Anti-inflammatory Activity of *Kaempferia parviflora* Rhizomes

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Thesis Title Anti-inflammatory activitiy of *Kaempferia parviflora* rhizomes

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บทคัดย่อ

จากการศึกษานี้ พบว่า ชั้น chloroform ของสารสกัดเหง้ากระชายดำยับยั้งการอักเสบได้ดีที่สุดโดยต้านการสร้าง nitric oxide (NO) ที่ค่า IC_{50}= 8.4 μg/ml ตามด้วยชั้น hexane ชั้นน้ำ และชั้น ethyl acetate ตามลำดับ โดยมีค่า IC_{50}= 13, 40 และ 61 μg/ml ตามลำดับ ชั้น hexane และชั้น chloroform มีฤทธิ์ต้านการอักเสบโดยผ่านการยับยั้งการสร้าง prostaglandin E_2 (PGE_2) เป็นหลัก โดยการลดการบวมของเท้าหนูหลังจาก 3 ชั่วโมงหลังการฉีดด้วย 1% carrageenan สารสกัดด้วย ethanol ยับยั้งการสร้าง PGE_2 ที่ IC_{50}= 9.6 μg/ml หนูขาวเล็กที่ถูกป้อนด้วยสารสกัดชั้น ethanol และ ชั้น chloroform ไม่แสดงความเป็นพิษทั้งในหนูเพศผู้และเพศเมีย นอกจากนี้ ชั้น chloroform สามารถยับยั้งการสร้าง TNF-α โดยมีค่า IC_{50}= 3.79 μg/ml และยับยั้งการแสดงออกของ iNOS, COX-2, NFKB, Rel A และ TNF-α mRNA

ผลการแยกสารสำคัญจากชั้น chloroform ด้วยวิธี open column chromatography (OCC) และทำให้บริสุทธิ์ด้วยเทคนิค high performance liquid chromatography (HPLC) พบสารในกลุ่ม methoxyflavonoid 12 ชนิด ดังนี้ techtochrysin (**1**), 5,7-dimethoxyflavone (**2**), 7,4′-dimethylapigenin (**3**), trimethylapigenin (**4**), tetramethylluteolin (**5**), 5-hydroxy-3,7-dimethoxyflavone (**6**), 3,5,7-trimethoxy-flavone (**7**), 3,7,4′-trimethylkaempferol (**8**), tetramethylkaempferol (**9**), ayanin (**10**), retusine (**11**) และ pentamethylquercetin (**12**) จากการตรวจสอบทางพฤกษเคมีของชั้น chloroform พบว่า สาร **4** เป็นสารหลัก (13.05% w/w) และยับยั้งการสร้าง NO ได้ดีโดยมีค่า IC_{50} = 4.6 μg/ml, ตามด้วย สาร **2** และสาร **5** โดยมีค่า IC_{50} = 5.1 และ 8.7 μg/ml ตามลำดับ และไม่เป็นพิษต่อเซลล์ ยกเว้นสาร **2** ที่มีความเป็นพิษที่ความเข้มข้น 100 μg/ml ดังนั้น การต้านการอักเสบในเท้าหนูที่บวมอาจเนื่องจากการยับยั้งการสร้าง PGE_2 และ NO ของสารออกฤทธิ์เหล่านี้ และได้มีการตรวจสอบการยับยั้ง TNF-α ของสาร **2**, **4** และ **5** ผลการทดลองพบว่าสาร **2**, **4** และ **5** มีฤทธิ์ปานกลางในการยับยั้งการหลั่งของ TNF-α โดยมีค่า IC_{50} = >30, 64 และ 100 μg/ml ตามลำดับ

ผลของสาร **2**, **4** และ **5** ต่อการแสดงออกของ iNOS, COX-2, NFKB, Rel A และ TNF-α mRNA ในระดับของการถอดรหัสโดยวิธี semi-quantitative polymerase chain reaction (PCR) พบว่า 5,7-dimethoxyflavone (**2**) ยับยั้ง iNOS, COX-2 และ Rel A mRNA แต่ไม่ยับยั้ง NFKB และ TNF-α mRNA การวิเคราะห์เชิงคุณภาพด้วย real time PCR พบว่าสาร **2** มีผลต่อการแสดงออกของ iNOS mRNA โดยให้ค่าการ

แสดงออกเท่ากับ 97, 71 และ 18% ที่ความเข้มข้น 3, 10 และ 30 μg/ml ตามลำดับ อีกทั้งสารนี้ยังยับยั้งการแสดงออกของโปรตีน iNOS แต่ไม่ยับยั้ง phophorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) และ phosphorylated c-Jun N-terminal kinase 1/2 (p-JNK1/2) ซึ่งเป็น transcription factors ในกระบวนการอักเสบ สาร **2** ที่ความเข้มข้นสูง (100 μg/ml) รบกวนการสร้าง β-actin mRNA จึงไม่ปลอดภัยการนำมาใช้ที่ความเข้มข้นดังกล่าว

Trimethylapigenin (**4**) ยับยั้ง iNOS, COX-2, NFKB และ Rel A mRNA โดยการยับยั้ง COX-2 mRNA เป็นหลัก ซึ่งบ่งบอกได้ว่าการยับยั้งการบวมของเท้าหนูมีสาเหตุมาจากการยับยั้ง COX-2 mRNA เป็นผลให้ PGE_2 ลดลง สาร **4** มีผลต่อการแสดงออกของ iNOS mRNA โดยให้ค่าการแสดงออกเท่ากับ 73 and 13% ที่ความเข้มข้น 10 และ 30 μg/ml ตามลำดับ และสารนี้สามารถยับยั้ง iNOS โปรตีน แต่ไม่ยับยั้ง p-ERK1/2 และ p-JNK1/2 นอกจากนี้ สาร **4** สามารถยับยั้งการแสดงออกของ spleen tyrosine kinase (SYK, NFKB activator) ได้ 52% ที่ความเข้มข้นของสาร 30 μg/ml และเป็นรายงานครั้งแรกในการยับยั้ง SYK ของสารในกลุ่ม methoxyflavonoid

Tetramethylluteolin (**5**) ยับยั้งการแสดงออกของ Rel A และ iNOS mRNA แต่ไม่มีผลต่อ COX-2, NFKB และ TNF-α mRNA สาร **5** มีผลต่อการแสดงออกของ iNOS mRNA โดยให้ค่าการแสดงออกเท่ากับ 60, 21 และ 4% ที่ความเข้มข้นของสาร 3, 10 และ 30 μg/ml ตามลำดับ สารนี้ยังสามารถยับยั้ง iNOS และ p-ERK1/2 โปรตีน ที่ความเข้มข้นของสาร 100 μg/ml และมีผลรบกวนการสร้าง β-actin mRNA จึงไม่ปลอดภัยในการนำมาใช้ในขนาดสูง (100 μg/ml)

การศึกษานี้ สนับสนุนการใช้เหง้ากระชายดำในการรักษาการอักเสบ เช่น ฝี หนอง แผลอักเสบในลำไส้ และเก๊าท์ trimethylapigenin (**4**) ซึ่งเป็นสารหลักจากชั้น chloroform แสดงศักยภาพในการต้านการอักเสบที่ดีที่สุดโดยไม่เป็นพิษต่อเซลล์ ดังนั้น จึงมีความปลอดภัยและมีศักยภาพสูงที่จะนำมาพัฒนาเป็นยารักษาโรคที่เกี่ยวข้องกับการอักเสบ

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ABSTRACT

In the present study, it was found that the chloroform fraction of *Kaempferia parviflora* extract exhibited the most potent inhibitory activity against NO production with an IC_{50} of 8.4 µg/ml, followed by the hexane, water, and ethyl acetate fractions with IC_{50} of 13, 40 and 61 µg/ml, respectively. The hexane and chloroform fractions showed potent anti-inflammatory activity mainly through the inhibition of PGE_2 production by decreasing the rat hind-paw edema after 3 h injected by 1% carrageenan. The ethanol extract inhibited PGE_2 production with an IC_{50} of 9.6 µg/ml. Treated mice with ethanol extract and chloroform fraction, did not show any signs of toxicity in both male and female mice. Moreover, the chloroform fraction inhibited TNF-α production with an IC_{50} of 1.37 µg/ml and suppressed iNOS, COX-2, NFKB, Rel A and TNF-α mRNA expression.

The compounds from chloroform fraction were isolated using the open column chromatograpy (OCC) and purified by high performance liquid chromatography (HPLC). Twelve methoxyflavonoids were obtained: techtochrysin (**1**), 5,7-dimethoxyflavone (**2**), 7,4′-dimethylapigenin (**3**), trimethylapigenin (**4**), tetramethylluteolin (**5**), 5-hydroxy-3,7-dimethoxyflavone (**6**), 3,5,7-trimethoxyflavone (**7**), 3,7,4′-trimethylkaempferol (**8**), tetramethylkaempferol (**9**), ayanin (**10**), retusine (**11**) and pentamethylquercetin (**12**). Phytochemical investigation of the chloroform fraction showed that compound **4** was the major compound (13.05% w/w) that inhibited NO production with an IC_{50} of 4.6 µg/ml, followed by compounds **2** and **5** with IC_{50} of 5.1 and 8.7 µg/ml, respectively without cytotoxic effect except compound **2** showed cytotoxicity at concentration of 100 µg/ml. Therefore, the anti-inflammation in rat paw edema may be due to the suppression of PGE_2 and NO production of these active compounds. The inhibition of TNF-α of compounds **2**, **4**, and **5** was also examined. The results showed that compound **2**, **4**, and **5** moderately inhibited the release of TNF-α with IC_{50} of >30, 64 and 100 µg/ml, respectively.

The result on the expression of iNOS, COX-2, NFKB, Rel A and TNF-α mRNA in transcriptional level using semi-quantitative polymerase chain reaction (PCR) of compounds **2**, **4**, and **5** found that 5, 7-dimethoxyflavone **(2)** inhibited iNOS, COX-2 and Rel A mRNA, but not that of NFKB and

TNF-α. Quantitative real time PCR analysis showed that the expression of iNOS mRNA by compound **2** was at 97, 71 and 18% at concentrations of 3, 10 and 30 μg/ml, respectively. This compound also inhibited the translational level of iNOS protein expression, but not that of phophorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and phosphorylated c-Jun N-terminal kinase 1/2 (p-JNK1/2), the transcription factors in inflammation process. However, compound **2** at high dose interfered the β-actin mRNA expression which implied that it is not safe for use.

Trimethylapigenin (**4**) showed dose dependently inhibited iNOS, COX-2, NF**K**B and Rel A mRNA which was mainly through COX-2 mRNA. The expression of iNOS mRNA by compound **4** was at 73 and 13% at concentration of 10 and 30 μg/ml, respectively. This compound also inhibited iNOS protein, but not inhibited p-ERK1/2 and p-JNK1/2. Moreover, spleen tyrosine kinase (SYK), an NF**K**B activator, was inhibited by compound **4** with 52% inhibition at the dose of 30 μg/ml. This is the first report on the inhibition of SYK by methoxyflavonoid.

Tetramethylluteolin (**5**) inhibited Rel A and iNOS mRNA expression, while COX-2, NF**K**B and TNF-α were not affected. The expression of iNOS mRNA by compound **5** was at 60, 21 and 4% at the dose of 3, 10 and 30 μg/ml, respectively. This compound also inhibited iNOS protein and p-ERK1/2. The highest dose (100 μg/ml) interfered β-actin mRNA expression.

The present study supports the traditional use of *K. parviflora* for the treatment of inflammation including abscess, duodenal ulcer and gout. Trimethylapigenin (**4**) which is the main compound from the chloroform fraction exhibited the most potent anti-inflammatory activity without any cytotoxic effects. Therefore, trimethylapigenin is safe for use and has a high potential for development as an anti-inflammatory drug.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACh	=	Acetylcholine
AChE	=	Acetylcholineesterase
ADAM 17	=	A disintegrin and metalloprotease domain 17
AKT	=	A serine/threonine protein kinase
ALT	=	Alanine aminotransferase
AP-1	=	Activating protein-1
AST	=	Aspatate aminotransferase
BChE	=	Butyrylcholinesterase
BW	=	Body weight
BUN	=	Blood urea nitrogen
CAM	=	Calmodulin
CAPE	=	Caffeic acid phenethyl ester
c.a	=	Circa (about, approximately)
CBC	=	Complete blood cell count
cDNA	=	Complementary deoxyribonucleic acid
CD 14	=	Cluster of differentiation 14
cGMP	=	Cyclic guanosine monophosphate
CHUK	=	Conserved helix-loop-helix ubiquitous kinase
COX-2	=	Cyclooxygenase-2
Cr	=	Creatinine
d	=	Doublet (for NMR signals)
DBD	=	DNA binding domain
DMEM	=	Dulbecco's modified Eagle's medium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
ECL	=	Enhanced chemiluminescence
EDRF	=	Endothelium-derived relaxing factor
EDTA	=	Ethylenediamine tetraacetic acid
Eg-1	=	Early growth response 1 protein
eNOS	=	Endothelial nitric oxide synthase

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

ERK	=	Extracellular signal-regulated kinase
et al	=	Et alibi
FBS	=	Fetal bovine serum
g	=	Gram
GTP	=	Guanosine triphosphate
h	=	Hour
IC_{50}	=	50% Inhibitory concentration
IF	=	Intromission frequency
IFN-γ	=	Interferon-γ
IGF1R	=	Insulin-like growth factor 1 receptor
IKBKB	=	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IKBKE	=	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
IKK	=	Inhibitor of nuclear factor kappa-B kinase
IL	=	Intromission latency
IL-1	=	Interleukin-1
IKB	=	Inhibitor of kappa-light-chain-enhancer of activated B cells
iNOS	=	Inducible nitric oxide synthase
i.p.	=	Intraperitoneal
IRAK	=	Interleukin-1 receptor-associated kinase
IRAK	=	Interleukin-1 receptor-associated kinase 4
J	=	Coupling constant
JAK 1	=	Janus kinase 1
JNK	=	c-Jun N-terminal kinase
Kg	=	Kilogram
L	=	Liter
LD_{50}	=	Lethal dose at 50%
LPS	=	Lipopolysaccharide
m	=	Meter

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

m/z	=	Mass-over-charge ratio
MAPK	=	Mitogen-activated protein kinase
MAPKAPK 2	=	Mitogen-activated protein kinase-activated protein kinase 2
MAP4K5	=	Mitogen-activated protein kinase kinase kinase kinase 5
MEK1/2	=	Mitogen-activated MAPK/ERK kinase 1/2
MF	=	Mount frequency
ML	=	Mount latency
MSRs	=	Murine scavenger receptors
mg	=	Milligram
MIC	=	Minimun inhibitory concentration
min	=	Minute
ml	=	Milliliter
mRNA	=	Messenger ribonucleic acid
MTT	=	3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazodium bromide
mult	=	Multiplicity
MyD88	=	Myeloid differentiation primary response gene 88
NADP	=	Nicotinamide adenine dinucleotide phosphate
NADPH	=	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	=	Sodium hydrogen carbonate
NANC	=	Non adrenergic, non cholinergic
NEK 1	=	NIMA (never in mitosis gene A)-related kinase 1
NFKB	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	=	Nuclear factor-kappa-beta-inducing kinase
nNOS	=	Neuronal nitric oxide synthase
NO	=	Nitric oxide
No.	=	Number
NOS	=	Nitric oxide synthase
NMR	=	Nuclear magnetic resonance

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

$ONOO^-$	=	Peroxynitrite
PBS	=	Phosphate-buffered saline
PCK	=	Protein kinase C
PDK 1	=	Pyruvate dehydrogenase kinase isozyme 1
PE	=	Phenylephrine
PGE_2	=	Prostaglandin E_2
pH	=	Potential of hydrogen
PI3K	=	Phosphoinositide 3-kinase
PKA	=	Protein kinase A
PKK	=	Protein kinase C-associated kinase
PKR	=	Protein kinase ribonucleic acid-activated
p.o.	=	Per oral
PRKACA	=	Protein kinase, cAMP-dependent, catalytic, alpha
PVDF	=	Polyvinylidene fluoride
RH	=	Rel homology
RPMI	=	Roswell park memorial institute
RT-PCR	=	Reverse transcription-polymerase chain reaction
s	=	Second
s.c.	=	Subcutaneous
S.E.M.	=	Standard error of the mean
sscDNA	=	Single-stranded complementary DNA
SYK	=	Spleen tyrosine kinase
TACE	=	Tumor necrosis factor –alpha-converting enzyme
TAD	=	Transactivation domain
TBK 1	=	TANK (TRAF family member associated NFKB activator)-binding kinase 1
TLR4	=	Toll-like receptor 4
TNF-α	=	Tumor necrosis factor alpha
TRAF 6	=	Through tumor necrosis factor receptor associated factor 6
TRD	=	Transrepression domain

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

T-TBS	=	Tris-tween buffer solution
µg	=	Microgram
µl	=	Microliter
µM	=	Micromolar
w/v	=	Weight by volume
w/w	=	Weight by weight
ZAP70	=	Zeta-chain-associated protein kinase 70
ºC	=	Degree celsius
/	=	Per
%	=	Percentage
®	=	Registered trade name
δ	=	Chemical shift in ppm

CHAPTER 1

INTRODUCTION

1. Background and Rationale

Inflammation is the body's response to tissue injury (Gould, 2002). Inflammation occurs as a defensive response, which induces profound physiological adaptions triggered in an attempt to limit tissue damage and remove the pathogenic insult. Such mechanisms involve a complex series of events including dilatation of arterioles, venules and capillaries with increased vascular permeability, exudation of fluids including plasma proteins and leukocyte migration into the inflammatory area. Although inflammation is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can be included, maintained or aggravated by many diseases (Gupta et al., 2003). Since disease characterized by inflammation is an important cause of morbidity and mortality in humans, the processes involved in the host defense in inflammation have been and continue to be the object of several experimental studies. The role of several mediators such as histamine, serotonin, bradykinin, prostaglandins and more recently, cytokines and nitric oxide have been evaluated, and a contribution for each one of these mediators has been proposed (Cuzzocrea, 2006).

Drugs of natural origin continue to be important for the treatment of many diseases worldwide and are believed to be an important source of new chemical substances with potential therapeutic effects. The investigation of the efficacy of plant-based drugs used in the traditional medicine has been paid great attention because they are cheap and have little side effects.

In the present study, isolated compounds, ethanol extract and fractions of *K. parviflora* were tested since it is commonly used in folklore medicine for treatment of gout, apthous ulcer, peptic ulcer and abscesses in patients of Thailand. Therefore, it is a great value to evaluate their effects on anti-inflammatory activities to confirm its therapeutic efficacy and to clarify anti-inflammatory mechanism of compounds isolated from this plant**.**

2. Objectives

1. To investigate anti-inflammatory activity of *Kaempferia parviflora* extract on both *in vitro* and *in vivo* assays
2. To purify and identify the pharmacological active compounds from *Kaempferia parviflora* rhizomes
3. To evaluate the effect of isolated compounds for anti-inflammatory activity of *Kaempferia parviflora* in murine macrophage cell line (RAW 264.7)
4. To study on anti-inflammatory mechanism of *Kaempferia parviflora* extract and its isolated compounds

CHAPTER 2

REVIEW OF LITERATURE

1. Botanical description of *Kaempferia parviflora* Wall. ex Baker

The genus *Kaempferia* L. (Zingiberaceae) is one of the important medicinal plant groups in Thailand. Many taxa are known locally for their medicinal properties (Picheansoonthon and Koonterm, 2009). It is a medium-sized genus with approximately 50 species, mostly distributed from India, South China, to Malaysia (Mabberley, 1993). The family Zingiberaceae is a large, important and well-known monocot family (ginger plants) that is conspicuous throughout the tropics. It comprises approximately 52 genera and 1400 species, with the centre of diversity being in South/Southeast Asia. In Thailand, there are 26 genera with more than 300 species of these plants (Larsen and Larsen 2006; Wichchulada et al., 2010) and sixteen taxa were previously accounted for Thailand (Sirirugsa, 1992; Picheansoonthon and Koonterm, 2009).

Kaempferia parviflora Wall. ex Baker, locally known in Thai as Kra-chai-dam (Figure 1) belongs to the Zingiberaceae family and sometimes referred to as Thai ginseng. This plant is a perennial ground herb that grows to 90 cm high with dark purple to black rhizomes with brown outside, and unique odor. Storage roots are blotchy while young plants have thin root which grow to be tuber, the color of light purple to black leads to the name Kra chai dam (Putiyanan et al., 2004).

Among local people in the Northeast of Thailand, the rhizomes of *K. parviflora* have been known as health-promoting herbs, and also frequently used for the treatment of colic disorder, peptic and duodenal ulcers. In Thailand, a tonic drink made from the rhizomes of *K. parviflora* is commercially available, and is believed to relieve impotent symptoms (Yenjai et al., 2004). Its rhizomes have been traditionally used in Thai folklore medicine for treatment of leucorrhea, oral diseases (Chomchalow et al., 2003; Sudwan et al., 2006), stomachache, flatulence, digestive disorders, gastric ulcer as well as diuresis and tonic (Wattanapitayakul et al., 2008).

(A) **(B)**

Figure 1 *Kaempferia parviflora* Wall. ex Baker**.** Whole plant **(A)**, Rhizomes **(B)**

2. Chemical constituents

The chemical analyses of different fractions of *K. parviflora* extracts showed that hexane and alcohol extracts yielded phenols and flavonoids but at different percentages. Phenols are the major component of the alcohol extract while flavonoids are present mainly in the hexane extract. The water extract contains only small amounts of phenols and flavonoids (Sutthanut et al., 2007; Chaturapanich et al., 2008).

Table 1 Chemical constituents found in various fractions of *K. parviflora*

Fraction	Constituent	Reference
Hexane	5-Hydroxy-3,7-dimethoxyflavone	Sawasdee et al., 2009
	Tectochrysin	Sawasdee et al., 2009
	5-Hydroxy-3,7-dimethoxyflavone	Tewtrakul et al., 2009
	5-Hydroxy-7-methoxyflavone	Tewtrakul et al., 2009
	5-Hydroxy-3,7,4'-trimethoxyflavone	Tewtrakul et al., 2009
	5-Hydroxy-7,4'-dimethoxyflavone	Tewtrakul et al., 2009
	5-Hydroxy-3,7,3',4'-tetramethoxyflavone	Tewtrakul et al., 2009
	3,5,7-Trimethoxyflavone	Tewtrakul et al., 2009
	3,5,7,4'-Tetramethoxyflavone	Tewtrakul et al., 2009

Table 1 Chemical constituents found in various fractions of *K. parviflora* (continued)

Fraction	Constituent	Reference
CH_2Cl_2	5-Hydroxy-7,3',4'-trimethoxyflavone	Sawasdee et al., 2009
	3,5,7-Trimethoxyflavone	Sawasdee et al., 2009
	3,5,7-Trimethoxyflavone	Azuma et al., 2008
	5-Hydroxy-7,3',4'-trimethoxyflavone	Sawasdee et al., 2009
	5- Hydroxy-7,3',4'-trimethoxyflavone	Azuma et al., 2008
	3,5,7,4'-Tetramethoxyflavone	Sawasdee et al., 2009
		Azuma et al., 2008
	5,7-Dimethoxyflavone	Sawasdee et al., 2009
	3,5,7,3',4'-Pentamethoxyflavone	Sawasdee et al., 2009
	3,5,7,3',4'-Pentamethoxyflavone	Azuma et al., 2008
	Genkwanin	Sawasdee et al., 2009
	5,7,4'-Trimethoxyflavone	Sawasdee et al., 2009
	5,7,4'-Trimethoxyflavone	Azuma et al., 2008
	5-Hydroxy-3,7-dimethoxyflavone	Azuma et al., 2008
	5-Hydroxy-3,7,4'-trimethoxyflavone	Azuma et al., 2008
	5-Hydroxy-7,4'-dimethoxyflavone	Azuma et al., 2008
	5-Hydroxy-3,7,3',4'-tetramethoxyflavone	Azuma et al., 2008
	5,3'-Dihydroxy-3,7,4'-trimethoxyflavone	Azuma et al., 2008
	5,7-Dimethoxyflavone	Azuma et al., 2008
	β-Sitosteryl myristate	Azuma et al., 2008
	(2*S*)-5-Hydroxy-7-methoxyflavanone	Azuma et al., 2008
	(*E*)-2'-Hydroxy-4',6'-dimethoxychalcone	Azuma et al., 2008
	(1*E*,6*E*)-1,7-Diphenyl-1,6-heptadiene-3,5-dione	Azuma et al., 2008
	5-Hydroxy-7-methoxyflavone	Azuma et al., 2008
	(2*S*)-5,7-Dimethoxyflavanone	Azuma et al., 2008

Table 1 Chemical constituents found in various fractions of *K. parviflora* (continued)

Fraction	Constituent	Reference
EtOAc	4'-Hydroxy-5,7-dimethoxyflavone	Azuma et al., 2008
	5,7,3',4'-Tetramethoxyflavone	Azuma et al., 2008
	1-*O*-β-Glucopyranosyl-(8*Z*)-2-(2-hydroxy tetracosanoylamino)-8-octadecene-1,3,4-triol	Azuma et al., 2008
	1-*O*-hexadecanoyl-3-*O*-[α-galactopyranosyl-(1$\rightarrow$6)-β -galactopyranosyl]-glycerol	Azuma et al., 2008
	1-*O*-Hexadecanoyl-2-*O*-(9*Z*,12*Z*-octadeca dienoyl)-3-*O*-[α-galactopyranosyl-(1$\rightarrow$6)-β-galactopyranosyl]-glycerol	Azuma et al., 2008
	Methyl 9*Z*,12*Z*-octadecadienoate 1-*O*-(9*Z*,12*Z*-octadecadienoyl)-3-*O*-[agalactopyranosyl-(1$\rightarrow$6)-β-galactopyranosyl]-glycerol	Azuma et al., 2008
H_2O	Isorhamnetin 3-*O*-[α-rhamnopyranosyl-(1$\rightarrow$6)-β-glucopyranoside]	Azuma et al., 2008
	Quercetin -3-*O*-[α-Rhamnopyranosyl-(1$\rightarrow$6)-β-glucopyranoside]	Azuma et al., 2008
	Rel-(5a*S*,10b*S*)-5a,10b-dihydro-1,3,5a,9-tetrahydroxy-8-methoxy-6*H*-benz[*b*]indeno [1,2-*d*]furan-6-one 5a-*O*-[α-*L*-rhamnopyra nosyl-(1$\rightarrow$6)-β-D-glucopyranoside]	Azuma et al., 2008
	Rel-(5a*S*,10b*R*)-5a,10b-dihydro-1,3,5a,9-tetrahydroxy-8-methoxy-6*H*-benz[*b*]indeno [1,2-*d*]furan-6-one 5a-*O*-[α-*L*-rhamno pyranosyl-(1$\rightarrow$6)-β-*D*-glucopyranoside]	Azuma et al., 2008
	(2*R*,3*S*,4*S*)-3-*O*-[α-*L*-Rhamnopyranosyl-(1$\rightarrow$6)-β-*D*-glucopyranosyl]- 3'-*O*-methyl-ent-epicatechin-(2 $\alpha\rightarrow O\rightarrow$3,4 $\alpha\rightarrow$4)-(5a*S*,10b*S*)- 5a,10b-dihydro-1,3,5a,9-tetrahydroxy-8-methoxy-6*H*-benz[*b*]indeno-[1,2-*d*] furan-6-one 5a-*O*-[α-*L*-rhamnopyranosyl-(1$\rightarrow$6)-β-*D*-glucopyranoside]	Azuma et al., 2008

3. Pharmacological activities

3.1 Anti-allergic activity

It was found that 5-hydroxy-3,7,3',4'-tetramethoxyflavone isolated from *K. parviflora* ethanolic extract possessed the highest anti-allergic activity against antigen-induced β-hexosaminidase release with an IC_{50} value of 8.0 μM, followed by 5-hydroxy-7-methoxyflavone (IC_{50} = 20.6 μM) and 5-hydroxy-7,4'-dimethoxyflavone (IC_{50} = 26.0 μM). The results indicated that the mechanism on inhibition of cell degranulation of compounds 5-hydroxy-3,7,3',4'-tetramethoxyflavone and 5-hydroxy-7-methoxyflavone mainly involve the inhibition of Ca^{2+} influx to the cells, whereas 5-hydroxy-7,4'-dimethoxyflavone may be partly due to this inhibition (Tewtrakul et al., 2007).

3.2 Anti-inflammatory activity

Tewtrakul and Subhadhirasakul (2008) reported that the hexane fraction of *K. parviflora* possessed anti-inflammatory effect through the inhibition of NO and PGE_2 releases, but partly due to that of TNF-α. The result suggested that the flavones isolated from this plant might involve in the suppression of iNOS and COX-2 genes. The anti-inflammatory mechanism in transcriptional level of active flavones from *K. parviflora* is still unknown.

3.3 Vasorelaxation and antispasmodic effects

Ethanolic extract of *K. parviflora* dose-dependently induced vascular and ileal relaxation in precontracted isolated tissue preparations. The spasmolytic effect of *K. parviflora* was not initiated by competitive binding at muscarinic receptors since no rightward shift of acetylcholine (ACh) dose–response curves was observed. Similar to the studies in aortic rings, it appears that *K. parviflora* antagonizes the given contractile stimuli phenylephrine (PE) and ACh with no specificity to their receptors and it may act through multiple mechanisms of actions (Wattanapitayakul et al., 2008).

3.4 Effects of *K. parviflora* extracts on reproductive parameters and spermatic blood flow

It has been reported on the effects of feeding three different *K. parviflora* extracts (alcohol, hexane, and water extracts) for 3-5 weeks on the reproductive organs, the aphrodisiac activity, fertility, sperm motility, and blood flow to the testis of male rats. Sexual performances (mount latency, mount frequency, ejaculatory latency, postejaculatory latency) and spermmotility were assessed by a video camera and computer-assisted spermanalysis respectively, while blood flow to the testis was measured by a directional pulsed Doppler flowmeter. The results showed that all extracts of *K. parviflora* had virtually no effect on the reproductive organ weights even after 5 weeks. However, administration of the alcohol extract at a dose of 70 mg/kg body weight (BW)/day for 4 weeks significantly decreased mount and ejaculatory latencies when compared with the control. Hexane and water extracts had no influence on any sexual behavior parameters. All types of extracts of *K. parviflora* had no effect on fertility or spermmotility. Alcohol extract produced a significant increase in blood flow to the testis without affecting the heart rate and mean arterial blood pressure. In a separate study, an acute effect of alcohol extract of *K. parviflora* on blood flow to the testis was investigated. Intravenous injection of *K. parviflora* at doses of 10, 20, and 40 mg/kg BW caused dose-dependent increases in blood flow to the testis. The results indicate that alcohol extract of *K. parviflora* had an aphrodisiac activity probably via a marked increase in blood flow to the testis (Chaturapanich et al., 2008).

3.5 Anti-gastic ulcer activity

Pretreatment rats with ethanol extract of *K. parviflora* at doses of 60 and 120 mg/kg but not 30 mg/kg significantly increased the amount of gastric mucus content in HCl/EtOH-ulcerated rats. *K. parviflora* failed to increase the gastric pH and decrease the gastric volume and acidity in pylorus-ligated rats suggests that anti-secretory action is unlikely ascribed to the anti-gastric ulcer effect of the *K. parviflora*. Hence, the gastroprotective effect of the *K. parviflora* is mediated only partly by preservation of gastric mucus secretion (Rujjanawate et al., 2005).

3.6 Anticholinesterase activity

K. parviflora methanol extract exhibited significant acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities. Among 11 flavones were examined, the most potent compounds toward AChE and BChE were 5,7,4′-trimethoxyflavone and 5,7-dimethoxyflavone, respectively. 5-hydroxy-3,7-dimethoxyflavone**,** tectochrysin, 5-hydroxy-3,7,4′-trimethoxyflavone, 5-hydroxy-3,7,3′,4′-tetramethoxyflavone and 3,5,7-trimethoxyflavone were not able to inhibit either AChE or BChE at 0.1 mg/ml, while 3,5,7,4′-tetramethoxyflavone, 3,5,7,3′,4′-pentamethoxyflavone, 5-hydroxy-7,4′-dimethoxyflavone, 5,3′-dihydroxy-3,7,4′-trimethoxyflavone showed low activities. Interestingly, 5,7-dimethoxyflavone exhibited strong selectivity for BChE over AChE which may be of great interest to modify as a treatment agent for Alzheimer's disease (Sawasdee et al., 2009).

3.7 Antiplasmodial activity

The study on anti plasmodial activity using microdilution radioisotope technique found that 5,7,4'-trimethoxyflavone and 5,7,3',4'-tetramethoxyflavone isolated from *K. parviflora* exhibited antiplasmodial activity against *Plasmodium falciparum*, with IC_{50} values of 3.70 and 4.06 µg/ml, respectively (Yenjai et al., 2004).

3.8 Antifungal activity

Antifungal activity was evaluated employing the modified formazan assay. It was found that 3,5,7,4'-tetramethoxyflavone and 5,7,4'-trimethoxyflavone isolated from *K. parviflora* rhizomes possessed antifungal activity against *Candida albicans* with respective IC_{50} values of 39.71 and 17.63 µg/ml (Yenjai et al., 2004).

3.9 Antimycobacterial activity

Antimycobacterial activity testing was followed the Micro Alamar Blue assay. Isolation of 3,5,7,4'-tetramethoxyflavone and 5,7,4'-trimethoxyflavone from rhizomes of *K. parviflora* showed mild antimycobacterial activity with the minimum inhibitory concentrations (MIC) of 200 and 50 µg/ml, respectively (Yenjai et al., 2004).

3.10 Toxicological study

K. parviflora extract treated male rats at doses of 60, 120, and 240 mg/kg BW and distilled water treated (control group) 1 ml/day per oral showed no significant difference body weight gain. All groups had a significant increase in body weight. The complete blood cell count (CBC) in all treated groups was not different from the controls. The male rats in all groups receiving *K. parviflora* extract had no significant difference in alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine (Cr), whereas the levels of aspartate aminotransferase (AST) of the male rats receiving *K. parviflora* extract at doses of 60 and 240 mg/kg were significantly lower than the control group (Sudwan et al., 2006).

Cytotoxicity was tested employing the colorimetric technique. Isolated flavonoids from *K. parviflora* rhizomes possessed no cytotoxicity against KB (oral human epidermoid carcinoma), BC (breast cancer) and NCI-H187 (human, small cell lung cancer) cell lines, and this cytotoxic information suggests that the rhizomes of *K. parviflora* may be safe when using as an ingredient in traditional medicine (Yenjai et al., 2004).

3.11 Sexual behavior

K. parviflora was administered to male rats to determine its effects on rat sexual behavior. No dose of *K. parviflora* produced any significant change in courtship behavior, mount frequency (MF), intromission frequency (IF), mount latency (ML) or intromission latency (IL) in the male rats. The courtship behavior in the first 10-minutes'observation of the male rats receiving 240 mg/kg BW was significantly lower than the control group. These results showed that *K. parviflora* extract did not enhance the sexual behaviors (courtship behavior, MF, IF, ML and IL) of male rats (Sudwan et al., 2006).

Since ancient time, *K. parviflora* has traditionally been used as a health promoting, stimulating and vitalizing agent (Yenjai et al., 2004; Sennil and Trichale, 2003; Vichitphan et al., 2007) and in Thai traditional medicine, the decoction of *K. parviflora* powder with alcohol has been reported to cure allergy, asthma, impotence, gout, diarrhea, dysentery, peptic ulcer, diabetes (เพิ่งเจริญ, 2002; Tewtrakul et al., 2009), stomachache, flatulence, digestive disorders, gastric ulcer and act as diuresis and tonic (Yenjai et al., 2004; Rujjanawate et al., 2005; Wattanapitayakul et al., 2007). Recently, various

bioactivities of extracts of *K. parviflora* and/or methoxyflavones isolated from this plant have been reported (Table 2 and Table 3).

Table 2 Pharmacological activities of the extract and fractions from *K. parviflora* rhizomes

Category	Pharmacological activity	Reference
Ethanol	Anti-gastric ulcer	Rujjanawate et al., 2005
	Sexual enhancement	Sudwan et al., 2006
	Anti-allergy	Tewtrakul and Subhadhirasakul, 2007
	Promote nitric oxide production	Wattanapitayakul et al., 2007
	Induced vascular and ileal relaxation	Wattanapitayakul et al., 2008
	Increase aphrodisiac activity	Chaturapanich et al., 2008

Table 3 Chemical constituents and pharmacological activities of methoxyflavones isolated from *K. parviflora* rhizomes

Pharmacological activity	Constituent	Reference
Anti-plasmodial	5,7,4'-Trimethoxyflavone	Yenjai et al., 2004
	5,7,3',4'-Tetramethoxyflavone	Yenjai et al., 2004
Anti-fungal	3,5,7,4'-Tetramethoxyflavone	Yenjai et al., 2004
	5,7,4'-Trimethoxyflavone	Yenjai et al., 2004
	5,7-Dimethoxyflavone	Yenjai et al., 2007
Anti-mycobacterial	3,5,7,4'-Tetramethoxyflavone	Yenjai et al., 2004
	5,7,3',4'-Tetramethoxyflavone	Yenjai et al., 2004
	5,7-Dimethoxyflavone	Yenjai et al., 2007
Inhibit P-glycoprotein	3,5,7,3',4'-Pentamethoxyflavone	Patanasehtanont et al., 2006
	5,7-Dimethoxyflavone	Patanasehtanont et al., 2006
Anti-allergy	5-Hydroxy-3,7,3',4'-tetramethoxyflavone	Tewtrakul et al., 2007
	5-Hydroxy-7-methoxyflavone	Tewtrakul et al., 2007
	5-Hydroxy-7,4'-dimethoxyflavone	Tewtrakul et al., 2007

4. Inflammation

4.1 Inflammation

Inflammation is the body's response to tissue injury (Gould, 2002) which clinically defined as a pathophysiological process characterized by redness, edema, fever, pain and loss of function. It is a defense system employed by organism to protect them from pathogenic invaders, to clean up damaged cell after injury and to prevent further damage. The main purpose of inflammation is to identify and eliminate injurious agents and to repair the surrounding tissue. The inflammation response involves several stages as follow;

1. Dilation of capillaries to increase blood flow
2. Microvascular structural change and escape of plasma proteins from the blood stream
3. Leukocyte-adhesion cascade
4. Elimination of possible pathogens
5. The resolution of inflammation

In the pathogenesis of several diseases, such as rheumatoid arthritis, inflammatory bowel diseases, artherosclerosis, multiple sclerosis, Alzheimer diseases, and transplant rejection, it is found that inflammation-mediated tissue injury plays an important role.

Inflammation is a defense reaction of the organism and its tissue to injurious stimuli that leads to the local accumulation of plasmatic fluid and blood cells. Although it is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can be included, maintained or aggravated by many diseases (Gupta et al., 2003).

4.2 Cause of inflammation

Inflammation is associated with many different types of tissue injury. Causes include direct damage (cuts, sprains), chemicals (acids), ischemia and cell necrosis or infarction, allergic reactions, physical agents (thermal injuries or burns, radiation), foreign bodies (splinters or dirt) and infection (Gould, 2002). Inflammation is grouped into two basic forms. Acute inflammation is of relatively short duration, lasting from a few minutes up to a few days, and is characterized by fluid and plasma protein exudation, and by a predominantly neutrophilic leukocyte accumulation. Chronic

inflammation is of longer duration (days to years) and is manifested histologically by influx of lymphocytes and macrophages and by tissue destruction and repair; the latter is associated with vascular proliferation and fibrosis (Kumar et al., 1997).

5. Nitric oxide (NO) and nitric oxide synthase (NOS)

NO is a small gaseous signaling molecule and short-lived free radical and a very small compound that diffuses freely within cells from its site of formation to its site of action (Aktan, 2003). NO is involved in various physiological and pathophysiological conditions and has both detrimental and beneficial effects in the human body. NO regulates vasorelaxation and platelet aggregation. It acts as a neurotransmitter in NANC (non adrenergic, non cholinergic) nerves and in the central nervous system. NO regulates neutrophil activation and cell growth and induces apoptosis. NO has an important role in host defence mechanisms. It mediates the toxicity of natural killer cells and regulates T cell activation. Nitrosylation of proteins by NO driven radicals regulates protein activity and function (Korbut and Guzik, 2005). In inflammation, NO modulates various vascular and cellular responses (Moilanen et al., 1999; Ricciardolo et al., 2004) and arthritis (Cuzzocrea, 2006; Vuolteenaho et al., 2007). NO is the factor regulating the relaxation of the endothelial smooth muscle cells which was first described as the endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980).

NO can be generated by three different forms of nitric oxide synthases (NOS): inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS). These enzymes catalyze L-arginine into L-citrulline and nitric oxide in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) (Figures 2 and 3) (Karpuzoglu and Ahmed, 2006).

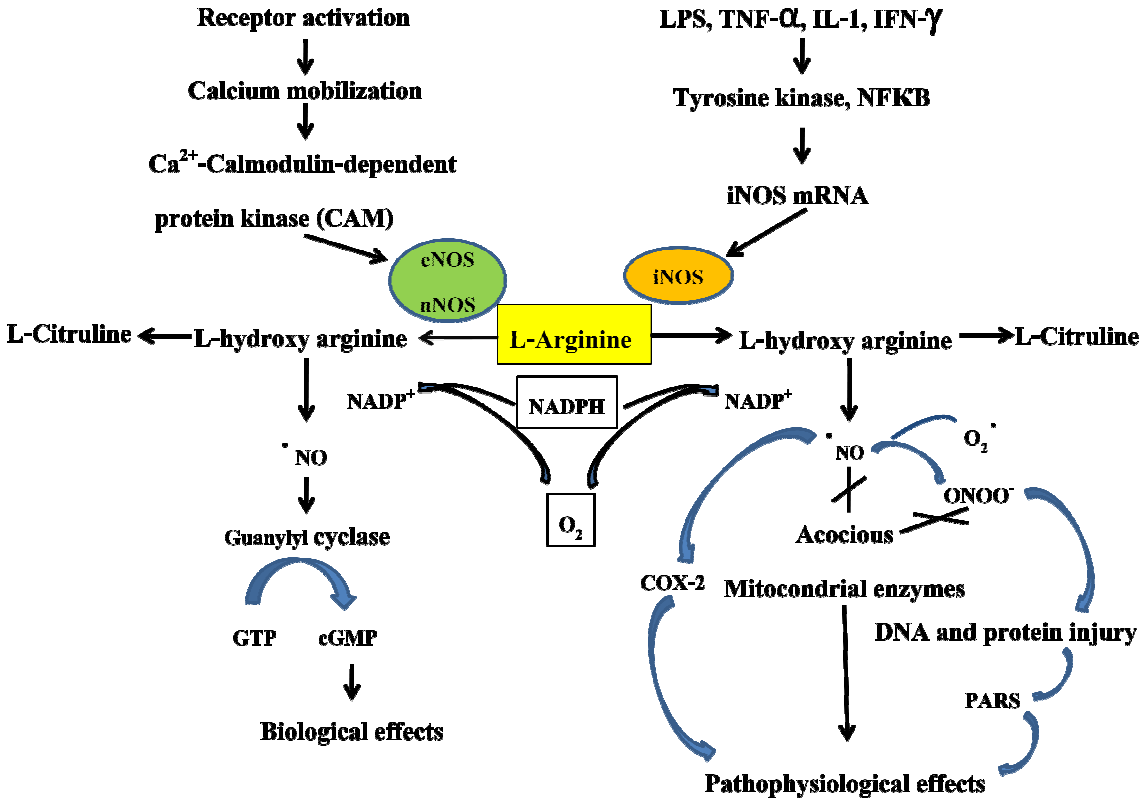


Figure 2 Synthesis of nitric oxide by NOS (Al-Sa□Doni and Ferro, 2000)

The endothelial nitric oxide synthase (eNOS) is present in endothelium, which is known to play an important role in the dynamic control of vascular tone. The neural nitric oxide synthase (nNOS) is mainly presents in neural tissue and serves as a neurotransmitter. eNOS and nNOS are constitutive isoforms of nitric oxide synthase and are also known as cNOS. The third nitric oxide synthase (iNOS) is induced by either bacterial lipopolysaccharide (LPS) or a number of cytokines including tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) in macrophages, hepatocytes, and endothelial cells. iNOS and nitric oxide are involved in host defense and immunity and modulate the inflammatory response (Vane et al., 1994; Jang and Murrell, 1998; Wang and Mazza, 2002). Key similarities and dissimilarities among the three NOS isoforms (nNOS, eNOS, and iNOS) are summarized in Table 4 (Karpuzoglu and Ahmed, 2006).

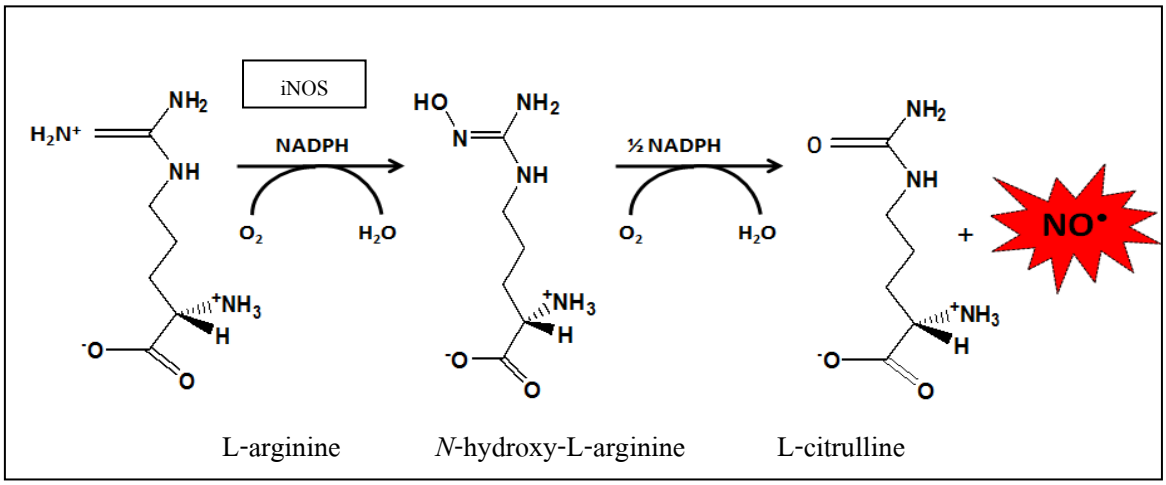


Figure 3 Nitric oxide generation from L-arginine by iNOS (Eijk et al., 2006)

Table 4 Biological comparison of three known isoforms of nitric oxide synthases (Karpuzoglu and Ahmed, 2006)

Characteristics	nNOS	eNOS	iNOS
Primary tissue or cellular source	Brain, peripheral (PNS) and central nervous system (CNS), and skeletal muscles.	Vascular endothelial cells, cardiomyocytes	Macrophages
Known functions	Neuromodulator in brain neurotransmittor in PNS regulation of smooth muscle activity and neuroendocrine functions in skeletal muscles	Vasodilator regulation of blood flow and pressure	Activation of APCs against infections and microbes cell death
Expression	Constitutively present	Constitutively present	Inducible
Concentration	In picomolar range	In picomolar range	In nanomolar range
	Calcium dependence	Ca^{2+} dependent	Ca^{2+} dependent
		Utilized only small	Quantities of Ca^{2+}.
			Mostly considered as Ca^{2+} independent
Sub-cellular location	Cytosol (not yet comfirmed)	Found in caveolae of membrane bound to calveolin-1	Cytoplasm
Association of dysregulated levels of nitric oxide with disorders	Dysregulation associated with neurodegenerative disorders	Hypertension, hypercholesterolemia, diabetes, heart failure. At low concentrations prevents apoptosis of endothelial cells, inhibits platelet aggregation, and smooth muscle proliferation	Infections, rheumatoid arthritis, Crohn's disease, asthma, septic shock

6. Prostaglandin E_2 (PGE_2) and cyclooxygenase-2 (COX-2)

Prostaglandin E_2 (PGE_2) is well known to be involved in the development of inflammation (Moncada et al., 1991; Lee et al., 1992; Nathan, 1997; Wheeler and Bernard, 1999; Sautebin, 2000). This inflammatory mediators is associated with the expression cyclooxygenase (COX). Two types of COX, constitutive cyclooxygenase-1 (COX-l) and inducible cyclooxygenase-2 (COX-2), have been isolated and characterized (Smith et al., 1996). The recognition that there are two cyclo-oxygenase enzymes, one predominating at sites of inflammation (COX-2) and one constitutively expressed in the gastrointestinal tract (COX-1), has led to the important therapeutic development of COX-2 inhibitors (Hawkey, 2001). COX-2 is induced by cytokines and outer bacterial toxins such as lipopolysaccharide (LPS) and lipoteichoic acid (Penglis et al., 2000; Yamashita et al., 2000). Besides, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 are interlinked with the production of small inflammatory mediators like NO and PGE_2, and thus contribute in eliciting inflammatory response (Feldmann et al., 1996; Dinarello, 1999; Mannel and Echtenacher, 2000; Straub et al., 2000). Accordingly, agents that block bacterial toxin-induced production of NO, PGE_2 or pro-inflammatory cytokines might be beneficial in the treatment of inflammatory responses. PGE_2 has a role in increasing vascular permeability, fever generation, and hyperalgesia. The importance of this function is highlighted by the wide clinical use of cyclooxgenase inhibitors to relieve inflammation (Chizzolini and Brembilla, 2009).

7. TNF-α

Tumour necrosis factor-alpha (TNF-α) is a multi-functional cytokine that can regulate many cellular and biological processes such as immune function, cell differentiation, proliferation, apoptosis and energy metabolism (Cawthorn and Sethi, 2008). TNF-α is a potent proinflammatory cytokine released primarily from stimulated macrophages. TNF-α was originally identified and isolated for two known characteristic activities: the ability to induce hemorrhagic necrosis of certain tumours and the ability to induce cachexia during states of chronic infection (Beutler and Cerami, 1989; Crisafulli and Galuppo, 2009). TNF-α now represents a key mediator of inflammatory responses. Many aspects of tissue damage following acute or chronic inflammatory reactions can be directly attributed to the concomitant induction of TNF biosynthesis and release, and provide the therapeutic rationale for developing TNF antagonists (Tracey and Cerami, 1993; Crisafulli and Galuppo, 2009).

LPS (coming from medium) bind to Toll-like receptor4 (TLR4). Binding activates two main pathways in the cells. One pathway leads to the activation of NFKB (through TNF receptor-associated factor 6 (TRAF6) and IKB kinase (IKK)). The other pathway passes through phosphorylated p38 MAP Kinase. Both pathways enable the activation of TNF-α transcription, followed by cleavage of the protein via a membrane metalloprotease, a disintegrin and metalloprotease domain 17 (ADAM17), also called tumor necrosis factor-α-converting enzyme (TACE), leading to the release of the soluble form of TNF-α. The phosphoinositide 3-kinase (PI3K) represents a third pathway, which activates NFKB and p38 MAPK. Another kinase, maybe the PI4K, plays an inhibitory role in the LPS activation of these two pathways. As far as the protein kinase C (PKC) is concerned, it probably activates IKK, or the p38 MAPKs, or both pathways. However, activation is only visible once the PI4K is inactive. It is therefore possible that PI4K constitutively inhibits PKC (Hoareau et al., 2010) (Figure 4).

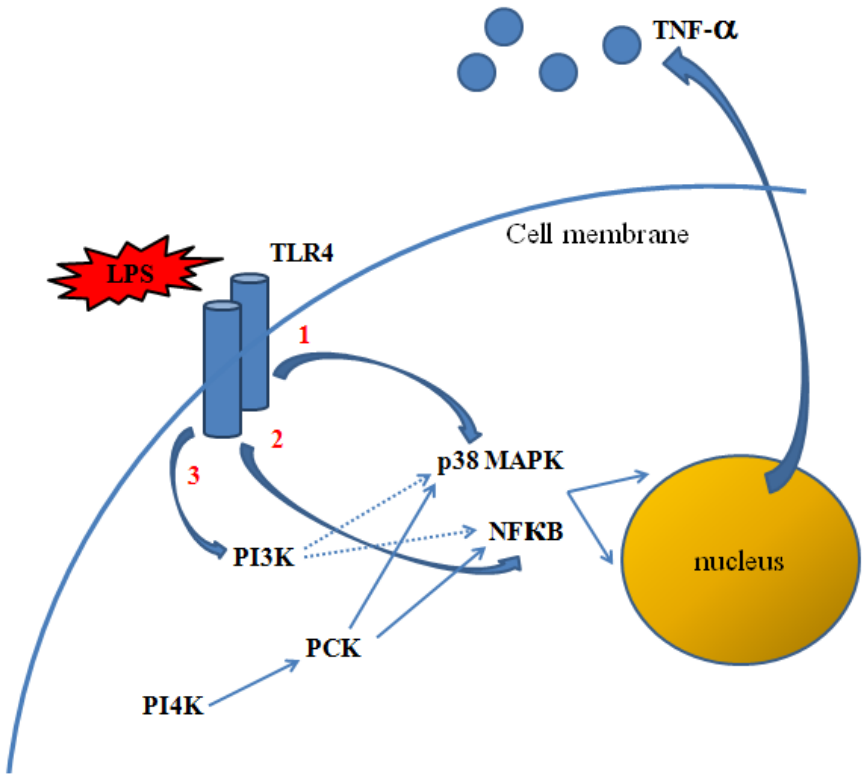
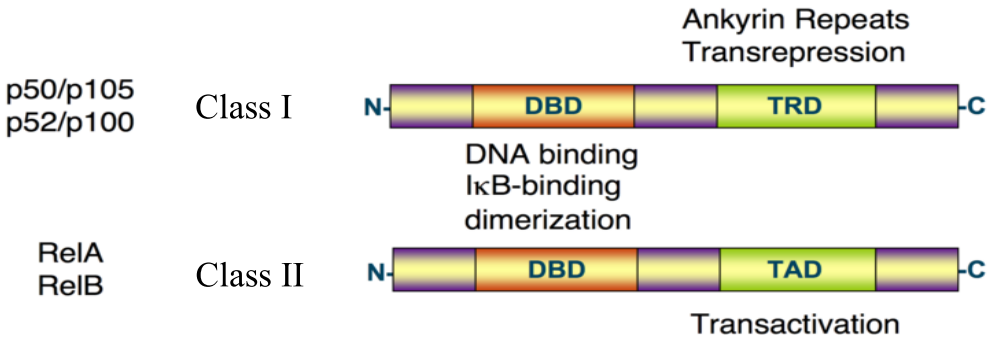


Figure 4 Signaling pathways involved in LPS-induced TNF-α production (Modify from Hoareau et al., 2006)

8. NFKB

Nuclear factor-kappa B (NFKB) plays an important role in controlling inflammatory gene activation. This transcription factor is usually found in the cytosol as a heterodimer complex with its inhibitory protein IKB (inhibitor of NFKB). When cells are stimulated with LPS, phorbol ester or inflammatory cytokines, IKB is phosphorylated by IKB kinase and degraded. IKB phosphorylation dissociates the dimer and allows NFKB to translocate to the nucleus, where it activates target genes, including iNOS (Baldwin, 1996; Lima et al., 2007). The family of NFKB transcription factors includes a collection of proteins, conserved from *Drosophila* to humans and related through a highly conserved DNA-binding and dimerization region, the Rel homology (RH) domain (Hoffmann et al., 1999). However, the NFKB family can be divided into two groups, based on differences in their structures, functions, and modes of synthesis (Baeuerle and Henkel, 1994; Siebenlist, Franzoso and Brown, 1994) Members of one group (p105, p100, and *Drosophila* Relish) have long C-terminal domains that contain multiple copies of ankyrin repeats, which act to inhibit these molecules. Members of this group give rise to active, shorter proteins that contain the Rel homology domain (p50 from p105, p52 from p100) (Figure 5) either by limited proteolysis (Lin and Ghosh, 1996; Chen, and Maniatis, 1998; Karin and Ben Neriah, 2000; Lee et al., 2001) or arrested translation (Lin, DeMartino and Greene, 1998; Lin, DeMartino and Greene, 2000). Members of this group do not function as transcription activators, except when they form dimers with members of the second group, which includes p65 (RelA), Rel (c-Rel), RelB, and the *Drosophila* Rel proteins (dorsal and Dif (Dorsal-related immunity factor)) (Baeuerle and Baltimore, 1996). These proteins are not synthesized as precursors, and in addition to the N-terminal Rel homology domain, they possess one or more C-terminal transcriptional activation domains. Members of both groups of NFKB proteins can form homodimers or heterodimers. NFKB was the original name for the p50-p65 heterodimer (Li and Stark, 2002) (Figure 6).



DBD = DNA binding domain, TRD = transrepression domain, TAD = transactivation domain

Figure 5 NFKB structure (Gilmor, 2006; Brasier, 2006; Perkins, 2007)

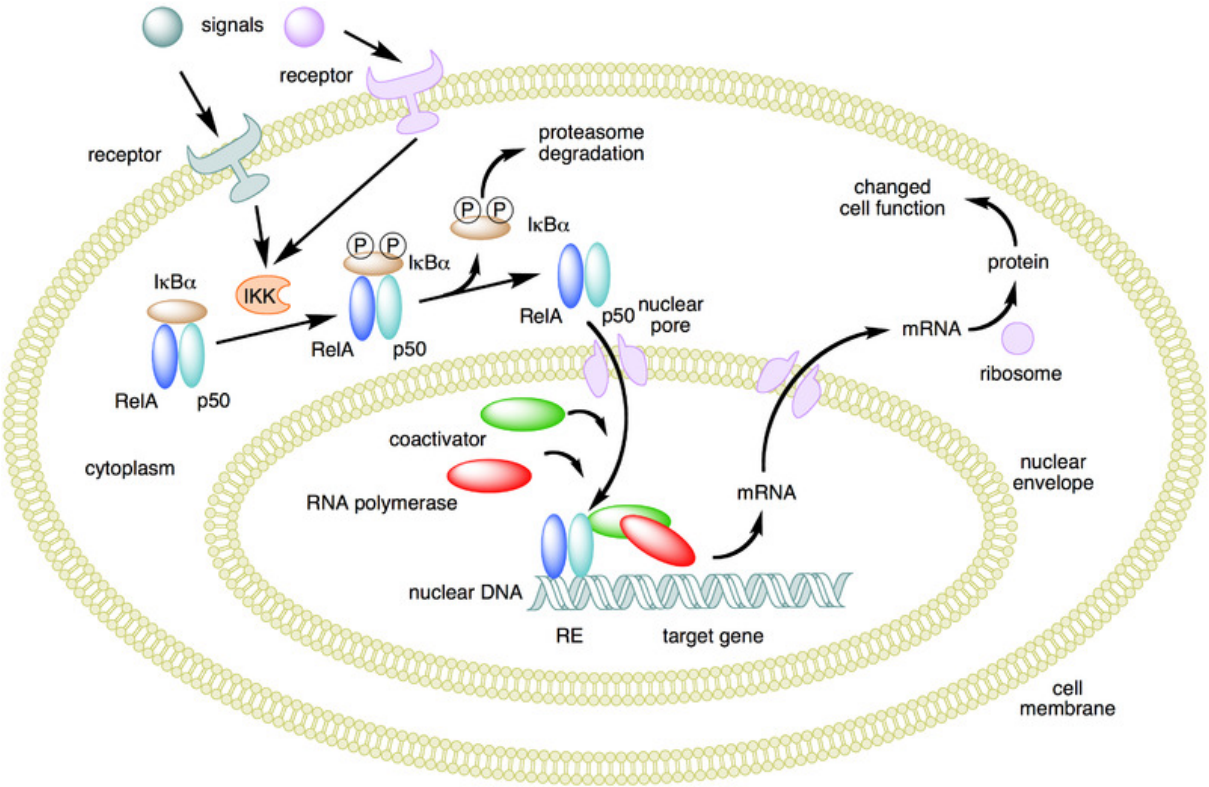


Figure 6 Mechanism of NFKB function (Gilmor, 2006; Brasier, 2006; Perkins, 2007)

In this figure, the NFΚB heterodimer between Rel A and p50 proteins is used as an example. While in an inactivated state, NFΚB is located in the cytosol complexed with the inhibitory protein IΚBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IΚB kinase (IKK). IKK, in turn, phosphorylates the IΚBα protein, which results in ubiquitination, dissociation of IΚBα from NFΚB, and eventual degradation of IΚBα by the proteosome. The activated NFΚB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE). The DNA/NFΚB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein, which results in a change of cell function (Gilmor, 2006; Brasier, 2006; Perkins, 2007).

9. MAPK kinase pathway

The mitogen-activated protein kinase (MAPK) superfamily of serine/threonine kinases is an important component of cellular signal transduction (Kieran and Zon, 1996; Kurosawa et al., 2000) and also appears to play important roles in inflammatory processes. At least three MAPK cascades; extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 are involved in the inflammation (Moon et al., 2007; Park et al., 2008) (Figure 7). ERKs function in the control of cell division, and inhibition of these enzymes are being explored as anticancer agents. JNKs are critical regulators of transcription, and JNK inhibitors may be effective in control of rheumatoid arthritis. The p38 MAPKs are activated by inflammatory cytokines and environmental stresses and may contribute to diseases like asthma and autoimmunity (Johnson and Lapadat, 2002). In macrophages, the inhibition of ERK1/2 and JNK phosphorylation, could lead to a decrease in activating protein-1 (AP-1) activation and production of pro-inflammatory cytokines.

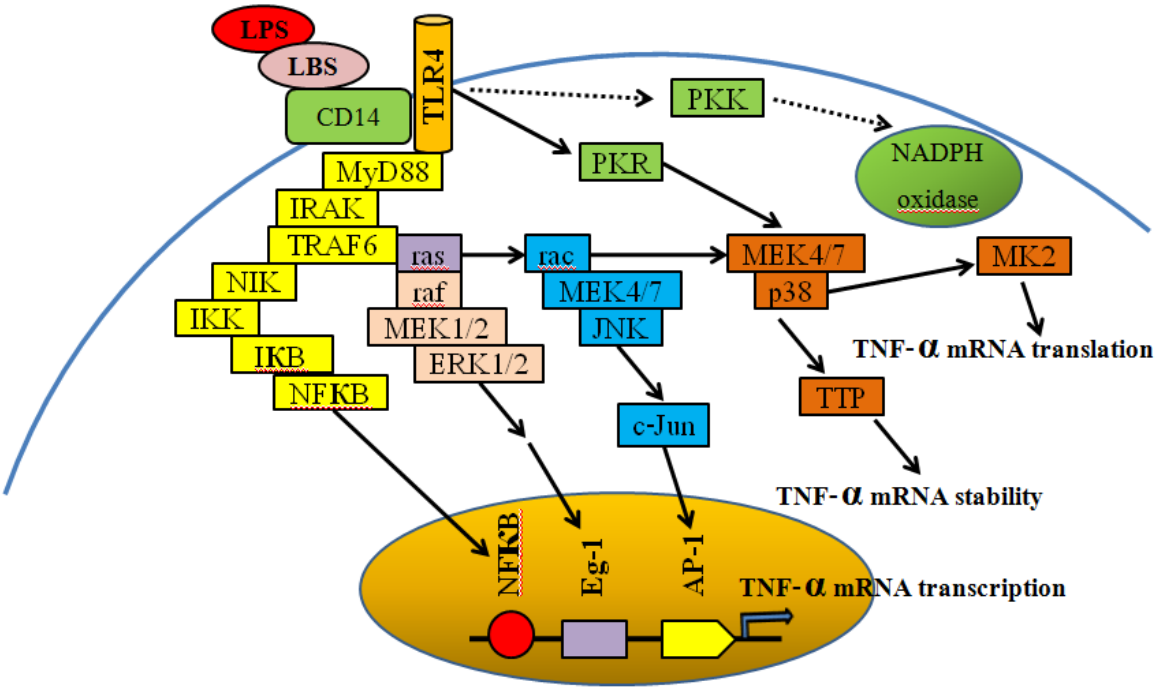


Figure 7 Lipopolysaccharide-stimulated signal transduction pathways which increase TNF-α production in macrophages. Figure represents of some of the signaling intermediates potentially involved in regulation of TNF-α expression in macrophages (Modify from Kishore et al., 2004).

CD14 = Cluster of differentiation 14, MyD88 = myeloid differentiation primary response gene 88, NIK = nuclear factor-kappa-beta-inducing kinase, PKR = protein kinase ribonucleic acid-activated, PKK = protein kinase-C associated kinase, MEK1/2 = mitogen-activated MAPK/ERK kinase ½, Eg-1 = early growth response 1 protein, AP-1 = activating protein-1

10. RAW 264.7 cell

RAW 264.7 cells are a macrophage-like, Abelson leukemia virus transformed cell line derived from BALB/c mice. Murine macrophages and macrophage–like cell lines such as RAW 264.7 adhere to tissue culture–grade plastic through cation–dependent integrin receptors and other cation-independent receptors, predominantly the murine scavenger receptors (MSRs) (Fraser, Hughes, and Gordon, 1993). In order to reduce adhesion during routine culture, the RAW 264.7 cells are grown on sterile non-tissue–grade plastic (ultra-dish Petri dishes). Adherence of macrophages to these plates is

mediated by $\alpha_M\beta_2$(CR3) integrins (Rosen and Gordon, 1987) and this interaction is readily reversed by using cation chelators such as ethylene diamine tetra acetic acid (EDTA). This adhesive interaction is also sufficiently weak that cells may be detached by the sheer force of media flowing over the cells.

Macrophages are extremely sensitive to lipopolysaccharide (LPS) endotoxin from Gram-negative bacteria. LPS has major effects on macrophage phenotype and function, including adhesion. All solutions, buffers, and media should be made with sterile, endotoxin-tested and distilled, deionized water (Figure 8).

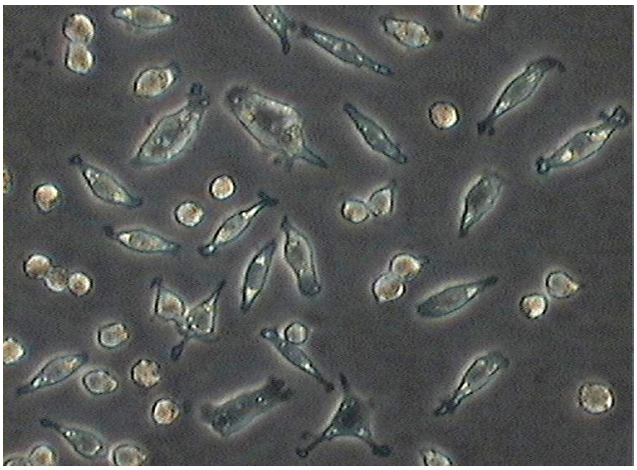


Figure 8 RAW 264.7 macrophage cells line

11. Toll like receptors (TLRs)

TLRs are transmembrane receptors that play a critical role in the detection of microbial infection and in the induction of inflammatory and immune responses against conserved microbial structures, called pathogen associated molecular patterns (Aderem and Ulevitch, 2000; Lima et al., 2007). Eleven members of the TLR family have been identified in humans to date and they are ubiquitously expressed in human tissues (Zarember and Godowski, 2002). Genetic and biochemical evidence have demonstrated that TLR4 confers the responsiveness to LPS (lipopolysaccharide) derived from Gram-negative bacteria (Qureshi et al., 1999; Rhee and Hwang, 2000).

CHAPTER 3

MATERIALS AND METHODS

1. Plant material

Kaempferia parviflora Wall. ex Baker rhizomes were bought from a Thai traditional drug store in Songkhla province, Thailand in May 2008. The plant material was identified by Assoc. Prof. Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. A voucher specimen number SKP2061116 of the plant material was deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla, Thailand.

2. Experimental animals

Male and female Swiss albino mice (30-40 g body weight) and Wistar rats (180-200 g body weight) were used in the experiments. All animals obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat-Yai, Songkhla, Thailand, were maintained in a standard environmental condition. They were fed with standard rodent diet and water *ad libitum*. Animal study protocol was approved by The Animal Ethic Committee, Prince of Songkla University (MOE 0521.11/375).

3. Cells type

The murine macrophage cells (RAW264.7, ATCC No. TIB-71) were obtained from Dainippon Pharmaceutical, Osaka, Japan and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma, USA). The cells were incubated at 37 □C in 5% CO_2/air.

4. Chemicals and reagents

4.1 Extraction and purification

- *n*-Hexane (Lab scan Asia, Thailand)
- Chloroform (LB Science, Thailand)
- Ethyl acetate (Lab scan Asia, Thailand)
- Ethanol (LB Science, Thailand)
- Methanol (LB Science , Thailand)

4.2 Animal-based assay

- Propylene glycol (Carlo ERBA reagents)
- Tween 80 (Fluka, UK)
- Tween 20 (Sigma, USA)
- Dimethyl sulfoxide (DMSO) (Lab scan Asia, Thailand)
- Sodium chloride (LAB-scan, Thailand)
- Indomethacin (Sigma, USA)
- Carrageenan type IV (Sigma, USA)

4.3 Cell-based assay

- SYBR® Safe DNA gel stain (10,000X concentrate in DMSO) (Invitrogen, USA)
- Dulbecco Modified Eagle Medium **(**DMEM) (Invitrogen, USA)
- RPMI 1640 medium (Gibco, USA)
- Lipopolysaccharide (LPS, from *Escherichia coli*) (Gibco, USA)
- Fetal bovine serum (FBS) (Gibco, USA)
- Penicillin–streptomycin (Invitrogen, USA)
- Trypsin-EDTA (Gibco, USA)
- Isopropanol (Lab scan Asia, Thailand)
- Sodium hydrogen carbonate ($NaHCO_3$) (Sigma, USA)
- Phosphate-buffered saline (PBS) (Sigma, USA)
- Trypan blue (Gibco, USA)
- 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazodium bromide (MTT) (Kumamoto, Japan)

- RT-PCR-kit, ReverTra Ace and KOD Dash (TOYOBO, Japan)
- Agarose (Invitrogen, Spain)
- Caffeic acid phenethylester (CAPE) (Sigma, USA)
- Parthenolide (Sigma, USA)
- Sodium dodesylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
- Phosphatase inhibitor cocktail tablet (Phos stop) (Mannheim, Germany)
- Blocking one-P (Nacalai tesque, Japan)
- Blocking one (Nacalai tesque, Japan)
- Can get signal® (TOYOBO, Japan)
    - Immunoreaction enhancer solution: solution for primary antibody
    - Immunoreaction enhancer solution: solution for secondary antibody
- SAPK/JNK antibody (Cell signaling technology, UK)
- P44/42 MAP kinase antibody (ERK) (Cell signaling technology, UK)
- Phospho-p44/42 kinase (Thr202/Tyr204) antibody (Cell signaling technology, UK)
- Phospho-SAPK/JNK (Thr183/Tyr185) antibody (Cell signaling technology, UK)
- β-Actin antibody (Cell signaling technology, UK)
- Purified mouse anti-iNOS/NOS Type II (BD transduction laboratories)
- Precision plus protein™ (Dual color standard) (BIO-RAD, USA)
- Restore™ plus Western blot (Sripping buffer) (Thermoscientific, USA)
- ECL plus Western blotting detection system (GE healthcare, UK)

5. Equipment, instruments and kits

The equipments used in this study were listed in Table 5.

Table 5 General information of equipments

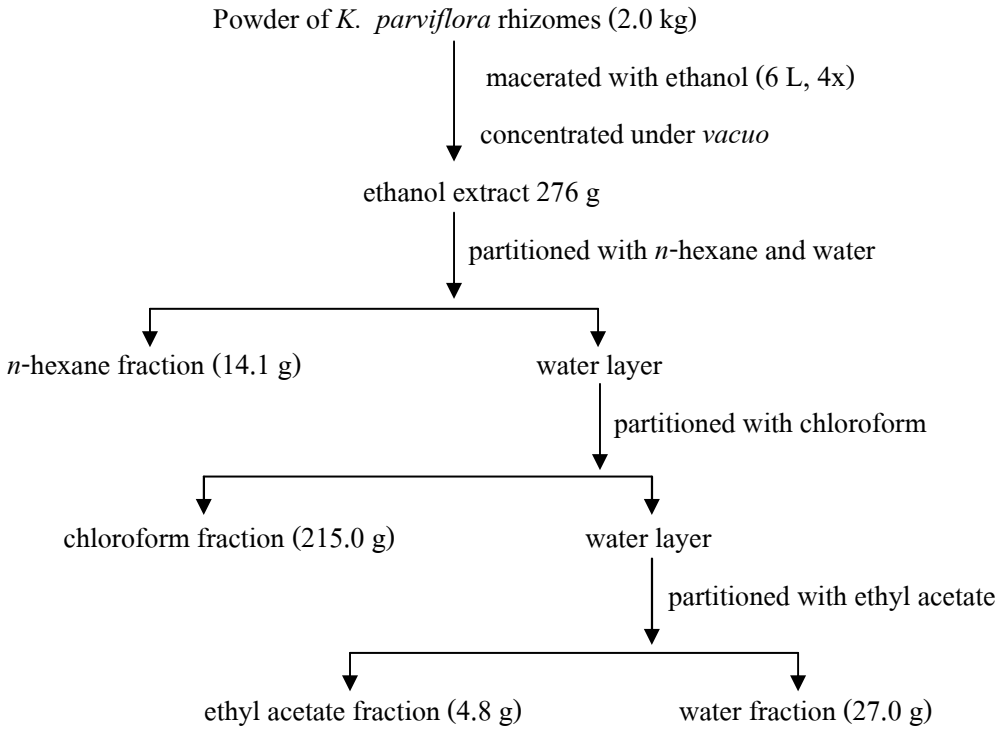
Instrument	Model	Company
Extraction and purification		
Hot air oven	DIN 12880-KI	Memmert, Germany
HPLC analysis column	Inertsil® ODS 3, 4.6 x 250 mm, i.d	GL Sciences, Japan
HPLC preparative column	Inertsil® ODS 3, 20 x 250 mm, i.d.	GL Sciences, Japan
Rotary evaporator	N-N Series	EYELA, Japan
NMR spectrometer (500 MHz)	JEOL JNM-LA500,	Tokyo, Japan
TLC (normal-phase)	gel 60F_{254}(0.25 mm)	Merck,USA
TLC (reversed-phase)	Silica gel RP-18	Merck, USA
	F_{254S} (0.25 mm)	
Animal-based assay		
Plethysmometer	UGO Basile	Italy
Needle	26G×½	Nipro, Thailand
Syringe	1 ml, 3 ml	Nipro, Thailand
Feeding needle	No.16, No. 18	Mahidol University, Thailand
Cell-based assay		
Laminar air flow	Faster Ultrasafe 48	FASTER, Italy
CO_2 incubator	Shel LAB	GIBTHAI, Thailand
Microplate	96-well	Nunc, Denmark
Microplate	6-well	Nunc, Denmark
Gene amplification	PTC-1148	BIO-RAD, USA
RNeasy Mini kit	-	Qiagen, USA
Centrifuge	SN ACF1-010G	Harikul, Thailand
Centrifuge	8880 series	Centurion Scientific, UK

Table 5 General information of equipments (continued)

Instrument	Model	Company
Microplate reader	PowerWaveX	BIO-TEK
Magnetic Stirrer	G-560E	Scientific industries, USA
pH Meter	SN AMS1-063H	Thailand
Auto-pipette	Transferpettt-8 (30-300 µl)	Germany
Pipette	Accu-jet	Germany
Pipette	Pipetman (2, 200, 1000 µl)	Gilson, France
Electrophoresis chamber	Socorex 2-20 µl	Swiss
Luminescent image analyzer	LAS-4000 mini	EYELA, Japan
ELISA kit	TNF-α	Invitrogen, Japan
ELISA kit	PGE_2	R&D systems, USA
Immuno-Blot PVDF Membrane	Mini Trans-Blot	BIO-RAD, USA
Polyacrylamide Gel - Electrophoresis	For protein blotting (0.2 µm) READY GELS J	BIO-RAD, USA

6. Extraction procedure

Two kilograms dried weight of *K. parviflora* rhizomes were ground and macerated with ethanol at room temperature, four times (6 L, 4x). The ethanol extract (267 g) was then concentrated and partitioned with water and hexane, and successively partitioned with chloroform and water. After that, the water layer was partitioned with ethyl acetate. Each partition was evaporated to dryness *in vacuo* to produce residues from the hexane (14.1 g, 5.28 % yield), chloroform (215.0 g, 80.52 % yield), ethyl acetate (4.8 g, 1.80 % yield) and water fractions (27.0 g, 10.11 % yield), respectively, (Scheme 1).



Scheme 1 Extraction procedure of *K. parviflora* rhizomes

7. Bioassay-guided isolation

The bioassay-guided fractionation of an ethanol extract led to the isolation of compounds using the inhibition of nitric oxide in RAW246.7 cells and carrageenan-induced rat paw edema models. The active fractions subsequently fractionated using silica gel column chromatography ($60F_{254,}$ Merck) and preparative reversed-phase HPLC (ODS column, Inersil® ODS3, 4.6×250 mm, RID-6A refractive index detector) until the pure compounds responsible for anti-inflammatory activity are obtained.

8. Isolation and purification

The $CHCl_3$ soluble fraction (200.0 g) was subjected to open column chromatography (Ø 12.5 cm) with normal-phase SiO_2. The amount of packing material (SiO_2) was calculated with the following equation:

SiO_2 packing material

$$h = \pi r^2 \times 0.43$$
$$= 3.14 \times (6.25)^2 \times 0.43$$
$$= 52.74 \text{ cm}$$

h = the height of SiO_2 in column, r = radius of column

ODS packing material

$$h = \pi r^2 \times 0.6$$

Sample was eluted by $CHCl_3$-MeOH gradient solvent system: $CHCl_3$, $CHCl_3$-MeOH (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 1:9). The eluted fractions were collected of 2 L/each and 100 fractions were obtained. After TLC checked, each fraction was grouped to afford 5 fractions [Fr. 1 (42.70 g), Fr. 2 (96.16 g), Fr. 3 (26.80 g), Fr. 4 (8.60 g), Fr. 5 (12.70 g)] (Figure 9). Each fraction was spotted on normal and reversed phase TLC for screening check the composition of substances of each fraction. The spots on TLC were detected by spraying with 1% $CeSO_4$ followed by heating on a hot plate. Fractions 1-4 were further purified by high performance liquid chromatography (HPLC), column chromatography or crystallization techniques (Scheme 2).

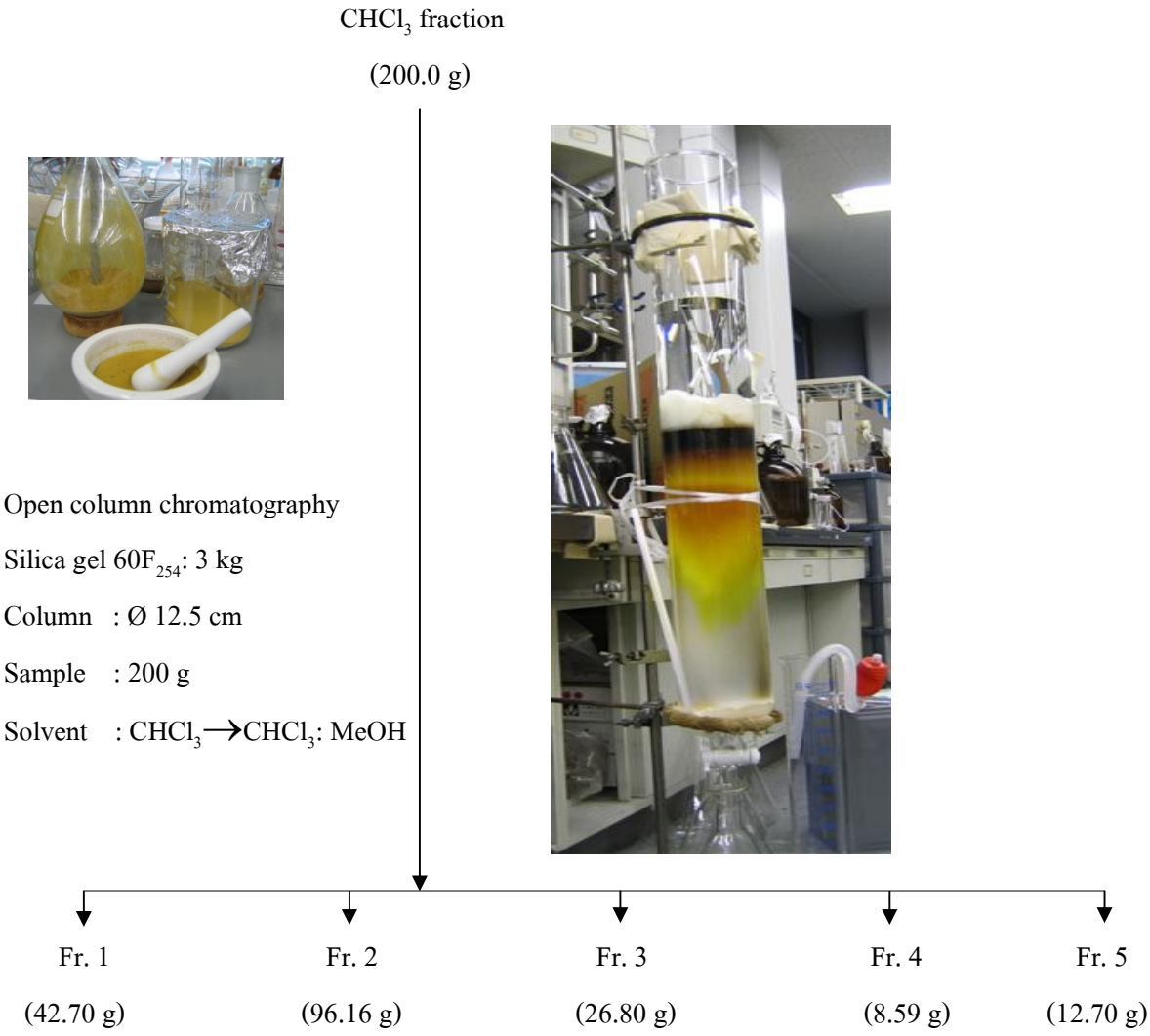
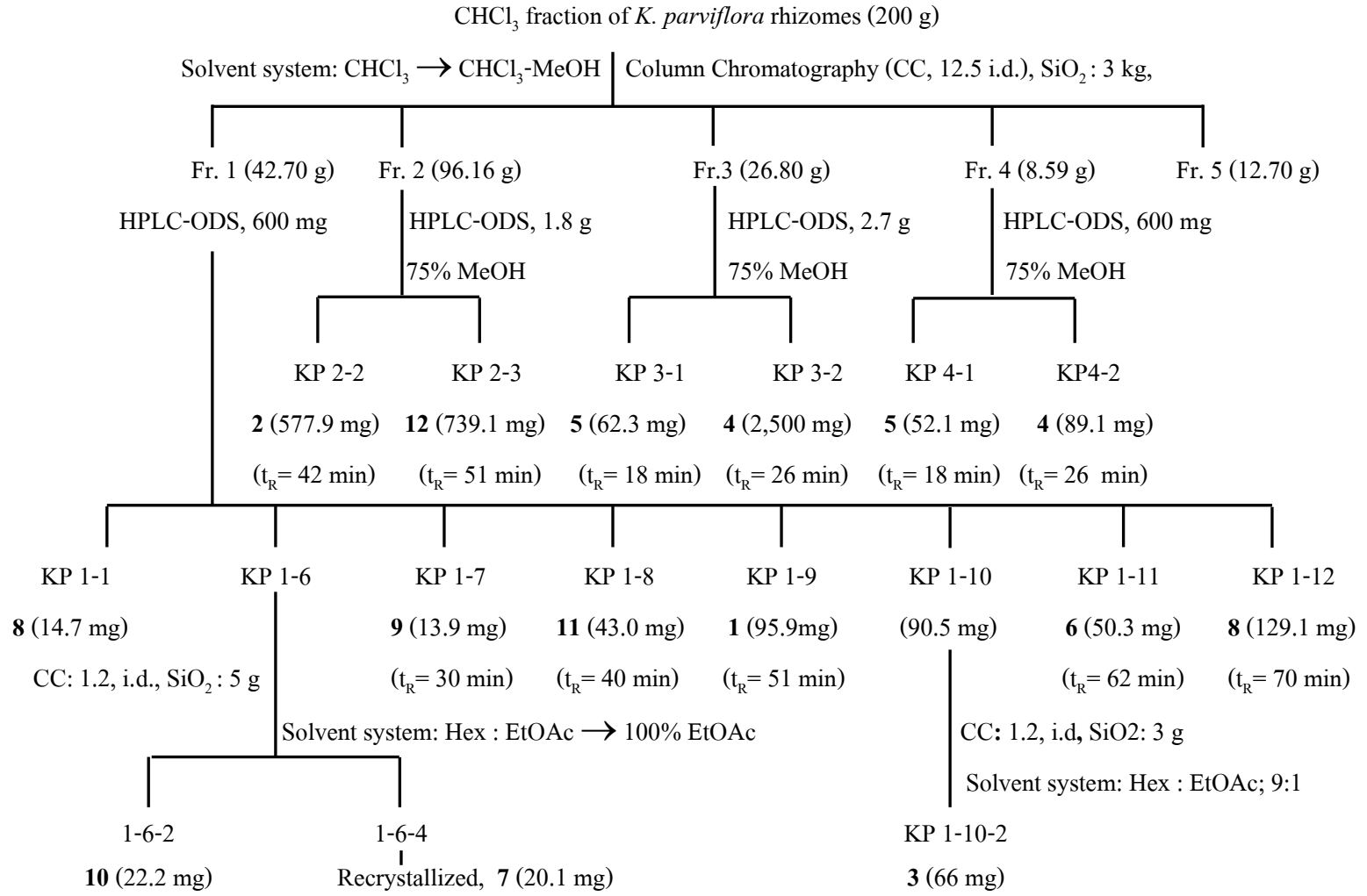


Figure 9 Extraction process of $CHCl_3$ fraction



Scheme 2 Isolation and purification of compunds from *K. parviflora* rhizomes

9. Sample preparation

9.1 Sample preparation for HPLC injection

Fifteen mg/ml of samples were dissolved in HPLC solvent system. The solution was filtered through a filter (0.45 μm) prior to use.

9.2 Sample preparation for carrageenan-induced rat paw edema test

Sample was prepared by fixed dose (150 mg/kg body weight). The 180-200 g of rats were used for this experiment. The volume of sample dissolving in cosolvent (10% DMSO: tween 80: propylene glycol: distilled water: 1:4:4:1) for feeding rat is 5 ml/kg.

150 mg/kg; 1,000 g of rat → is treated with 150 mg sample

200 g of rat → is treated with [200 g (150 mg)]/1,000 g = 30 mg sample

10 ml/kg; 1,000 g of rat → is treated with 10 ml cosolvent

200 g of rat → is treated with [200 g (10 ml)]/1,000 g = 2 ml solution

200 g of rat → is treated with 30 mg sample/2 ml cosolvent

Hence, 10 rats/each group → 300 mg sample/20 ml cosolvent was prepared.

9.3 Sample preparation for acute toxicity test in mice

Sample was prepared by fixed dose (maximum dose 2 g/kg body weight). The 30-40 g of mice were used for this experiment. The volume of sample dissolving in cosolvent (10% DMSO: tween 80: propylene glycol: distilled water: 1:4:4:1) for feeding rat is 10 ml/kg.

2 g/kg; 1,000 g of rat → is treated with 2,000 mg sample

40 g of rat → is treated with [40 g (2,000 mg)]/1,000 g = 80 mg sample

10 ml/kg; 1,000 g of rat → is treated with 10 ml sample dissolving solution

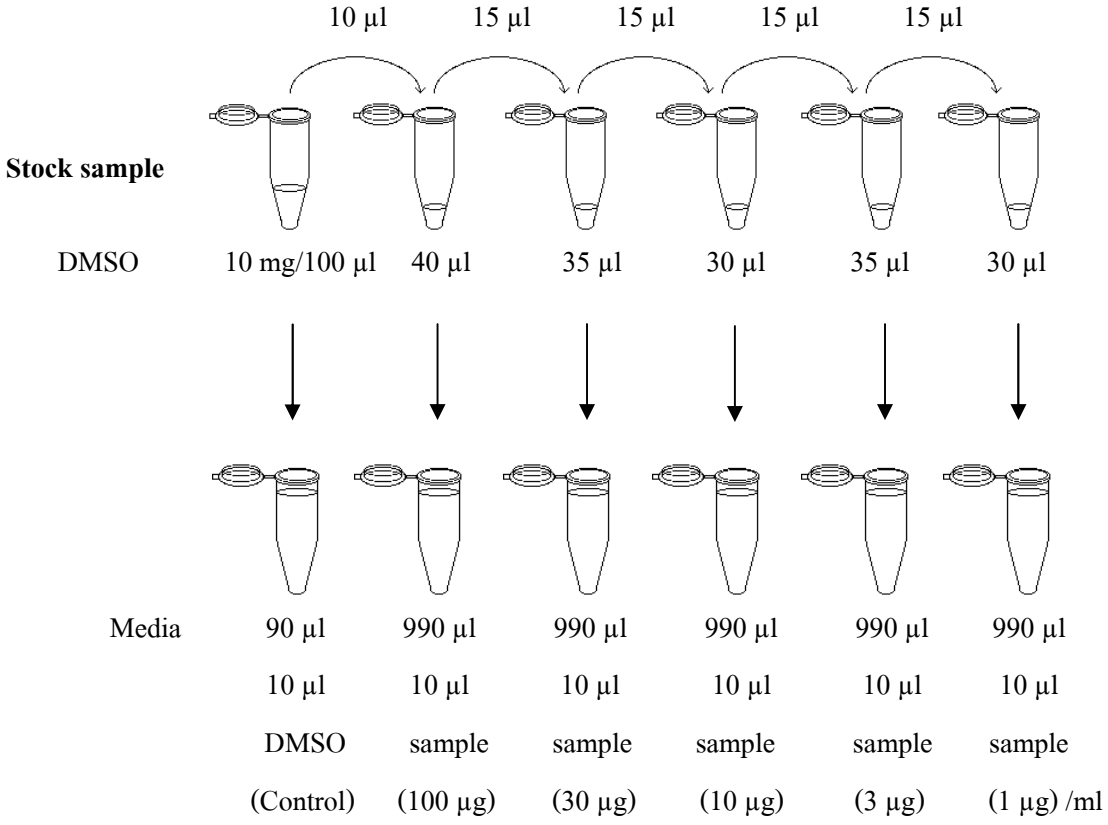
40 g of rat → is treated with [40 g (10 ml)]/1,000 g = 0.4 ml solution

40 g of rat → is treated with 80 mg sample/0.4 ml sample dissolving solution

Approximately, 3 rats/each group → 240 mg sample/1.2 ml cosolvent was prepared.

9.4 Sample preparation for cell-based assay

Stock samples (10 mg/100 µl DMSO) were prepared and made in a serial dilution (Scheme 3).



Scheme 3 Schematic dilution of sample preparation

10. Media, solution and sample preparation for cell-based assay

10.1 Roswelle Park Memorial Institute-1640 (RPMI-1640) medium

RPMI-1640 powder was dissolved in distilled water. The solution was adjusted to pH 7 with 1 N HCl. Final volume was adjusted to 1 L by distilled water. After that, the process was carried on laminar airflow; 90 ml of fetal calf serum (FCS) and 10 ml pen-strep were added to 900 ml of RPMI-1640 solution. Then, the solution was filtrated through 0.2 µm filter for sterile and keep at 12 °C until use.

10.2 Griess's reagent

Griess's reagent was prepared by mixing of 1.0 g sulfanilamide, 0.1 g *N*-(1-napthyl) ethylene diamine and 2.5 g H_3PO_4 (Table 6). The volume was adjusted to 100 ml with distilled water.

Table 6 Stock solution for Griess's reagent

Reagents	Amount
Sulfanilamide	1.0 g
N-(1-napthyl) ethylene diamine	0.1 g
H_3PO_4	2.5 g
Distilled water q.s.	100 ml

10.3 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution

The 200 mg of thiazolyl blue tetrazollium bromide was dissolved in 40 ml phosphate buffer (PBS).

10.4 SYBR® Safe DNA Staining Solution

The SYBR® Safe DNA gel stain 10,000X concentrate 25 µl was diluted with 250 ml running buffer. For larger gels, volumes can be increased proportionally, ensuring that the entire gel is fully immersed during staining.

10.5 10×T-TBS stock solution

The 10×T-TBS stock solution was prepared by mixing of 58.4 g NaCl, 12.1 g Tris and 10 ml Tween 20 (Table 7). The volume was adjusted to 1 L with distilled water.

Table 7 10×T-TBS stock solution

Reagents	Amount
NaCl	58.4 g
Tris	12.1 g
Tween 20	10 ml
Distilled water q.s.	1 L

10.6 Transfer buffer

Transfer buffer was prepared by mixing of 58.1 g of 480 mM Tris and 29.3 g of 390 mM of glycine (Table 8). The volume was adjusted to 1 L with distilled water.

Table 8 Transfer buffer

Reagents	Amount
480 mM Tris	58.1 g
390 mM Glycine	29.3 g
Distilled water q.s.	1L

10.7 Sample buffer for cells scraping

Sample buffer was prepared by mixing of 8.4 ml distilled water, 100 µl Protease inhibitor cocktail and 1 ml sample buffer. The mixture was adjusted to pH 7.4 before 100 µl 22% triton X and one Tablet Phos stop was added (Table 9).

Table 9 Sample buffer

Reagents	**Volume**
Distilled water	8.4 ml
Protease inhibitor cocktail	100 µl
Sample buffer - NaCl 0.877 g - Tris 0.121 g - β-glycerophate 0.612 g - EDTA 0.076 g - Distilled water 100 ml	1 ml
Adjust to pH 7.4	
22% triton X	100 µl
Phos stop	1 tablet

10.8 Sample dissolving solution

Sample dissolving solution was prepared by mixing of 342.3 mg EDTA, 6.0 g SDS, 3.634 g Tris, 100 ml ultrapure water and 8 ml glycerol. The mixture was adjusted to pH 6.8 prior to 0.03 g bromophenol blue and 120 µl 2-mercaptoethanol were added (Table 10).

Table 10 Sample dissolving solution

Reagents	**Amount**
EDTA	342.3 mg
SDS	6.0 g
Tris	3.634 g
Ultrapure water	100 ml
Glycerol	8 ml
Adjust to pH 6.8	
Bromophenol blue	0.03 g
2-Mercaptoethanol	120 µl

10.9 Solution for Plethysmometer (Use within 3 days after preparation)

Solution for plethysmometer was prepared by mixing of 0.25 g NaCl and 0.125 ml tween 20. The mixture was adjusted to 500 ml by distilled water.

11. Procedure for cell thawing

Stock cell line of RAW264.7 (1×10^4 cells/ml) kept at -80 ºC was thawed by heating up at 37 ºC using water bath. Cells were transferred to 5 ml media tube and centrifuged at 1,000 rpm for 5 min. The supernatant was removed and 10 ml of media were added. Cells were mixed well by pipetting and transferred to 75 cm^2 culture flask. The adherence cells were subcultured when the cells grow up to 80 % confluence.

12. Concentration-dependent effects of LPS-induced NO production in RAW 264.7 cells

To study the suitable concentration of LPS-activated RAW264.7 cells for nitric oxide production without any cytotoxic effect, the following procedure was then carried out. Briefly, RAW264.7 cells were cultured in DMEM, and the suspension cells were seeded into a 96-well microplate at 2.5×10^6 cells/100 µl/well. After 6 h, the cells were treated with various concentrations (0.1, 0.3 1, 3, 10 and 30 µg/ml) of LPS from *E. coli*, 055: B5. After 18 h incubation, nitrite concentration was measured from the supernatant by Griess's reaction. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent (Figure 10). The optical density was measured with a microplate reader at 570 nm. Nitric oxide production was determined graphically comparing with the standard nitrite curve.

The Griess reaction under acidic conditions, nitrite reacts with amino group of sulfanilamide to form the diazonium cation, which couples to *N*-(1-naphthyl) ethylenediamine in para-position to form the corresponding azo dye (Tsikas, 2007).

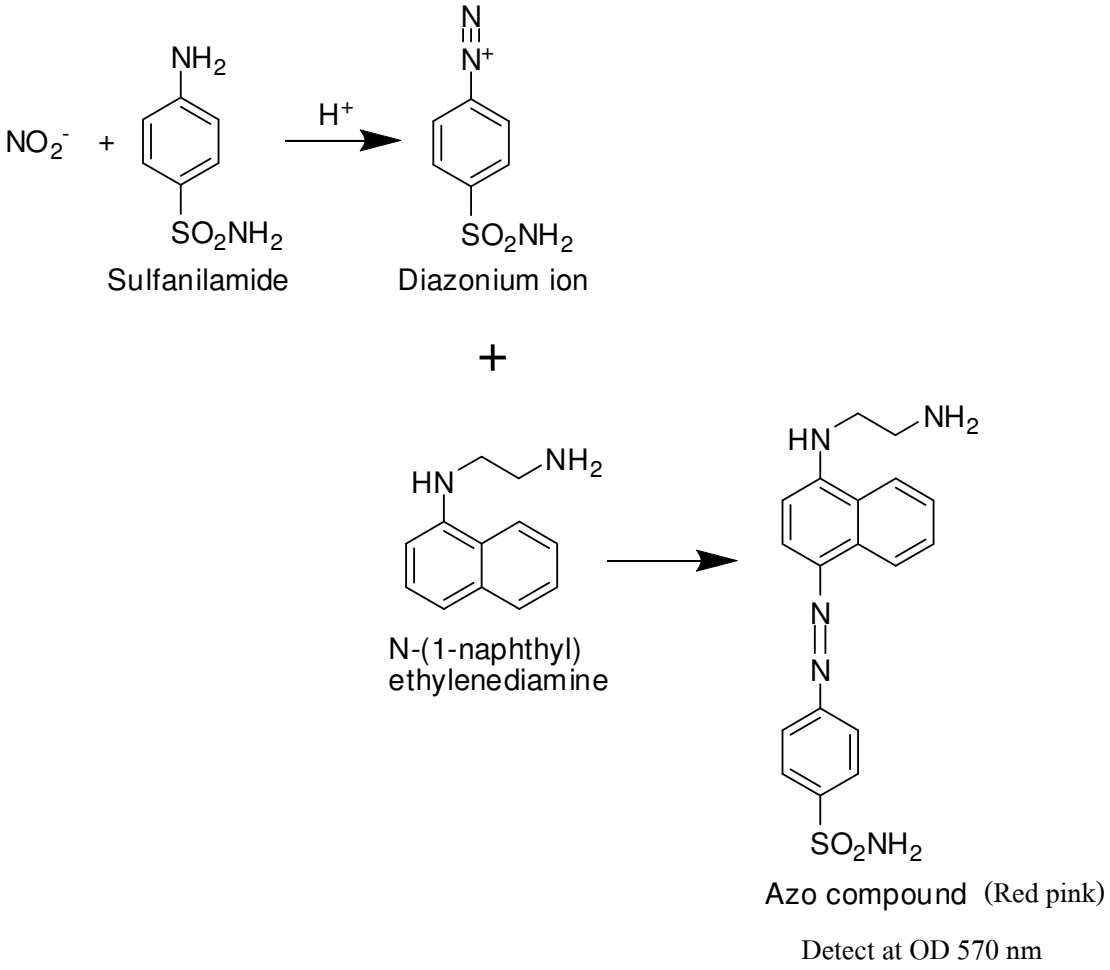


Figure 10 Greiss reaction for the determination of nitrite (Tsikas, 2007)

13. Effects on production of NO in LPS-stimulated macrophage RAW264.7 cells

Total nitrite in medium is used as an indicator of NO synthesis (Crisafulli et al., 2009). Briefly, RAW 264.7 cells were cultured in DMEM, and the suspension cells were seeded into a 96-well microplate at 2.5×10^6 cells/100 µl/well. After 6 h, the cells were treated with various concentrations of compounds for 10 min and then stimulated for 18 h with 10 µg/ml of LPS (from *E. coli*, 055: B5, Sigma). Nitrite concentration was measured from the supernatant by Griess's reaction. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent.

14. Cytotoxicity test

Cytotoxicity was evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric assay. Briefly, after 18 h of incubation with test compounds, a MTT (10 µl, 5 mg/ml in PBS) solution was added to the wells. After incubation for 4 h at 37 ºC, the medium was removed, and 0.04 M HCl in isopropanol was added to dissolve the formazan produced in the cells. The optical density (OD) of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm) (Figure 11). If the OD of the sample-treated group dropped below 80% of that in the vehicle-treated group, the test compound was considered cytotoxic (Matsuda et al., 2003).

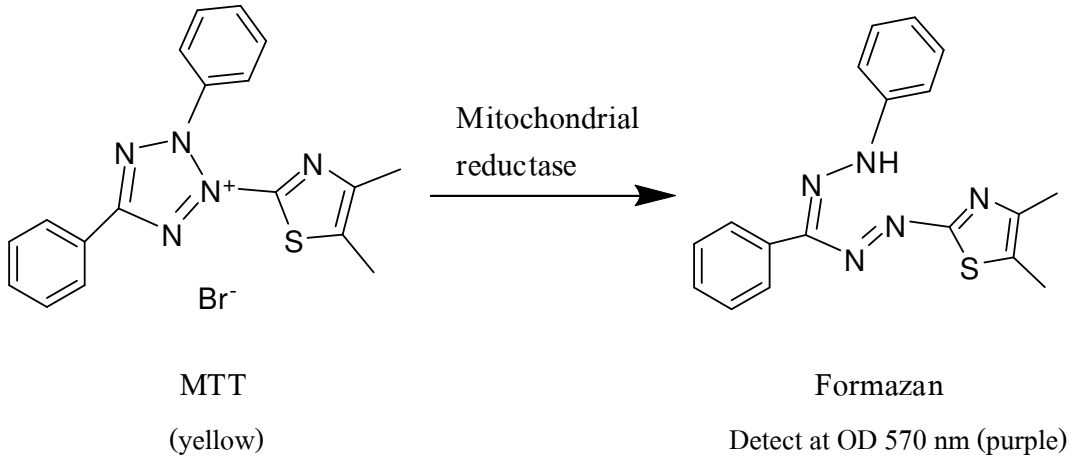


Figure 11 Structure of MTT and formazan

15. Carrageenan-induced rat paw edema

This experiment was performed according to the method described by Winter et al., (1962). The initial right hind paw volume of the rats was measured using a Plethysmometer (UGO Basile). Male Wistar rats, weighing 150-200 g, were divided into 7 groups of 10 rats each.

Group 1: Control (cosolvent 10 ml/kg, p.o.)

Group 2: Indomethacin (10 mg/kg, p.o.)

Group 3: Ethanol extract of *K. parviflora* (150 mg/kg, p.o.)

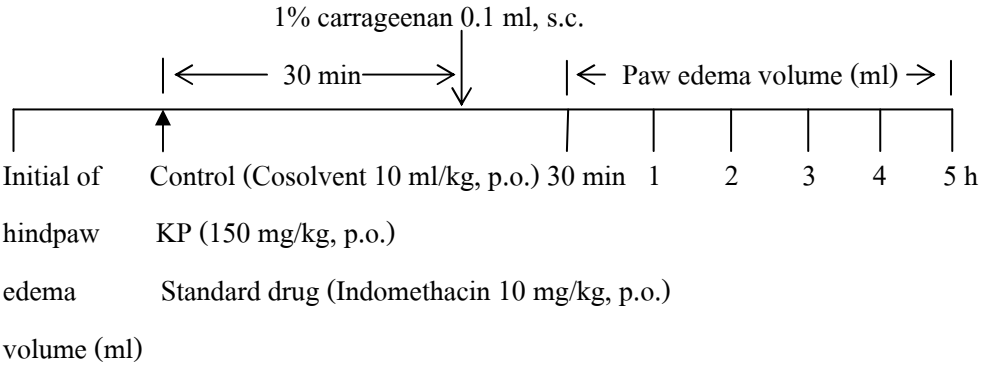
Group 4: Water fraction of *K. parviflora* (150 mg/kg, p.o.)

Group 5: Ethyl acetate fraction of *K. parviflora* (150 mg/kg, p.o.)

Group 6: Chloroform fraction of *K. parviflora* (150 mg/kg, p.o.)

Group 7: *n*-Hexane fraction of *K. parviflora* (150 mg/kg, p.o.)

The ethanol extract and fractions of *K. parviflora* rhizomes were tested in animal model, carrageenan-induced rat paw edema, as described by Winter and co-workers (1963). Briefly, male Wistar rats (body weight in the range of 180-200 g), were randomly divided into 7 groups. The ethanol extract and fractions of *K. parviflora* rhizomes were suspended in vehicle. The doses of 150 mg/kg was administered orally to rats for 30 minutes before subcutaneous injection of 1% carrageenan in NSS into the plantar surface of the rat hind paw. The control group was treated with vehicle (10 ml/kg, 10 % DMSO: tween 80: propylene glycol: water, 1:4:4:1) and the positive control group was treated by indomethacin (10 mg/kg). The measurements of paw volume were taken before carrageenan injection and in every 0.5, 1, 2, 3, 4 and 5 h using a plethysmometer (UGO Basile, Italy). The inhibition of the inflammation was calculated by measuring the volume difference between the control and the tested paw. The schematic plan of the carrageenan-induced paw edema experiment was illustrated in Scheme 4.



Scheme 4 Schematic plan of the carrageenan-induced paw edema

The volume of hind paw was evaluated for anti-inflammatory activity and was expressed as % inhibition of the hind paw volume. The percentage of inhibition was determined for each experimental group as following equation.

$$\text{Inhibition (\%)} = \frac{(V_t\text{-}V_o)_{\text{control}} - (V_t\text{-}V_o)_{\text{treated}}}{(V_t\text{-}V_o)_{\text{control}}}$$

V_t = volume of hind paw after carrageenan injection

V_o = volume of hind paw before carrageenan injection

16. Acute toxicity test of *K. parviflora* extract in mice

The up-and-down method described by Bruce (1985) was used in this study. The method used for acute toxicity test has been developed and statistically evaluated and permitted a reduction in the number of animal used. The first dose was begun at 300 mg/kg and adjusted by a constant multiplicative factor of 1.5 up to 2 g/kg. The crude extract of *K. parviflora* was orally administered to a group of mice both male and female. Behavior parameters such as convulsion, hyperactivity, sedation, grooming, loss of righting reflex and increased or decreased respiration were observed during a period of 8 h and everyday until 7 days after administration. Food and water were given *ad libitum*.

17. Effects on LPS-induced PGE_2 release from RAW264.7 cells

The mouse macrophage cell line (RAW264.7 cells) was purchased from Cell Lines Services (CLS). The cells were grown and maintained in RPMI-1640 medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 U/ml), streptomycin (100 µg/ml) and 10% FBS in culture flasks at 37 °C, 5% CO_2, in a fully humidified incubator. The cells were harvested with 0.25% trypsin-EDTA and re-suspended in a fresh medium. Cell counts were measured using a standard trypan blue cell counting technique. The cell concentration was adjusted to 1×10^6 cells/ml in the same medium. 100 µl Cells suspension were seeded in 96-well plate and incubated for 1 h at 37 °C. The medium was replaced with LPS in RPMI-1640 (10 µg/ml) which is the triggering agent for PGE_2 production. After that, *K. parviflora* extract in various concentrations (3-100 µg/ml) were dispensed into

the wells. The cells were incubated for 8 h at the same condition. The supernatant was transferred into 96-well ELISA plate and then PGE_2 concentrations were determined using commercial ELISA kits according to the manufacturer's instruction. The absorbance at 450 nm was recorded using a microplate reader (Bio-Tek instruments).

18. Determination of TNF-α production by LPS-activated macrophages

RAW 264.7 cells were seeded in the 96-well microplate at a density of 2.5×10^6 cells/100 µl/well and incubated for 6 h, and then the cells were treated with various concentrations of compounds for 10 min. After that, LPS was added to each well (final concentration: 10 µg/ml) and the cells were incubated for 4 h. The supernatant (5 µl) was transferred to ELISA plate and levels of TNF-α in the culture media were determined using a commercial kit (Mouse TNF-α ELISA kit, Invitrogen) according to the manufacturer's instructions and the absorbance was read at 450 nm within 30 min.

19. Study of iNOS, p-JNK1/2 and p-ERK1/2 protein expression

19.1 Time-courses of iNOS, p-JNK and p-ERK protein expression

19.1.1 Protein extraction from RAW264.7 cells

RAW 264.7 cells (5.0×10^6 cells/2 ml/well) were seeded into a 6-well multiplate and allowed to adhere for 6 h at 37 ºC in a humidified atmosphere containing 5% CO_2. After that, cells were treated with various concentrations of samples and stimulated for 10, 30 min and 1, 2, 4, 6, 8 and 12 h with or without LPS. The adherence cells were scrapped in a lysis buffer with the following components: 8.4 ml distilled water, 100 µl protease inhibitor cocktail (Thermo Scientific), 100 µl 22% triton X-100, phosphatase inhibitor cocktail (PhosSTP, Roche), and 1 ml sample buffer (0.877 g NaCl, 0.121 g Tris, 0.612 g β-glycerophate, 0.076 g EDTA, and 100 ml H_2O, pH 7.4). Then, cells were disrupted three times (Microson™ ultrasonic cell disruptor, USA) for 30 s, and centrifuged at 2,000 *g* for 2 min.

The supernatant was used to detect UV absorbance for calculation of the protein loading volume and for protein sample preparation. For the UV absorbance detection; 10 µl of supernatant was added to 190 µl of 0.9 % normal saline solution (NSS) and vortexed. The 10 µl portions of the supernatant was then transferred to a 96-well plate and 200 µl of working reagent (BCA™ protein assay kit) was added. After 30 min incubation at 37 ºC, the UV absorbance was measured at 570 nm. Protein concentrations of cell lysates were determined using the BCA™ protein assay kit. Bovine serum albumin was used for a standard curve. The UV absorbance data was then used to calculate the appropriate volume of protein loading. For protein sample preparation; the 100 µl of supernatant was transferred to 50 µl sample dissolving agent. Then, the samples were heated in boiled water for 5 min. After cooling down, the samples were kept at -80 ºC until use.

19.1.2 SDS-PAGE electrophoresis and Western blot analysis

A positive control, caffeic acid phenethyl ester (CAPE), and test samples were loaded onto polyacrylamide gels using a BIO-RAD ready gel J. For western blot analysis, β-actin was used as an internal standard. After electrophoresis, all gels from each experiment were transferred onto polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA). The membrane was then soaked in Tris-buffered saline containing 0.1% Tween 20 (T-TBS) with gentle shaking at 75 rpm for 10 min, three times. For blocking the nonspecific site, membrane was soaked in the Blocking One-P (for phosphorated protein: p-ERK1/2, p-JNK1/2; Nacalai Tesque, Japan) or Blocking One (for others protein: iNOS, ERK1/2, JNK1/2, β-actin) by shaking at 40 rpm for 1 h. The membrane was then rinsed with T-TBS and incubated with specific primary antibodies as follow: p-ERK1/2, p-JNK1/2, ERK1/2, JNK1/2, iNOS and β-actin (dilution 1:1000, Cell Signaling Technology). After incubation overnight at 4 ºC, membrane was rinsed in T-TBS, and incubated in secondary antibodies (HRP-conjugated goat anti-mouse and anti-rabbit, 1:5000) in an immunoreaction enhancer solution (Can Get Signal (TOYOBO, Japan) for 1 h. Then, the membranes were shaken in T-TBS at 75 rpm 10 min, three times. The proteins were detected using an enhanced chemiluminescence (ECL) plus western blotting detection system (Amersham™ GE Healthcare, Biosciences). The images of membranes were recorded using a luminescent image analyzer LAS-4000 mini (Fuji film, Japan).

19.2 Determination of iNOS, p-JNK1/2 and p-ERK1/2 protein expression

RAW 264.7 cells (5.0×10^6 cells/2 ml/well) were seeded into a 6-well multiplate and allowed to adhere for 6 h at 37 ºC in a humidified atmosphere containing 5% CO_2. After that, cells were treated with various concentrations of samples and stimulated for 0.5 and 12 h with or without LPS. The adherence cells were scrapped in a lysis buffer with the following components: 8.4 ml distilled water, 100 µl protease inhibitor cocktail (Thermo Scientific), 100 µl 22% triton X-100, phosphatase inhibitor cocktail (PhosSTP, Roche), and 1 ml sample buffer (0.877 g NaCl, 0.121 g Tris, 0.612 g β-glycerophate, 0.076 g EDTA, and 100 ml H_2O, pH 7.4). Then, cells were disrupted three times (Microson™ ultrasonic cell disruptor, USA) for 30 s, and centrifuged at 2,000 *g* for 2 min.

The supernatant was UV absorbance detected for calculation of the protein loading volume and for protein sample preparation. For the UV absorbance detection; 10 µl of supernatant was added to 190 µl of 0.9 % normal saline solution (NSS) and vortexed. The 10 µl portions of the supernatant was then transferred to a 96-well plate and 200 µl of working reagent (BCA™ protein assay kit) was added. After 30 min incubation at 37 ºC, the UV absorbance was measured at 570 nm. Protein concentrations of cell lysates were determined using the BCA™ protein assay kit. Bovine serum albumin was used for a standard curve. The UV absorbance data was then used to calculate the appropriate volume of protein loading. For protein sample preparation; the 100 µl of supernatant was transferred to 50 µl sample dissolving agent. Then, the samples were heated in boiled water for 5 min. After cooling down, the samples were kept at -80 ºC until use. The SDS-PAGE electrophoresis and the Western blot analysis were follows **19.1.2**

20. Semi-quantitative analysis of RT-PCR

20.1 Total RNA extraction

The 1.5 ml of RAW 264.7 cells (1×10^6 cells/ml) were added to 6-well culture plate and allowed to adhere for 1 h at 37 ºC in a humidified atmosphere containing 5% CO_2. After that, cells were harvested after 20 h incubation with various concentrations of samples. The cells were removed from the culture flask by scrapping and were then isolated to obtain RNA using RNeasy Mini Kit (Qiagen Operon, USA). Briefly, the cells were centrifuged at 4,000 *g* for 5 min. The supernatant were removed and 350 µl of buffer RLT supplemented with 3.5 µl of β-mercaptoethanol was added to the cells and

mixed well by pipetting. Then, 350 µl of 70% ethanol was added and mixed by pipetting. After that, 700 µl of the lysate was loaded into the RNeasy mini column in a 2 ml collection tube and centrifuged at 10,000 *g* for 30 s. The flow-through was discarded and 700 µl of buffer RW1 was added to the RNeasy column and centrifuged at 10,000 *g* for 30 s. The RNeasy mini column was transferred to a new collection tube. Buffer RPE 500 µl was added to RNeasy mini column and centrifuged at 10,000 *g* for 30 s. The flow-through was discarded. The 500 µl of buffer RPE was again added to RNeasy mini column and centrifuged at 10,000 *g* for 2 min 30 s. After that, the RNeasy mini column was placed in a 1.5 ml microcentrifuge tube. The 55 µl of RNase free water was added into a mini column and centrifuged at 10,000 *g* for 30 s to obtain total RNA. The isolated RNA was stored at -20 ºC until use.

20.2 Detection of iNOS and COX-2 mRNA by RT-PCR

In order to acquire the mechanism for anti-inflammatory activity of *K. parviflora* extract, the suppression on mRNA expression of iNOS and COX-2 genes was carried out. Single stranded complementary DNA (cDNA) was generated using cDNA synthesis kit (Rever Tra Ace-α kit, Toyobo, Japan). The standard procedure for a PCR reaction was performed in a 20 µl mixture (Tables 11 and 12).

Table 11 Standard procedure for PCR

Reagents	**Volume/reaction (µl)**
5xRT buffer	4
dNTP mixture (10 mM)	2
RNase inhibitor (10 U/µl)	1
Oligo (dT_{20})	1
Total RNA solution	11
Rever Tra Ace	1
Total volume	20

Table 12 PCR conditions for changing iNOS mRNA and COX-2 mRNA to cDNA

Segment	Step	Temperature (°C)	Time (min)	Cycle
1	Denaturing	42	20	1
2	Annealing	99	5	
3	Extension	4	5	

The resulting cDNA was used as a template for subsequent PCR. The iNOS, COX-2 and β-actin genes were amplified by PCR kit (Rever Tra Dash, TOYOBO, Japan). The standard procedure for a PCR reaction was performed in a 100 µl mixture (Tables 13 and 14). The β-actin, a constitutively expressed gene, was analyzed as an internal standard. Primers used in this study are shown in Table 15.

The PCR mixture is consisted of cDNA 2 µl, dH_2O (RNase free water) 85 µl, 10x PCR buffer 10 µl, forward primer 1 µl, reverse primer 1 µl (10 pmol/µl each) and KOD Dash DNA polymerase (2.5 U) 1 µl, to give a final volume of 100 µl. Amplification was performed for 30 cycles using Takara PCR Thermal Cycler Dice TP600 (Takara, Japan) with the following programme: denaturation at 98 ºC for 30 s, annealing at 60 ºC for 30 s, and extension at 74 ºC for 1 min.

Table 13 Standard procedure for amplification cDNA

Reagents	Volume/reaction (µl)
dH_2O	42.5
10X PCR buffer	5
Primer F	0.5
Primer R	0.5
KOD Dash	0.5
Rever Tra Product	1
Total volume	50

Table 14 PCR conditions for iNOS and COX-2 cDNA amplification

Segment	Step	Temperature (°C)	Time (second)	Cycle
1	Denaturing	98	30	30
2	Annealing	60	30	
3	Extension	74	60	
4	Holding	4	∞	

Table 15 Primers used for detection of iNOS and COX-2, NFKB, Rel A, TNF-α and β-actin mRNA

Gene	Base pair	Sequence of primers (5' to 3')
iNOS	580 bp	Forward -ATCTGGATCAGGAACCTGAA
		Reverse-CCTTTTTTGCCCCATAGGAA
COX-2	860 bp	Forward-GGAGAGAC TATCAAGATAGTGATC
		Reverse-ATGGTCAGTAGACTTTTACAGCTC
NFKB	501 bp	Forward-GTG AGAATGGACAGAACAGC
		Reverse-GAATGTAATCCCACCGTAGG
Rel A	619 bp	Forward-ATGACTTGAATGCAGTGCGC
		Reverse-TTTGGAACAGGTGCAGACAG
TNF-α	347 bp	Forward-TCTGTCTACTGAACTTCGGG
		Reverse-AGATAGCAAATCGGCTGACG
β-actin	514 bp	Forward-TGTGATGGTGGGAATGGGTCAG
		Reverse-TTTGATGTCACGCACGATTTCC

20.3 Agarose gel electrophoresis

The 580 base pairs (bp) of iNOS, 860 bp of COX-2 and 514 bp of β-actin DNA fragments were obtained from **20.2** and separated on 1.2% (w/v) agarose gel electrophoresis for determining an amount of DNA. Agarose (0.72 g) was added into 10xTAE buffer (60 ml) and heated up in the microwave until clear solution and poured on a plastic tray. After the agarose gel has completely set (30-45 minutes at room temperature), the comb was carefully removed and the gel was installed on the platform in the electrophoresis tank containing 1x TAE buffer. The 10 µl PCR products mixed with

5 µl DNA ladder were loaded into the wells of 1.2% w/v agarose gel using micropipette. The electrophoresis was run at 100 volts. The electrophoresis process was stopped until the loading dye has migrated to three fourth of the agarose gel. The agarose gel was stained with SYBR® Safe DNA gel stain solution for 30 minutes under dark condition. After that the DNA pattern was observed under UV 312 nm using a UV light box (Vilber Lourmet) and photographed.

21. Quantitative analysis of real-time RT-PCR

RAW 264.7 cells (5.0×10^6 cells/2 ml/well) were seeded into 6-well multiplates. After 6 h, the cells were then incubated with LPS, and test compounds at the concentrations of 3, 10 and 30 µg/ml for 10 min, and then stimulated for 8 h with LPS. The cells were removed from the culture flask by scrapping and total RNA was extracted from the cells using RNeasy™ mini Kit (Qiagen) according to the manufacturer's instructions. In Brief, 350 µl of buffer RLT supplemented with β-mercaptoethanol were added to the well and cells were scrapped and mixed well by syringe. Then, 350 µl of 70% ethanol was added and mixed by vortex for 1 min. After that, 700 µl of the lysate was loaded into the RNeasy mini column and centrifuged at 10,800 *g* for 15 s. The flow-through was discarded and the RNeasy mini column was transferred to a new collection tube. The 700 µl of buffer RW1 was added to the RNeasy column and centrifuged at 10,800 *g* for 15 s. The RNeasy mini column was transferred to a new collection tube. Buffer RPE 500 µl was added to RNeasy mini column and centrifuged at 10,800 *g* for 15 s. The flow-through was discarded. The 500 µl of buffer RPE was again added to RNeasy mini column and centrifuged at 10,800 *g* for 2 min. After that, the RNeasy mini column was placed in a 1.5 ml microcentrifuge tube. The 30 µl of RNase free water was added into a mini column and centrifuged at 10,800 *g* for 1 min. Replicated this step twice times to obtain 60 µl total RNA. The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and determining the ratio of the readings at 260 nm and 280 nm. Isolated RNA was subsequent to determined nucleic acid concentration for submitted to quantitative analysis mRNA and to ensure that positive results were not due to poor RNA extraction. In brief, RNA extract was 10 times diluted by diethyl dicarbonate (DEPC) water containing 0.1 M Tris-HCl and were then spectrophotometrically using spectrophotometer (Life Science UV/Vis spectrophotometer Du®530 BECKMAN).

Single-stranded complementary DNA (sscDNA) was generated from 1 µg total RNA using cDNA synthesis kit (Rever Tra Ace-α, TOYOBO, Japan) consisting of RNA 1 µg, 5xRT buffer 4 µl, RT enzyme mix 1 µl, Primer mix 1 µl for total volume 20 µl according to the manufacturer's instructions. Reverse-transcription was performed at 37 ºC for 15 min and 98 ºC for 5 min. The cDNA obtained were used as the following real-time PCR.

The iNOS and β-actin genes were amplified. The β-actin, a constitutively expressed gene, was analyzed as an internal standard. The primers for each gene were designed follow (Table 16):

Table 16 Primers used for detection of iNOS mRNA

Gene	**Sequence of primers (5' to 3')**
iNOS	Forward-CAGCTGGGCTGTACAAACCTT
	Reverse- CATTGGAAGTGAAGCGTTTCG
β-actin	Forward-AGTGGTACGACCAGAGGCATAC
	Reverse-ATGGGTCAGAAGGACTCCTACG

To the complete real-time PCR mixture, the following components were added for the quantitative assay, each 50 µl PCR mixture contained 22 µl distilled-deionized water, 25 µl THUNDERBIRD™ SYBR® qPCR mix (TOYOBO Co, Japan), 1 µl each of iNOS forward primers and reverse primers for testing samples and β-actin primers for internal standard, and 1 µl cDNA were submitted to the following thermal profile: 2 min at 95 ºC (pre-PCR step), 60 cycles at 95 ºC for 30 s, 64 ºC for 30 s, and 72 ºC for 30 s (Table 17). Amplification was performed using MJ Mini™ personal Thermal Cycler with a MiniOpticon™ Real-time PCR System (BIO-RAD). The analysis software was MJ Opticon Monitor™ version 3.1.

Table 17 PCR conditions for iNOS cDNA amplification

Segment	**Step**	**Temperature (°C)**	**Time**	**Cycle**
1	Pre-PCR	95	2 min	1
2	Denaturing	95	30 s	60
3	Annealing	64	30 s	
4	Extension	72	30 s	
5	Holding	4	∞	

22. Effects on protein kinases

Effects on enzyme activities of protein tyrosine kinases involved in the expression of iNOS, were examined by a SelectScreen® Kinase Profiling Service (Life Technologies Corp., Carlsbad, CA, USA) and using Z'-LYTE® and Adapta® assay methods.

23. Statistical analysis

All data were expressed as means ± SEM. The data analysis was performed by one-way analysis of variance (1-WAY ANOVA), followed by *Bonferroni*'s test. The *p*-value less than 0.05 was considered to be significant.

CHAPTER 4

RESULTS

1. Concentration-dependent effects of LPS on NO production in RAW 264.7 cells

The concentration-dependent effects of LPS on production of NO comparing with the standard curve (accumulation of NO_2^-) was observed (Figure 12).

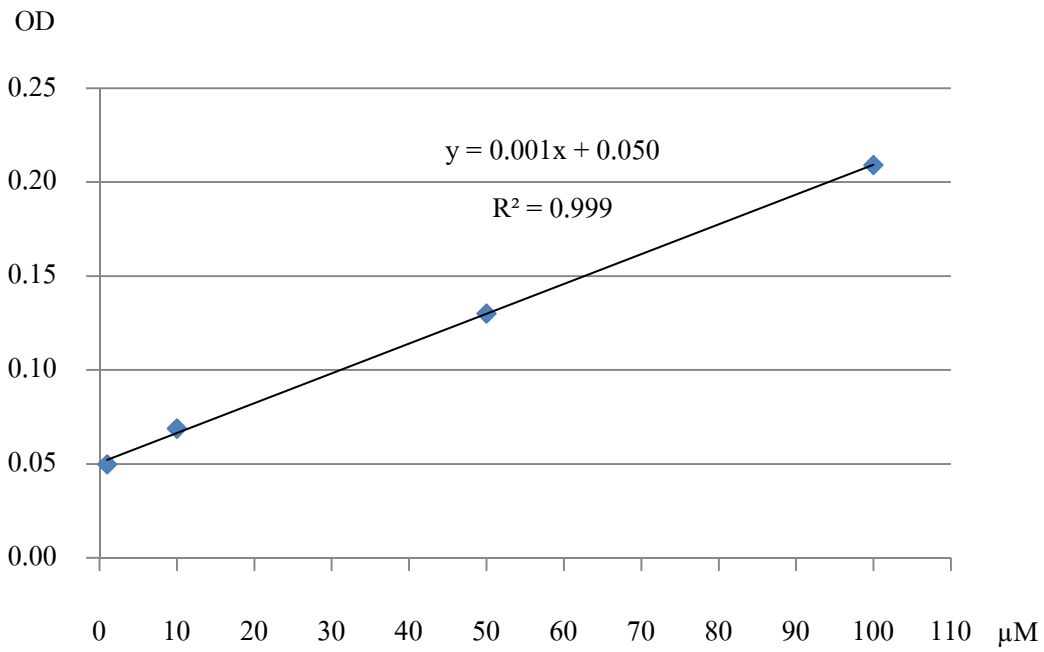


Figure 12 Standard curve of NO_2^- production

Table 18 Concentration-dependent effects of LPS on NO production in RAW 264.7 cells

LPS	0.1	0.3	1.0	3.0	10	30	(µg/ml)
NO_2^-	11.3±0.2	16.4±0.4	22.1±0.6	24.4±0.5	27.2±0.3	26.2±0.8	(µM)

The result showed that the increasing of LPS concentration cause an increase of NO production, but not all ranges. The highest concentration of LPS (30 µg/ml) showed lower amount of NO_2^- production (26.2±0.8 µM) than concentration of 10 µg/ml (27.2±0.3 µM).

2. Inhibition on NO production of various fractions from *K. parviflora* and its ethanol extract

After treating cells with EtOH extract and various fractions of *K. parviflora* (n-hexane, $CHCl_3$, EtOAc and water fractions), the results showed that the chloroform fraction and ethanol extract of *K parviflora* had potent inhibitory effect on the release of NO with IC_{50} values of 8.4 and 8.1 µg/ml, respectively, and cytotoxic effects were not observed at concentration less than 30 µg/ml. n-Hexane fraction also significantly inhibited NO production with IC_{50} of 13 µg/ml. Whereas EtOAc and water fraction showed moderate inhibitory effect (IC_{50} of 61 and 40 µg/ml, respectively) (Table 19).

3. Carrageenan-induced rat paw edema

From the carrageenan-induced rat paw edema test, the average right hind paw volumes and percentages of inhibition by the EtOH extract, fractions from *K. parviflora* and standard drugs are showed in Table 20. For the control group, the injection of the phlogistic agent caused localized edema starting at 0.5 h after injection. The swelling increased progressively to a maximum volume of 7.29 ± 0.13 ml at 5 h after the carrageenan injection. Hexane and chloroform fractions (150 mg/kg) showed potent activity on anti-inflammatory effect by reducing the paw edema volumes. Hexane fraction significantly reduced paw edema volume at 1-5 h (p<0.01) with percentage of inhibition being 8.74, 15.14, 20.30, 24.69, 25.21 and 25.31, respectively when compared to the control. Moreover, the chloroform fraction was more potent than the hexane fraction, which significantly decreased paw edema volume at 0.5-5 h (p<0.01) with percentage of inhibition being 13.47, 19.94, 24.79, 25.39, 24.24 and 24.23, respectively compared to the control. Rats pre-treated with indomethacin (10 mg/kg, p.o.) had a significant reduction of the paw edema at 2 h up to 3 h and inhibition of rat paw edema was dramatically decrease until 5 h. After the administration of indomethacin, the percentage of inhibition being 17.03, 18.32, 16.60 and 10.75 respectively compared to the control. The EtOH extract also decreased the volume of paw edema at 1 h (p<0.05), 2 h (p<0.01), 3 h (p<0.01), 4 h (p<0.01) and 5 h (p<0.01) with % inhibition of 9.02, 10.83, 12.91, 13.86 and 11.36, respectively. The EtOAc fraction showed inhibitory

effect only at 1 h (p<0.05) after administration (9.59 % inhibition). While, the water fraction did not show any inhibitory effect. Therefore, the results indicated that chloroform fraction possessed anti-inflammatory activity and had maximum inhibitory effects at 3 h similar to that of the standard indomethacin.

4. Acute toxicity test of *K. parviflora* extract in mice

No death of mice was observed during the treatment period in the control and treated groups which received the ethanol extract and chloroform fraction of *K. parviflora* up to 2 g/kg orally in both male and female mice. The animals did not show any changes in general behaviors or signs of toxicities or other abnormal physiological activities during the observation period.

5. Effect of *K. parviflora* extract on PGE_2 production

The ethanol extract highly inhibited PGE_2 production with an IC_{50} of 9.26 µg/ml, while the reference drug, indomethacin, showed more potent with an IC_{50} of 0.5 µg/ml.

6. Isolation and structure elucidation

6.1 Isolation

From bioassay-guided isolation, the chloroform fraction had a marked effect on rat paw edema. To identify its active components, the chloroform fraction was subjected to silica gel column chromatography and HPLC, giving 12 known methoxyflavonoids: techtochrysin (**1**, 3.42% w/w from $CHCl_3$ fraction), 5,7-dimethoxyflavone (**2**, 15.44%), 7,4′-dimethylapigenin (**3**, 2.35%), trimethylapigenin (**4**, 13.05%), tetramethylluteolin (**5**, 0.68%), 5-hydroxy-3,7-dimethoxyflavone (**6**, 1.79%), 3,5,7-trimethoxyflavone (**7**, 0.72%), 3,7,4′-trimethylkaempferol (**8**, 5.12%), tetramethyl- kaempferol (**9**, 0.49%), ayanin (**10**, 0.79%), retusine (**11**, 1.53%), and pentamethylquercetin (**12**, 19.74%) (Figures 13 and 14). The structure of the isolated compounds **1**-**12** were elucidated by comparing with the previous literatures (Wang et al., 1989, Sutthanut et al., 2007). Phytochemical investigation of chloroform

fraction showed that pentamethylquercetin (**12**, 19.74%w/w), 5,7-dimethoxyflavone (**2**, 15.44%w/w) and trimethylapigenin (**4**, 13.05%w/w) were the major compounds (Figure 13).

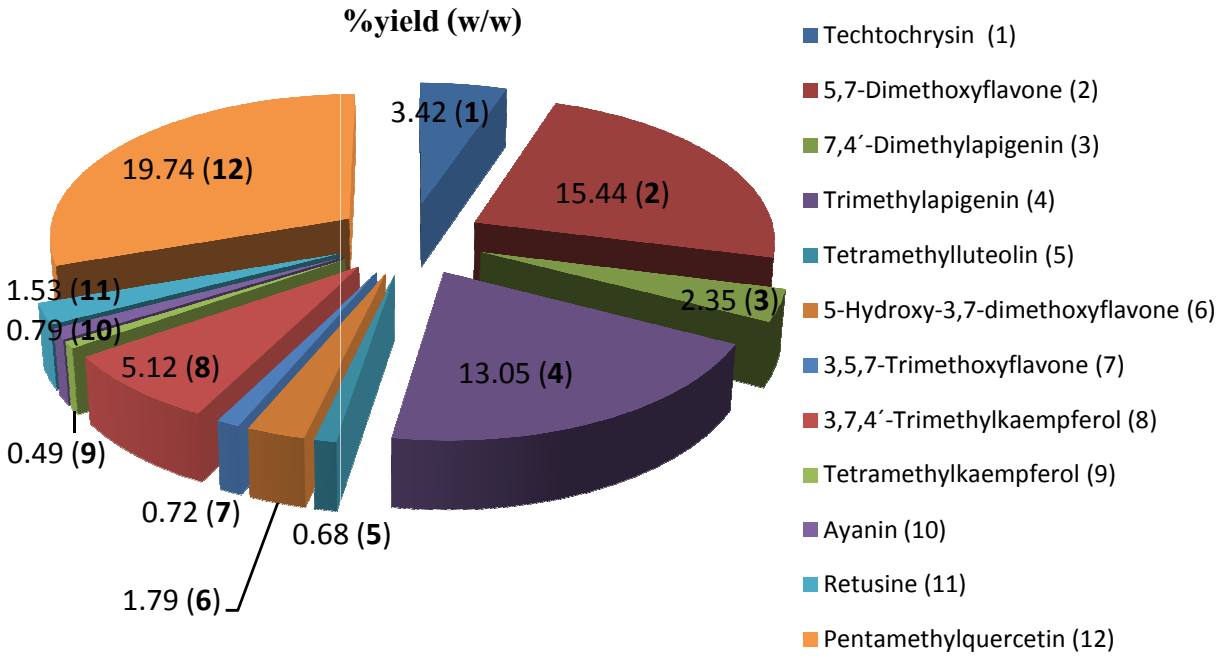
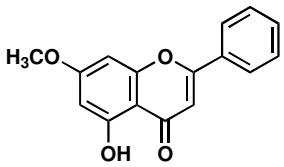
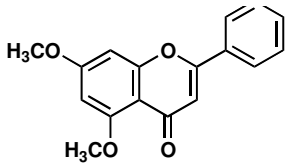


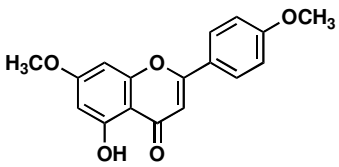
Figure 13 % Yield (w/w) of the isolated compounds (**1-12**) from *K. parviflora* rhizomes



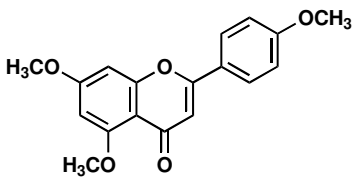
Techtochrysin (**1**)



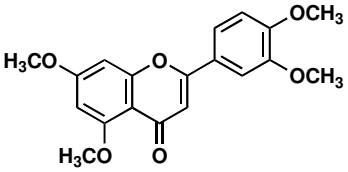
5,7-Dimethoxyflavone (**2**)



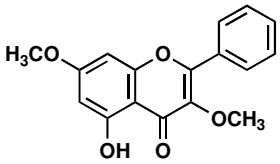
7,4'-Dimethylapigenin (**3**)



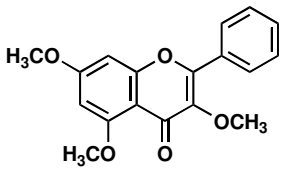
Trimethylapigenin (**4**)



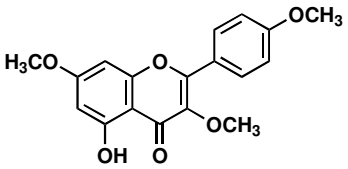
Tetramethylluteolin (**5**)



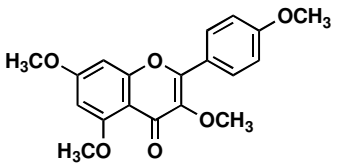
5-Hydroxy-3,7-dimethoxyflavone (**6**)



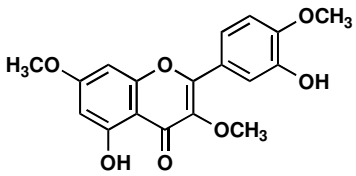
3,5,7-Trimethoxy-flavone (**7**)



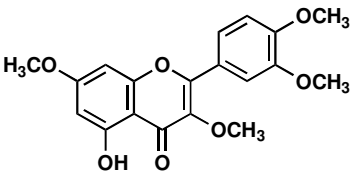
3,7,4'-Trimethylkaempferol (**8**)



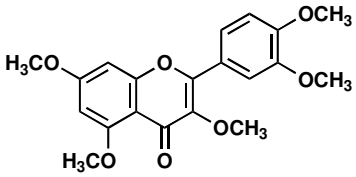
Tetramethylkaempferol (**9**)



Ayanin (**10**)



Retusine (**11**)



Pentamethylquercetin (**12**)

Figure 14 Chemical structures of **1–12** isolated from the rhizomes of *K. parviflora*

Table 19 Inhibitory effects of the ethanolic extract and fractions of *K. parviflora* rhizomes on NO production in LPS-activated RAW264.7 cells

	Concentration of test sample (μg/ml)						
Sample	0	1	3	10	30	100	IC_{50} (μg/ml)
	Inhibition (%)						
EtOH extract	0.0±1.7	4.6±1.5	16.5±0.7**	61.4±0.9**	91.1±0.3**	97.4±0.2**, a (0.44±0.0)	8.1
n-Hexane fraction	0.0±2.0	5.5±0.7	9.4±2.0**	41.3±0.7**	74.2±0.8**	96.5±0.3**	13
$CHCl_3$ fraction	0.0±1.3	2.9±1.4	13.4±0.9**	64.9±0.8**	89.0±0.2**	98.2±0.3**, a (0.19±0.0)	8.4
EtOAc fraction	0.0±1.5	4.4±0.6	3.7±1.5	17.1±0.6**	28.1±1.1**	77.5±1.0**	61
Water fraction	0.0±2.1	7.1±1.2	7.9±2.6	27.5±1.3**	41.7±1.3**	84.3±0.2**	40

Values represent means±S.E.M. (*N*=4). Significantly different from the control (0 μg/ml), **p<0.01.

[a]Cytotoxic effect was observed and values in parentheses indicate %cell viability in MTT assay at 100 μg/ml

Table 20 Effect of each fraction of *K. parviflora* and indomethacin on carrageenan-induced rat paw edema

Treatment	Dose (mg/kg, p.o.)	Initial paw volume (ml)	Paw edema volume (ml)						Inhibition of paw edema (%)					
			0.5 h	1 h	2 h	3 h	4 h	5 h	0.5 h	1h	2 h	3 h	4 h	5 h
Cosolvent	10[a]	4.45±0.05	5.02±0.08	5.61±0.06	6.37±0.09	6.93±0.10	7.22± 0.12	7.29± 0.13						
Indomethacin	10	4.57±0.05	5.26±0.08	5.29±0.08	5.29±0.05**	5.66±0.11**	6.02±0.12**	6.50±0.11**	-4.70	5.76	17.03	18.32	16.60	10.75
EtOH extract	150	4.42±0.16	4.98±0.12	5.10±0.11*	5.68±0.16**	6.04±0.17**	6.21±0.16**	6.46±0.12**	0.88	9.02	10.83	12.91	13.86	11.36
Hexane fraction	150	4.34±0.12	4.58±0.08	4.76±0.09**	5.08±0.08**	5.22±0.07**	5.40±0.08**	5.44±0.08**	8.74	15.14	20.30	24.69	25.21	25.31
$CHCl_3$ fraction	150	4.17±0.08	4.35±0.04**	4.49±0.05**	4.79±0.07**	5.17±0.13**	5.47±0.10**	5.52±0.09**	13.47	19.94	24.79	25.39	24.24	24.23
EtOAC fraction	150	4.49±0.15	4.89±0.14	5.07±0.15*	5.87±0.22	6.54±0.28	6.57±0.23	6.85±0.25	2.68	9.59	7.79	5.64	8.91	6.01
Water fraction	150	4.44± 0.09	5.07±0.11	5.23±0.15	6.00±0.24	6.50±0.20	6.72±0.19	6.79±0.20	-0.85	6.75	5.78	6.23	6.87	6.84

Values were presented as mean ± S.E.M. (N=10), Control = 10% DMSO: tween 80: propylene glycol: distilled water, 1:4:4:1)

[a]Dose in ml/kg, $^{*}p<0.05$, $^{**}p< 0.01$, significantly different compared to the control group (Bonferroni's test)

6.2 Structure identification

All isolated compounds were identified as 7-methoxyflavonoids which showed structure backbone and C-position as figure 15. All compounds were run on 500 MHz of ^{1}H NMR and ^{13}C NMR , samples were dissolved in DMSO. Twelve compounds (**1**-**12**) were purified and elucidated by comparison with spectral data (^{1}H NMR and ^{13}C NMR) previously reported (Wang et al., 1989; Sutthanutetal., 2007 (Table 21)).

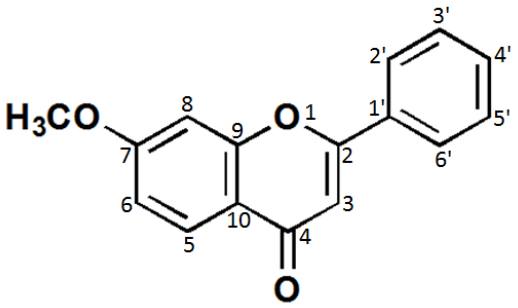


Figure 15 Backbone structure of 7-methoxyflavonoid

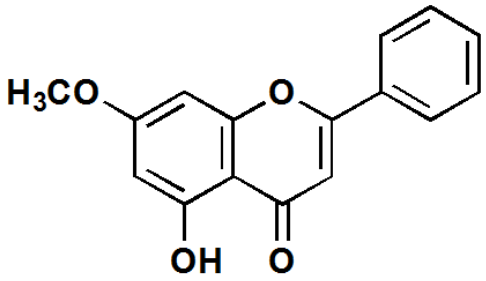
Table 21 ^{1}H NMR and ^{13}C NMR spectral data of 5,7-dimethoxyflavone (**2**) and the reference

Positions	**5,7-Dimethoxyflavone** (Sutthanut et al., 2007)		**(2)**	
	^{13}C ($CDCl_3$; 100 MHz)	**^{1}H (mult.; *J* in Hz)** ($CDCl_3$; 400 MHz)	**^{13}C** (DMSO-d_6, 500 MHz)	**^{1}H (mult.; *J* in Hz)** (DMSO-d_6, 500 MHz)
1	-	-	-	-
2	160.70	-	159.48	-
3	109.10	6.67 (s)	108.15	6.78 (s)
4	177.50	-	175.57	-
5	161.00, -OCH_3; 56.30	-OCH_3; 3.90 (s)	160.22, -OCH_3; 55.99	-OCH_3; 3.84 (s)
6	96.20	6.37(d; 2.2)	96.20,	6.51 (d; 2.5)
7	164.10, -OCH_3; 55.70	-OCH_3; 3.94 (s)	163.69, -OCH_3; 55.88	-OCH_3; 3.91 (s)
8	92.90	6.56 (d; 2.2)	93.28	6.86 (d; 2.5)
9	159.90	-	159.12	-
10	109.40	-	108.29	-
1'	131.60	-	131.29	-
2'	125.90	7.86 (m)	125.81	8.04 (dd; 2, 7)
3'	128.90	7.49 (m)	128.94	7.57 (m)
4'	131.10	7.49 (m)	130.81	7.57 (m)
5'	128.90	7.49 (m)	128.94	7.57 (m)
6'	125.90	7.86 (m)	125.81	8.04 (dd; 2, 7)

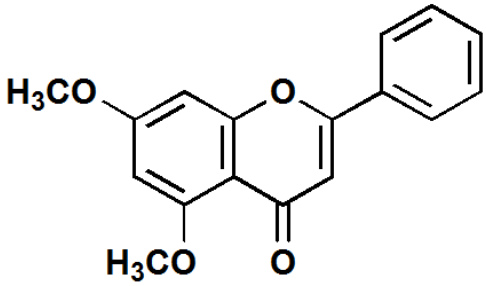
6.2.1 Identification of techtochrysin (1) and 5,7-dimethoxyflavone (2)

Techtochrysin (1): Pale yellow, amorphous powder, $C_{16}H_{121}O_4$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 22).

5,7-Dimethoxyflavone (2): Amorphous, white powder, $C_{17}H_{14}O_4$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 22).



Compound 1. Techtochrysin



Compound 2. 5,7-Dimethoxyflavone

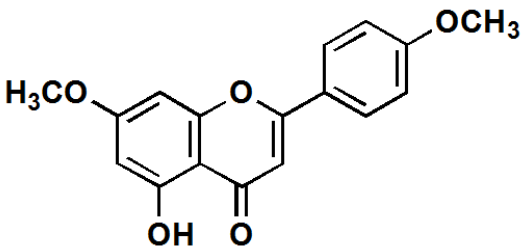
Table 22 ^{1}H NMR and ^{13}C NMR (DMSO-d_6; 500 MHz) spectral data of techtochrysin and 5,7-dimethoxyflavone

Positions	(1)		(2)	
	^{13}C	^{1}H (mult.; *J* in Hz)	^{13}C	^{1}H (mult.; *J* in Hz)
1	-	-	-	-
2	157.30	-	159.48	-
3	104.85	7.04 (s)	108.15	6.78 (s)
4	182.01	-	175.57	-
5	163.39	-OH; 12.82 (s)	160.22, -OCH_3;55.99	-OCH_3; 3.84 (s)
6	98.07	6.40 (d; 2.05)	96.20,	6.51 (d;2.5)
7	165.29, -OCH_3;56.02	-OCH_3; 3.88 (s)	163.69, -OCH_3;55.88	-OCH_3; 3.91 (s)
8	92.74	6.82 (d; 2.05)	93.28	6.86 (d;2.5)
9	161.10	-	159.12	-
10	105.27	-	108.29	-
1'	132.06	-	131.29	-
2'	126.37	8.10 (d, 7.95)	125.81	8.04 (dd; 2, 7)
3'	129.06	7.60 (m)	128.94	7.57 (m)
4'	130.51	7.60 (m)	130.81	7.57 (m)
5'	129.06	7.60 (m)	128.94	7.57 (m)
6'	126.37	8.10 (2, 7.95)	125.81	8.04 (dd; 2, 7)

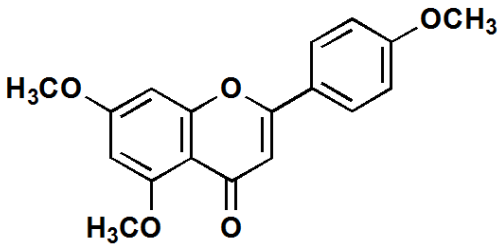
6.2.2 Identification of 7,4´-dimethylapigenin (3) and trimethylapigenin (4)

7, 4'-Dimethylapigenin (3): Pale yellow, amorphous powder, $C_{17}H_{14}O_5$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 23).

Trimethylapigenin (4): Amorphous, white powder, $C_{18}H_{16}O_5$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 23).



ompound 3. 7, 4'-Dimethylapigenin



Compound 4. Trimethylapigenin

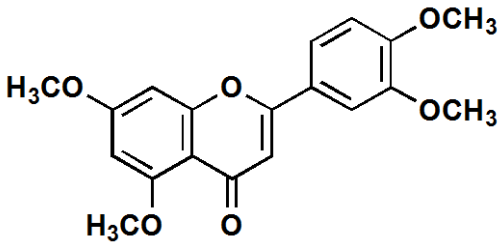
Table 23 ^{1}H NMR and ^{13}C NMR (DMSO-d_6; 500 MHz) spectral data of 7,4´-dimethylapigenin and trimethylapigenin

Positions	(3)		(4)	
	^{13}C	^{1}H (mult.; *J* in Hz)	^{13}C	^{1}H (mult.; *J* in Hz)
1	-	-	-	-
2	138.95	-	159.03	-
3	131.08	7.58 (s)	106.67	6.67 (s)
4	178.25	-	175.53	-
5	165.27	-OH; 12.55 (s)	161.67, -OCH_3; 55.84	-OCH_3; 3.90 (s)
6	97.85	6.41 (d; 2)	96.09	6.49 (d; 2.5)
7	156.46, -OCH_3; 56.13	-OCH_3; 3.87 (s)	163.57, -OCH_3; 55.38	-OCH_3; 3.83 (s)
8	92.40	6.78 (d; 2)	93.25	6.84 (d; 2.5)
9	155.43	-	159.55	-
10	105.37	-	108.19	-
1'	128.66	-	122.94	-
2'	129.88	8.05 (m)	127.60	7.9 (dd; 8.5, 3)
3'	128.13	7.60 (m)	114.35	7.09 (dd; 8.5, 2.5)
4'	160.88	- OCH_3; 3.83 (s)	160.15, -OCH_3; 55.96	-OCH_3;3.85 (s)
5'	128.13	7.60 (m)	114.35	7.9 (dd; 8.5, 3)
6'	129.88	7.30 (m)	127.60	7.09 (dd; 8.5, 2.5)

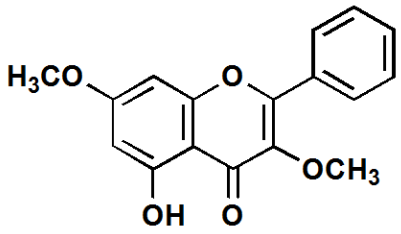
6.2.3 Identification of tetramethylluteolin (5) and 5-hydroxy-3,7-dimethoxyflavone (6)

Tetramethylluteolin (5): Pale yellow, amorphous powder, $C_{19}H_{18}O_6$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 24).

5-Hydroxy-3,7-dimethoxyflavone (6): Pale yellow, amorphous powder, $C_{17}H_{14}O_5$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 24).



Compound 5. Tetramethylluteolin



Compound 6. 5-Hydroxy-3,7-dimethoxyflavone

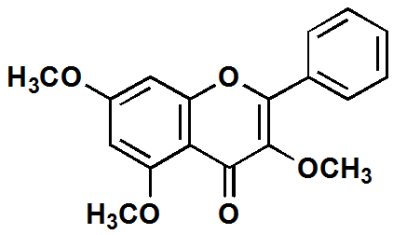
Table 24 ^{1}H NMR and ^{13}C NMR (DMSO-d_6; 500 MHz) spectral data of tetramethylluteolin and 5-hydroxy-3,7-dimethoxyflavone

Positions	(5)		(6)	
	^{13}C	^{1}H (mult.; *J* in Hz)	^{13}C	^{1}H (mult.; *J* in Hz)
1	-	-	-	-
2	148.92	-	155.40	-
3	106.90	6.77 (s)	138.94	-OCH_3; 3.87 (s)
4	175.53	-	178.24	-
5	160.18, -OCH_3; 55.94	-OCH_3; 3.91 (s)	160.88	-OH; 12.5 (s)
6	96.21	6.51 (d; 3)	97.84	6.40 (d; 3)
7	163.55, -OCH_3; 55.59	-OCH_3; 3.83 (s)	165.26, -OCH_3; 56.04	-OCH_3;3.83 (s)
8	93.34	6.87 (d; 3)	92.38	6.76 (d; 3)
9	151.50	-	156.44	-
10	109.05	-	105.37	-
1'	123.10	-	131.07	-
2'	119.16	7.65 (dd; 8.5, 2.5)	128.12	8.04 (m)
3'	111.63	7.12 (d; 8.5)	129.88	7.60 (m)
4'	159.61, -OCH_3; 55.87	-OCH_3; 3.88 (s)	128.66	7.60 (m)
5'	159.03, -OCH_3; 55.76	-OCH_3; 3.85 (s)	129.88	7.60 (m)
6'	108.26	7.54 (d; 2.5)	128.12	8.04 (m)

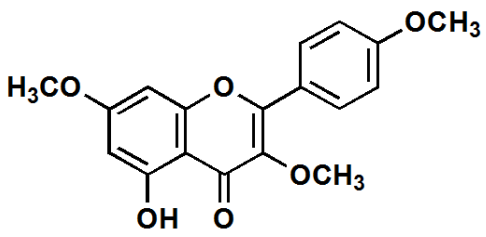
6.2.4 Identification of 3,5,7-trimethoxyflavone (7) and 3,7,4′-trimethylkaempferol (8)

3,5,7-trimethoxyflavone (7): White crystal, $C_{18}H_{16}O_5$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 25).

3,7,4′-trimethylkaempferol (8): Pale yellow, amorphous powder, $C_{18}H_{16}O_6$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 25).



Compound 7. 3,5,7-Trimethoxyflavone



Compound 8. 3,7,4′-Trimethylkaempferol

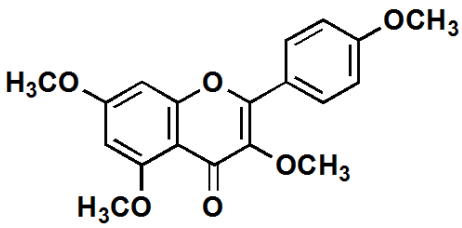
Table 25 ^{1}H NMR and ^{13}C NMR (DMSO-d_6; 500 MHz) spectral data of 3,5,7-trimethoxyflavone and 3,7,4′-trimethylkaempferol

Positions	(7)		(8)	
	^{13}C	^{1}H (mult.; *J* in Hz)	^{13}C	^{1}H (mult.; *J* in Hz)
1	-	-	-	-
2	151.66	-	155.44	-
3	141.01, -OCH_3; 59.44	-OCH_3; 3.76 (s)	138.08, -OCH_3; 59.68	-OCH_3; 3.87 (s)
4	172.19	-	178.03	-
5	160.34, -OCH_3; 56.11	-OCH_3; 3.89 (s)	161.37	-OH; 12.5 (s)
6	92.97	6.51 (d; 2.51)	97.73	6.75 (d; 2)
7	163.80, -OCH_3; 55.98	-OCH_3; 3.86 (s)	165.10, -OCH_3; 56.01	-OCH_3; 3.33 (s)
8	95.96	6.82 (d; 2.5)	92.32	6.38 (d; 2)
9	158.27	-	156.27	-
10	108.56	-	105.18	-
1'	130.48	-	122.01	-
2'	127.80	8.03 (dd; 7.75, 2)	129.96	8.07 (dd; 8, 2)
3'	130.33	7.56 (m)	114.18	7.15 (d, 8)
4'	128.62	7.56 (m)	160.86	-OCH_3; 3.81 (s)
5'	130.33	7.59 (m)	114.18	7.15 (d; 8)
6'	127.80	8.03 (dd; 7.75, 2)	129.96	8.07 (dd; 8, 2)

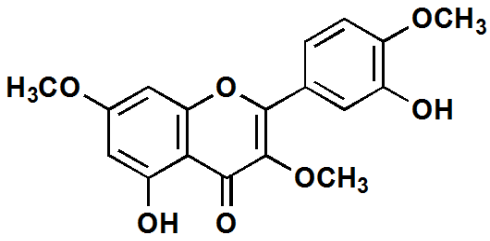
6.2.5 Identification of tetramethylkaempferol (9) and ayanin (10)

Tetramethylkaempferol (9): Pale yellow, amorphous powder, $C_{19}H_{18}O_6$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 26).

Ayanin (10): Yellow crystal, $C_{18}H_{16}O_7$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 26).



Compound 9. Tetramethylkaempferol



Compound 10. Ayanin

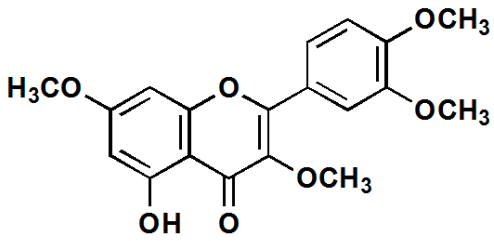
Table 26 ^{1}H NMR and ^{13}C NMR (DMSO-d_6; 500 MHz) spectral data of tetramethylkaempferol and ayanin

Positions	(9)		(10)	
	^{13}C	^{1}H (mult.; *J* in Hz)	^{13}C	^{1}H (mult.; *J* in Hz)
1	-	-	-	-
2	151.61	-	155.56	-
3	140.14, -OCH_3; 59.15	-OCH_3; 3.85 (s)	138.13, -OCH_3; 59.66	-OCH_3; 3.81 (s)
4	172.02	-	178.02	-
5	160.78	-OCH_3; 3.89 (s)	160.85	-OH; 12.65 (s)
6	95.80	6.48 (d; 2)	97.71	6.38 (d; 2.1)
7	163.58, -OCH_3; 55.88	-OCH_3; 3.85 (s)	165.09, -OCH_3; 56.00	-OCH_3; 3.87 (s)
8	92.88	6.80 (d; 2)	92.21	6.73 (d, 2.1)
9	158.08	-	156.23	-
10	108.40	-	105.15	-
1'	-	-	122.09	-
2'	129.45	8.03 (dd; 9.75, 2.75)	120.33	7.59 (dd;9.5, 2.2)
3'	114.03	7.12 (dd; 9.75, 3)	111.80	7.12 (d, 9.5)
4'	160.78	-OCH_3; 3.74 (s)	146, -OCH_3; 55.58	-OCH_3; 3.87 (s)
5'	114.03	7.12 (dd; 9.75, 3)	150	-OH; 9.5 (s)
6'	129.45	8.03 (dd; 9.75, 2.75)	114.99	7.58 (d; 2.2)

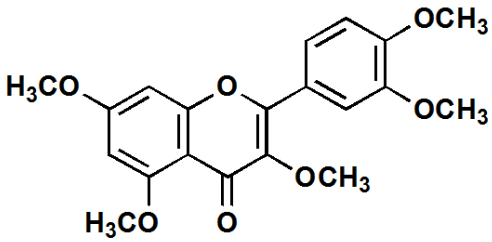
6.2.6 Identification of retusine (11) and pentamethylquercetin (12)

Retusine (11): Pale yellow, amorphous powder, $C_{19}H_{18}O_6$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 27).

Pentamethylquercetin (12): Pale yellow, amorphous powder, $C_{18}H_{16}O_7$, ^{1}H NMR (DMSO-d_6, 500 MHz), ^{13}C NMR (DMSO-d_6, 500 MHz) (Table 27).



Compound 11. Retusine



Compound 12. Pentamethylquercetin

Table 27 ^{1}H NMR and ^{13}C NMR (DMSO-d_6; 500 MHz) spectral data of retusine and pentamethylquercetin

Positions	(11)		(12)	
	^{13}C	**^{1}H (mult.; *J* in Hz)**	**^{13}C**	**^{1}H (mult.; *J* in Hz)**
1	-	-	-	-
2	155.40	-	151.59	-
3	138.21, -OCH_3; 59.71	-OCH_3; 3.83 (s)	140.25, -OCH_3; 3.89 (s)	-OCH_3; 3.85 (s)
4	178.02	-	172.01	-
5	160.83	-OH; 12.6 (s)	160.20, -OCH_3; 59.17	-OCH_3; 3.90 (s)
6	97.76	6.39 (d;, 2.5)	95.79	6.48 (d; 2.5)
7	165.12, -OCH_3; 56.04	-OCH_3; 3.87 (s)	163.58, -OCH_3; 55.52	-OCH_3; 3.75 (s)
8	92.41	6.80 (d; 2.5)	92.95	6.80 (d; 2.5)
9	156.25	-	158.05	-
10	105.17	-	110.99	-
1'	111.51	-	122.43	-
2'	121.98	7.74 (dd; 8.5, 3)	121.31	7.68 (dd; 8, 2)
3'	111.19	7.16 (d; 8.5)	108.37	7.15 (d; 8)
4'	148.40	-OCH_3; 3.88 (s)	148.00, -OCH_3; 55.90	-OCH_3; 3.85 (s)
5'	151.26	-OCH_3; 3.86 (s)	150.00	-OCH_3; 3.85 (s)
6'	111.19	7.66 (d; 3)	111.44	7.64 (d; 2)

7. Effects on production of NO in LPS-stimulated macrophage RAW264.7 cells

The results showed that compounds **2**, **4**, and **5** significantly inhibited NO production in LPS-activated RAW 264.7cells with IC_{50} values of 5.1, 4.6, and 8.7 μg/ml, respectively, without cytotoxic effects except for **2** at 100 μg/ml; while compounds **7**, **8**, and **10** possessed moderate to mild activity (IC_{50} = 44-60 μg/ml) and compounds **3**, **6**, **11**, and **12** showed moderate cytotoxic effects (Table 28). Whereas, the reference compounds, parthenolide and CAPE, showed strong activity with IC_{50} values of 0.31 and 0.92 μg/ml.

8. Determination of TNF-α production by LPS-activated macrophages

The result revealed that ethanol extract, *n*-hexane, chloroform and ethyl acetate fractions suppressed TNF-α production in macrophage cells with IC_{50} of 22.28, 12.64, 3.79 and 34.72 μg/ml, respectively, while the water fraction could not inhibit TNF-α production. The active compounds responsible for the inhibitory effect on LPS-induced TNF-α release were also examined.

The results showed that compounds **2**, **4**, and **5** moderately inhibited the release of TNF-α with IC_{50} of >30, 64 and 100 μg/ml, respectively (Table 29). Because the toxicity of compound **2** had been observed at the highest concentration (100 μg/ml) on the study of effects on NO production in LPS-stimulated macrophage RAW264.7 cells (Table 28) and inhibitory effect of compound **2** on TNF-α production at the concentration of 30 μg/ml showed %inhibition lower than 50 %. Thus, the IC_{50} value of compound **2** was then reported as >30 μg/ml.

Table 28 Inhibitory effects of **1**-**12** and reference compounds (parthenolide and CAPE) on NO production in LPS-activated RAW264.7 cells

	Concentration of test sample (μg/ml)							
	0	1	3	10	30	100	IC_{50} (μg/ml)	IC_{50} (μM)
Sample	Inhibition (%)							
Techtochrysin (**1**)	0.0±0.8	5.5±0.8*	9.3±1.4**	9.7±2.1**	20.8±2.4**	33.2±0.3**	>100	-
5,7-Dimethoxyflavone (**2**)	0.0±1.3	13.0±0.4**	19.4±1.3**	65.5±1.2**	90.8±0.5**	99.4±0.1$^{**, a}$ (0.1±0.0)	5.1	18
7,4′-Dimethylapigenin (**3**)	0.0±0.6	10.3±0.6** (77.7±0.0)	12.2±1.0$^{**, a}$ (68.4±0.0)	22.1±0.6$^{**, a}$ (59.9±0.0)	36.1±0.4$^{**, a}$ (52.1±0.0)	78.2±0.4$^{**, a}$ (39.8±0.0)	56	188
Trimethylapigenin (**4**)	0.0±1.5	22.0±0.7**	38.4±1.6**	67.5±0.5**	80.7±0.4**	98.7±0.0**	4.6	15
Tetramethylluteolin (**5**)	0.0±0.5	17.3±0.4**	29.2±0.2**	55.2±1.0**	62.7±0.8**	95.4±0.3**	8.7	26
5-Hydroxy-3,7-dimethoxyflavone (**6**)	0.0±0.5	11.2±0.4**	17.3±0.5**	25.0±0.9**	35.5±0.5$^{**, a}$ (77.1±0.0)	84.5±1.0$^{**, a}$ (68.8±0.1)	51	169
3,5,7-Trimethoxyflavone (**7**)	0.0±1.3	5.5±1.0*	8.5±0.7**	23.0±0.5**	31.4±1.0**	75.6±1.2**	60	193
3,7,4′-Trimethylkaempferol (**8**)	0.0±1.1	19.6±0.6**	23.1±0.9**	33.1±0.6**	44.4±1.4**	82.7±0.6**	44	135

Values represent means±S.E.M. (N=4). Significantly different from the control (0 μg/ml), *p<0.05, **p<0.01.

[a]Cytotoxic effect was observed and values in parentheses indicate %cell viability in MTT assay.

Table 28 Inhibitory effects of **1**-**12** and reference compounds (parthenolide and CAPE) on NO production in LPS-activated RAW264.7 cells (continued)

	Concentration of test sample (μg/ml)							
	0	1	3	10	30	100	IC_{50} (μg/ml)	IC_{50} (μM)
Sample	Inhibition (%)							
Tetramethylkaempferol (**9**)	0.0±2.2	-10.5±1.7	-1.6±2.5	5.6±1.5	9.1±1.5**	20.0±2.1**	>100	-
Ayanin (**10**)	0.0±1.5	2.4±0.7	13.1±0.7**	52.7±0.3**	47.7±0.8**	56.7±1.5**	49	129
Retusine (**11**)	0.0±1.1	11.8±0.9*	9.1±0.8**	8.8±1.9$^{**, a}$	25.5±1.2$^{**, a}$	73.3±1.9$^{**, a}$	66	183
			(76.8±0.0)	(79.6±0.0)	(78.0±0.0)	(70.6±0.0)		
Pentamethylquercetin (**12**)	0.0±8.8	0.7±0.5	-4.1±0.3	9.4±0.8	29.3±2.3$^{**, a}$	50.7±4.3$^{**, a}$	96	258
					(54.6±0.1)	(57.7±0.1)		

	Concentration of test sample (μg/ml)						
	0	0.1	0.3	1	3	IC_{50} (μg/ml)	IC_{50} (μM)
Sample	Inhibition (%)						
Parthenolide	0.0±1.5	17.1±3.4**	42.2±0.6**	97.8±0.5**	100.1±0.2**	0.31	1.1
CAPE	0.0±1.5	1.4±1.3	8.8±0.5**	51.6±1.0**	95.6±0.2**	0.92	3.7

Values represent means±S.E.M. (N=4). Significantly different from the control (0 μg/ml), *p<0.05, **p<0.01.

[a]Cytotoxic effect was observed and values in parentheses indicate %cell viability in MTT assay.

9. Determination of iNOS, p-JNK1/2 and p-ERK1/2 protein expression

9.1 Time-course of iNOS, p-JNK1/2 and p-ERK1/2 protein expression

In this study, it is aimed to investigate the effect of isolated active compounds on the expression of iNOS, p-ERK1/2 and p-JNK1/2 proteins. The result revealed that during the short time periods (10 and 30 min) and long time periods (1, 2, 4, 6, 8 and 12 h) activated macrophage cells with LPS, the expressions of iNOS, p-ERK1/2 and p-JNK1/2 protein were in the similar level (Figure 16).

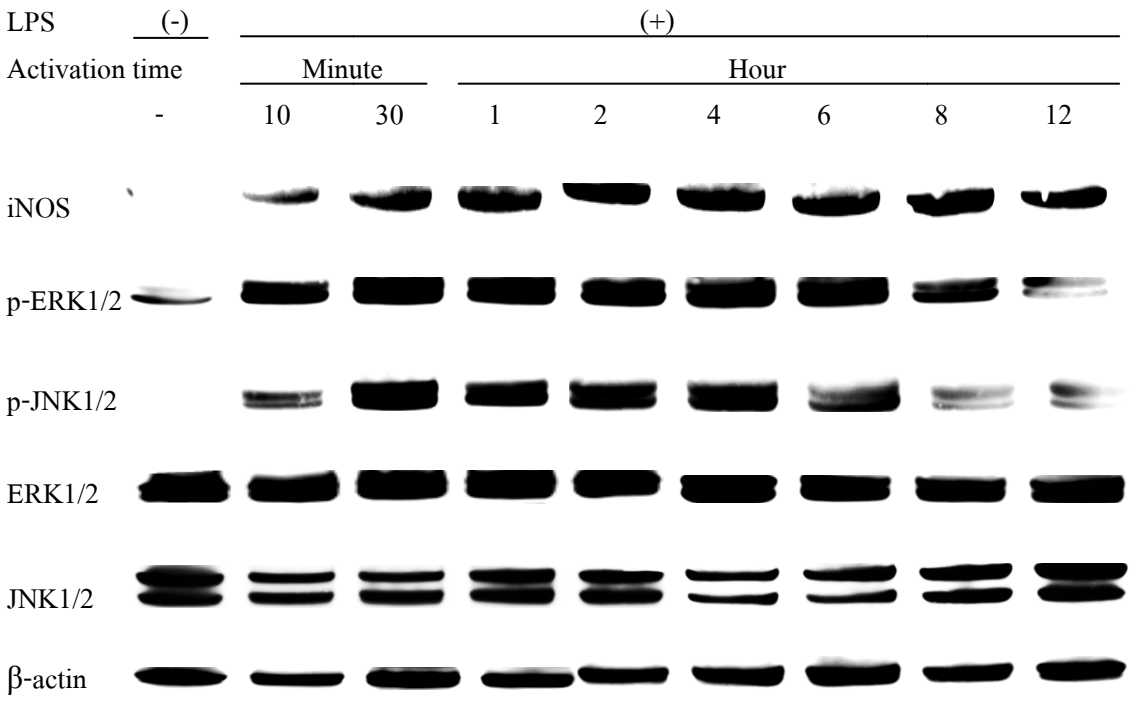


Figure 16 Time-course study of iNOS, p-ERK1/2, p-JNK1/2, ERK1/2 and JNK1/2 protein expression

Table 29 Effects of **2**, **4**, and **5** and reference compounds (parthenolide and CAPE) on the release of TNF-α in LPS-activated RAW264.7 cells

	Concentration of test sample (μg/ml)							
	0	1	3	10	30	100	IC_{50} (μg/ml)	IC_{50} (μM)
Sample	Inhibition (%)							
5,7-Dimethoxyflavone (**2**)	0.0±1.9	2.9±3.6	14.2±1.8	25.4±3.2**	37.0±3.8**	-	>30	-
Trimethylapigenin (**4**)	0.0±1.9	-	11.6±4.5	13.9±3.6	39.0±1.6**	68.8±2.0**	64	206
Tetramethylluteolin (**5**)	0.0±1.9	-	12.2±0.7	10.6±4.6	23.8±4.5**	50.3±2.5**	100	292
Parthenolide	0.0±1.5	27.6±1.0**	99.0±0.2**	100.4±0.1**	-	-	ca. 1.6	ca. 5.2
CAPE	0.0±1.5	10.4±0.8**	18.8±3.8**	32.5±3.9**	66.8±2.9**	87.1±2.9**	21	85

Values represent means±S.E.M. (*N*=4). Significantly different from the control (0 μg/ml), **p<0.01, Ca. represent circa (about, approximately).

Table 30 Effects of a MEK1 inhibitor (PD98059) and a JNK inhibitor (SP600125) on production of NO in LPS-activated RAW264.7 cells

	Concentration (μM)				
Sample	0	10	30	100	IC_{50} (μM)
PD98059	0.0±0.8	1.0±0.6	4.0±2.1	-2.4±1.1	>100
SP6100125	0.0±1.1	32.4±1.3**	74.6±0.6**	84.1±0.5**	17

Values represent as means±S.E.M. (*N*=4), Significantly different from the control (0 μM), **p<0.01

9.2 Determination of iNOS, p-JNK1/2 and p-ERK1/2 protein expression

The active compounds (**2**, **4** and **5)** were further investigated on the Western blot analysis to clarify the anti-inflammatory mechanism. Compounds **2**, **4** and **5** exhibited strong inhibitory activity against iNOS protein expression at an initial dose of 10 μg/ml, and CAPE also inhibited iNOS protein expression as same as the positive control, CAPE (Figure 17A-D). Moreover compound **5** also inhibited p-ERK1/2 protein which may come from other secondary mediator. This result was confirmed by activating cells with LPS and compound **5** for 30 minute. The result showed that compound **5** could not inhibit p-ERK1/2 protein at 30 minute (Figure 17E).

10 Semi-quantitative analysis of RT-PCR

Since NFKB (NFKB-p50 and Rel A) has been reported to be the transcription factor of iNOS, COX-2 and TNF-α production (Zhang et al., 2009; Slomiany and Amalia Slomiany, 2004), the ethanol extract and chloroform fraction of *K. parviflora* were then screened on the expression of iNOS, COX-2, NFKB, Rel A and TNF-α mRNA in order to clarify the mechanism of action. The results revealed that EtOH extract and $CHCl_3$ fraction inhibited the expression of iNOS, COX-2, NFKB, Rel A and TNF-α mRNA in a dose-dependent manner (Figures 18 A and B). In addition, $CHCl_3$ fraction showed strong activity than the EtOH extract. However, cytotoxic effect was observed after treatment with $CHCl_3$ fraction at concentration of 100 μg/ml (Figure 18 B).

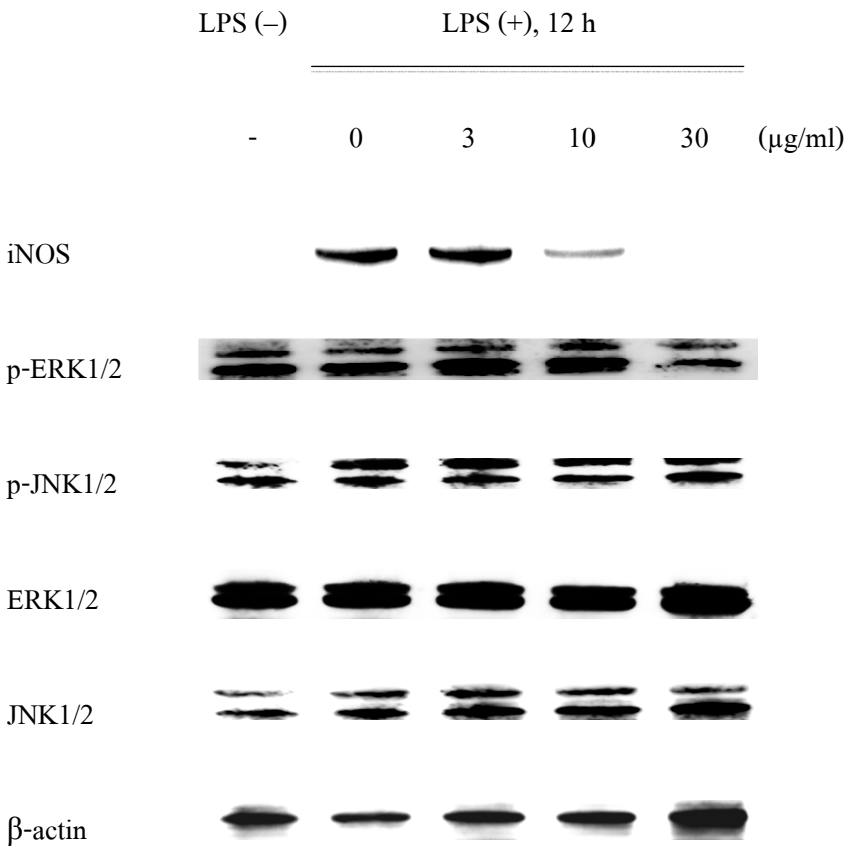


Figure 17 (A) Effect of 5,7-dimethoxyflavone (**2**) on iNOS, p-ERK1/2 and p-JNK1/2 protein levels

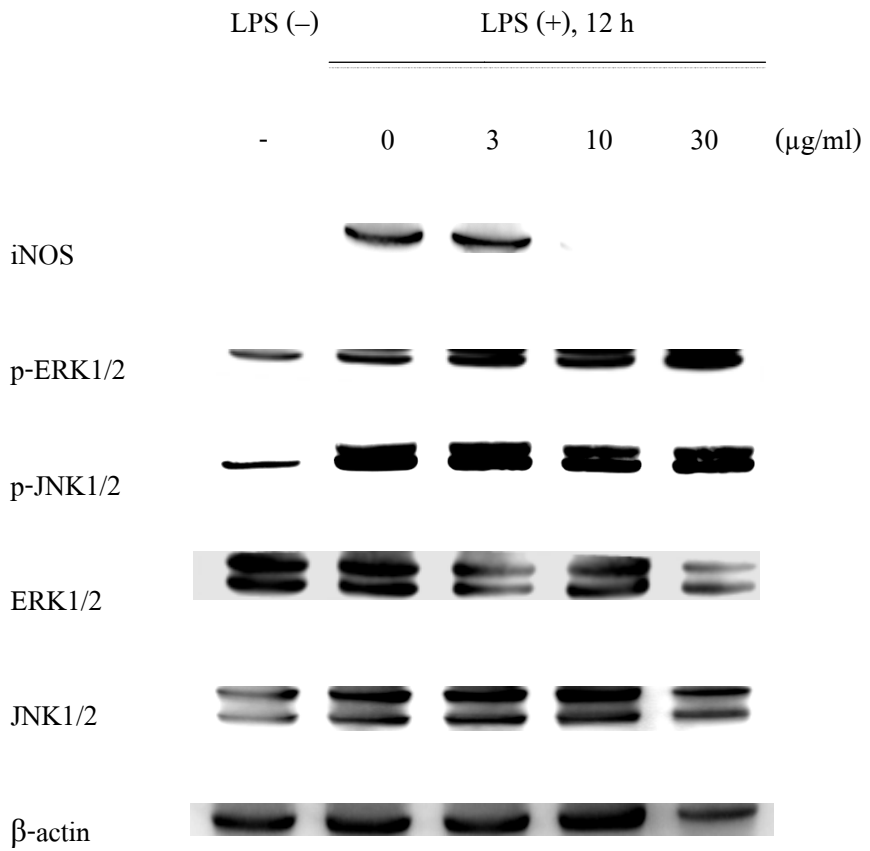


Figure 17 (B) Effect of trimethylapigenin (**4**) on iNOS, p-ERK1/2 and p-JNK1/2 protein levels

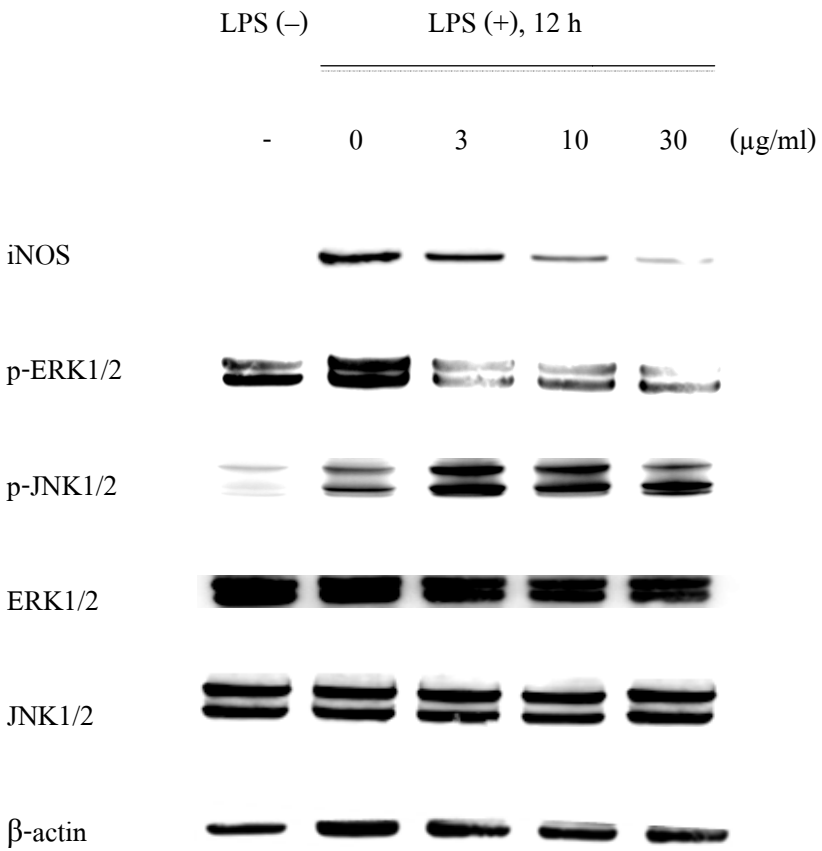


Figure 17 (C) Effect of tetramethylluteolin (**5**) on iNOS, p-ERK1/2 and p-JNK1/2 protein levels

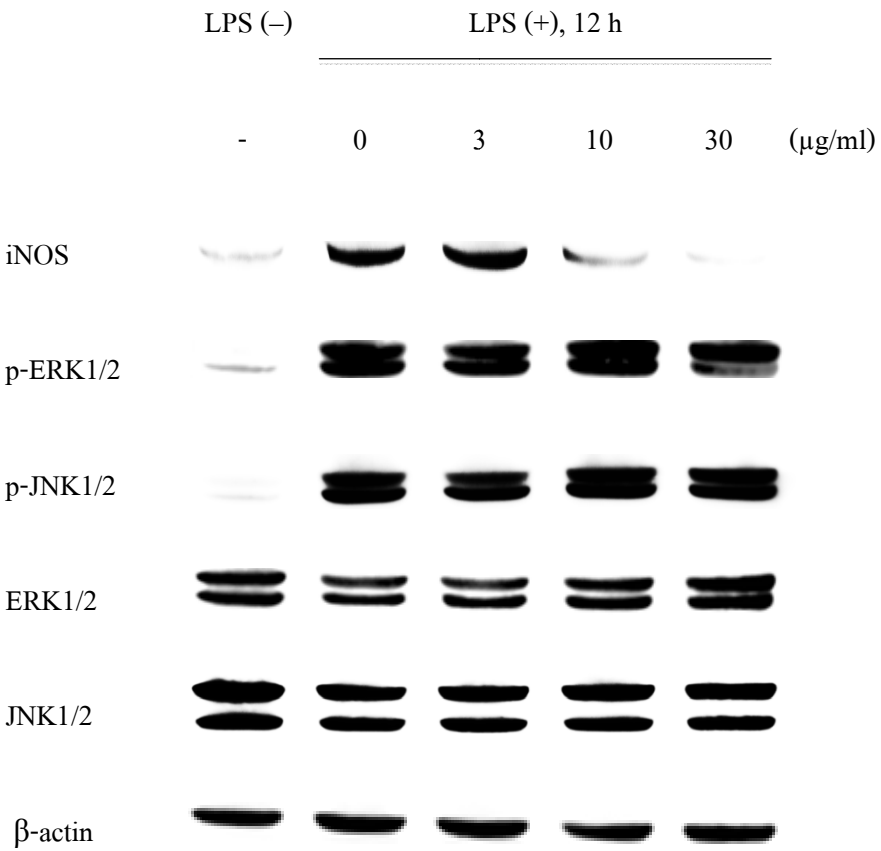


Figure 17 (D) Effect of CAPE on iNOS, p-ERK1/2 and p-JNK1/2 protein levels

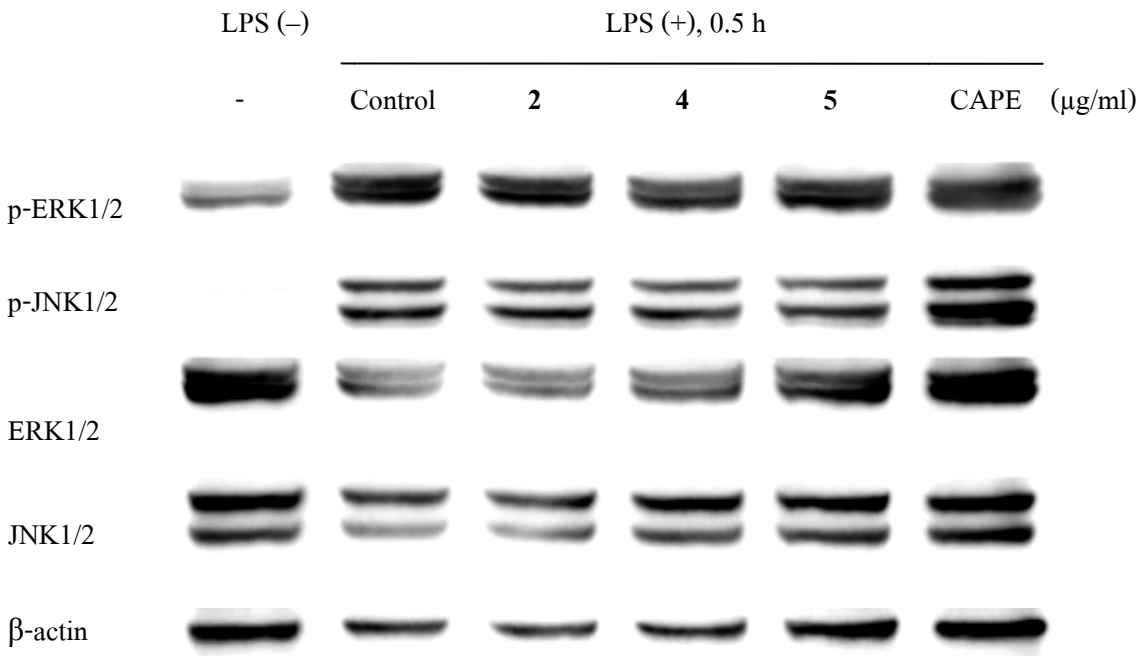


Figure 17 (E) Effect of tetramethylluteolin (**5**) on iNOS, p-ERK1/2 and p-JNK1/2 protein levels

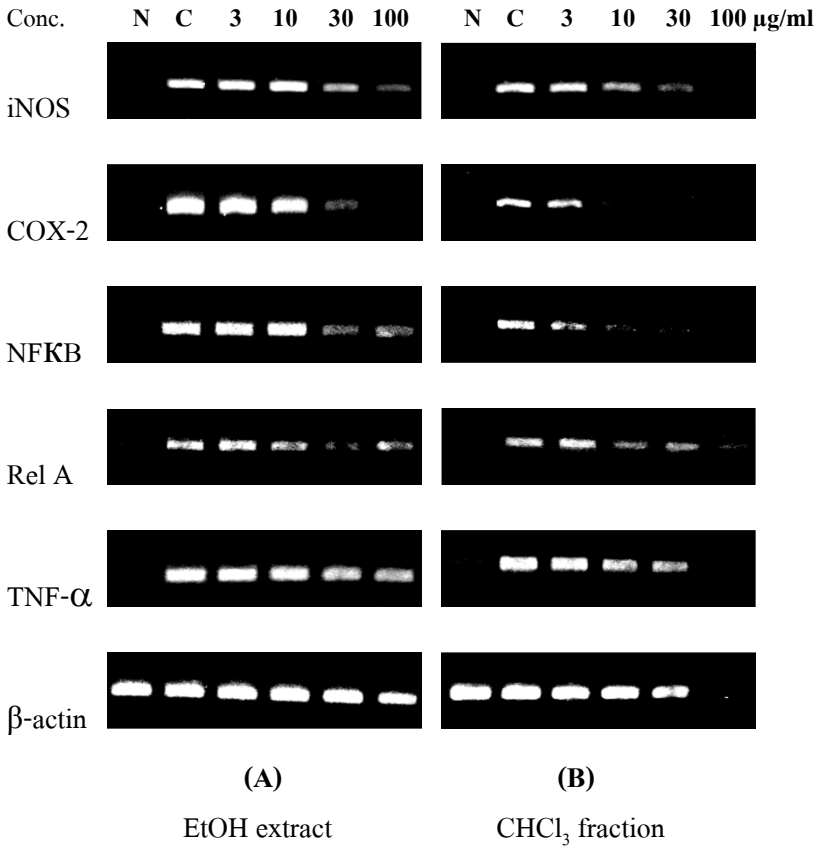


Figure 18 Effect of EtOH extract **(A)** and $CHCl_3$ fraction **(B)** on the expression of mRNA

From the screening test of the EtOH extract and $CHCl_3$ fraction on mRNA expression, it is indicated that isolated methoxyflavonoids (**2**, **4**, and **5**) from $CHCl_3$ fraction which are active compounds against NO production might have a potential to inhibit iNOS, COX-2, NFKB, Rel A and TNF-α mRNA expression. To clarify the anti-inflammatory mechanism of these active compounds, the effects of **2**, **4** and **5** on the expression of mRNA were also examined. The result showed that compound **2** decreased cellular iNOS, COX-2 and Rel A mRNA level and this compound also affected the β-actin mRNA expression. Compound **4** dose-dependently suppressed iNOS, COX-2 and Rel A mRNA level, while inhibitory effect on NFKB was partly affected at the dose of 100 μg/ml. Compound **5** slightly inhibited iNOS mRNA, but strongly inhibited Rel A. While the expression of COX-2, NFKB and TNF-α was not affected (Figure 19).

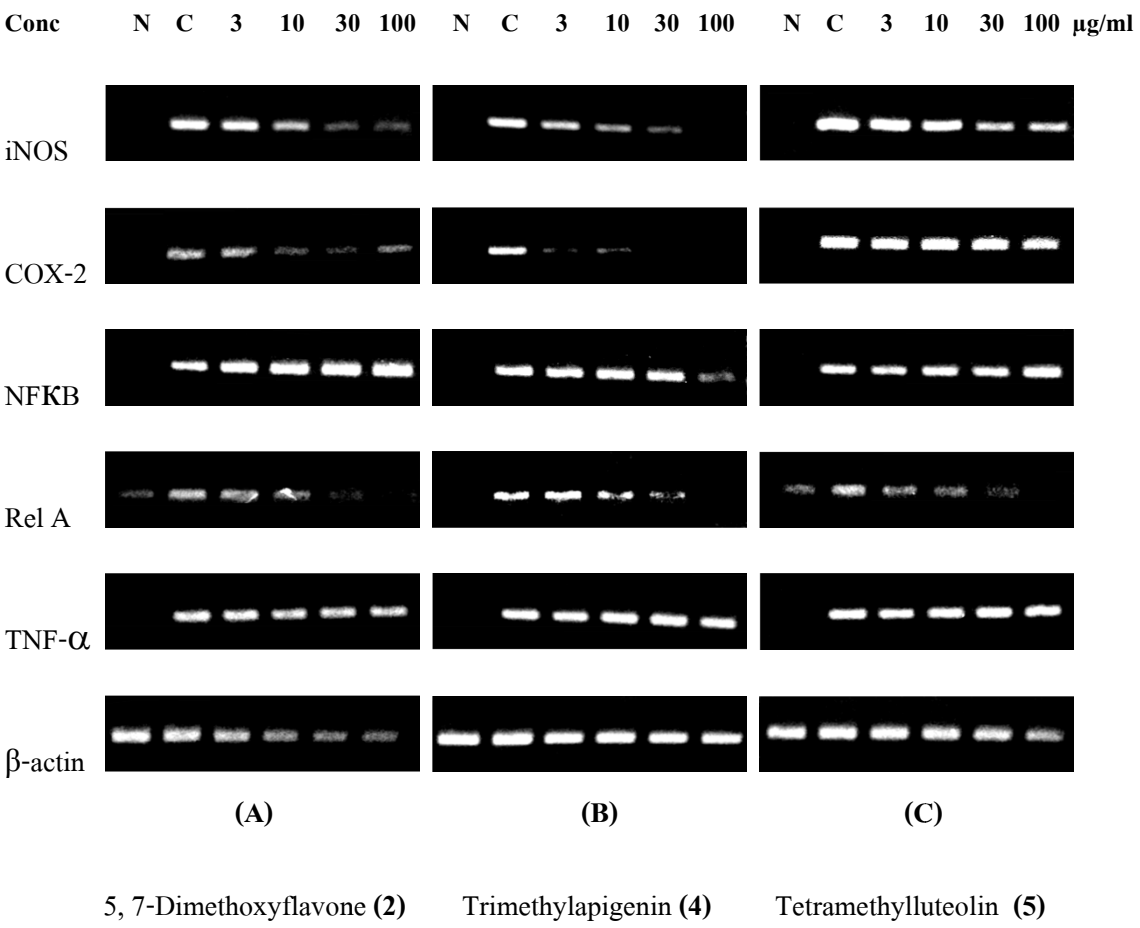


Figure 19 Effect of 5, 7-dimethoxyflavone **(A)**, trimethylapigenin **(B)** and tetramethylluteolin **(C)** on the expression of mRNA

11. Quantitative analysis of real-time RT-PCR

11.1 Time-course study of the effect of LPS incubation time on iNOS mRNA expression

To clarify the mechanism of the effect of methoxyflavonoids (**2**, **4**, and **5**) on LPS-induced NO production, the effect on LPS-induced expression of iNOS mRNA was examined. Firstly, the time-course dependent effect of LPS incubation time on iNOS mRNA expression was studied. The result showed that iNOS mRNA was dramatically increased at 1, 4, and 8 h incubated with LPS. After 8 h incubated RAW264.7 cells with LPS, the expression of iNOS mRNA exhibited the highest level, while the expression of mRNA at 12 h incubation time was decreased (Figure 20). Therefore, the suitable incubation time of RAW264.7 cells with LPS is 8 h.

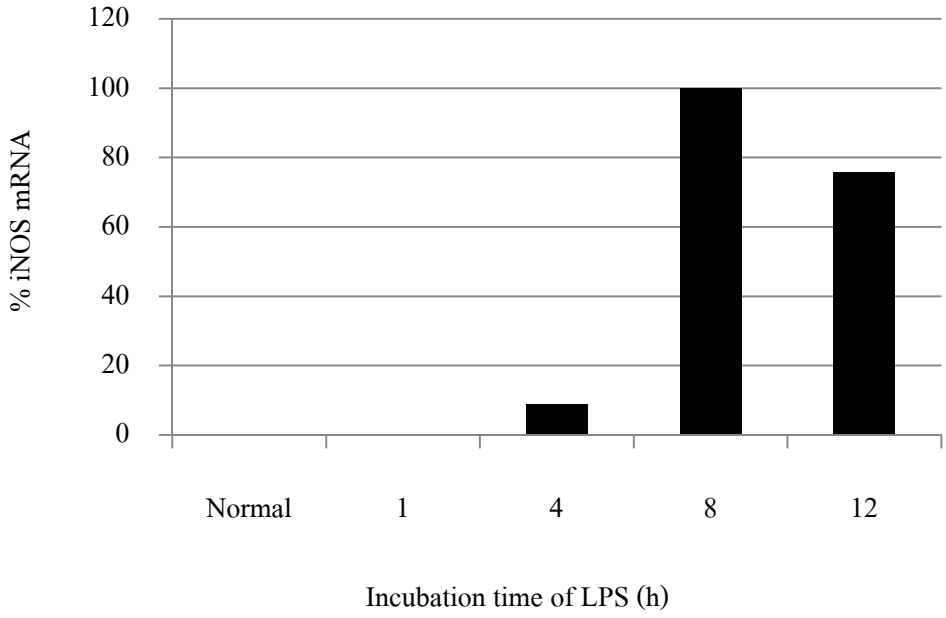
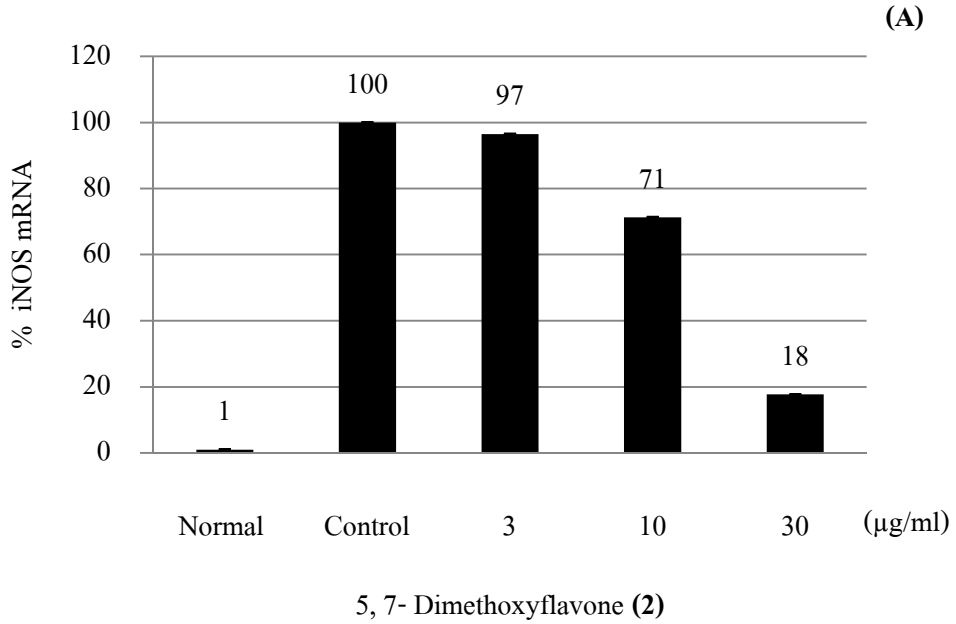


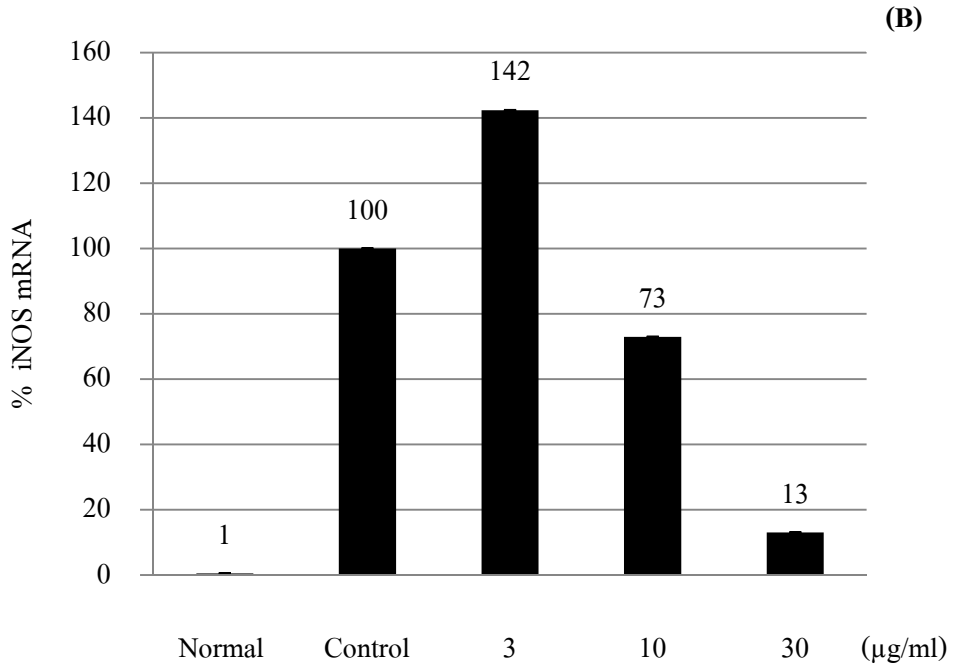
Figure 20 Time-course dependent on LPS-induced iNOS mRNA expression

11.2 Effect of compounds 2, 4 and 5 and CAPE on iNOS mRNA expression

More than 80% of iNOS mRNA was inhibited by compounds **2**, **4** and **5** at the dose of 30 μg/ml. Compound **5** also showed potent inhibitory effect than that of the reference drug (CAPE) (Figure 21).

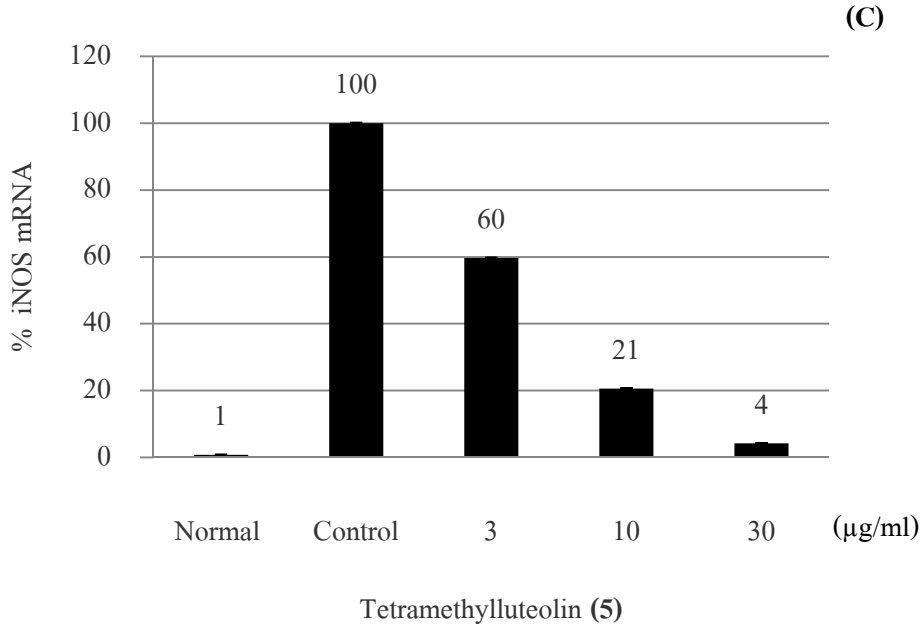


5, 7- Dimethoxyflavone **(2)**



Trimethylapigenin **(4)**

Figure 21 Quantitative analysis of iNOS mRNA expression of compound **2 (A)**, **4 (B)**, **5 (C)** and CAPE **(D)** in macrophage RAW264.7 cells



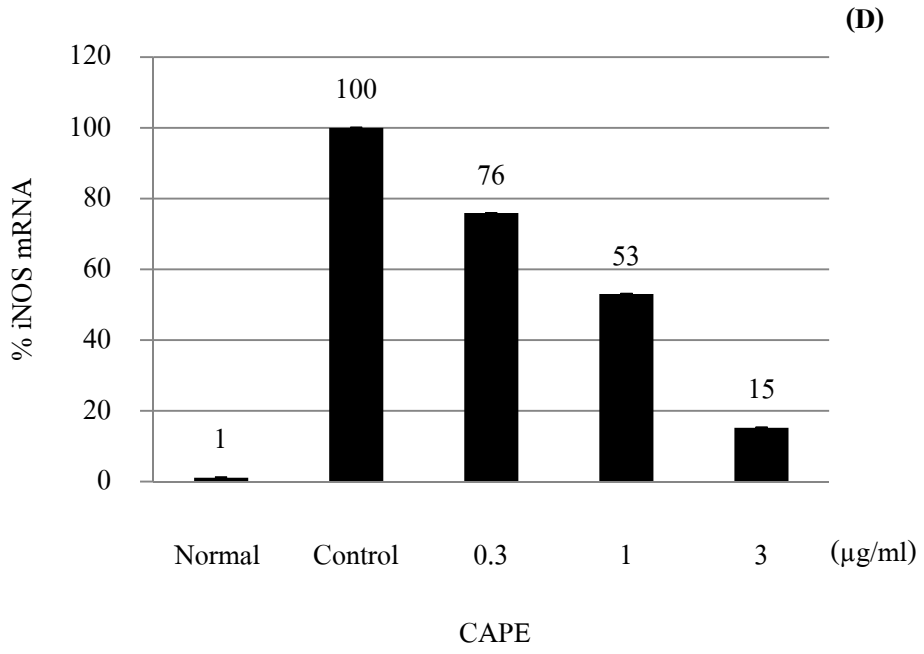


Figure 21 Quantitative analysis of iNOS mRNA expression of compound **2 (A)**, **4 (B)**, **5 (C)** and CAPE **(D)** in macrophage RAW264.7 cells (continued)

12. Effects on protein kinases

Effects of the most potent compound, trimethylapigenin (**4**), on the activities of protein kinases involved in the expression of iNOS including MAPK, were examined using the SelectScreen® Kinase Profiling Service with Z'-LYTE® and Adapta® assay methods. SYK was inhibited by **4**, but other important protein kinases including IκB kinases (IKK), which phosphorylates IκB to activate NFκB, and MAPK were not markedly inhibited (less than 21% inhibition at 30 μg/ml) (Table 31).

Table 31 Effects of trimethylapigenin **(4)** on several protein kinases

Protein tyrosine kinases [a)]	ATP (concentration)[a)]	Concentration (μg/ml)	
		3	30
		Inhibition (%)[b)]	
AKT1(PKBα)	75 μM (Km app.)	7	15
BTK	36 μM (Km app.)	7	19
CHUK (IKKα)	9 μM (Km app.)	15	17
IGF1R	140 μM (Km app.)	10	12
IKBKB (IKKβ)	5 μM (Km app.)	0	11
IKBKE (IKKε)	16 μM (Km app.)	2	3
IRAK4	34 μM (Km app.)	3	-3
JAK1	87 μM (Km app.)	-10	-5
JAK2	31 μM (Km app.)	-7	11
MAP4K5 (KHS1)	55 μM (Km app.)	5	3
MAPK1 (ERK2)	100 μM (Km app.)	0	-5
MAPK3 (ERK1)	45 μM (Km app.)	3	8
MAPK8 (JNK1)	100 μM	-5	-6
MAPK9 (JNK2)	100 μM	5	11
MAPK10 (JNK3)	100 μM	3	9
MAPKAPK2	3 μM (Km app.)	2	8
NEK1	119 μM (Km app.)	5	6
NEK2	150 μM (Km app.)	1	1
PDK1 Direct	27 μM (Km app.)	7	21
PRKACA (PKA)	4 μM (Km app.)	1	10
SYK	25 μM (Km app.)	35	52
TBK1	31 μM (Km app.)	17	19
ZAP70	2 μM (Km app.)	3	7

a) Effects on the protein kinases except for CHUK (IKKα) were examined with Z'-LYTE® assay, and that on CHUK (IKKα) were examined with Adapta® assay. For abbreviations and experimental conditions, refer to the Invitrogen site (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Services/Screening-and-Profiling-Services/SelectScreen-Profiling-Service/SelectScreenKinase-Profiling-Service.html). b) The measurements were done in duplicate.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

1. In *vitro* study

1.1 Concentration-dependent effects of LPS on NO production in RAW 264.7 cells

From the study of concentration-dependent effects of LPS on NO production in RAW 264.7 cells, cells were activated with 1 to 30 µg/ml of LPS, the result showed that the accumulation of NO_2^- was different at various concentrations without cytotoxicity. Activated cells with 10 µg/ml of LPS showed the highest amount of NO_2^- production. Therefore, 10 µg/ml of LPS is considered to be a suitable concentration for activation macrophages to produce NO.

1.2 Effects on production of NO in LPS-stimulated macrophage RAW264.7 cells

In murine macrophage RAW264.7 cells, LPS alone induced the transcription and protein synthesis of iNOS, and increased NO production (Yoon et al., 2009). Using the Griess reaction, a spectrophotometric determination of nitrite (NO_2^-) was determined in the conditioned medium of RAW 264.7 cells treated with LPS. This cell-based assay system has been used for drug screening and the evaluation of potential inhibitors of the pathways leading to the induction of iNOS and NO production. The results showed that ethanol extract and chloroform fraction showed good activity against NO production with IC_{50} of 8.1 and 8.4 µg/ml, respectively followed by hexane fraction (IC_{50}=13.0 µg/ml**),** while ethylacetate and water fractions showed moderate inhibitory effect (IC_{50}= 61.0 and 40.0 µg/ml, respectively). These results suggested that the major active compounds from *K. parviflora* may come from chloroform fraction. After purify compounds from this fraction and tested all isolated compounds on NO production, the result revealed that compounds **2**, **4** and **5** significantly inhibited NO production in LPS-activated RAW 264.7cells with IC_{50} values of 5.1, 4.6, and 8.7 µg/ml, respectively, without cytotoxic effects except for **2** at 100 µg/ml; while **7**, **8**, and **10** possessed moderate to mild activity (IC_{50}

= 44-60 µg/ml) and **3**, **6**, **11**, and **12** showed moderate cytotoxic effects (Table 28). The reference compounds, parthenolide (IKKα inhibitor) and CAPE (NFKB inhibior), showed strong activity with IC_{50} values of 0.31 and 0.92 µg/ml. It was indicated that the active compounds of *K. parviflora* on the inhibition of NO production were compounds **2**, **4** and **5**. Previously, Matsuda and co-workers (2003) reported the effects of 73 flavonoids including methoxyflavonoids (**1**, **5**, **10**, and **12**) on the production of NO in LPS-activated mouse peritoneal macrophages and several structure-activity relationships of flavonoids, and **5** had the strongest effect inhibiting iNOS expression without affecting the enzyme activity.

1.3 Effect of *K. parviflora* extract on PGE_2 production

PGE_2 is synthesized in substantial amounts at sites of inflammation where it acts as a potent vasodilator and synergist with other mediators such as histamine and bradykinin which finally causes an increase in vascular permeability and edema (Davies et al., 1984). From the effect of *K. parviflora* extract on PGE_2 production, the result revealed that the ethanol extract strongly inhibited PGE_2 production with an IC_{50} of 9.26 µg/ml. It was indicated that ethanol extract and chemical constituents from this extract possess therapeutic effect on anti-inflammation by inhibit PGE_2 production and decrease an edema, while the reference drug, indomethacin, showed more potent with an IC_{50} of 0.5 µg/ml. Hence, carrageenan-induced rat paw edema, *in vivo* study, is necessary to confirm this therapeutic effect.

1.4 Phytochemical study

After partitioned 267 g of ethanol extract with *n*-hexane, chloroform, ethylacetate and water, the chloroform fraction was obtained the highest amount (215 g). Therefore, it was indicated that major active compounds against NO production may come from chloroform fraction (IC_{50} = 8.4 µg/ml). The phytochemical investigation of chloroform fraction using bioassay-guided isolation showed that all isolated compounds were 7-methoxyflavonoids which pentamethylquercetin (**12**, 19.74%w/w), 5,7-dimethoxyflavone (**2**, 15.44%w/w) and trimethylapigenin (**4**, 13.05%w/w) were the major compounds. This finding thus suggests that the inhibition of NO and PGE_2 productions were due to 7-methoxyflavonoids which were the high %w/w from *K. parviflora*.

1.5 Determination of TNF-α production by LPS-activated macrophages

TNF-α induces the gene expression of various inflammatory cytokines and chemokines, either dependently or independently of the activation of transcription factors, such as NFKB and AP-1 (activator protein 1) (Zhang et al., 2009). Ethanol extract and chloroform fraction of *K. parviflora* were screened on the inhibition of TNF-α cytokine. The result showed that ethanol extract and chloroform fraction strongly inhibited TNF-α production with IC_{50} of 6.09 µg/ml and 1.37 µg/ml, respectively. This result was concurrent with the study on inhibition of NO and PGE_2 production of *K. parviflora*. However, the isolated compounds (**2**, **4** and **5**) showed moderate inhibitory effect against TNF-α (IC_{50} = >30-100 µg/ml). This may due to the synergistic effect of several methoxyflavonoids that promote anti-inflammatory activity or may due to the effect of minor active compounds from chloroform fraction that could not be isolated in this study.

1.6 Study of iNOS, p-JNK1/2 and p-ERK1/2 protein expression

1.6.1 Time-course study of iNOS, p-JNK1/2 and p-ERK1/2 protein expression

In macrophages, the inhibition of ERK1/2 and JNK1/2 phosphorylation, could lead to a decrease in AP-1 activation and production of pro-inflammatory cytokines. Therefore, in this study, it is aimed to investigate the effect of isolated active compounds on the expression of iNOS, p-ERK1/2 and p-JNK1/2 proteins. Firstly, the time-course of LPS-activated protein expression was investigated. During short time periods (10 and 30 min) and long time periods (1, 2, 4, 6, 8 and 12 h) activated macrophage cells with LPS, the expressions of iNOS, p-ERK1/2 and p-JNK1/2 protein were in the similar level (Figure 16). In addition, time-course study of iNOS mRNA expression showed that after 8 h incubated RAW264.7 cells with LPS, the expression of iNOS mRNA exhibited the highest level and iNOS mRNA was decreased after 12 h incubation. Therefore, the suitable time for activation of iNOS, p-ERK1/2 and p-JNK1/2 protein expression is 12 h.

1.6.2 Determination of iNOS, p-JNK1/2 and p-ERK1/2 protein expression

The MAPK pathway is known to play an important role in the transcriptional regulation of LPS-induced iNOS and COX-2 expression via suppression of the activation of transcription factor NFKB (Park et al., 2008). To investigate the involvement of MAPK pathway in the inhibitory effect by isolated compounds (**2**, **4** and **5**) from *K. parviflora* on NO and PGE_2 production, the activation of MAP kinase (phosphorylation of ERK1/2 and JNK1/2) induced by LPS was evaluated in RAW 264.7 cells. It is generally accepted that ERK and JNK are involved in inflammation (Moon et al., 2007; Park et al., 2008). Recently, inhibitors of the phosphorylation of JNK, but not of ERK, were reported to reduce LPS-stimulated NO production (Lin et al., 2009). In contrast, Hwang et al. (2010) reported that the inhibitors of phosphorylation of ERK and p-38, but not of JNK, reduced LPS-stimulated NO production. In the present study, a MAPK-ERK kinase 1 (MEK 1) inhibitor (PD98059) acting on the phosphorylation of ERK and an ERK inhibitor (FR180204) showed less inhibition against the production of NO with IC_{50} of >100 μM, while a JNK inhibitor (SP600125) significantly inhibited the NO production (IC_{50} =17 μM) (Table 30) which was consistent with the previous report by Lin et al. (2009). MEK1 inhibitor (PD98059) did not inhibit the production of NO by LPS in the cells. The inhibitory effect on the p-ERK1/2 and p-JNK1/2 proteins by **2** and **4** was marginal (Figure 17 A and B). Compound **5** reduced both iNOS and p-ERK1/2 protein levels in a dose-dependent manner at 3, 10 and 30 μg/ml, but had no inhibitory effect on p-JNK1/2 (Figure 17 C). However, the inhibitory effect of **5** on the phosphorylation of ERK1/2 was not observed at 30 min after the treatment with LPS (Figure 17 E), suggesting that the inhibition by compound **5** after treatment with LPS (for 12 h) may come from secondary effects by other mediators. Therefore, ERK pathway is not involved in anti-inflammatory mechanism. JNK inhibitor (SP6100125) inhibited the production of NO, hence, this pathway is also important. However, compound **5** could not inhibit the phosphorylation of JNK1/2 in the Western blot analysis. Therefore, JNK pathway is also not involved in anti-inflammatory mechanism of compound **5**. These findings include the effects of PD98059, FR180204, and SP600125 suggesting that the ERK and JNK signaling pathways are not involved in the inhibition of NO production by methoxyflavonoids (**2**, **4**, and **5**), and it can be concluded that the inhibitory effect on NO production occurs through iNOS protein synthesis which is similar to that of the reference compound, CAPE (Figure 17 D).

1.7 Semi-quantitative analysis of RT-PCR

To clarify the anti-inflammatory mechanism of the active compounds from *K. parviflora*, the effects of compounds **2**, **4** and **5** on the expression of mRNA were also examined. Compound **2** decreased cellular iNOS, COX-2 and Rel A mRNA level and this compound also affected the β-actin mRNA expression. β-actin is a relatively stable cytoskeletal protein generally thought to be present at a constant level in cells, regardless (in most cases) of experimental treatment or technical procedure. For this reason, measurement of β-actin is generally used as an internal control for experimental error. Compound **4** dose-dependently suppressed iNOS, COX-2 and Rel A mRNA level, while inhibitory effect on NFKB was partly affected at the dose of 100 μg/ml. Therefore, inhibition of NO production of **4** may cause by the suppressive effect of nuclear transcription factor NFKB-p50 and Rel A that are influence iNOS mRNA expression. It is suggested that the potent inhibitory effect on COX-2 of compound **4** related to *in vivo* test on carrageenan-induced rat paw edema. The mediator of which is suspected to be PGE_2 occur from 2.5 to 6 h after carrageenan injection (Vinegar et al., 1969). It is agreed with the result of chloroform fraction that markedly decreased paw edema at 3 and 5 h after carrageenan injection by 25.4 and 25.3% inhibition, respectively; and showed higher effect than that of the standard drug, indomethacin (10 mg/kg, p.o., 18.3% inhibition at 3 h). This result indicated that compound **4** inhibited PGE_2 production through suppression of COX-2 mRNA. Moreover, compound **4** is the main compound isolated from the chloroform fraction and exhibited strong anti-inflammatory activity both *in vivo* and *in vitro* assays. Compound **5** inhibited Rel A, which is a transcription factor of iNOS mRNA. Thus, the result of NO inhibition of **5** could also be directly mediated by inhibition of Rel A mRNA expression, while the expression of COX-2, NFKB and TNF-α was not affected. Compounds **2**, **4** and **5** could not inhibit TNF-α mRNA. Hence, the inhibition of TNF-α on ELISA assay might be caused by the reduction of other mediators or on the inhibition of TNF-α protein expression (Figure 19).

1.8 Quantitative analysis of real-time RT-PCR

1.8.1 Time-course study of the effect of LPS incubation time on iNOS mRNA expression

From time-course study, the result showed that iNOS mRNA was dramatically increased at 1, 4, and 8 h incubated with LPS. The expression of iNOS mRNA exhibited the highest level at 8 h incubation time, while the expression of mRNA at 12 h incubation time was decreased (Figure 20). Therefore, the suitable incubation time of RAW264.7 cells with LPS to activate iNOS mRNA expression is 8 h.

1.8.2 Effect of compounds 2, 4 and 5 and CAPE on iNOS mRNA expression

Compounds **2**, **4** and **5** strongly inhibited iNOS mRNA expression. Therefore, it can be suggested that inhibition on NO production of these three compounds is due to the suppression of the transcription level of iNOS mRNA.

1.9 Effects on protein kinases

SYK is an important component of intracellular signaling cascades. It is activated following cross-linking of Fc$\gamma$and Fcε receptors on macrophages, mast cells, and other cells, ultimately leading to inflammatory events (Darby et al., 1994; Costello et al., 1996). SYK also induces the activation of NFκB which regulates the transcription of genes encoding pro-inflammatory molecules (Takada and Aggarwal, 2004). Evidence of the involvement of SYK in the production of NO is still unknown, but SYK is involved in the regulation of LPS-induced signaling in macrophages (Ulanova et al., 2007). In this study, trimethylapigenin strongly inhibited SYK, the initiation process of inflammation which mean that pathology of inflammation can be cured suddenly. Therefore, trimethylapigenin may show rapid therapeutic effect on inflammation. This point is interesting for further study on *in vivo* to confirm the therapeutic use. Recently, several flavonoids (e.g. luteolin, apigenin) were reported to inhibit SYK (Shichijo et al., 2003). However, to the best of our knowledge, inhibition of SYK by methoxyflavonoids has not been reported so far.

The inhibitory mechanism of isolated compounds can be suggested that *K. parviflora* possessed anti-inflammatory activity by inhibited NO and PGE_2 production at both of transcription and translation levels through inhibition of iNOS, COX-2, NFKB, Rel A and TNF-α mRNA expression and inhibition of iNOS and ERK proteins as well as strongly inhibited SYK which is an NFKB activator (Figure 22).

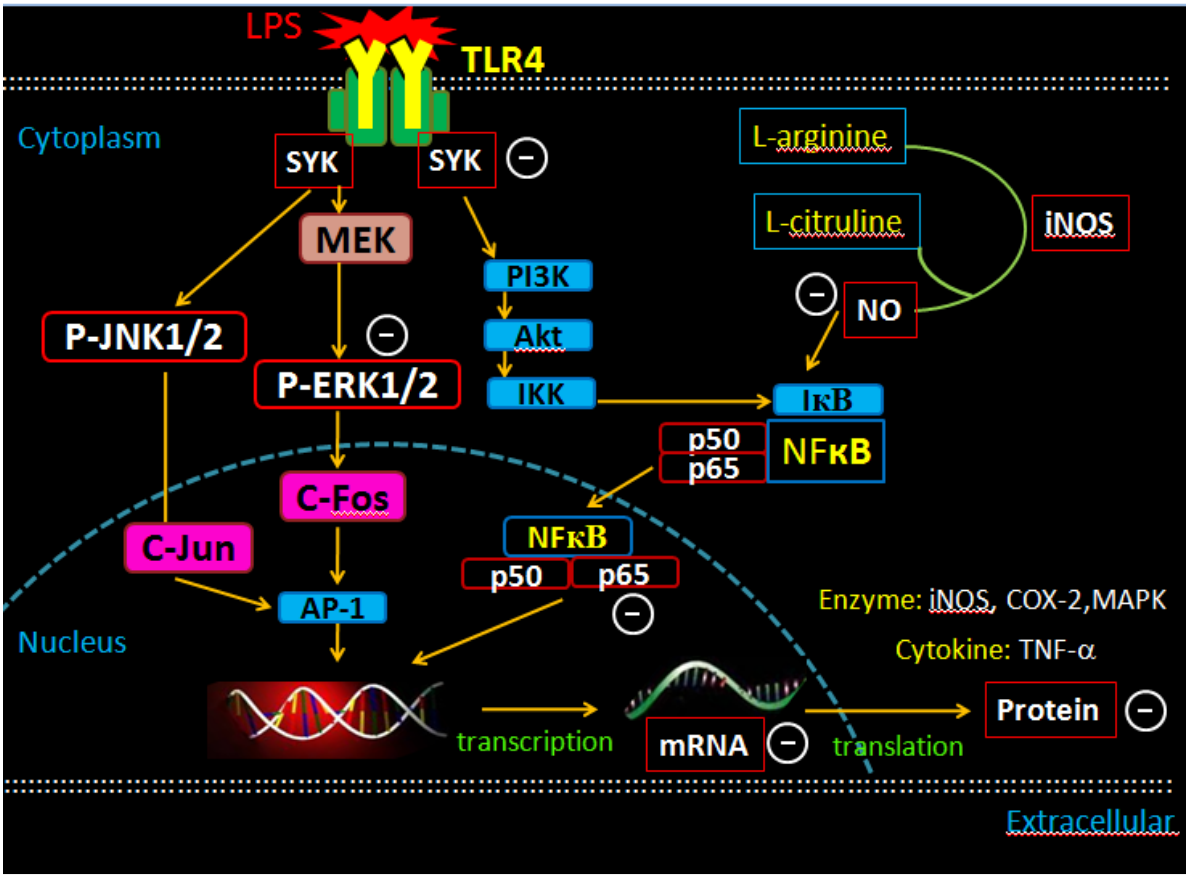


Figure 22 Inhibitory mechanism of *K. parviflora* on inflammatory pathway

2. *In vivo* study

2.1 Carrageenan-induced rat paw edema

Acute inflammation is a short-term process characterized by swelling, redness, pain, heat generation and loss of cell function caused by infiltration of plasma and leukocytes at the inflammatory site (Sarkar et al., 2008). The local injection of carrageenan-induced inflammation process in the rat involves three phases by several mediators released in ordinary sequence (DiRosa, 1972). An initial phase during the first 1.5 h, is caused by the release of histamine and serotonin, a second phase is mediated by bradykinin from 1.5 to 2.5 h and finally, a third phase, the mediator of which is suspected to be PGE_2 occur from 2.5 to 6 h after carrageenan injection (Vinegar et al., 1969). The result revealed that chloroform and hexane fractions (150 mg/kg, p.o.) markedly decreased paw edema at 3 and 5 h after carrageenan injection by 25.4 and 25.3% inhibition, respectively; and showed higher effect than that of the standard drug indomethacin (10 mg/kg, p.o., 18.3% inhibition at 3 h). The crude ethanol extract, ethyl acetate and water fractions had low potency at 3 h with % inhibition of 12.9, 5.6 and 6.2, respectively (Table 20). Our result of *in vivo* study indicated that chloroform and hexane fractions showed potent anti-inflammatory activity mainly through the inhibition of PGE_2 production.

2.2 Acute toxicity test of *K. parviflora* extract in mice

In the acute toxicity test, *K. parviflora* extract and chloroform fraction up to 2 g/kg, orally as a single dose did not produce any clinical sign of toxicity during 7 days, including convulsion, hyperactivity, sedation, respiratory depression and loss of righting reflex. This result indicated that *K. parviflora* extract has low toxicity. This result is agreed with Chivapat and co-worker (2004), after treating rat with suspension powder of *K. parviflora* in water, the result revealed that LD_{50} of *K. parviflora* powder was more than 13.33 g/kg in mice and at this dose, no abnormal of histopathological change was found in various visceral organs. Chronic toxicity has been studied in Wistar rat. Both male and female rats received *K. parviflora* at the doses of 20, 200, 1,000 and 2,000 mg/kg /day for 6 months and withdrawn for 2 weeks. The result showed that all *K. parviflora* treated groups has no difference of body weight and health when comparing with the control group. Histopathological examination of visceral organs did not show any abnormality due to toxicity. Therefore, *K parviflora* is safety for use.

5.2 CONCLUSION

In the present study, it was found that the chloroform fraction exhibited the most potent inhibitory activity against NO production (IC_{50} = 8.4 µg/ml), followed by *n*-hexane (IC_{50} = 13 µg/ml), water (IC_{50} = 40 µg/ml) and ethyl acetate (IC_{50} = 61 µg/ml) fractions, respectively. Hexane and chloroform fractions showed potent anti-inflammatory activity mainly by decreasing edema of rat hind-paw. Moreover, the chloroform fraction highly inhibited TNF-α production (IC_{50} = 3.79 µg/ml) and suppressed iNOS, COX-2, NFKB, Rel A and TNF-α mRNA expression. Treated mice with ethanol extract and chloroform fraction, did not show any sign of toxic in both male and female mice. Thus, both ethanol extract and chloroform fraction are safe for use. The chloroform fraction was further selected for the isolation and purification of active compounds.

Phytochemical investigation of chloroform fraction led to the isolation of twelve methoxyflavonoids, including techtochrysin (**1**), 5,7-dimethoxyflavone (**2**), 7,4′-dimethylapigenin (**3**), trimethylapigenin (**4**), tetramethylluteolin (**5**), 5-hydroxy-3,7-dimethoxyflavone (**6**), 3,5,7-trimethoxy-flavone (**7**), 3,7,4′-trimethylkaempferol (**8**), tetramethylkaempferol (**9**), ayanin (**10**), retusine (**11**) and pentamethylquercetin (**12**). Trimethylapigenin (**4**) was the main compound (13.05% w/w from $CHCl_3$ fraction and 1.3% w/w from *K. parviflora* dried weight) with the highest activity against NO production, followed by 5,7-dimethoxyflavone (**2**) and tetramethylluteolin (**5**) without cytotoxic effect except for compound **2** at the highest dose (100 µg/ml) that showed some toxicity. Therefore, the anti-inflammation in rat paw edema may be due to the suppression of PGE_2 and NO production of these active compounds. The inhibition on TNF-α of compounds **2**, **4**, and **5** was also examined. The results showed that **2**, **4**, and **5** moderately inhibited the release of TNF-α with IC_{50} values of >30, 64 and 100 µg/ml, respectively.

5,7-Dimethoxyflavone (**2**) inhibited the expression of iNOS, COX-2 and Rel A mRNA but did not inhibit NFKB and TNF-α mRNA. Compound **2** also inhibited iNOS protein at a translational level, but did not inhibit p-ERK1/2 and p-JNK1/2. However, this compound interfered β-actin mRNA expression which is not safe for use at a high dose (100 µg/ml).

Trimethylapigenin (**4**) dose dependently inhibited iNOS, COX-2, NFKB and Rel A mRNA, mainly through COX-2 mRNA inhibition which can reduce PGE_2 production. The expression of iNOS mRNA and iNOS protein was inhibited by compound **4**, but not to p-ERK1/2 and p-JNK1/2.

Moreover, SYK was also inhibited by this compound and this is the first report on inhibition of SYK by methoxyflavonoid.

Tetramethylluteolin (**5**) inhibited Rel A and iNOS mRNA expression, while COX-2, NFKB and TNF-α were not affected. This compound also inhibited iNOS and p-ERK1/2 protein.

In summary, the present study using a macrophage cell line supports the traditional use of *K. parviflora* for the treatment of inflammation including abscesses, duodenal ulcers and gout. Compound **4** which is the main compound from the chloroform fraction exhibited the most potent anti-inflammatory activity without cytotoxic effect. Therefore, compound **4** is safe for use and has a high potential to be developed as an anti-inflammatory agent.

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APPENDIX

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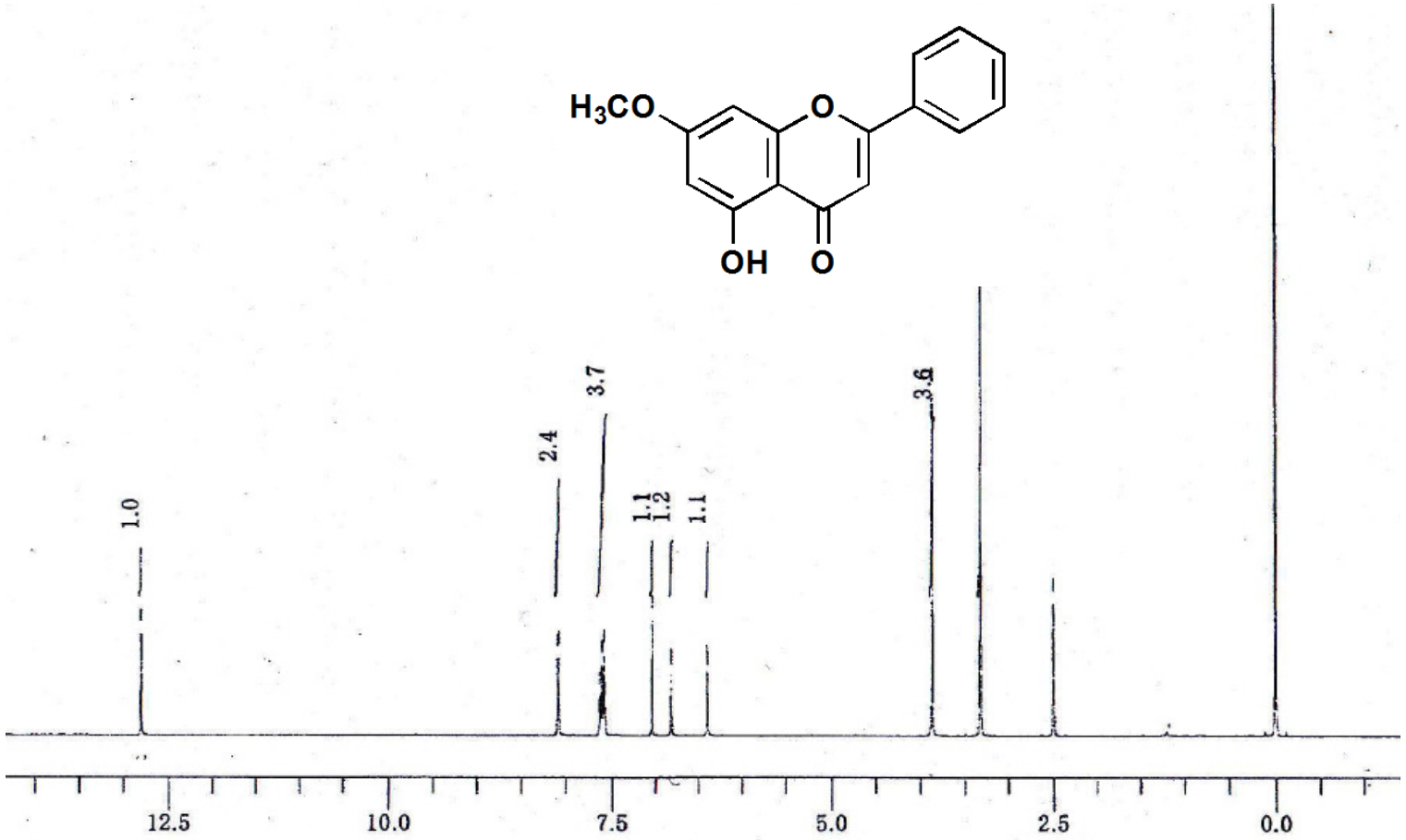
MOE 0521.11/375 Ref. 26/51

September 29, 2008

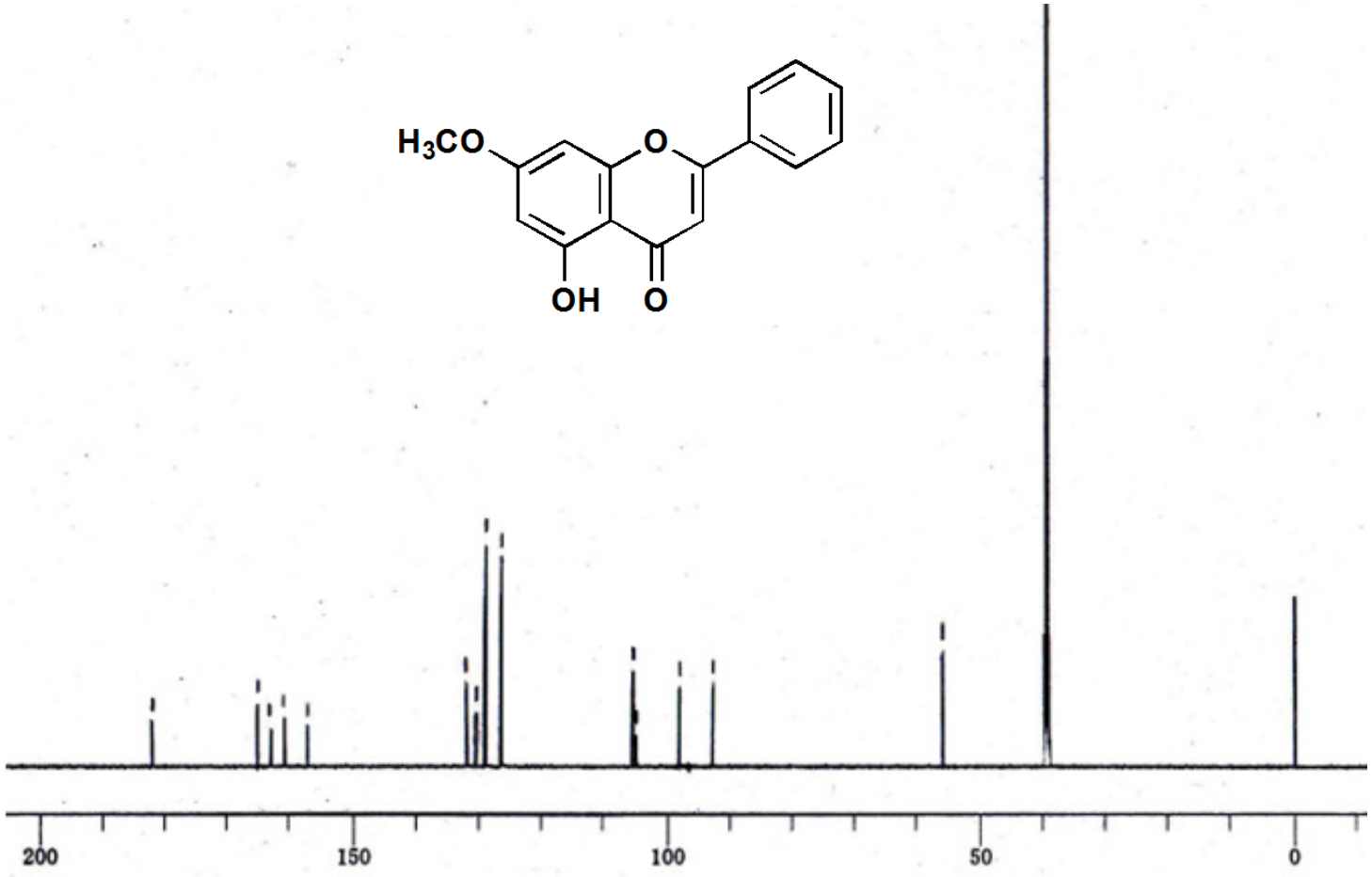
This is to certify that the research project entitled "Evaluation of anti-inflammatory activity of *Kaempferia parviflora* in animal model and in murine macrophage cell line RAW 264.7" which was conducted by Assoc.Prof.Dr. Supinya Tewtrakul, Faculty of Pharmaceutical Sciences, Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.

K. Sawangjaroen

Kitja Sawangjaroen, Ph.D.
Chairman,
The Animal Ethic Committee, Prince of Songkla University



^{1}H NMR spectrum of techtochrysin (**1**) (DMSO-d_6; 500 MHz)



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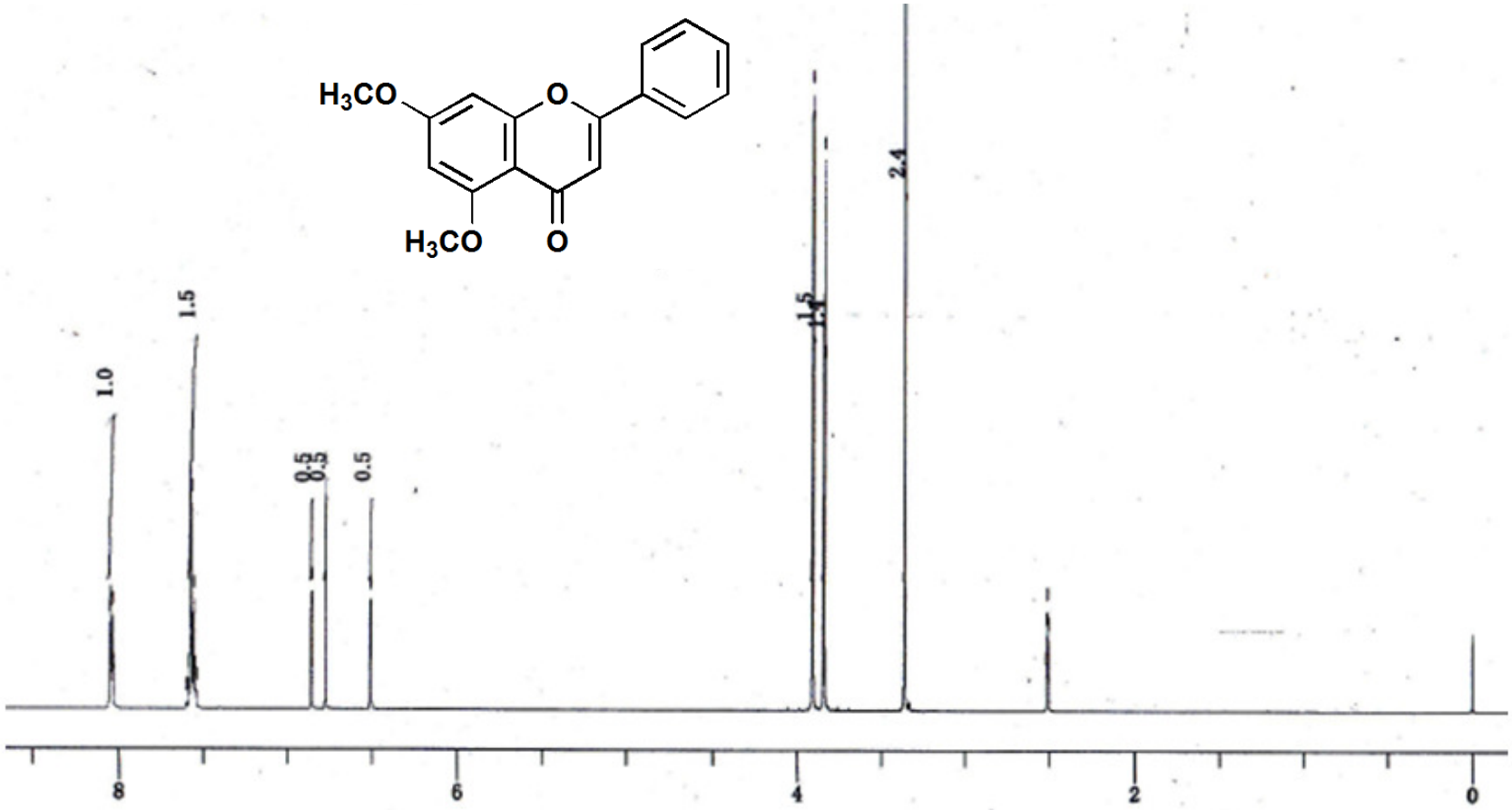
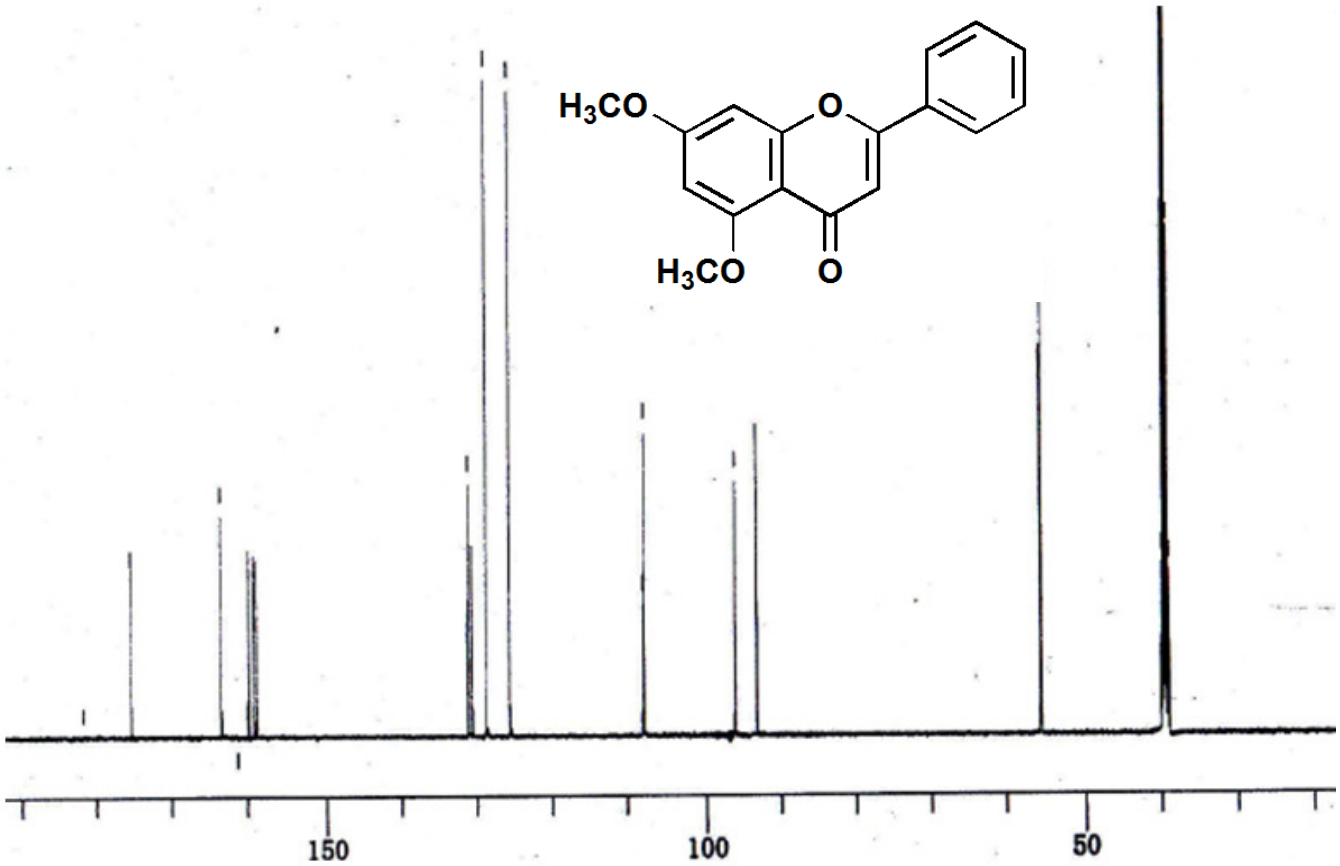


[1]H NMR spectrum of 5,7-dimethoxyflavone (**2**) (DMSO-d_6; 500 MHz)



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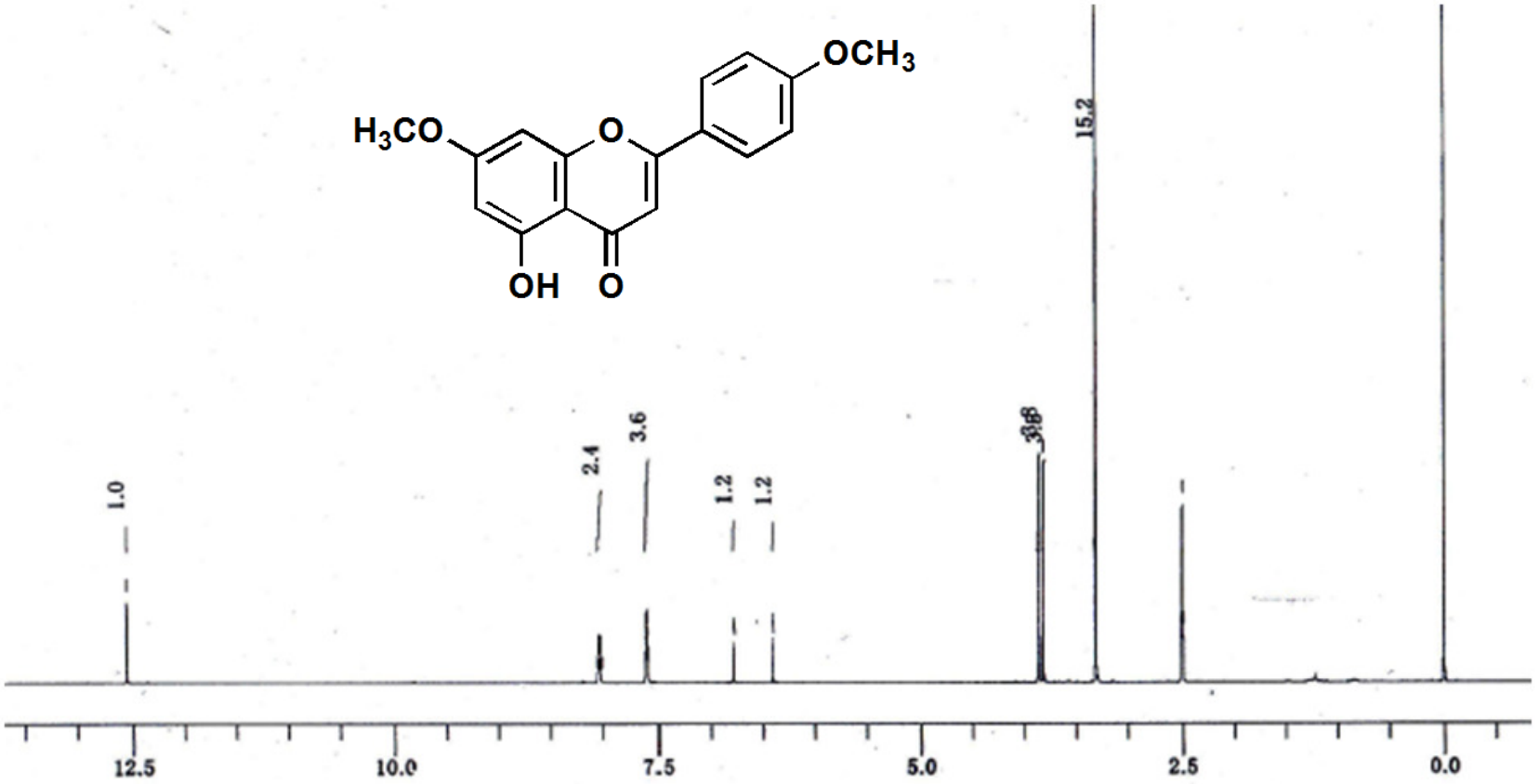
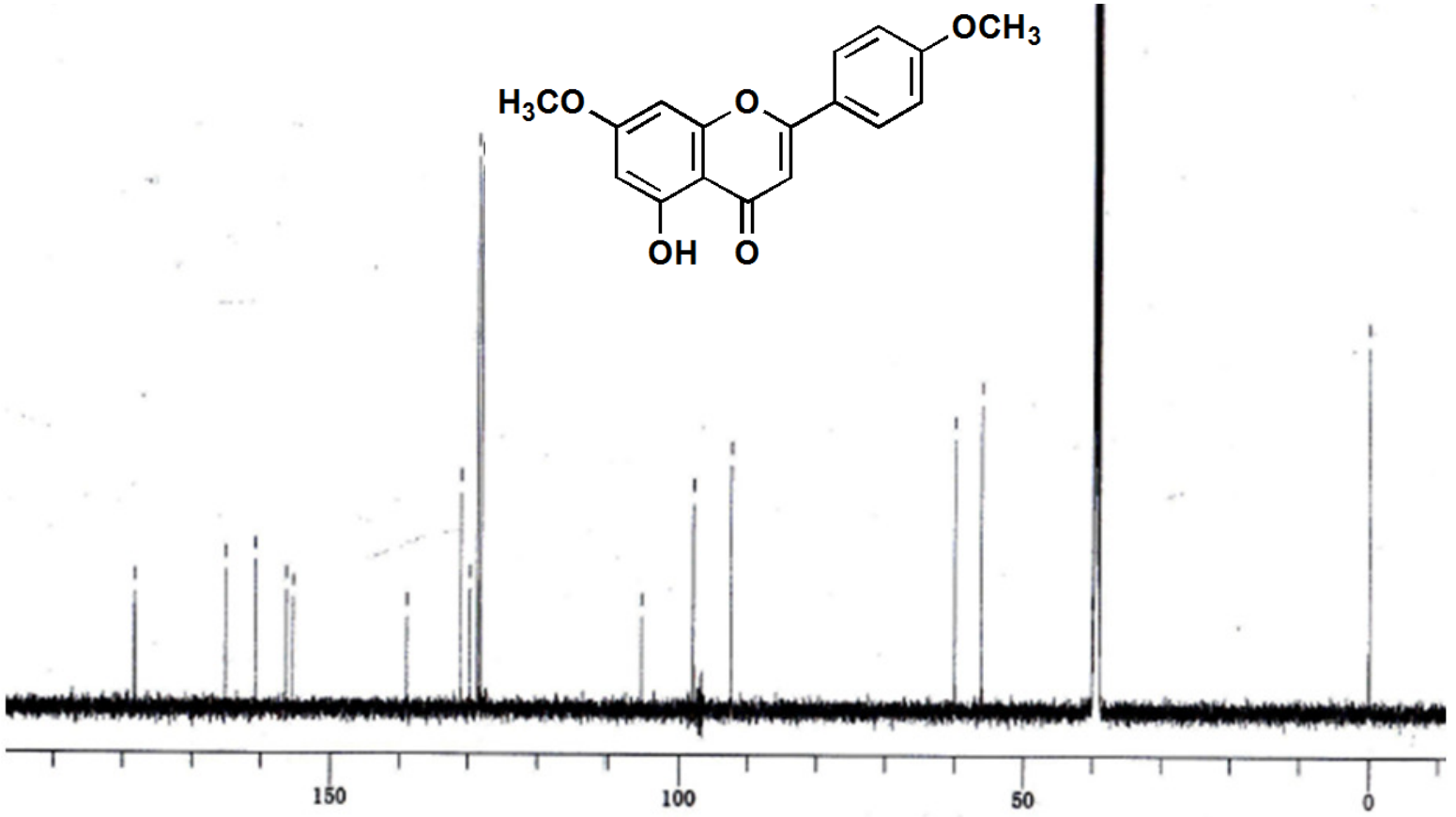


[1] H NMR spectrum of 7, 4'-dimethylapigenin (**3**) (DMSO-d_6; 500 MHz)



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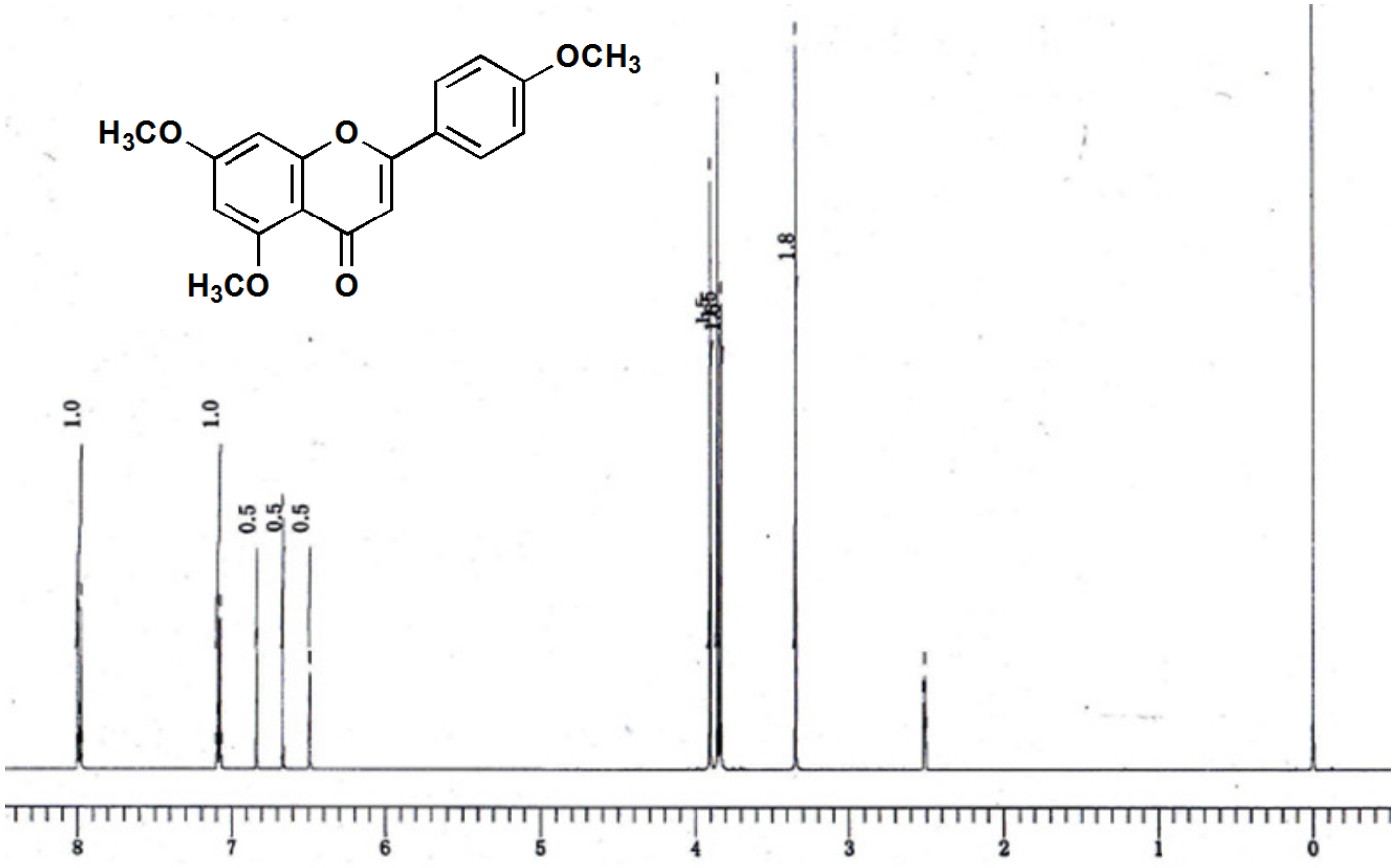

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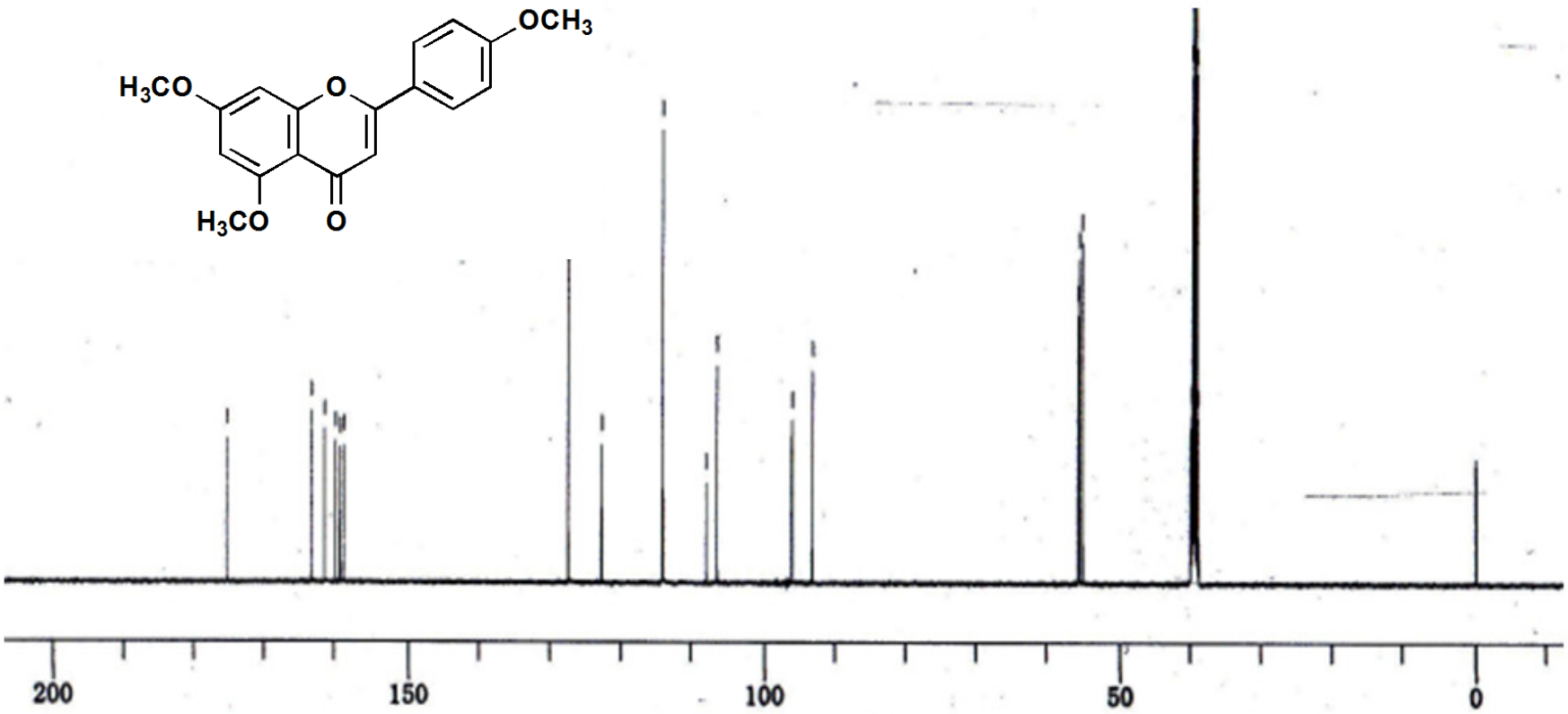

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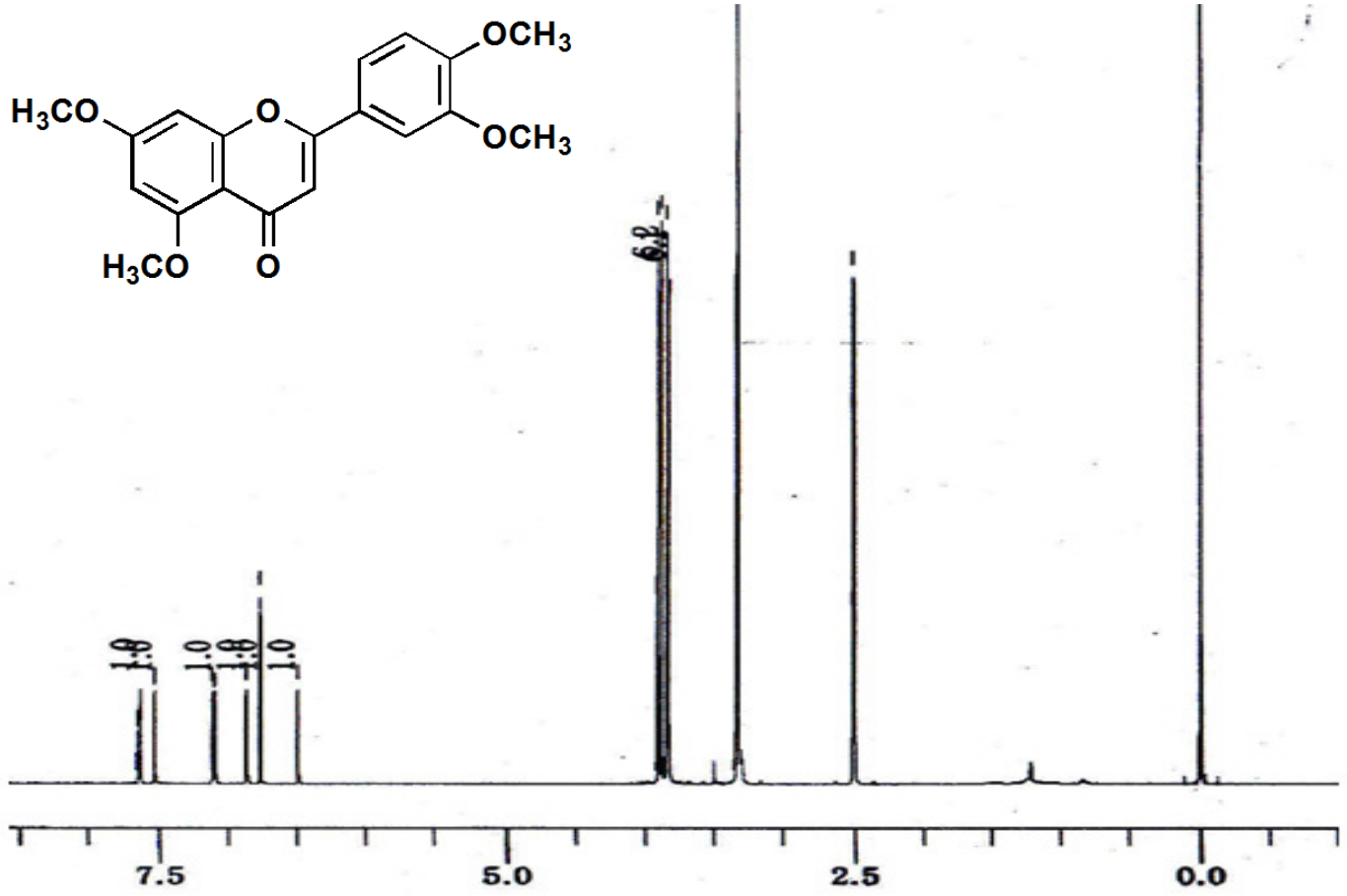

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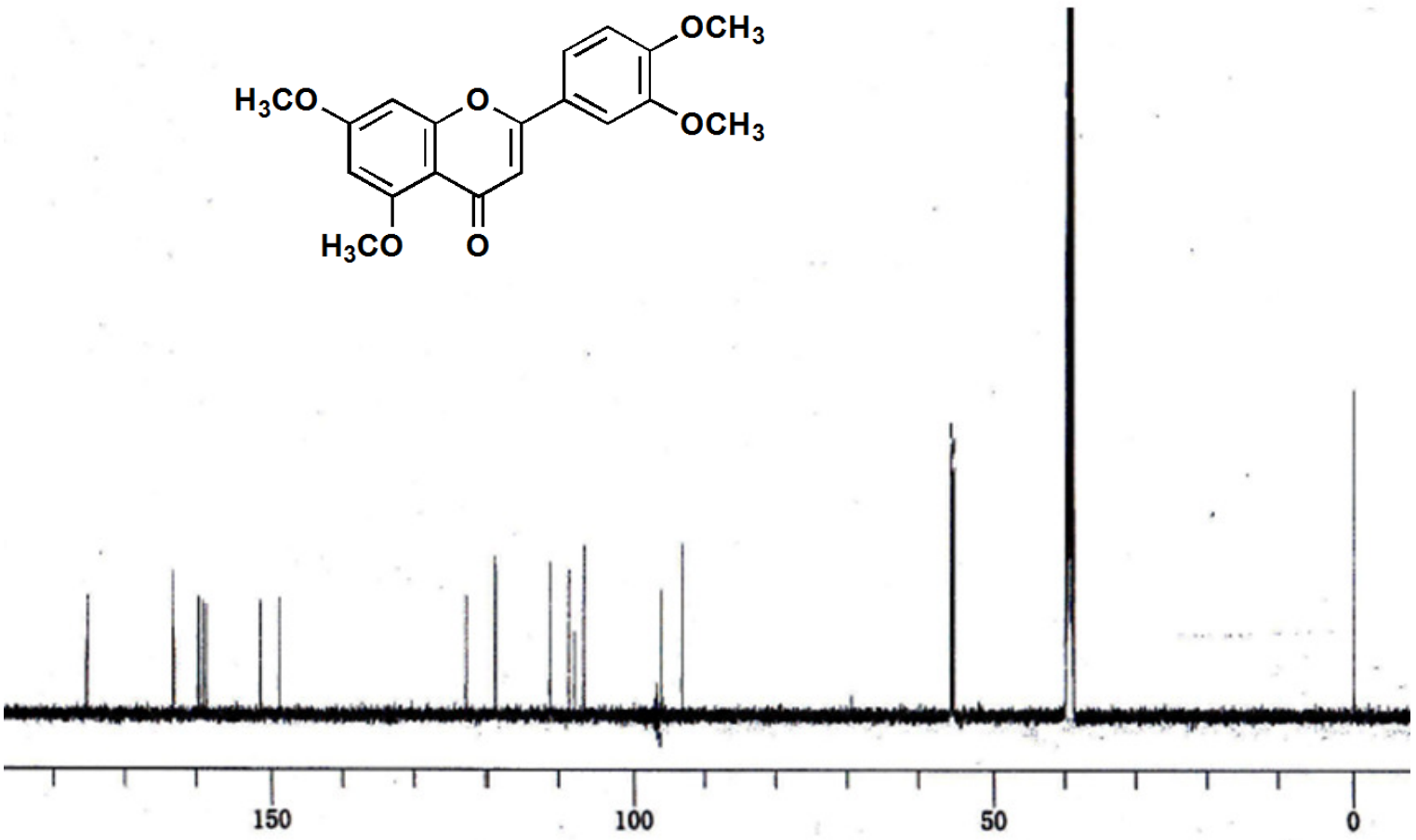

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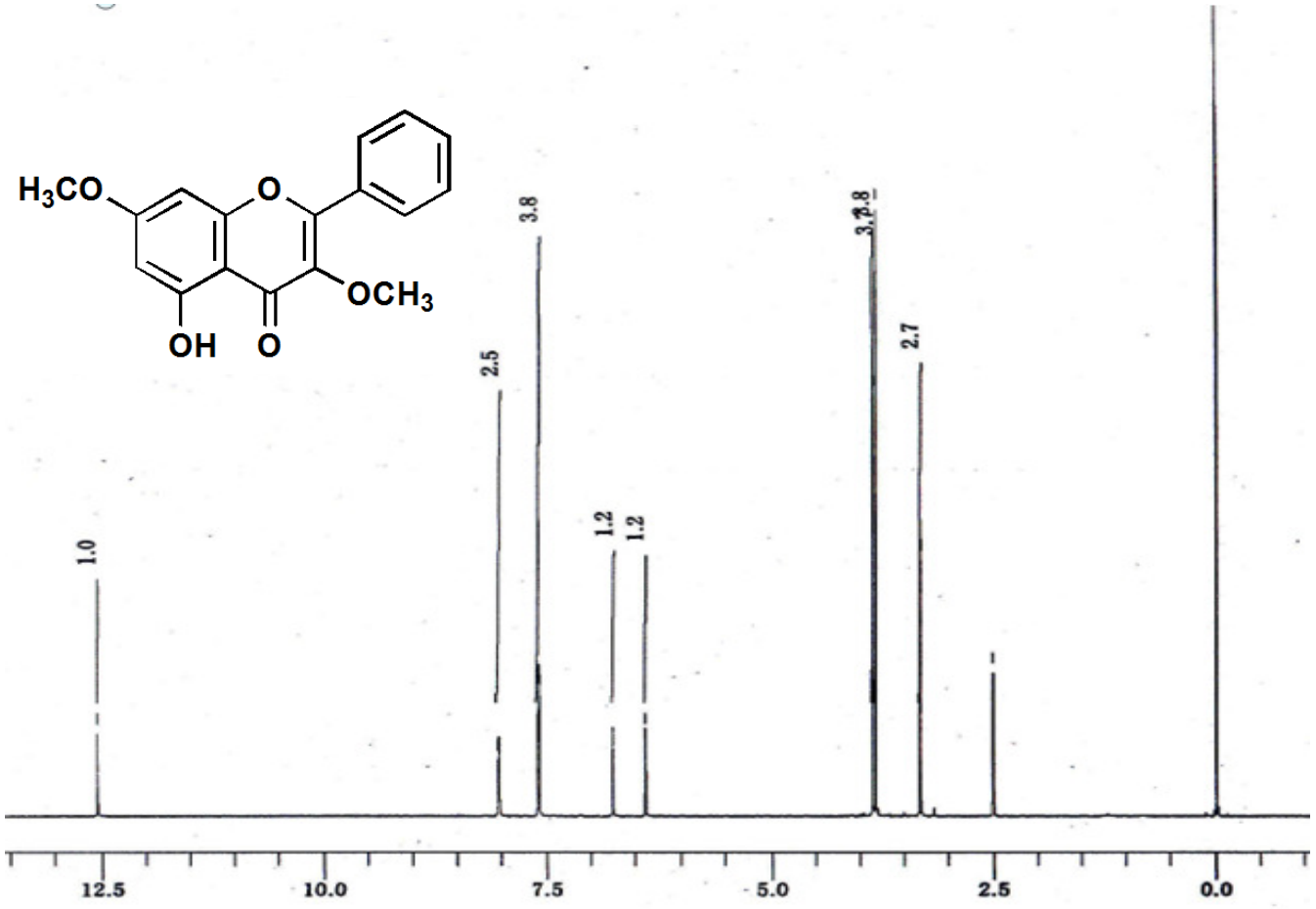
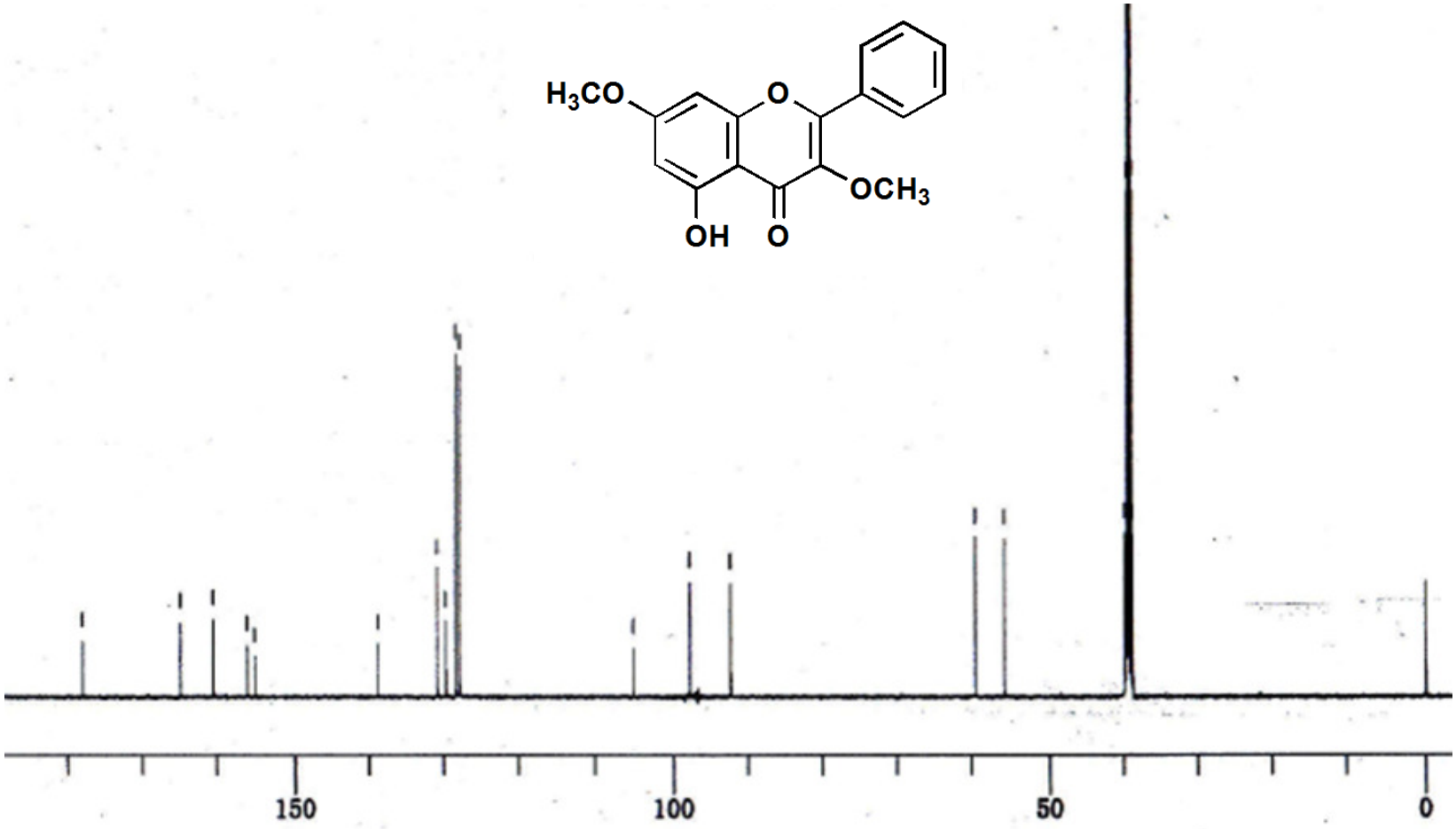


[1]H NMR spectrum of 5-hydroxy-3,7-dimethoxyflavone (**6**) (DMSO-d_6; 500 MHz)



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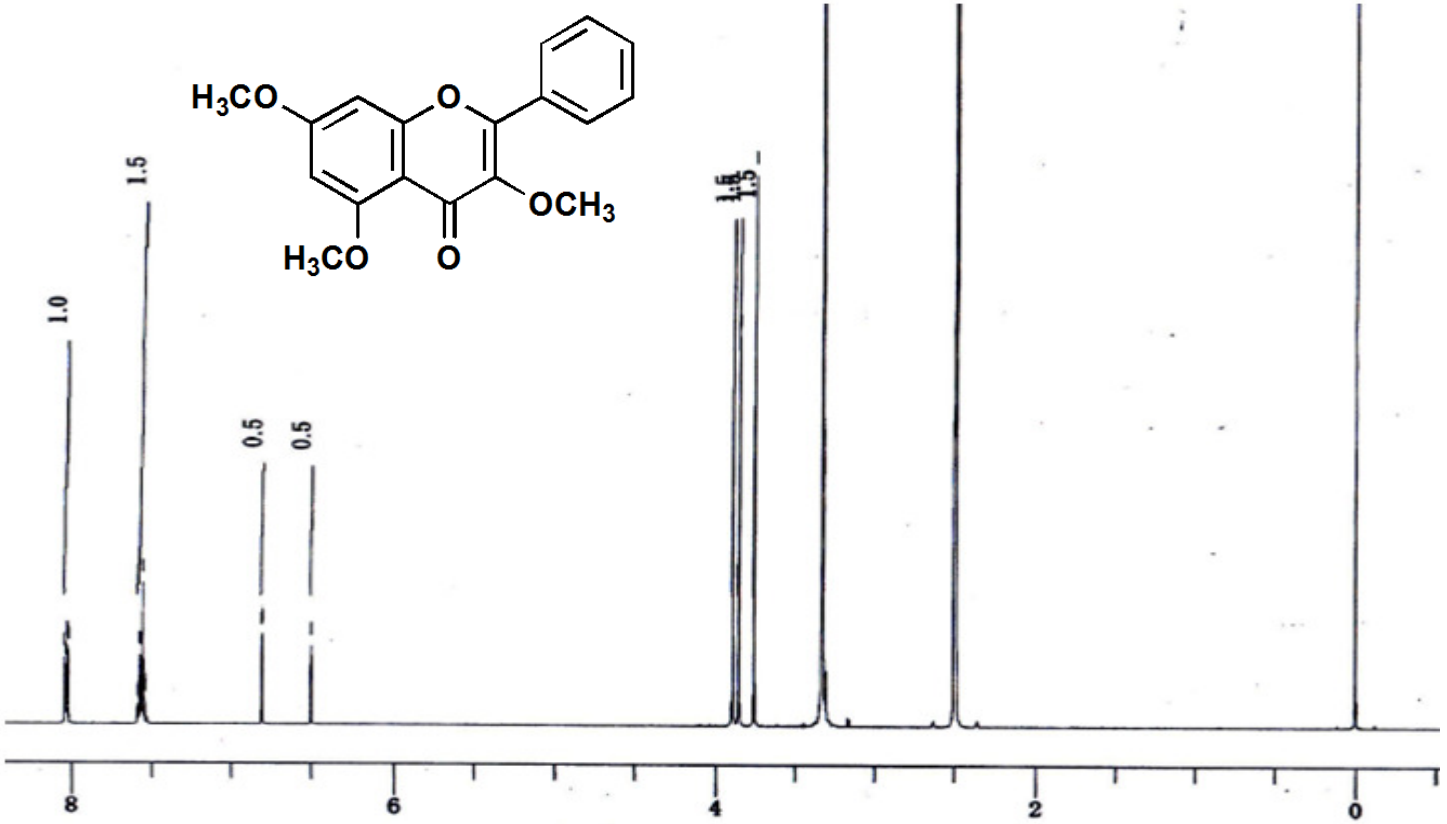

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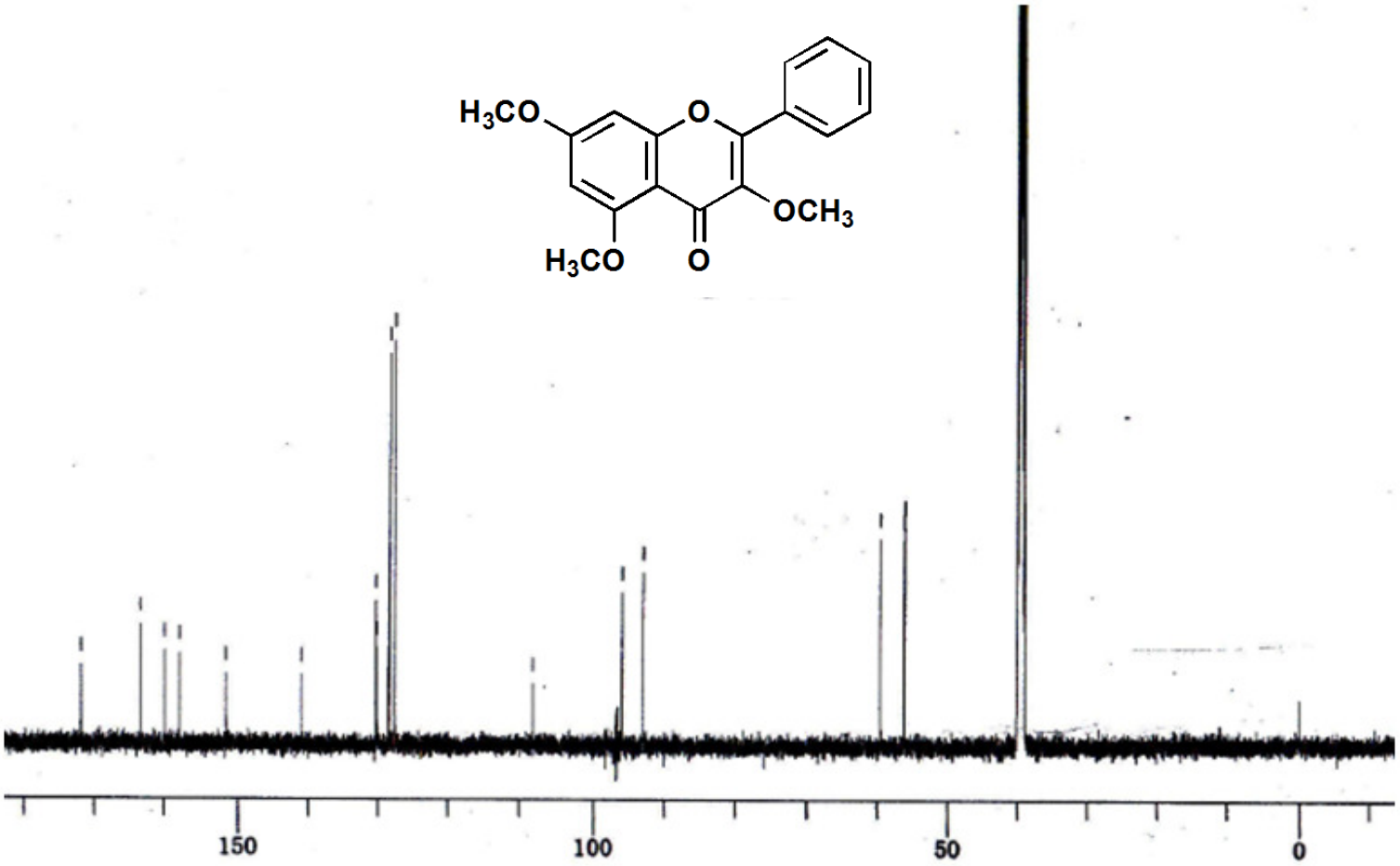

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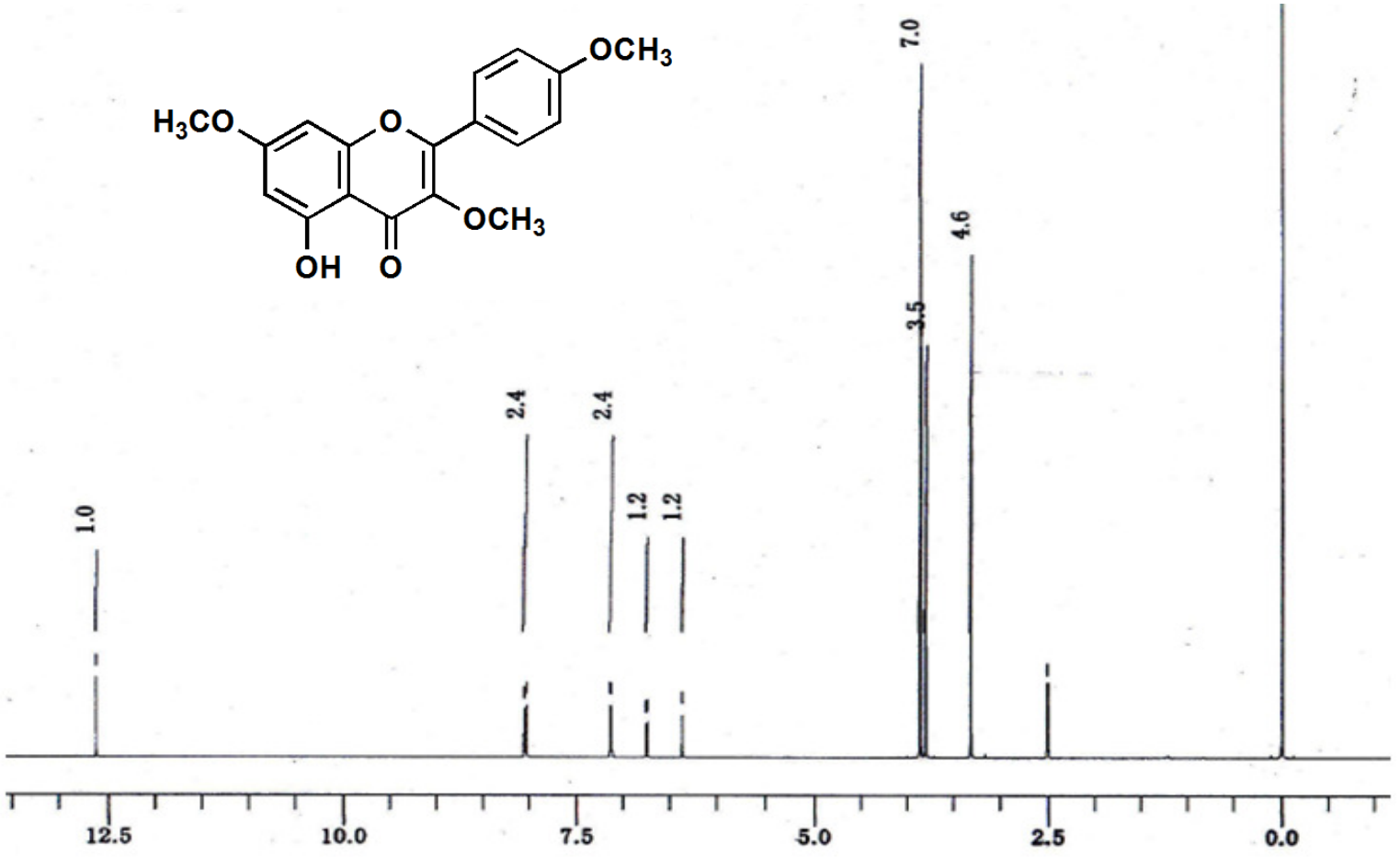
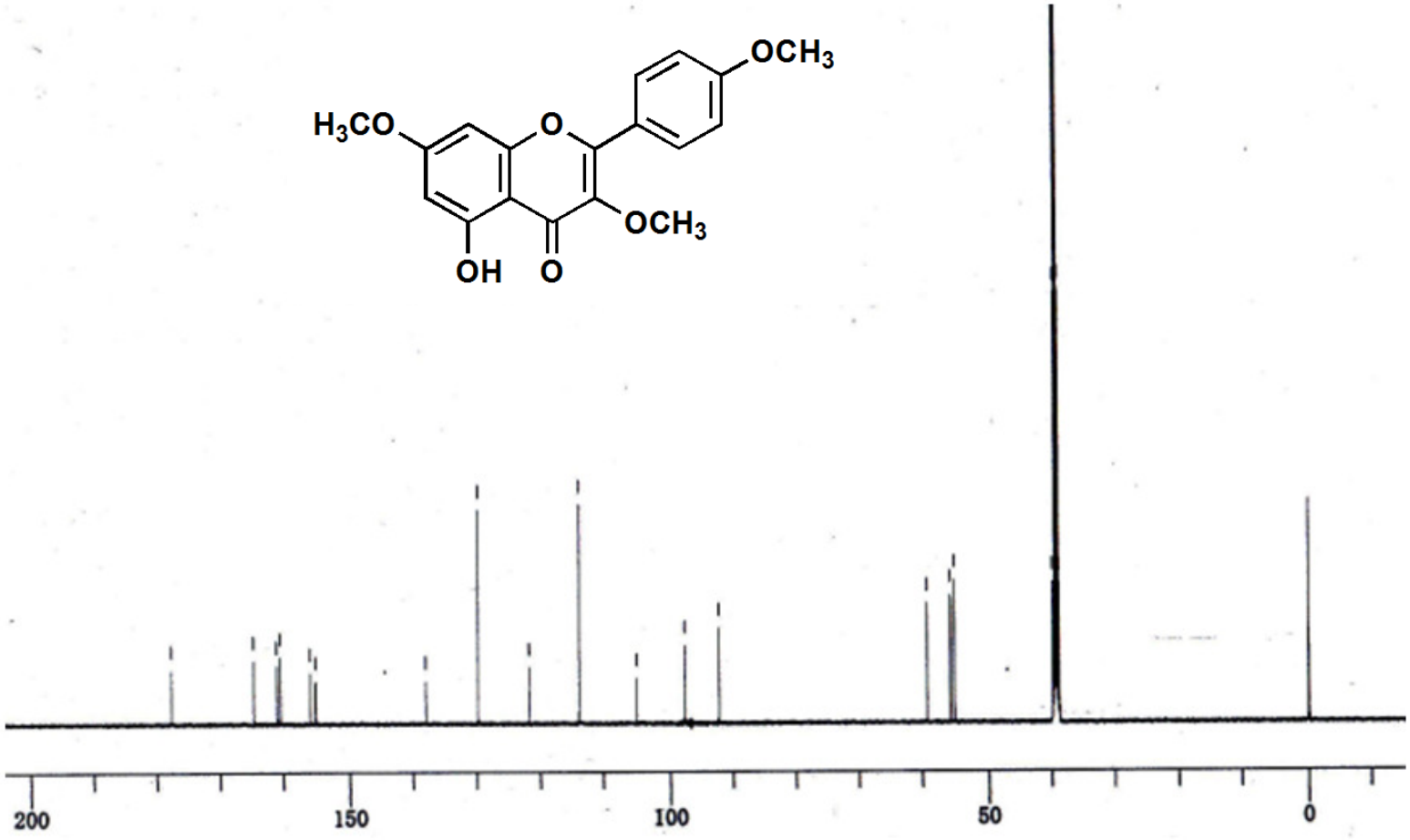


[1]H NMR spectrum of 3,7,4′-trimethylkaempferol (**8**) (DMSO-d_6; 500 MHz)



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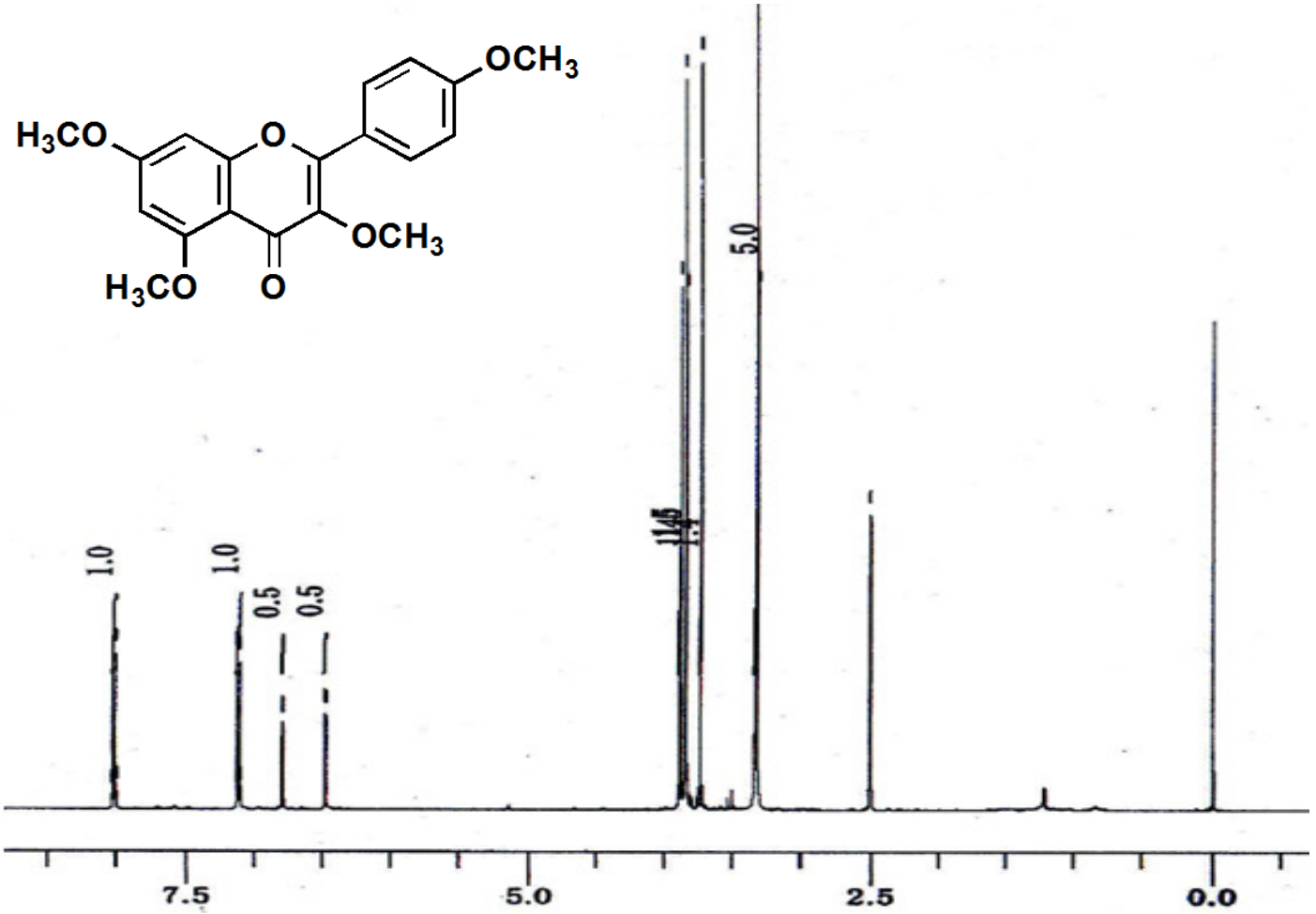

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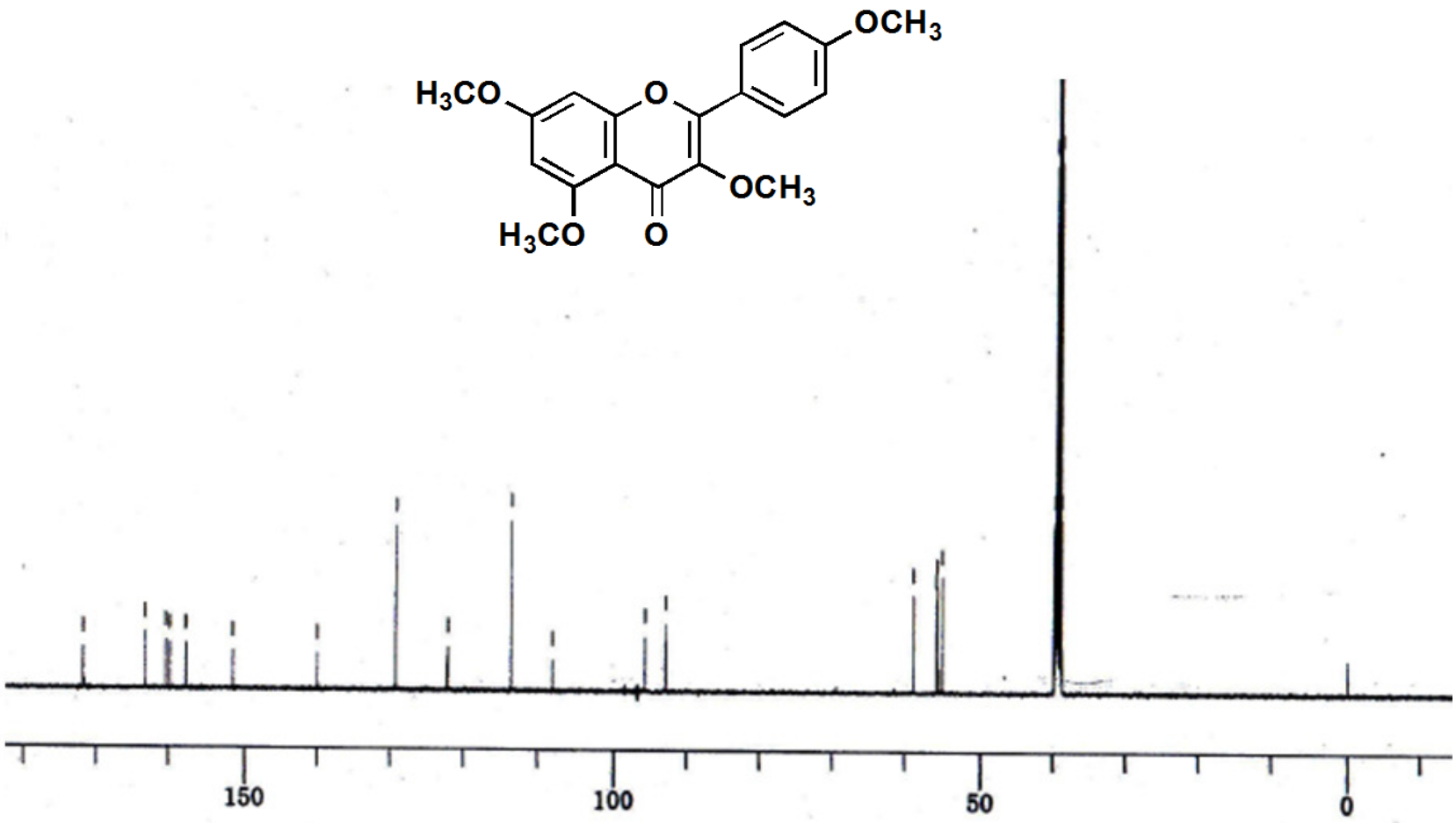

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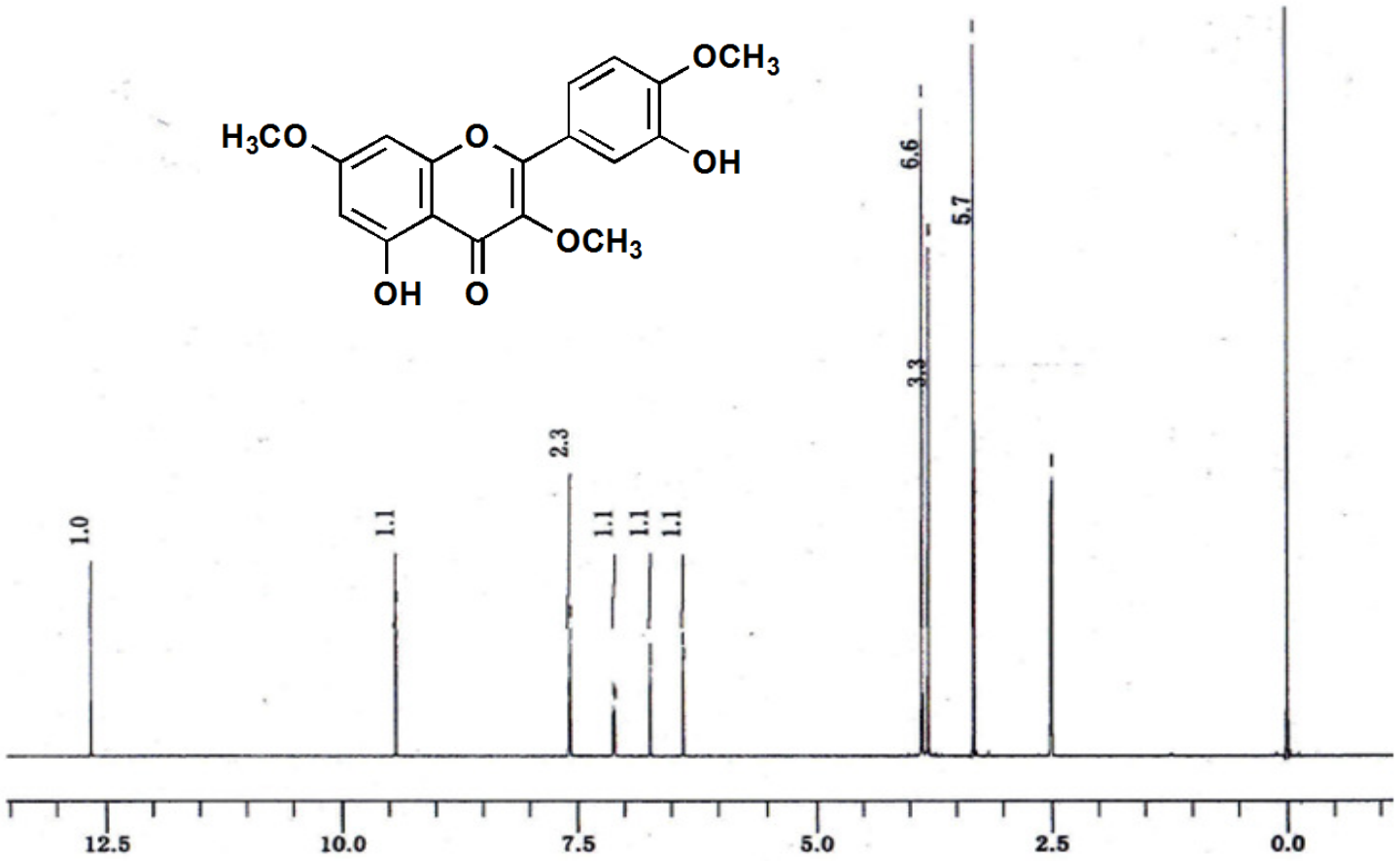
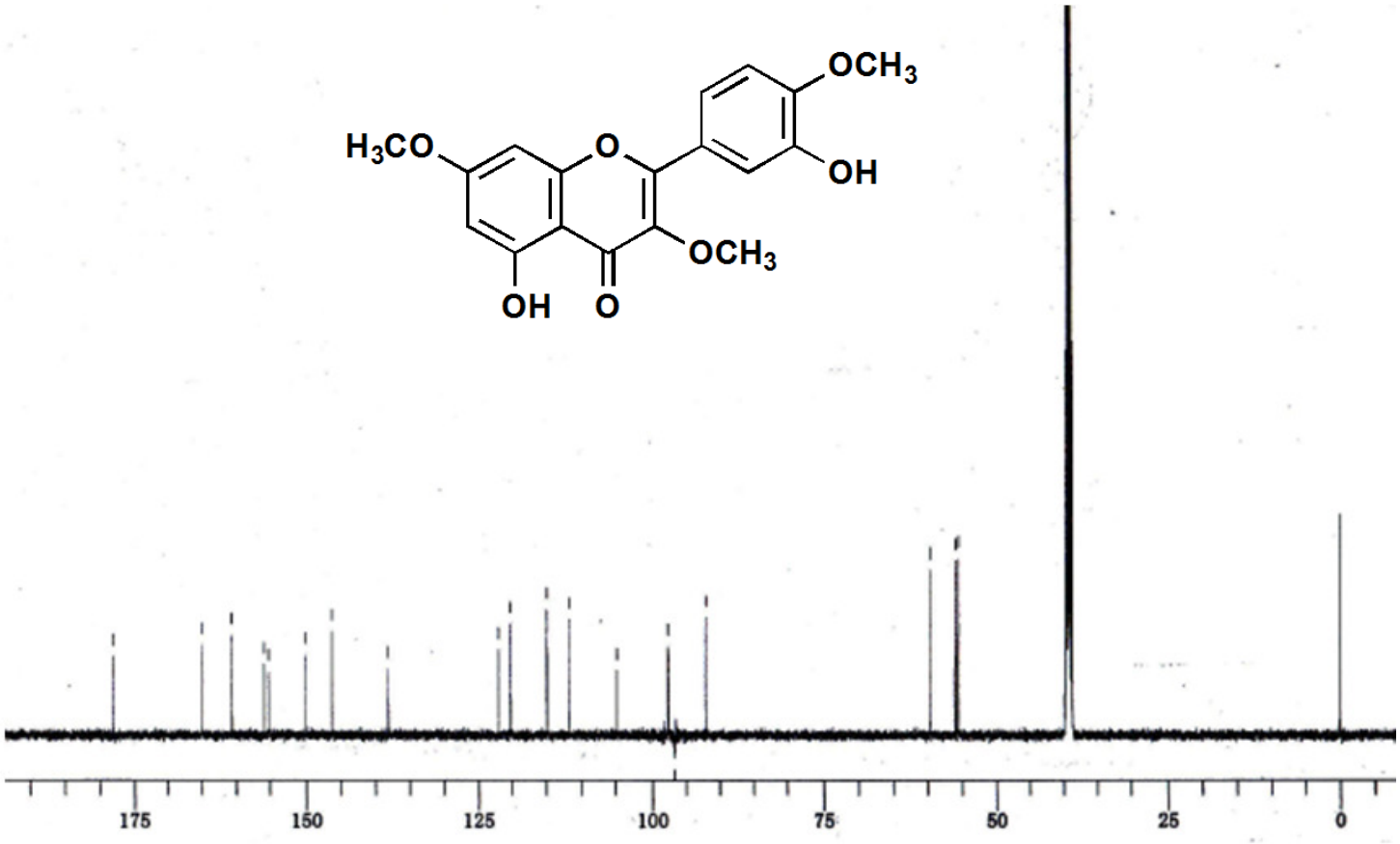


[1]H NMR spectrum of ayanin (**10**) (DMSO-d_6; 500 MHz)



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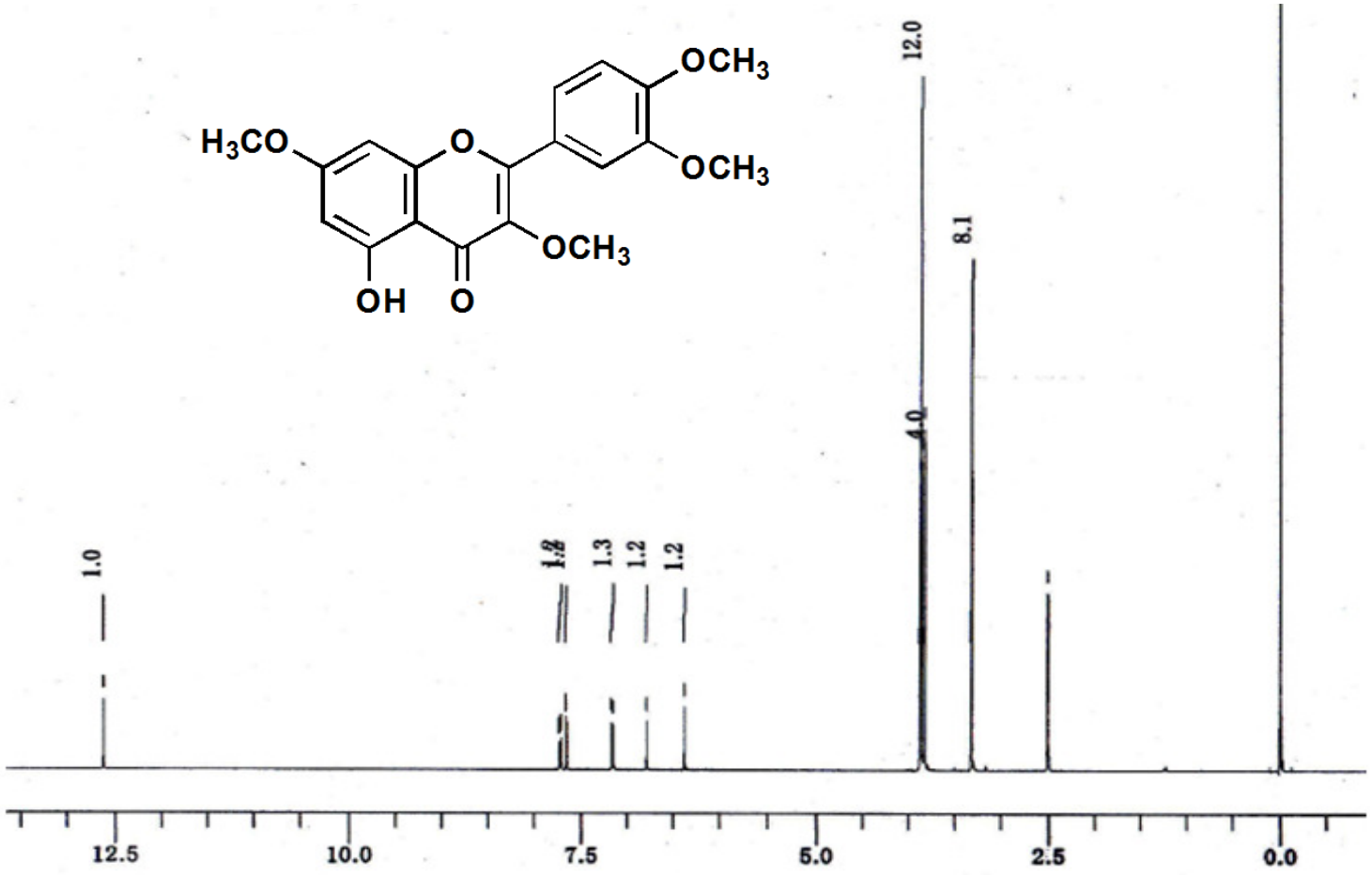

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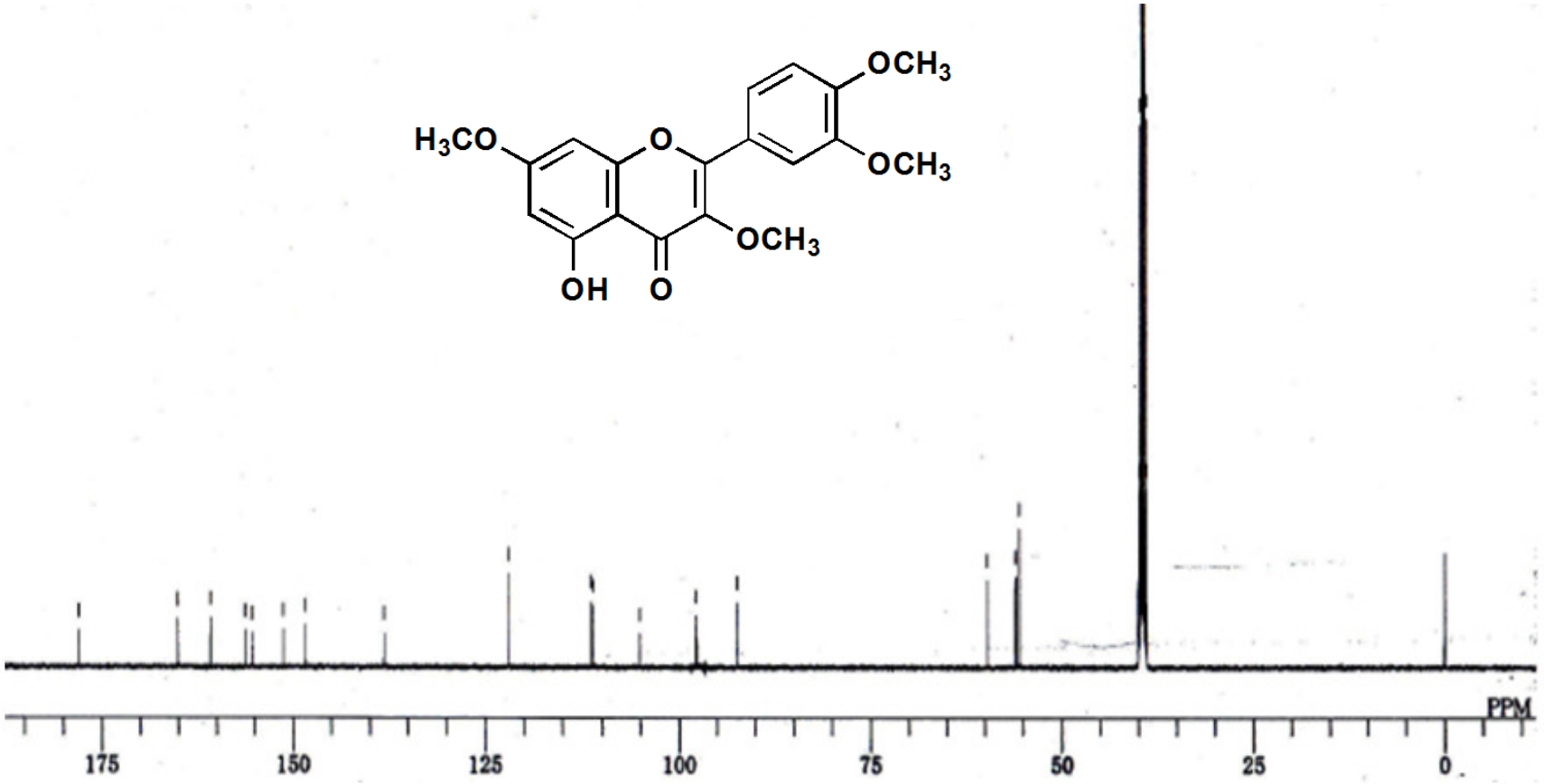

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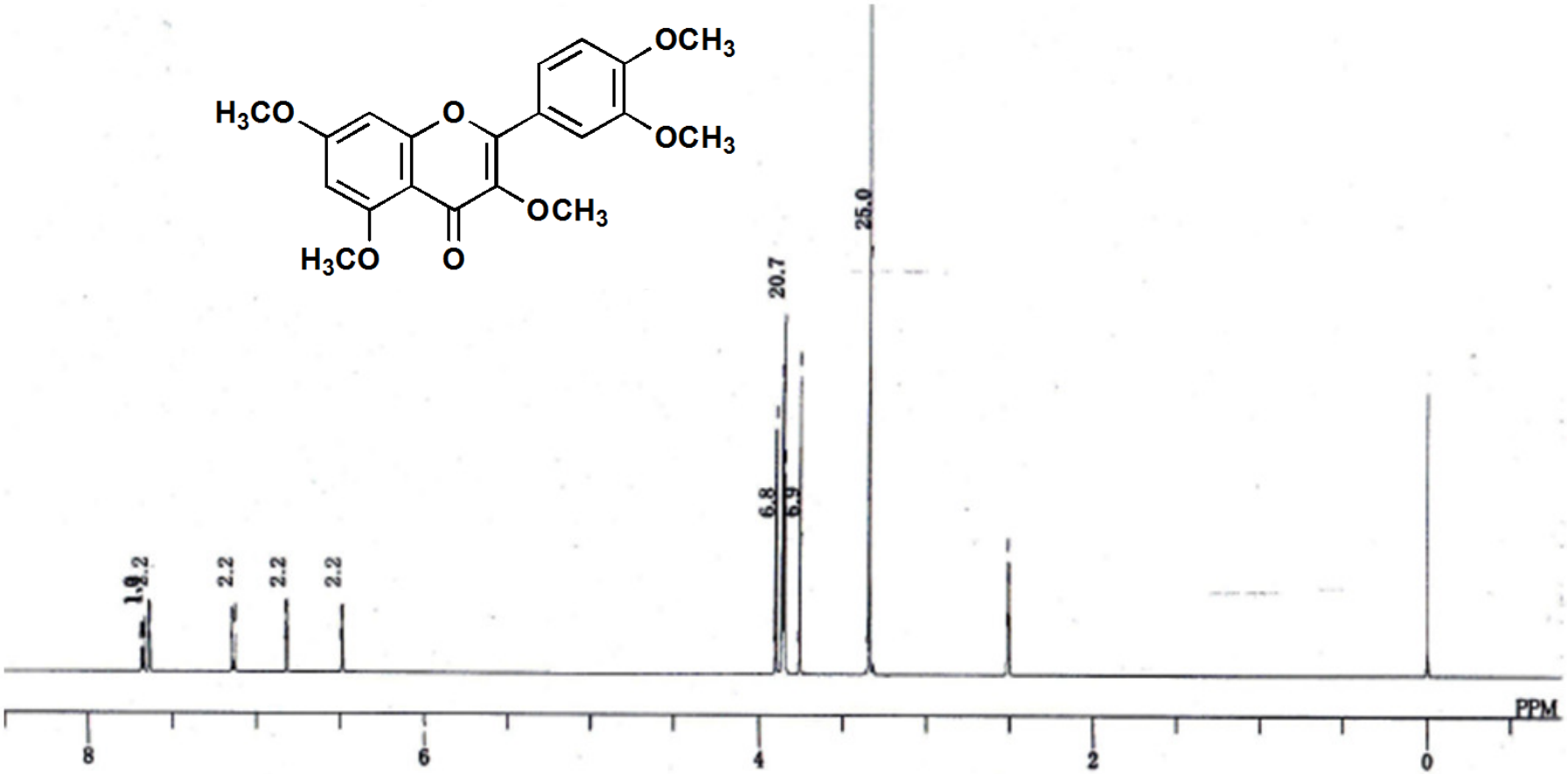

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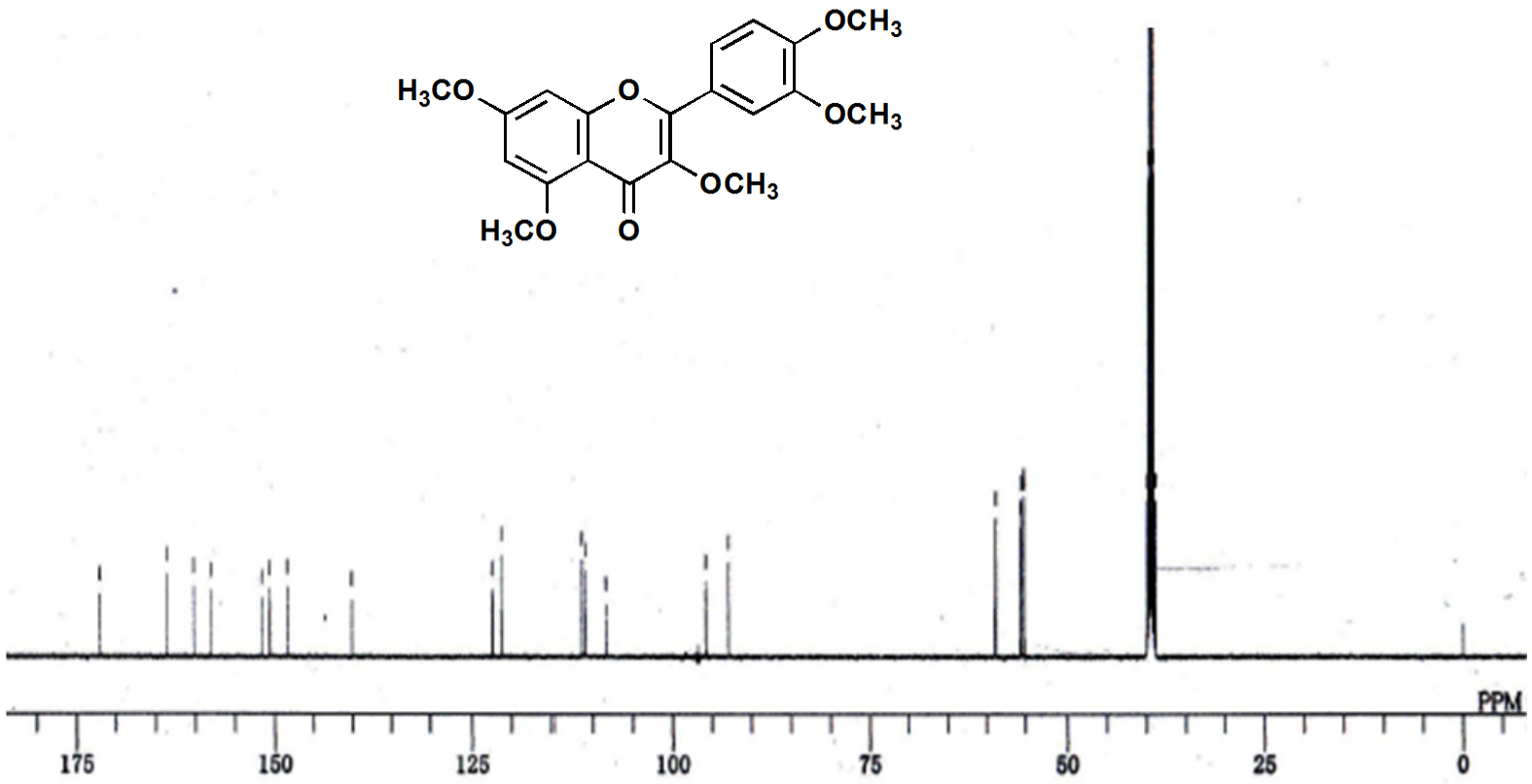

^{13}C NMR spectrum of pentamethylquercetin (**12**) (DMSO-d_6; 500 MHz)

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1. The Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0175/2550)
2. Academic Excellence Program in Pharmaceutical Sciences, Prince of Songkla University.

List of Publications and Proceedings

Publications

1. Sae-wong, C., Tewtrakul, S., Tansakul, P. 2009. Anti-inflammatory mechanism of *Kaempferia parviflora* in murine macrophage cells (RAW264.7) and in experimental animals. Journal of Ethnopharmacology 124, 576-580.
2. Sae-wong, C., Matsuda, H., Tewtrakul, S., Tansakul, P., Nakamura, S., Nomura, Y., Yoshikawa, M. 2011. Suppressive effects of methoxyflavonoids isolated from *Kaempferia parviflora* on inducible nitric oxide synthase (iNOS) expressions in RAW 264.7 cells. Journal of Ethnopharmacology. In press.

Proceedings

1. Sae-wong, C., Tewtrakul, S., Matsuda, H., and Yoshikawa, M. Inhibition on nitric oxide release and iNOS mRNA expression of methoxyflavonoids isolated from *Kaempferia parviflora* rhizomes. The 23rd Federation of Asian Pharmaceutical Associations Congress. 2010 FAPA Congress in Taipei, Taiwan, 5-8 November, 2010.

2. Sae-wong, C., Tewtrakul, S., Matsuda, H., and Yoshikawa, M. Anti-inflammatory activities and phytochemical study of *Kaempferia parviflora* Wall Ex. Baker. The 9th NRCT-JSPS Joint Seminar, Chulalongkorn University, Bangkok Thailand, 8-9 December, 2010.
3. Sae-wong, C., Tewtrakul, S., Matsuda, H., and Yoshikawa, M. Inhibition of iNOS protein expression by methoxyflavonoids isolated from *Kaempferia parviflora* rhizomes. RGJ-Ph.D. Congress XII. Chonburi, Thailand, 1-3 April, 2011.