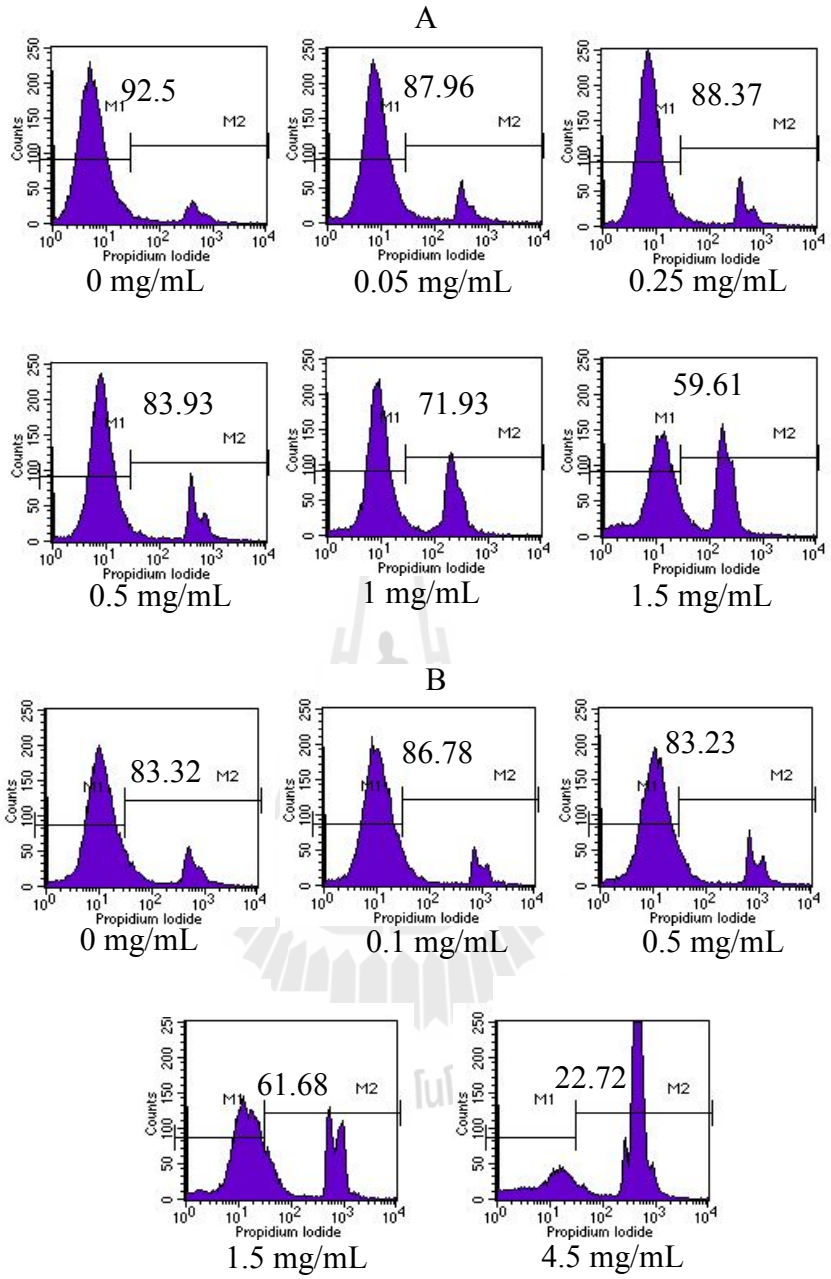

Figure 4.4 Effects of 95EE-FLP (A) and WE-FLP (B) on cell viability of RAW264.7 cells were assessed by PI staining. Data were expressed as the percentage of viable cells and are representative of at least two independent experiments.

4.4.2 Suppression of NO product by extracts of PP leaves in LPS plus IFN-*γ*-activated RAW264.7 cells

RAW264.7 cells were pretreated with antioxidant, vitamin C, 95EE-FLP or WE-FLP for 1 h, then stimulated with LPS plus IFN-*γ*, and measured for NO production

using the Griess assay. As shown in Figure 4.5, Naïve RAW264.7 cells (NA) secreted basal levels of NO, while the production of NO was increased to about 43 μM in LPS-activated RAW264.7 cells. The antioxidant control, 500 μM vitamin C, decreased the NO production by almost 35%. Pretreated RAW264.7 cells with 95EE-FLP or WE-FLP significantly suppressed (p<0.05) the induction of NO production in a dose-dependent manner (Figure. 4.5A and B), and the suppression was observed in all 95EE-FLP- and WE-FLP-treated groups. These results also clearly indicated that 95EE-FLP was more potent in suppressing NO production than WE-FLP. The concentrations of 50 μg/mL 95EE-FLP, and 150 and 200 μg/mL WE-FLP were required to exhibit about the same efficiency as 500 μM vitamin C in NO suppression.

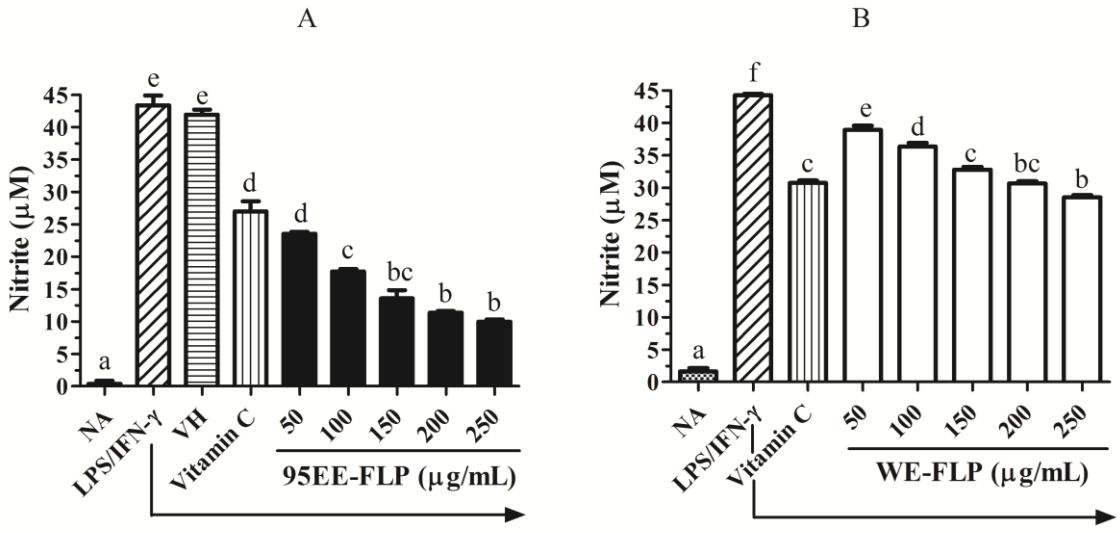

Figure 4.5 95EE-FLP (A) and WE-FLP (B) suppressed LPS plus IFN-γ-induced nitrite production in RAW264.7 cells. RAW264.7 cells were incubated for 24 h with LPS (1 μg/mL) plus IFN-γ (25 U/mL) in the presence or absence of indicated concentrations of vitamin C (500 μM), 95EE-FLP or WE-FLP. Accumulated nitrite in the culture medium was determined by the Griess reaction. The values are means ± SEM (n=3) of representative of three independent experiments with a similar results. Bars marked with different letters are significantly different at p<0.05 as determined by one-way ANOVA.

4.4.3 Suppression of iNOS and COX-2 protein expressions by extracts of PP leaves in LPS plus IFN-γ-activated RAW264.7 cells

To determine whether suppression of NO production by 95EE-FLP and WE-FLP was related to changes in iNOS as well as COX-2 protein levels, Western blotting analysis was performed. RAW264.7 cells were pretreated with antioxidants, trolox (50 μg/mL) or vitamin C (500 μM), and PP extracts, 95EE-FLP or WE-FLP, at 50 to 250 μg/mL for 1 h prior subsequently activated with LPS (1 μg/mL) plus IFN-γ (25 U/mL) for 18 h. Total proteins were extracted and analyzed by Western blotting for the expressions of iNOS and COX-2. LPS plus IFN-γ-induced increases in iNOS (Figure 4.6A and B) and COX-2 (Figure 4.6C and D) expression compared to unstimulated naïve culture. Anti-oxidant controls (trolox and vitamin C) decreased LPS plus IFN-γ-induced iNOS and COX-2 protein levels. The data also suggested that the suppression of 500 μM vitamin C was more pronounced than 50 μg/mL trolox. Compared to the corresponding controls, both 95EE-FLP and WE-FLP produced a dose-dependent suppression of iNOS level in LPS plus IFN-γ activated RAW264.7 cells (Figure 4.6A and B) suggesting the suppression of NO production by 95EE-FLP and WE-FLP was mediated by decreasing the expression of iNOS. In parallel to the result of NO suppression, 95EE-FLP at 50 to 200 μg/mL was more efficient than WE-FLP in iNOS suppression. The iNOS expression was almost completely abolished at 200 μg/mL 95EE-FLP and was barely observed at 250 μg/mL WE-FLP. The anti-inflammatory activity of 95EE-FLP and WE-FLP was also further supported by the dose-dependent suppression of COX-2 level by both 95EE-FLP and WE-FLP (Figure 4.6C and D) in the activated RAW264.7 cells. Notably, 95EE-FLP and WE-FLP exhibited higher suppressive effect on iNOS than COX-2.

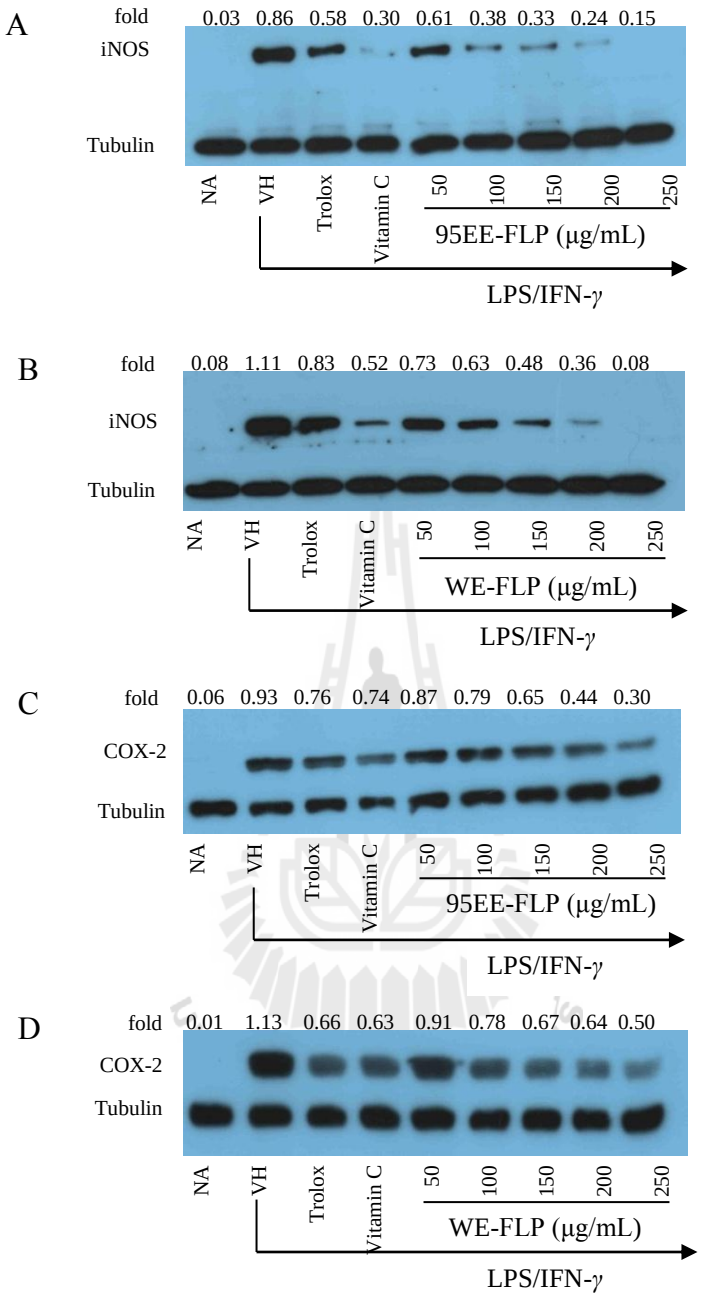

Figure 4.6 Effect of 95EE-FLP on LPS plus IFN-γ-induced iNOS (A) and COX-2 (C) and WE-FLP on LPS plus IFN-γ-induced iNOS (B) and COX-2 (D) protein levels in RAW26.7 cells. The relative expression of proteins was quantified densitometrically using ImageJ software and normalized according to tubulin reference bands. Data are a representative of at least two independent experiments (NA = naïve, VH = vehicle, Trolox = 50 μg/mL, Vitamin C = 500 μM).

4.5 Discussion and conclusion

Although PP leaves have long been used in folk medicine for curing inflammatory diseases, their anti-inflammatory effect has not been reported until now. To determine anti-inflammatory effects of extracts from PP leaves using RAW264.7 *in vitro* model, RAW264.7 cell line toxicity test was conducted to investigate concentrations of 95EE-FLP and WE-FLP that cause no detrimental effects on cells or cell viability. Therefore, the number of viable cells was estimated using different methods to ensure that these concentrations do not adversely affect RAW264.7 cells. The number of viable cells showed a similar trend of decreased viability after RAW264.7 cell treatment for 24 h with 95EE-FLP or WE-FLP as assessed by MTT, trypan blue, and PI staining. Moreover, this study suggested that the concentration range of 0-0.25 mg/mL both of 95EE-FLP and WE-FLP does not affect cell viability. Therefore, these concentrations were selected for RAW264.7 cell treatment in the anti-inflammatory test.

Inflammatory disorders are characterized, among other events, by the production of significant amounts of free radicals, nitrogen reactive species as well as proinflammatory cytokines (Martin et al., 1997). Therefore, this study aimed to investigate the anti-inflammatory effects of extracts from PP leaves on the suppression of NO production in LPS plus IFN-γ-activated RAW264.7 cells. Both 95EE-FLP and WE-FLP at concentrations ranging from 50-250 µg/mL showed concentration-dependent suppression of NO production, and the suppression was also more pronounced in 95EE-FLP than WE-FLP (Figure 4.5). These results are consistent with the observation that 95EE-FLP was also a better scavenger of intracellular ROS than WE-FLP (Chapter III). To rule out the possibility that NO suppression by 95EE-FLP and WE-FLP was due to the direct scavenging effect on NO alone, the effect of 95EE-FLP and WE-FLP on the suppression of iNOS, the inducible isoform of nitric oxide synthase, was also investigated. The result clearly indicated that the mechanism of NO suppression by 95EE-FLP and WE-FLP was

mediated through the suppression of iNOS expression (Figure 4.6A and B). In agreement with the findings of NO suppression, the suppressive effect of 95EE-FLP (50-200 µg/mL) on iNOS was more remarkable than WE-FLP. Excess ROSs have been implicated in causing extensive oxidative damage to cells leading to tissue damage, cell death or degenerative processes, including aspects of cancer, atherosclerosis, diabetes, aging and other chronic inflammatory diseases (Valko, Rhodes, Moncol, Izakovic, and Mazur, 2006; Fang, Seki, and Maeda, 2009). High NO concentrations can combine with superoxide to form peroxynitrite ion ($OONO^-$) which is responsible for cell and tissue damages in inflammation (Valko et al., 2006). The present result strongly suggested that 95EE-FLP and WE-FLP could play an important role in attenuating cell damage through suppression of NO production by macrophages in response to inflammatory stimuli of LPS plus IFN-γ. Increased level of COX-2 expression has been detected in various tumor types, and may account for the excessive production of inflammatory PGH_2 (Subbaramaiah and Dannenberg, 2003). This study showed that COX-2 production induced by LPS plus IFN-γ-activated RAW264.7 cells was suppressed by increasing concentrations of both 95EE-FLP and WE-FLP (Figure 4.6C and D). Therefore, 95EE-FLP and WE-FLP can also exhibit anti-inflammatory effects by suppression of high COX-2 protein level. It is well known that there is a link between the overproduction of NO, iNOS, and COX-2 in response to inflammatory stimuli. Many inflammatory conditions are associated with production of large amounts of NO (Liu and Hotchkiss, 1995) in parallel with increased expression of inducible enzymes of both iNOS and COX-2 (Dannhardt and Kiefer, 2001; Calixto, Otuki, and Santos, 2003). Therefore, extracts of PP leaves might exert their anti-inflammatory effects by decreasing NO production, iNOS and COX-2 protein expression in LPS plus IFN-γ-stimulated RAW264.7 cells.

Several natural compounds derived from plants have been demonstrated to directly suppress the expression of NO, iNOS and COX-2. For example, phenolic compounds from

the roots of *Ulmus macroparpa* (Kwon, Kim, Park, and Lee, 2011), certain flavonols (Wang et al., 2006), and arctigenin (Kou, Qi, Dai, Luo, and Yin, 2011) displayed antioxidant activity and directly suppressed the expression of NO, iNOS, and/or COX-2. PP as part of the Acanthaceae plant family like *Andrographis paniculata* (AP) and the extract of AP displayed a wide spectrum of bioactive including anti-inflammatory. For example, ethyl acetate extract (EtOAc) from AP suppressed NO production in LPS plus IFN-γ-stimulated RAW264.7 macrophages (Chao, Kuo, Hsieh, and Lin, 2011). A mixture of β-sitosterol and stigmasterol isolated from EtOAc fraction of AP also significantly decreased NO production in LPS plus IFN-γ-stimulated RAW264.7 cells (Chao, Kuo, and Lin, 2010). Andrograpanin, isolated from AP also suppresed NO production in LPS-activated macrophage cells (Liu, Wang, and Ge, 2008). In addition, neoandrographolind, one of the bioactive components isolated from AP, suppressed the production of NO, iNOS, and COX-2 proteins in LPS-stimulated murine macrophages (Liu, Wang, Ji, and Ge, 2007). Thus, NO, iNOS, and COX-2 expression were suppressed by AP, mirroring the suppression by PP in LPS plus IFN-γ-activated RAW264.7 macrophages. Liang and coworkers demonstrated that pretreatement of RAW264.7 cells with flavonoids such as apigenin, genistein, and kaempherol suppressed LPS-induced expressions of NO secretion, and iNOS and COX-2 protein productions (Liang et al., 1999). Major chemical constituents of PP leaves consist of β-sitosterol, stigmasterol, kaempferol 3-methyl ether 7-O-β-glucoside and apigenin 7-O-β-glucoside (Phan, Ha, and Phan, 2003) including flavonoids (Nguyen and Eun, 2011). These chemical constituents have been shown to possess anti-inflammatory properties. Both kaempferol 3-methyl ether 7-O-β-glucoside and apigenin-7-O-β-glucoside may be metabolized into kaempferol and apigenin which also have antioxidant and anti-inflammatory activities. Thus, considering their long historic use in folk medicine and their chemical composition, PP leaves have a high potential for treatment of inflammatory diseases. Results from this study also supported the anti-inflammatory roles of PP leaves.

Whether the regulation of crucial proinflammatory cytokines is the underlying mechanism of anti-inflammation of PP needs further investigation.

In summary, non-cytotoxic concentrations (0-250 µg/mL) of 95EE-FLP and WE-FLP exerted anti-inflammatory effects by suppressing the expressions of NO production, iNOS, and COX-2 protein levels in LPS plus IFN-γ activated RAW264.7 cells. Therefore, 95EE-FLP and WE-FLP could play an anti-inflammatory role in response to endotoxin. In addition, increasing concentrations of 95EE-FLP also decreased NO production and iNOS protein levels in LPS plus IFN-γ-activated RAW264.7 cells better than that of WE-FLP. These novel findings provide addition support for the anti-inflammatory action of PP leaves.

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CHAPTER V

IMMUNE MODULATION OF PROINFLAMMATORY CYTOKINES TNF-α AND IL-6 BY ETHANOL EXTRACT FROM FRESH LEAVES OF *PSEUDERANTHEMUM PALATIFERUM*

5.1 Abstract

Extracts of *Pseuderanthemum palatiferum* (PP) leaves have been reported to possess immune modulatory properties. In the present study, the anti-inflammatory potential and underlying mechanisms of 95% ethanol extract of fresh leaves of PP (95EE-FLP) was investigated using LPS-activated macrophages. Specifically, the suppressive effect of 95EE-FLP was examined on the production of proinflammatory mediators, TNF-α and IL-6. The results showed a concentration-dependent suppression by 95EE-FLP of LPS-induced TNF-α and IL-6 secretion by mouse peritoneal macrophages. 95EE-FLP also suppressed LPS-induced TNF-α and IL-6 protein and mRNA levels in the RAW264.7 murine macrophage cell line. To further elucidate the molecular mechanisms responsible for impaired TNF-α and IL-6 regulation, the activation of the transcription factors, nuclear factor-kappaB (NF-κB), CCAAT/enhancer-binding protein (C/EBP), and activator protein-1 (AP-1), was monitored by the gel mobility shift assay. 95EE-FLP suppressed LPS-induced NF-κB DNA binding activity within both the TNF-α and IL-6 promoters in RAW264.7 cells with impairment being more pronounced in the IL-6 promoter. In

addition, 95EE-FLP exhibited a concentration-dependent suppression of C/EBP, and AP-1 DNA binding activity within the IL-6 promoter. Concordantly, IL-6 luciferase promoter reporter activity was also suppressed by 95EE-FLP in transiently transfected RAW264.7 cells upon LPS activation. Collectively, these results suggest that the anti-inflammatory effects of 95EE-FLP are mediated, at least in part, by modulating TNF-α and IL-6 expression through down regulation of NF-κB, C/EBP, and AP-1 activity.

5.2 Introduction

In both Vietnam and Thailand, *Pseuderanthemum palatiferum* (PP) leaves have been traditionally used for the prevention or treatment of hypertension, diabetes, cancer, as well as inflammation associated with wound healing, general trauma, colitis, and nephritis (Dieu, Loc, Yamasaki, and Hirata, 2006; Padee, Nualkaew, Talubmook, and Sakuljaitrong, 2010). Phan, Ha, and Phan (2003) found that fractional extracts of PP leaves contain several compounds, such as phytosterols, lipids, saponins, and flavonoids. Additionally, flavonoids from PP display antioxidant activity (Phan, Ha, and Phan, 2005). Likewise, previous study have also found that ethanolic extract of PP leaves which contain phenols and flavonoids exhibited potent antioxidant activity as assessed by various *in vitro* models (chapter III). Many investigators have demonstrated that numerous antioxidant medicinal plants also possess anti-inflammatory property (Heras et al., 1998; Sheeja, Shihab, and Kuttan, 2006; Dufour et al., 2007). For example, green tea polyphenols are potent antioxidants and have anti-inflammatory effects. Specifically, these polyphenols can suppress lipopolysaccharide (LPS)-induced proinflammatory tumor necrosis factor alpha (TNF-α) mRNA expression, its protein synthesis, and nuclear factor-kappaB (NF-κB)-binding activation in RAW264.7 cells (Yang, DE Villiers, McClain, and Varilek, 1998).

LPS is an endotoxin that activates a variety of mammalian cells types including macrophages to produce proinflammatory cytokines (Guha and Mackman, 2001). TNF-α

and IL-6 are among the most important cytokines released by activated macrophages. TNF is a pleiotropic and multifunctional proinflammatory cytokine. It can mediate both growth promotion and inhibitory activities in many cell types (Liu and Han, 2001), and exerts a wide spectrum of biological effects, including inflammation, lipid metabolism, coagulation, insulin resistance, and endothelial functions (Founds et al., 2008). TNF-α is a prime mediator involved in many inflammatory mediated diseases, such as septic shock, cancer, multiple sclerosis, diabetes, and rheumatoid arthritis (Vassalli, 1992). Several studies have shown the rapid elevation of TNF-α level with inflammation, which, if specifically blocked either through release or receptor binding, can alleviate severity of inflammatory response (Mohler et al., 1993; Colón et al., 2001; Scallon et al., 2002). Similarly, IL-6 is a cytokine that is also induced by a variety of stimuli, including LPS, and is also a major initiating stimuli of the acute phase response. In addition, IL-6 plays important roles in the immune response during chronic inflammation. Elevated IL-6 levels have also been identified in many inflammatory diseases including rheumatoid arthritis (RA), systemic juvenile idiopathic arthritis, systemic lupus erythematosus, ankylosing spondylitis, psoriasis, and Crohn's disease (Gabay, 2006; Kishimoto, 2010).

LPS induces the expression of numerous genes involved in the inflammatory process by activating several types of transcription factors (Van Miert, 2002). NF-κB is an important transcription factor playing crucial roles in the inflammatory response by regulating the gene expressions of proinflammatory cytokines (e.g., IL-1, IL-2, IL-6, TNF-α, etc), chemokines, adhesion molecules, inducible enzymes (COX-2 and iNOS), growth factors, some acute phase proteins, and immune receptors (Calixto, Otuki, and Santos, 2003). The mouse TNF promoter contains several LPS-inducible NF-κB binding sites (Kuprash et al., 1999). Recently, numerous plant-derived substances have been investigated for their potential to impair NF-κB binding with the intent to identify their therapeutic benefits in treating various inflammatory diseases (Calixto et al., 2003). Also

involved in the regulation of inflammatory responses are CCAAT/enhancer binding proteins (C/EBPs), which are a family of six proteins containing basic leucine Zipper (bzip) motifs. The C/EBPs are critical regulators of cellular differentiation and functions in multiple tissues. Among these proteins, C/EBP β and C/EBP δ are involved in the regulation of gene expression during inflammation (Poli, 1998). A third transcription factor also critically involved in regulating the inflammatory response is activator protein-1 (AP-1). AP-1 consists of either Jun homodimers or Fos/Jun heterodimeric complexes. This transcription factor binds to the TPA DNA response element (TRE) appearing in various mammalian promoters, including those of acute phase proteins and cytokines involved in the inflammatory state (Koj, 1996). Furthermore, NF-κB, C/EBP, and AP-1 are critical for transcriptional regulation of the mouse IL-6 promoter (Baccam, Woo, Vinson, and Bishop, 2003). Mouse IL-6 promoter also contained the same DNA biding sequences for NF-κB and AP-1 as human IL-6 promoter (Allen et al., 2010).

Currently, natural substances derived from plants are of potential interest for therapeutic interventions in the treatment of a variety of inflammatory diseases. To date, the molecular mechanisms responsible for the anti-inflammatory properties of PP leaves ethanolic extract remains unknown. The objective of the present study was to investigate the anti-inflammatory effects of PP by examining its modulation of macrophage-derived TNF-α and IL-6. Here the present study demonstrated for the first time that 95EE-FLP decreases the levels of TNF-α and IL-6 at the protein and mRNA level in LPS-activated macrophage as well as provides a partial mechanism for the observed immunumodulatory activity.

5.3 Materials and methods

5.3.1 Chemicals and reagents

LPS (*Escherichia coli* O111:B4), streptavidin peroxidase, Phenylmethanesulfonyl fluoride (PMSF), Igepal, HEPES, bicinchoninic acid (BCA) solution, and copper (II) sulfate solution, were purchased from Sigma-Aldrich (St. Louis, MO). RPMI medium 1640, Dulbecco's Modified Eagle Medium (DMEM), high glucose, Hank's balanced salt solution (HBSS), penicillin-streptomycin, 0.25% trypsin-EDTA, UltraPure™ Trist, and UltraPure™ TEMED (N, N, N′, N′-tetramethylethylenediamine), were obtained from Gibco Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, Utah). Ammonium persulfate, boric acid, $MgCl_2$, NaCl, and glycerol, were purchased from Baker Analyzed Reagent (Phillipsburg, NJ). Autoradiography film was purchased from Denville Scientific Inc. (Metuchen, NJ). DNA binding sequences of transcription factors, NF-κB, AP-1, and C/EBP, were purchased from Integrated DNA Technologies (Coralville, IA). Bovine serum albumin (BSA) was obtained from Calbiochem (La Jolla, CA). Poly dI-dC was obtained from Roche (Indianapolis, IN).

5.3.2 Cell lines

The mouse macrophage RAW264.7 cell line was used to investigate the effects of 95% ethanol extract of fresh leaves of PP (95EE-FLP) on anti-inflammatory mechanism. Cells were cultured as previously described in Chapter III: Cell lines.

5.3.3 Animals

Pathogen-free female C57BL/6 mice, 5-8 weeks of age, were purchased from Charles River Breeding Laboratories (Portage, MI). On arrival, mice were randomized, transferred to plastic cages containing sawdust bedding (5 animals/cage), and acclimatized for at least 1 week prior experiments. Mice were provided food (Purina Certified Laboratory Chow) and water *ad libitum* and were not used for experimentation until their body weight was 17-20 g. Animal holding rooms were kept at 21-24 °C and 40-60%

relative humidity with a 12-h light/dark cycle. All procedures involving mice were in accordance with the Michigan State University Institutional Animal Care and Use Committee.

5.3.4 Plant material

Fresh leaves of PP were purchased from Yasothon province, Thailand. The plant was identified and authenticated by Dr. Kongkanda Chayamarit, the Forest Herbarium, Royal Forest Department, Bangkok, Thailand. A voucher specimen (BKF 174009) was deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

5.3.5 Plant extract preparation

95EE-FLP was prepared as previously described in Chapter III: Plant extract preparation. 95EE-FLP was dissolved in DMSO, and then diluted to 0.125% (v/v) DMSO in cell culture medium when preparing the indicated concentrations.

5.3.6 Sample preparations for mRNA and ELISA studies

To determine the effects of 95EE-FLP on LPS induced proinflammatory cytokine gene and protein expression from RAW264.7 cells. RAW264.7 cells were seeded in a 6-well culture plate at $2x10^6$ cells/well overnight. After incubation, cells were pretreated with various concentrations of 95EE-FLP (50, 150, or 250 µg/mL) or DMSO vehicle control (0.125%) for 1 h. RAW264.7 cells were activated with 100 ng/mL of LPS for 8 h. The supernatants were then collected by centrifugation at 300 x *g* for 5 min and stored at -80 °C for further analysis of TNF-*α* and IL-6 by ELISA. For RNA isolation, the adherent cells were lysed with 175 µL lysis buffer from SV Total RNA Isolation kit (Promega, Madison, WI) in a 6-well plate and stored at -80 °C until further use.

5.3.7 Isolation and culture of mouse peritoneal macrophages

To determine the effects of 95EE-FLP on LPS induced proinflammatory cytokine production from murine peritoneal macrophages, murine peritoneal macrophages

were obtained as described previously (Kaminski, Roberts, and Guthrie, 1982). In brief, 10 mL of HBSS were injected into the peritoneal cavity of C57BL/6 mice. The abdominal area was gently massaged for 1 min to ensure uniform distribution of free cells in the HBSS and peritoneal cells were harvested by inserting a 16-gauge (B-D, Becton-Dickinson, Franklin Lakes, NJ), 1-inch needle into the lower right quadrant of the peritoneum. A polyethylene tube, i.d. 0.58 mL, o.d. 0.965 mL, (Clay Adam Division Becton Dickinson Co, Parsippany, NJ) was then passed through a 16-gauge needle into the cavity. The end of the tubing inserted into the cavity contained multiple perforations while the other end was attached to a 5 mL syringe (B-D) with a 23-gauge needle (B-D). A 4 mL aliquot of collected peritoneal cell suspension was aspirated into the syringe and immediately transferred to a 50 mL tube on ice. The isolated cells were then washed and resuspended in complete culture medium. Cells were seeded in a 96-well culture plate at $2.5x10^5$ cells/well in a final volume of 200 µL. The cells were cultured at 37 °C for 2 h to allow macrophages to adhere to the plate. Non-adherent cells were removed by washing once with warm HBSS and the adherent cells were cultured in complete RPMI 1640 medium. After an overnight culture at 37 °C, 5% CO_2 in a humidified incubator, culture medium was removed, replaced with fresh medium, and then the adhered peritoneal macrophages were exposed to various concentrations of 95EE-FLP or vehicle control for 1 h before stimulation with 100 ng/mL LPS. After 8 h incubation, the supernatants were isolated and stored at -80 °C for quantification the levels of TNF-α, and IL-6 cytokines.

5.3.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a very popular technique in clinical diagnosis, biological, and medical scientific research. ELISA can be used to detect and quantify the antigens, antibodies, hormones or cytokines presented in the sample. Figure 5.1 shows the principle of an ELISA assay. A specific monoclonal antibody (mAb) against the specific cytokine of interest is coated on a microtiterplate. After adding samples, a second mAb which binds

a different epitope on the cytokine is added. Commonly, the second mAb is labeled with biotin, which allows subsequent binding of a streptavidin-conjugated enzyme. Unbound reagents are washed away in each step. When substrate is added, a color reaction, which is proportional to the amount of cytokine bound, will develop. The concentration of cytokine is determined from a standard curve prepared from the known amount of standard cytokine of interest (Mabtech, www, 2012).

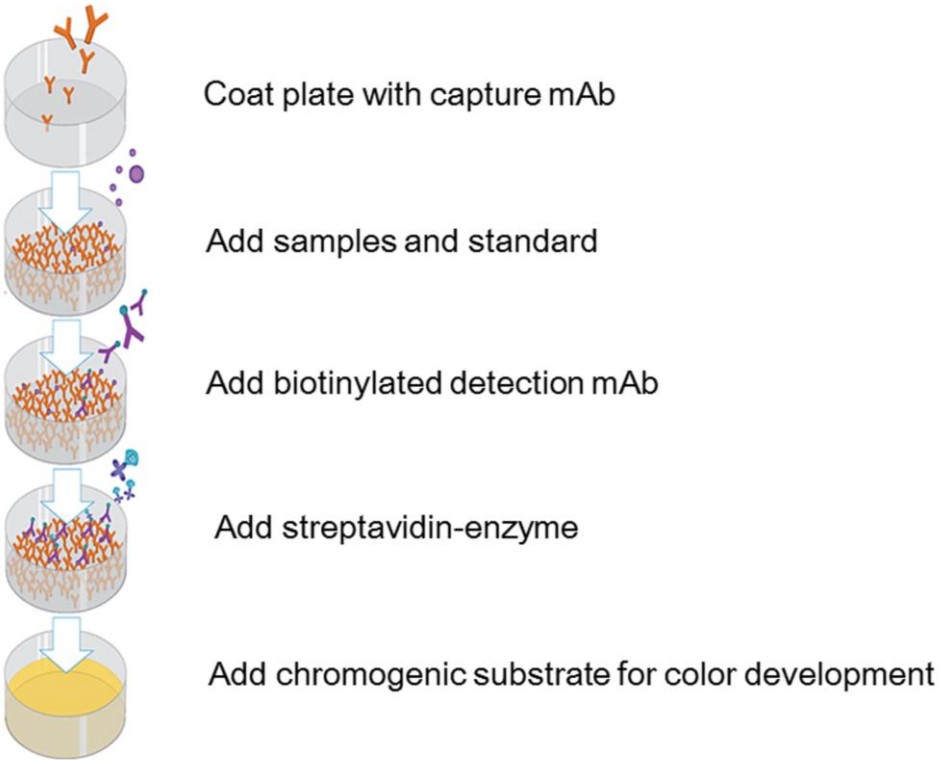

Figure 5.1 ELISA assay procedure. After coating with monoclonal antibody (mAb), unknown sample or standard are added to the plate. Biotinylated detection mAb and streptavidin-enzyme are added, respectively. When substrate is added, a color reaction will be developed (Mabtech, www, 2012).

Cytokine levels were quantified by ELISA as previously described (Condie, Herring, Koh, Lee, and Kaminski, 1996). Briefly, Immunolon strip plates (Thermo, York, PA) were coated with 50 µL/well of 1 µg/mL purified anti-mouse/rat TNF-α (clone#TN3-

19.12, eBioscience, San Diego, CA) or purified anti-mouse IL-6 (clone#MP5-20F3, eBioscience) and incubated overnight at 4 °C. Wells were then washed three times with 250 µL PBST (0.02% Tween 20 in phosphate buffered saline, pH 7.4) to remove excess unbound antibody. The non-specific sites were blocked with 300 µL/well of 3% BSA in PBST for 1 h at room temperature followed by three additional washes. Fifty µL/well of sample or standard, mouse TNF-α recombinant protein (eBioscience) or mouse IL-6 recombinant protein (eBioscience), were added to respective wells. After washing, 1 µg/mL biotinylated anti-mouse/rat TNF-α (Polyclonal Ab, eBioscience) or biotinylated anti-mouse IL-6 (clone#MP5-32C11, eBioscience) diluted in 3% BSA-PBST, was added to the corresponding wells and allowed to incubate at room temperature for 1 h. After washing, 50 µL of streptavidin peroxidase diluted 333.3-fold was added followed by incubation at room temperature for 1 h. After washing, TMB substrate [12.5 mL citric-phosphate buffer pH 5.0 + 200 µL of 3, 3', 5, 5'-tetramethyl-benzidine stock solution (6 mg/mL in DMSO) + 50 µL 1% H_2O_2] was added. The reaction was terminated by the addition of 100 µL 6 N H_2SO_4. Optical density was determined at 450 nm using an EL-808 microplate reader (Bio-Tek Instruments, Winooski, VT).

5.3.9 Quantitative reverse transcription PCR (qRT-PCR)

Real-Time qRT-PCR (Real Time quantitative Reverse Transcrtiption Polymerase Chain Reaction) is a major development of PCR technology that enables reliable detection and quantification of product generated during each cycle using SYBR Green or TaqMan probes for detection. TaqMan Gene Expression Assay reaction steps are shown in Figure 5.2. At the start of the RT-PCR reaction, the temperature is increased to denature the double stranded cDNA. The signal from the fluorescent dye on the 5′ end of TaqMan probe is quenched by the non-fluorescent quencher on the 3′ end of the probe. The reaction temperature is decreased to allow the primers and probe to anneal to their specific target sequences. In the next step, Taq polymerase synthesizes a complementary

DNA strand using the unlabeled primers and template as a guide. When the polymerase reaches the TaqMan probe, its endogenouse 5′ nuclease activity cleaves the probe, separating the dye from the quencher. When more dye molecules are released in each cycle of PCR, these accumulated dye molecules lead to increased fluorescence intensity, which in turn, is proportional to the synthesis amount of amplicons (NCBI, www, 2012).

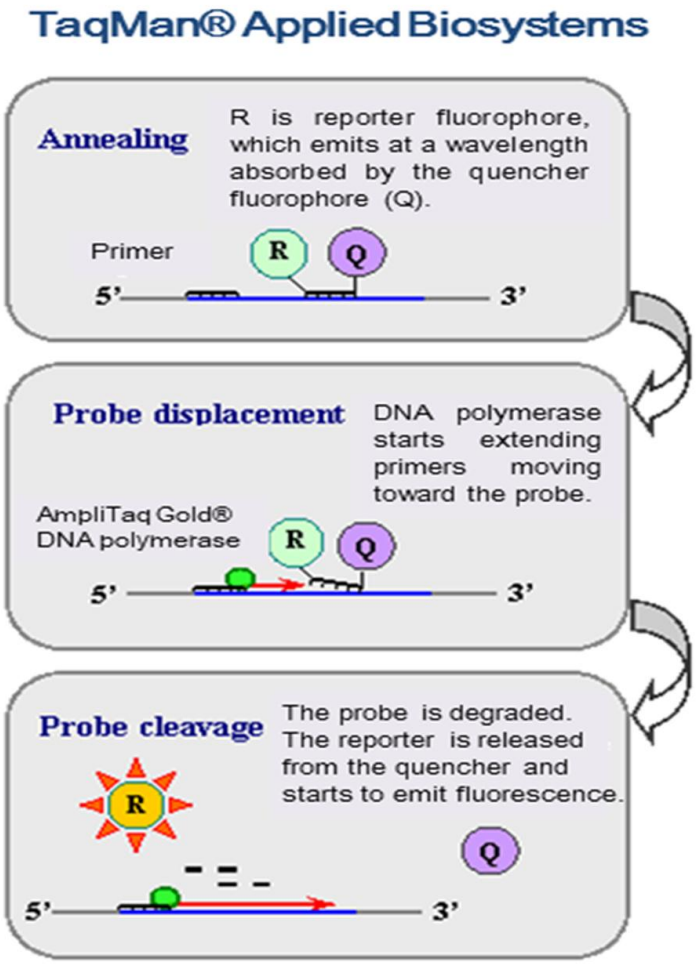

Figure 5.2 TaqMan gene expression assay reaction steps (NCBI, www, 2012).

Total RNA was isolated from RAW264.7 cells using SV Total RNA Isolation kits (Promega) according to the manufacturer's instructions. The isolated RNA was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). qRT-PCR was performed on an Applied Biosystems 7900 PRISM Thermocycler (ABI, Foster City, CA) using a Taqman assay system. Briefly, total RNA (250 ng) in a total reaction volume of 25 µL was reverse transcribed using High Capacity cDNA Reverse Transcription kit (AB Applied Biosystems) as described by the manufacturer's instructions. Two µL of the generated cDNA were used as a template in a 20 µL PCR reaction containing 1 µL of the target gene primers (TNF-*α*, and IL-6 AB Applied Biosystems), 1 µL of endogenous reference primers (18S ribosomal RNA, Applied Biosystem), 10 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), and 6 µL of water to bring final volume to 20 µL. The amount of target gene mRNA was normalized to 18S ribosomal RNA and relative to the calibrator was calculated by using the formula $2^{-\Delta\Delta CT}$ and was expressed as fold change compared to naïve control.

5.3.10 Electrophoretic mobility shift assay (EMSA)

The electrophoretic mobility shift assay (EMSA) is a powerful, widely used method that tests the capacity of purified proteins or proteins in cell extracts to bind selectively to specific double stranded DNA sequences. Electrophoretic separation of a protein-DNA mixture on a polyacrylamide gel was indicated in Figure 5.3. If specific protein-DNA adducts form, they will migrate more slowly than an unbound radioactive probe and are thus visualized by discrete bands of radioactivity near the top of an acrylamide gel image (Kothinti, Tabatabai, and Petering, 2011).

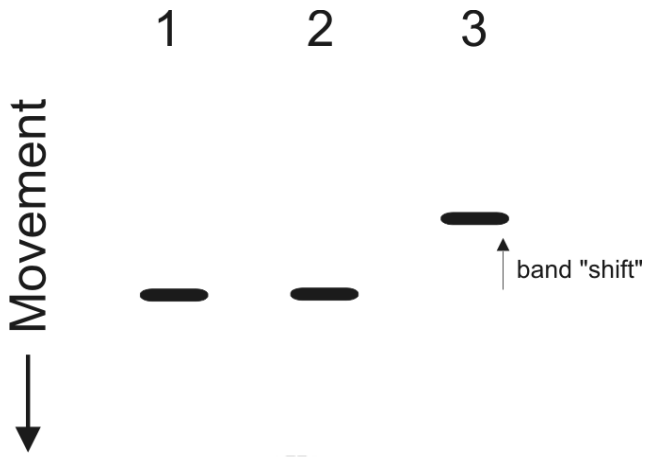

Figure 5.3 Electrophoretic separation of a protein-DNA mixture on a polyacrylamide gel for a short period of time. Lane 1 is a negative control containing only DNA. Lane 2 contains protein as well as a DNA fragment that, based on its sequence, does not interact. Lane 3 contains protein and a DNA fragment that does react. The resulting protein-DNA complex is larger, heavier, and slower-moving than the unbound probe, and these appear as a shifted up band in the autoradiographic film (Molecular station, www, 2008).

5.3.10.1 Preparation for protein lysate

Nuclear extracts from RAW264.7 cells were prepared as described previously (Condie et al., 1996). Briefly, cells were seeded at $1x10^7$ cells/mL in a 150 mm^2 petri dish and cultured for 48 h. Culture medium was removed at the end of 48 h and replaced with fresh media. Cells were pretreated with 95EE-FLP (50, 150, or 250 µg/mL) or vehicle for 1 h and then stimulated with 100 ng/mL of LPS for 30 min (for NF-κB in mTNF-*α*, NF-κB in mIL-6 and AP-1 in mIL-6 promoters) or 1 h (for C/EBP in mIL-6 promoter). Cells were then isolated by scraping, resuspended in fresh media, transferred to 50 mL tubes, and centrifuged at 300 x *g* for 5 min. Cell pellets were resuspended in cold 1X PBS, centrifuged at 300 x *g* for 5 min, and resuspended in 500 µL of hypotonic buffer (10 mM HEPES, and 1.5 mM $MgCl_2$) supplemented with 1 mM dithiothreitol, 0.1 mM

phenylmethylsulfonyl, 0.2 µg/mL aprotinin, and 0.2 µg/mL leupeptin, and transferred to a 1.5 mL eppendorf tube. After 15 min on ice, cell pellets were centrifuged at 1,000 x *g* for 5 min at 4 °C. The cell pellets were vortex mixed in 100 µL of ice cold buffer C (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA, 10% glycerol, and 0.1% Igepal) added with 1 mM DTT, 0.1 mM PMSF, and 0.2 µg/mL of leupeptin and aprotinin, and incubated on ice for 30 min. Cellular debris was removed by centrifugation at 14,500 rpm, 15 min at 4 °C. Then, 100 µL supernatant was transferred and combined with 100 µL buffer D (30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, and 10% glycerol). These supernatant combined with buffer D was collected and kept in -80 °C for further analysis. The aliquot part was used for protein determination.

5.3.10.2 Determination of protein concentration

The protein concentration of the supernatant was quantified using the BCA assay (Stoscheck, 1990). The protein lysate was diluted in nuclease free water (1:12). Thirty microliters of diluted protein lysate was mixed with 200 µL of copper BCA solution in a 96-well plate. The reaction was incubated at 37 °C for 30 min and measured the absorbance of 562 nm. BSA was used as a standard curve for protein determination.

5.3.10.3 Electrophoretic mobility shift assay

The binding reaction was performed by adjusting the final NaCl concentration to 50 mM (for NF-κB in mTNF-α, and NF-κB and C/EBP in mIL-6 promotors) and 100 mM (for AP-1 in mIL-6 promoter) followed by incubation on ice for 10 min with nuclear protein (1 µg for NF-κB, C/EBP and AP-1 in mIL-6 promoters, and 0.5 µg for NF-κB in mTNF-α promoter) and poly dI-dC (1 µg for NF-κB in mTNF-α and mIL-6 promoters and 0.2 µg for AP-1 and C/EBP in mIL-6 promoters). After incubation with poly dI-dC, 45,000 cpm of the double-stranded ^{32}P-labeled probes were added to the reaction and incubated at room temperature for another 20 min. Pairs of complementary oligonucleotides with 2 nucleotide overhangs were used to generate double-stranded DNA

probes (Table 5.1). To assess the specificity of DNA binding activity, the nuclear extracts were incubated with a 25-fold excess of unlabeled probe (NF-κB in mTNF-α and AP-1 in mIL-6 promoters), or a 10-, 25-, 50-, 100- and 250-fold excess of unlabeled probe (NF-κB and C/EBP in mIL-6 promoters) prior to addition of the radiolabeled probe. The resulting protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.5X TBE buffer (1X = 89 mM Tris, 89 mM borate, and 2 mM EDTA). The gel was then dried on 3 mm filter paper (Whatman, Hillsboro, OR) and autoradiographed.

Table 5.1 Sequences of oligonucleotide probes used in the EMSA assay.

Transcription factors	Mouse promoter	Oligonucleotide probes	Position relative to transcription start
NF-κB	TNF-α	5'-GT AAA CA**GGGGGCT TTCCC**T CCT CAA-3'	-510
		5'-GT TTG AGG A**GGGAAAGCCCC C**TG TTT-3'	
NF-κB	IL-6	5'-TC AAA T**GTGGG ATT TTCCCA** TGA G-3'	-64
		5'-GA CTC A**TGGGA AAA TCCCAC** ATT T-3'	
C/EBP	IL-6	5'-AG C**ACATT GTG CAA TCT** TAA-3'	-147
		5'-TA TTA **AGA TTG CAC AATGT**G-3'	
AP-1	IL-6	5'-TT CCC A**TG AGT CT**C AAA ATT-3'	-55
		5'-CT AAT TTT G**AGACTCA**T GGG-3'	

The putative transcription factor binding sequence is in bold for each probe.

5.3.11 Transient transfection and luciferase assay

5.3.11.1 Preparation of competent cells and their transformation with plasmid DNA

A genetically engineered highly transformable strain *E. coli* DH5α, pGL2-basic luciferase reporter gene vector, pmIL-6.Luc(-231) plasmid, and RAW264.7 cell were used in this study. Two plasmids DNA were obtained as a kind gift from Dr. Gail Bishop from the University of Iowa. Competent *E. coli* cells for DNA uptake were

prepared using the standard method of calcium chloride ($CaCl_2$) treatment (Sharma, Singh, and Gill, 2007) with minor modification. Briefly, a single *E. coli* DH5*α* colony was inoculated into 5 mL of Luria broth (LB) media in a 15 mL conical tube and incubated overnight at 37 °C in shaking water bath (GFL Shaking Water Baths-1086, Labsource, Manchester, UK) with a rotation speed of 150 rpm. Then, 1 mL of the bacterial suspension was inoculated into 100 mL of pre-warmed LB broth medium in a 500 mL flask and continuously incubated under the same condition until the bacterial cells grew to log phase (OD_{600} = 0.35-0.5 in LB broth medium), which was determined by a Cecil 1000 series spectrophotometer (Cecil Instruments, Cambridge, UK). Twenty milliliters of bacterial suspension was transferred to 50 mL conical tubes (20 mL/tubes). Bacterial suspension in 50 mL conical tubes were pelleted by centrifugation for 5 min at 1,500 x *g* at 4 °C, resuspended in 6 mL of sterile ice-cold 100 mM $MgCl_2$ solution (6 mL/tubes), and then incubated for 10 min on ice. The bacteria solution was pelleted by centrifugation at 1,500 x *g* for 5 min at 4 °C, resuspended in 6 mL of sterile ice-cold 100 mM $CaCl_2$ solution (6 mL/tubes), and incubated on ice for another 30 min. All bacteria solutions were pooled into one 50 mL conical tube. After another centrifugation step as above, the resulting cells pellet was ultimately resuspended in 5 mL ice cold 100 mM $CaCl_2$ plus 15% glycerol. The competent cell suspension was aliquoted into eppendorf tubes and stored at -80 °C until needed.

For transformation, the competent cell suspension and plasmid DNA of pGL2-basic and pmIL-6.Luc(-231) were thawed on ice. Afterwards, 50 µL of the competent cell suspension and 1-3 µL (5-15 ng) of the plasmid DNA were directly loaded into the ice-chilled microcentrifuge tube and was allowed to incubate on ice for 30 min. The cell-DNA complex was then heat shocked at 42 °C for exactly 90 s. The mixture was returned to ice for 3 min before 1 mL of fresh LB broth medium was added, and the mixture was incubated for 1 h at 37 °C in a shaking water bath with a rotation speed of 150 rpm. At

this time, cultures were serially diluted and 200 µL aliquots were spread on agar-LB broth with and without ampicillin (100 µg/mL) to obtain the number of transformants and viable cells respectively. After overnight incubation at 37 °C, a single colony was inoculated into 5 mL of LB broth with ampicillin (100 µg/mL) in a 50 mL conical tube and continuously incubated under the same condition. 1 mL of bacteria solution was transferred to sterile cryo-storage tubes and 1 mL of sterile 100% glycerol was added. After mixing, the glycerol stocks were stored at -80 °C until use. Bacteria solution was then pelleted by centrifugation at 2,400 x *g* for 10 min at 4 °C and plasmid DNA was isolated from bacteria using the QIAprep Spin Miniprep Kit and a Microcentrifuge (Qiagen, Valencia, CA). DNA concentration (pGL2-basic: 535.4 ng/µL, pmIL-6.Luc(-231): 692 ng/µL) in the extract was quantified using the NanoDrop ND-1000 Spectrophotometer and digested with HindIII-HF and BamHI (New England Biolabs Inc., Ipswich, MA). DNA sequencing was performed for confirmation of the plasmid DNA.

5.3.11.2 Bacterial culture

Bacterial cells were streaked from glycerol stock, immediately transferred to 5 mL of LB broth with ampicillin (100 µg/mL) in a 50 mL conical tube, and incubated overnight at 37 °C in shaking water bath with a rotation speed of 150 rpm. Bacteria solution were harvested by centrifugation at 2,400 x *g* for 10 min at 4 °C, and plasmid DNA was isolated from bacteria using the QIAprep Spin Miniprep Kit and a Microcentrifuge. Extracted DNA was then pooled into one microcentrifuge tube and concentrated using Savant DNA 110 Speed Vac (Global Medical Instrumentation, Inc., Ramsey, MN). Consequently, the concentrated DNA (pGL2-basic: 578.2 ng/µL, pmIL-6.Luc(-231): 2,822.2 ng/µL) was quantified and stored at -20 °C until needed.

5.3.11.3 Transfecting plasmid DNA into RAW264.7 cells using Lipofectamine 2000

RAW264.7 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, RAW264.7 cells were preseeded in a 6-well plate at $5x10^5$ cells/well in high glucose DMEM medium with 10% FBS followed by overnight incubation. Culture medium was then removed and replaced with fresh medium with 5% FBS. After 24 h incubation, culture medium was removed, replaced with fresh medium without serum and antibiotics, and incubated overnight. Before transfection, culture medium was removed and replaced with fresh medium without serum and antibiotics at 2 mL/well. Cells were then incubated for 4 h with transfection mixture [8 µg of pGL2-basic in 16 µL of lipofectamine 2000 or 8 µg of pmIL-6.Luc(-231) (generously provided by Dr. Gail Bishop) in 16 µL of lipofectamine 2000 per well]. The medium containing the transfection mixture was removed by centrifugation for 3 min at 700 rpm and replaced with fresh medium containing 2% FBS, and the cells were allowed to recover for 4 h. Then, transfected cells were scraped and distributed to a 48-well plate at $4x10^5$ cells/1 mL/well. After overnight incubation, culture medium was removed by centrifugation and replaced with fresh medium containing 2% FBS. The transfected RAW264.7 cells were then pretreated with increasing concentrations of 95EE-FLP (50, 150, or 250 µg/mL) or vehicle for 1 h before stimulation with 700 ng/mL LPS. Treatments were performed in 4 replicates. Eighteen hours after stimulation, the medium was removed and cells were washed twice with 1X PBS by centrifugation for 5 min at 300 x *g* at 4 °C. Then, 40 µL of reporter lysis 1X buffer were added to wells of a 48-well plate and the plate was kept at -80 °C overnight.

5.3.11.4 Luciferase assay

Reporter gene system is a technology where a reporter gene is synthesized in response to activation of a specific signaling cascade of interests. Thus, reporter protein expression of interest will be monitored by its enzymatic activities linked with a variety of colorimetric, fluorescent, or luminescent read-outs (Cheng et al., 2010).

Cell lysates in a 48-well plate were thawed at room temperature. The plate was rocked and vortexed for several times to ensure the complete coverage of the cells with lysis buffer. Lysed cells were then transferred to a 500 µL eppendorf tube on ice and vortexed. Twenty microliter of lysed cells were added to a 96-luminometer plate and the luminometer was read with 100 µL luminescent substrate using Wallac Microbeta 1450 Trilux Luminometer Liquid Scintillation Counter (Perkin Elmer Life Sciences, Turku, Finland). Protein determinations were performed using the Bradford protein assay. The luciferase activity was normalized to the determined amount of total protein. Results of luciferase expression were expressed as a relative light units (RLU)/µg protein.

5.3.12 Statistical analysis

Statistical significance of all variables (GraphPad Prism 5, USA) was determined by performing a one-way analysis of variance (ANOVA) with a *post-hoc* Turkey' s analysis to determine differences between treatments and control groups. Values were considered statistically significant when $p<0.05$. Data were presented as mean ± SEM (n=3 for ELISA and qRT-PCR analysis, n=4 for transient transfection analysis) and were representative of at least three independent experiments. EMSA analysis experiments were repeated at least 2 times with similar results.

5.4 Results

5.4.1 Suppression by 95EE-FLP of TNF-α and IL-6 release in LPS-stimulated macrophages

To investigate the anti-inflammatory activity of 95EE-FLP, freshly isolated peritoneal macrophages were activated with LPS in the presence of 95EE-FLP. ELISA was performed to quantify secreted proinflammatory cytokines TNF-α and IL-6. 95EE-FLP at 150 and 250 µg/mL significantly decreased TNF-α production induced by LPS when compared to the vehicle control (Figure 5.4A). Likewise, at the same concentrations, 95EE-FLP also significantly suppressed LPS-induced IL-6 production (Figure 5.4B). The suppressive effect of 95EE-FLP on TNF-α and IL-6 was not due to direct cytotoxicity as cell viability of peritoneal murine macrophages was ≥ 80%, and there was no difference in cell viability among naïve (NA), vehicle (VH), and all 95EE-FLP treatment groups as assessed by trypan blue exclusion staining (data not shown). Thus, 95EE-FLP suppressed both LPS-induced TNF-α and IL-6 secretions by mouse peritoneal macrophages with IL-6 exhibiting more sensitivity to suppression. Reduced TNF-α and IL-6 production by 95EE-FLP treatment was also observed in the LPS-activated mouse macrophage cell line RAW264.7 (Figure 5.5A and B). Cellular viability of RAW264.7 cells was ≥ 85% for all treatment groups and no differences in cell viability were observed among NA, VH, and 95EE-FLP (data not shown). Thus, 95EE-FLP was capable of impairing proinflammatory cytokine levels of TNF-α and IL-6 produced by both peritoneal macrophages and RAW264.7 macrophage cell line.

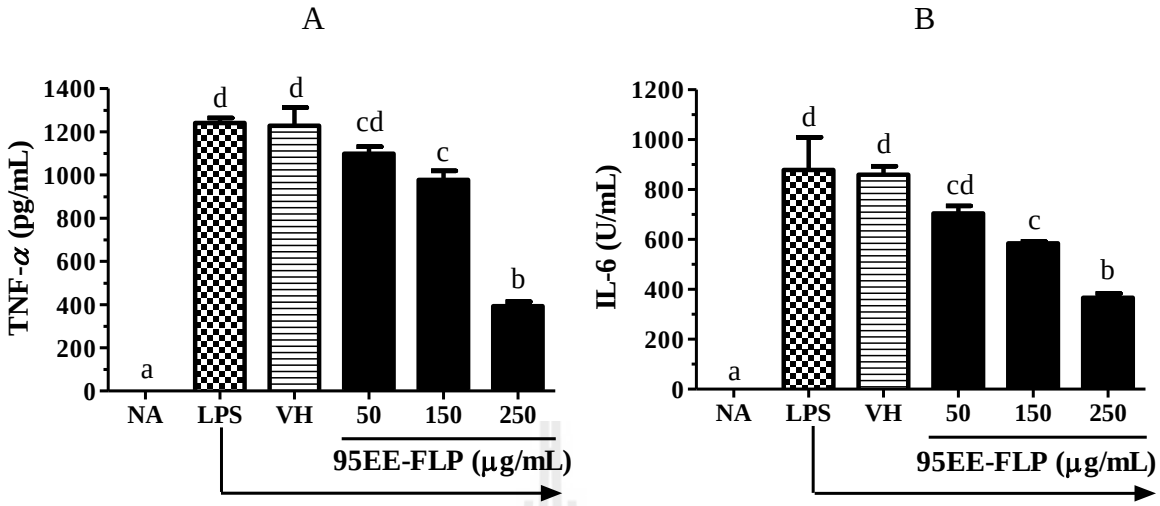

Figure 5.4 95EE-FLP suppressed TNF-α (A) and IL-6 (B) release in LPS-activated mouse peritoneal macrophages. Mouse peritoneal macrophages ($2.5x10^5$ cells/well) were pretreated with various concentrations of 95EE-FLP (50, 150, or 250 µg/mL) or vehicle control for 1 h. Cells were then activated with 100 ng/mL LPS for 8 h. The supernatants were harvested and measured for TNF-α and IL-6 production by ELISA. Values shown are the mean ± SEM (n=3) and are representative for three independent experiments. Bars marked with different letters are significantly different at $p<0.05$.

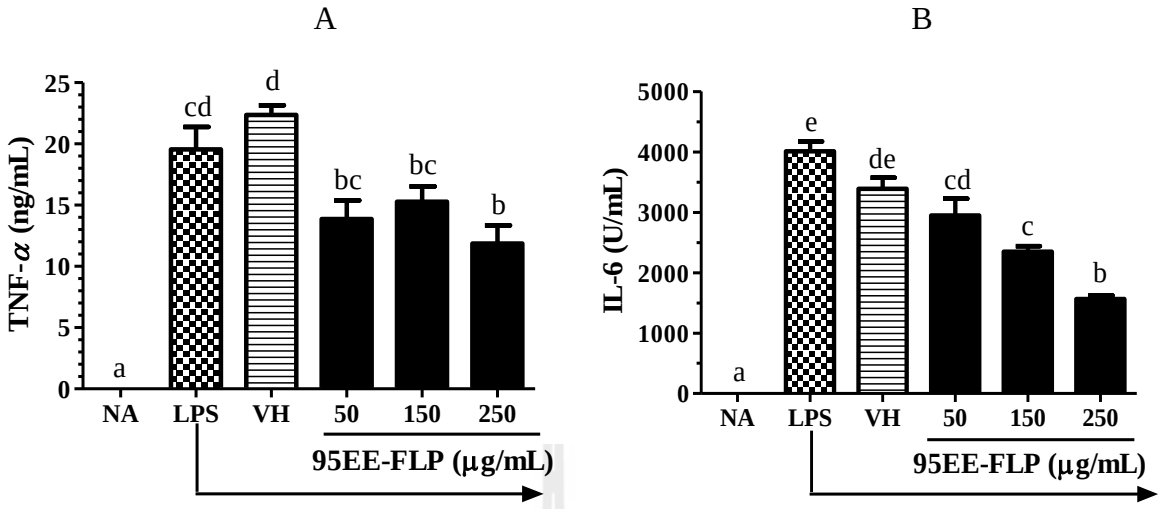

Figure 5.5 95EE-FLP suppressed TNF-α (A) and IL-6 (B) release in LPS-activated RAW264.7 cells. RAW264.7 cells ($2x10^6$ cells/well) were pretreated with various concentrations of 95EE-FLP (50, 150, or 250 μg/mL) or vehicle control for 1 h prior to activation with 100 ng/mL LPS for 8 h. The supernatants were harvested, and TNF-α and IL-6 production were quantified by ELISA. Data presented as mean ± SEM (n=3) and are representative for three independent experiments. Bars marked with different letters are significantly different at $p<0.05$.

5.4.2 95EE-FLP suppressed TNF-α and IL-6 proinflammatory cytokines mRNA levels in LPS-stimulated RAW264.7 cells

To determine if suppression of TNF-α and IL-6 production by 95EE-FLP was related to changes in mRNA levels for both inflammatory cytokines, qRT-PCR was performed. Increasing concentrations of 95EE-FLP produced a concomitant decrease in mRNA levels for both TNF-α and IL-6 in LPS-activated RAW264.7 cells (Figure 5.6A and B). The profile of TNF-α and IL-6 suppression by 95EE-FLP at both the protein and mRNA levels was remarkably similar with 95EE-FLP exerting more pronounced effect on IL-6 than TNF-α. In order to gain further insight into the molecular mechanisms of

95EE-FLP-mediated suppression of LPS-induced TNF-α and IL-6 production, EMSAs were conducted to examine 95EE-FLP modulation of transcription factor DNA binding activity of NF-κB, C/EBP, and AP-1.

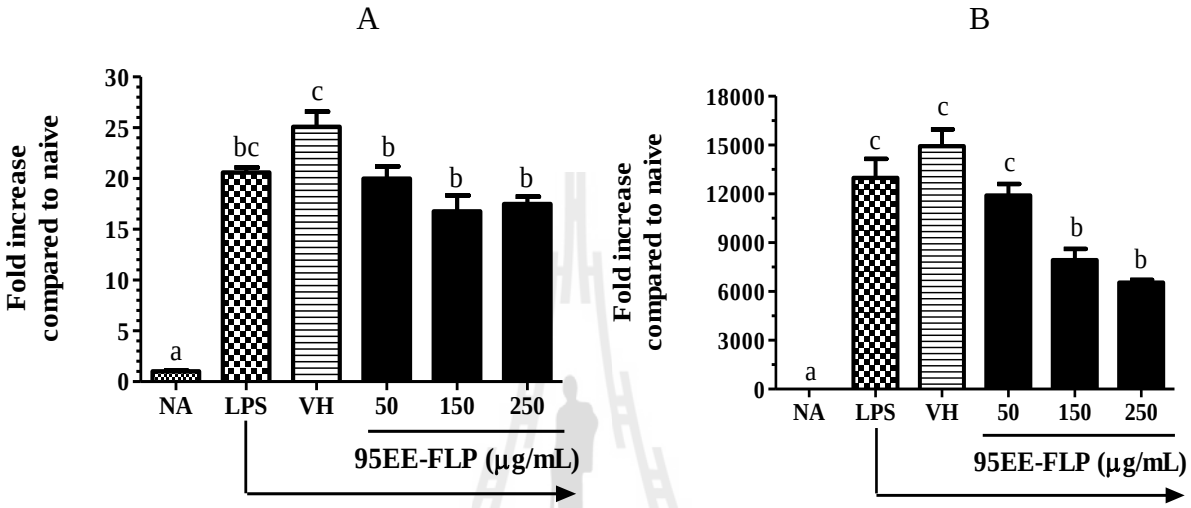

Figure 5.6 95EE-FLP suppressed TNF-α (A) and IL-6 (B) proinflammatory cytokine gene expression in LPS-activated RAW264.7 cells. RAW264.7 cells ($2x10^6$ cells/well) were pretreated with various concentrations of 95EE-FLP (50, 150, or 250 µg/mL) or vehicle control for 1 h followed by activation with 100 ng/mL LPS. Cells were then incubated for 8 h after-activation, at which time total RNA was isolated. TNF-α and IL-6 mRNA levels were determined by qRT-PCR using Taqman primers and probe. Endogenous 18S rRNA was used as a control and fold changes were obtained using $2^{-\Delta\Delta Ct}$ method. Data are presented as mean ± SEM (n=3) and are representative of three independent experiments. Bars marked with different letters are significantly different at $p<0.05$.

5.4.3 95EE-FLP suppressed LPS-induced NF-κB DNA binding activity in the TNF-α and the IL-6 promoters

As previously reported (Kuprash et al., 1999), the mouse TNF-α promoter contains several key NF-κB elements that are critically involved in regulating transcription (-860, -660, -630, and -510). Of these four κB sites, the κB element located at -510 was reported to exhibit the strongest DNA binding activity. Based on these prior findings, the effect of 95EE-FLP on LPS-induced NF-κB DNA-binding activity was investigated by EMSA using nuclear extracts from RAW264.7 cells. Initially, kinetic studies were conducted to identify the peak time of κB binding after LPS activation of RAW264.7 cells. As shown in Figure 5.7A and 5.8A, NF-κB DNA binding activity in the mouse TNF-α and mouse IL-6 promoter was similarly induced with 100 ng/mL of LPS within 30 min and remained elevated throughout the 4 h time period. Increasing concentrations of 95EE-FLP (50, 150, or 250 μg/mL) for 1 h prior to LPS (100 ng/mL) activation suppressed the increased NF-κB binding activity observed at 30 min post activation, in a concentration-dependent manner (Figure 5.7B and 5.8B).

5.4.4 95EE-FLP suppressed LPS-induced C/EBP DNA binding activity in IL-6 promoter

To further investigate transcription factor responses modulated by 95EE-FLP, gel shift assays of C/EBP binding to the IL-6 promoter was performed on RAW264.7 cell extracts. The present study first examined the peak time of C/EBP DNA binding activity in 100 ng/mL of LPS-stimulated RAW264.7 cells. As presented in Figure 5.9A, C/EBP DNA binding activity in the mIL-6 promoter was induced by LPS within 30 min, reaching a peak at 2 h, decreased thereafter and almost returned to basal level by 4 h. Exposure to increasing 95EE-FLP concentrations (50, 150, or 250 μg/mL) produced a concentration-dependent suppression of C/EBP DNA binding activity in mIL-6 promoter (Figure 5.9B).

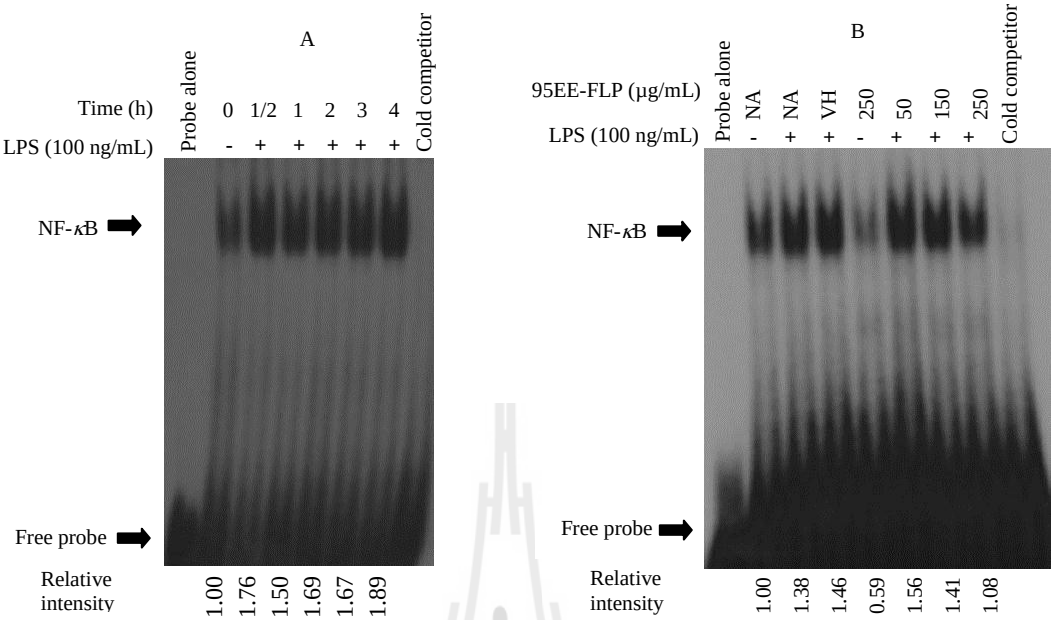

Figure 5.7 Electrophoretic mobility shift assay (EMSA) of NF-κB DNA binding activity in mouse TNF-α promoter. (A) Time course for LPS-induced NF-κB DNA binding activity in RAW264.7 cells. (B) The effect of 95EE-FLP on NF-κB binding activity in LPS-activated RAW264.7 cells. Cells were pretreated with increasing concentrations of 95EE-FLP (50, 150, or 250 μg/mL) for 1 h and then activated with 100 ng/mL LPS for 30 min. The nuclear protein (0.5 μg) was resolved by EMSA as described in the materials and methods. 25-fold excess of unlabeled probe was used to compete with the labeled oligonucleotide and their NF-κB binding activity was compared at 30 min time point. The data shown is representative of three independent experiments.

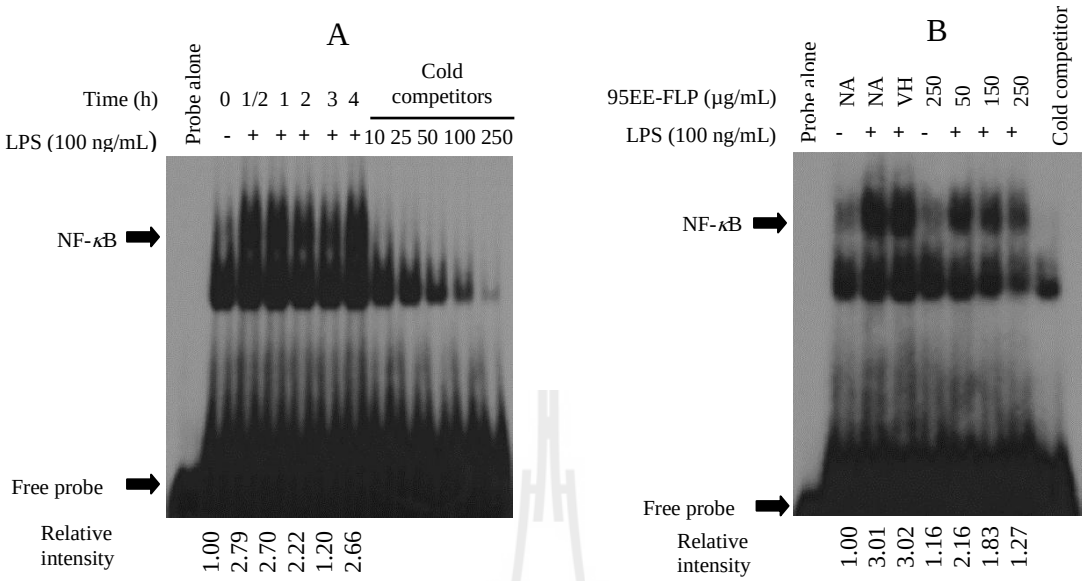

Figure 5.8 Electrophoretic mobility shift assay (EMSA) of NF-κB DNA binding activity in mouse IL-6 promoter. (A) Time course for LPS-induced NF-κB DNA binding activity in RAW264.7 cells. (B) The effect of 95EE-FLP on NF-κB binding activity in RAW264.7 cells. RAW264.7 cells were pretreated with increasing concentrations of 95EE-FLP (50, 150, or 250 μg/mL) for 1 h and then activated with 100 ng/mL LPS for 30 min. The nuclear protein (1 μg) was resolved by EMSA as described in the materials and methods. The binding specificity was determined using an unlabeled probe (10 - 250 fold in excess) to compete with the labeled oligonucleotide and their NF-κB binding activity was compared at 30 min time point. The data shown is representative of three independent experiments.

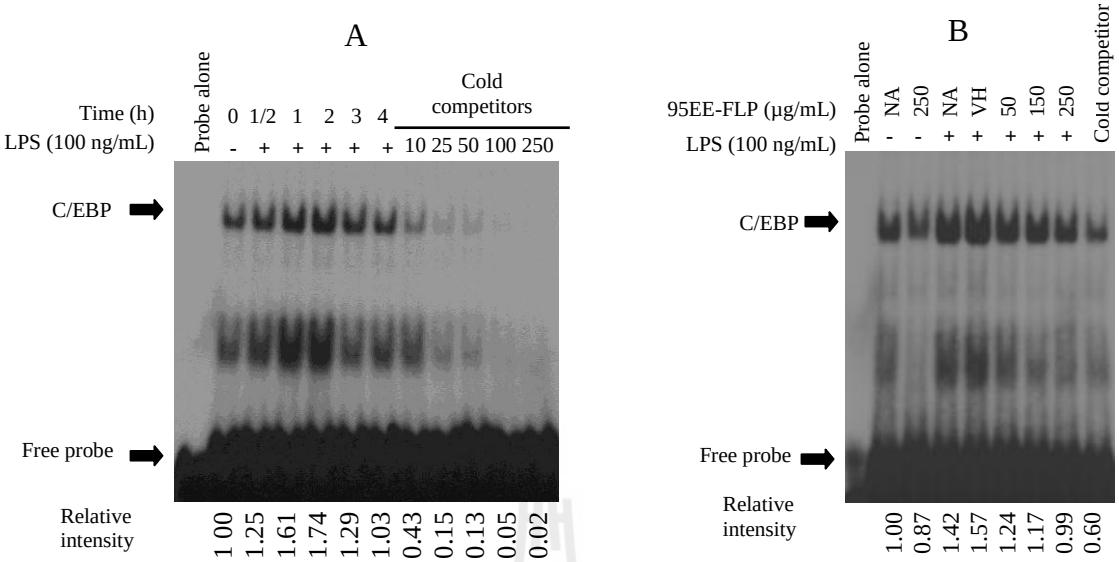

Figure 5.9 Electrophoretic mobility shift assay (EMSA) of C/EBP DNA binding activity in mouse IL-6 promoter. (A) Time course for LPS-induced C/EBP DNA binding activity in RAW264.7 cells. (B) The effect of 95EE-FLP on C/EBP binding activity in LPS-activated RAW264.7 cells. RAW264.7 cells were pretreated with increasing concentrations of 95EE-FLP (50, 150, or 250 μg/mL) for 1 h and then activated with 100 ng/mL LPS for 2 h. The nuclear protein (1 μg) was resolved by EMSA as described in the materials and methods. The binding specificity was determined using an unlabeled probe (10 – 250 fold in excess) to compete with the labeled oligonucleotide and their C/EBP binding activity was compared at 30 min time point. The data shown is representative of three independent experiments.

5.4.5 95EE-FLP suppressed LPS-induced AP-1 DNA binding activity in IL-6 promoter

Gel shift analysis was conducted to determine whether 95EE-FLP alters AP-1 DNA binding activity in LPS-activated RAW264.7 cells. Kinetic analysis revealed the peak time of AP-1 DNA binding activity was at 30 min (Figure 5.10A). The potency of 95EE-FLP on AP-1 DNA binding activity in activated RAW264.7 cells was evidenced by a

concentration-dependent decrease of both upper and lower bands of AP-1 DNA binding activity (Figure 5.10B).

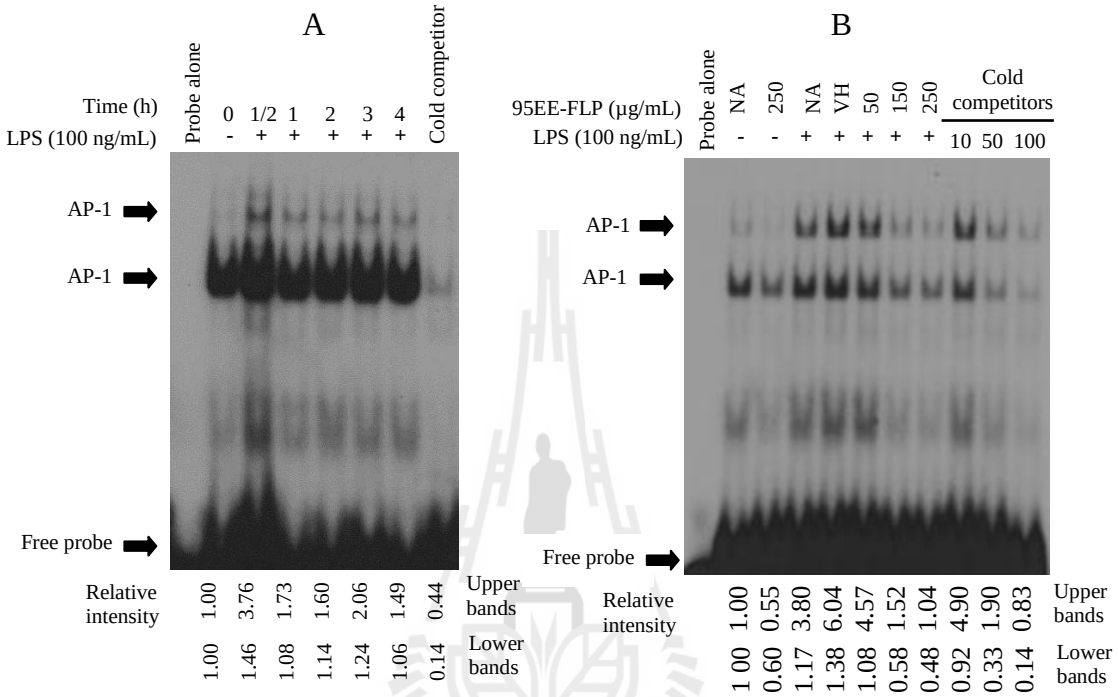

Figure 5.10 Electrophoretic mobility shift assay (EMSA) of AP-1 DNA binding activity in mouse IL-6 promoter. (A) Time course for LPS-activated AP-1 DNA binding activity in RAW264.7 cells. (B) The effect of 95EE-FLP on AP-1 binding activity in LPS-activated RAW264.7 cells. RAW264.7 cells were pretreated with increasing concentrations of 95EE-FLP (50, 150, or 250 µg/mL) for 1 h and then activated with 100 ng/mL LPS for 30 min. The nuclear protein (1 µg) was resolved by EMSA as described in the materials and methods. The binding specificity was determined using an unlabeled probe (10-100 fold in excess) to compete with the labeled oligonucleotide and their AP-1 binding activity was compared to VH control. The data shown is representative of three independent experiments.

5.4.6 95EE-FLP-mediate suppression of pmIL-6.Luc(-231) reporter activity in LPS-activated RAW264.7 cells

To further investigate whether the impairment of NF-κB, C/EBP, and AP-1 DNA binding activity in LPS activated RAW264.7 cells by 95EE-FLP resulted in suppression of IL-6 transcription, transient transfection experiments were performed using IL-6 promoter reporter. Previously, Baccam and coworkers showed the activation of 5'truncation mutants of the mIL-6 [pmIL-6.Luc(-231), pmIL-6.Luc(-161), pmIL-6.Luc (-84)] promoter/luciferase reporter with CD40 in CD154.CH12.LX B cells resulted in strongest luciferase activity with pmIL-6.Luc(-231). In fact, pmIL-6.Luc(-231) exhibited comparable activity to the original full length mIL-6 promoter pmIL-6.Luc(-1277) (Baccam et al., 2003). Based on these prior findings, we investigated whether 95EE-FLP suppression on proinflammatory IL-6 induction in LPS-stimulated RAW264.7 cells was at the level of transcription by using the IL-6 promoter-reporter possessing the 5' truncation, pmIL-6.Luc(-231) (Figure 5.11A) (Baccam et al., 2003). As presented in Figure 5.11B, pmIL-6.Luc(-231)-transfected RAW264.7 cells expressed higher luciferase activity upon LPS induction for 18 h, suggesting that the LPS regulatory elements present within the 231 bp upstream of transcription start site were functionally active. However, preliminary LPS concentration-response range finding studies using 100 to 1,000 ng/mL did not produce significant differences in luciferase activity in RAW264.7 cells. Figure 5.11C shows that 95EE-FLP treatment of transiently transfected RAW264.7 cells with pmIL-6 followed by activation with increasing concentrations of LPS demonstrated a dependent decrease in luciferase activity. It is noteworthy that pmIL-6.Luc(-231) activity was greatly reduced with 95EE-FLP treatment (150 and 250 μg/mL).

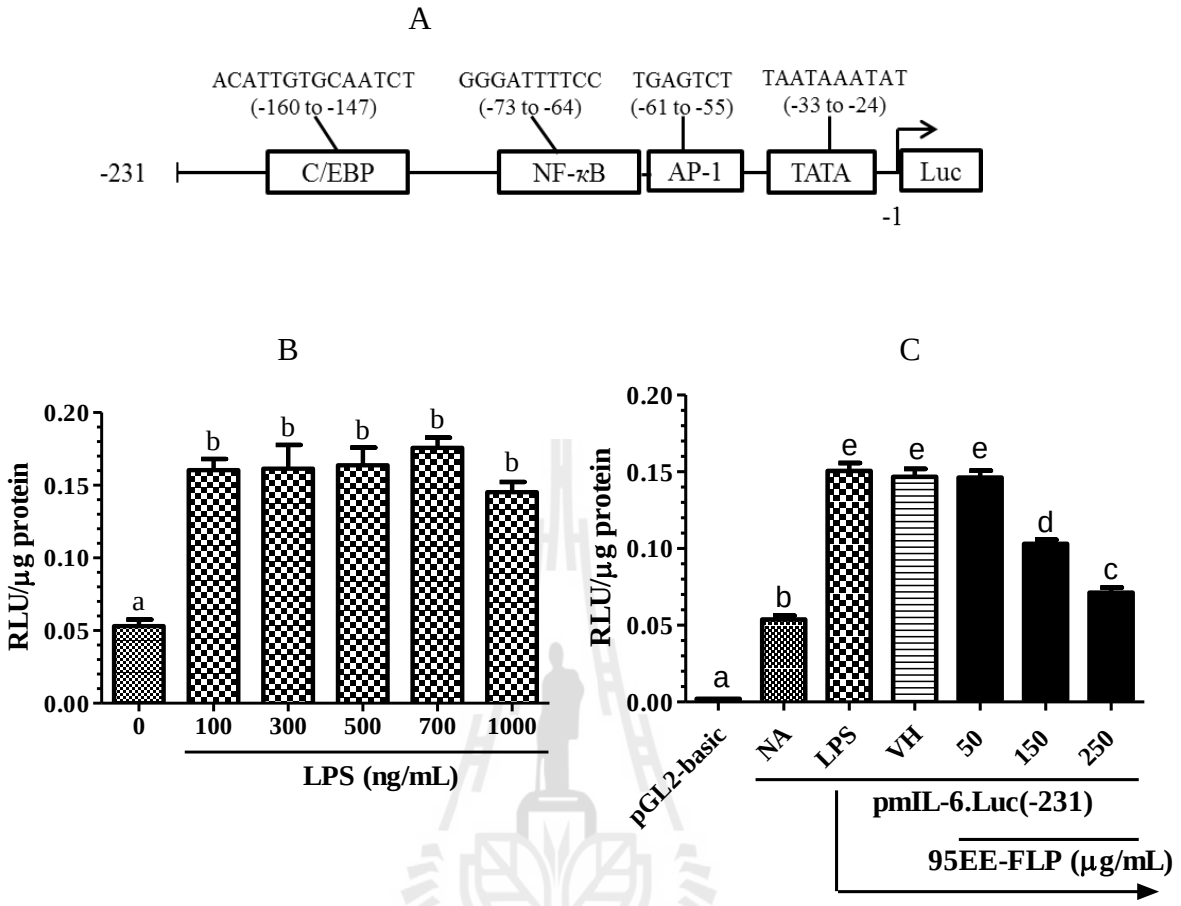

Figure 5.11 IL-6 promoter reporter luciferase activity in transient transfected RAW264.7 cells. (A) a schematic diagram of -231 IL-6 promoter reporter construct. Transcriptional response elements of AP-1, NF-κB, and C/EBP. (B) IL-6 promoter reporter activity in RAW264.7 cells transiently transfected with pmIL-6.Luc(-231) construct and treated with or without LPS at indicated concentrations for 18 h. (C) pmIL-6.Luc(-231) reporter activity in RAW264.7 cells treated with 95EE-FLP. RAW264.7 cells ($5x10^5$ cells/well) were preseeded in a 6-well plate and then transiently transfected with pGL2-basic and pmIL-6. Luc(-231). Transfected cells were scraped, distributed to a 48-well plate ($4x10^5$ cells/well) and incubated overnight. Cells were then pretreated with indicated concentrations of 95EE-FLP or vehicle for 1 h, followed by addition of 700 ng/mL LPS. Eighteen hours after LPS treatment, luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. The luciferase activity was then normalized to the determined amount of total protein. Results of luciferase expression are expressed as a RLU/µg protein. The results are presented as mean ± SEM (n=4) and are representative of three independent experiments. Bars marked with different letters are significantly different at $p<0.05$.

5.5 Discussion and conclusion

Natural products derived from plants have long been used as potentially important sources of anti-inflammatory drugs (Gautan and Jachak, 2009). The present study reports on the immune modulatory activity of 95EE-FLP as evidenced by suppression of proinflammatory cytokines, TNF-*α* and IL-6, in LPS-activated peritoneal murine macrophages and RAW264.7 cells. Because high levels of TNF-*α* and IL-6 play a critical role in acute and chronic inflammatory diseases, both cytokines are prime targets for intervention by anti-inflammatory therapeutic agents. Thus, suppression of elevated TNF-*α* and IL-6 induced by LPS has served as a biological model for evaluating anti-inflammatory drug candidates (Hüll et al., 1996; Wang et al., 2007; Zhiyuan and Wu, 2009). Phytochemicals, especially flavonoids have been shown to possess immune suppressive activity against these two inflammatory mediators. Pretreatment of RAW264.7 cells with flavonoids such as luteolin, luteolin-7-glucoside, quercetin, and the isoflavonoid genistein suppressed LPS-stimulated TNF-*α* and IL-6 production (Xagorari et al., 2001). Likewise, pretreatment of RAW264.7 cells with 7-*O*-methylnaringenin extract (flavonone class of polyphenols) from *Rhododendron speciferum* suppressed LPS-induced TNF-*α* and IL-6 production (Soromou et al., 2012). Analytical analyses in a previous study (Chapter III) identified considerable levels of flavonoids in ethanol extracts from PP leaves. It is possible that flavonoids in 95EE-FLP represent one of the active constituents responsible for anti-inflammatory activity by down regulating TNF-*α* and IL-6 released from myeloid lineage cells. Interestingly, PP belongs to the Acanthaceae plant family like *Andrographis paniculata* (AP) whose extracts displayed a wide spectrum of bioactivities, including anti-inflammation. Indeed, TNF-*α* and IL-6 mRNA expression were down regulated by andrograpanin isolated from AP (Liu, Wang, and Ge, 2008), mirroring the profile of suppression by 95EE-FLP in LPS-activated macrophages observed in the present study.

To further gain insight into the molecular mechanisms by which 95EE-FLP impairs TNF-α and IL-6, the effects of 95EE-FLP on the activation of key transcription factors involved in TNF-α and mIL-6 regulation, principally NF-κB, C/EBP, and AP-1, were examined. The present study demonstrated that 95EE-FLP decreased activation of NF-κB DNA binding activity in both mTNF-α and mIL-6 promoters occurred in a concentration-dependent manner. The decreased NF-κB DNA binding activity to its cognate DNA response element in both the mTNF-α and mIL-6 promoters was consistent with the decreased TNF-α and IL-6 protein synthesis and mRNA expression in LPS-stimulated RAW264.7 cells. In addition, the *cis*-acting element identified within the promoter region of murine IL-6 also contains C/EBP and AP-1 response elements (Baccam et al., 2003). The result shown here suggested that C/EBP and AP-1 are required for IL-6 induction in LPS-stimulated RAW26.7 macrophages as evidenced by dominant-positive suppression of C/EBP and AP-1 in the mIL-6 promoters by 95EE-FLP. It is important to emphasize that although many studies have shown that various natural products can impair LPS-induced DNA binding activity of AP-1 and C/EBP to probes containing consensus sequences (Cho, Lee, and Kim, 2003; Lee, Sung, Kim, and Kim, 2003; Cho, Jang, Kim, and Kim, 2004), the results here have demonstrated decreased DNA binding activity to bona fided NF-κB binding sites present in mTNF-α promoter and NF-κB, C/EBP, and AP-1 binding sites present in mIL-6 promoters. In the absence of LPS, 95EE-FLP-exposed RAW264.7 cells also displayed lower background mRNA levels of both mTNF-α and mIL-6 when compared to naïve resting cells (Figure 5.7B, 5.9B, and 5.10B). These results suggested that 95EE-FLP may have therapeutic utility by decreasing transcription of proinflammatory cytokines. Furthermore, the present study also showed that 95EE-FLP suppressed LPS-induced pmIL-6.Luc(-231) luciferase expression in a concentration-dependent manner suggesting the suppression of IL-6 occurred at the transcription level. As the activation of NF-κB, C/EBP, and AP-1 results in the expression of gene encoding mediator IL-6

(Baccam et al., 2003), the suppression of IL-6 mRNA expression by 95EE-FLP could result from decreased transcriptional activity of NF-κB, C/EBP, and AP-1. The effect of medicinal plants on suppressing transcriptional activity with consequent reduction of proinflammatory cytokines has been shown previously. The ethyl acetate extract from AP significantly suppressed NF-κB luciferase activity resulting in reduction of TNF-*α* and IL-6 production in LPS/IFN-*γ* stimulated RAW264.7 macrophages (Chao, Kuo, Hsieh, and Lin, 2011). In addition, a mixture of *β*-sitosterol and stigmasterol isolated from AP significantly suppressed NF-κB luciferase activity and TNF-*α* and IL-6 productions in LPS/IFN-*γ* stimulated RAW264.7 macrophages (Chao, Kuo, and Lin, 2010). PP contains *β*-sitosterol, stigmasterol, and apigenin 7-*O*-*β*-glucoside (Phan et al., 2003) and other flavonoid compounds (Phan et al., 2005; Nguyen and Eun, 2011). All these constituents have known or putative anti-inflammatory activity. Apigenin-7-*O*-*β*-glucoside can metabolize into apigenin which also has antioxidant and anti-inflammatory activity. Apigenin suppressed both protein and mRNA expression of COX-2 and iNOS and blocks NF-κB activation in LPS-activated RAW264.7 macrophages through suppression of inhibitory kappaB (IκB) and NF-κB luciferase activity (Liang et al., 1999). Another study also demonstrated that apigenin suppressed the production of proinflammatory cytokines IL-1*β*, IL-6 and TNF-*α* and NF-κB luciferase activity in LPS stimulated RAW264.7 macrophages (Nicholas et al., 2007).

In conclusion, this study demonstrates that 95EE-FLP-mediated suppression of LPS-induced TNF-*α* and IL-6 protein synthesis and mRNA expression in murine macrophages occurs, at least in part, through suppression of NF-κB(-510) in the mTNF-*α* and NF-κB, C/EBP and AP-1 activation, and DNA binding in the mIL-6 promoters. Furthermore, the LPS activation of pmIL-6.Luc(-231) promoter/luciferase reporter gene in transfected RAW264.7 cells was also suppressed by 95EE-FLP. Collectively, the partial mechanism of 95EE-FLP-mediated suppression of proinflammatory cytokines in LPS-induced

macrophages was summarized in Figure 5.12. Though anti-inflammatory leaves of PP, as well as various herbal medicine, have been anecdotal in nature rather than scientifically validated, PP leaves have long been traditionally used by Thai and Vietnamese people for prevention and treatment of various inflammatory diseases. Considering the long historical use in folk medicine, its potential bioactive constituents, and the anti-inflammatory evidence provided by this study, PP leaves possess high potential to be developed as a new medicinal drug for the prevention or treatment of acute or chronic inflammatory diseases.

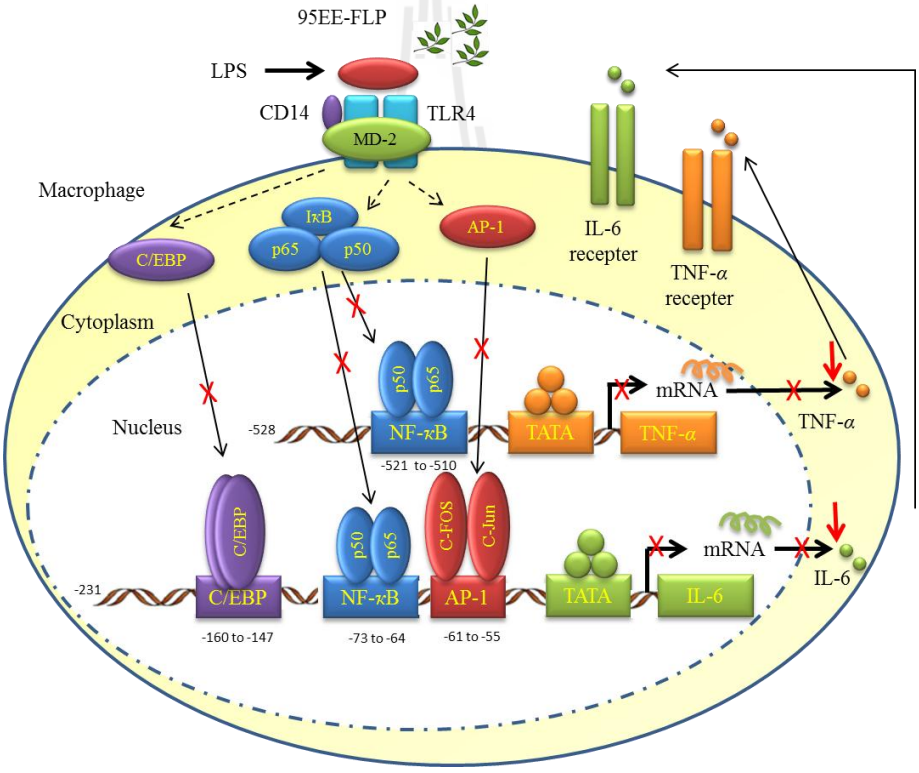

Figure 5.12 Partial mechanism of 95EE-FLP on suppression of proinflammatory cytokines TNF-α and IL-6 in LPS-induced macrophages. In unstimulated macrophages, C/EBP, NF-κB, and AP-1 are presented in the cytoplasm. After TLR4 binds with LPS on the surface of macrophage, adaptor molecules associate with TLR4 in the cytoplasm. These trigger phosphorylation or activations of C/EBP, NF-κB, or AP-1 transcription factor proteins to enter the nucleus and bind to their response elements in the mTNF-α and mIL-6 promoters. The DNA/CEBP, NF-κB, or AP-1 complexes then recruit other proteins to transcribe downstream DNA into mRNA, which inturn, is translated into high levels of TNF-α or IL-6 protein. Then, TNF-α and IL-6 proteins bind to their receptors, initiate their signal and cellular functions. Pretreatment of 95EE-FLP down regulated TNF-α and IL-6 mRNA and protein expressions by decreasing binding activity of NF-κB to the response element in mTNF-α promoter, and the binding activities of NF-κB, C/EBP, and AP-1 in the mIL-6 promoters. The LPS activation of plasmid mIL-6.Luc(-231) was also suppressed by 95EE-FLP treatment.

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CHAPTER VI

CONCLUSIONS

The present study demonstrated that various extracts of *Pseuderanthemum palatiferum* (PP) leaves (95EE-FLP, 80EE-FLP, 80EE-DLP, WE-FLP, and WE-DLP) had phenolic and flavonoid compounds as well as antioxidant properties as evaluated by chemical antioxidant assays (DPPH and FRAP). However, the extracts from PP fresh leaves (95EE-FLP, 80EE-FLP, and WE-FLP) displayed higher contents of phenolic and flavonoids as well as antioxidant activities than that of extracts from dried leaves (80EE-DLP and WE-DLP). No significantly different levels of phenolic and flavonoids as well as ferric reducing antioxidant power between 95EE-FLP and 80EE-FLP was observed. Having highest contents of phenolic and flavonoids and strongest antioxidant activity, 95EE-FLP and WE-FLP were selected for further determination of cellular antioxidant activity in macrophage RAW264.7 cells. The range-finding study suggested that the concentration range of 0-250 µg/mL of both 95EE-FLP and WE-FLP did not affect cell viability of RAW264.7, as assessed by three different cell viability tests. At non-cytotoxic concentration (50-250 µg/mL), 95EE-FLP and WE-FLP effectively diminished intracellular both in cellular antioxidant and anti-inflammatory activity. ROS generation in tBuOOH induced oxidative stress in RAW264.7 cells, which monitored by DCFH-DA fluorescent probe. The cytoprotective effect of both extracts against oxidative damage induced by tBuOOH was observed as early as 30 min and throughout the incubation period. This cytoprotective effect of both extracts was dose- and time-dependent and the profile of scavenging activity were similar to other antioxidant positive controls (250 µM catechin, 20 µM resveratrol, and 10 µM quercetin). The present study suggested that both 95EE-FLP

and WE-FLP possess anti-inflammatory property. Increasing concentrations (50, 100, 150, 200, or 250 µg/mL) of both 95EE-FLP and WE-FLP as well as various reference antioxidants positive controls [500 µM vitamin C (for NO, iNOS and COX-2) or 50 µg/mL trolox (for iNOS and COX-2)], significantly lowered the production of NO ($p<0.05$), iNOS, and COX-2, in LPS (1 µg/mL) plus IFN-γ (25 U/mL)-stimulated RAW264.7 cells. This suppressive effect was, however, more pronounced with 95EE-FLP than with WE-FLP.

95EE-FLP, the most potent suppressor of inflammatory factors was selected for further investigation of its immune modulation of two most prominent proinflammatory cytokines, TNF-α and IL-6. The present study demonstrated that 95EE-FLP dose-dependently suppressed LPS (100 ng/mL)-induced TNF-α and IL-6 secretion in mouse (C57BL/6) peritoneal macrophages. The similar suppression of LPS-induced up regulation of TNF-α and IL-6 protein synthesis and mRNA expression by 95EE-FLP was also observed in RAW264.7 cells. To investigate the molecular mechanisms of 95EE-FLP-mediated impairment of LPS-induced TNF-α and IL-6, gel shift assays were conducted to examine the modulation of transcription factor DNA binding activity of NF-κB, C/EBP, and AP-1. 95EE-FLP exhibited a concentration-dependent suppression of NF-κB binding activity within both the mTNF-α and mIL-6 promoters, when RAW264.7 cells were activated with LPS for 30 min. 95EE-FLP also exhibited a concentration-dependent suppression of C/EBP and AP-1 DNA binding activity within the mIL-6 promoters after activation of RAW264.7 cells with LPS for 30 min and 2 h, respectively. The transient transfection of RAW264.7 cells with IL-6 promoter-luciferase reporter plasmid was performed to confirm that suppression of IL-6 transcription by 95EE-FLP was actually caused by impairment of NF-κB, C/EBP, and AP-1 DNA binding activity in LPS-activated RAW264.7 cells. The result clearly indicated that 95EE-FLP reduced IL-6 luciferase promoter reporter activity in a concentration-dependent manner in transiently transfected RAW264.7 cells, upon LPS activation. Therefore, the potent anti-inflammatory effects of

95EE-FLP are mediated, at least in part, by modulating TNF-α and IL-6 expression through down regulation of NF-κB, C/EBP, and AP-1 activity.

Collectively, these results suggest that extracts of PP leaves contain an abundance of phenolic compounds, especially flavonoids, and possess significant antioxidant and anti-inflammatory activities. However, further research in animal models and identification of PP therapeutic components are still required for the successful development of PP as new therapeutic agent against diseases with overproduction of ROSs and inflammatory disorders.

APPENDICES

APPENDIX A

PREPARATION OF REAGENTS

A.1 Nitrite assay

- Griess reagent
  - Phosphoric acid 3.5 mL
  - Sulfanilamide 1 g
  - N-(1-napthyl)-ethylenediamine (NED) 0.1 g

  Adjust volume to 100 mL with DI water, and filter (store at 4 °C).

A.2 Western blot

- RIPA buffer
  - PBS (1X) 100 mL
  - NP-40 1 mL
  - Sodium dodecyl sulfate (SDS) 0.1 g

  (Store at 4 °C)

- Lysis buffer
  - RIPA buffer 1 mL
  - Phenylmethanesulfonyl fluoride (PMSF, 200 mM) 10 µL
  - Leupeptin (2 mM) 1 µL
  - E-64 (1 mM) 1 µL

  (Freshly prepared)

- Lowry reagents
  - Reagent A (2% Na_2CO_3 in 0.1 N NaOH)

- Na_2CO_3 5 g
- NaOH (0.1 N) 250 mL

  (Store at 4 °C)

- Reagent B (0.5% $CuSO_45H_2O$ in 1% sodium citrate)
  - $CuSO_45H_2O$ 0.05 g
  - Sodium citrate 0.1 g
  - DI water 10 mL
- Reagent C (1 N Folin phenol reagent)
  - 2 N Folin phenol reagent diluted with DI water (1:1, v/v)

    (Freshly prepared)
- Reagent D
  - Reagent A : Reagent B (50 : 1) (Freshly prepared)

- Sample buffer (6X)
  - Tris-base 0.59 g
  - DI water 8.5 mL
  - SDS 1.5 g
  - 2-Mercaptoethanol (2ME) 0.6 mL
  - Glycerol 7.5 mL
  - Bromophenol blue 7.5 mg

    (Store at 4 °C)

- SDS (10%, w/v)
  - SDS 10 g
  - DI water 100 mL

    (Store at RT)

- Ammonium persulfate (Aps) solution (10%, w/v)
    - Aps 0.1 g
    - DI water 1 mL
- Acrylamide (30%, w/v)
    - Acrylamide 30 g
    - Bis-acrylamide 0.8 g
    - DI water 100 mL

      (Filtrate, store at 4 °C)
- Tris-Cl (1.5 M, pH 8.8)
    - Tris-base 18.165 g
    - DI water 80 mL

      Adjust pH to 8.8 with HCl and bring to 100 mL with DI water.

      (Filtrate, store at 4 °C)
- Tris-Cl (0.5 M, pH 6.8)
    - Tris-base 6 g
    - DI water 80 mL

      Adjust pH to 6.8 with HCl and bring to 100 mL with DI water.

      (Filtrate, store at 4 °C)
- Running buffer (10X)
    - Tris-base 30 g
    - Glycine 14.4 g
    - SDS 10 g
    - DI water 1 L

      (Filtrate, store at 4 °C)
- Running buffer (1X)
    - Running buffer (10X) 100 mL

- DI water 900 mL

- Blotting buffer (1X)
  - Tris-base 3 g
  - Glycine 14.4 g
  - Methanol 200 mL

  Adjust volume to 1 L with DI water, and filter (store at 4 °C).

- TPBS 0.1% Tween 20
  - PBS (1X) 1000 mL
  - Tween 20 1 mL

  (Filtrate, store at 4 °C)

- Nonfat milk (5%, w/v)
  - Nonfat milk 0.75 g
  - TPBS 0.1% Tween 20 15 mL

  (Freshly prepared)

- Coomassie blue solution
  - Coomassie blue 0.05 g
  - Methanol 80 mL
  - Glacial acetic acid 14 mL

  Adjust volume to 100 mL with DI water (store at RT).

- Destaining solution
  - Methanol 5 mL
  - Glacial acetic acid 7 mL

  Adjust volume to 100 mL with DI water (store at RT).

- Resolving gel (7.5%) (for 2 gels)
  - DI water 4.84 mL
  - 1.5 M Tris-Cl (pH 8.8) 2.5 mL

- Acrylamide (30%, w/v) 2.5 mL
- SDS (10%, w/v) 100 µL
- Aps (10%, w/v) 50 µL
- Tetramethylethylenediamine (TEMED) 10 µL

- Resolving gel (10%) (for 2 gels)
  - DI water 4 mL
  - Tris-Cl (1.5 M, pH 8.8) 2.5 mL
  - Acrylamide (30%, w/v) 3.3 mL
  - SDS (10%, w/v) 100 µL
  - Aps (10%, w/v) 50 µL
  - TEMED 10 µL
- Stacking gel (4%) (for 2 gels)
  - DI water 3 mL
  - Tris-Cl (0.5 M, pH 6.8) 1.25 mL
  - Acrylamide (30%, w/v) 665 µL
  - SDS (10%, w/v) 50 µL
  - Aps (10%, w/v) 25 µL
  - TEMED 10 µL

A.3 ELISA

- Citric-phosphate buffer (pH 5.0)
  - Diabasic sodium phosphate (0.2 M) 25.7 mL
  - Ctric acid (0.1 M) 24.3 mL
  - DI water 50 mL

    (Adjust pH to 5.0)

- 3, 3', 5, 5'-tetramethyl-benzidine stock solution (6 mg/mL)
  - 3, 3', 5, 5'-tetramethyl-benzidine (TMB) 30 mg
  - DMSO 5 mL

    (Stock solution can be store up to 1 month at RT)
- H_2O_2 (1%)
  - H_2O_2 (30%) 0.1 mL
  - DI water 2.9 mL
- TMB substrate
  - Citric-phosphate buffer pH 5.0 12.5 mL
  - TMB stock solution (6 mg/mL) 200 µL
  - H_2O_2 (1%) 50 µL

    (Freshly prepared)
- H_2SO_4 (6 N)
  - H_2SO_4 (18 M) 166.67 mL

    Adjust volume to 1 L with DI water

A.4 EMSA

- DNA annealing solution
  - Prepared stock DNA solution in nuclease free water (5 pmoles/µL).
  - Stock DNA in eppendorf tube are heated 2 min at 88 °C (usually use heat block).
  - Stand at RT and wait until it is at RT.
  - Aliquot 5 µL/tubes.

    (Store at -20 °C)
- Protease inhibitors
  - DTT (1 M) 10 µL

- Aprotinin (0.5 mg/mL) 4 µL
- Leupeptin (0.5 mg/mL) 4 µL
- PMSF (200 mM) 5 µL

(In 10 mL hypotonic buffer and in 10 mL buffer C)

- Hypotonic buffer (HB buffer)
  - HEPES (0.5 M) 1 mL
  - $MgCl_2$ (1 M) 75 µL

  Adjust volume to 50 mL with MQ water
- Buffer C
  - HEPES (0.5 M) 1.5 mL
  - $MgCl_2$ (1 M) 37.5 µL
  - EDTA (0.5 M) 15 µL
  - Glycerol 2.5 mL
  - NaCl (5 M) 2.25 mL
  - Igepal 25 µL

  Adjust volume to 25 mL with MQ water
- Buffer D
  - HEPES (0.5 M) 1.5 mL
  - $MgCl_2$ (1 M) 37.5 µL
  - EDTA (0.5 M) 15 µL
  - Glycerol 2.5 mL

  Adjust volume to 25 mL with MQ water
- Poly[d(I-C)]
  - Poly[d(I-C)] 1 bottom $10A_{260}$ units
  - HEPES (30 mM) 1 mL

  Measure absorbance (ng/mL)

- Polyacrylamide gel (5%) in TBE buffer (0.5X)
    - MQ water 40.75 mL
    - Acrylamide : Bis-Acrylamide solution (29:1) 6.25 mL
    - TBE (10X) 2.5 mL
    - Aps (10%) 0.5 mL
    - TEMED 50 µL
- TBE buffer (10X)
    - Tris (890 mM) 54 g
    - Boric acid (890 mM) 27.5 g
    - EDTA (0.5 M) 20 mL

      Adjust volume to 500 mL with MQ water
- Running buffer (TBE buffer, 0.5X)
    - TBE buffer (10X) 25 mL

      Adjust volume to 500 mL with DI water

A.5 Transfection and luciferase

- LB broth
    - NaCl 10 g
    - Yeast extract 5 g
    - Tryptone 10 g

      Adjust volume to 1 L with DI water

      (Adjust pH 7.0 ± 0.2 and the medium was autoclaved at 121 °C for 15 min)
- LB broth agar
    - LB broth 200 mL
    - Bacto agar 4 g
    - Melt agar into LB broth in the microwave
    - Wait until the solution is cool enough to be held in the hands

- Ampicillin (100 mg/mL) 200 µL
- Pour the LB broth agar onto the plate

- Bradford's reagent
  - Coomassie Blue-G 0.01 g
  - phosphoric acid (85%) 10 mL
  - Ethyl alcohol 5 mL
  - Adjust volume to 100 mL with DI water
  - Filtrate with Whatman No. 1 (5-6 times)
  - Keep in dark bottom in 4 °C
- Bradford protein assay
  - Add 20 µL of BSA standard or sample to each well of a 96-well plate (2 µL sample + 18 µL DI water).
  - Add 180 µL of Bradford's reagent to each well of a 96-well plate and gently mix.
  - Incubate for 5 min at RT.
  - Measure the absorbance at 595 nm on a microplate reader.

APPENDIX B

REAGENTS FOR CELL CULTURE

B.1 Phosphate buffered saline (PBS)

- PBS (1X)
  - KH_2PO_4 0.21 g
  - NaCl 9 g
  - Na_2HPO_4 0.38 g
  - DI water 1 L

  Adjust pH to 7.2 ± 0.1, and filter sterile (store at 4 °C).

B.2 Trypsin/EDTA preparation

- Trypsin/EDTA
  - Trypsin 0.25 g
  - EDTA 0.04 g
  - PBS (1X) 100 mL

  (Filter sterile, aliquot and store at -20 °C)

B.3 Culture media preparation

- FBS (inactivated)
  - Slowly thaw the frozen FBS in a beaker filled with water.
  - Put in water bath at 37 °C till completely thaw.
  - Heat inactivate (56 °C, 20 min), gentle mix every 5-10 min.
  - Aliquot 50 mL into conical tubes.

  (Store at -20 °C)

- HEPES buffer (1 M)
  - HEPES 23.83 g
  - DI water 100 mL

  (Filter sterile, aliquot and store at -20 °C)
- Penicillin/Streptomycin (100X)
  - Penicillin 0.6 g
  - Streptomycin 1.34 g
  - PBS (1X) 100 mL

  (Filter sterile, aliquot and store at -20 °C)
- RPMI 1640, 1X (incomplete medium)
  - RPMI-1640 (1X) with L-glutamine, and phenol red 1 pack
  - $NaHCO_3$ 2 g
  - DI water 1 L

  Adjust pH to 7.2-7.4 (Filter sterile, store at 4 °C)
- RPMI 1640, 1X (complete medium)
  - Inactivated FBS 20 mL
  - Penicillin/Streptomycin 2 mL
  - HEPES buffer (1 M) 3 mL

  Adjust volume to 200 mL with RPMI-1640, 1X, incomplete medium.

  (Store at 4 °C)
- DMEM, high glucose, 1X (incomplete medium)
  - DMEM, high glucose, 1X with L-glutamine, with phenol red 1 pack
  - $NaHCO_3$ 3.7 g
  - DI water 1 L

  Adjust pH to 7.0-7.4 (Filter sterile, store at 4 °C)

- DMEM, high glucose, 1X (complete medium)
    - Inactivated FBS 20 mL
    - Penicillin/Streptomycin 2 mL
    - HEPES buffer (1 M) 3 mL

    Adjust volume to 200 mL with DMEM, high glucose, 1X, incomplete medium (store at 4 °C)

APPENDIX C

DETECTION KITS

C.1 mRNA analysis (qRT-PCR)

- RNA isolation using SV Total RNA Isolation kits (Promega, Madison, WI)
    - Remove supernatant (collect supernatant and freeze at -80 °C for ELISA analysis) and wash the cells with ice-cold, sterile 1X PBS.
    - Add 175 µL of RNA Lysis Buffer (RLA) to the washed cells in a 6-well plate, disperse the pellet and mix well by vortexing and/or pipetting.
    - Transfer to 1.5 mL microcentrifuge tubes (or freeze at -80 °C until needed).
    - Add 350 µL of RNA Dilution Buffer (blue) to 175 µL of lysate. Mix by inverting the tube 3-4 times. Place in a water bath or heating block at 70 °C for 3 min. Incubation longer than 3 minutes may compromise the integrity of the RNA.
    - Centrifuge at 12,000-14,000 x *g* for 10 min at 20-25 °C.
    - Transfer the cleared lysate solution to a fresh microcentrifuge tube by pipetting. Avoid disturbing the pellet debris.
    - Add 200 µL 95% ethanol to the cleared lysate, and mix by pipetting 3-4 times. Transfer this mixture to the spin column assembly. Centrifuge at 12,000-14,000 x *g* for 1 min.
    - Take the spin basket from the spin column assembly, and discard the liquid in the collection tube. Put the spin basket back into the collection tube.

Add 600 µL of RNA Wash Solution to the spin column assembly. Centrifuge at 12,000-14,000 x *g* for 1 minute.

- Empty the collection tube as before and place it in a rack. For each isolation to be performed, prepare the DNase incubation mix by combining 40 µL Yellow Core Buffer, 5 µL 0.09 M $MnCl_2$ and 5 µL of DNase I enzyme per sample in a sterile tube (in this order). Prepare only the amount of DNase incubation mix required and pipet carefully. Mix by gentle pipetting; do not vortex. Keep the DNase I on ice while it is thawed. Apply 50 µL of this freshly prepared DNase incubation mix directly to the membrane inside the spin basket. Make sure that the solution is in contact with and thoroughly covering the membrane. The incubation solution is yellow to make this easier to visualize.
- Incubate for 15 minutes at 20-25 °C. After this incubation, add 200 µL of DNase Stop Solution to spin basket, and centrifuge at 12,000-14,000 x *g* for 1 minute. There is no need to empty the collection tube before the next step.
- Add 600 µL RNA Wash Solution (with ethanol added) and centrifuge at 12,000-14,000 x *g* for 1 minute.
- Empty the collection tube, and add 250 µL RNA Wash Solution (with ethanol added); centrifuge at high speed (20,000 x *g*) for 2 min.
- If you have not already done so, remove the cap from the spin basket by using a twisting motion.
- For each sample, remove one capped 1.5 mL elution tube. Transfer the spin basket from the collection tube to the elution tube, and add 100 µL nuclease-free water to the membrane. Be sure to completely cover the surface of the membrane with the water. Place the spin basket assemblies

in the centrifuge with the lids of elution tube facing out. Centrifuge at 12,000-14,000 x *g* for 1 minute. Remove the spin basket and discard. Cap the elution tube containing the purified RNA and store at -70 °C to convert RNA into cDNA. (If you want to determine purified RNA). After that remove the spin basket and discard. Cap the elution tube and put on ice to determine RNA using NanoDrop ND-1000 spectrophotometer.

- Reverse transcription (RT) of mRNA to cDNA using High Capacity cDNA Reverse Transcription kit (AB Applied Biosystems, Foster City, CA)
  - Prepare the 2X Reverse Transcription Master Mix on ice.

Components	Volume/Reaction (µL)
10X RT Buffer	2.5
25X dNTP Mix	1
10X RT Random Primers	2.5
MultiScribe Reverse Transcriptase (50U/µL)	1.25
RNase-free H_2O	5.25
Final Volume	12.5

  - Pipette 12.5 µL of 2X RT master mix into each well.
  - Pipette 12.5 µL of RNA sample into each well, pipette up and down two times to mix.
  - Seal the plate and incubate at 25 °C for 10 min, 37 °C for 2 h, and 85 °C for 5 min.
  - Store at 4 °C for 24 h before use.

C.2 EMSA

- EMSA probe preparation using illustra ProbeQuant G-50 Micro Column (GE Healthcare, Piscataway, NJ)
    - Use 10 pmole annealed DNA in total volume of 46 µL 1X buffer.
    - Add 2 µL ^{32}P-ATP (stock 10 µCi/µL) and mix gently.
    - Add 2 µL T4 kinase and mix gently.
    - Incubate 30 min at 37 °C (usually use water bath).
    - Proceed with column purification using GE Healthcare Illustra ProbeQuant G50 microcolumns.
    - Vortex column to suspend resin.
    - Snap off bottom and place column in a 2 mL eppendorf tube.
    - Spin at 735 x *g*, 1 min at RT. Discard eppendorf.
    - Place column in fresh collection tube from GE ProbeQuant kit.
    - Apply 50 µL sample to resin without disturbing it.
    - Spin at 735 x *g*, 2 min at RT. Discard column and retain flow-through.
    - Acess CPM with Liquid Scintillation Counter (LSC). Place 2 µL sample on filter paper and read in 10 mL scintillation fluid.

C.3 Transfection and luciferase

- Plasmid DNA purification using the QIAprep Spin Miniprep kit and a Microcentrifuge (Qiagen, Valencia, CA)
    - Resuspend pelleted bacterial cells in 250 µL Buffer P1 and transfer to a microcentrifuge tube.
    - Add 250 µL Buffer P2 and mix thoroughly by inverting the tube 4-6 times.
    - Add 350 µL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.

- Centrifuge for 10 min at 13,000 rpm (~ 17,900 x g) in a table-top microcentrifuge.
- Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.
- Centrifuge for 30-60 s. Discard the flow-through.
- Recommended: Wash the QIAprep spin column by adding 0.5 mL Buffer PB and centrifugation for 30-60 s. Discard the flow-through.
- Wash QIAprep spin column by adding 0.75 mL Buffer PE and centrifuging for 30-60 s.
- Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
- Place the QIAprep column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 50 µL Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin, let stand for 1 min, and centrifuge for 1 min.

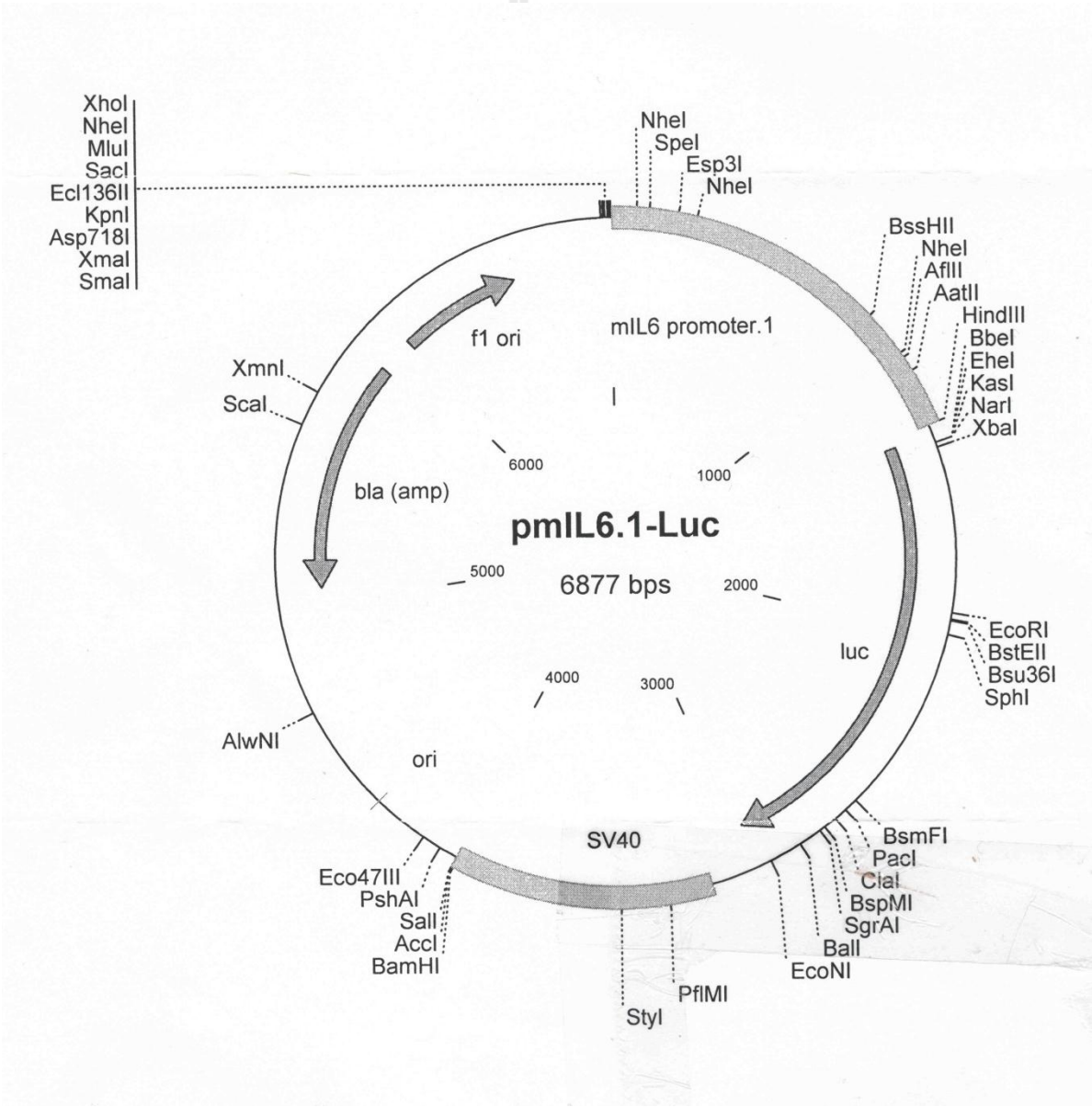
- Preparation of transfection mixture using Lipofectamine 2000 (Invitrogen, Carlsbad, CA)
  - Dilute DNA in 250 µL of Opti-MEM reduced serum medium 1X and mix gently.
  - Dilute Lipofectamine 2000 in 250 µL of Opti-MEM reduced serum medium, mix gently, and incubate for 5 min at RT.
  - After 5 min incubation, combine the diluted DNA with Lipofectamine 2000, mix gently, and incubate for 20 min at RT.
  - Add 500 µL complexes to each well (6-well plate) in a drop wise fashion and mix gently.

- Preparation reporter lysis 1X buffer from Reporter Lysis 5X Buffer (Promega)
    - Add 4 volumes of water to 1 volumes of Reporter Lysis 5X Buffer. Equilibrate 1X lysis buffer to room temperature before use.
    - Carefully remove the growth medium from cells to be assayed. Rinse the cells twice with PBS, being careful not to dislodge attached cells. Remove as much of the final PBS rinse as possible.
    - Add enough 1X reporter lysis buffer to cover the cells. Freeze-thaw once to ensure complete lysis.
    - Rock culture dishes several times to ensure complete coverage of the cells with lysis buffer. Transfer the cell lysates to a microcentrifuge tube. Place the tube on ice.
    - Vortex the microcentrifuge tube and transfer cell lysates to 96 luminator plate.
    - The cell lysates may be assayed directly or store at-70 °C.
- Luciferase assay reagent preparation (Luciferase Assay System 10-Pack, Promega)
    - To prepare the luciferase assay reagent, add 10 mL of Luciferase Assay Buffer to the vial containing the lyophilized Luciferase Assay Substrate.
    - Avoid exposure of the luciferase assay reagent to multiple freeze-thaw cycles by dispensing the reconstituted reagent into working aliquots.
    - Store any unused luciferase assay reagent at -70 °C.
    - Equilibrate luciferase assay reagent to RT before each used.
    - Each reaction requires 100 µL of reagent to initiate enzyme activity.

APPENDIX D

RESTRICTION DIGESTION OF DNA

D.1 Mapping of pmIL6.1-Luc (6877 bps or full length mIL-6 prompter)



D.2 The amount of DNA for using in restriction digestion of DNA

- pGL2 (535.4 ng/µL), need 500 ng/lane 0.93 µL (~0.9 µL)
- pmIL-6.Luc(-231) (692 ng/µL), need 500 ng/lane 0.72 µL (~0.7 µL)
- The table shows working reaction of DNA restriction enzyme digestion

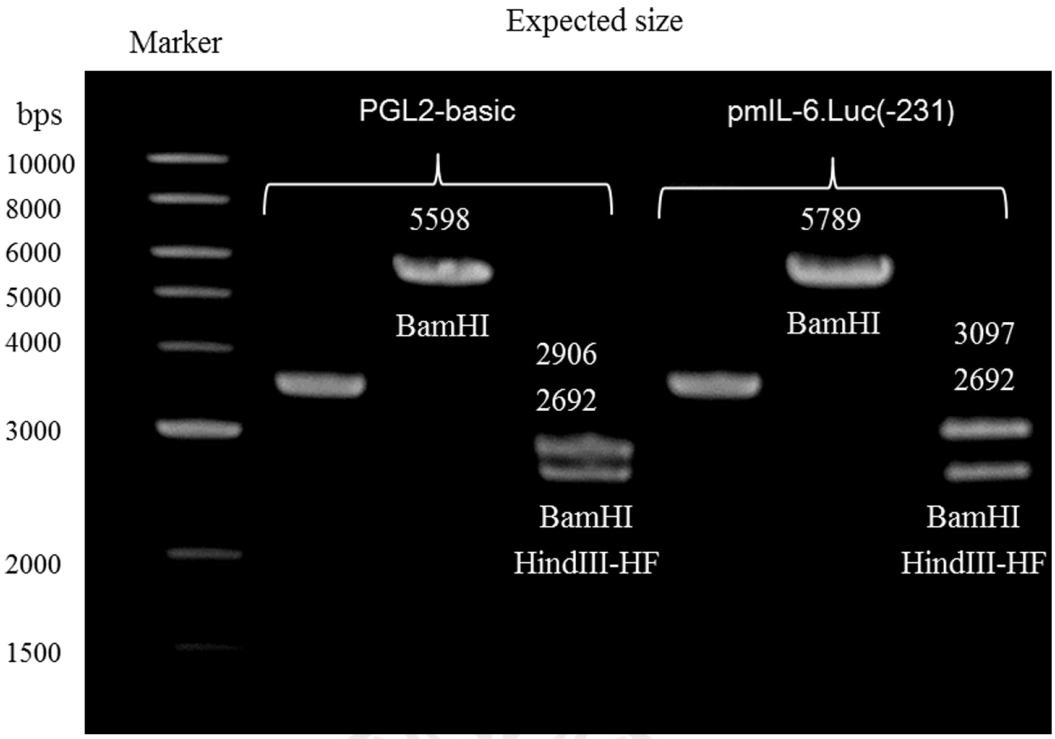
Samples	pGL2 (0.5 µg/Lane)			pmIL-6.Luc(-231) (0.5 µg/Lane)		
	Uncut (µL)	1 cut (µL)	2 cut (µL)	Uncut (µL)	1 cut (µL)	2 cut (µL)
Template (DNA)	0.9	0.9	0.9	0.7	0.7	0.7
10X NEB Buffer3	-	2	-	-	2	-
10X NEB Buffer4	-	-	2	-	-	2
10X BSA	-	2	2	-	2	2
BamHI	-	2	2	-	2	2
HindIII-HF	-	-	2	-	-	2
DI water	19.1	13.1	11.1	19.3	13.3	11.3
Total volume	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL

D.3 Expected size

The table shows expected size of interested plasmid DNA

Plasmid DNA	Size (bp)	BamHI	HindIII-HF
pGL2-basic	5598	5598	2906 and 2692
pmIL-6.Luc(-231)	5789	5789	3097 and 2692

D.4 Result of DNA restriction analysis



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