ข้อมูลการเผยแพร่ผลงานวิทยานิพนธ์

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2. **หลักสูตรระดับ** ☑ ปริญญาเอก ○ แบบ 1 ☑ แบบ 2
☐ ปริญญาโท ○ แผน ก แบบ ก 1 ○ แผน ก แบบ ก 2
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☑ รับทุนอุดหนุนจากบัณฑิตวิทยาลัยภายใต้เงื่อนไข -
☑ รับทุนผู้ช่วยวิจัย โครงการปริญญาเอกกาญจนาภิเษกภายใต้เงื่อนไข จะต้องตีพิมพ์ผลงานวิจัยในวารสารนานาชาติ อย่างน้อย 1 ฉบับ
○ รับทุนบัณฑิตวิทยาลัยร่วมด้านพลังงานและสิ่งแวดล้อมภายใต้เงื่อนไข.....
○ รับทุนอื่น ๆ ทุนความเป็นเลิศ สาขาวิชาเภสัชศาสตร์
ภายใต้เงื่อนไข.....

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3.1 ชื่อผู้เขียน Sae-wong, C, Tewtrakul, S., Tansakul

ชื่อบทความ Anti-inflammatory mechanism of *Kaempferia parviflora* in murine macrophage cells (RAW264.7) and in experimental animals

ชื่อวารสาร Journal of Ethnopharmacology

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เป็นวารสารระดับ ○ ชาติ ☑ นานาชาติ **อยู่ในฐานข้อมูล** ○ TCI ○ สกอ. ☑ ISI

สถานภาพของบทความ ☑ ตีพิมพ์แล้ว ○ อยู่ระหว่างการตีพิมพ์ ○ ได้รับการตอบรับให้ตีพิมพ์

3.2 ชื่อผู้เขียน Sae-wong, C., Matsuda, H., Tewtrakul, S., Tansakul, P., Nakamura, S., Nomura, Y. and Yoshikawa, M.

ชื่อบทความ Suppressive effects of methoxyflavonoids isolated from *Kaempferia parviflora* on inducible nitric oxide synthase (iNOS) expressions in RAW 264.7 cells.

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เป็นวารสารระดับ ○ ชาติ ☑ นานาชาติ **อยู่ในฐานข้อมูล** ○ TCI ○ สกอ. ☑ ISI

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ชื่อเรื่องผลงานที่นำเสนอ Inhibition on Nitric Oxide Release and iNOS mRNA Expression of Methoxyflavonoids Isolated from *Kaempferia parviflora* rhizomes.

ชื่อการประชุม The 23rd Federation of Asian Pharmaceutical Associations Congress. 2010 FAPA Congress

วันเดือนปีและสถานที่จัดประชุม 5-8 November 2010. Taipei, Taiwan

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ชื่อเรื่องผลงานที่นำเสนอ Anti-inflammatory Activities and Phytochemical Study of *Kaempferia parviflora* Wall Ex. Baker.

ชื่อการประชุม The 9th NRCT-JSPS Joint Seminar

วันเดือนปีและสถานที่จัดประชุม 8-9 December 2010, Chulalongkorn University, Bangkok Thailand

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เป็นการประชุมระดับ ○ ชาติ ☑ นานาชาติ **รูปแบบผลงาน** ☑ Proceedings ○ Abstract

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ชื่อเรื่องผลงานที่นำเสนอ Inhibition of iNOS Protein Expression by Methoxyflavonoids Isolated from *Kaempferia parviflora* Rhizomes.

ชื่อการประชุม RGJ-Ph.D. Congress XII

วันเดือนปีและสถานที่จัดประชุม 1-3 April 2011, Chonburi, Thailand

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6. ขณะนี้ยังไม่สามารถเผยแพร่หรือตีพิมพ์ผลงานได้ เนื่องจาก (โปรดระบุรายละเอียด เช่น มีการจดสิทธิบัตร หรือเป็นเงื่อนไขของการรับทุนจากแหล่งทุน (ระบุแหล่งทุน))

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ข้าพเจ้าขอรับรองว่า ได้ตรวจสอบความถูกต้องของข้อมูลดังกล่าวแล้วทุกประการ หากบัณฑิตวิทยาลัยตรวจสอบพบในภายหลังว่าข้อมูลไม่เป็นความจริง ข้าพเจ้ายินดีให้บัณฑิตวิทยาลัยตัดสิทธิ์ในการสำเร็จการศึกษาโดยไม่ขออุทธรณ์ใด ๆ ทั้งสิ้น

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ความเห็นคณะกรรมการ บริหารหลักสูตร สาขาวิชาเภสัชศาสตร์

คณะกรรมการ บริหารหลักสูตร สาขาวิชาเภสัชศาสตร์ ในคราวประชุมครั้งที่ 2 / 2554 เมื่อวันที่ 26 เมษายน 2554 ให้ความเห็นชอบการเผยแพร่ผลงานวิทยานิพนธ์ของ (นาย/นาง/นางสาว) จุฑา แซ่ว่อง ว่าครบถ้วนตามมาตรฐานการประกันคุณภาพของหลักสูตรระดับ

☑ ปริญญาเอก สาขาวิชา เภสัชศาสตร์

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Anti-inflammatory mechanism of *Kaempferia parviflora* in murine macrophage cells (RAW 264.7) and in experimental animals

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ABSTRACT

Ethnopharmacological relevance: The rhizomes of *Kaempferia parviflora* Wall. ex Baker have been used in Thailand for treatment of gout, apthous ulcer, peptic ulcer and abscesses.
Aim of the study: In our previous study, the crude ethanol extract of *Kaempferia parviflora* and its compound (**5**, 5-hydroxy-3,7,3′,4′-tetramethoxyflavone), was reported to show nitric oxide (NO) inhibition in RAW 264.7 cells. The present study is thus investigated the anti-inflammatory mechanism of *Kaempferia parviflora* extract and compound **5** against inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expressions.
Materials and methods: The extract of *Kaempferia parviflora* and its compound were tested against NO and prostaglandin E_2 (PGE_2) releases using RAW264.7 cells as well as studied on anti-inflammatory activity in carrageenan-induced rat paw edema and acute toxicity in mice.
Results: The results revealed that the ethanol extract of *Kaempferia parviflora* markedly inhibited PGE_2 release with an IC_{50} value of 9.2 μg/ml. This plant extract and compound **5** also suppressed mRNA expression of iNOS in dose-dependent manners, whereas COX-2 mRNA expression was partly affected. According to the *in vivo* study, chloroform and hexane fractions greater decreased rat paw edema than ethanol, ethyl acetate and water fractions.
Conclusion: The mechanisms for anti-inflammatory activity of *Kaempferia parviflora* and compound **5** are mainly due to the inhibition of iNOS mRNA expression but partly through that of COX-2 mRNA.

1. Introduction

Kaempferia parviflora Wall. ex Baker or kra-chai-dam, a plant in a family of Zingiberaceae and sometimes referred as Thai ginseng, is very popular for health promotion in Thailand. This plant is a perennial herb that grows to 90 cm height with dark purple to black rhizomes and these colors lead to the name kra-chai-dam (Putiyanan et al., 2004). Among local people in the northeast of Thailand, the rhizomes of *Kaempferia parviflora* have been known as health-promoting herbs, and also frequently used for treatment of gout, abscesses, colic disorder, peptic- and duodenal ulcers. In Thailand, a tonic drink made from the rhizomes of *Kaempferia 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., 2007).

Macrophages are known to play an important role in host defense mechanism (Krol et al., 1995). RAW264.7 cells stimulated by lipopolysaccharide (LPS) produce a variety of pro-inflammatory mediators, including interleukin, cytokines, nitric oxide and prostaglandin E_2 (PGE_2) (Chien et al., 2008). Thus, agents that down-regulate these pro-inflammatory mediators would be beneficial in the treatment of inflammation (Chen et al., 2001; Wang and Mazza, 2002; Chien et al., 2008).

Previously, Tewtrakul and Subhadhirasakul (2008) reported the effects of 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**5**) from *Kaempferia parviflora* on nitric oxide (NO), PGE_2 and tumor necrosis factor-alpha (TNF-α) productions in RAW264.7 macrophage cells. It was found that compound **5** exhibited potent anti-inflammatory effect against LPS-induced NO and PGE_2 release in RAW264.7 cells with IC_{50} values of 16.1 and 16.3 μM, respectively. Hence, the present study is aimed to investigate the mechanism in transcriptional level of this plant on the suppression of iNOS and COX-2 genes as well as the anti-inflammatory effect in animal model

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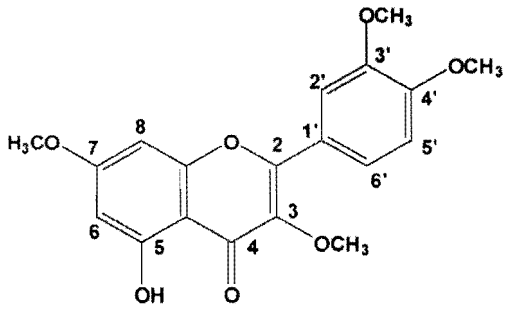

Fig. 1. Structure of compound **5** isolated from *Kaempferia parviflora* rhizomes.

(carrageenan-induced rat paw edema) and its acute toxicity in mice.

2. Materials and methods

2.1. Chemicals and reagents

Lipopolysaccharide (LPS) from *Escherichia coli*, RPMI-1640 medium, indomethacin, phosphate buffer saline (PBS), carrageenan type 4, Tween 80, and propylene glycol were purchased from Sigma–Aldrich (Sigma–Aldrich, MO, USA). Fetal bovine serum (FBS) was bought from Gibco (Invitrogen, CA, USA). Penicillin–streptomycin was purchased from Invitrogen (Invitrogen, CA, USA). The 6-well microplates were obtained from Nunc (Nunc, Birkrød, Denmark).

2.2. Plant materials and preparation of the plant extract

Kaempferia parviflora Wall. ex Baker rhizomes were bought from a Thai traditional drug store in Songkhla province, Thailand in 2006. The plant material was identified by Assoc. Prof. Dr. Sanan Subhadhirasakul. The voucher specimen (specimen no. SKP2061116) was deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla, Thailand.

Two kilograms dried weight of *Kaempferia parviflora* were ground and macerated with ethanol at room temperature, four times (6 l, 4×). The ethanol extract (267 g) was then concentrated and partitioned between 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 give residues of hexane (14.1 g), chloroform (215.0 g), ethyl acetate (4.8 g) and water fractions (27.0 g), respectively.

2.3. Isolation of compound 5 from the hexane fraction

The hexane fraction (5.0 g) which possessed the highest NO inhibitory activity (IC_{50} = 3.6 μg/ml) (Tewtrakul and Subhadhirasakul, 2008) was chromatographed on silica gel (230–400 mesh) using hexane and ethyl acetate (95:5 to ethyl acetate 100%, 8000 ml) to obtain compound **5** (5-hydroxy-3,7,3′,4′-tetramethoxyflavone, 54 mg, 1.0%, w/w). The structure of **5** was elucidated by comparing the ^1H and ^{13}C NMR spectral data with those reported (Jaipetch et al., 1983; Harborne et al., 1988; Agrawal, 1989) (Fig. 1).

2.4. Experimental animals

Male Swiss albino mice (30–40 g) and Wistar rats (180–200 g) 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).

2.5. Inhibitory 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. Cells suspension 100 μl were seeded in 96-well plate and incubated for 1 h at 37 °C. The medium was replaced with LPS in RPMI-1640 (300 μg/ml) which is the triggering agent for PGE_2 production. After that, *Kaempferia parviflora* extract in various concentrations (3–100 μg/ml) were dispensed into the wells. The cells were incubated for 48 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, Inc.).

2.6. Total RNA isolation from RAW264.7 cells

RAW 264.7 cells 1.5 ml (1×10^6 cells/ml) were added to 6-well culture plate and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that, cells were harvested after 20 h incubation with various concentrations of samples (3, 10, 30 and 100 μM for compound **5** and 3, 10, 30 and 100 μg/ml for *Kaempferia parviflora* extract). The cells were removed from the culture flask by scrapping and were then isolated to obtain RNA using RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). Briefly, the cells were centrifuged at 4000 rpm 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 rpm 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 rpm 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 rpm 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 rpm 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 rpm for 30 s to obtain total RNA. The isolated RNA was stored at −20 °C until use.

2.7. Detection of iNOS and COX-2 mRNA by RT-PCR

In order to investigate the mechanisms in transcriptional level of *Kaempferia parviflora* extract and compound **5**, 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, Toyobo Co., Ltd., Japan) consisting of RNA solution 11 μl, 5× RT buffer 4 μl, dNTP mixture (10 mM) 2 μl, RNase inhibitor (10 U/μl) 1 μl, Oligo (dT) 20 1 μl and Rever Tra Ace (reverse transcriptase enzyme) 1 μl for a 20 μl reaction. Reverse-

transcription was performed at 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. 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 Co., Ltd., Japan). The β-actin, a constitutively expressed gene, was analyzed as an internal standard. The primers for each gene were as follows: forward primer for iNOS: 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGAC TATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGC TC-3′; forward primer for β-actin: 5′-TGTGATGGTGGGAATGGGTCAG-3′ and its reverse primer: 5′-TTTGATGTCACGCACGATTTCC-3′.

The PCR mixture is consisted of cDNA 2 μl, dH_2O (RNase free water) 85 μl, 10× 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 °C for 30 s, annealing at 60 °C for 30 s, and extension at 74 °C for 1 min. The 580 base pairs (bp) of iNOS, 860 bp of COX-2 and 514 bp of β-actin DNA fragments were obtained and separated on 1.2% (w/v) agarose gel electrophoresis. The bands of DNA were detected by staining with SYBR® Safe DNA staining solution for 30 min and were observed under a UV light box at wavelength 312 nm (Gel Doc model 1000, Bio-Rad, USA).

2.8. Acute toxicity test of *Kaempferia 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 *Kaempferia 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 7 days after administration. Food and water were given *ad libitum*.

2.9. 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 rat was measured using a plethysmometer (Ugo basile). Male Wistar rats (180–200 g each) were divided into 7 groups of 10 rats each. The control group (group 1) received cosolvent (10% DMSO:propylene glycol:Tween 80:distilled water at the ratio of 1:4:4:1, respectively) at 10 ml/kg, p.o. Group 2 received the standard drug indomethacin (10 mg/kg, p.o.), whereas groups 3–7 were that of *Kaempferia parviflora* extract and its fractions (150 mg/kg, p.o.). After 30 min, they were subcutaneously injected with 0.1 ml of 1% (w/v) carrageenan in 0.9% normal saline into the subplantar region of the right-hind paw. The volume of right-hind paw was measured at 0.5, 1, 2, 3, 4 and 5 h after carrageenan injection with plethysmometer. The percentage of inhibition was determined from the following formula:

$$\text{Inhibition } (\%) = \frac{(V_t - V_0)_{\text{control}} - (V_t - V_0)_{\text{treated}}}{(V_t - V_0)_{\text{control}}}$$

V_t = volume of right-hind paw after carrageenan injection; V_0 = volume of right-hind paw before carrageenan injection

2.10. Statistical analysis

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

3. Results and discussion

The result showed that *Kaempferia parviflora* extract inhibited LPS-induced PGE_2 production in RAW 264.7 cells with an IC_{50} value of 9.2 μg/ml, which is in agreement with a previous report (Tewtrakul and Subhadhirasakul, 2008) on anti-inflammatory effect of *Kaempferia parviflora* against NO release, a pro-inflammatory mediator (IC_{50} = 3.6 μg/ml). Since, *Kaempferia parviflora* possessed potent NO and PGE_2 inhibitory activities in RAW264.7 cells, the carrageenan-induced rat paw edema of various *Kaempferia parviflora* fractions was further investigated. 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 1).

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). 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.

In the acute toxicity test, *Kaempferia parviflora* extract up to 2 g/kg, orally as a single dose did not produce any clinical sign of toxicity, including convulsion, hyperactivity, sedation, respiratory depression and loss of righting reflex. This result indicated that *Kaempferia parviflora* extract has low toxicity.

In order to determine the mechanism of *Kaempferia parviflora* on LPS-induced NO and PGE_2 productions, we therefore examined the effect of *Kaempferia parviflora* and compound **5** on LPS-induced expression of iNOS and COX-2 genes. The result showed that *Kaempferia parviflora* extract and compound **5** decreased cellular iNOS mRNA level, while inhibitory effect on COX-2 mRNA expression was partly affected (Fig. 2A and B). Indomethacin which is the reference drug showed dose-dependent manner inhibited iNOS mRNA expression but mildly affected on COX-2 mRNA (Fig. 2C). An excessive NO has been shown to interact with oxygen radicals and form highly reactive peroxynitrite, which in turn induce inflammatory cellular cytokines and COX-2 (Tannous et al., 2001; Cho et al., 2004). Thus, the result of PGE_2 inhibition could also be directly mediated by down-regulation of iNOS expression. Moreover, it has been reported that the inhibition on iNOS mRNA expression might involve in the blockade of NFκB, an essential transcription factor for iNOS gene transcription (Diaz-Guerra et al., 1996; Jung et al., 2007).

In summary, the present study on both *in vitro* (macrophage cell line) and *in vivo* (carrageenan-induced rat paw edema) supports the traditional use of *Kaempferia parviflora* for treatment of inflammation. The anti-inflammatory mechanism of *Kaempferia parviflora* and compound **5** in RAW 264.7 macrophages is attributed mainly through down-regulation of iNOS mRNA expression but

Table 1
Effect of ethanol extract and fractions of *Kaempferia 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	1 h	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**	−4.77	5.76	17.03	18.32	16.60	10.75
Ethanol 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	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
Ethyl acetate	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
Chloroform	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
Water	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 + cosolvent (propylene glycol:Tween 80:distilled water, 4:1:4).
[a] Dose in ml/kg.
* $p < 0.05$.
** $p < 0.01$, significantly different compared to the control group (Bonferroni's test).

Fig. 2. Inhibition on iNOS and COX-2 mRNA expressions of *Kaempferia parviflora* extract (A), compound **5** (B) and indomethacin (C) in various concentrations using RAW264.7 cells. (−) = LPS (−), Sample (−); (0) = LPS (+), Sample (−); 3–100 = LPS (+), Sample (+).

partly through that of COX-2 mRNA. The isolation of compounds from chloroform fraction (IC_{50} against NO release = 8.8 μg/ml) that are responsible for anti-inflammatory activity will be further investigated.

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Suppressive effects of methoxyflavonoids isolated from *Kaempferia parviflora* on inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells

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ABSTRACT

Ethnopharmacological relevance: The rhizomes of *Kaempferia parviflora* Wall. ex Baker have been traditionally used in Thailand to treat abscesses, gout, and peptic ulcers.
Aim: Previously, we reported that the chloroform fraction of a *Kaempferia parviflora* extract had an inhibitory effect on rat paw-edema. In the present study, we isolated the constituents of this fraction and investigated the anti-inflammatory mechanism against nitric oxide (NO) production, tumor necrosis factor-α (TNF-α) and the expression of inducible nitric oxide synthase (iNOS) as well as phosphorylated extracellular signal-regulated kinase (p-ERK), and phosphorylated c-Jun N-terminal kinase (p-JNK). In addition, effects of trimethylapigenin (**4**) on the enzyme activities of protein kinases possibly leading to iNOS expression were examined to clarify the targets.
Materials and methods: The chloroform fraction was isolated using silica gel column chromatography and HPLC. Isolated compounds were tested against NO and TNF-α using RAW264.7 cells. Cytotoxicity and iNOS, p-ERK and p-JNK expression were also examined.
Results: Three active components, 5,7-dimethoxyflavone (**2**), trimethylapigenin (**4**), and tetramethylluteolin (**5**), markedly inhibited the production of NO in lipopolysaccharide (LPS)-activated RAW264.7 cells. Compounds **2**, **4**, and **5** moderately inhibited production of TNF-α. Compounds **2**, **4**, and **5** strongly inhibited expression of iNOS mRNA and iNOS protein in a dose-dependent manner, but did not inhibit p-ERK or p-JNK protein expression. The most active compound, **4**, did not inhibit the enzyme activity of inhibitor of κB kinases or mitogen-activated protein kinases, but inhibited that of spleen tyrosine kinase (SYK).
Conclusion: The mechanism responsible for the anti-inflammatory activity of methoxyflavonoids from the chloroform fraction of the rhizomes of *Kaempferia parviflora* is mainly the inhibition of iNOS expression, and the inhibition of SYK by **4** may be involved in the suppression of LPS-induced signaling in macrophages.

1. Introduction

Inflammation is a systemic response aimed to decrease the toxicity of harmful agents and repair damaged tissue (Kontush and Chapman, 2006). A key feature of the inflammatory response is the activation of phagocytic cells involved in host defense, which produce an oxidative burst of reactive oxygen, chlorine, and nitrogen species (Hansson, 2005; Kontush and Chapman, 2006).

Macrophages play major roles in the immunity and inflammatory responses involved in host defence. Once activated, they initiate the production of cytokines, oxygen and nitrogen species, and eicosanoids. In macrophages, bacterial lipopolysaccharide (LPS) is best able to induce the transcription of genes encoding pro-inflammatory proteins. The stimulation results in the release of cytokines and synthesis of enzymes such as inducible nitric oxide synthase (iNOS). The nitric oxide (NO) radical is known to play a central role in inflammatory and immune reactions (MacMicking et al., 1997; Rao et al., 2005). It is synthesized through the L-arginine pathway by three types of nitric oxide synthase (NOS): endothelial NOS (eNOS), neural NOS (nNOS) and inducible NOS (iNOS) (Qu and Zheng, 1997; Luo et al., 2001). eNOS and nNOS are constitutively expressed at low levels. Under normal physiological conditions,

Abbreviations: CAPE, caffeic acid phenethyl ester; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; IFNγ, interferon-γ; IκB, inhibitor of κB; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK1, MAPK-ERK kinase 1; nNOS, neural nitric oxide synthase; NO, nitric oxide; p-ERK, phosphorylated extracellular signal-regulated kinase; p-JNK, phosphorylated c-Jun N-terminal kinase; SYK, spleen tyrosine kinase; T-TBS, Tris-buffered saline containing 0.1% Tween 20; TNF-α, tumor necrosis factor-α.

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iNOS is dormant in resting cells, but under pathological conditions, it produces a large amount of NO leading to a 10-fold higher level of eNOS by interferon-γ (IFNγ) and LPS (Bogdan, 2001; Kumar et al., 2005) and plays a dual role in chronic infection, inflammation and carcinogenesis (Zang and Liu, 1998; Luo et al., 2001).

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 inflammation (Moon et al., 2007; Park et al., 2008). ERKs function in the control of cell division, and inhibitors of these enzymes are being explored as anticancer agents. JNKs are critical regulators of transcription, and JNK inhibitors may be effective in the control of rheumatoid arthritis (Johnson and Lapadat, 2002).

Kaempferia parviflora Wall. ex Baker belongs to the Zingiberaceae family. Its rhizomes are a dark purple to black color, and have been traditionally used in Thai folklore medicine for the treatment of leucorrhea, oral diseases (Chomchalow et al., 2003; Sudwan et al., 2006), stomachache, flatulence, digestive disorders and gastric ulcer (Wattanapitayakul et al., 2007). The rhizomes have also been used as a health-promoting agent, and for the treatment of gout, abscesses and colic disorder (Yenjai et al., 2007). In fact, a tonic drink made from *Kaempferia parviflora* rhizomes is commercially available (Yenjai et al., 2004). The major phytoconstituents of *Kaempferia parviflora* are methoxyflavone derivatives (Wattanapitayakul et al., 2007).

Previously, we found that the chloroform fraction of a *Kaempferia parviflora* extract displayed an appreciable anti-inflammatory effect against carrageenan-induced edema in rat paw (Sae-Wong et al., 2009). The components of this fraction were isolated using chemical means and the mechanism of action of the compounds was investigated. In the present study, we examined the effects of **1–12** isolated from the fraction on the production of NO and TNF-α as well as on iNOS mRNA expression, iNOS, phosphorylated ERK (p-ERK) and phosphorylated JNK (p-JNK) protein expression, in an attempt to understand the possible anti-inflammatory mechanism of *Kaempferia parviflora* rhizomes. In addition, effects of the most potentially active compound (**4**) on the enzyme activities of protein tyrosine kinases possibly leading to iNOS expression were examined to clarify the targets.

2. Materials and methods

2.1. Plant materials and preparation of the plant extract

Kaempferia parviflora Wall. ex Baker rhizomes were bought from a Thai traditional drug store in Songkhla province, Thailand on May 2008. The plant material was identified by one of the authors, Dr. Supinya Tewtrakul. A voucher specimen (SKP2061116) has been deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla, Thailand.

The dried rhizomes of *Kaempferia parviflora* (2.0 kg) were ground and macerated with ethanol at room temperature, four times (6 l, 4×). The ethanol extract (267 g) was then concentrated and partitioned between water and *n*-hexane, and successively partitioned with chloroform and water. The water layer was partitioned with ethyl acetate. Each partition was evaporated to dryness in vacuo to give the *n*-hexane (14.1 g), chloroform (215.0 g), ethyl acetate (4.8 g) and water (27.0 g) fractions.

2.2. Purification of compounds

Bioassay-guided fractionation of the ethanol extract led to the isolation of **1–12** (Fig. 1) from the chloroform fraction, which exhibited the greatest inhibitory effect on carrageenan-induced rat paw edema (Sae-Wong et al., 2009). Briefly, the chlorofrom fraction (200.0 g) was subjected to chromatography on silica gel using a step gradient solvent system starting with chloroform, methanol and ending with water to afford 7 fractions and then each fraction was purified by HPLC (Inertsil® ODS 3, 20 mm × 250 mm, GL Sciences Inc., Japan) using a 75% methanol solvent. Twelve compounds (**1–12**) were purified and elucidated by comparison with spectral data (^{1}H NMR, ^{13}C NMR and MS) previously reported (Wang et al., 1989; Sutthanut et al., 2007).

2.3. Cell culture

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, high glucose) supplemented with 5% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma Chemical Co., St. Louis, MO, USA). The cells were incubated at 37 °C in 5% CO_2/air.

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

The total amount of nitrite in a medium is used as an indicator of NO synthesis (Crisafulli et al., 2009). The screening test for NO production using RAW264.7 cells was described previously (Sae-Wong et al., 2009) with a slight modification. Briefly, RAW264.7 cells were cultured in DMEM, and the suspension of the cells were seeded into a 96-well microplate at 2.5×10^5 cells/100 μl/well. After 6 h, the cells were treated with various concentrations of compounds for 10 min and stimulated for 18 h with 10 μg/ml of LPS (from *E. coli*, 055: B5, Sigma). The nitrite concentration was measured from the supernatant by Griess's reaction. Inhibition (%) was calculated using the following formula and the IC_{50} was determined graphically ($N = 4$).

$$\text{Inhibition}\,(\%) = \left[\frac{(A-B)}{(A-C)}\right] \times 100$$

where $A - C$: nitrite concentration (μg/ml); *A*: LPS (+), Sample (−); B: LPS (+), Sample (+); C: LPS (−), Sample (−).

2.5. Determination of cytotoxic effects

Cytotoxicity was evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-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 4-h incubation at 37 °C, the medium was removed, and isopropanol containing 0.04 M HCl 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). 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).

2.6. Effects on TNF-α release in LPS-activated macrophages

RAW264.7 cells were seeded into the 96-well microplate at a density of 2.5×10^5 cells/100 μl/well and incubated for 6 h, and then treated with various concentrations of compounds for 10 min. LPS was added to each well (final concentration: 10 μg/ml)

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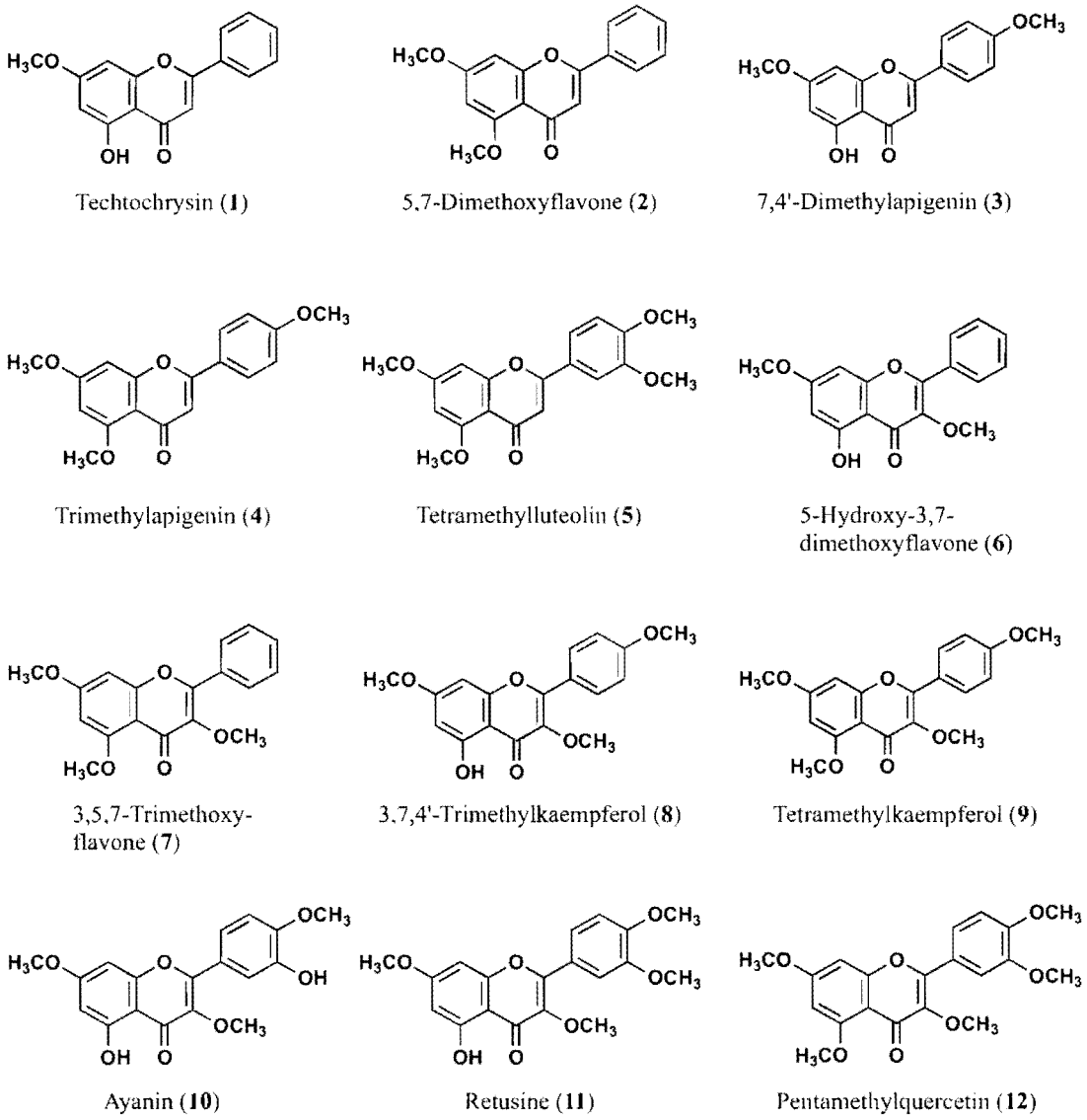

Fig. 1. Chemical structures of **1–12** isolated from the rhizomes of *Kaempferia parviflora*.

and the cells were incubated for 4 h. The supernatant (5 μl) was transferred to an ELISA plate and TNF-α levels in the culture medium were determined using a commercial kit (Mouse TNF-α ELISA kit, Invitrogen) according to the manufacturer's instructions.

2.7. Effects on expression of iNOS mRNA

RAW264.7 cells (5.0×10^6 cells/2 ml/well) were seeded into 6-well multiplates. After 6 h, the cells were incubated with the test compounds at the concentrations of 3, 10 and 30 μg/ml for 10 min

Table 1
Inhibitory effects of the ethanolic extract and fractions of *Kaempferia parviflora* rhizomes on NO production in LPS-activated RAW264.7 cells.

	Concentration of test sample (μg/ml)						IC_{50} (μg/ml)
	0	1	3	10	30	100	
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 (0.4 ± 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 (0.2 ± 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
H_2O 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.

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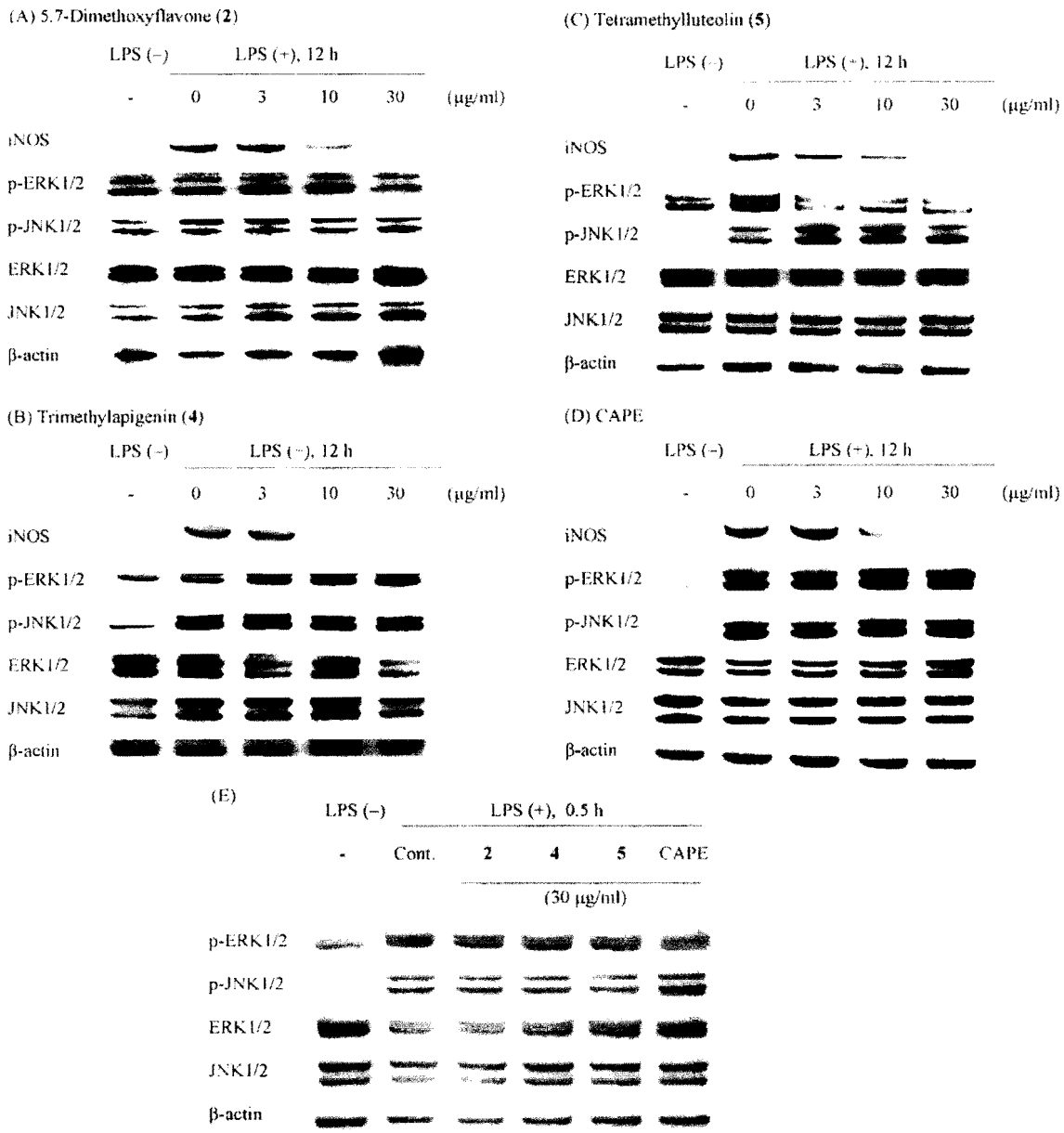

Fig. 2. (A–E) Effects of **2**, **4**, and **5** and CAPE on iNOS, p-ERK, and p-JNK protein levels.

and then with LPS (10 μg/ml) for 8 h. Total RNA was extracted from the cells using the RNeasy™ mini Kit (Qiagen) according to the manufacturer's directions. The concentration and purity of the RNA were determined by measuring absorbance at 260 nm and determining the ratio of the readings at 260 and 280 nm. Single-stranded complementary DNA (cDNA) was generated from 1 μg of total RNA using a cDNA synthesis kit (ReverTra Ace® qPCR RT Kit, TOYOBO Co. Ltd., Japan) according to the instructions. The cDNA obtained was used for the following real-time PCR.

The iNOS and β-actin genes were amplified. The β-actin gene, a constitutively expressed gene, was analyzed as an internal standard. The primers for each gene were as follows:

iNOS: forward primer; 5′-CAGCTGGGCTGTACAAACCTT-3′
reverse primer; 5′-CATTGGAAGTGAAGCGTTTCG-3′
β-actin: forward primer; 5′-AGTGGTACGACCAGAGGCATAC-3′
reverse primer; 5′-ATGGGTCAGAAGGACTCCTACG-3′

The following components were added to the PCR mixture (50 μl) for the quantitative assay: 22 μl of PCR mixture contained distilled-deionized water (DW-DI), 25 μl of THUNDERBIRD™ SYBR® qPCR mix (TOYOBO Co. Ltd.), 1 μl of the forward and reverse primers, and 1 μl of cDNA. The thermal profile was 2 min at 95 °C (pre-PCR step), and 60 cycles at 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s. Amplification was performed using a MJ Mini™ personal Thermal Cycler with a MiniOpticon™ Real-time PCR System (BIO-RAD). The analysis software was MJ Opticon Monitor™ version 3.1.

In preliminary experiments, iNOS mRNA levels were markedly increased 4–20 h after the treatment with LPS (data not shown). Therefore, effects of test compounds on the expression of iNOS mRNA were determined 8 h after the treatment with LPS.

2.8. Extraction of proteins 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 °C in a humid-

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Table 2
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)							IC_{50} (μg/ml)	IC_{50} (μM)
	0	1	3	10	30	100			
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
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**,a (76.8 + 0.0)	8.8 + 1.9**,a (79.6 + 0.0)	25.5 + 1.2**,a (78.0 ± 0.0)	73.3 ± 1.9**,a (70.6 ± 0.0)		66	183
Pentamethylquercetin (**12**)	0.0 ± 8.8	0.7 ± 0.5	−4.1 ± 0.3	9.4 ± 0.8	29.3 ± 2.3**,a (54.6 ± 0.1)	50.7 ± 4.3**,a (57.7 ± 0.1)		96	258

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

ified atmosphere containing 5% CO_2. The cells were then treated with various concentrations of the samples and stimulated for 0.5 h or 12 h with LPS (10 μg/ml). The adhered cells were collected using a cell scraper in a lysis buffer [8.4 ml of distilled water, 100 μl of protease inhibitor cocktail (Thermo Scientific), 100 μl of 22% triton X-100, phosphatase inhibitor cocktail (PhosSTP, Roche), and 1 ml of 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 2000 rpm for 2 min. Protein concentrations of cell lysates were determined using the BCA™ protein assay kit. For protein sample preparation; 100 μl of supernatant was transferred to 50 μl of a dissolving agent (0.3423 g EDTA, 6 g SDS, 3.634 g Tris, 100 ml H_2O, 8 ml glycerol and 0.03 g bromophenol blue). Then, the samples were heated in boiling water for 5 min. After cooling down, the samples were kept at −80 °C until used.

2.9. SDS-PAGE 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 the Western blot analysis, β-actin was used as an internal standard. After electrophoresis, the proteins from each experiment were transferred onto a 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 the blocking of the nonspecific sites, the membrane was soaked in Blocking One-P (for phosphorylated proteins: p-ERK, p-JNK; Nacalai Tesque, Japan) or Blocking One (for others: iNOS, ERK, JNK, β-actin) by shaking at 40 rpm for 1 h. The membrane was then rinsed with T-TBS and incubated with specific primary antibodies: p-ERK, p-JNK, ERK, JNK, iNOS and β-actin (1:1000, Cell Signaling Technology). After incubation overnight at 4 °C, the 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 membrane was shaken in T-TBS at 75 rpm for 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). In our preliminary experiments, the amount of iNOS protein markedly increased 6 h after the treatment with LPS, and levels of p-ERK and p-JNK increased after 10 min and remained high for 12 h (data not shown). Therefore, the effects of test compounds on iNOS, p-ERK, and p-JNK protein levels were determined 0.5 or 12 h after the treatment with LPS.

2.10. Effects on protein kinases

Effects on the enzyme activities of protein kinases (Table 5) involved in the expression of iNOS were examined using a SelectScreen® Kinase Profiling Service (Life Technologies Corp., Carlsbad, CA, USA) with Z′-LYTE® and Adapta® assay methods.

2.11. Statistical analysis

All data are expressed as means ± S.E.M. The data analysis was performed with a one-way analysis of variance (1-ANOVA), followed by Bonferroni's test. The p value of less than 0.05 was considered to be significant.

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Table 3
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)						IC_{50} (μg/ml)	IC_{50} (μM)
	0	1	3	10	30	100		
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), **$p < 0.01$.

3. Results and discussion

The chloroform fraction and ethanol extract of *Kaempferia parviflora* had a 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 less than 30 μg/ml (Table 1). The results are consistent with our previous findings (Sae-Wong et al., 2009) that 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**, 0.131% yield from the material), 5,7-dimethoxyflavone (**2**, 0.289%), 7,4′-dimethylapigenin (**3**, 0.0453%), trimethylapigenin (**4**, 1.29%), tetramethylluteolin (**5**, 0.0312%), 5-hydroxy-3,7-dimethoxyflavone (**6**, 0.0252%), 3,5,7-trimethoxyflavone (**7**, 0.0101%), 3,7,4′-trimethylkaempferol (**8**, 0.0719%), tetramethylkaempferol (**9**, 0.0070%), ayanin (**10**, 0.0111%), retusine (**11**, 0.0215%), and pentamethylquercetin (**12**, 0.391%) (Fig. 1).

In murine macrophage RAW264.7 cells, LPS alone induces 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 carried out to quantify the levels in the conditioned medium of RAW264.7 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. Our results showed that **2**, **4**, and **5** significantly inhibited NO production in LPS-activated RAW264.7 cells 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 2). The reference compounds, parthenolide and CAPE, showed strong activity with IC_{50} values of 0.31 and 0.92 μg/ml.

Previously (Matsuda et al., 2003), we 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 that **5** had the stronger effect inhibiting iNOS expression without affecting the enzyme activity. In the present study using RAW264.7 cells, **5** also showed a strong effect, but the additional structure–activity relationships could not be clarified.

Next, the active compounds responsible for the inhibitory effect on LPS-induced TNF-α release were examined. The results showed that **2**, **4**, and **5** moderately inhibited the release of TNF-α (Table 3).

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 (MEK1) inhibitor (PD98059) acting on the phosphorylation of ERK and an ERK inhibitor (FR180204) showed less inhibition against the production of NO; −2.4 ± 1.1% inhibition at 100 μM and 7.2 ± 2.2% inhibition at 10 μM, respectively. While a JNK inhibitor (SP600125) significantly inhibited the production of NO (74.6 ± 0.6% inhibition at 30 μM, IC_{50} = 17 μM) consistent with the previous report by Lin et al. (2009).

To clarify the mechanism of the effect of methoxyflavonoids (**2**, **4**, and **5**) on LPS-induced NO production, we examined the effect on the LPS-induced expression of iNOS mRNA and induction of iNOS, p-ERK and p-JNK proteins. The results showed that **2**, **4**, and **5** reduced both iNOS mRNA and iNOS protein levels in a concentration-dependent manner 8 and 12 h after the incubation with LPS (Table 4, Fig. 2(A)–(C)), while the inhibitory effect on the p-ERK and p-JNK proteins by **2** and **4** was marginal (Fig. 2(A) and (B)). CAPE inhibited iNOS protein expression but not p-ERK or p-JNK protein expression (Fig. 2(D)). Compound **5** reduced both iNOS and p-ERK protein levels in a dose-dependent manner at 3, 10 and 30 μg/ml, but had no inhibitory effect on p-JNK (Fig. 2(C)). However, the inhibitory effect of **5** on the phosphorylation of ERK was not observed 30 min after the treatment with LPS (Fig. 2(E)), suggesting the inhibition by **5** after treatment with LPS for 12 h to be secondary effects by other mediators. These findings including the effects of PD98059, FR180204, and SP600125 suggest that the ERK and JNK signaling pathways are not involved in the inhibition of NO production by methoxyflavonoids (**2**, **4**, and **5**), and we can conclude that the inhibitory effect on NO production occurs through iNOS protein synthesis similar to that of the reference compound CAPE, although the precise mechanisms of action including nuclear factor κB (NF-κB) should be examined further.

Finally, effects of the most potent compound, **4**, on the activities of protein kinases involved in the expression of iNOS including

Table 4
Effects of **2**, **4**, and **5** and CAPE on iNOS mRNA expression in RAW264.7 cells.

	LPS (−)	LPS (+)			
		0 (μg/ml)	3 (μg/ml)	10 (μg/ml)	30 (μg/ml)
5,7′-Dimethoxyflavone (**2**)	1.0 ± 0.1**	100.0 ± 5.1	96.5 ± 7.2**	71.3 ± 10.2**	17.7 ± 3.6**
Trimethylapigenin (**4**)	0.5 ± 0.0**	100.0 ± 10.9	142.4 ± 23.6	73.0 ± 5.4**	13.1 ± 2.6**
Tetramethylluteolin (**5**)	0.8 ± 0.1**	100.0 ± 8.7	59.7 ± 7.2**	20.6 ± 1.0**	4.2 ± 0.6**
CAPE	1.1 ± 0.0**	100.0 ± 3.0	75.9 ± 3.4**	53.1 ± 3.6**	15.2 ± 1.1**

The iNOS mRNA was normalized to β-actin, and the iNOS/β-actin ratio in the control (0 μg/ml) was expressed as 100%. Values represent mean ± S.E.M. ($N = 4$). Significantly different from the control group, **$p < 0.01$.

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Table 5
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 pp.)	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/SelectScreen-Kinase-Profiling-Service.html).
[b] The measurements were done in duplicate.

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 NF-κB, and MAPK were not inhibited markedly (less than 21% inhibition at 30 μg/ml) (Table 5).

SYK is an important component of intracellular signaling cascades. It is activated following cross-linking of Fcγ 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 NF-κB 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 lacking, but SYK is involved in the regulation of LPS-induced signaling in macrophages (Ulanova et al., 2007). Recently, several flavonoids (e.g. luteolin, apigenin) were reported to inhibit SYK (Shichijo et al., 2003). But, to the best of our knowledge, inhibition by methoxyflavonoids has not been reported.

In summary, the present study using a macrophage cell line supports the traditional use of *Kaempferia parviflora* for the treatment of inflammation including abscesses, duodenal ulcers and gout. The anti-inflammatory effect of *Kaempferia parviflora* in RAW 264.7 macrophages was mainly due to a down-regulation of iNOS protein expression.

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INHIBITION ON NITRIC OXIDE RELEASE AND INOS MRNA EXPRESSION OF METHOXYFLAVONOIDS ISOLATED FROM KAEMPFERIA PARVIFLORA RHIZOMES

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Nitric oxide (NO) is produced from nitric oxide synthase by activating macrophages defence mechanism. Excesscive of NO production causes inflammatory reaction(1). In Thailand, Kaempferia parviflora Wall. ex Baker (Zingiberaceae) rhizomes have been used for treatment of gout, arthritis, peptic ulcer and abscesses(2). The main aims of this study were therefore to isolate the active constituents from this plant and assessed their mode of action on anti-inflammatory activities. According to the in vivo study, chloroform and hexane extracts exhibited greater in decreasing of rat paw edema than ethanol extract, ethyl acetate and water extracts. The chloroform extract was isolated and 13 compounds were obtained. Isolated compounds were tested on LPS-activated NO release in RAW264.7 cells and iNOS mRNA expression. The results revealed that compounds 10, 11 and 12 significantly inhibited NO production; whereas other compounds showed moderate to mild activity. Moreover, compound 11 showed stronger activity than 10 and 12 by reducing iNOS mRNA expression. The mechanisms for anti-inflammatory activity of isolated compounds of K. parviflora are due to the inhibition of iNOS mRNA expression.

	C-3	C-5	C-7	C-3'	C-4'
1	OH	OH	OCH3	OCH3	OCH3
2	OCH3	OCH3	OCH3	H	H
3	OCH3	OCH3	OCH3	H	OCH3
4	OCH3	OH	OCH3	OCH3	OCH3
5	H	OH	OCH3	H	H
6	H	OH	OCH3	H	OCH3
7	OCH3	OH	OCH3	H	H
8	OCH3	OH	OCH3	H	OCH3
9	OCH3	OCH3	OCH3	OCH3	OCH3
10	H	OCH3	OCH3	H	H
11	H	OCH3	OCH3	OCH3	OCH3
12	H	OCH3	OCH3	H	OCH3

Keywords: Kaempferia parviflora, nitric oxide, iNOS

Acknowledgements: The Royal Golden Jubilee scholarship of Thailand(PHD/0175/2550).
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ANTI-INFLAMMATORY ACTIVITIES AND PHYTOCHEMICAL STUDY OF *KAEMPFERIA PARVIFLORA* WALL EX. BAKER

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KEYWORDS: *Kaempferia parviflora*, Carrageenan-induced rat paw edema, Inflammation

INTRODUCTION

Kaempferia parviflora Wall. ex Baker belongs to the Zingiberaceae family. Its rhizomes have been traditionally used in Thai folklore medicine for the treatment of gastric ulcer [1], gout and abscesses [2]. Therefore, it might be great values to evaluate its anti-inflammatory activity and studied on chemical constituents from the active fraction to support the use in Thai traditional medicine.

MATERIALS AND METHODS

Extraction and fractionation. Dried powder of *K. parviflora* (2 kg) was macerated with ethanol (6L × 4). The extract was then partitioned between *n*-hexane and water to obtain *n*-hexane fraction. The water layer was partitioned with $CHCl_3$ to obtain $CHCl_3$ fraction. After that, the water layer was partitioned with EtOAc to obtain EtOAc fraction and water fraction, respectively.

Carrageenan-induced rat paw edema test. 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 [3]. Briefly, male Wistar rats (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 were administered orally to rats for 30 minutes before subcutaneous injection of carrageenan (0.9% in NSS) into the plantar surface of the rat hind paw. The control group was treated with vehicle (5 ml/kg, propylene glycol: tween 80: % DMSO: water- 4:4:1:1)) 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. The inhibition of the inflammation was calculated by measuring the volume difference between the control and the tested paw.

Effects of methoxyflavnoids on nitric oxide (NO) production. The total amount of nitrite in a medium is used as an indicator of NO synthesis [4]. Briefly, RAW264.7 cells were cultured in DMEM, and the suspension of the cells were seeded into a 96-well microplate at 2.5×10^5 cells/100 µl/well. After 6 h, the cells were treated with various concentrations of compounds for 10 min and stimulated for 18 h with 10 µg/ml of LPS. The 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.

Phytochemical study. The $CHCl_3$ fraction was subsequently fractionated using silica gel column chromatography and were then subjected to preparative reversed phase HPLC.

RESULTS

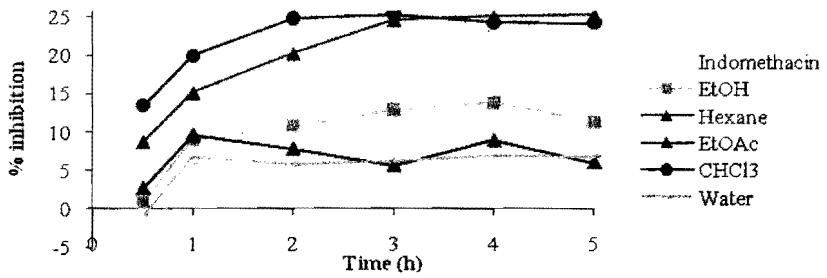

Fig. 1 Effect of ethanol extract and fractions of *K. parviflora* on carrageenan-induced rat paw edema

From the phytochemical study, 12 methoxyflavonoids were obtained from chloroform fraction to afford 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 pentamethyl quercetin (**12**) (Table 1).

Table 1. % yield and inhibition of NO production of isolated compounds (1-12) in LPS-stimulated macrophage RAW 264.7 cells

Compound	% yield	IC_{50} (μM)		Compound	% yield	IC_{50} (μM)
1	10.44	>100		**7**	2.20	193
2	7.01	18		**8**	15.71	135
3	4.70	188		**9**	1.52	>100
4	23.22	15		**10**	2.43	129
5	5.83	26		**11**	4.70	183
6	9.88	169		**12**	12.37	258

DISCUSSION

The local injection of carrageenan-induced inflammation process in the rat involves three phases by several mediators released in ordinary sequence. 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 [5]. Our result indicated that chloroform and *n*-hexane fractions showed potent anti-inflammatory activity mainly through the inhibition of PGE_2 production. Phytochemical investigation of chloroform fraction showed that trimethylapigenin (**4**) was the main compound (23.22% yield) with an IC_{50} value of 15 μM, follow by 5,7-dimethoxyflavone (**2**) and tetramethylluteolin (**5**) with IC_{50} of 18 and 26 μM, respectively. Hence, the anti-inflammation in rat paw edema may be due to the suppression of PGE_2 and NO production of these active compounds.

CONCLUSION

In this study, the chloroform fraction exhibited potent anti-inflammatory activity in reduction of rat paw edema which maybe due to the effect of trimethylapigenin, 5,7-dimethoxyflavone and tetramethylluteolin, through an inhibition of PGE_2 and NO productions.

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Inhibition of iNOS Protein Expression by Methoxyflavonoids Isolated from *Kaempferia parviflora* Rhizomes

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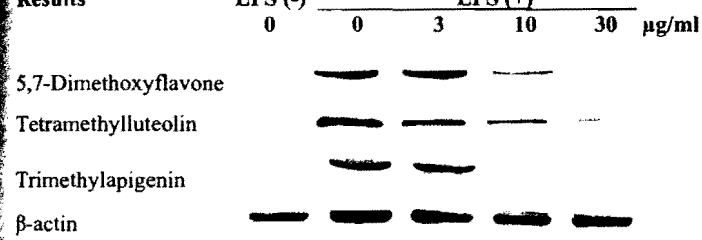
Introduction and Objective

5,7-Dimethoxyflavone, tetramethylluteolin and trimethylapigenin are active compounds isolated from chloroform fraction of *Kaempferia parviflora* rhizomes [1]. Previously, we reported that the chloroform fraction of a *Kaempferia parviflora* extract had an inhibitory effect on rat paw-edema [2]. In the present study, we investigated the isolated constituents of this fraction on anti-inflammatory mechanism through the inhibition of iNOS protein expression.

Methods

The chloroform fraction was isolated using silica gel column chromatography and High performance liquid chromatography. Isolated compounds were tested against iNOS protein expression using LPS (10 μg/ml)-activated RAW264.7 cells. Western blot technique was used to determine the expression of iNOS protein.

Results



Conclusion

The mechanism responsible for the anti-inflammatory activity of methoxyflavonoids from *Kaempferia parviflora* rhizomes may due to the inhibition of iNOS protein expression.

Keywords: iNOS, methoxyflavonoid, *Kaempferia parviflora*, western blot

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