Part II. Anti-inflammatory activity of compounds from *Kaempferia parviflora*

1. Introduction

Nitric oxide (NO) is one of the inflammatory mediators that have been implicated in a variety of pathophysiological conditions including inflammation, carcinogenesis and atherosclerosis (Mordan et al., 1993; Ohshima and Bartsch, 1994; Krönche et al., 1998). NO acts as a host defense by damaging pathogenic DNA, and as a regulatory molecule with homeostatic activities (Kou and Schroder, 1995). This free radical can combine with the superoxide anion to yield potent antimicrobial substances (Goldsby et al., 2002). However, excessive production of this free radical is pathogenic to the host tissue itself, since NO can bind with other superoxide radicals and acts as a reactive radical which directly damages the function of normal cells (Moncada et al., 1991). NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). In the NOS family, inducible NOS (iNOS) is particularly well known to be involved in the overproduction of NO in cells. Prostaglandin E_2 (PGE_2) and tumor necrosis factor-alpha (TNF-α) are also inflammatory mediators that involved in various pathophysiological processes including increased vascular permeability, vascular dilation and neutrophil chemotaxis. PGE_2 is derived from the catalyzation of arachidonic acid by cyclooxygenase-2 enzyme (COX-2). The cells that produce large amount of PGE_2 are monocytes and macrophages, whereas those for TNF-α are macrophages, mast cells and basophils. The macrophages can be activated by lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). LPS is a major component of the cell walls of Gram-negative bacteria. This antigen can activate macrophages to release some inflammatory mediators such as NO, TNF-α, PGE_2 and so on (Goldsby et al., 2002). Therefore, the inhibition of NO, PGE_2 and TNF-α production is an important therapeutic consideration in development of anti-inflammatory agents.

K. parviflora Wall. ex Baker, is one of the plants in the Zingiberaceae family, locally known in Thai as kra-chai-dam. The rhizome of this plant has been used for treatment of gout, apthous ulcer, abscesses, allergy and gastrointestinal disorders, as well as an aphrodisiac (Pengcharoen, 2002). *K. parviflora* has recently been reported to possess anti-allergic (Tewtrakul et al., 2008), antimycobacterial, antiplasmodial (Yenjai et al., 2004), anti-peptic ulcer (Rujjanawate et al., 2005) and anti-viral protease effects (Sookkongwaree et al., 2006). Moreover, it has been reported that the ethanolic extract of this plant promoted NO production in human umbilical vein endothelial cells (Wattanapitayakul et al., 2007). Since *K. parviflora* rhizomes have long been used for treatment of inflammation and possessed marked anti-NO activity, we thus investigated

the inhibitory activity of compounds isolated from this plant against NO, PGE_2 and TNF-α releases using RAW264.7 macrophage cells.

2. Materials and methods

2.1 Reagents

Lipopolysaccharide (LPS, from *Escherichia coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), L-nitroarginine (L-NA), caffeic acid phenethylester (CAPE), indomethacin and phosphate buffer saline (PBS) were purchased from Sigma Aldrich (Sigma Aldrich, Missouri, USA). Fetal calf serum (FCS) was bought from Gibco (Invitrogen, California, USA). Penicillin-streptomycin was purchased from Invitrogen (Invitrogen, California, USA). 96-Well microplates were obtained from Nunc (Nunc, Birkrød, Denmark). ELISA test kits of PGE_2 and TNF-α were from R&D systems (R&D systems, Minnesota, USA). Other chemicals were from Sigma Aldrich (Sigma Aldrich, Missouri, USA).

2.2 Plant material and preparation of extracts

The rhizomes of five selected Zingiberaceous plants including *C. mangga* Val. & Zijp., *K. galanga* Linn., *Z. officinale* Roscoe and *Z. zerumbet* (L.) Sm. were collected in June 2005 in Songkhla province, Thailand. *K. parviflora* Wall ex Baker rhizomes were bought from a Thai traditional drug store in Songkhla province, Thailand. The voucher specimens are SKP 2060313, SKP 2061107, SKP 2062615, SKP 2062616 and SKP2061116, respectively. The plant materials were identified by Assoc. Prof. Dr. Sanan Subhadhirasakul and the voucher specimens are kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

Ten grams of each dried plant was powdered and extracted successively by reflux for 3 h with 200 ml of ethanol (EtOH) and water, separately. The solvents were removed under reduced pressure to give ethanolic and water extracts, respectively. The yields of ethanolic extracts of *C. mangga*, *K. galanga*, *K. parviflora*, *Z. officinale* and *Z. zerumbet* were found to be 15.6, 17.3, 16.9, 11.4 and 8.7 %w/w, respectively; whereas the water extracts were 15.1, 15.5, 10.0, 10.4 and 24.6 %w/w, respectively. Stock solutions (10 mg/ml) of the extracts were prepared in DMSO and stored at 4 °C until use.

2.3 Isolation of compounds from *K. parviflora* extract

Two kilograms dried weight of *K. parviflora* were ground and macerated with EtOH at room temperature, 4 times (6 L, each). The EtOH 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 (EtOAc). Each partition was evaporated to dryness in vacuo to give residues of hexane (14.1 g), chloroform (215.0 g), EtOAc (4.8 g) and water fractions (27.0 g), respectively. The hexane fraction (5.0 g) which possessed the highest NO inhibitory activity (IC_{50} = 3.6 μg/ml) was chromatographed on silica gel using hexane and EtOAc (95:5 to EtOAc 100%, 8,000 ml) to afford compound **1** (5-hydroxy-3, 7-dimethoxyflavone, 370 mg, 7.4%w/w), **2** (5-hydroxy-7-methoxyflavone, 230 mg, 4.6% w/w), **3** (5-hydroxy- 3, 7, 4'-trimethoxyflavone, 280 mg, 5.6%w/w), **4** (5-hydroxy-7, 4'-dimethoxyflavone, 125 mg, 2.5%w/w), **5** (5-hydroxy-3, 7, 3', 4'-tetramethoxyflavone, 54 mg, 1.0%w/w), **6** (3, 5, 7-trimethoxyflavone, 50 mg, 1.0%w/w) and **7** (3, 5, 7, 4'-tetramethoxyflavone, 70 mg, 1.4%w/w), respectively. The structures of **1-7** were elucidated by comparing the ^{1}H and ^{13}C-NMR spectral data with those reported (Agrawal, 1989; Harborne et al., 1988; Jaipetch et al., 1983).

2.4 Assay for NO inhibitory effect from RAW264.7 cells

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota et al., 2003). Briefly, the RAW264.7 cell line (purchased from Cell Lines Services) was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/ml), streptomycin (100 μg/ml) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1 x 10^5 cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 100 μg/ml of LPS together with the test samples at various concentrations (3-100 μg/ml for crude extracts and 3-100 μM for pure compounds) and was then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the MTT colorimetric method. Briefly, after 48 h incubation with the test samples, MTT solution (10 μl, 5 mg/ml in PBS) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. L-NA (NO

synthase inhibitor), CAPE (NF-κB inhibitor) and indomethacin (non-steroidal anti-inflammatory drug, NSAID) were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1%). Inhibition (%) was calculated using the following equation and IC_{50} values were determined graphically (n = 4):

$$\text{Inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

A-C : NO_2^- concentration (μM) [*A* : LPS (+), sample (-); *B* : LPS (+), sample(+); *C* : LPS (-), sample (-)].

2.5 Inhibitory effects on LPS-induced PGE_2 and TNF-α release from RAW264.7 cells

Briefly, the RAW264.7 cell line was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/ml), streptomycin (100 μg/ml) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1.0×10^5 cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 100 μg/ml of LPS together with the test samples at various concentrations (10-100 μM) and was then incubated for 48 h. The supernatant was transferred into 96 well ELISA plate and then PGE_2 and TNF-α concentrations were determined using commercial ELISA kits. The test sample was dissolved in DMSO, and the solution was added to RPMI. The inhibition on PGE_2 and TNF-α releases was calculated and IC_{50} values were determined graphically.

2.6 Statistics

For statistical analysis, the values are expressed as mean ± S.E.M of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

3. Results and discussion

EtOH and water extracts from the rhizomes of five selected Zingiberaceous plants used for treatment of inflammation in Thai traditional medicine, including *C. mangga*, *K. galanga*, *K. parviflora*, *Z. officinale* and *Z. zerumbet* were investigated for their anti-inflammatory activities using RAW264.7 cell line. Among these, the EtOH extract of *K. parviflora* exhibited the most appreciable anti-inflammatory effect against LPS-induced NO release in RAW264.7 cells, with an IC_{50} value of 7.8 μg/ml (Table 1).

From bioassay-guided fractionation of *K. parviflora* (Table 2), the hexane fraction showed high activity against NO release with an IC_{50} value of 3.6 μg/ml, followed by $CHCl_3$ fraction (IC_{50} = 8.8 μg/ml), EtOAc- and water fractions (IC_{50} > 100 μg/ml), respectively. The hexane fraction was then chromatograpged further to obtain seven methoxyflavones (Figure 1) and were tested for their NO inhibitory effects (Table 3A). The result indicated that compound **5** (5-hydroxy-3,7,3′,4′-tetramethoxyflavone) exhibited the highest activity against NO release with an IC_{50} value of 16.1 μM, followed by **4** (IC_{50} = 24.5 μM) and **3** (IC_{50} = 30.6 μM). These three compounds exhibited higher effect than L-NA, a positive control (NO synthase inhibitor, IC_{50} = 61.8 μM). Moreover, compounds **5** (IC_{50} = 16.1 μM) and **2** (IC_{50} = 24.5 μM) conferred higher effect than that of indomethacin (IC_{50} = 25.0 μM), a clinical used drug. Compound **5** was also tested on LPS-induced PGE_2 and TNF-α releases from RAW264.7 cells (Table 3B). It was revealed that **5** again exhibited appreciable inhibitory effect on PGE_2 release (IC_{50} = 16.3 μM), but inactive on the release of TNF-α (IC_{50} > 100 μM).

The structure-activity trends of *K. parviflora* upon NO inhibition could be summarized as follow: 1) 3,7,3′,4′-tetramethoxyflavone was essential for NO inhibitory activity; and 2) 4′-methoxyl group on B-ring increased the activity as shown in **4** (IC_{50} = 24.5 μM) versus **2** (IC_{50} = 64.3 μM). The result of the present study are concurrent with the previous report that the methoxylation at position 3 or 4′ enhanced the activity. It has also been reported that some active flavonoids suppressed the iNOS induction in a dose-dependent manner (Matsuda et al., 2003). Nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone), which was purified from the fruit peel of *Citrus sunki* at concentration 6-50 μM significantly suppressed NF-κB transcriptional activation, NO and PGE_2 production in LPS-activated RAW 264.7 cells. This result revealed that the methoxyl flavones may exert anti-inflammatory effect (Choi et al., 2007).

In conclusion, the present study may support the use in Thai traditional medicine of *K. parviflora* for treatment of inflammatory-related diseases through the inhibition of NO and PGE_2 releases, but partly due to that of TNF-α. It is 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* will be further investigated.

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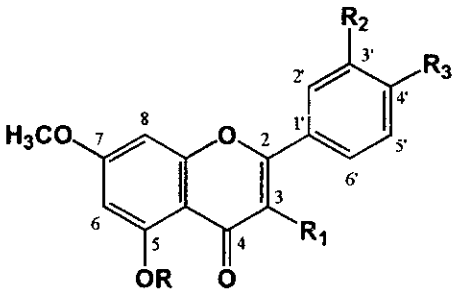

1: R, R_2, R_3 = H; R_1 = OCH_3
2: R, R_1, R_2, R_3 = H
3: R, R_2 = H; R_1, R_3 = OCH_3
4: R, R_1, R_2 = H; R_3 = OCH_3
5: R = H; R_1, R_2, R_3 = OCH_3
6: R = CH_3; R_1 = OCH_3; R_2, R_3 = H
7: R = CH_3; R_1, R_3 = OCH_3; R_2 =H

Figure 1. Structures of compounds **1**-**7** isolated from *K. parviflora* rhizomes

Table 1. Inhibition on NO production in RAW264.7 cells[a] of some selected plants in the Zingiberceae family

Plant species	% Inhibition at various concentrations (μg/ml)					IC_{50} (μg/ml)
	0	3	10	30	100	
Curcuma mangga (EtOH)	0.0 ± 3.5	-12.0 ± 3.7	40.3 ± 5.7**	93.6 ± 3.7**	99.8 ± 1.4[b]**	11.8
C. mangga (Water)	0.0 ± 6.9	-	24.2 ± 4.2*	31.4 ± 5.9**	87.6 ± 1.4**	49.7
Kaempferia galanga (EtOH)	0.0 ± 3.5	-14.1 ± 3.9	16.1 ± 9.3	67.5 ± 1.4**	97.3 ± 2.3**	22.2
K. galanga (Water)	0.0 ± 6.9	-	33.7 ± 4.4**	41.9 ± 6.1**	59.6 ± 7.5**	48.0
Kaempferia parviflora (EtOH)	0.0 ± 3.5	-12.3 ± 6.5	92.4 ± 7.9**	93.5 ± 5.3**	100.3 ± 3.4[b]**	7.8
K. parviflora (Water)	0.0 ± 6.9	-	23.1 ± 7.2	32.9 ± 10.3*	90.4 ± 2.8**	48.2
Zingiber officinale (EtOH)	0.0 ± 3.5	-21.2 ± 7.2	32.9 ± 11.3*	95.7 ± 2.4**	101.9 ± 2.7[b]**	12.7
Z. officinale (Water)	0.0 ± 6.9	-	-7.9 ± 13.1	32.1 ± 6.2**	42.2 ± 4.2**	>100
Zingiber zerumbet (EtOH)	0.0 ± 3.5	-14.2 ± 13.8	25.6 ± 10.7*	101.9 ± 5.8**	101.6 ± 3.4[b]**	12.3
Z. zerumbet (Water)	0.0 ± 6.9	-	39.5 ± 6.1**	57.6 ± 2.8**	88.6 ± 9.9**	17.8

[a]Each value represents mean ± S.E.M. of four determinations

Statistical significance, *$p< 0.05$; ** $p< 0.01$

[b] Cytotoxic effect was observed.

Table 2. Inhibition on NO production of EtOH extract and fractions from *K. parviflora* rhizomes[a]

Fractions	% Inhibition at various concentrations (μg/ml)					IC_{50} (μg/ml)
	0	3	10	30	100	
Kaempferia parviflora (EtOH)	0.0 ± 3.5	12.3 ± 6.5	62.4 ± 7.5**	93.5 ± 5.3**	100.3 ± 3.4[b]**	7.8
K. parviflora (Hexane fr.)	0.0 ± 4.1	35.0 ± 2.1*	83.0 ± 2.0**	91.5 ± 1.7**	100.0 ± 0.1[b]**	3.6
K. parviflora ($CHCl_3$ fr.)	0.0 ± 4.1	18.1 ± 3.2	53.0 ± 2.4**	93.2 ± 1.4**	103.7 ± 1.5[b]**	8.8
K. parviflora (EtOAc fr.)	0.0 ± 3.5	-	2.6 ± 1.1	2.2 ± 0.8	6.8 ± 1.8	> 100
K. parviflora (Water fr.)	0.0 ± 3.5	-	0.9 ± 1.1	10.2 ± 1.1	34.4 ± 2.4**	>100

[a]Each value represents mean ± S.E.M. of four determinations

Statistical significance, *$p< 0.05$; ** $p< 0.01$

[b] Cytotoxic effect was observed.

Table 3. Inhibitory effects on NO production[a] of compounds isolated from *K. parviflora* rhizomes (A) and inhibition on PGE_2 and TNF-α releases of compound **5** (B)

A

Compound	% Inhibition at various concentrations					IC_{50} (μM)
	0	3	10	30	100	
1	0.0 ± 6.0	-	38.8 ± 1.8**	45.9 ± 3.7**	70.6 ± 1.9**	41.6
2	0.0 ± 6.0	-	7.9 ± 2.2	23.8 ± 3.2*	79.9 ± 2.1**	64.3
3	0.0 ± 6.0	-	21.7 ± 3.4	50.9 ± 3.4**	78.5 ± 1.7**	30.6
4	0.0 ± 3.7	-	29.4 ± 2.8**	49.5 ± 2.7**	89.8 ± 1.2[b]**	24.5
5	0.0 ± 3.7	-	28.3 ± 3.9**	64.3 ± 3.6**	92.3 ± 0.4[b]**	16.1
6	0.0 ± 3.7	-	-	1.3 ± 2.8	59.7 ± 1.9[b]**	81.8
7	0.0 ± 6.0	-	-	2.5 ± 1.2	46.8 ± 1.9[b]**	>100
L-NA	0.0 ± 9.9	11.7 ± 4.6	20.2 ± 5.9	34.7 ± 1.8 *	71.6 ± 2.6**	61.8
CAPE	0.0 ± 9.9	30.7 ± 3.2	68.6 ± 3.4[b]**	98.7 ± 1.2[b]**	98.9 ± 2.1[b]**	5.6
Indomethacin	0.0 ± 3.6	14.5 ± 2.7	30.2 ± 1.6**	47.6 ± 2.3**	80.3 ± 1.5**	25.0

B

Inflammatory-mediator	% Inhibition at various concentrations (μM) of compound **5**					IC_{50} (μM)
	0	3	10	30	100	
PGE_2	0.0 ± 5.5	-	45.8 ± 1.0**	55.8 ± 0.2**	60.7 ± 0.8**	16.3
TNF-α	0.0 ± 3.2	-	3.1 ± 2.0	14.0 ± 1.7	21.8 ± 0.9**	>100

[a]Each value represents mean ± S.E.M. of four determinations

Statistical significance, * $p<0.05$, ** $p<0.01$

[b]Cytotoxic effect was observed.