รายงานวิจัยฉบับสมบูรณ์

โครงการ : ฤทธิ์ต้านการอักเสบและกลไกการออกฤทธิ์ของสารสำคัญจากต้นกะเม็ง ขันทองพยาบาท และ หงอนไก่ทะเล

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Contents

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บทคัดย่อ

สารสกัดจากต้นกะเม็ง ขันทองพยาบาท และหงอนไก่ทะเล รวมทั้งสารสำคัญที่แยกได้ ถูกนำมาทดสอบฤทธิ์ต้านการอักเสบในการยับยั้งการหลั่ง nitric oxide (NO) และ prostaglandin E_2 (PGE_2) โดยใช้ cell RAW264.7 ผลการทดลองพบว่า ต้นกะเม็ง แยกได้สารที่ชื่อ orobol มีฤทธิ์ยับยั้งการหลั่งสาร NO ได้ดีมาก โดยให้ค่า IC_{50} = 4.6 μM และยับยั้ง PGE_2 ได้ปานกลาง (IC_{50} = 49.6 μM)

สำหรับเปลือกขันทองพยาบาท สารสำคัญที่ออกฤทธิ์ต้าน NO ได้ดีคือ helioscopinolide A โดยให้ค่า IC_{50} = 9.1 μM และยับยั้ง PGE_2 ได้ปานกลาง (IC_{50} = 46.3 μM) เช่นกัน ส่วนสารสกัดจากเปลือกหงอนไก่ทะเล แยกได้สารที่ชื่อ ergosterol peroxide มีฤทธิ์ยับยั้งการหลั่งสาร NO ได้ดีที่สุด โดยให้ค่า IC_{50} = 2.5 μM และยับยั้ง PGE_2 ได้ดี (IC_{50} = 28.7 μM) กลไกการออกฤทธิ์ของสาระสำคัญทั้งสามสารพบว่า มีฤทธิ์ยับยั้งการแสดงออกของ mRNA ของ iNOS และ COX-2 ในรูปแบบ dose-dependence

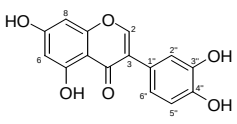
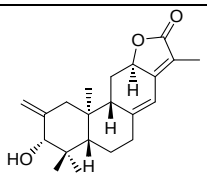
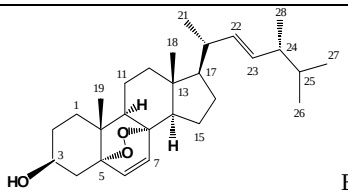
การศึกษาในครั้งนี้สามารถสนับสนุนการใช้ต้นไม้ทั้งสามชนิดนี้ในการรักษาโรคที่เกี่ยวข้องกับการอักเสบได้

Abstract

Eclipta prostrata, Suregada multiflora, Heritiera littoralis extracts and their isolated compounds were tested for anti-inflammatory effects against lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostraglandin E_2 (PGE_2) release from RAW264.7 cells.

Among the isolated compounds of *Eclipta prostrata*, orobol exhibited the highest activity against NO release with an IC_{50} value of 4.6 μM. The IC_{50} value of orobol against PGE_2 release was found to be 49.6 μM. For *Suregada multiflora*, helioscopinolide A exhibited the highest activity against NO release with an IC_{50} value of 9.1 μM. The IC_{50} value of helioscopinolide A against PGE_2 production was found to be 46.3 μM. Whereas ergosterol peroxide from *Heritiera littoralis* exhibited the highest activity against NO release with an IC_{50} value of 2.5 μM. It was also found that ergosterol peroxide possessed marked activity against PGE_2 release with an IC_{50} value of 28.7 μM. The mechanism in transcriptional level of orobol, helioscopinolide A and ergosterol peroxide was found to down regulate mRNA expressions of iNOS and COX-2 in a dose-dependent manner.

The present study supports the uses of these three plants for treating inflammatory-related diseases.

Orobol 	 Helioscopinolide A	 Ergosterol peroxide

Keywords: NO; PGE_2; *Eclipta prostrata*; *Suregada multiflora*; *Heritiera littoralis*

Executive summary

Anti-inflammatory activity of *Eclipta prostrata, Suregada multiflora* and *Heritiera littoralis* using RAW264.7 macrophage cells

Introduction

Nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs and it has potent antimicrobial activity (Goldsby et al, 2002). NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). This inorganic free radical has been implicated in physiological and pathological processes, such as vasodilation, non-specific host defense and acute or chronic inflammation. NO acts as a host defense by damaging pathogenic DNA, and as a regulatory molecule with homeostatic activities (Kou and Schroder, 1995). In the NOS family, inducible NOS (iNOS) is particularly well known to be involved in the overproduction of NO in cells. 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).

Cyclooxygenase-2 (COX-2) is an inducible enzyme catalyzing the conversion of arachidonic acid to prostaglandins. Recent studies have suggested that increased levels of prostaglandins and cyclooxygenase activity may play important roles in multiple epithelial cancers. COX-2-derived bioactive lipids, including prostaglandin E_2, are potent inflammatory mediators (Pan et al., 2006).

Since the extracts of *Eclipta prostrata*, *Suregada multiflora*, *Heritiera littoralis* possessed potent NO inhibitory effect (IC_{50} < 30 μg/ml), the compounds from these three plants were further isolated and tested for NO and PGE_2 inhibitory activities, as well as the mechanism in transcriptional level of active compounds (orobol, helioscopinolide A and ergosterol peroxide) against iNOS and COX-2 mRNA expression using RAW264.7 cells.

Material and Methods

Assay for NO and PGE_2 inhibitory effects from RAW264.7 cells

Inhibitory effect on NO and PGE_2 production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota et al., 2003).

Results and discussions

Anti-inflammatory activity of compounds from *Eclipta prostrata*

The whole plant extract of *Eclipta prostrata* and its isolated compounds were tested for their anti-inflammatory effects against lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E_2 (PGE_2) and tumor necrosis factor-alpha (TNF-α) releases in RAW264.7 cells as well as the anti-inflammatory mechanism on mRNA expression of the active compound. Among the isolated compounds, orobol (**5**) exhibited the highest activity against NO release with an IC_{50} value of 4.6 μM, followed by compounds **1**, **2** and **4** with IC_{50} values of 12.7, 14.9 and 19.1 μM, respectively. The IC_{50} value of **5** against PGE_2 release was found to be 49.6 μM, whereas it was inactive towards TNF-α (IC_{50} > 100 μM). The mechanism of orobol (**5**) was found to down regulate iNOS and COX-2 mRNA expression in concentration-dependent manners. The present study may support the traditional use of *Eclipta prostrata* for treatment of inflammatory-related diseases.

Anti-inflammatory activity of compounds from *Suregada multiflora*

A CH_2Cl_2 extract from the bark of *Suregada multiflora* and its isolated compounds were tested for their anti-inflammatory effects against lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostraglandin E_2 (PGE_2) release from RAW264.7 cells. Among the isolated compounds, helioscopinolide A (**5**) exhibited the highest activity against NO release with an IC_{50} value of 9.1 μM, followed by helioscopinolide C (**6**) and suremulol D (**2**) with IC_{50} values of 24.5 and 29.3 μM, respectively. The IC_{50} value of **5** against PGE_2 production was found to be 46.3 μM. Compound **5** inhibited the production of iNOS and COX-2 mRNA in a dose-dependent manner. The present study supports the traditional use of *Suregada multiflora* bark for treating inflammatory-related diseases.

Anti-inflammatory activity of compounds from *Heritiera littoralis*

Compounds from the hexane, dichloromethane and acetone extracts of *Heritiera littoralis* bark were investigated for their nitric oxide (NO) inhibitory effects using RAW264.7 macrophage cells. The result indicated that ergosterol peroxide (**13**) exhibited the highest activity against NO release with an IC_{50} value of 2.5 μM, followed by 6-α-hydroxystigmast-4-en-3-one (**11**, IC_{50} = 9.5 μM) and stigmast-4-en-

3-one (**9**, IC_{50} = 15.9 μM), whereas other compounds showed moderate and mild effects (25.4- > 100 μM). Ergosterol peroxide (**13**) and 6-α-hydroxystigmast-4-en-3-one (**11**) were also tested against prostaglandin E_2 (PGE_2) and tumor necrosis factor alpha (TNF-α) releases. It was found that ergosterol peroxide (**13**) possessed marked activity against PGE_2 release with an IC_{50} value of 28.7 μM, while 6-α-hydroxystigmast-4-en-3-one (**11**) was 86.7 μM. However, these two compounds were inactive towards TNF-α release (IC_{50} > 100 μM). The mechanism in transcriptional level of ergosterol peroxide (**13**) was found to down regulate mRNA expressions of iNOS and COX-2 in dose-dependent manners.

In conclusion, the present study may support the use of these three plants for treatment of inflammatory-related diseases through the inhibition of NO and PGE_2 releases. The mechanism might involve in the suppression of iNOS and COX-2 genes.

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Research work

Part I. Anti-inflammatory activity of compounds from *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 treatment of skin diseases. The stem has been used as a blood tonic and for treatment of abscess, itching, haemorrhoid, 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 immunomodulatory effect on T-lymphocytes (Liu *et al*., 2001), anti-HIV-1 integrase and HIV-1 protease (Tewtrakul *et al*., 2007), anti-inflammatory (Kobori *et al*., 2004), hepatoprotective (Han *et al*., 1998) and antimicrobial activities (Wiart *et al*., 2004).

Since the CH_2Cl_2 and MeOH extracts of *Eclipta prostrata* possessed marked NO inhibitory effect, the compounds from this plant were further isolated and tested for NO, PGE_2 and TNF-α inhibitory activities. In addition, the mechanism in transcriptional level of active compounds against iNOS and COX-2 mRNA using lipopolysaccharide(LPS)-stimulated RAW264.7 cells was also examined.

Material and methods

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).

Plant material

Whole plants of *Eclipta prostrata* were collected in August 2004 at the botanical garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand and identified by Assoc. Prof. Dr. Sanan Subhadhirasakul. The voucher specimen (No. SN 4412025) is deposited at the Southern center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Extraction and isolation

The dried whole plants (250.0 g) of *Eclipta prostrata* were extracted sequentially with CH_2Cl_2 and MeOH (2 L x 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 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 by 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 literatures (Das and Chakravarty, 1991; Jain and Singh, 1988; Sashida *et al*., 1983; Kosuge *et al*., 1985).

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 200 μg/mL of LPS together with the test samples at various concentrations (3-100 μg/mL for crude extract 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, CAPE and indomethacin 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 (-)].

Inhibitory effects on LPS-induced PGE_2 and TNF-α releases 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 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 200 μg/mL of LPS together with the test samples at various concentrations (3-100 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 graphically determined.

Total RNA isolation and RT-PCR

In order to acquire the mechanism of action on cytokine release of orobol (**5**), the assays for mRNA expression of iNOS and COX-2 were carried out. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with samples in various concentrations (3, 10, 30, 100 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis using first strand cDNA synthesis kit (Rever Tra Ace-α, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers for iNOS and COX-2 were used (forward primer for iNOS : 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGCTC-3′; forward primer for β-actin (an internal standard): 5´-TGTGATGGTGGGAATGGGTCAG-3´ and reverse primer: 5´-TTTGATGTCACGCACGATTTCC-3´.

The solution for cDNA synthesis consisted of RNA solution 11 μL, 5 x 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 (reverese transcriptase enzyme) 1 μL for a 20

μL reaction. The condition for cDNA synthesis was as follow; 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. After that, 1/10 times (2 μL) of cDNA product was used further for PCR. The PCR mixture consisted of RT reaction mixture (cDNA product) 2 μL; sterilized water 85 μL, 10 x PCR buffer 10 μL, forward primer (10 pmol/ μL) 1 μL, reverse primer (10 pmol/ μL) 1 μL and KOD Dash (polymerase enzyme)1 μL for final volume of 100 μL. The condition for PCR was as follow; denaturation at 94 °C for 1 min, 98 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were analyzed in 1.2 % agarose gel electrophoresis and visualized by SYBR safe staining and UV irradiation.

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 programme. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

Results and discussions

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 methanol fraction were orobol (**5**) and wedelolactone (**6**) (Figure 1). Among these isolated compounds, orobol (**5**) exhibited the most potent activity against NO release with an IC_{50} value of 4.6 μM, followed by **1**, **2** and **4** with IC_{50} values of 12.7, 14.9 and 19.1 μM, respectively; whereas those of other compounds (**3** and **6**) showed moderate effects ranging from 23.3 to 27.2 μM (Table 1A). The effect of **5** against NO release was higher than that of CAPE, an NF-κB inhibitor (IC_{50} = 5.0 μM), and indomethacin, a non-steroidal anti-inflammatory drug (NSAID, IC_{50} = 20.1 μM) as well as L-NA, a NO synthase inhibitor (IC_{50} = 59.0 μM). The IC_{50} value of **5** on PGE_2 release was found to be 49.6 μM, whereas it was inactive towards TNF-α (IC_{50} > 100 μM) (Table 1B). Orobol (**5**), the most potent compound, was further investigated for its anti-inflammatory mechanism. The result showed that the

mechanism in transcriptional level of compound **5** was found to inhibit iNOS and COX-2 mRNA expression in concentration-dependent manners (Figure 2). Regarding biological activities of *Eclipta prostrata*, the extract of this plant has been reported to scavenge hydroxyl and peroxyl radicals in *in vitro* system (Yang *et al*., 2008) and possessed antiproliferative effect against hepatic stellate cells which is a key role in the pathogenesis of liver fibrosis (Lee *et al*., 2008). Orobol (**5**), an isoflavone derivative, has also been reported to show marked HIV-1 IN inhibitory activity (Tewtrakul *et al*., 2007). However, the inhibitory effect of orobol on inflammatory mediators including NO, PGE_2 and TNF-α releases have not yet been reported so far.

In conclusion, the present study may support the traditional use of *Eclipta prostrata* for treatment of inflammatory-related diseases. The anti-inflammatory effect of this plant is due to the inhibition on NO and PGE_2 releases through down regulation of iNOS and COX-2 mRNA expressions, whereas it does not affect on TNF-α release.

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Table 1. NO inhibitory production[a] of compounds isolated from *Eclipta prostrata* (A) and inhibition on PGE_2 and TNF-α production of orobol (**5**) using RAW264.7 cells (B)

A

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
5-Hydroxymethyl-(2, 2´:5´, 2´´)-terthienyl tiglate (**1**)	0.0 ± 2.4	-	43.9 ± 2.4**	68.9 ± 1.6**	86.3 ± 2.3[b]**	12.7
5-Hydroxymethyl-(2, 2´: 5´, 2´´)-terthienyl agelate (**2**)	0.0 ± 2.4	-	35.0 ± 3.6*	76.3 ± 2.2**	102.4 ± 4.4[b]**	14.9
5-Hydroxymethyl-(2, 2´: 5´, 2´´)-terthienyl acetate (**3**)	0.0 ± 3.5	-	23.3 ± 2.9	57.4 ± 2.8**	96.4 ± 6.8[b]**	23.3
Ecliptal (**4**)	0.0 ± 3.5	-	34.8 ± 3.6*	65.4 ± 3.8**	74.9 ± 5.2**	19.1
Orobol (**5**)	0.0 ± 4.8	36.5 ± 2.4*	74.8 ± 2.7**	82.0 ± 1.7**	107.5 ± 0.8**	4.6
Wedelolactone (**6**)	0.0 ± 4.8	-	13.3 ± 2.3	69.6 ± 3.8**	78.2 ± 3.4**	27.2
L-Nitroarginine (L-NA)	0.0 ± 5.6	15.3 ± 2.8	21.4 ± 2.5	35.6 ± 2.1**	73.2 ± 3.5**	59.0
Caffeic acid phenethylester (CAPE)	0.0 ± 5.6	35.2 ± 3.0*	70.3 ± 2.7**	97.6 ± 2.4[b]**	99.5 ± 2.7[b]**	5.0
Indomethacin	0.0 ± 4.2	16.6 ± 2.9	32.7 ± 2.6**	53.4 ± 3.0**	85.6 ± 1.8**	20.1

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

Statistical significant, *p<0.05, **p<0.01

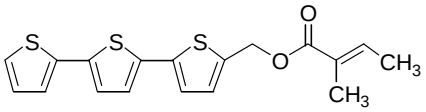
[b]Cytotoxic effect was observed.

B

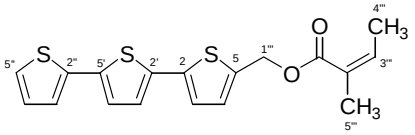
Inflammatory mediators	% Inhibition at various concentrations (μM) of orobol (**5**)					IC_{50} (μM)
	0	3	10	30	100	
PGE_2	0.0 ± 0.8	-	32.2 ± 1.4**	44.5 ± 1.0**	57.7 ± 1.2**	49.6
TNF-α	0.0 ± 2.8	-	4.7 ± 0.6	5.9 ± 0.8	7.1 ± 3.6	>100

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

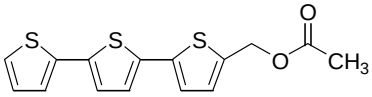
Statistical significant, *p<0.05, **p<0.01



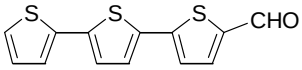
5-Hydroxymethyl-(2,2':5',2'')-terthienyl tiglate **(1)**



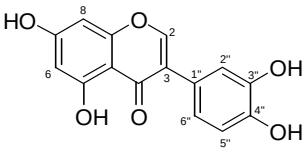
5-Hydroxymethyl-(2,2':5',2'')-terthienyl agelate **(2)**



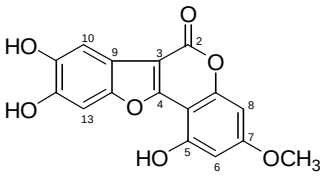
5-Hydroxymethyl-(2,2':5',2'')-terthienyl acetate **(3)**



Ecliptal **(4)**



Orobol **(5)**



Wedelolactone **(6)**

Figure 1. Structures of compounds from *Eclipta prostrata*

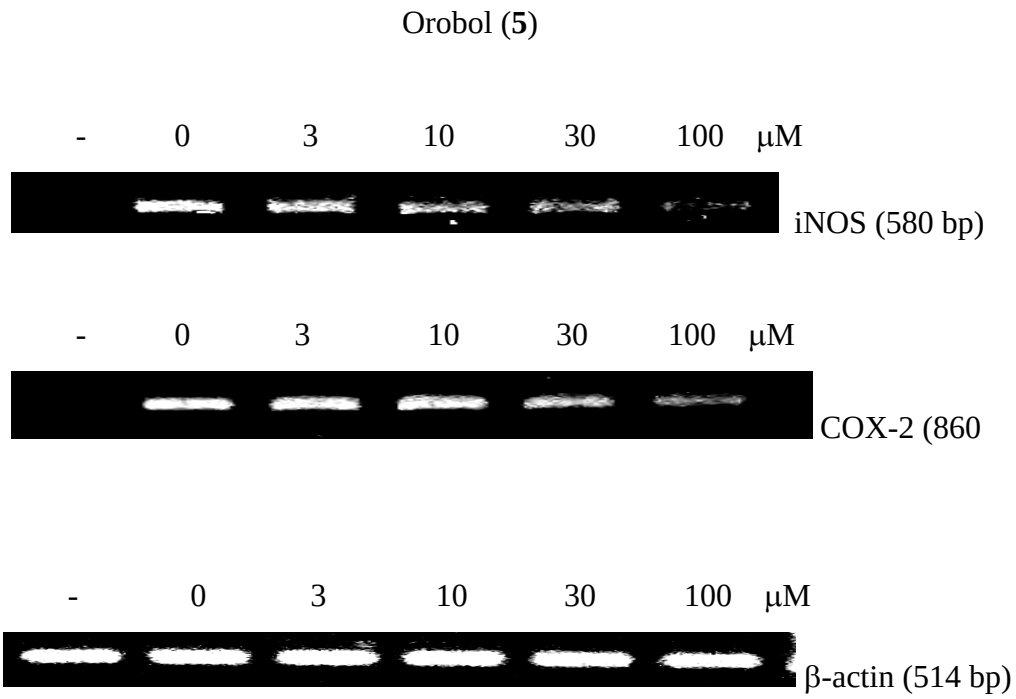


Figure 2. Effect of orobol (**5**) at various concentrations (0, 3, 10, 30, 100 μM) on mRNA expressions of iNOS and COX-2 by LPS-induced NO and PGE_2 releases in RAW264.7 cells.

(-) = LPS (-), sample (-)

(+) = LPS (+), sample (-)

3-100 μM = LPS (+), sample (+)

Part II. Anti-inflammatory activity of compounds from *Suregada multiflora*

Introduction

Suregada multiflora A. Juss. (syn. *Gelonium multiflorum*), is a plant in the Euphorbiaceae family. It grows widely throughout tropical and subtropical areas especially in Asia and Africa. In Thai traditional medicine, the bark of this plant has been used to treat hepatitis, lymphatic disorders, skin diseases, venereal diseases, fungal infections and leprosy. The wood has been used to treat pyretic, eczema and veneral diseases, whereas the roots have been used for treating skin infections and lymphatic disorders (Wutthithamavet, 1997). This plant has been reported to possess anti-HIV activity and has an inhibitory effect on the infection and replication of herpes simplex virus (HSV) (Bourinbaiar and Huang, 1996). The crude (CH_2Cl_2-MeOH, 1:1) extract of this plant exhibits selective cytotoxic activity against different human tumor cell lines (Jahan et al. 2002).

Since the CH_2Cl_2 extract of *Suregada multiflora* bark possessed a potent NO inhibitory effect (IC_{50} = 8.6 μg/ml), the compounds from this plant were further isolated and tested for NO and PGE_2 inhibitory activities In addition, the mechanism at a transcriptional level of one isolated active compound (helioscopinolide A) was also investigated using RAW264.7 cells.

Materials and methods

General experimental procedures

Melting points were determined on a Fisher-John melting point apparatus. The specific rotation $[\alpha]_D$ values were determined with a JASCO P-1020 polarimeter. UV spectra were obtained with a SPECORD S 100 (Analytikjena). The IR spectra were measured with a Perkin-Elmer FTS FT-IR spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded using a 300 MHz Bruker FTNMR Ultra Shield^{TM} spectrometer. Chemical shifts are recorded in parts per million (δ) in $CDCl_3$ with tetramethylsilane (TMS) as an internal reference. The EIMS was obtained from a MAT 95 XL mass spectrometer. Quick column chromatography (QCC) and column chromatography (CC) were carried out on silica gel 60 F_{254} (Merck) and silica gel 100 (Merck), respectively. Precoated plates of silica gel 60 F_{254} were used for analytical purposes.

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 for 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).

Plant material

Barks of *Suregada multiflora* was collected from Songkhla province, Thailand in November 2004. Identification was made by Prof. Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University and a specimen (No. SC04) deposited at Prince of Songkla University Herbarium.

Extraction and isolation

Air-dried and ground bark (5.9 kg) of *Suregada multiflora* was extracted with hexane and CH_2Cl_2 (2 x 7.5 L, for 5 days each) at room temperature. The crude extracts were evaporated under reduced pressure to afford hexane and CH_2Cl_2 extracts. The CH_2Cl_2 extract (27.3 g) was further purified by QCC using hexane as eluent with an increasing polarity obtained with acetone and MeOH to give seven fractions (F1-F7). Fraction F2 (702.9 mg) was subjected to CC with EtOAc-hexane (1:3, v/v) followed by preparative TLC with MeOH-CH_2Cl_2 (1:49, v/v) to give **2** (3.3 mg) and **4** (7.2 mg). Fraction F4 (1.8 g) was purified by CC with acetone-CH_2Cl_2 (1:49, v/v) to afford four subfractions. Subfraction F4b (67.4 mg) was separated by CC with EtOAc-hexane (3:7, v/v) to afford **5** (12.6 mg). Subfraction F4d (370.2 mg) was purified by CC with EtOAc-hexane (3:7, v/v) and followed by preparative TLC with EtOAc-hexane (3:7, v/v) to give **3** (6.2 mg), **6** (9.1 mg) and **7** (8.3 mg). Fraction F6 (1.2 g) was subjected to CC with MeOH-CH_2Cl_2 (1:19, v/v) to afford three subfractions (F6a-F6c). Subfraction F6c (327.7 mg) was further purified by CC with

acetone-CH_2Cl_2 (1:9, v/v) to give **1** (22.5 mg). The structures of the isolated compounds were elucidated and compared with the previous literatures (Cheenpracha et al. 2006; Das et al. 1994; Agrawal et al. 1995; Borghi et al. 1991; Crespi-Perellino et al. 1996).

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 by elution with trypsin-EDTA and diluted to make a suspension in a fresh medium. The cells were seeded into 96-well plates with 1 x 10^5 cells/well. They were allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. The medium was then replaced with a fresh medium containing 200 μg/ml of LPS together with the test samples at various concentrations (3-100 μg/ml for crude extract 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, CAPE and indomethacin were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the RPMI medium (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 (-)].

Inhibitory effects on LPS-induced PGE_2 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 by elution with trypsin-EDTA and diluted to make a suspension in a fresh medium. The cells were seeded in 96-well plates with 1.0 x 10^5 cells/well. Cells were allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. The medium was then replaced with a fresh medium containing 200 μg/ml of LPS together with the test samples at various concentrations (3-100 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 releases was calculated and IC_{50} values were determined graphically.

Total RNA isolation and RT-PCR

In order to understand the effect of helioscopinolide A (**5**) on cytokine release, assays for mRNA expression of iNOS and COX-2 were carried out. Total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with various concentrations of helioscopinolide A (3, 10, 30, 100 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis using the first strand cDNA synthesis kit (Rever Tra Ace-α, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers used for iNOS and COX-2 were used (forward primer for iNOS : 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGCTC-3′; forward primer for β-actin (an internal standard): 5´-TGTGATGGTGGGAATGGGTCAG-3´ and reverse primer: 5´-TTTGATGTCACGCACGATTTCC-3´.

The solution for cDNA synthesis consisted of the RNA solution 11 μl, 5 x 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 (reverese transcriptase enzyme) 1 μl for a 20 μl reaction. The conditions for cDNA synthesis were as follow; 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. After that, 1/10 times (2 μl) of the total cDNA product was used for PCR analysis. The PCR mixture consisted of the RT reaction mixture (cDNA product) 2 μl; sterilized water 85 μl, 10 x PCR buffer 10 μl, forward primer (10 pmol/ μl) 1 μl, reverse primer (10 pmol/ μl) 1 μl and KOD Dash (polymerase enzyme)1 μl in a final volume of 100 μl. The condition for PCR was as follow; denaturation at 94 °C for 1 min, 98 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were analyzed by electrophoresis in a 1.2 % agarose gel and visualized by SYBR safe staining and UV irradiation at a wavelength of 312 nm.

Statistics

For statistical analysis, the values are expressed as a mean ± S.E.M 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 the Dunnett's test.

Results and discussion

Two *ent*-kaurene diterpenoids (**1**-**2**) together with five diterpenoids (**3**-**7**) were isolated from the bark of *Suregada multiflora*. There are suremulol C (**1**), suremulol D (**2**), *ent*-kaurene-3*β*,15*β*-diol (**3**), abbeokutone (**4**), helioscopinolide A (**5**), helioscopinolide C (**6**) and helioscopinolide I (**7**) (Figure 1). Among these isolated compounds, helioscopinolide A (**5**) exhibited the most potent activity against NO release with an IC_{50} value of 9.1 μM, followed by helioscopinolide C (**6**) and suremulol D (**2**) with IC_{50} values of 24.5 and 29.3 μM, respectively, whereas other compounds showed moderate effects ranging from 30.1 to 56.3 μM (Table 1A). The effect of compound **5** against NO release was comparable to that of CAPE, an NF-κB inhibitor (IC_{50} = 5.6 μM) but higher than those of indomethacin, a non-steroidal anti-inflammatory drug (NSAID, IC_{50} = 25.0 μM) and L-NA, a NO synthase inhibitor (IC_{50} = 61.8 μM). The IC_{50} value of **5** on PGE_2 release was found to be 46.3 μM

(Table 1B). Helioscopinolide A (**5**), the most potent compound, was further investigated for anti-inflammatory mechanism at the transcriptional level. The result showed that helioscopinolide A (**5**) inhibited the expression of iNOS and COX-2 mRNA in a dose-dependent manner (Figure 2). Helioscopinolide A (**5**) has been reported to show marked anti-allergic effect in RBL-2H3 cells (Cheenpracha et al. 2006), anti-cancer activity against HeLa and MDA-MB-231 cells (Lu et al. 2008), anti-bacterial effect against *Staphylococcus aureus* (Valenta et al. 2004) and acts as a CNS stimulant (Speroni et al. 1991). However, the inhibitory effect of this compound on inflammatory mediators including NO and PGE_2 releases has not yet been investigated.

In conclusion, the present study supports the traditional use of *Suregada multiflora* for treatment of inflammatory-related diseases. The anti-inflammatory effect of this plant is most likely due to its inhibition of NO and PGE_2 releases through down regulation of iNOS and COX-2 mRNA expressions.

Acknowledgements

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Table 1. Inhibition on NO production of compounds isolated from *Suregada multiflora* (A) and inhibition on PGE_2 production of helioscopinolide A (**5**) using RAW264.7 cells (B)

A

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
Suremulol C (**1**)	0.0 ± 3.1	-	19.0 ± 3.1*	43.4 ± 2.4**	89.0 ± 2.8**	30.6
Suremulol D (**2**)	0.0 ± 3.1	-	19.6 ± 2.0**	44.5 ± 2.9**	91.1 ± 2.8**	29.3
ent-Kaurene-3β, 15β-diol (**3**)	0.0 ± 3.1	-	23.2 ± 2.2*	45.6 ± 1.3**	83.6 ± 3.3**	30.1
Abbeokutone (**4**)	0.0 ± 3.0	-	19.1 ± 3.0*	28.3 ± 2.6**	93.9 ± 4.3**	50.0
Helioscopinolide A (**5**)	0.0 ± 3.0	23.6 ± 4.1*	50.7 ± 2.6**	82.9 ± 2.5**	100.1 ± 0.7**	9.1
Helioscopinolide C (**6**)	0.0 ± 3.0	-	26.3 ± 4.3*	53.9 ± 6.9**	89.0 ± 3.3**	24.5
Helioscopinolide I (**7**)	0.0 ± 4.0	-	13.8 ± 2.1	25.1 ± 0.7**	87.0 ± 3.5**	56.3
L-nitroarginine (L-NA)	0.0 ± 9.9	11.7 ± 4.6	20.2 ± 5.9	34.7 ± 1.8*	71.6 ± 2.6**	61.8
Caffeic acid phenethylester (CAPE)	0.0 ± 9.9	30.7 ± 3.2*	68.6 ± 3.4**	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

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
Helioscopinolide A (**5**)	0.0 ± 0.8	-	31.8 ± 0.9**	48.0 ± 0.2**	57.0 ± 0.8**	46.3

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

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

[b]Cytotoxic effect was observed.

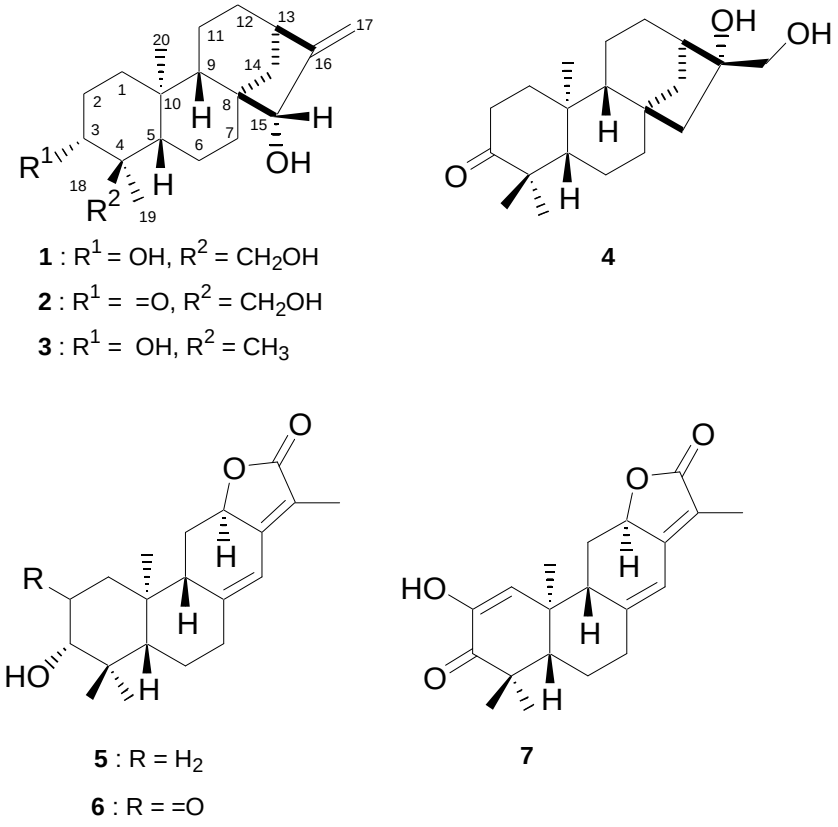

Figure 1. Structures of compounds from *Suregada multiflora*

mRNA expression of helioscopinolide A (**5**)

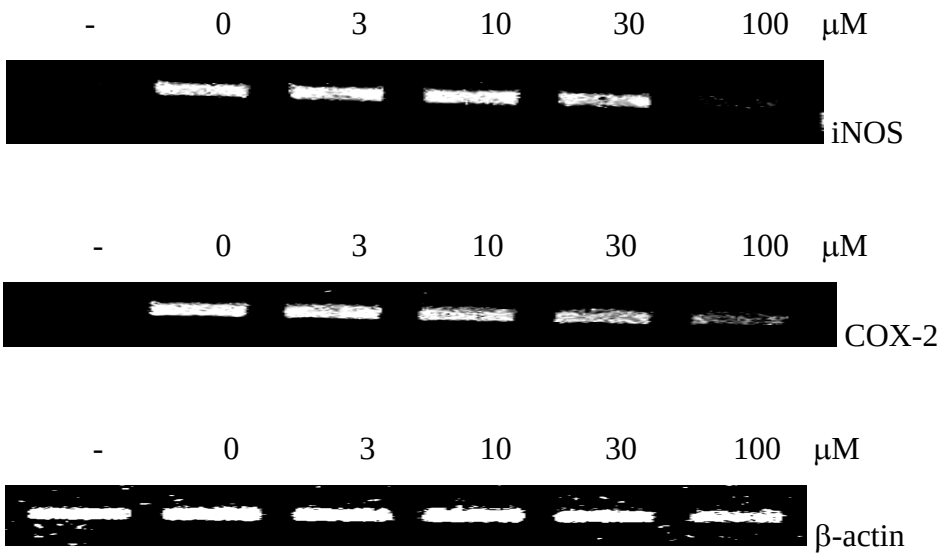

Figure 2. Effect of helioscopinolide A (**5**) at various concentrations (0, 3, 10, 30, 100 μM) on mRNA expressions of iNOS and COX-2 by LPS-induced NO and PGE_2 releases in RAW264.7 cells.

(-) = LPS (-), sample (-)

(+) = LPS (+), sample (-)

3-100 μM = LPS (+), sample (+)

Part III. Anti-inflammatory activity of compounds from *Heritiera littoralis*

Introduction

Heritiera littoralis Dry., locally known in Thai as Ngon kai thale, is the mangrove that widely distributed in East Africa and Madagascar (Tomlinson, 1986). In Thailand, *Heritiera littoralis* has been found in the eastern and southern parts. This plant is a substantial tree (20 to 25 m height) and is typically found in the mangrove zones which are upstream and low salinity areas. The bark is grayish, fissured and scaly. In terms of medicinal uses, the Vietnameses use the seeds to treat diarrhea and dysentery by decoction (Bamroongrugsa, 1999), whereas the local fishermen in Philippines use the sap as fish poison (Miles, 1991).

Since the extract of *Heritiera littoralis* possessed high NO inhibitory effect (18.8 μg/ml), the compounds from this plant were then isolated and tested for NO, PGE_2 and TNF-α inhibitory activities, as well as the mechanism on iNOS and COX-2 mRNA expressions of active compounds using RAW264.7 macrophage cells.

Materials and methods

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).

Plant material

Heritiera littoralis bark was collected from Songkhla province, Thailand in November 2004 and was identified by Prof. Puangpen Sirirugsa, Department of

Biology, Faculty of Science, Prince of Songkla University. The specimen (No. CD01) was deposited at Prince of Songkla University Herbarium.

Extraction and isolation of compounds from Heritiera littoralis extract

The air-dried bark of *H. littoralis* (6.0 kg) was extracted with hexane (2 x 30 L), CH_2Cl_2 (2 x 25 L) and acetone (2 x 25 L) successively for 5 days at room temperature. The extract was filtered and concentrated under *vacuo* to give crude extract of hexane (30.7 g), dichloromethane (32.0 g) and acetone fractions (44.0 g), respectively.

The hexane fraction (30.7 g) was further isolated by quick column chromatography (QCC) using silica gel and eluted with hexane and increasing polarity with CH_2Cl_2 and MeOH to obtain 8 fractions (A1-A8). Fraction A2 was further isolated by column chromatography (CC) using silica gel and eluted with hexane to give compounds **1** (936.6 g), **14** (4.5 mg) and **15** (20.2 mg), respectively. Fraction A4 was purified by CC using silica gel and washed with hexane which finally afforded compounds **7** and **8** (mixture, 7.6 mg) and **9** (43.3 mg), respectively. Fraction A6 was further isolated by CC using silica gel and eluted with 90% CH_2Cl_2 in hexane to obtain compound **2** (10.4 mg). Fraction A7 was separated by QCC, and Sephadex LH20 to afford compounds **5** (4.6 mg), **10** (13.2 mg) and **11** (9.4 mg), respectively.

Dichloromethane fraction (32.0 g) was separated by QCC using silica gel and eluted with hexane and increasing polarity with dichloromethane and methanol, successively to afford seven fractions B1-B7. Fraction B2 was subjected to CC using 15% acetone and hexane to give compound **1** (115.7 mg). Fraction B3 was washed with hexane to afford compound **2** (14.0 mg). Fraction B4 was subjected to QCC and eluted with hexane and increasing polarity with ethyl acetate to give seven subfractions. Subfraction B4 was washed with hexane to yield compounds **3** (128.6 mg) and **6** (4.5 mg). Fraction B6 was separated by CC using hexane and increasing polarity with ethyl acetate to afford six subfractions. Subfraction B6 was purified by CC using 10% ethyl acetate in dichloromethane to give compounds **13** (8.3 mg) and **16** (14.4 mg).

Acetone fraction (44.0 g) was purified by QCC using silica gel and washed with gradient eluent of hexane, dichloromethane and methanol to obtain six fractions (C1-C6. Fraction C2 was separated by QCC using 70% dichloromethane in hexane to

afford three subfractions. Subfraction C2 was washed with hexane to obtain compounds **3** (33.3 mg) and **4** (6.6 mg). Fraction C3 was purified by QCC using 3% methanol in dichloromethane to obtain three subfractions. Subfraction C3 was rechromatographed on CC using 30% ethyl acetate in hexane as eluent to afford compound **17** (9.4 mg). Fraction C4 was washed with hexane and crystallized with 50% methanol in dichloromethane to give compound **12** (31.5 mg). Fraction C5 was purified by QCC using hexane and increasing polarity with acetone as eluent to afford five subfractions. Subfraction C5 was crystallized with 80% methanol in dichloromethane to afford compound **18** (30.0 mg). All these compounds were identified by comparison of their spectroscopic data with those reported in the literatures (Ahad et al. 1991; Arai et al. 1988; Ali et al. 2001; Castola et al. 2002; Cheenpracha et al. 2004; Chu et al. 2005; David et al. 2004; Deachathai, 2005; Dela Greca et al. 1990; Elix et al. 1997; Macias et al. 1994; Martinez et al. 1988; Miles et al. 1991; Moiteiro et al. 2001; Rosecke et al. 2000; Thongdeeying et al. 2005; Vardamidesa et al. 2003; Yue et al. 2001).

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 200 μg/ml of LPS together with the test samples at various concentrations (3-100 μg/ml for crude extract 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, CAPE and indomethacin 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 (-)].

Inhibitory effects on LPS-induced PGE_2 and TNF-α releases 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 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 200 μg/ml of LPS together with the test samples at various concentrations (3-100 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.

Total RNA isolation and RT-PCR

In order to acquire the mechanism of action on cytokine release of ergosterol peroxide (**13**), the assays for mRNA expression of iNOS and COX-2 were carried out. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of

incubation with samples in various concentrations (3, 10, 30, 100 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis using first strand cDNA synthesis kit (Rever Tra Ace-α, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers for iNOS and COX-2 were used (forward primer for iNOS : 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGCTC-3′; forward primer for β-actin (an internal standard): 5´-TGTGATGGTGGGAATGGGTCAG-3´ and reverse primer: 5´-TTTGATGTCACGCACGATTTCC-3´.

The solution for cDNA synthesis consisted of RNA solution 11 μl, 5 x 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 (reverese transcriptase enzyme) 1 μl for a 20 μl reaction. The condition for cDNA synthesis was as follow; 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. After that, 1/10 times (2 μl) of cDNA product was used further for PCR. The PCR mixture consisted of RT reaction mixture (cDNA product) 2 μl; sterilized water 85 μl, 10 x PCR buffer 10 μl, forward primer (10 pmol/ μl) 1 μl, reverse primer (10 pmol/ μl) 1 μl and KOD Dash (polymerase enzyme)1 μl for final volume of 100 μl. The condition for PCR was as follow; denaturation at 94 °C for 1 min, 98 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were analyzed in 1.2 % agarose gel electrophoresis and visualized by SYBR safe staining and UV irradiation under a wavelength of 312 nm.

Statistical analysis

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 programme. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

Results and discussion

Compounds (Figure 1) from the hexane, dichloromethane and acetone extracts of *Heritiera littoralis* bark were investigated for their nitric oxide (NO) inhibitory effects using RAW264.7 macrophage cells. The result indicated that ergosterol peroxide (**13**) exhibited the highest activity against NO release with an IC_{50} value of 2.5 μM, followed by 6-α-hydroxystigmast-4-en-3-one (**11**, IC_{50} = 9.5 μM) and stigmast-4-en-3-one (**9**, IC_{50} = 15.9 μM), whereas other compounds showed moderate and mild effects (25.4- > 100 μM) (Table 1). The effect of ergosterol peroxide (**13**) against NO release was higher than that of CAPE, an NF-κB inhibitor (IC_{50} = 5.6 μM), indomethacin, a non-steroidal anti-inflammatory drug (NSAID, IC_{50} = 25 μM) and L-NA, a NO synthase inhibitor (IC_{50} = 61.8 μM). Ergosterol peroxide (**13**) and 6-α-hydroxystigmast-4-en-3-one (**11**) were also tested against prostaglandin E_2 (PGE_2) and tumor necrosis factor alpha (TNF-α) releases. It was found that ergosterol peroxide (**13**) possessed marked activity against PGE_2 release with an IC_{50} value of 28.7 μM, while 6-α-hydroxystigmast-4-en-3-one (**11**) was 86.7 μM (Table 2). However, these two compounds were inactive towards TNF-α release (IC_{50} > 100 μM). Compound **13** was also examined for its anti-inflammatory mechanism against mRNA expressions. The mechanism in transcriptional level of ergosterol peroxide (**13**) was found to down regulate mRNA expressions of iNOS and COX-2 in dose-dependent manners (Figure 2). Ergosterol peroxide (**13**), a steroidal derivative, has been reported to show marked anti-cancer activity against MCF-7 human breast cancer cells (Ioannou et al. 2007). However, the inhibitory effect of this compound on inflammatory mediators including NO, PGE_2 and TNF-α releases have not yet been studied.

The present study may support the use of *Heritiera littoralis* bark for treatment of inflammatory-related diseases. The anti-inflammatory effect of this plant is mainly due to the inhibition on NO and PGE_2 releases through down regulation of iNOS and COX-2 mRNA expressions.

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Table 1. NO inhibitory activity of compounds **1-16** from *Heritiera littolaris* bark

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
(1) Friedelin	0.0 ± 3.1	-	-	-	47.8 ± 3.1**	>100
(**2**) 3-α-Hydroxyfriedelan-2-one	0.0 ± 4.2	-	-	-	6.4 ± 2.5	>100
(**3**) Cerin	0.0 ± 4.2	-	-	-	36.4 ± 1.9**	>100
(**4**) Friedelan-3-one-29-ol	0.0 ± 3.1	-	9.2 ± 2.4	66.8 ± 4.1**	97.9 ± 1.1[b]**	25.4
(**5**) Betulinic acid	0.0 ± 3.1	-	-10.3 ± 1.8	37.0 ± 3.1**	84.4 ± 1.9**	42.5
(**6**) 3-β-*O*-E-feruloyl oleanolic acid	0.0 ± 4.5	-	15.3 ± 2.7	33.4 ± 3.6*	65.1 ± 5.6**	54.1
(**7**) β-Sitosterol + (**8**) Stigmasterol	0.0 ± 6.3	-	1.0 ± 7.5	6.0 ± 4.1	85.8 ± 1.3**	64.7
(**9**) Stigmast-4-en-3-one	0.0 ± 4.5	-	26.6 ± 2.6*	87.1 ± 2.6**	94.7 ± 1.5[b]**	15.9
(**10**) 6-β-Hydroxystigmast-4-en-3-one	0.0 ± 4.2	-	-	-	36.4 ± 1.9**	>100
(**11**) 6-α-Hydroxystigmast-4-en-3-one	0.0 ± 3.8	25.2 ± 3.1*	50.4 ± 4.0**	65.4 ± 6.5**	89.7 ± 0.6[b]**	9.5
(**12**) β-Sitosterol glucopyranoside	0.0 ± 4.5	-	10.2 ± 2.1	23.5 ± 2.6	58.5 ± 4.9**	77.4
(**13**) Ergosterol peroxide	0.0 ± 4.5	50.2 ± 2.3**	85.4 ± 2.2**	98.7 ± 1.2[b]**	99.1 ± 1.0[b]**	2.5
(**14**) Physcion	0.0 ± 1.6	-	-	-	24.6 ± 1.5**	>100
(**15**) Methyl β-orinol carboxylate	0.0 ± 1.6	-	-	-	23.6 ± 1.9**	>100
(**16**) Vallapin	0.0 ± 3.8	-	19.9 ± 4.3	32.4 ± 4.6	66.4 ± 0.5**	51.9
(**17**) 5-Propylresorcinol	0.0 ± 1.6	-	-	-	22.9 ± 1.9**	>100
(**18**) (-)-Epicatechin	0.0 ± 3.8	-	-	-	9.7 ± 1.8	>100
L-Nitroarginine (L-NA)	0.0 ± 9.9	11.7 ± 4.6	20.2 ± 5.9	34.7 ± 1.8 *	71.6 ± 2.6**	61.8
Caffeic acid phenethylester (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

[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. Anti-PGE_2 and TNF-α production of compounds **11** and **13** from *Heritiera littoralis* bark

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
Against PGE_2						
(**11**) 6-α-Hydroxystigmast-4-en-3-one	0.0 ± 3.0	10.7 ± 0.7	20.4 ± 1.1	36.3 ± 0.8**	51.6 ± 1.5**	86.7
(**13**) Ergosterol peroxide	0.0 ± 3.0	31.6 ± 1.6	46.2 ± 0.2**	48.0 ± 0.3**	59.3 ± 1.0**	28.7
Against TNF-α						
(**11**) 6-α-Hydroxystigmast-4-en-3-one	0.0 ± 2.8	-	4.1 ± 1.3	3.2 ± 1.5	7.7 ± 1.0	>100
(**13**) Ergosterol peroxide	0.0 ± 2.8	-	4.5 ± 1.2	4.1 ± 0.9	16.0 ± 1.5*	>100

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

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

Legend of figures

Figure 1. Structures of compounds from *Heritiera littoralis* bark

Figure 2. Effect of ergosterol peroxide (**13**) at various concentrations (0, 3, 10, 30, 100 μM) on mRNA expressions of iNOS and COX-2 by LPS-induced NO and PGE_2 releases in RAW264.7 cells.

(-) = LPS (-), sample (-)

(+) = LPS (+), sample (-)

3-100 μM = LPS (+), sample (+)

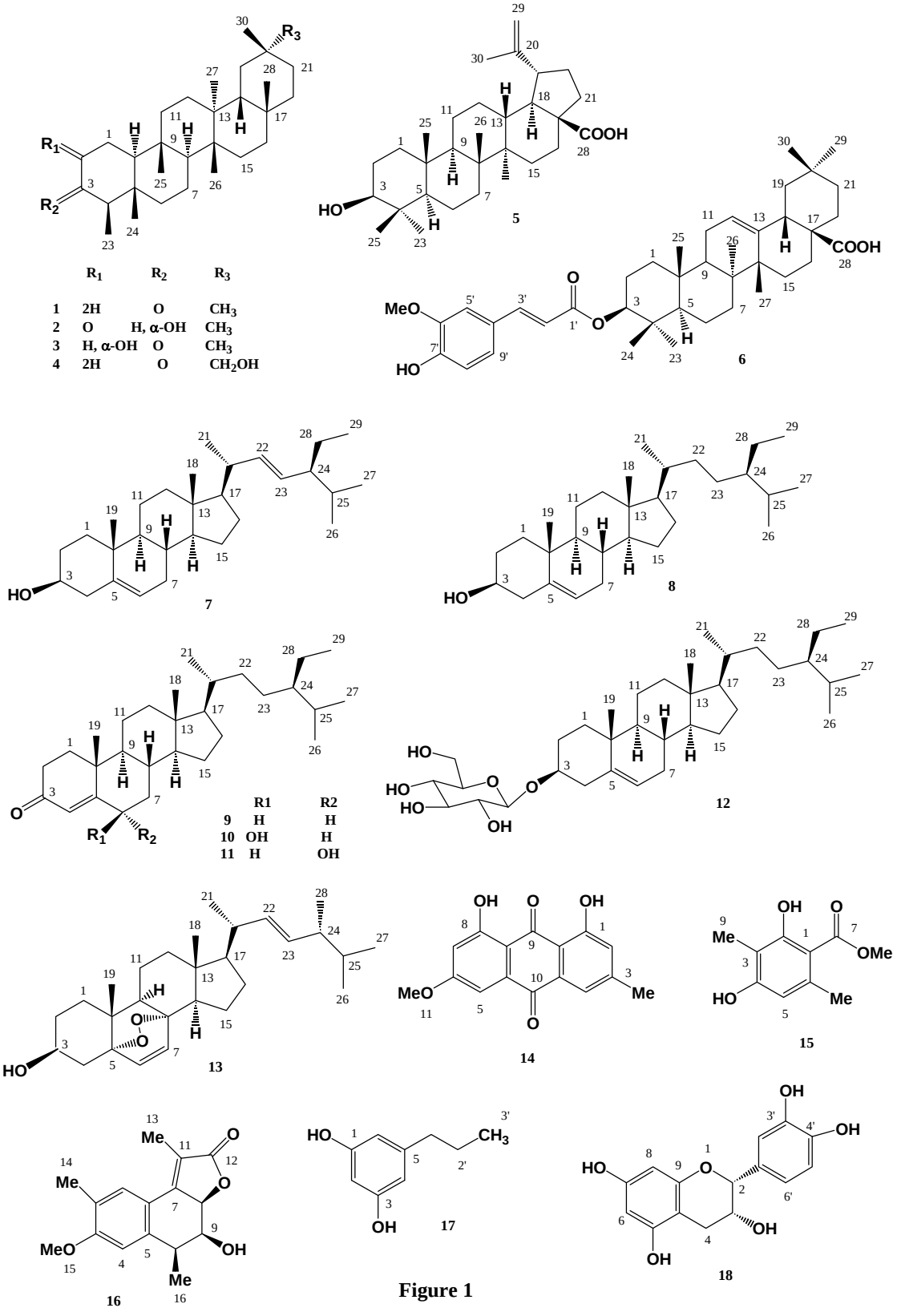

Figure 1

Figure 1. Structures of compounds from *Heritiera littoralis* bark

mRNA expression of ergosterol peroxide (**13**)

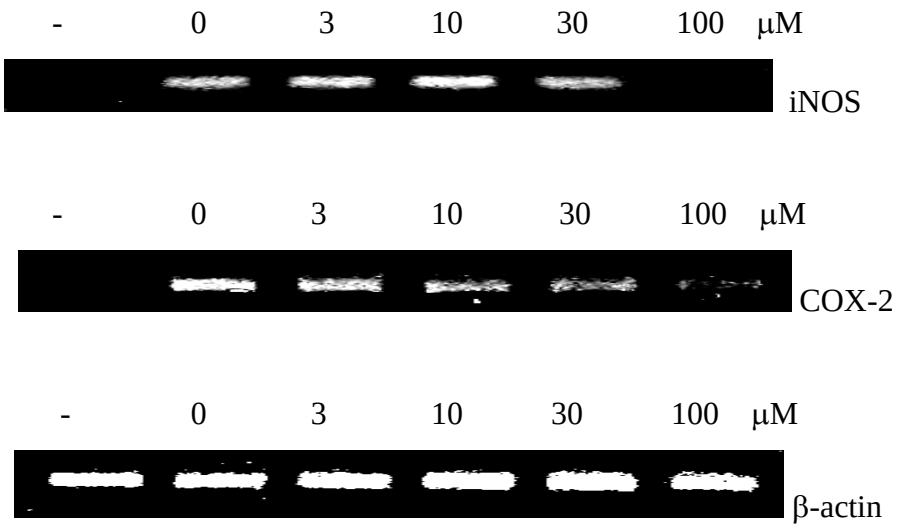

Figure 2. Effect of ergosterol peroxide (**13**) at various concentrations (0, 3, 10, 30, 100 μM) on mRNA expressions of iNOS and COX-2 by LPS-induced NO and PGE_2 releases in RAW264.7 cells.

(-) = LPS (-), sample (-)

(+) = LPS (+), sample (-)

3-100 μM = LPS (+), sample (+)

Outputs from this research work

1. Among the isolated compounds of *Eclipta prostrata*, orobol exhibited the highest activity against NO release with an IC_{50} value of 4.6 μM. The IC_{50} value of orobol against PGE_2 release was found to be 49.6 μM. For *Suregada multiflora*, helioscopinolide A exhibited the highest activity against NO release with an IC_{50} value of 9.1 μM. The IC_{50} value of helioscopinolide A against PGE_2 production was found to be 46.3 μM. Whereas ergosterol peroxide from *Heritiera littoralis* exhibited the highest activity against NO release with an IC_{50} value of 2.5 μM. It was also found that ergosterol peroxide possessed marked activity against PGE_2 release with an IC_{50} value of 28.7 μM.
2. The mechanism in transcriptional level of orobol, helioscopinolide A and ergosterol peroxide was found to down regulate mRNA expressions of iNOS and COX-2 in a dose-dependent manner.
3. The present study supports the uses of these three plants for treating inflammatory-related diseases.

Publications from this research work

1. Tewtrakul S, Subhadhirasakul S, Tansakul P, Cheenpracha S, Karalai C. *Anti-inflammatory constituents from Eclipta prostrata using RAW264.7 macrophage cells.* Phytotherapy Research. **2011**; 25: 1313-1316.
2. Tewtrakul S, Subhadhirasakul S, Cheenpracha S,Yodsaoue O, Ponglimanont C, Karalai C. *Anti-inflammatory principles of Suregada multiflora against nitric oxide and prostaglandin E_2 releases.* Journal of Ethnopharmacology. **2011**; 133:63-66.
3. Tewtrakul S, Tansakul P, Daengrot C, Ponglimanont C, Karalai C. *Anti-inflammatory principles from Heritiera littoralis bark.* Phytomedicine. **2010**; 17: 851-855

Appendix

PHYTOTHERAPY RESEARCH
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Antiinflammatory Constituents from *Eclipta prostrata* using RAW264.7 Macrophage Cells

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The whole plant extract of *Eclipta prostrata* and its isolated compounds were tested for their antiinflammatory effects against lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E_2 (PGE_2) and tumor necrosis factor-alpha (TNF-α) release in RAW264.7 cells, as well as for the antiinflammatory mechanism of the active compound on mRNA expression. Among the isolated compounds, orobol (5) exhibited the highest activity against NO release with an IC_{50} value of 4.6 μM, followed by compounds 1, 2 and 4 with IC_{50} values of 12.7, 14.9 and 19.1 μM, respectively. The IC_{50} value of compound 5 against PGE_2 release was found to be 49.6 μM, whereas it was inactive towards TNF-α (IC_{50}>100 μM). The mechanism of orobol (5) was found to down-regulate iNOS and COX-2 mRNA expression in a concentration-dependent manner. The present study may support the traditional use of *Eclipta prostrata* for the treatment of inflammatory-related diseases.

Keywords: NO; inflammatory mediators; *Eclipta prostrata*; Compositae; orobol.

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 the treatment of skin diseases. The stem has been used as a blood tonic and for the treatment of abscess, itching, haemorrhoid, anaemia, tuberculosis, amoebiasis and asthma, whereas the root has been used as an 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), anti-HIV-1 integrase and HIV-1 protease (Tewtrakul *et al*., 2007), antiinflammatory (Kobori *et al*., 2004), hepatoprotective (Han *et al*., 1998) and antimicrobial activities (Wiart *et al*., 2004).

Since the CH_2Cl_2 and MeOH extracts of *Eclipta prostrata* possessed a marked NO inhibitory effect, compounds from this plant were further isolated and tested for NO, PGE_2 and TNF-α inhibitory activities. In addition, the mechanism at the transcriptional level of the active compounds against iNOS and COX-2 mRNA using lipopolysaccharide(LPS)-stimulated RAW264.7 cells was also examined.

MATERIALS AND METHODS

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). The 96-well microplates were obtained from Nunc (Nunc, Birkrød, Denmark). The ELISA test kits for 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).

Plant material. Whole plants of *Eclipta prostrata* were collected in August 2004 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 the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Extraction and isolation. The dried whole plants (250.0 g) of *Eclipta 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 separated by CC with 30% CH_2Cl_2: hexane to afford **4** (4.6 mg).

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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 Singh, 1988; Sashida *et al*., 1983; Kosuge *et al*., 1985).

Assay for NO inhibitory effect from RAW264.7 cells. The inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that reported previously (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 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 (3–100 μg/mL for crude extract and 3–100 μM for pure compounds) and it was then incubated for 48 h. The NO production was determined by measuring the accumulation of nitrite in the culture supernatant using 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 0.04M 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, CAPE and indomethacin 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):

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

Where $A-C$ is the NO_2^- concentration (μM), A is LPS (+), sample (-); B is LPS (+), sample (+); C is LPS (-), sample (-).

Inhibitory effects on LPS-induced PGE_2 and TNF-α releases 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 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 fresh medium containing 200 μg/mL of LPS together with the test samples at various concentrations (3–100 μM) and 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 sample was dissolved in DMSO, and the solution was added to RPMI. The inhibition of PGE_2 and TNF-α release was calculated and the IC_{50} values were determined graphically.

Total RNA isolation and RT-PCR. In order to determine the mechanism of action of cytokine release by orobol (**5**), assays for mRNA expression of iNOS and COX-2 were carried out. The total RNA was isolated from RAW264.7 cells and harvested after 20 h of incubation with samples at various concentrations (3, 10, 30, 100 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd, USA). The total RNA from each sample was used for cDNA synthesis using a first strand cDNA synthesis kit (Rever Tra Ace-α, Toyobo Co., Ltd, Japan), followed by RT-PCR (Rever Tra Dash, Toyobo Co., Ltd, Japan). The primers for iNOS and COX-2 were used (forward primer for iNOS: 5′-ATCTG GATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTA CAGCTC-3′; forward primer for β-actin (an internal standard): 5′-TGTGATGGTGGGAATGGGTCAG-3′ and reverse primer: 5′-TTTGATGTCACGCACGATTTCC-3′.

The solution for cDNA synthesis consisted 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. The condition for cDNA synthesis were as follows: 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. After that, 1/10 times (2 μL) of cDNA product was used further for PCR. The PCR mixture consisted of RT reaction mixture (cDNA product) 2 μL; sterilized water 85 μL, 10×PCR buffer 10 μL, forward primer (10 pmol/ μL) 1 μL, reverse primer (10 pmol/ μL) 1 μL and KOD Dash (polymerase enzyme) 1 μL for final volume of 100 μL. The conditions for PCR were as follows: denaturation at 94 °C for 1 min, 98 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were analysed in 1.2% agarose gel electrophoresis and visualized by SYBR safe staining and UV irradiation.

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.

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 methanol fraction were orobol (**5**) and wedelolactone (**6**) (Fig. 1). Among these isolated compounds, orobol (**5**) exhibited the most potent activity against NO release with an IC_{50} value of 4.6 μM, followed by **1**, **2** and **4** with IC_{50} values of 12.7, 14.9 and 19.1 μM, respectively; whereas those of other compounds (**3** and **6**) showed moderate effects ranging from 23.3 to 27.2 μM (Table 1). The effect of **5** against NO release was higher than that of CAPE, an NF-κB inhibitor (IC_{50}=5.0 μM), and indomethacin, a non-steroidal antiinflammatory drug (NSAID, IC_{50}=20.1 μM) as well as L-NA, a NO synthase inhibitor (IC_{50}=59.0 μM). The IC_{50} value of **5** on PGE_2 release was found to be 49.6 μM, whereas it was inactive towards TNF-α (IC_{50}>100 μM) (Table 2). Orobol (**5**), the most potent compound, was further investigated for its antiinflammatory mechanism. The results showed that the mechanism in the transcriptional level of compound **5** was found to inhibit iNOS and COX-2 mRNA expression in a concentration-dependent manner (Fig. 2). Regarding the biological activities of *Eclipta prostrata*, the extract of this plant has been reported to scavenge hydroxyl and peroxyl radicals in an *in vitro* system (Yang *et al*., 2008) and possessed an antiproliferative effect against hepatic stellate cells which is a key role in the pathogenesis of liver fibrosis (Lee *et al*., 2008). Orobol (**5**), an isoflavone derivative, has also been reported to show marked HIV-1 IN inhibitory activity (Tewtrakul *et al*., 2007). However,

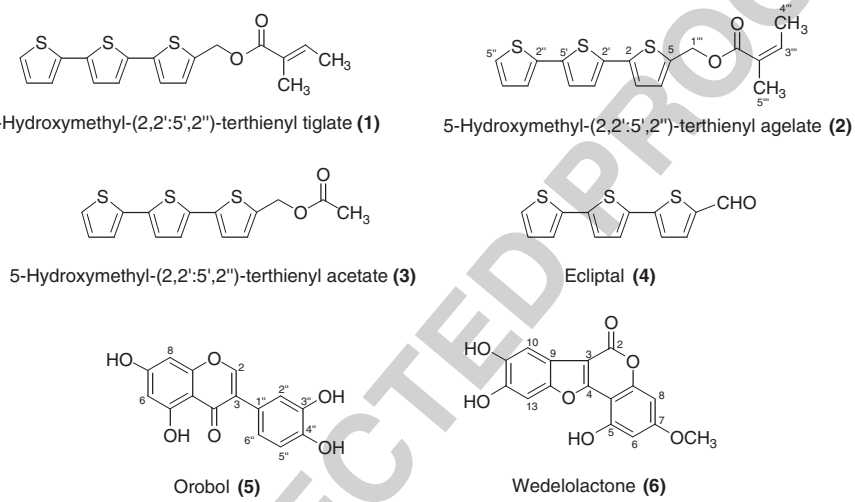

Figure 1. Structures of compounds from *Eclipta prostrate*.

Table 1. NO inhibitory production of compounds isolated from *Eclipta prostrata* (A)

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
5-Hydroxymethyl-(2,2´:5´,2´´)-terthienyl tiglate (**1**)	0.0±2.4	—	43.9±2.4[b]	68.9±1.6[b]	86.3±2.3[b,c]	12.7
5-Hydroxymethyl-(2,2´:5´,2´´)-terthienyl agelate (**2**)	0.0±2.4	—	35.0±3.6[a]	76.3±2.2[b]	102.4±4.4[b,c]	14.9
5-Hydroxymethyl-(2,2´:5´,2´´)-terthienyl acetate (**3**)	0.0±3.5	—	23.3±2.9	57.4±2.8[b]	96.4±6.8[b,c]	23.3
Ecliptal (**4**)	0.0±3.5	—	34.8±3.6[a]	65.4±3.8[b]	74.9±5.2[b]	19.1
Orobol (**5**)	0.0±4.8	36.5±2.4[a]	74.8±2.7[b]	82.0±1.7[b]	107.5±0.8[b]	4.6
Wedelolactone (**6**)	0.0±4.8	—	13.3±2.3	69.6±3.8[b]	78.2±3.4[b]	27.2
L-Nitroarginine (L-NA)	0.0±5.6	15.3±2.8	21.4±2.5	35.6±2.1[b]	73.2±3.5[b]	59.0
Caffeic acid phenethylester (CAPE)	0.0±5.6	35.2±3.0[a]	70.3±2.7[b]	97.6±2.4[b,c]	99.5±2.7[b,c]	5.0
Indomethacin	0.0±4.2	16.6±2.9	32.7±2.6[b]	53.4±3.0[b]	85.6±1.8[b]	20.1

Each value represents mean±SEM of four determinations.
Statistically significant, [a]$p < 0.05$, [b]$p < 0.01$.
[c]Cytotoxic effect was observed.

Table 2. Inhibition on PGE_2 and TNF-α production of orobol (5) using RAW264.7 cells

Inflammatory mediator	% Inhibition at various concentrations (μM) of orobol (**5**)					IC_{50} (μM)
	0	3	10	30	100	
PGE_2	0.0±0.8	—	32.2±1.4[b]	44.5±1.0[b]	57.7±1.2[b]	49.6
TNF-α	0.0±2.8	—	4.7±0.6	5.9±0.8	7.1±3.6	>100

Each value represents mean±SEM of four determinations.
Statistically significant, [a]$p < 0.05$, [b]$p < 0.01$.

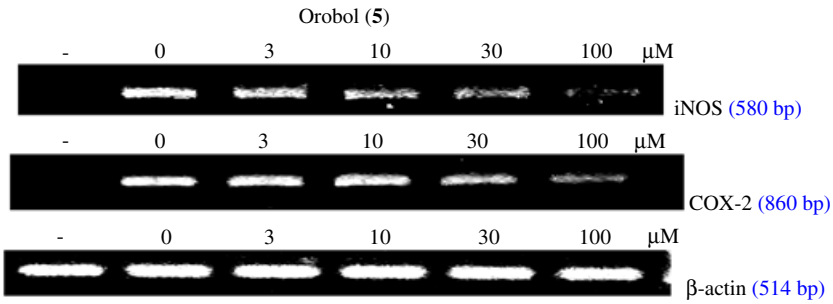

Figure 2. Effect of orobol (**5**) at various concentrations (0, 3, 10, 30, 100 μM) on mRNA expression of iNOS and COX-2 by LPS-induced NO and PGE_2 releases in RAW264.7 cells. (-) is LPS (-), sample (-); (+) is LPS (+), sample (-); 3–100 μM is LPS (+), sample (+). Q5

the inhibitory effect of orobol on inflammatory mediators including NO, PGE_2 and TNF-α releases have not yet been reported so far.

In conclusion, the present study may support the traditional use of *Eclipta prostrata* for the treatment of inflammatory-related diseases. The antiinflammatory effect of this plant is due to the inhibition on NO and PGE_2 release through down-regulation of iNOS and COX-2 mRNA expressions, whereas it does not affect the TNF-α release.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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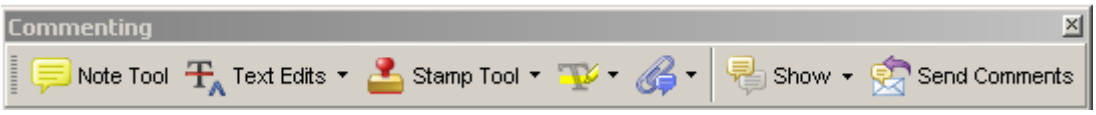

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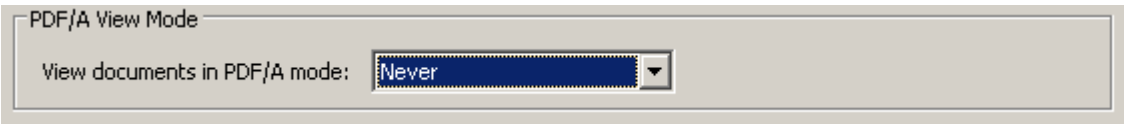

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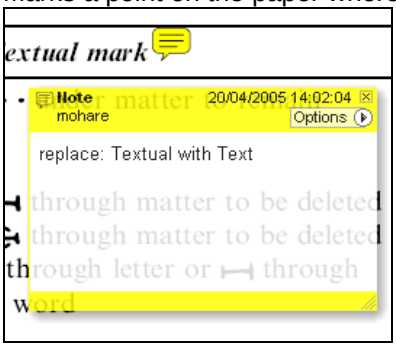

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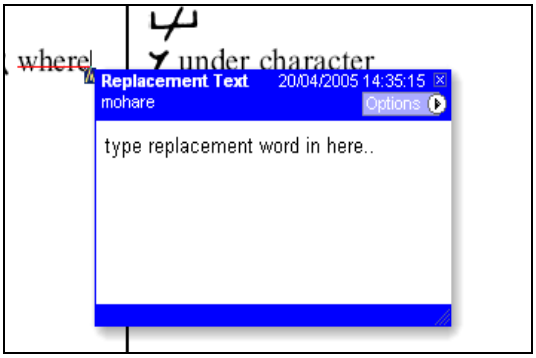

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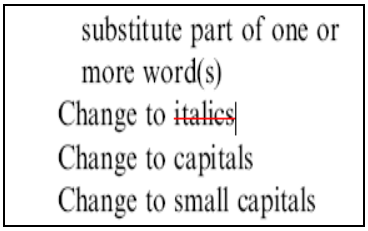

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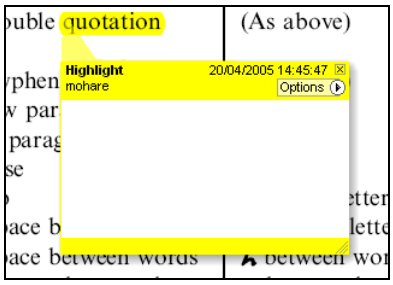

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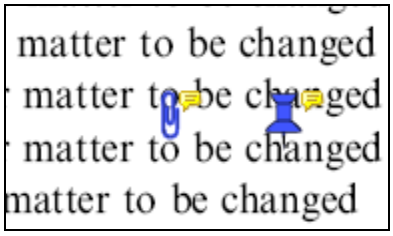

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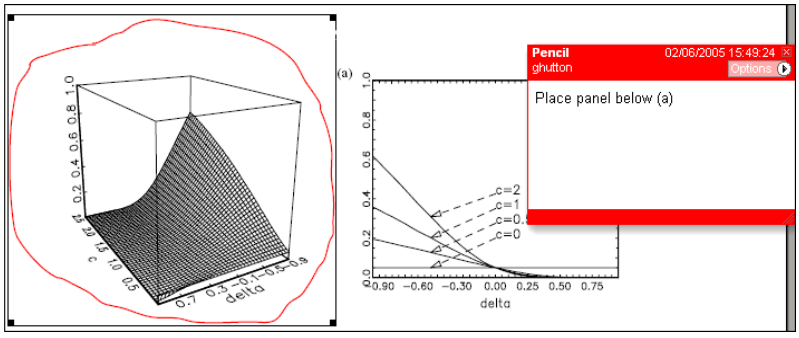

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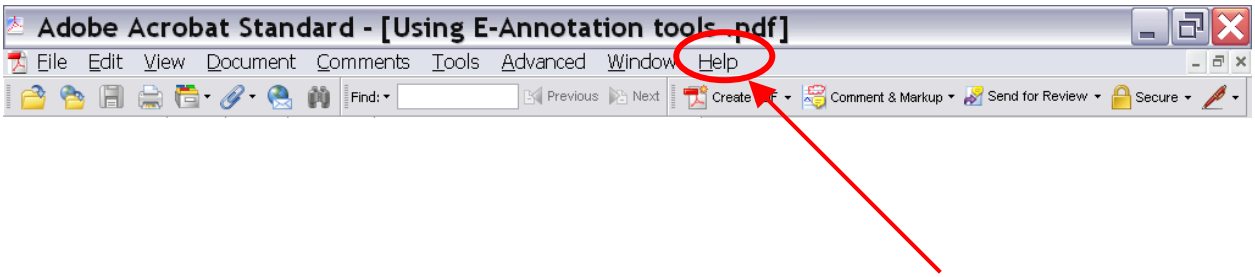

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Anti-inflammatory principles from *Heritiera littoralis* bark

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ABSTRACT

Compounds from the hexane, dichloromethane and acetone extracts of *Heritiera littoralis* bark were investigated for their nitric oxide (NO) inhibitory effects using RAW264.7 macrophage cells. The result indicated that ergosterol peroxide (**13**) exhibited the highest activity against NO release with an IC_{50} value of 2.5 μM, followed by 6-α-hydroxystigmast-4-en-3-one (**11**, IC_{50} = 9.5 μM) and stigmast-4-en-3-one (**9**, IC_{50} = 15.9 μM), whereas other compounds showed moderate and mild effects (25.4- > 100 μM). Ergosterol peroxide (**13**) and 6-α-hydroxystigmast-4-en-3-one (**11**) were also tested against prostaglandin E_2 (PGE_2) and tumor necrosis factor alpha (TNF-α) releases. It was found that ergosterol peroxide (**13**) possessed marked activity against PGE_2 release with an IC_{50} value of 28.7 μM, while 6-α-hydroxystigmast-4-en-3-one (**11**) was 86.7 μM. However, these two compounds were inactive towards TNF-α release (IC_{50} > 100 μM). The mechanism in transcriptional level of ergosterol peroxide (**13**) was found to down regulate mRNA expressions of iNOS and COX-2 in dose-dependent manners.

Introduction

Heritiera littoralis Dry., locally known in Thai as Ngon kai thale, is the mangrove that widely distributed in East Africa and Madagascar (Tomlinson 1986). In Thailand, *H. littoralis* has been found in the eastern and southern parts. This plant is a substantial tree (20–25 m height) and is typically found in the mangrove zones which are upstream and low salinity areas. The bark is grayish, fissured and scaly. In terms of medicinal uses, the Vietnameses use the seeds to treat diarrhea and dysentery by decoction (Bamroongrugsa 1999), whereas the local fishermen in Philippines use the sap as fish poison (Miles et al. 1991).

Since the extract of *H. littoralis* possessed high NO inhibitory effect (18.8 μg/ml), the compounds from this plant were then isolated and tested for NO, PGE_2 and TNF-α inhibitory activities, as well as the mechanism on iNOS and COX-2 mRNA expressions of active compounds using RAW264.7 macrophage cells.

Materials and methods

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).

Plant material

H. littoralis bark was collected from Songkhla province, Thailand in November 2004 and was identified by Prof. Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University. The specimen (No. CD01) was deposited at Prince of Songkla University Herbarium.

Extraction and isolation of compounds from H. littoralis extract

The air-dried bark of *H. littoralis* (6.0 kg) was extracted with hexane (2 × 30 l), CH_2Cl_2 (2 × 25 l) and acetone (2 × 25 l) successively for 5 days at room temperature. The extract was filtered and concentrated under *vacuo* to give crude extract of hexane (30.7 g), dichloromethane (32.0 g) and acetone fractions (44.0 g), respectively.

The hexane fraction (30.7 g) was further isolated by quick column chromatography (QCC) using silica gel and eluted with hexane and increasing polarity with CH_2Cl_2 and MeOH to obtain 8 fractions

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(A1–A8). Fraction A2 was further isolated by column chromatography (CC) using silica gel and eluted with hexane to give compounds **1** (936.6 g), **14** (4.5 mg) and **15** (20.2 mg), respectively. Fraction A4 was purified by CC using silica gel and washed with hexane which finally afforded compounds **7** and **8** (mixture, 7.6 mg) and **9** (43.3 mg), respectively. Fraction A6 was further isolated by CC using silica gel and eluted with 90% CH_2Cl_2 in hexane to obtain compound **2** (10.4 mg). Fraction A7 was separated by QCC, and Sephadex LH20 to afford compounds **5** (4.6 mg), **10** (13.2 mg) and **11** (9.4 mg), respectively.

Dichloromethane fraction (32.0 g) was separated by QCC using silica gel and eluted with hexane and increasing polarity with dichloromethane and methanol, successively to afford seven fractions B1–B7. Fraction B2 was subjected to CC using 15% acetone and hexane to give compound **1** (115.7 mg). Fraction B3 was washed with hexane to afford compound **2** (14.0 mg). Fraction B4 was subjected to QCC and eluted with hexane and increasing polarity with ethyl acetate to give seven subfractions. Subfraction B4 was washed with hexane to yield compounds **3** (128.6 mg) and **6** (4.5 mg). Fraction B6 was separated by CC using hexane and increasing polarity with ethyl acetate to afford six subfractions. Subfraction B6 was purified by CC using 10% ethyl acetate in dichloromethane to give compounds **13** (8.3 mg) and **16** (14.4 mg).

Acetone fraction (44.0 g) was purified by QCC using silica gel and washed with gradient eluent of hexane, dichloromethane and methanol to obtain six fractions (C1–C6). Fraction C2 was separated by QCC using 70% dichloromethane in hexane to afford three subfractions. Subfraction C2 was washed with hexane to obtain compounds **3** (33.3 mg) and **4** (6.6 mg). Fraction C3 was purified by QCC using 3% methanol in dichloromethane to obtain three subfractions. Subfraction C3 was rechromatographed on CC using 30% ethyl acetate in hexane as eluent to afford compound **17** (9.4 mg). Fraction C4 was washed with hexane and crystallized with 50% methanol in dichloromethane to give compound **12** (31.5 mg). Fraction C5 was purified by QCC using hexane and increasing polarity with acetone as eluent to afford five subfractions. Subfraction C5 was crystallized with 80% methanol in dichloromethane to afford compound **18** (30.0 mg). All these compounds were identified by comparison of their spectroscopic data with those reported in the literatures (Ahad et al. 1991; Arai et al. 1998; Ali et al. 2001; Castola et al. 2002; Cheenpracha 2004; Chu et al. 2005; David et al. 2004; Deachathai 2005; Della Greca et al. 1990; Elix and Wardlaw 1997; Macias et al. 1994; Martinez et al. 1988; Miles et al. 1991; Moiteiro et al. 2001; Rosecke and Konig 2000; Thongdeeying 2005; Vardamidesa et al. 2003; Yue et al. 2001).

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 U/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 (3–100 μg/ml for crude extract 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, CAPE and indomethacin 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 (−)].

Inhibitory effects on LPS-induced PGE_2 and TNF-α releases 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 U/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 (3–100 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.

Total RNA isolation and RT-PCR

In order to acquire the mechanism of action on cytokine release of ergosterol peroxide (**13**), the assays for mRNA expression of iNOS and COX-2 were carried out. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with samples in various concentrations (3, 10, 30, 100 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis using first strand cDNA synthesis kit (Rever Tra Ace-α, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers for iNOS and COX-2 were used (forward primer for iNOS: 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGCTC-3′; forward primer for β-actin (an internal standard): 5′-TGTGATGGTGGGAATGGGTCAG-3′ and reverse primer: 5′-TTTGATGTCACGCACGATTTCC-3′.

The solution for cDNA synthesis consisted 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 (reverese transcriptase enzyme) 1 μl for a 20 μl reaction. The condition for cDNA synthesis was as follow; 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. After that, 1/10 times (2 μl) of cDNA product was used further for PCR. The PCR mixture consisted of RT reaction mixture (cDNA product) 2 μl; sterilized water 85 μl, 10× PCR buffer 10 μl, forward primer (10 pmol/μl) 1 μl, reverse primer (10 pmol/μl) 1 μl and KOD Dash (polymerase enzyme)1 μl for final volume of 100 μl.

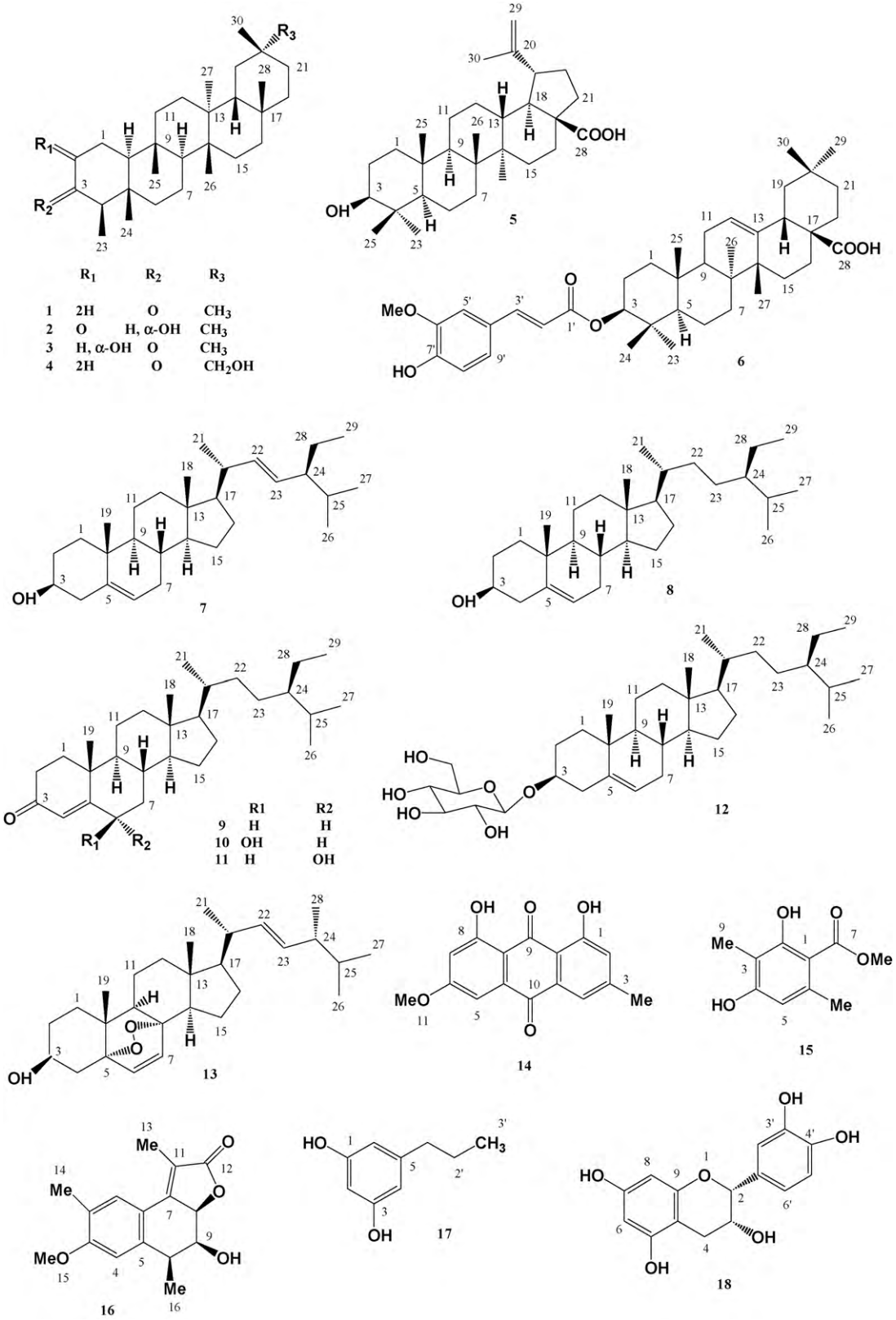

Fig. 1. Structures of compounds from *Heritiera littoralis* bark.

The condition for PCR was as follow; denaturation at 94 °C for 1 min, 98 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were analyzed in 1.2% agarose gel electrophoresis and visualized by SYBR safe staining and UV irradiation under a wavelength of 312 nm.

Statistical analysis

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 programme. The statistical significance was cal-

Table 1
NO inhibitory activity of compounds **1–16** from *Heritiera littolaris* bark.

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
(**1**) Friedelin	0.0 ± 3.1	–	–	–	47.8 ± 3.1**	>100
(**2**) 3-α-Hydroxyfriedelan-2-one	0.0 ± 4.2	–	–	–	6.4 ± 2.5	>100
(**3**) Cerin	0.0 ± 4.2	–	–	–	36.4 ± 1.9**	>100
(**4**) Friedelan-3-one-29-ol	0.0 ± 3.1	–	9.2 ± 2.4	66.8 ± 4.1**	97.9 ± 1.1b**	25.4
(**5**) Betulinic acid	0.0 ± 3.1	–	−10.3 ± 1.8	37.0 ± 3.1**	84.4 ± 1.9**	42.5
(**6**) 3-β-*O*-E-feruloyl oleanolic acid	0.0 ± 4.5	–	15.3 ± 2.7	33.4 ± 3.6*	65.1 ± 5.6**	54.1
(7) β-Sitosterol + (**8**) Stigmasterol	0.0 ± 6.3	–	1.0 ± 7.5	6.0 ± 4.1	85.8 ± 1.3**	64.7
(**9**) Stigmast-4-en-3-one	0.0 ± 4.5	–	26.6 ± 2.6*	87.1 ± 2.6**	94.7 ± 1.5b**	15.9
(**10**) 6-β-Hydroxystigmast-4-en-3-one	0.0 ± 4.2	–	–	–	36.4 ± 1.9**	>100
(**11**) 6-α-Hydroxystigmast-4-en-3-one	0.0 ± 3.8	25.2 ± 3.1*	50.4 ± 4.0**	65.4 ± 6.5**	89.7 ± 0.6b**	9.5
(**12**) β-Sitosterol glucopyranoside	0.0 ± 4.5	–	10.2 ± 2.1	23.5 ± 2.6	58.5 ± 4.9**	77.4
(**13**) Ergosterol peroxide	0.0 ± 4.5	50.2 ± 2.3**	85.4 ± 2.2**	98.7 ± 1.2b**	99.1 ± 1.0b**	2.5
(**14**) Physcion	0.0 ± 1.6	–	–	–	24.6 ± 1.5**	>100
(**15**) Methyl β-orinol carboxylate	0.0 ± 1.6	–	–	–	23.6 ± 1.9**	>100
(**16**) Vallapin	0.0 ± 3.8	–	19.9 ± 4.3	32.4 ± 4.6	66.4 ± 0.5**	51.9
(**17**) 5-Propylresorcinol	0.0 ± 1.6	–	–	–	22.9 ± 1.9**	>100
(**18**) (−)-Epicatechin	0.0 ± 3.8	–	–	–	9.7 ± 1.8	>100
L-Nitroarginine (L-NA)	0.0 ± 9.9	11.7 ± 4.6	20.2 ± 5.9	34.7 ± 1.8*	71.6 ± 2.6**	61.8
Caffeic acid phenethylester (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

[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
Anti-PGE_2 and TNF-α production of compounds **11** and **13** from *Heritiera littoralis* bark.

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
Against PGE_2 (**11**) 6-α-Hydroxystigmast-4-en-3-one	0.0 ± 3.0	10.7 ± 0.7	20.4 ± 1.1	36.3 ± 0.8**	51.6 ± 1.5**	86.7
(**13**) Ergosterol peroxide	0.0 ± 3.0	31.6 ± 1.6	46.2 ± 0.2**	48.0 ± 0.3**	59.3 ± 1.0**	28.7
Against TNF-α (**11**) 6-α-Hydroxystigmast-4-en-3-one	0.0 ± 2.8	–	4.1 ± 1.3	3.2 ± 1.5	7.7 ± 1.0	>100
(**13**) Ergosterol peroxide	0.0 ± 2.8	–	4.5 ± 1.2	4.1 ± 0.9	16.0 ± 1.5*	>100

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

culated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

Results and discussion

Compounds (Fig. 1) from the hexane, dichloromethane and acetone extracts of *H. littoralis* bark were investigated for their nitric oxide (NO) inhibitory effects using RAW264.7 macrophage cells. The result indicated that ergosterol peroxide (**13**) exhibited the highest activity against NO release with an IC_{50} value of 2.5 μM, followed by 6-α-hydroxystigmast-4-en-3-one (**11**, IC_{50} = 9.5 μM) and stigmast-4-en-3-one (**9**, IC_{50} = 15.9 μM), whereas other compounds showed moderate and mild effects (25.4 to >100 μM) (Table 1). The effect of ergosterol peroxide (**13**) against NO release was higher than that of CAPE, an NF-κB inhibitor (IC_{50} = 5.6 μM), indomethacin, a non-steroidal anti-inflammatory drug (NSAID, IC_{50} = 25 μM) and L-NA, a NO synthase inhibitor (IC_{50} = 61.8 μM). Ergosterol peroxide (**13**) and 6-α-hydroxystigmast-4-en-3-one (**11**) were also tested against prostaglandin E_2 (PGE_2) and tumor necrosis factor alpha (TNF-α) releases. It was found that ergosterol peroxide (**13**) possessed marked activity against PGE_2 release with an IC_{50} value of 28.7 μM, while 6-α-hydroxystigmast-4-en-3-one (**11**) was 86.7 μM (Table 2). However, these two compounds were inactive towards TNF-α release (IC_{50} > 100 μM). Compound **13** was also examined for its anti-inflammatory mechanism against mRNA expressions. The mechanism in transcriptional level of ergosterol peroxide (**13**) was found to down regulate mRNA expressions of iNOS and COX-2 in dose-dependent manners (Fig. 2). Ergosterol peroxide (**13**), a steroidal derivative, has been reported to show marked anti-cancer activity against MCF-7 human breast cancer cells (Ioannou et al. 2009). However, the inhibitory effect of this compound on inflammatory mediators including NO, PGE_2 and TNF-α releases have not yet been studied.

The present study may support the use of *H. littoralis* bark for treatment of inflammatory-related diseases. The anti-inflammatory effect of this plant is mainly due to the inhibition on NO and PGE_2 releases through down regulation of iNOS and COX-2 mRNA expressions.

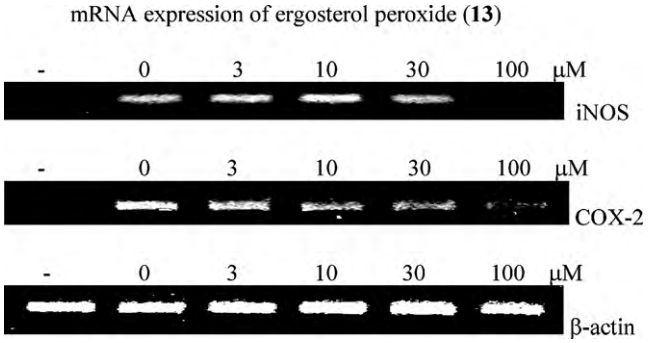

Fig. 2. Effect of ergosterol peroxide (**13**) at various concentrations (0, 3, 10, 30, 100 μM) on mRNA expressions of iNOS and COX-2 by LPS-induced NO and PGE_2 releases in RAW264.7 cells.
(−) = LPS (−), sample (−).
(+) = LPS (+), sample (−).
3–100 μM = LPS (+), sample (+).

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Anti-inflammatory principles of *Suregada multiflora* against nitric oxide and prostaglandin E_2 releases

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ABSTRACT

Aim of the study: The stem bark of *Suregada multiflora* and the isolated compounds were carried out to investigate for anti-inflammatory activity.
Materials and methods: The stem bark of *Suregada multiflora* and its isolated compounds were tested for their anti-inflammatory effects against lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostraglandin E_2 (PGE_2) releases in RAW264.7 cells as well as the anti-inflammatory mechanism on mRNA expression of the active compound (**5**, helioscopinolide A).
Results: The extract of *Suregada multiflora* possessed potent NO inhibitory effect with an IC_{50} value of 8.6 μg/ml. Among the isolated compounds, helioscopinolide A (**5**) exhibited the highest activity against NO release with an IC_{50} value of 9.1 μM, followed by helioscopinolide C (**6**) and suremulol D (**2**) with IC_{50} values of 24.5 and 29.3 μM, respectively. The IC_{50} value of **5** against PGE_2 production was found to be 46.3 μM. The mechanism in transcriptional level of compound **5** was found to inhibit iNOS and COX-2 mRNA expressions in dose-dependent manners.
Conclusions: The present study may support the traditional use of *Suregada multiflora* stem bark for treatment of inflammatory-related diseases.

1. Introduction

Suregada multiflora A. Juss. (syn. *Gelonium multiflorum*) is a plant in the Euphorbiaceae family. It grows widely throughout tropical and subtropical areas especially in Asia and Africa. In Thai traditional medicine, the bark of this plant has been used to treat hepatitis, lymphatic disorders, skin diseases, venereal diseases, fungal infections and leprosy. The wood has been used for treatment of pyrexia, eczema and venereal diseases, whereas the roots have been used for treating skin infection and lymphatic disorders (Wutthithamavet, 1997). This plant has been reported to possess anti-HIV activity and also exhibited the inhibitory effect on the infection and replication of *Herpes simplex* virus (HSV) (Bourinbaiar and Lee-Huang, 1996). The crude (CH_2Cl_2–MeOH, 1:1) extract of this plant exhibited selective cytotoxic activity against different human tumor cell lines (Jahan et al., 2002).

Since the CH_2Cl_2 extract of *Suregada multiflora* bark possessed potent NO inhibitory effect (IC_{50} = 8.6 μg/ml), the compounds from this plant were further isolated and tested for NO and PGE_2 inhibitory activities, as well as the mechanism in transcriptional level of active compound (helioscopinolide A) against iNOS and COX-2 mRNA expression using RAW264.7 cells.

2. Materials and methods

2.1. General experimental procedures

Melting points were determined on a Fisher–John melting point apparatus. The specific rotation $[\alpha]_D$ values were determined with a JASCO P-1020 polarimeter. UV spectra were measured with a SPECORD S 100 (Analytikjena). The IR spectra were measured with a Perkin-Elmer FTS FT-IR spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded using 300 MHz Bruker FTNMR Ultra Shield™ spectrometer. Chemical shifts are recorded in part per million (δ) in $CDCl_3$ with tetramethylsilane (TMS) as an internal reference. The EIMS was obtained from a MAT 95 XL mass spectrometer. Quick column chromatography (QCC) and column chromatography (CC) were carried out on silica gel 60 F_{254} (Merck) and silica gel 100 (Merck), respectively. Precoated plates of silica gel 60 F_{254} were used for analytical purposes.

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2.2. 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, 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 kit of PGE_2 was from R&D systems (R&D systems, Minnesota, USA). Other chemicals were from Sigma–Aldrich, Missouri, USA.

2.3. Plant material

Bark of *Suregada multiflora* was collected from Songkhla province, Thailand in November 2004. Identification was made by Prof. Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University and a specimen (No. SC04) deposited at Prince of Songkla University Herbarium.

2.4. Extraction and isolation

Air-dried and ground bark (5.9 kg) of *Suregada multiflora* was extracted with hexane and CH_2Cl_2 (2 × 7.5 l, for 5 days each) at room temperature. The crude extracts were evaporated under reduced pressure to afford hexane and CH_2Cl_2 extracts. The CH_2Cl_2 extract (27.3 g) was further purified by QCC using hexane as eluent with an increasing polarity obtained with acetone and MeOH to give seven fractions (F1–F7). Fraction F2 (702.9 mg) was subjected to CC with EtOAc–hexane (1:3, v/v) followed by preparative TLC with MeOH–CH_2Cl_2 (1:49, v/v) to give **2** (3.3 mg) and **4** (7.2 mg). Fraction F4 (1.8 g) was purified by CC with acetone–CH_2Cl_2 (1:49, v/v) to afford four subfractions. Subfraction F4b (67.4 mg) was separated by CC with EtOAc–hexane (3:7, v/v) to afford **5** (12.6 mg). Subfraction F4d (370.2 mg) was purified by CC with EtOAc–hexane (3:7, v/v) and followed by preparative TLC with EtOAc–hexane (3:7, v/v) to give **3** (6.2 mg), **6** (9.1 mg) and **7** (8.3 mg). Fraction F6 (1.2 g) was subjected to CC with MeOH–CH_2Cl_2 (1:19, v/v) to afford three subfractions (F6a–F6c). Subfraction F6c (327.7 mg) was further purified by CC with acetone–CH_2Cl_2 (1:9, v/v) to give **1** (22.5 mg). The structures of the isolated compounds were elucidated and compared with the previous literatures (Borghi et al., 1991; Das et al., 1994; Agrawal et al., 1995; Crespi-Perellino et al., 1996; Cheenpracha et al., 2006).

2.5. 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 U/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 50 μg/ml of LPS together with various concentrations of test samples (3–100 μg/ml for crude extract 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, CAPE and indomethacin 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 (−)].

2.6. Inhibitory effects on LPS-induced PGE_2 releases 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 U/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 50 μg/ml of LPS together with the test samples at various concentrations (3–100 μM) and was then incubated for 48 h. The supernatant was transferred into 96-well ELISA plate and then PGE_2 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 releases was calculated and IC_{50} values were determined graphically.

2.7. Total RNA isolation and RT-PCR

In order to understand the mechanism of action on cytokine release of helioscopinolide A (**5**), the assays for mRNA expression of iNOS and COX-2 were carried out. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with various concentrations (3, 10, 30, and 100 μM) of samples using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis using first strand cDNA synthesis kit (Rever Tra Ace-, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers used for iNOS and COX-2 were as follows: forward primer for iNOS: 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGCTC-3′; forward primer for β-actin (an internal standard): 5′-TGTGATGGTGGGAATGGGTCAG-3′ and its reverse primer: 5′-TTTGATGTCACGCACGATTTCC-3′.

The solution for cDNA synthesis consisted 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 (reverese transcriptase enzyme) 1 μl for a 20 μl reaction. The condition for cDNA synthesis was as follow: 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. After that, 1/10 times (2 μl) of cDNA product was used further for PCR. The PCR mixture consisted of RT reaction mixture (cDNA product) 2 μl; sterilized water 85 μl, 10× PCR buffer 10 μl, forward primer (10 pmol/μl) 1 μl, reverse primer (10 pmol/ μl) 1 μl and KOD Dash (polymerase enzyme) 1 μl for final volume of 100 μl.

The condition for PCR was as follows: denaturation at 94 °C for 1 min, 98 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were analyzed by electrophoresis in a 1.2% agarose gel and visualized by SYBR safe staining and UV irradiation at a wavelength of 312 nm.

2.8. Statistics

For statistical analysis, the values are expressed as a mean ± S.E.M. 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

Seven diterpenoids (**1**–**7**) were isolated from the bark of *Suregada multiflora* including suremulol C (**1**), suremulol D (**2**), *ent*-kaurene-3β,15β-diol (**3**), abbeokutone (**4**), helioscopinolide A (**5**), helioscopinolide C (**6**) and helioscopinolide I (**7**) (Fig. 1). Among these isolated compounds, helioscopinolide A (**5**) exhibited the most potent activity against NO release with an IC_{50} value of 9.1 μM, followed by helioscopinolide C (**6**) and suremulol D (**2**) with IC_{50} values of 24.5 and 29.3 μM, respectively, whereas other compounds showed moderate effects ranging from 30.1 to 56.3 μM (Table 1A). The effect of **5** against NO release was comparable to that of CAPE, an NF-κB inhibitor (IC_{50} = 5.6 μM) but higher than those of indomethacin, a non-steroidal anti-inflammatory drug (NSAID, IC_{50} = 25 μM) and L-NA, a NO synthase inhibitor (IC_{50} = 61.8 μM). The IC_{50} value of **5** on PGE_2 release was found to be 46.3 μM (Table 1B). Helioscopinolide A (**5**), the most potent compound, was further investigated for its anti-inflammatory mechanism in transcriptional level. It is showed that the mechanism in transcriptional level of compound **5** was found to inhibit iNOS and COX-2 mRNA expression in dose-dependent manners (Fig. 2). Helioscopinolide A (**5**) has been reported to exhibit marked anti-allergic effect in RBL-2H3 cells (Cheenpracha et al., 2006), anti-cancer activity against HeLa and MDA-MB-231 cells (Lu et al., 2008), anti-bacterial effect against *Staphylococcus aureus* (Valente et al., 2004) and acts as a CNS stimulant (Speroni et al., 1991). However, the inhibitory effect of this compound on inflammatory mediators including NO and PGE_2 releases has not been investigated.

In conclusion, the present study may support the traditional use of *Suregada multiflora* for treatment of inflammatory-related dis-

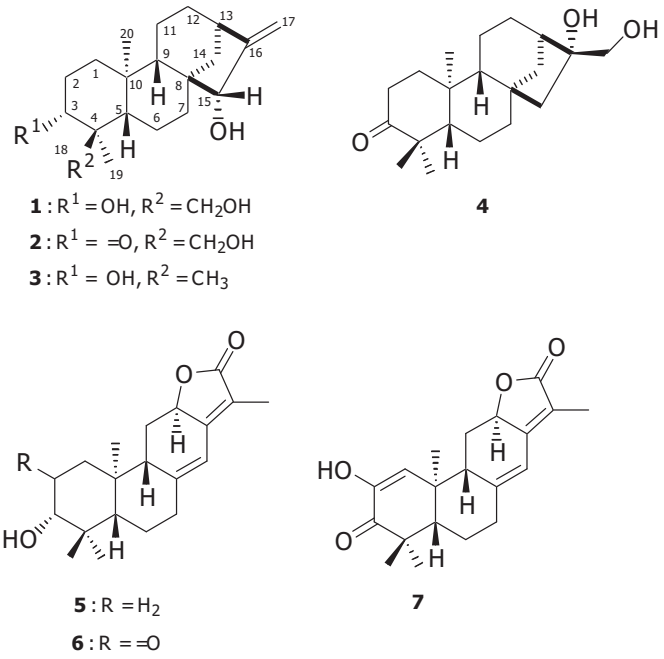

Fig. 1. Structures of compounds from *Suregada multiflora*.

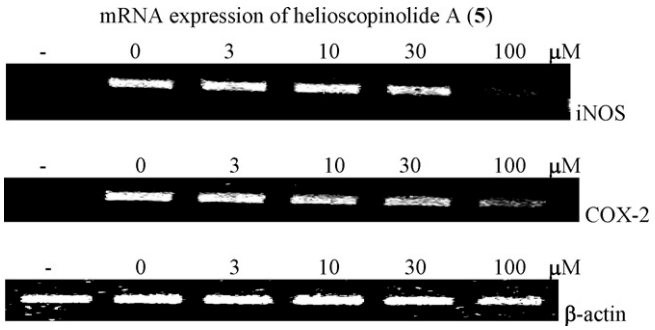

Fig. 2. Effect of helioscopinolide A (**5**) at various concentrations (0, 3, 10, 30, and 100 μM) on mRNA expressions of iNOS and COX-2 by LPS-induced NO and PGE_2 releases in RAW264.7 cells. (−) = LPS (−), sample (−); (+) = LPS (+), sample (−); 3–100 μM = LPS (+), sample (+).

Table 1
NO inhibitory production[a] of compounds isolated from *Suregada multiflora* (A) and inhibition on PGE_2 production[a] of helioscopinolide A (**5**) using RAW264.7 cells (B).

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
A						
Suremulol C (**1**)	0.0 ± 3.1	–	19.0 ± 3.1*	43.4 ± 2.4**	89.0 ± 2.8**	30.6
Suremulol D (**2**)	0.0 ± 3.1	–	19.6 ± 2.0**	44.5 ± 2.9**	91.1 ± 2.8**	29.3
ent-Kaurene-3β,15β-diol (**3**)	0.0 ± 3.1	–	23.2 ± 2.2*	45.6 ± 1.3**	83.6 ± 3.3**	30.1
Abbeokutone (**4**)	0.0 ± 3.0	–	19.1 ± 3.0*	28.3 ± 2.6**	93.9 ± 4.3**	50.0
Helioscopinolide A (**5**)	0.0 ± 3.0	23.6 ± 4.1*	50.7 ± 2.6**	82.9 ± 2.5**	100.1 ± 0.7**	9.1
Helioscopinolide C (**6**)	0.0 ± 3.0	–	26.3 ± 4.3*	53.9 ± 6.9**	89.0 ± 3.3**	24.5
Helioscopinolide I (**7**)	0.0 ± 4.0	–	13.8 ± 2.1	25.1 ± 0.7**	87.0 ± 3.5**	56.3
L-Nitroarginine (L-NA)	0.0 ± 9.9	11.7 ± 4.6	20.2 ± 5.9	34.7 ± 1.8*	71.6 ± 2.6**	61.8
Caffeic acid phenethylester (CAPE)	0.0 ± 9.9	30.7 ± 3.2*	68.6 ± 3.4**	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						
Helioscopinolide A (**5**)	0.0 ± 0.8	–	31.8 ± 0.9**	48.0 ± 0.2**	57.0 ± 0.8**	46.3

(−): not determined.
[a] Each value represents mean ± S.E.M. of four determinations.
[b] Cytotoxic effect was observed.
* Statistical significance, $p < 0.05$.
** Statistical significance, $p < 0.01$.

eases. The anti-inflammatory effect of this plant is most likely due to the inhibition on NO and PGE_2 releases through down regulation of iNOS and COX-2 mRNA expressions.

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