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-acetoxyandara-copimaradiene-2-one (1) and 1α-acetoxyandaracopimaradiene (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 IC₅₀ 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 E₃ (PGE₃). 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 (Chiu et al., 2000; Kim et al., 1999; Konkimala 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 PGE2. 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 & Koontner, 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 & Koontner, 2008). Bioactive compounds isolated from several Kaempferia species are cinnamon derivatives, cyclohexene oxide derivatives, chalcone derivatives, monoterpenes, diterpenes and flavonoids (Hwang et al., 2003; Jiangguang et al., 2001; Patanasethanon 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, cinnamonate derivatives, flavones, benzoparyanes and cyclohexene oxide derivatives (Jingguang et al., 2001; Kaewkroe 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, Birkroed, 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 H4 was purified by recrystallization to give 1 (white crystals, 0.0302 g, 0.0378% w/w). Fraction H19 was purified by recrystallization to give 2 (white powder, 0.0205 g, 0.0256% w/w). Fraction H14 (2.78 g) was separated by silica gel column chromatography using CHCl3/MeOH (0-30%) to afford seven subfractions (H14-1–H14-7). Subfraction H14-2 was separated by silica gel column chromatography using 1% MeOH/CHCl3 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 CHCl3/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 marginatal (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/CHCl3 (60-100%), followed by CHCl3/MeOH (0-30%) to afford seven subfractions (H8-1–H8-7). Subfraction H8-5 (0.06 g) was separated by silica gel column chromatography using CHCl3/MeOH (0-10%) to afford six fractions (H8-5.1–H8-5.6). Subfraction H8-4 (0.20 g) was further subjected to column chromatography and nine 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 diarylethanol 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-acetoxyandracopimaradien-2-one (1) and 1α-acetoxyandracopimaradienone (4) (Figure 1).

1α-Acetoxyandracopimaradien-2-one (1): White crystals (30.2 mg); [α]D +14.1 (c 0.1, CHCl3); IR (KBr) νmax 3300, 2952, 2870, 2949, 2839, 1782, 1713, 1634, 1368, 1231, 1030, 904 and 880 cm−1; EIMS [M]+ m/z 343.9; HREIMS: m/z [M]+ calcd for C22H29O8: 344.2346; found: 344.2346; 1H NMR (500 MHz, CDCl3) and 13C NMR (125 MHz, CDCl3) data are shown in Table 1.

1α-Acetoxyandracopimaramadienone (4): Colorless oil (17.4 mg); [α]D +11.4 (c 0.56, CHCl3); IR (KBr) νmax 2949, 2870, 1731, 1636, 1373, 1244, 1030, 990, 911 and 860 cm−1; FABMS [M]+ 330.0; 1H NMR (500 MHz, CDCl3) and 13C NMR (125 MHz, CDCl3) data are shown in Table 1.

2.5 Experimental animals

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

\[
\text{Inhibition (\%)} = \frac{(V_{t} - V_{0})_{\text{control}} - (V_{t} - V_{0})_{\text{treated}}}{(V_{t} - V_{0})_{\text{control}}} \times 100
\]

\(V_{t}\) = 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 (\(n = 4\)):

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Table 1. \(^1\)H NMR (500 MHz) and \(^13\)C NMR (125 MHz) spectral data of compounds 1 and 4 in CDCl\(_3\).

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<td>21.3</td>
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Inhibitory effects on LPS-induced TNF-\(\alpha\) release using RAW264.7 cells

The concentration of RAW264.7 cells was adjusted to 1×10^5 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):

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

A-C: NO\(^-\) concentration (\(\mu\)M) [A: LPS (+), sample (-); B: LPS (+), sample (+); and C: LPS (-), sample (-)].

2.7.2 Inhibitory effects on LPS-induced TNF-\(\alpha\) release using RAW264.7 cells

Statistical analysis was performed using 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 [1\(\alpha\)-acetoxysandaracopimaradien-2-one (1) and 1\(\alpha\)-acetoxysandaracopimaradiene (4)], two known pimarane-type diterpenes [marginatol (5) and sandaracopimaradiene (8)], one kavalactone (desmethoxyyangonin, 3), three steroids [sitosterol-\(\beta\)-D-glucoside (2) and the mixture of stigmasterol and \(\beta\)-sitosterol (6 + 7)] and one diarylheptanoid (bis-demethoxycurcumin, 9).

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., 2001; Khatun et al., 2012; Pérez-Almeida et al., 2005; Touche et al., 1997). Two new compounds (1 and 4) were identified as follows:

- Compound 1 was isolated as white crystal and [\(\alpha\)]_D^\text{20} +40 (c 0.1, CHCl\(_3\)). The molecular formula of C_{32}H_{52}O_8 was determined from high resolution electron capture mass spectrometry observed at \(m/z\) 434.2436 [M\(^+\)]. The IR spectrum showed absorptions of carbonyl (\(\nu_{\text{max}}\) 1752 cm\(^{-1}\)). Absorption bands at \(\nu_{\text{max}}\) 1634, 990 and 904 cm\(^{-1}\) suggested the presence of a vinyl group, and a trisubstituted olefinic absorption appeared at \(\nu_{\text{max}}\) 880 cm\(^{-1}\). The \(1^\text{C}\) NMR spectrum indicated 22 carbon signals, including five methyls, six methylenes, five methines and six quaternary carbons.
- The \(1^\text{H}\) NMR spectrum showed the presence of four methyl groups (\(\delta_{\text{H}}\) 1.03, 0.88, 0.82), an olefinic proton as singlet (\(\delta_{\text{H}}\) 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 \(\delta_{\text{H}}\) 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 \(\delta_{\text{H}}\) 5.34. The signal at \(\delta_{\text{H}}\) 0.15 (s) in the \(1^\text{H}\) NMR spectrum indicated that 1 was an acetate, an acetoxy group was present at \(\delta_{\text{H}}\) 4.59 (\(\delta_{\text{C}}\) 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 \(1^\text{H}\) and \(1^\text{C}\) NMR data, it was apparent that three rings were present in the molecule. The long-range \(1^\text{H}-1^\text{C}\) NMR correlations between a methyl proton signal at \(\delta_{\text{C}}\) 1.10 and the \(1^\text{C}\) signal at \(\delta_{\text{C}}\) 148.8 as well as correlations between a vinylic proton at \(\delta_{\text{C}}\) 4.89 and 4.92 and a quaternary \(1^\text{C}\) signal at \(\delta_{\text{C}}\) 37.3 indicated a pimarane diterpenes skeleton with a vinyl group attached to C-13. The COSY spectrum is shown in Figure 2A. An acetoxy group at C-1 and a keto group (\(\delta_{\text{C}}\) 207.2) at C-2 were implied from the \(1^\text{H}-1^\text{C}\) HMBC NMR correlations between H-1/C-2, C-3, C-5, C-9 and C-10; H-7a/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 acetoxy group at C-1 was found to be \(\alpha\)-oriented due to the NOE between H-1/H-\_20 and H-\_17/H-\_20, indicated that H-1
was β-oriented. From these spectral data, compound 1 was therefore elucidated as 1α-acetoxyandsandaracopimaradien-2-one.

Compound 4 was obtained as a colorless oil. Its specific rotation was [α]D +14.1 (c 0.56, CHCl3). FABMS showed [M]+ ion at m/z 330.0, corresponding to the molecular formula C22H24O2. The IR spectrum showed absorptions of carbonyl (ν max 1731 cm⁻¹). Absorption bands at ν max 1636, 990 and 911 cm⁻¹ suggested the presence of a vinyl group and a trisubstituted olefinic absorption appeared at ν 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 (δH 1.75 and 1.31-1.39). The 1H and 13C NMR spectra showed the presence of a methine carbon at δC 75.0 (δH 4.82, br s), a trisubstituted olefin at δC 129.3 and δH 4.90, dd, J = 17.4, 10.5 Hz, δH 110.1 and δC 148.8 (δH 5.75, dd, J = 17.4, 10.5 Hz and δH 4.87, dd, J = 10.5, 1.5 Hz) and four tertiary methyl groups singlet signals at δH 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 acetoxy group was present at δH 4.82 (δC 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-20/C-1, C-5, C-9 and C-10 (Figure 2B). Detailed assignments of 1H and 13C chemical shifts were based on the COSY, HMOC and HMBC NMR experiments. The NOESY spectrum of 4 showed correlations between H-1/H-16, indicating the β-orientation of H-1 and the α-orientation of an acetoxy group at C-1. Compound 4 was thus assigned as 1α-acetoxyandsandaracopimaradieniene.

### 3.2 Carrageenan-induced hind paw edema

The anti-inflammatory 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 for the release of inflammatory mediators, generally the mediators of inflammation are histamine, PGs, 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 (Kaeuwkroek 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-inflammatory activity through the inhibition of NO and PGE2 with mild effect towards TNF-α productions.

### 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 IC₅₀ value of 6.8 µM, followed by desmethoxyyangonin (3, IC₅₀ = 10.1 µM), marginalin (5, IC₅₀ = 12.1 µM), 1α-acetoxyandsandaracopimaradienie (4, IC₅₀ = 13.9 µM), sandaracopimaradienie (8, IC₅₀ = 18.6 µM), 1α-acetoxyandsandaracopimaradien-2-one (1, IC₅₀ = 40.3 µM), the mixture of stigmasterol and β-sitosterol (6 + 7, IC₅₀ = 81.7 µM) and sitosterol-β-D-glucoside (2, IC₅₀ = 94.8 µM), respectively. The inhibitory activity of 3, 4, 5, 8 and 9 exhibited higher effect than that of indomethacin (IC₅₀ = 39.9 µM), a clinically used drug, while compound 1 (IC₅₀ = 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 isolated from *Kaempferia marginata* rhizomes.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (µM)</th>
<th>NO</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>1α-Acetoxysandaracopimaradien-2-one (1)</td>
<td>40.3 ± 0.4</td>
<td>&gt;100 (2.0)$^b$</td>
<td></td>
</tr>
<tr>
<td>Sitosterol-β-D-glucoside (2)</td>
<td>94.8 ± 1.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Desmethoxyyangonin (3)</td>
<td>10.1 ± 0.6</td>
<td>&gt;100 (6.1)$^b$</td>
<td></td>
</tr>
<tr>
<td>1α-Acetoxysandaracopimaradien (4)</td>
<td>13.9 ± 0.3</td>
<td>&gt;100 (7.2)$^b$</td>
<td></td>
</tr>
<tr>
<td>8(14),15-Isopimaradiene-6α-ol (5)</td>
<td>12.1 ± 0.3</td>
<td>&gt;100 (17.2)$^b$</td>
<td></td>
</tr>
<tr>
<td>Mixture of stigmasterol and β-sitosterol (6 + 7)</td>
<td>81.7 ± 0.4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sandaracopimaradiene (8)</td>
<td>18.6 ± 0.3</td>
<td>&gt;100 (2.1)$^b$</td>
<td></td>
</tr>
<tr>
<td>Bisdemethoxycurcumin (9)</td>
<td>6.8 ± 0.5</td>
<td>&gt;100 (10.1)$^b$</td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>39.9 ± 0.6</td>
<td>&gt;100 (2.5)$^b$</td>
<td></td>
</tr>
<tr>
<td>CAPE</td>
<td>3.2 ± 0.4</td>
<td>75.0 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Each value represents mean ± S.E.M. of four determinations.  
$^b$Values in parenthesis are %inhibition at 100 µM.

Compounds 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.

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-inflammatory 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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