Appendix

PHYTOTHERAPY RESEARCH
Phytother. Res. **21**, 1092–1095 (2007)
Published online 15 August 2007 in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.2252

HIV-1 Protease and HIV-1 Integrase Inhibitory Substances from *Eclipta prostrata*

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The bioassay-guided fractionation for anti-HIV-1 integrase activity led to the isolation of six compounds from the whole plant extract of *Eclipta prostrata* extract. They were identified as 5-hydroxymethyl-(2,2′:5′,2″)-terthienyl tiglate (1), 5-hydroxymethyl-(2,2′:5′,2″)-terthienyl agelate (2), 5-hydroxymethyl-(2,2′:5′,2″)-terthienyl acetate (3), ecliptal (4), orobol (5) and wedelolactone (6). Of these, compound 6 showed the highest activity against HIV-1 integrase (IN) with an IC_{50} value of 4.0 ± 0.2 μM, followed by compound 5 (IC_{50} = 8.1 ± 0.5 μM), whereas the four terthiophene compounds (1–4) were inactive (IC_{50} > 100 μM). Regarding HIV-1 protease (PR) inhibitory activity, compound 1 exhibited appreciable activity against HIV-1 PR with an IC_{50} of 58.3 ± 0.8 μM, followed by compound 4 (IC_{50} = 83.3 ± 1.6 μM) and compound 3 (IC_{50} = 93.7 ± 0.8 μM), while compounds 2, 5 and 6 were inactive against HIV-1 PR (IC_{50} > 100 μM). This is the first report of anti-HIV-1 IN activities for wedelolactone (6), a coumarin derivative, and orobol (5), an isoflavone derivative. This study supports the use of *E. prostrata* in AIDS patients, which is in accord with its traditional use by Thai traditional doctors for curing blood related diseases. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: HIV-1 protease; HIV-1 integrase; inhibitory activities; *Eclipta prostrata*.

INTRODUCTION

Eclipta prostrata Linn. (syn: *E. alba* Hassk., *E. erecta* Hassk.) is a plant in the Compositae family. It is a perennial herb that grows widely throughout tropical areas especially in Asia. In Thai traditional medicine, the leaf of this plant has been used for hair dying and the treatment of skin diseases. The stem has been used as a blood tonic and for treatment of anaemia, tuberculosis, amoebiasis and asthma, whereas the root has been used as antibacterial agent, hepatoprotectant and tonic (Tungtrongjit, 1978; Wutthithamavet, 1997). It has been reported that *Eclipta prostrata* exhibits an immunomodulatory effect on T-lymphocytes (Liu *et al.*, 2001), antiinflammatory (Kobori *et al.*, 2004), antimicrobial (Wiart *et al.*, 2004) and hepatoprotective activities (Han *et al.*, 1998).

AIDS has been a major problem in Thailand since the late 1980s (58 000 deaths/year, 21 000 new HIV positives reported/year and 570 000 total HIV-positive patients to date). Specific drug treatment is expensive, and only a very small number of AIDS patients have access to the cocktail of modern antiviral agents. Therefore the majority of AIDS patients resort to using Thai traditional doctors, who prescribe a range of plant-based products. However, scientific studies supporting this use (efficacy, specificity, toxicity) have in most cases not yet been carried out.

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Contract/grant sponsor: Thailand Research Fund; Royal Thai Government Budget.

A preliminary screening of Thai medicinal plants that are used as self-medication by AIDS patients revealed that the extract of *E. prostrata* exhibited high inhibitory activity against HIV-1 IN with an IC_{50} of 21.1 μg/mL (Tewtrakul *et al.*, 2006). This study therefore reports the isolation of active principles from *E. prostrata* and their HIV-1 PR and HIV-1 IN inhibitory activities.

MATERIALS AND METHODS

Plant material. Whole plants of *Eclipta prostrata* were collected at the botanical garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand and identified by Associate Professor Dr Sanan Subhadhirasakul. A voucher specimen (No. SN 4412025) is deposited at Southern Center of Traditional Medicine at the Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Extraction and isolation. The dried whole plants (250.0 g) of *E. prostrata* were extracted sequentially with CH_2Cl_2 and MeOH (2 L × 2, 5 days with each solvent) at room temperature. The extracts were filtered and concentrated under reduced pressure to afford CH_2Cl_2 and MeOH crude extracts.

The CH_2Cl_2 extract (1.95 g) was subjected to quick column chromatography (QCC) over silica gel and eluted with a gradient of hexane: ethyl acetate (EtOAc) and EtOAc: MeOH to give four fractions (D1–D4). Fraction D2 (441.4 mg) was further purified by column chromatography (CC) with 5% EtOAc: hexane to yield **1** (10.0 mg), **2** (2.3 mg) and **3** (2.6 mg). Fraction D3 was

Received 19 March 2006
Accepted 17 May 2007

separated by CC with 30% CH_2Cl_2: hexane to afford **4** (4.6 mg).

The MeOH extract (1.0 g) was fractionated by CC with hexane and the polarity increased with CH_2Cl_2 and MeOH, respectively, to afford four fractions (M1–M4). Fraction M3 (12.5 mg) was further purified by preparative thin layer chromatography (preparative silica TLC) with 5% MeOH: 95% CH_2Cl_2 to give **5** (5.4 mg). Fraction M4 (10.0 mg) was subjected to preparative silica TLC with 5% MeOH : 95% CH_2Cl_2 to afford **6** (6.2 mg). These compounds were identified by comparison of their spectroscopic data with those reported in the literature (Das and Chakravarty, 1991; Jain and Sigh, 1988; Sashida *et al.*, 1983; Kosuge *et al.*, 1985).

Enzymes and chemicals. Recombinant HIV-1 PR, substrate peptides and acetyl pepstatin, were purchased from Sigma Chemical Co., St Louis, USA.

Recombinant HIV-1 IN was expressed in *Escherichia coli*, purified according to the method described in a previous publication (Jenkins *et al.*, 1996), except that after removal of the His-tag with thrombin, chromatography on a Sephadex 200 column was the final purification step. The integrase enzyme was stored at −80 °C until use.

Assay of HIV-1 PR inhibitory activity. This assay was modified from the previously reported method (Tewtrakul *et al.*, 2003). In brief, the recombinant HIV-1 PR solution was diluted with a buffer composed of a solution containing 50 mM of sodium acetate (pH 5.0), 1 mM ethylenediamine disodium (EDTA.2Na) and 2 mM 2-mercaptoethanol (2-ME) and mixed with glycerol in a ratio of 3: 1. The substrate peptide, Arg-Val-Nle-(*p*NO_2-Phe)-Glu-Ala-Nle-NH_2, was diluted with a buffer solution of 50 mM sodium acetate (pH 5.0). Two microlitres of plant extract and 4 μL of HIV-1 PR solution (0.025 mg/mL) were added to a solution containing 2 μL of 50 mM buffer solution (pH 5.0) and 2 μL of substrate solution (2 mg/mL), and the reaction mixture (10 μL) was incubated at 37 °C for 1 h. A control reaction was performed under the same conditions but without the plant extract. The reaction was stopped by heating the reaction mixture at 90 °C for 1 min. Subsequently, 20 μL of sterile water was added and an aliquot of 10 μL was analysed by HPLC using RP-18 column (4.6 × 150 mm i.d., Supelco 516 C-18-DB 5 μm, USA). Ten microlitres of the reaction mixture was injected to the column and gradiently eluted with acetonitrile (15–40%) and 0.2% trifluoroacetic acid (TFA) in water, at a flow rate of 1.0 mL/min. The elution profile was monitored at 280 nm. The retention times of the substrate and *p*-NO_2-Phe-bearing hydrolysate were 11.33 and 9.47 min, respectively. The inhibitory activity on HIV-1 PR was calculated as follows:

$$\% \text{ inhibition } (A_{\text{control}} - A_{\text{sample}}) \times 100/A_{\text{control}}$$

where A is a relative peak area of the product hydrolysate. Acetyl pepstatin was used as a positive control.

Assay for HIV-1 IN inhibitory activity. Oligonucleotides of long terminal repeat donor DNA (LTR-D) and target substrate (TS) DNA were purchased from QIAGEN Operon, USA and stored at −25 °C before use. The sequence of biotinylated LTR donor DNA and its unlabelled complement were 5′-biotin-ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3′ (LTR-D1) and 3′-GAAAATCAGTCACACCTTTTAGAGATCGTCA-5′ (LTR-D2), respectively. Those of the target substrate DNA (digoxigenin-labelled target DNA, TS-1) and its 3′-labelled complement were 5′-TGACCAAGGGCTAATTCACT-digoxigenin and digoxigenin-ACTGGTTCCCGATTAAGTGA-5′ (TS-2), respectively.

Multiplate integration assay (MIA). The integration reaction was evaluated according to the method previously described (Tewtrakul *et al.*, 2001). A mixture (45 μL), composed of 12 μL of IN buffer [containing 150 mM 3-(*N*-morpholino) propane sulfonic acid, pH 7.2 (MOPS), 75 mM $MnCl_2$, 5 mM dithiothritol (DTT), 25% glycerol and 500 μg/mL bovine serum albumin], 1 μL of 5 pmol/mL digoxigenin-labelled target DNA and 32 μL of sterilized water were added into each well of a 96-well plate. Subsequently, 6 μL of sample solution and 9 μL of 1/5 dilution of integrase enzyme were added to each well and incubated at 37 °C for 80 min. The wells were then washed with PBS 4 times, and 100 μL of 500 mU/mL alkaline phosphatase (AP) labelled anti-digoxigenin antibody then added to all wells and incubated at 37 °C for 1 h. The plate was washed again with washing buffer containing 0.05% Tween 20 in PBS 4 times and with PBS 4 times. Then, AP buffer (150 μL) containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ and 10 mM p-nitrophenyl phosphate were added to each well and incubated at 37 °C for 1 h. Finally, the plate was measured with a microplate reader at a wavelength of 405 nm. A control was composed of a reaction mixture, 50% DMSO and an integrase enzyme, while a blank was buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA. 2Na), 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1 mM DTT and 4 M urea without the integrase enzyme. Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control. The % inhibition against HIV-1 IN was calculated as follows:

$$\% \text{ Inhibition against HIV-1 IN} = [(\text{OD control} - \text{OD sample})/\text{OD control}] \times 100$$

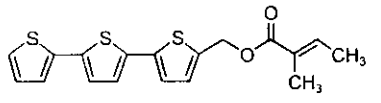
where OD is the absorbance detected from each well.

Statistics. For statistical analysis, the results of anti-HIV-1 PR activity were expressed as mean ± SD of three determinations, while anti-HIV-1 IN were as mean ± SD of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. Dunnett's test was used versus control for calculation of statistical significance.

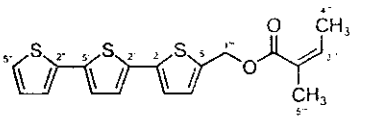
RESULTS AND DISCUSSION

Four compounds belonging to terthiophene derivatives were isolated from the CH_2Cl_2 extract of the whole plants of *Eclipta prostrata*. They were found to be 5-hydroxymethyl-(2,2′:5′,2″)-terthienyl tiglate (**1**), 5-hydroxymethyl-(2,2′:5′,2″)-terthienyl agelate (**2**), 5-hydroxymethyl-(2,2′:5′,2″)-terthienyl acetate (**3**), ecliptal (**4**); whereas those of the methanol fraction were orobol (**5**) and wedelolactone (**6**) (Fig. 1). Of these compounds, wedelolactone (**6**) possessed the highest activity against

DOI: 10.1002/ptr



5-Hydroxymethyl-(2,2′:5′,2″)-terthienyl tiglate (**1**)



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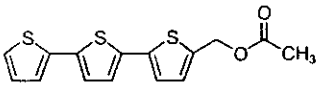

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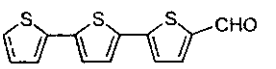
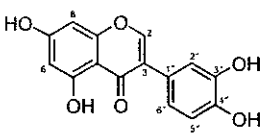
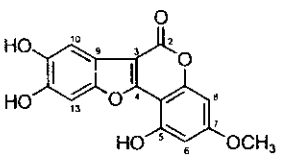


Ecliptal (**4**)



Orobol (**5**)



Wedelolactone (**6**)

Figure 1. Chemical structures of compounds isolated from the whole plants of *Eclipta prostrata*.

HIV-1 IN with an IC_{50} value of 4.0 μM, followed by orobol (**5**) (IC_{50} = 8.1 μM), while all isolated terthiophene compounds were apparently inactive (IC_{50} > 100 μM). Wedelolactone exhibited potent activity, comparable to that of the positive control, suramin (IC_{50} = 2.4 μM). In the case of HIV-1 PR inhibitory activity, **1** exhibited appreciable activity with an IC_{50} value of 58.3 μM, followed by **4** (IC_{50} = 83.3 μM) and **3** (IC_{50} = 93.7 μM), while compounds **2**, **5** and **6** were inactive. This result implies that wedelolactone (**6**) and orobol (**5**) are selective inhibitors for HIV-1 IN, but not for HIV-1 PR; whereas the inhibitory activity of the thiophene derivatives is selective for HIV-1 PR, but not for HIV-1 IN (Table 1, Figs 2 and 3).

Several classes of photosensitizers including sulphur-containing compounds and some terthiophenes have been reported to possess anti-HIV-1 activities. In the present study, compound **1** (*cis*-form), a terthiophene

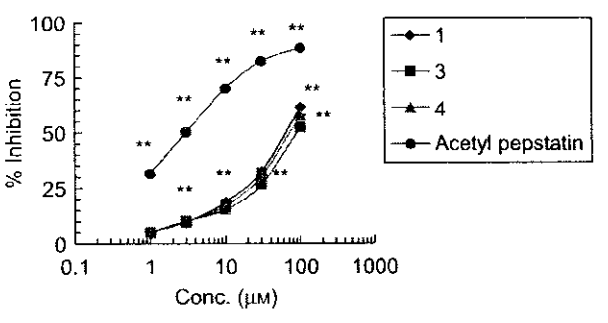


Figure 2. Dose-dependence of anti-HIV-1 PR activity of compounds **1**, **3**, **4** and acetyl pepstatin. Each value represents the mean ± SD of three determinations.

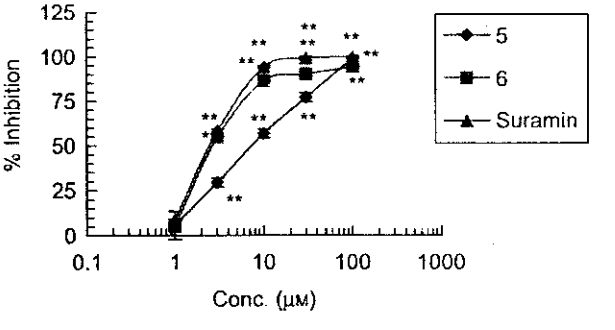


Figure 3. Dose-dependence of anti-HIV-1 IN activity of compounds **5**, **6** and suramin. Each value represents the mean ± SD of four determinations.

Table 1. Anti-HIV-1 PR and HIV-1 IN activities of compounds isolated from the whole plants of *Eclipta prostrata*

Compound	IC_{50} (μM) ± SD	
	HIV-1 PR[a]	HIV-1 IN[b]
5-Hydroxymethyl-(2,2′:5′,2″)-terthienyl tiglate (**1**)	58.3 ± 0.8	>100 (2.3%)
5-Hydroxymethyl-(2,2′:5′,2″)-terthienyl agelate (**2**)	>100 (24.2%)	>100 (8.9%)
5-Hydroxymethyl-(2,2′:5′,2″)-terthienyl acetate (**3**)	93.7 ± 0.8	>100 (4.0%)
Ecliptal (**4**)	83.3 ± 1.6	>100 (11.2%)
Orobol (**5**)	>100 (43.8%)	8.1 ± 0.5
Wedelolactone (**6**)	>100 (32.7%)	4.0 ± 0.2
Acetyl pepstatin (Positive control for HIV-1 PR)	3.4 ± 0.2	–
Suramin (Positive control for HIV-1 IN)	–	2.4 ± 0.1

Values in parenthesis are % inhibition at 100 μM.
[a] Each value represents the mean ± SD of three determinations.
[b] Each value represents the mean ± SD of four determinations.

DOI: 10.1002/ptr

derivative, displayed appreciable activity against HIV-1 PR (IC_{50} = 58.3 μM); whereas its isomer (**2**, *trans*-form) was inactive (IC_{50} > 100 μM).

Coumarins, such as calanolides and inophyllums, have been established as non-nucleotide specific inhibitors of HIV-1 reverse transcriptase (RT). Calanolides A and B isolated from *Calophyllum lanigerum* have been reported to be potent anti-HIV-1 agents (Cardellina *et al.*, 1995). Two coumarins isolated from *Glycyrrhiza glabra*, namely glycocoumarin and licopyranocoumarin, were reported to inhibit giant cell formation in HIV infected cell cultures (Hatano *et al.*, 1988). However, wedelolactone (**6**) which is also a coumarin derivative, has not previously been reported for anti-HIV-1 activity, as well as orobol (**5**), an isoflavone derivative.

In conclusion, of the isolated compounds from *E. prostrata*, wedelolactone (**6**) and orobol (**5**) are responsible for anti-HIV-1 IN activity; whereas 5-hydroxymethyl-(2,2′ 5′ 2″)-terthienyl tiglate (**1**), ecliptal (**4**) and 5-hydroxymethyl-(2 2′ 5′ 2″)-terthienyl acetate (**3**) act as HIV-1 PR inhibitors. These lead compounds could form the basis of future research in the search for potent selective and non-toxic anti-HIV molecules for the treatment of HIV patients worldwide. This finding also supports the use of *E. prostrata* in AIDS treatment which agrees with its traditional use for treatment of blood-related diseases.

Acknowledgements

This work was financially supported by the Thailand Research Fund and the Royal Thai Government Budget. We also thank Dr Robert Craigie, the National Institute of Health, Bethesda, Maryland, USA for providing an HIV-1 integrase enzyme.

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Journal of Ethnopharmacology 120 (2008) 81–84

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Effects of compounds from *Kaempferia parviflora* on nitric oxide, prostaglandin E_2 and tumor necrosis factor-alpha productions in RAW264.7 macrophage cells

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ARTICLE INFO

Article history:
Received 9 February 2008
Received in revised form 30 June 2008
Accepted 26 July 2008
Available online 3 August 2008

Keywords:
NO
PGE_2
Kaempferia parviflora
Zingiberaceae
Anti-inflammatory compounds

ABSTRACT

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, apthous ulcer and abscesses. Since *K. parviflora* rhizomes have long been used for treatment of inflammation and possessed marked nitric oxide (NO) inhibitory activity (IC_{50} = 7.8 μg/ml), we thus investigated the inhibitory activity of compounds isolated from this plant against lipopolysaccharide (LPS)-induced NO release in RAW264.7 cells.

From bioassay-guided fractionation of *K. parviflora*, seven methoxyflavones were isolated from the hexane fraction and were tested for their anti-inflammatory effects. Among the isolated compounds, 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). Compound 5 was also tested on LPS-induced prostaglandin E_2 (PGE_2) and tumor necrosis factor-alpha (TNF-α) releases from RAW264.7 cells. It was revealed that 5 showed appreciable inhibitory effect on PGE_2 release (IC_{50} = 16.3 μM), but inactive on TNF-α (IC_{50} > 100 μM). These findings 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.

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.

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, apthous ulcer, abscesses, allergy and gastrointestinal disorders, as well as an aphrodisiac (Pengcharoen, 2002). *Kaempferia parviflora* has recently been reported to possess anti-allergic (Tewtrakul et

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doi:10.1016/j.jep.2008.07.033

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 *Kaempferia 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 phenethyl ester (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 *Curcuma mangga* Val. & Zijp., *Kaempferia galanga* Linn., *Zingiber officinale* Roscoe and *Zingiber zerumbet* (L.) Sm. were collected in June 2005 in Songkhla province, Thailand. *Kaempferia 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 *Curcuma mangga*, *Kaempferia galanga*, *Kaempferia parviflora*, *Zingiber officinale* and *Zingiber 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 Kaempferia parviflora extract

Two kilograms dried weight of *Kaempferia parviflora* were ground and macerated with EtOH at room temperature, four 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%, 8000 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 ^1H and ^{13}C NMR spectral data with those reported (Jaipetch et al., 1983; Harborne et al., 1988; Agrawal, 1989).

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 1×10^5 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

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
Curcuma 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
Kaempferia 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
Kaempferia 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
Zingiber 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
Zingiber zerumbet (Water)	0.0 ± 6.9	–	39.5 ± 6.1**	57.6 ± 2.8**	88.6 ± 9.9**	17.8

Statistical significance, *$p < 0.05$; **$p < 0.01$.
[a] Each value represents mean ± S.E.M. of four determinations.
[b] Cytotoxic effect was observed.

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.

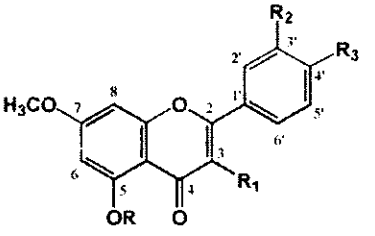
2.6. Statistics

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 *Curcuma mangga*, *Kaempferia galanga*, *Kaempferia parviflora*, *Zingiber officinale* and *Zingiber zerumbet* were investigated for their anti-inflammatory activities using RAW264.7 cell line. Among these, the EtOH extract of *Kaempferia 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 *Kaempferia 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 (Fig. 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



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.

(NO synthase inhibitor, IC_{50} = 61.8 μM). Moreover, compounds **5** (IC_{50} = 16.1 μM) and **4** (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 *Kaempferia parviflora* upon NO inhibition could be summarized as follows: (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

Table 2
Inhibition on NO production of EtOH extract and fractions from *Kaempferia 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
Kaempferia 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
Kaempferia 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
Kaempferia parviflora (EtOAc fr.)	0.0 ± 3.5	–	2.6 ± 1.1	2.2 ± 0.8	6.8 ± 1.8	>100
Kaempferia parviflora (Water fr.)	0.0 ± 3.5	–	0.9 ± 1.1	10.2 ± 1.1	34.4 ± 2.4**	>100

Statistical significance, *$p < 0.05$; **$p < 0.01$.
[a] Each value represents mean ± S.E.M. of four determinations.
[b] Cytotoxic effect was observed.

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

Compound	% Inhibition at various concentrations					IC_{50} (μM)
	0	3	10	30	100	
(A)						
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 ± 1.2b,**	24.5
5	0.0 ± 3.7	–	28.3 ± 3.9**	64.3 ± 3.6**	92.3 ± 0.4b,**	16.1
6	0.0 ± 3.7	–	–	1.3 ± 2.8	59.7 ± 1.9b,**	81.8
7	0.0 ± 6.0	–	–	2.5 ± 1.2	46.8 ± 1.9b,**	>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 ± 3.4b,**	98.7 ± 1.2b,**	98.9 ± 2.1b,**	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

Inflammatory-mediator	% Inhibition at various concentrations (μM) of compound **5**					IC_{50} (μM)
	0	3	10	30	100	
(B)						
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

Statistical significance, *$p < 0.05$, **$p < 0.01$.
[a] Each value represents mean ± S.E.M. of four determinations.
[b] Cytotoxic effect was observed.

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 *Kaempferia 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 *Kaempferia parviflora* will be further investigated.

Acknowledgements

The authors are grateful to the Thailand Research Fund (TRF) and the Commission on Higher Education (CHE) for financial support. We also thank the Faculty of Pharmaceutical Sciences for providing laboratory facilities.

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