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Anti-inflammatory effects of compounds from *Kaempferia parviflora* and *Boesenbergia pandurata*

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ABSTRACT

Kaempferia parviflora and *Boesenbergia pandurata* are perennial herbs in the Zingiberaceae family. The rhizomes of these two plants have been used as food ingredients and in Thai traditional medicine for treatment of several inflammatory-related diseases, such as gout, allergy, apthous ulcer and peptic ulcer. The compounds isolated from the rhizomes of *K. parviflora* and *B. pandurata* were, therefore, examined for their inhibitory activities against nitric oxide (NO) production. For *K. parviflora*, compound **5** (5-hydroxy-3,7,3′,4′-tetramethoxyflavone) exhibited the highest activity against the NO inhibitory effect, with an IC_{50} value of 16.1 μM, followed by **4** (IC_{50} = 24.5 μM) and **3** (IC_{50} = 30.6 μM). Regarding the NO inhibitory activity of *B. pandurata*, compound **2** (panduratin A) displayed the most potent effect with an IC_{50} value of 5.3 μM, followed by **3** (hydroxypanduratin A, IC_{50} = 13.3 μM) and **7** (cardamonin, IC_{50} = 24.7 μM), respectively. The 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**5**), panduratin A (**2**) and hydroxypanduratin A (**3**), were also tested on prostaglandin E_2 (PGE_2) and tumour necrosis factor-alpha (TNF-α) production. 5-Hydroxy-3,7,3′,4′-tetramethoxyflavone (**5**) exhibited a potent inhibitory effect on PGE_2 production (IC_{50} = 16.3 μM), but a mild effect on TNF-α (IC_{50} > 100 μM). Panduratin A and hydroxypanduratin A showed strong activity against PGE_2 with IC_{50} values of 10.5 and 12.3 μM, respectively, and a moderate effect on TNF-α (IC_{50} = 60.3 and 57.3 μM, respectively). This study indicated that compound **5** (5-hydroxy-3,7,3′,4′-tetramethoxyflavone) is responsible for anti-inflammatory activity of *K. parviflora*, while **2** (panduratin A) and **3** (hydroxypanduratin A), the prenylated chalcones, are responsible for that of *B. pandurata*.

The present study, therefore, supports the traditional use of *K. parviflora* and *B. pandurata* rhizomes for treatment of inflammatory-related diseases through the inhibition of NO, PGE_2 and TNF-α release.

1. Introduction

Kaempferia parviflora Wall. ex Baker is one of the plants in the Zingiberaceae family, locally known in Thai as Kra-chai-dam. The rhizome of this plant has been used for treatment of gout, allergy and gastrointestinal disorders, as well as an aphrodisiac (Pengcharoen, 2002) and it is well-known as Thai ginseng. *Boesenbergia pandurata* Holtt is locally known, in Thai, as Kra-chai, which also belongs to the Zingiberaceae family. The fresh rhizomes have a characteristic aroma and a slightly pungent taste. It is commonly used in southeast Asia as a food ingredient and a folk medicine for the treatment of several diseases, such as aphthous ulcer, dry mouth, stomach discomfort, leukorrhea and dysentery (Saralamp, Chuakul, Temsirirkkul, & Clayton, 1996).

Nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs. This inorganic free radical has been implicated in physiological and pathological processes, such as vasodilation, non-specific host defence and acute or chronic inflammation. NO acts as a host defence by damaging pathogenic DNA, and as a regulatory molecule with homeostatic activities (Kou & Schroder, 1995). 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, Palmer, & Higgs, 1991). Prostaglandin E_2 (PGE_2) and tumour necrosis factor-alpha (TNF-α) are also inflammatory mediators that are involved in various pathophysiological processes including inflammation and carcinogenesis (Yun, Kwon, & Hwang, 2003). Therefore, the inhibition of NO, PGE_2 and TNF-α production is an important therapeutic consideration in development of anti-inflammatory agents.

K. parviflora and *B. pandurata* rhizomes have long been used for treatment of inflammation (Pengcharoen, 2002; Wutthithamavet,

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1997) and were previously thought to be the same species. Moreover, their extracts possessed potent anti-NO activity in our preliminary study (IC_{50} < 20 μg/ml). We, therefore, investigated the inhibitory activity of compounds isolated from these two plants against NO, PGE_2 and TNF-α production.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS, from *Salmonella enteritidis*), 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) and phosphate-buffered saline (PBS) were purchased from Sigma. Foetal calf serum (FCS) was bought from Gibco. Penicillin–streptomycin was purchased from Invitrogen. 96-Well microplates were obtained from Nunc. ELISA test kits of PGE_2 and TNF-α were from R&D systems, USA. Other chemicals were from Sigma.

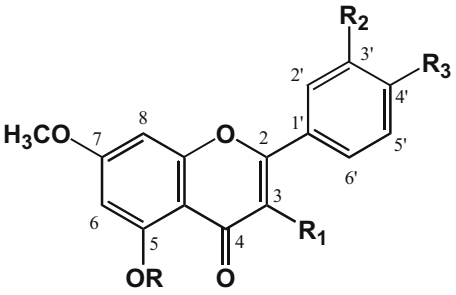
2.2. Plant material and preparation of extracts

K. parviflora rhizomes were bought from a Thai traditional drug store in Songkhla Province, Thailand. The voucher specimen is SKP 2061116. The fresh rhizomes of *B. pandurata* Holtt. were bought from the Hat-Yai market, Hat-Yai, Thailand and the voucher specimen was number: SN 4412015. Both plant materials were identified by Assoc. Prof. Dr. Sanan Subhadhirasakul and kept in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

2.3. Isolation of compounds from K. parviflora and B. pandurata extracts

Two kilogrammes (dried weight) of *K. parviflora* were ground and macerated with ethanol four times (6 l × 4) at room temperature. The ethanolic (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). The hexane fraction (5.0 g) which possessed a potent NO inhibitory effect was chromatographed on silica gel, using hexane and EtOAc (95:5 to EtOAc 100%, 8000 ml), to afford compounds **1** (5-hydroxy-3,7-dimethoxyflavone, 370 mg), **2** (5-hydroxy-7-methoxyflavone, 230 mg), **3** (5-hydroxy-3,7,4′-trimethoxyflavone, 280 mg), **4** (5-hydroxy-7,4′-dimethoxyflavone, 125 mg), **5** (5-hydroxy-3,7,3′,4′-tetramethoxyflavone, 54 mg), **6** (3,5,7-trimethoxyflavone, 50 mg) and **7** (3,5,7,4′-tetramethoxyflavone, 70 mg). The structures of **1**–**7** were elucidated by comparing the ^1H and ^{13}C NMR spectral data with those reported (Agrawal, 1989; Harborne, Agrawal, & Bansal, 1988; Jaipetch, Reutrakul, Tuntiwachwuttikul, & Santisuk, 1983) (Figs. 1 and 2).

Chopped-dried rhizomes (10.0 kg) of *B. pandurata* were extracted with $CHCl_3$ and MeOH (30 l × 3, seven days each) at room temperature and the solvent was evaporated under reduced pressure to afford the $CHCl_3$ (608.40 g) and MeOH (211.70 g) extracts. A part of the MeOH extract (140 g) which possessed marked NO inhibitory effect was further subjected to quick column chromatography (QCC) on silica gel (200 g), eluting with hexane–CH_2Cl_2–MeOH (9:1:0, 1:1:0, 0:100:0, 0:19:1, 0:17:1, 0:1:1, 0:0:100, each 1500 ml) to yield seven fractions (F1–F7). Fraction F2 (hexane–CH_2Cl_2, 1:1, 18.7 g) was chromatographed on silica gel (180 g) by QCC, eluting with hexane–CH_2Cl_2 (1:1, 2000 ml) to give three subfractions (F2a–F2c). Subfraction F2c (1.03 g) was recrystallised from CH_2Cl_2 to give **2** (715.2 mg). Fraction F3 (CH_2Cl_2–MeOH, 19:1, 300 mg) was separated by CC on silica gel (18 g) with CH_2Cl_2–MeOH (19:1, 1000 ml) to afford four subfractions (F3a–F3d). Subfraction F3b (10.3 mg) was purified by preparative TLC with hexane–EtOAc (3:2) to obtain **4** (8.3 mg). Subfraction F3c (130.0 mg) was separated by CC on silica gel (8 g) with hexane–EtOAc (13:7, 400 ml) to give **3** (36.6 mg) and **1** (6.2 mg). Fraction F4 (CH_2Cl_2–MeOH, 17:1, 1.2 g) was purified by CC on silica gel (60 g) and eluted with hexane–EtOAc (13:7, 1500 ml) to give four subfractions (F4a–F4d). Subfraction F4c (49.3 mg) was purified by reversed-phase preparative TLC with MeOH–H_2O (3:1) to afford **5** (25.2 mg). Subfraction F4d (898.0 mg) was subjected to CC on silica gel (60 g) with hexane–EtOAc (13:7, 1000 ml), to give **6** (21.0 mg). The structures of **1**–**6** were elucidated by comparing the spectral data with those reported (Hufford & Oguntimein, 1980; Tanaka, Ichino, & Ito, 1984; Tuchinda et al., 2002; Tuntiwachwuttikul, Pancharoen, Reutrakul, & Byrne, 1984; Van Puyvelde et al., 1989). Compound **7** (cardamonin) was previously isolated by our group (Tewtrakul, Subhadhirasakul, Puripattanavong, & Panphadung, 2003).



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

Fig. 1. Structures of compounds **1**–**7** isolated from *Kaempferia parviflora* rhizomes.

2.4. Assay for NO inhibitory effect using RAW264.7 cells

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a method modified from that previously reported (Banskota et al., 2003). Briefly, the RAW264.7 cell line [purchased from cell lines service (CLS)] 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 1×10^5 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 200 μg/ml of LPS, together with the test samples at various concentrations, 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 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric method. Briefly, after 48 h of incubation with the test samples, MTT solution (10 μl,

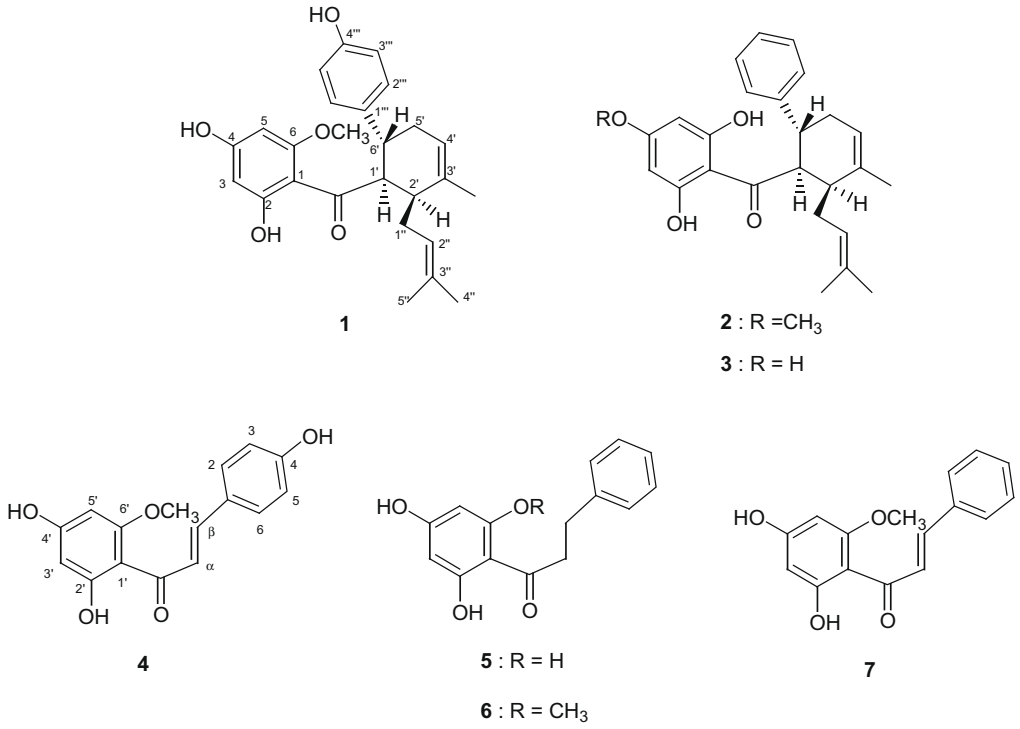


Fig. 2. Structures of compounds **1**–**7** isolated from *Boesenbergia pandurata* rhizomes.

5 mg/ml in PBS) was added to the wells. After 4 h of 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 and caffeic acid phenethylester (CAPE) 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 (+); and *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 [purchased from cell lines service (CLS)] 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 200 μg/ml of LPS, together with the test samples at various concentrations, and was then incubated for 48 h. The supernatant was transferred into a 96-well ELISA plate and then PGE_2 and TNF-α concentrations were determined using commercial ELISA kits. The test samples were dissolved in DMSO, and the solution was added to RPMI. The inhibitions of PGE_2 and TNF-α production were calculated and IC_{50} values were determined graphically.

2.6. Statistics

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

3. Results and discussion

The compounds isolated from the rhizomes of *K. parviflora* and *B. pandurata* were examined for their inhibitory activities against nitric oxide (NO) production. For *K. parviflora*, compound **5** (5-hydroxy-3,7,3′,4′-tetramethoxyflavone) exhibited the highest activity against NO inhibitory effect with an IC_{50} value of 16.1 μM, followed by **4** (IC_{50} = 24.5 μM) and **3** (IC_{50} = 30.6 μM), whereas other compounds possessed moderate or weak activity. The NO inhibition

Table 1
Inhibition of NO production of compounds isolated from *K. parviflora* rhizomes on LPS-induced NO release from RAW264.7 cells.

Compounds	IC_{50} (μM)
5-Hydroxy-3,7-dimethoxyflavone (**1**)	41.6
5-Hydroxy-7-methoxyflavone (**2**)	64.3
5-Hydroxy-3,7,4′-trimethoxyflavone (**3**)	30.6
5-Hydroxy-7,4′-dimethoxyflavone (**4**)	24.5
5-Hydroxy-3,7,3′,4′-tetramethoxyflavone (**5**)	16.1
3,5,7-Trimethoxyflavone (**6**)	88.5
3,5,7,4′-Tetramethoxyflavone (**7**)	>100
L-Nitroarginine (L-NA)	61.8
Caffeic acid phenethylester (CAPE)	5.6

Table 2
Inhibition of NO production of compounds isolated from *B. pandurata* rhizomes on LPS-induced NO release from RAW264.7 cells.

Compounds	IC_{50} (μM)
Panduratin C (**1**)	67.8
Panduratin A (**2**)	5.3
Hydroxypanduratin A (**3**)	13.3
Helichrysetin (**4**)	62.3
2′,4′,6′-Trihydroxyhydrochalcone (**5**)	96.2
Uvangoletin (**6**)	74.7
Cardamonin (**7**)	24.7
L-Nitroarginine (L-NA)	61.8
Caffeic acid phenethylester (CAPE)	5.6

activity of compound **5** (IC_{50} = 16.1 μM) was three times weaker than that of caffeic acid phenethylester (CAPE, IC_{50} = 5.6 μM), an NF-κB inhibitor, but four times higher than that of L-nitroarginine (L-NA, IC_{50} = 61.8 μM), a nitric oxide synthase inhibitor (Table 1). *K. parviflora* has been reported to have antimycobacterial, anti-peptic ulcer (Rujjanawate, Kanjanapothi, Amornlerdpison, & Pojanagaroon, 2005), anti-viral protease (Sookkongwaree, Geitmann, Roengsumran, Petsom, & Danielson, 2006) and anti-allergic activities (Tewtrakul, Subhadhirasakul, & Kummee, 2007). However, anti-NO production of compounds isolated from this plant has not so far been studied.

Regarding NO inhibitory activity of *B. pandurata*, compound **2** (panduratin A) displayed the most potent effect against NO production, with an IC_{50} value of 5.3 μM, followed by **3** (hydroxypanduratin A, IC_{50} = 13.3 μM) and **7** (cardamonin, IC_{50} = 24.7 μM), whereas other compounds showed moderate or mild effects (62.3–74.7 μM). The NO inhibition activity of compound **2** (IC_{50} = 5.3 μM) was comparable to that of CAPE (IC_{50} = 5.6 μM) and very much higher than that of L-NA (IC_{50} = 61.8 μM) (Table 2).

The 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**5**) isolated from *K. parviflora* and also panduratin A (**2**) and hydroxypanduratin A (**3**) from *B. pandurata* were tested on PGE_2 and TNF-α production (Table 3). 5-Hydroxy-3,7,3′,4′-tetramethoxyflavone (**5**) exhibited a potent inhibitory effect on PGE_2 production (IC_{50} = 16.3 μM), but a mild effect on TNF-α (IC_{50} > 100 μM). Panduratin A and hydroxypanduratin A showed strong activities against PGE_2 release, with IC_{50} values of 10.5 and 12.3 μM, respectively, and moderate effects on TNF-α (IC_{50} = 60.3 and 57.3 μM, respectively). Panduratin A (**2**) and hydroxypanduratin A (**3**), isolated from *B. pandurata*, have been reported to show a topical anti-inflammatory activity on TPA-induced ear oedema in rats (Tuchinda et al., 2002). Panduratin A was previously reported to show inhibitory effect on NO production in RAW264.7 cells through the inhibition of inducible nitric oxide synthase (iNOS) expression (Yun et al., 2003). However, the anti-inflammatory activity and structure–activity trends of compounds isolated from this plant have not so far been reported.

Table 3
Inhibition of PGE_2 and TNF-α production of 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**5**) isolated from *K. parviflora* rhizomes and panduratin C (**1**), panduratin A (**2**) and hydroxypanduratin A (**3**) isolated from *B. pandurata* rhizomes on LPS-induced PGE_2 and TNF-α releases from RAW264.7 cells.

Compounds	IC_{50} (μM)	
	PGE_2	TNF-α
5-Hydroxy-3,7,3′,4′-tetramethoxyflavone (**5**)	16.3	>100
Panduratin C (**1**)	>100	>100
Panduratin A (**2**)	10.5	60.3
Hydroxypanduratin A (**3**)	12.3	57.3

The structure–activity trends of *K. parviflora* upon NO inhibition could be summarised as follows: (1) 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); and (2) vicinal methoxylation at positions 3′ and 4′ conferred higher activity, as observed in **5** (IC_{50} = 16.1 μM) versus **3** (IC_{50} = 30.6 μM).

Structure–activity trends of *B. pandurata* against NO production were found in that: (1) prenylation of chalcones is essential for the activity, as observed in **2** (IC_{50} = 5.3 μM) and **3** (IC_{50} = 13.3 μM) versus **4**–**7** (IC_{50} = 24.7–96.2 μM); (2) methoxylation at position 4 conferred a greater effect than did hydroxylation, as seen in **2** (IC_{50} = 5.3 μM) versus **3** (IC_{50} = 13.3 μM); (3) hydroxylation at position 4″ reduced activity, as shown in **1** (IC_{50} = 67.8 μM) versus **2** (IC_{50} = 5.3 μM) and **3** (IC_{50} = 13.3 μM); and (4) introduction of the α,β-double bond increased activity, as observed in **7** (IC_{50} = 24.7 μM) versus **6** (IC_{50} = 74.7 μM).

In conclusion, compound **5** (5-hydroxy-3,7,3′,4′-tetramethoxyflavone) is responsible for the anti-inflammatory activity of *K. parviflora*, while **2** (panduratin A) and **3** (hydroxypanduratin A), the prenylated chalcones, are responsible for that of *B. pandurata*. The present study therefore supports the traditional use of *K. parviflora* and *B. pandurata* rhizomes for treatment of inflammatory-related diseases through the inhibition of NO, PGE_2 and TNF-α releases.

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