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*Original Article*

# Anti-inflammatory activity of compounds from *Kaempferia marginata* rhizomes

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**Abstract**

Two new pimarane diterpenes were obtained from *Kaempferia marginata* rhizomes, which are 1α-acetoxysandaracopimaradien-2-one (**1**) and 1α-acetoxysandaracopimaradiene (**4**), along with seven known compounds from the hexane and chloroform fractions including two pimarane-type diterpenes [marginatol (**5**), sandaracopimaradiene (**8**)], one kavalactone [desmethoxyyangonin (**3**)], three steroids [sitosterol-β-D-glucoside (**2**), the mixture of stigmasterol and β-sitosterol (**6** + **7**)] and one diarylheptanoid [bisdemethoxycurcumin (**9**)]. Compounds **3** and **9** exhibited potent effect against NO production with $IC_{50}$ of 10.1 and 6.8 μM, respectively. Compound **3** inhibited iNOS mRNA expression in a dose-dependent manner, while **9** suppressed both of iNOS and COX-2 genes. Moreover, compounds **2**, **3**, **6** + **7** and **9** were isolated for the first time from *K. marginata.* These results revealed that diterpenes, diarylheptanoid and kavalactone are components of *K. marginata* that afford anti-inflammatory effect through a mechanism involving a decrease in inflammatory mediators.

**Keywords:** *Kaempferia marginata*, diterpenes, diarylheptanoid, kavalactone, anti-inflammatory activity

## 1. Introduction

Macrophages play a central role in inflammatory diseases relating to over production of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukins (IL-6 and IL-1β), and generation of inflammatory mediators in response to microbial products (LPS, lipopolysaccharide), such as nitric oxide (NO) and prostaglandin $E_2$ ($PGE_2$). Thus, inhibition of the production of these inflammatory mediators is an important target in the treatment of inflammatory diseases (Gordon *et al.*, 2001; Heo *et al.*, 2010; Lin *et al.*, 2010; Lyons *et al.*, 1992; Pan *et al.*, 2011).

Nitric oxide (NO) is synthesized from L-arginine by NOS in various animal cells and tissues. The excessive production of NO also destroys functional normal tissues during acute and chronic inflammation. Therefore, inhibition of NO by phytochemicals may have potential therapeutic value related to inflammation (Chiou *et al.*, 2000; Kim *et al.*, 1999; Konkimalla *et al.*, 2010; Nakamura *et al.*, 2009; Won *et al.*, 2005). After exposure to endogenous and exogenous stimulators, iNOS can be induced quantitatively in various cells such as macrophages, smooth muscle cells and hepatocytes to trigger several disadvantageous cellular responses and cause some diseases including inflammation, sepsis, and

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stroke. (Chen *et al.*, 2001; Ko *et al.*, 2003). TNF-α plays a pivotal role in inflammation and host defense. TNF-α is a potent activator of macrophages and can stimulate the production or expression of IL-6, IL-1 and $PGE_2$. Persistent or inappropriately high TNF-α expression contributes to the inflammatory conditions, including septic shock, rheumatoid arthritis, multiple sclerosis and AIDS. Many experimental results have indicated that overproduction of TNF-α and NO resulted in excess inflammatory reactions of the human body in the inflammation process (Li *et al.*, 2007; Yang *et al.*, 2010). NF-κB regulates various genes involved in immune and acute phase inflammatory responses. NF-κB activation, in response to pro-inflammatory stimuli, involves the rapid phosphorylation of inhibitors of kappa-B (IκBs) by the IκB kinase (IKK). Free NF-κB produced by this process translocates to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes. It then induces the transcription of pro-inflammatory mediators such as iNOS, cyclooxygenase-2 (COX- 2), TNF-α, IL-1β, IL-6 and IL-8 (Lee *et al.*, 2013; Matsuda *et al.*, 2003; Yang *et al.*, 2010; Yun *et al.*, 2003).

The genus *Kaempferia* (Zingiberaceae) is one of the important medicinal plant genera in Thailand. It is a medium-sized genus with approximately 60 species, mostly distributed in India, Myanmar, China, Thailand, Laos and Cambodia. All species in this genus are used to flavor rice and as a medicine. In Thailand, the root and leaves are used by local people for curries as a flavoring and the plant is used as a medicine (Kaushita *et al.*, 2014, 2015; Picheansoonthon & Koonterm, 2008; Picheansoonthon *et al.*, 2009).

Thailand appears to be the richest biodiversity region with more than 20 extant species. Several *Kaempferia* species such as *Kaempferia galanga* rhizomes possesses a carminative effect, *K. marginata rhizomes* as an antipyretic effect, the blended leaves and rhizomes of *K. grandifolia* Saensouk and Jenjitt with husked rice acts as an anti-herpes. The rhizomes of *K. filifolia* K. Larsen are used orally for treatment of leucorrhoea by boiling with water. Rhizomes of *K. larsenii* Sirirugsa are used topically for treatment of inflammation caused by insect bite (Jenjittikul *et al.*, 2010; Picheansoonthon & Koonterm, 2008). Bioactive compounds isolated from several *Kaempferia* species are cinnamate derivatives, cyclohexane oxide derivatives, chalcone derivatives, monoterpenes, diterpenes and flavonoids (Hwang *et al.*, 2003; Jingguang *et al.*, 2001; Patanasethanont *et al.*, 2007; Techaprasan *et al.*, 2010; Thongnest *et al.*, 2005; Vincent *et al.*, 1992).

*Kaempferia marginata* Carey (Zingerberaceae, local name: Proh pa or Tup mup) is a Thai medicinal plant and its roots have been used for treatment of allergy, fever and swollen leg. The whole plant decoction of *K. marginata* is used for treatment of fever (Chuakul *et al.*, 2002; Thongnest *et al.*, 2005). Chemical constituents of *K. marginata* are reported to be diterpenes, monoterpenes, cinnamate derivatives, flavones, benzopyranes and cyclohexane oxide derivatives (Jingguang *et al.*, 2001; Kaewkroek *et al.*, 2013; Thongnest *et al.*, 2005). Our previous report demonstrated that diterpenes from *K. marginata* rhizomes inhibited NO and TNF-α releases in RAW264.7 cells. Thus, this study aimed to investigate other chemical constituents of this plant as anti-inflammatory agents, as well as the anti-inflammatory mechanism using mRNA expression.

## 2. Materials and Methods

### 2.1 Reagents

Lipopolysaccharide (LPS, from *Escherichia coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 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). Other chemicals were from Sigma Aldrich (Sigma-Aldrich, Missouri, USA).

### 2.2 Plant material and preparation of the plant extract

*K. marginata* rhizomes were bought from a local market in Bangkok in May 2010. The voucher specimens (SKP 206111301) are kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

Three kilograms dried weight of *K. marginata* rhizomes were ground and macerated with ethanol (EtOH) at room temperature, four times (6L, 4x). The EtOH extract (971.5 g, 32.38% w/w) was then concentrated and partitioned between water and hexane, and successively partitioned with chloroform and water. After that, the water layer was partitioned with ethyl acetate (EtOAc). Each partition was evaporated to dryness *in vacuo* to give residues of hexane (560.9 g, 57.33% w/w), chloroform (199.7 g, 20.55% w/w), EtOAc (57.3 g, 5.89% w/w) and water fractions (24.0 g, 2.47% w/w), respectively.

### 2.3 Isolation of compounds

The *n*-hexane fraction (80 g) was subjected to silica gel column chromatography (2400 g) by elution with *n*-hexane/EtOAc (0-100%), followed by EtOAc/MeOH (0-50%) to give twenty six fractions (H1–H26). Fraction H 4 was purified by recrystallization to give **1** (white crystals, 0.0302 g, 0.0378% w/w). Fraction H19 was purified by recrystallization to give sitosterol-β-D-glucoside (**2**, white powder, 0.0205 g, 0.0256% w/w). Fraction H14 (2.78 g) was separated by silica gel column chromatography using $CHCl_3$/MeOH (0-30%) to afford seven subfractions (H14-1–H14-7). Subfraction H14-2 was separated by silica gel column chromatography using 1% MeOH/$CHCl_3$ to afford five fractions (H14-2.1–H14-2.5). Fraction H14-2.3 was purified by preparative thin layer chromatography (PLC) using 1% MeOH/$CHCl_3$ to give desmethoxyyangonin (**3**, colorless solid, 0.0275 g, 0.0343%

w/w). Fraction H5 (1.02 g) was separated by silica gel column chromatography using $CHCl_3$/MeOH (0.5-20%) to afford twelve subfractions (H5-1–H5-12). Subfraction H5-4 gave compound **4** (colorless oil, 0.0174 g, 0.0218% w/w). Subfraction H5-10 gave marginatol (**5**, white powder, 0.0999 g, 0.1249% w/w). Fraction H8 (1.92 g) was separated by silica gel column chromatography using *n*-hexane/$CHCl_3$ (60-100%), followed by $CHCl_3$/MeOH (0-30%) to afford seven subfractions (H8-1–H8-7). Subfraction H8-5 (0.06 g) was purified by silica gel column chromatography using $CHCl_3$/MeOH (0-10%) to afford six fractions (H8-5.1–H8-5.6). Fraction H8-5.5 gave the mixture of stigmasterol and β-sitosterol **6** + **7** (white powder, 0.0148 g, 0.0185% w/w). Fraction H1 (1.60 g) was separated by silica gel column chromatography using *n*-hexane/$CHCl_3$ (1-100%), followed by $CHCl_3$/MeOH (0-20%) to afford twelve subfractions (H1-1–H1-12). Subfraction H1-3 gave sandaracopimaradiene (**8**, white powder, 0.4305 g, 0.5381% w/w).

The chloroform fraction (100.00 g) was chromatographed on silica gel (400.00 g) by vacuum liquid chromatography (VLC). The column was eluted with $CHCl_3$/MeOH (0-60%) to afford eight fractions (C1–C8). Fraction C5 (6.00 g) was subjected to silica gel column chromatography by elution with $CH_2Cl_2$/EtOAc (0-100%), followed by EtOAc/MeOH (0-30%) to afford ten fractions (C5-1-C5-10). Subfraction C5-4 (0.20 g) was separated by sephadex LH-20 using 10% $CHCl_3$/MeOH to afford 6 fractions (C5-4.1-C5-4.6). Subfraction C5-4.3 (0.08 g) was purified by silica gel column chromatography using 1% MeOH/$CHCl_3$ to afford five fractions (C5-4.3a-C5-4.3e). Fraction C5-4.3b gave bisdemethoxycurcumin (**9**, orange crystal, 0.0500 g, 0.0500% w/w).

## 2.4 Structure elucidation of compounds 1-9

The hexane and chloroform fractions of *K. marginata* rhizomes exhibited potent NO inhibitory activity with $IC_{50}$ values of 3.0 and 3.8 μg/ml, respectively. Thus, these fractions were further subjected to column chromatography and nine compounds were isolated, two new pimarane-type diterpene from the hexane fraction (**1** and **4**), along with seven known compounds including two pimarane-type diterpene (**5**, **8**), one kavalactone (**3**), three steroids (**2**, **6** + **7**) and one diarylheptanoid (**9**) from the chloroform fraction (Figure 1). These structures were elucidated by spectroscopic methods (1D and 2D NMR experiments and MS analysis) and were confirmed by comparison with published data in the literature.

Seven known compounds were assigned as 8(14),15-isopimaradiene-6α-ol (**5**; Jingguang *et al.*, 2001), sandaracopimaradiene (**8**; Touche *et al.*, 1997), desmethoxyyangonin (**3**; Dharmaratne *et al.*, 2002), sitosterol-β-D-glucoside (**2**; Khatun *et al.*, 2012), the mixture of stigmasterol and β-sitosterol (**6** + **7**; Chaturvedula *et al.*, 2012), bisdemethoxycurcumin (**9**; Jayaprakasha *et al.*, 2002; Péret-Almeida *et al.*, 2005) and two new compounds were identified as 1α-acetoxysandaracopimaradien-2-one (**1**) and 1α-acetoxysandaracopimaradiene (**4**) (Figure 1).

1α-Acetoxysandaracopimaradien-2-one (**1**): White crystals (30.2 mg); $[\alpha]_D$:+40 (*c* 0.1, $CHCl_3$); IR (KBr) $n_{max}$ 2925, 2868, 1752, 1634, 1368, 1231, 1030, 990, 904 and 880 $cm^{-1}$; EIMS $[M]^+$ *m/z* 343.9; HREIMS: *m/z* $[M]^+$ calcd for $C_{22}H_{32}O_3$: 344.2346; found: 344.2346; $^1H$ NMR (500 MHz, $CDCl_3$) and $^{13}C$ NMR (125 MHz, $CDCl_3$) data are shown in Table 1.

1α-Acetoxysandaracopimaradiene (**4**): Colorless oil (17.4 mg); $[\alpha]_D$:+14.1 (*c* 0.56, $CHCl_3$). IR (KBr) $n_{max}$ 2949, 2870, 1731, 1636, 1373, 1244, 1030, 990, 911 and 860$cm^{-1}$; FABMS $[M]^+$ 330.0; $^1H$ NMR (500 MHz, $CDCl_3$) and $^{13}C$ NMR (125 MHz, $CDCl_3$) data are shown in Table 1.

## 2.5 Experimental animals

Wistar rats (180-200 g) were used in the experiments.

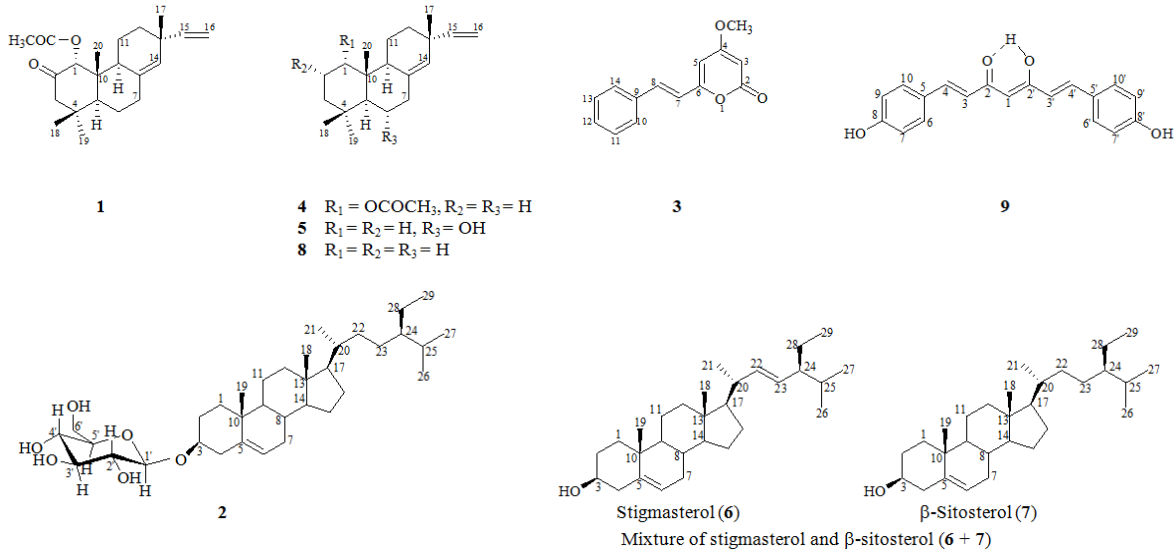



Figure 1. Structures of compounds **1-9** isolated from *Kaempferia marginata* rhizomes.

Table 1. $^{1}$H NMR (500 MHz) and $^{13}$C NMR (125 MHz) spectral data of compounds **1** and **4** in $CDCl_3$.

| Position | **1** | | | **4** | | |
|---|---|---|---|---|---|---|
| | $\delta_C$ | $\delta_H$ | HMBC | $\delta_C$ | $\delta_H$ | HMBC |
| 1 | 81.3 | 4.59 (s) | 2, 3, 5, 9, 10 | 75.0 | 4.82 (br s) | 3, 21 |
| 2 | 207.2 | - | | 22.3 | 1.75 (m), a<br>1.31-1.39 (m), b | |
| 3 | 50.9 | 2.74 (d, 12.0), a<br>1.95 (d, 12.0), b | 2, 4, 19<br>1, 2, 4, 5, 19 | 34.7 | 1.54 (td 13.7, 4.1), a<br>1.17(dt, 13.7, 3.4), b | |
| 4 | 40.5 | - | | 33.5 | - | |
| 5 | 48.5 | 1.90 (dd, 12.6, 2.7) | 10, 19, 20 | 48.7 | 1.41-1.50 (m) | 1, 6, 20 |
| 6 | 22.5 | 1.68 (m), a<br>1.35-1.46 (m), b | 7 | 22.3 | 1.70 (m), a<br>1.31-1.39 (m), b | |
| 7 | 35.2 | 2.32 (ddd, 14.1, 4.5, 2.2), a<br>2.08 (m), b | 5, 8, 9, 14<br>6, 8, 14 | 35.6 | 2.25 (ddd, 13.7, 4.0, 2.0), a<br>2.03(m), b | 8, 9, 14<br>6, 8, 14 |
| 8 | 136.9 | - | | 136.9 | - | |
| 9 | 42.6 | 2.41(t, 7.5) | 8, 10, 11, 14, 20 | 43.4 | 2.14 (t, 7.5) | 8, 10, 14 |
| 10 | 46.3 | - | | 41.0 | - | |
| 11 | 18.0 | 1.35-1.46 (m, 2H), a,b | 9, 13 | 18.1 | 1.41-1.50 (m), a<br>1.31-1.39(m), b | |
| 12 | 34.0 | 1.35-1.46 (m, 2H), a,b | 13, 14, 15 | 34.2 | 1.60 (m), a<br>1.41-1.50 (m), b | 11 |
| 13 | 37.3 | - | | 37.3 | - | |
| 14 | 129.3 | 5.34 (s) | 7, 9, 13, 17 | 129.3 | 5.26 (br s) | 7, 9, 13, 15 |
| 15 | 148.8 | 5.78 (dd, 17.7, 10.2) | 12, 13, 17 | 148.8 | 5.75 (dd, 17.4, 10.5) | 13, 17 |
| 16 | 110.1 | 4.92 (dd, 17.7, 1.3), a<br>4.89 (dd, 10.2, 1.3), b | 13 | 110.1 | 4.90 (dd, 17.4, 1.5), a<br>4.87 (dd, 10.5, 1.5), b | 13, 15<br>13 |
| 17 | 26.2 | 1.10 (s) | 12, 13, 14, 15 | 26.1 | 1.02 (s) | 12, 13, 14, 15 |
| 18 | 23.0 | 0.88 (s) | 3, 4, 5, 19 | 22.3 | 0.88 (s) | |
| 19 | 33.5 | 1.03 (s) | 3, 4, 5, 18 | 32.9 | 0.93 (s) | 4, 5, 18 |
| 20 | 13.2 | 0.82 (s) | 1, 5, 9, 10 | 15.1 | 0.85 (s) | 1, 5, 9, 10 |
| OAc | 170.6 | - | | 170.6 | - | |
| | 20.8 | 2.15 (s) | | 21.3 | 2.07 (s) | |

Animals were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Animal study protocol was approved by The Animal Ethic Committee, Prince of Songkla University (MOE 0521.11/326).

### 2.6 Carrageenan-induced hind paw edema

This model was performed according to the method described by Winter *et al.* (1962). The percent inhibition is calculated for each experiment group using the formula as follows:

$$\text{Inhibition (\%)} = \frac{(V_t - V_0)_{control} - (V_t - V_0)_{treated}}{(V_t - V_0)_{control}} \times 100$$

$V_t$ = volume of hind paw after carrageenan injection
$V_0$ = volume of hind paw before carrageenan injection

### 2.7 Cell culture

Inhibitory effect on NO production was investigated using murine macrophage-like RAW264.7 cells (purchased from Cell Lines Services) 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.

#### 2.7.1 Inhibitory effects on LPS-induced NO release using RAW264.7 cells

NO production was determined by measuring the accumulation of nitrite ($NO_2^-$) in the culture medium using Griess reagent (Tewtrakul *et al.*, 2009). CAPE (NF-κB inhibitor) and indomethacin were used as positive controls. Inhibition (%) was calculated using the following equation and $IC_{50}$ values were determined graphically ($n = 4$):

Inhibition (%) = (A – B) / (A – C) × 100
A-C: $NO_2^-$ concentration (μM) [A: LPS (+), sample (-);
B: LPS (+), sample (+); and C: LPS (-), sample (-)].

### 2.7.2 Inhibitory effects on LPS-induced TNF-α release using RAW264.7 cells

The concentration of RAW264.7 cells was adjusted to $1\times10^5$ cells/ml in the same medium as described above (Tewtrakul *et al.*, 2009). The Inhibition (%) was calculated using the following equation and $IC_{50}$ values were determined graphically ($n = 4$):

Inhibition (%) = (A – B) / (A – C) × 100
A-C: TNF-α concentration (pg/ml) [A: LPS (+), sample (-);
B: LPS (+), sample (+); and C: LPS (-), sample (-)].

### 2.7.3 Determination of iNOS, COX-2 and TNF-α mRNA expression

In order to understand the mechanism of action on cytokine release of the compounds, the assays for mRNA expression of iNOS, COX-2 and TNF-α were carried out. The primers used for iNOS, COX-2, TNF-α and β-actin were as follows:

iNOS: forward primer;
5′-ATCTGGATCAGGAACCTGAA-3′
reverse primer;
5′-CCTTTTTTGCCCCATAGGAA-3′

COX-2: forward primer;
5′- GGAGAGACTATCAAGATAGTGATC-3′
reverse primer:
5′-ATGGTCAGTAGACTTTTACAGCTC-3′

TNF-α: forward primer;
5′-TCTGTCTACTGAACTTCGGG-3′
reverse primer;
5′-AGATAGCAAATCGGCTGACG-3′

β-actin: forward primer;
5′-TGTGATGGTGGGAATGGGTCAG-3′
reverse primer;
5′-TTTGATGTCACGCACGATTTCC-3′

### 2.8 Statistical analysis

The results were expressed as a mean ± S.E.M of 4 determinations for each sample. The $IC_{50}$ values were calculated using the Microsoft Excel program. Statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

## 3. Results and Discussion

### 3.1 Isolation and structure elucidation

The hexane and chloroform fractions of *K. marginata* rhizomes were subjected to silica gel column chromatography to obtain nine compounds including two new pimarane-type diterpenes [1α-acetoxysandaracopimaradien-2-one (**1**) and 1α-acetoxysandaracopimaradiene (**4**)], two known pimarane-type diterpenes [marginatol (**5**) and sandaracopimaradiene (**8**)], one kavalactone (desmethoxyyangonin, **3**), three steroids [sitosterol-β-D-glucoside (**2**) and the mixture of stigmasterol and β-sitosterol (**6** + **7**)] and one diarylheptanoid (bis-demethoxycurcumin, **9**). This is the first report of compounds **2**, **3**, **6** + **7** and **9** from the rhizomes of *K. marginata.*

Seven known compounds **2**, **3**, **5**, **6** + **7**, **8** and **9** were identified by their spectroscopic data (1D and 2D NMR experiments and MS analysis), and are in agreement with those reported in the literatures (Chaturvedula & Prakash, 2012; Dharmaratne *et al.*, 2002; Jayaprakasha *et al.*, 2002; Jingguang *et al.*, 2001; Khatun *et al.*, 2012; Péret-Almeida *et al.*, 2005; Touche *et al.*, 1997). Two new compounds (**1** and **4**) were identified as follow;

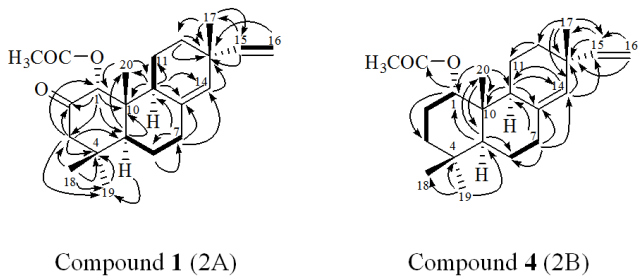
Compound **1** was isolated as white crystal and $[\alpha]_D$: +40 (*c* 0.1, $CHCl_3$). The molecular formula of $C_{22}H_{32}O_3$ was determined from high resolution electron capture mass spectrometry observed at *m/z* 344.2346 $[M]^+$. The IR spectrum showed absorptions of carbonyl ($\nu_{max}$ 1752 $cm^{-1}$). Absorption bands at $\nu_{max}$ 1634, 990 and 904 $cm^{-1}$ suggested the presence of a vinyl group, and a trisubstituted olefinic absorption appeared at $\nu_{max}$ 880 $cm^{-1}$. The $^{13}C$ NMR spectrum indicated 22 carbon signals, including five methyls, six methylenes, five methines and six quaternary carbons. The $^1H$ NMR spectrum showed the presence of four methyl groups (δ 1.10, 1.03, 0.88, 0.82), an olefinic proton as singlet (δ 5.34) which could be assigned to the trisubstituted olefinic proton (H-14) and displayed signals characteristic for the vinylic protons (H-15, H-16a and H-16b) at $\delta_H$ 5.78 (1H, dd, $J$ = 17.7, 10.2 Hz, H-15), 4.92 (1H, dd, $J$ =17.7, 1.3 Hz, H-16a) and 4.89 (1H, dd, $J$ = 10.2, 1.3 Hz, H-16b) as well as an olefinic proton at $\delta_H$ 5.34. The signal at δ2.15 (s) in the $^1H$ NMR spectrum indicated that **1** was an acetate, an acetoxyl group was present at $\delta_H$ 4.59 ($\delta_C$ 81.3), in addition to four tertiary methyl group singlet signals. On the basis of the molecular formula and the presence of two unsaturated double bonds inferred from the $^1H$ and $^{13}C$ NMR data, it was apparent that three rings were present in the molecule. The long-range $^1H$-$^{13}C$ NMR correlations between a methyl proton signal at $\delta_H$ 1.10 and the $^{13}C$ signal at $\delta_C$ 148.8 as well as correlations between a vinylic proton at $\delta_H$ 4.89 and 4.92 and a quaternary $^{13}C$ signal at $\delta_C$ 37.3 indicated a pimarane diterpene skeleton with a vinyl group attached to C-13. The COSY spectrum is shown in Figure 2A. An acetoxyl groups at C-1 and a keto group ($\delta_C$ 207.2) at C-2 were implied from the $^1H$-$^{13}C$ HMBC NMR correlations between H-1/C-2, C-3, C-5, C-9 and C-10; $H_3$-20/C-1, C-5, C-9 and C-10 and H-3a/C-2, C-4 and C-19; H-3b/C-1, C-2, C-4, C-5 and C-19, respectively. The correlations between H-14/C-7, C-9, C-13 and C-17 indicated a double bond at C-8(14) (Figure 2A). The relative stereochemistry of **1** was assigned from the NOESY experiment. The location of an acetoxyl group at C-1 was found to be α-oriented due to the NOE between H-1/$H_3$-20 and $H_3$-17/$H_3$-20, indicated that H-1

was β-oriented. From these spectral data, compound **1** was therefore elucidated as 1α-acetoxysandaracopimaradien-2-one.

Compound **4** was obtained as a colorless oil. Its specific rotation was $[\alpha]_D$:+14.1 (*c* 0.56, $CHCl_3$). FABMS showed $[M]^+$ ion at *m/z* 330.0, corresponding to the molecular formula $C_{22}H_{34}O_2$. The IR spectrum showed absorptions of carbonyl ($\nu_{max}$ 1731 $cm^{-1}$). Absorption bands at $\nu_{max}$ 1636, 990 and 911 $cm^{-1}$ suggested the presence of a vinyl group and a trisubstituted olefinic absorption appeared at $\nu_{max}$ 860 $cm^{-1}$. The $^1H$ NMR spectrum of compound **4** was similar to that of compound **1** but there were additional signals for methylene proton at C-2 position ($\delta_{H-2}$ 1.75 and 1.31-1.39). The $^1H$ and $^{13}C$ NMR spectra showed the presence of a methine carbon at $\delta_C$ 75.0 ($\delta_{H-1}$, 4.82, br s), a trisubstituted olefin at $\delta_{C-14}$ 129.3 and $\delta_{C-8}$ 136.9 ($\delta_{H-14}$, 5.26, br s), a vinyl group at $\delta_{C-16}$ 110.1 and $\delta_{C-15}$ 148.8 ($\delta_{H-15}$ 5.75, dd, *J* = 17.4, 10.5 Hz, $\delta_{H-16a}$ 4.90, dd, *J* = 17.4, 1.5 Hz and $\delta_{H-16b}$ 4.87, dd, *J* = 10.5, 1.5 Hz) and four tertiary methyl groups singlet signals at $d_H$ 1.02, 0.93, 0.88 and 0.85. The signal at δ 2.07 (s) in the $^1H$ NMR spectrum suggested that **4** was acetate protons. An acetoxyl group was present at $\delta_H$ 4.82 ($\delta_C$ 75.0). The COSY spectrum is shown in Figure 2B. Long-range correlations in the HMBC spectrum were observed between H-1/C-3, C-21 and $H_3$-20/C-1, C-5, C-9 and C-10 (Figure 2B). Detailed assignments of $^1H$ and $^{13}C$ chemical shifts were based on the COSY, HMQC and HMBC NMR experiments. The NOESY spectrum of **4** showed correlations between H-1/$H_3$-20, indicating the β-orientation of H-1 and the α-orientation of an acetoxyl groups at C-1. Compound **4** was thus assigned as 1α-acetoxysandaracopimaradiene.

### 3.2 Carrageenan-induced hind paw edema

The anti-inammatory activity was evaluated as inhibition of the carrageenan-induced rat paw edema. The result indicated that the hexane fraction of *K. marginata* rhizomes potently reduced paw edema at 5 h after carrageenan injection with 82.3% inhibition and showed higher effect than that of indomethacin, standard drug, at 10 mg/kg (58.3% inhibition at 5 h). Inflammation may be acute and chronic, the course of acute inflammation is biphasic. The initial phase is due to the release of histamine, serotonin and kinins. The second phase is related to the release of prostaglandins, protease and lysosome (Binny *et al.*, 2010). Carrageenan is a strong chemical use for the release of inflammatory mediators, generally the mediators of inflammation are histamine, PGs, LTB4, NO, platelet-activation factor (PAF), bradykinin, serotonin, lipoxins, cytokines and growth factors. Carrageenan injection into the rat paw produces inflammation resulting from plasma extravasation, increased tissue water and plasma protein exudation, along with neutrophil extravasation, due to the metabolism of arachidonic acid (Winter *et al.*, 1968). The first phase begins immediately after injection of carrageenan and diminishes in two hours, while the second phase begins at the end of first phase and remains through three to five hours. Second phase is sensitive to both the clinically useful steroidal and nonsteroidal anti-inflammatory agent. Prostaglandins are the main culprit responsible for acute inflammation (Amdekar *et al.*, 2012; Hajhashemi *et al.*, 2010; Ratheesh *et al.*, 2007). The present results suggest that hexane fraction of *K. marginata* rhizomes suppressed carrageenan-induced paw edema. In addition, our previous study demonstrated that the ethanol extract of *K. marginata* rhizomes inhibited the mRNA expression of iNOS, COX-2 and TNF-α genes in dose dependent manners (Kaewkroek *et al.*, 2013), thus we could assume that our result of *in vivo* study indicated that the hexane fraction of *K. marginata* rhizomes showed potent anti-inammatory activity through the inhibition of NO and $PGE_2$ with mild effect towards TNF-α productions.




Compound **1** (2A) Compound **4** (2B)

Figure 2. Long-range (H→C; arrows) and $^1H$-$^1H$ (bold lines) correlations of **1** (2A) and **4** (2B).

### 3.3 Inhibitory effects on LPS-induced NO production of compounds isolated from *K. marginata* rhizomes

Compounds **1**-**9** were tested for their anti-inflammatory activity using RAW264.7 cell line. The results showed that bisdemethoxycurcumin (**9**) exhibited the most potent inhibitory effect with an $IC_{50}$ value of 6.8 μM, followed by desmethoxyyangonin (**3** , $IC_{50}$ = 10.1 μM), marginatol (**5**, $IC_{50}$ = 12.1 μM), 1α-acetoxysandaracopimaradiene (**4**, $IC_{50}$ = 13.9 μM), sandaracopimaradiene (**8**, $IC_{50}$ = 18.6 μM), 1α-acetoxysandaracopimaradien-2-one (**1**, $IC_{50}$ = 40.3 μM), the mixture of stigmasterol and β-sitosterol (**6** + **7**, $IC_{50}$ = 81.7 μM) and sitosterol-β-D-glucoside (**2**, $IC_{50}$ = 94.8 μM), respectively. The inhibitory activity of **3**, **4**, **5**, **8** and **9** exhibited higher effect than that of indomethacin ($IC_{50}$ = 39.9 μM), a clinically used drug, while compound **1** ($IC_{50}$ = 40.3 μM) was comparable to that of indomethacin and compounds **2** and **6** + **7** showed weak activity (Table 2). Cytotoxicity was determined using the MTT colorimetric assay. The result showed that at the highest dose (100 μM), the cell viability was less than 80% when treated with compounds **2**, **3**, **4**, **5**, **8** and **9**; whereas compounds **1** and **6** + **7** at 30 and 100 μM showed cytotoxicity in MTT assay.

### 3.4 Inhibitory effects on LPS-induced TNF-α release using RAW264.7 cells

In investigation of the effect on TNF-α release, com-

Table 2. Inhibition of NO and TNF-α production of compounds[a] isolated from *Kaempferia marginata* rhizomes.

| Compounds | $IC_{50}$ (μM) | |
|---|---|---|
| | NO | TNF-α |
| 1α-Acetoxysandaracopimaradien-2-one (**1**) | 40.3 ± 0.4 | > 100 (2.0)[b] |
| Sitosterol-β-D-glucoside (**2**) | 94.8 ± 1.7 | - |
| Desmethoxyyangonin (**3**) | 10.1 ± 0.6 | > 100 (6.1)[b] |
| 1α-Acetoxysandaracopimaradiene (**4**) | 13.9 ± 0.3 | > 100 (7.2)[b] |
| 8(14),15-Isopimaradiene-6α-ol (**5**) | 12.1 ± 0.3 | > 100 (17.2)[b] |
| Mixture of stigmasterol and β-sitosterol (**6** + **7**) | 81.7 ± 0.4 | - |
| Sandaracopimaradiene (**8**) | 18.6 ± 0.3 | > 100 (2.1)[b] |
| Bisdemethoxycurcumin (**9**) | 6.8 ± 0.5 | > 100 (10.1)[b] |
| Indomethacin | 39.9 ± 0.6 | > 100 (2.5)[b] |
| CAPE | 3.2 ± 0.4 | 75.0 ± 0.4 |

[a]Each value represents mean ± S.E.M. of four determinations.
(-) = not determined
[b]Values in parenthesis are %inhibition at 100 μM.

pounds **1**, **3**, **4**, **5**, **8** and **9** showed weak activity ($IC_{50}$ > 100 μM) (Table 2).

### 3.5 Determination of iNOS, COX-2 and TNF-α mRNA expression

Desmethoxyyangonin (**3**) and bisdemethoxycurcumin (**9**) showed potent activity against NO production with $IC_{50}$ values of 10.1 and 6.8 μM, respectively. Hence, the mRNA expressions of iNOS, COX-2 and TNF-α were determined by RT-PCR. Desmethoxyyangonin (**3**) suppressed the mRNA expressions of iNOS gene in a dose-dependent manner, whereas bisdemethoxycurcumin suppressed the effect on transcription of iNOS and COX-2 genes with a higher effect than that of TNF-α (Figure 3). The result is concurrent with the *in vitro* test on anti-NO production.

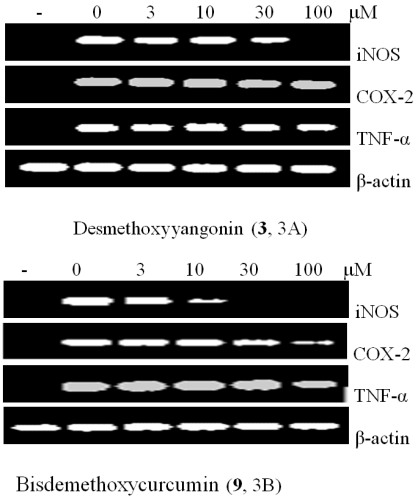



Figure 3. Effects of desmethoxyyangonin (**3**, 3A) and bisdemethoxycurcumin (**9**, 3B) at various concentrations (0, 3, 10, 30 and 100 μM) on mRNA expression of β-actin (514 bp), iNOS (580bp), COX-2 (860bp) and TNF-α (347 bp) by LPS-induced NO, $PGE_2$ and TNF-α productions in RAW264.7 cells. (-) = LPS (-), sample (-); (0) = LPS (+), sample (-); 3-100 μM = LPS (+), sample (+).

## 4. Conclusions

In the present study, the hexane fraction of *K. marginata* rhizomes strongly reduced the carrageenan-induced rat paw edema. Desmethoxyyangonin (**3**) decreased the mRNA expression of only iNOS gene in a dose-dependent manner, whereas bisdemethoxycurcumin (**9**) from the chloroform fraction had marked inhibitory effects on transcription of both iNOS and COX-2 genes with mild effect towards TNF-α. The isolated compounds (**3**-**5**, **8**-**9**) showed potent anti-inammatory activity. These findings may support the pharmacological basis of *K. marginata* as a Thai herbal medicine for the treatment of inflammatory-related diseases.

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