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Analgesic, Antipyretic, Anti-Inflammatory and Toxic Effects of Andrographolide Derivatives in Experimental Animals

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Andrographolide (**1**) and 14-deoxy-11,12-didehydroandrographolide (**2**) are active constituents of *Andrographis paniculata* (Burm. f.), family Acanthaceae. *A. paniculata* extracts are reported to have antiviral, antipyretic, immunostimulant and anticancer activities. In this study, **1** and its 14-acetyl- (**4**) and 3,19-isopropylidenyl- (**3**) derivatives, as well as **2** and its 3,19-dipalmitoyl-derivative (**5**), were intraperitoneally tested for their analgesic, antipyretic, anti-inflammatory and acute toxicity effects in animal models. Analgesic effects were tested in mice using hot plate and writhing tests to distinguish the central and peripheral effects, respectively. The results showed that, at 4 mg/kg, all tested substances have significant analgesic effects, and the highest potency was seen with **3**, **4** and **5**. Increasing the dose of **3** and **5** to 8 mg/kg did not increase the analgesic effect. In the writhing test, **3** and **5**, but not **1**, showed significant results. In a baker's yeast-induced fever model, **3** and **5** significantly reduced rats' rectal temperature ($p < 0.05$). In a carrageenan-induced inflammation model, **1**, **3** and **5** significantly reduced rats' paw volume. Doses of **3** and **5** up to 100 mg/kg did not show any serious toxic effects. From this study, **3** and **5** are the most interesting derivatives, showing much greater potency than their parent compounds. These could be further developed as analgesic, antipyretic and anti-inflammatory agents, without any serious toxicity.

Key words: Andrographolide derivatives, Antipyretic, Analgesic, Anti-inflammatory, Acute toxicity

INTRODUCTION

Andrographis paniculata (Burm. f.) Wall ex. Nees, (Acanthaceae) is a traditional medicine widely used in Asian countries for its antipyretic, analgesic, protozoacidal, antihepatotoxic, anti-HIV, immunostimulant, anticancer (Nanduri et al., 2004). The main constituents in *A. paniculata* are andrographolide (**1**), and 14-deoxy11,12-didehydroandrographolide (**2**). There are more than 20 other constituents that have been isolated from *A. paniculata*, including their stereoisomers and glycosides (Shen et al., 2006). *A. paniculata* is known for its reputation as the "king of the bitter". The antipyretic and anti-inflammatory effects of **1**, **2**, neoandrographolide (19-O-glucoglycoside of **1**, and 14-deoxyandrographolide – when tested on mice, rats and rabbits – have been reported with varying degrees of effect (Deng et al., 1982). The pharmacological effect was highest with **2** followed by **1**, neoandrographolide, and 14-deoxyandrographolide. However, they exerted less activity than corticosteroid and nonsteroidal drugs. The anti-inflammatory effect of the four compounds was not detected in adrenalectomized animals, indicating possible involvement of the pituitary-adrenal system in the compounds' anti-inflammatory action, which differs from other conventional drugs. Madav et al. (1995) studied the analgesic, antipyretic and anti-ulcerogenic activities of **1** at oral doses of 30, 100 and 300 mg/kg. It was found that **1** did not show any analgesic activity in the hot plate test in mice, while it showed significant ($p < 0.05$) analgesic activity in acetic acid-

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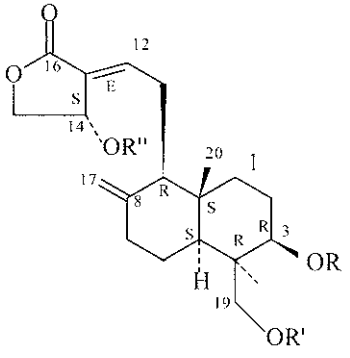
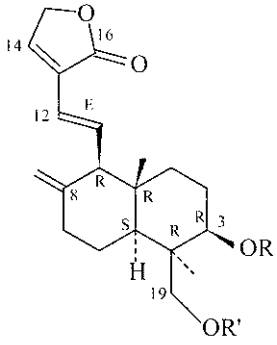
induced writhing in mice, as well as in the Randall-Selitto test in rats at 300 mg/kg dose. For antipyretic effect, oral doses of 100 and 300 mg/kg produced significant ($p < 0.05$) activity in brewer's yeast-induced pyrexia in rats. Significant antiulcerogenic activity was also found at doses of 100 and 300 mg/kg. Moreover, **1** significantly inhibited the weight of cotton-pellet-induced granuloma, and decreased edema in adjuvant-induced arthritis (Madav et al., 1996). Iruretagoyena et al. (2005) found the ability of **1** to inhibit T cell activation, which was applied to interfere with the onset of experimental autoimmune encephalomyelitis (EAE), an inflammatory demyelinating disease of the central nervous system that is primarily mediated by $CD4^+$ T cells and serves as an animal model for human multiple sclerosis. Treatment with **1** was able to significantly reduce EAE symptoms in mice by inhibiting T cell and antibody responses directed to myelin antigens. Thus it was suggested that **1** was able to efficiently block T cell activation *in vitro*, as well as *in vivo*, a feature that could be useful for interfering with detrimental T cell responses.

Sheeja et al. (2006) explored the antioxidant and anti-inflammatory properties of methanolic extract of *A. paniculata*, and found a complete inhibition of carrageenan-induced inflammation compared with control models. It was recently reported to inhibit NF-kB binding to DNA, thus reducing the expression of pro-inflammatory proteins such as cycloxygenase-2 (COX-2) (Shen et al., 2002). Inducible COX-2 is thought to act in pathological processes such as inflammation, to sensitize pain receptors on the skin and to regulate hypothalamic temperature control (Nakano et al., 2007). NF-kB activity was potently inhibited by **1**. Mechanistically, it formed a covalent adduct with reduced cysteine (62) of p50, thus blocking the binding of NF-kB oligonucleotide to nuclear proteins. **1** suppressed the activation of NF-kB in stimulated endothelial cells, which reduced the expression of the cell adhesion molecule E-selectin, and prevented E-selectin-mediated leukocyte adhesion under flow. It also abrogated the cytokine- and endotoxin-induced peritoneal deposition of neutrophils, attenuated septic shock, and prevented allergic lung inflammation *in vivo*. Notably, it had no suppressive effect on IkBα degradation, p50 and p65 nuclear translocation, or cell growth rates (Xia *et. al.,* 2004).

1 is an *ent*-labdane containing an a-alkylidene-g-butyrolactone moiety; two double bonds $\Delta^{8(17)}$, $\Delta^{12(13)}$; and three hydroxyls at C-3 (a secondary), C-19 (a primary), and C-14 (an allylic), as shown in Graphic 1. The stability of the amorphous form of **1** is temperature-dependent, and changes into **2** (Lomlim et al., 2003).

1 is metabolized very quickly in biological systems. The t_{max} is around 1-2 hours (He et al., 2003; Suo et al., 2007; Panossian et al., 2000; Cui et al., 2005), and the C_{max} of various doses ranged from 63-1620 ng/mL. About 55% of andrographolide is bound to plasma proteins, and only a limited amount can enter the cells (Panossian et al., 2000). Ten metabolites of andrographolide were found in urine and feces, which were sulfonic acid adducts and sulfate compounds, 3-O-sulfate conjugates, and one 3-O-sulfate-12-S-cysteine conjugate (He et al., 2003). Cui et al. (2005) further investigated the fate of andrographolide. They found seven glucuronides in urine: andrographolide-19-O-β-D-glucuronide, isoandrographolide-19-O-β-D-glucuronide, 14-deoxy-12-hydroxy-andrographolide-19-O-β-D-glucuronide, andrographolide-19-O-[6'-methyl-β-D-glucuronide], 14-deoxy-12(13)-en-andrographolide-19-O-β-





Andrographolide and derivatives
1 R, R', R'' = H
3 Isopropylidene R, R' = *i*-Pr, R'' = H
4 R, R' = H, R'' = Ac

14-Deoxy-11,12 didehydroandrographolide
2 R, R' = H
5 R, R' = palmitoyl- ($CH_3(CH_2)_{14}C$=O-)

Graphic 1. Structures of andrographolide and andrographolide derivatives

D-glucuronide, 14-deoxyandrographolide-19-O-β-D-glucuronide, and 3-oxoandrographolide-19-O-β-D-glucuronide.

From the metabolites, the hydroxyl groups at positions 3- and 19- are also the sites of biotransformation of **1**. Thus protection of the 3- and 19-OHs might prolong the drug being metabolized at these two positions. Besides that, increasing the lipophilicity of **1** by conjugating these two polar groups with isopropylidenyl, or esterifying with palmitic acid, would enhance their absorptivity to target cells, and possibly cause less protein binding, as seen in andrographolide. Another advantage is the decreasing degree of bitterness of the compound, much less than **1**.

In this era of nanotechnology, bipartate drugs are now strategically applied in many methods of drug delivery chemotherapy (Ganesh, 2007), skin delivery, and other routes of administration. Palmitate is one of the most frequently used prodrugs (e.g. chloramphenicol palmitate, ascorbyl palmitate, retinyl palmitate, zidovudine palmitate (AZT palmitate) (Heiati, 1997), paclitaxel palmitate (Goldstein, 2007), and paliperidone palmitate (Nasrallah, 2008), an injectable formulation recently approved by the U.S. FDA for treatment of schizophrenia.

In this study, bipartrate prodrugs were synthesised. Two known compounds **3** and **4**, and a new compound **5** were synthesised from **1**. These compounds were investigated for their original pharmacological activities in animal models by comparing them with their parent compounds, **1** and **2**, see Graphic 1. The baker's yeast-induced fever test (Tomazetti et al., 2005) and the hot plate test (Brochet et al., 1986) were evaluated. Compounds with interesting results were selected for a dose-pain response relationship test; a writhing test (Madav et al., 1995); a carrageenan-induced paw edema test; anti-inflammation effects (Winter et al.,1962); and an acute toxicity test.

MATERIALS AND METHODS

1 and **2** were isolated from dried and powdered *A. paniculata* purchased from an Ubon Ratchathani community agricultural agent. The plant's voucher (No. ISB 003) was deposited at the Faculty of Pharmaceutical Sciences, Khon Kaen University, and had been compared to the authentic plant, DMSc Herbarium No. 821. Structures of all compounds were elucidated by NMR (Varian, Mercury 400, $CDCl_3$ or CD_3OD), IR (Perkin Elmer Series 1600, KBr Disc), and LCMS (Bruker Daltonics, Billerica,). Melting temperature was determined by a melting point apparatus (Electrothermal Engineering Ltd.; IA9900, Series no. 9808). LCMS samples were introduced by flow injection and electrospray ionization for positive mass, and detected with a TOF detector from 50 m/z to 3000 m/z.

1 and its derivatives were detected by a TLC system using silica gel GF 254 precoated plates as stationary phases, and mixtures of methanol in dichloromethane as mobile phases. The plates were examined under UV light at 366 nm. The existence of intact γ-lactone was confirmed by spraying with Kedde's reagent. Reaction mixtures were partitioned in water and dichloromethane. The dichloromethane phase was dried with anhydrous sodium sulfate and chromatographed on a silica column gradiently eluted with mixtures of hexane and ethyl acetate.

Isolation and semisynthesis of andrographolide derivatives

Isolation of andrographolide (1) and 14-deoxy-11,12-didehydroandrographolide (2): **1** and **2** were isolated from dried and powdered *A. paniculata* as described by Fujita et al. (1984) with 2 and 0.5% yields, respectively.

Isopropylideneandrographolide (3): **3** was prepared from **1** and dimethoxypropane using pyridinium toluene sulfonate as a catatyst, as described by Nanduri et al. (2004) in 80% yield.

14-acetyl andrographolide (4): **4** was prepared from **1** as described by Jada et al. (2007), the yield was 60%.

14-deoxy-11,12-didehydro-3,19-dipalmitoylandrographolide (5): **1**, 0.0760 g (0.216 mmole), was dissolved in 6 ml of benzene. Palmitic anhydride 1.4901 g (3.011 mmole) was added, and 2 mg of 4-pyrolidinylpyridine (4-PPY) was used as a catalyst. The mixture was stirred for 12 h, and then partitioned with water. The benzene layer was separated, dried, and chromatographed on a silica column, and then gradiently eluted with mixtures of hexane and ethyl acetate. **5** was obtained with 50% yield.

Pharmacological activities

Animals: Experiments were conducted on male IRC mice and Sprague Dawley rats obtained from the Animal House, Faculty of Medicine, Khon Kaen University. The animals were housed in groups in the Animal House and had free access to laboratory chow and tap water. Before starting the experiments, the animals were left in the experimental room for at least 1 hour. The experiments were approved by the Animal Ethics Committee, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand (ethical number AEKKU014/06). Substances were dissolved in 5% DMSO.

Dimethyl sulfoxide (DMSO) and sodium chloride (analytical-grade reagents), carrageenan (Sigma, USA), paracetamol injection (TP Drug Co. Ltd., Thailand) and baker's yeast (Fermipan, Netherlands) were used in this study. Equipment included a hot plate for analgesics (Clinical Scientific Equipment Co., USA); plethysmometer (Ugo Basile S.R.L., Italy; No. 7140); digital thermometer (True Line Med Co., Ltd., Taiwan; MT B122); and a digital balance (Soehnle, Switzerland).

Statistical methods: The data were expressed as mean ± S.D. One-way analysis of variance (ANOVA) and a supplementary Tukey test for pairwise comparison were performed to determine any significant difference at $p < 0.05$.

Preparation of tested compounds for animals

All studies were performed at room temperature. The suspension of andrographolide and its semisynthetic derivatives were prepared in 5% dimethyl sulfoxide (DMSO) in saline. Concentrations of substances were prepared so that the volume of injection was 0.05 ml to 10 g body weight.

Antipyretic effect: Baker's yeast-induced fever test (Tomazetti et al., 2005)

Rectal temperature (T_R) was recorded by inserting a lubricated digital thermometer (external diameter 3 mm) into the rectum of each animal. A digital device displayed the temperature at the tip with a 0.1°C precision. After measuring basal T_R, rats were injected subcutaneously with pyrogenic doses of baker's yeast (0.135 g/kg), and T_R was recorded every hour. Two hours after yeast injection, 5% DMSO, 125 mmole/kg paracetamol, or 4 mg/kg of **1**, **2**, **3**, **4** or **5** was injected intraperitoneally. T_R was recorded every hour for at least 6 h. Results are expressed as the changes of the T_R from basal T_R.

Analgesic effect: The hot plate test (Brochet et al., 1986)

Male ICR mice were injected intraperitoneally with either 5% DMSO or 4 mg/kg of **1**, **2**, **3**, **4** or **5**. Thirty minutes after injection, mice were placed on a hot plate that was thermostatically maintained at 50°C, with a four-wall plexiglass container to confine the animals on the hot plate. The time that each mouse spent on the hot plate until it licked or jumped in response to pain was recorded as the reaction time. Cut-off time of the test was set at 30 seconds in order to prevent tissue damage. From the preliminary results of the hot plate test, all compounds showing high analgesic effect were selected to test for the dose-pain response relationship and acute toxicity.

Dose-pain response relationship test

The experiment was carried out as described previously, with doses of 0.5, 1, 4, 8 mg/kg of **3** and **5**; 5% DMSO was used as control.

Writhing test (Madav et al., 1995)

Male ICR mice were injected intraperitoneally with either 5% DMSO, or 20 mg/Kg^{-1} diclofenac, or 4 mg/kg of **1**, **3** or **5**. Thirty minutes after injection of each test solution, 1% acetic acid was injected intraperitoneally at a dose of 100 mg/kg. At 5, 15, 25, 35 and 45 min after acetic acid injection, the number of writhing responses observed during a 5-minute period were counted and recorded.

Anti-inflammation effect: Carrageenan-induced paw edema test (Winter et al., 1962)

Rats were divided into 6 groups with 6 rats in each group. The animals received intraperitoneal injections with 5% DMSO (as a control) or 4 mg/kg^{-} of **1**, **3** or **5.** One hour later, acute inflammation of the paw was induced by subplantar injection of 0.1 mL of 0.1 % w/v of carrageenan in normal saline. The paw volume was measured by using a plethysmometer at 0, 1, 2 and 3 h after carrageenan injection.

Acute toxicity test

For the toxicity study the test compounds **3** and **5**, at either 0.5, 1, 4, 8, 50 or 100 mg/kg, or 5% DMSO, were intraperitoneally administered to 13 groups of 5 mice each. Animal behavioral changes were observed for 6 h, and mortality rates were observed at 24 h after injection.

RESULTS

Andrographolide (1)

White or colourless crystals (CH_2Cl_2), mp 225-227°C uncorrected, lit 218-221°C (Fujita et al., 1984); Rf 0.35, silica gel $60F_{254}$, 10% methanol in CH_2Cl_2. IR (KBr) ν_{max}: 3399 with shoulder at 3322 (broad and s), 3152 (w), 2932 (m), 2849, 1732 (very s), 1675(m), 1221, 1033 cm^{-1}. ^1H NMR (CD_3OD) δ: 6.84 (1H, *dt*, *J* = 7.02, 1.56 Hz, H-12), 5.00 (1H, *d, J* = 5.5 Hz, H-14), 4.88 (1H, *s*, H-17a), 4.66 (1H, *s*, H-17b), 4.15 (1H, *dd*, *J* = 10.1, 1.95, Hz, H-15a), 4.11 (1H, *d*, *J* = 10.9 Hz, H-15b), 3.40 (1H, *m, J* = 7.03, H-3), 3.3 (1H, *d*, *J* = 8.2, H-19), 1.21 (3H, *s*, H-18), 0.74 (3H, *s*, H-20). ^{13}C NMR (CD_3OD) δ: 172.7 (C, C-16), 149.4 (CH, C-12), 148.9 (C, C-8), 129.8 (C, C-13), 109.2 (CH2, C-17), 80.9 (CH, C-3), 76.2 (CH2, C-15), 66.7 (CH, C-14), 65.0(CH2, C-19), 57.4 (CH, C-9), 56.3 (CH, C-5), 43.7 (C, C-4), 40.0 (C, C-10), 39.0 (CH2, C-7), 38.1 (CH2, C-1), 29.0 (CH2, C-2), 25.73 (CH2, C-

11), 25.2 (CH2, C-6), 23.4 (CH3, C-18), 15.5 (CH3, C-20).

14-Deoxy-11,12-didehydroandrographolide (2)

White or colourless crystals (CH_2Cl_2), mp 198-200ºC , lit 203-204ºC (Fujita et al., 1984), Rf 0.58, silica gel 60F254, 10% methanol in CH2Cl2. IR(KBr) ν_{max}: 3304 (brd,s), 3082 (m, C=C), 2934 (m), 2851, 1732 (s), 1605 (w), 1100, 1040 cm^{-1}. ^1H NMR (CD_3OD) δ: 7.43 (1H, *t*, *J* = 1.76 Hz, H-14), 6.85 (1H, *dd*, *J* = 10.1 and 15.8 Hz, H-12), 6.15 (1H, *d*, *J* = 15.8 Hz, H-11), 4.86 (2H, *d*, *J* = 1.3 Hz, H-15), 4.75 (1H, *d*, *J* = 1.8 Hz, 17a), 4.49 (1H, *d*, *J* = 1.8 Hz, 17b), 4.12 (1H, *d*, *J* = 11.0 Hz, H-19a), 3.39 (1H, *t*, *J* = 5.3 Hz, H-3), 3.38 (1H, *d*, *J* = 11.4 Hz, H-19b), 1.22 (3H, *s*, H-18), 0.83 (3H, *s*, H-20). ^{13}C NMR (CD_3OD) δ: 172.2 (C=O, C-16), 148.0 (C, C-8), 142.8 (CH, C-12), 136.0 (CH, C-11), 129.2 (C, C-13), 121.1 (CH, C-14), 109.2 (CH2, C-17), 80.8 (CH, C-3), 69.5 (CH2, C-15), 64.2 (CH2, C-19), 61.7 (CH, C-9), 54.7 (CH, C-5), 43.0 (C, C-4), 38.5 (C, C-10), 38.2 (CH2- C-1), 36.6 (CH2, C-7), 28.1(CH2, C-2), 22.9 (CH2., C-6), 22.6 (CH3, C-18), 15.9 (CH3, C-20). ESI *m/z*: 355.19 (M+Na)+, 687(2M+Na)$^+$.

Isopropylideneandrographolide (3)

Crystallised powder from hexane and ethyl acetate (1:1); mp 194-196ºC (lit 194-196ºC, Jada et al., 2007). IR (KBr) ν_{max}: 3409, 2939, 1785, 1683, 1222 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 6.97 (1H, td, *J* = 1.5 and 8.0 Hz, H-12), 5.05 (1H, *d*, *J* = 5.9 Hz, H-14), 4.91 (1H, *s*, H-17a), 4.62 (1H, *s*, H-17b), 4.45 (1H, *dd*, *J* = 6.2 Hz and 10.5 Hz, H-15a), 4.26 (1H, *dd*, *J* = 2.0 and 10.5 Hz, H-15b), 3.96 (1H, *d*, *J* = 11.3 Hz, H-19a), 3.50 (1H, *dd*, *J* = 3.1 and 8.6 Hz, H-3), 3.18 (1H, *d*, *J* = 11.7 Hz, H-19b), 2.57 (2H, *t,* *J* = 6.8Hz, H-11), 1.41 (3H, *s*, gem-Mt), 1.37 (3H, *s*, gem-Mt), 1.20 (3H, *s*, H-18), 0.97 (3H, *s*, H-20). ^{13}C NMR ($CDCl_3$) δ: 169.7 (C, C-16), 149.0 (C, C-12), 147.1 (CH, C-8), 127.9 (C, C-13), 108.9 (CH2, C-17), 99.2 (C, spiro *i-pr*), 76.1 (CH, C-3), 74.2 (CH2, C-15), 66.2 (CH2, C-14,), 63.9 (CH, C-19), 56.1 (CH, C-9), 38.4 (C, C-4), 37.9 (CH, C-10), 37.6 (C, C-7), 34.5 (CH2, C-1), 27.0 (CH3, gem Mt), 26.1 (CH3, gem M), 25.3 (CH2, C-11), 25.0 (CH3, C-18), 24.9 (CH2, C-2), 23.2 (CH2, C-6), 16.2 (CH3, C-20). EIMS *m/z*: 412.8 (M+Na)$^+$.

14-acetyl andrographolide (4)

14-Acetyl andrographolide (**4**) was obtained with 60% yield. White powder (CH_2Cl_2); mp 168.29ºC (onset 163.65, endset 170.41ºC, lit 168-170ºC, Jada et al. 2007); Rf 0.35, silica gel $60F_{254}$, 5% methanol in CH_2Cl_2, IR (KBr) ν_{max} : 3363, 1772, 1751, 1209, 1021 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 7.00 (1H, *dt*, *J* = 6.63, 0.39 Hz, H-12), 5.91 (1H, *d,* *J* = 5.5 Hz, H-14), 4.87 (1H, *s*, H-17a), 4.49 (1H, *s*, H-17b), 4.23(1H, *d*, *J* = 10.9 Hz, H-15a), 4.16 (1H, *d*, *J* = 10.9 Hz, H-15b), 3.47 (1H, *t,* *J* = 7.03, H-3), 3.3 (1H, *d*, *J* = 8.2, H-19), 2.83 (2H,2s), 2.11 (3H, *s*, OAc), 1.25 (3H, *s*, H-18), 0.66 (3H, *s*, H-20). ^{13}C NMR ($CDCl_3$) δ: 170.5 (C, Ac), 169.1 (C, C-16), 150.5 (CH, C-12), 146.7 (C, C-8), 123.8 (C, C-13), 108.7 (CH2, C-17), 80.4 (CH, C-3), 71.6 (CH2, C-15), 67.7 (CH, C-14), 64.1 (CH2, C-19), 55.8 (CH, C-9), 55.2 (CH, C-5), 42.8 (C, C-4), 38.8 (C, C-10), 37.7 (CH2, C-1), 37.0 (CH2, C-7), 28.1 (CH2, C-2), 25.3 (CH2, C-11), 23.7 (CH3, C-18), 22.7 (CH2, C-6), 20.7 (CH3, Ac), 15.1 (CH3, C-20). ESI MS m/z: 415.2106 [M+ Na]$^+$

14-deoxy-11,12-didehydro-3,19-dipalmitoylandrographolide (5)

White powder or colorless crystals (CH_2Cl_2), mp 57-61ºC, IR(KBr): 3080 (very weak, C=C), 2956 (C-H), 2916 (very str, C-H), 2851 (C-H), 1748 (str, C=O), 1727 (very str, C=O), 1472 (m, CH2), 804 (wk, brd, CH bending of long chain hydrocarbon) cm^{-1}.^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.09 (1H, *s*, H-14), 6.85 (1H, *q*, *J* = 9.8 and 15.8 Hz, H-12), 6.05 (1H, *d*, *J* = 15.6 Hz, H-11), 4.74 (2H, *s*, H-15), 4.73 (1H, *s*, 17a), 4.53 (1H, *t*, *J* = 8.6 and 7.8 Hz, H-3), 4.49 (1H, *s*, H-17b), 4.23 (1H, *d*, *J* = 11.7 Hz, H-19a), 4.14 (1H, *d*, *J* = 11.7 Hz, H-19b), 0.94 (3H, *s*, H-20), 0.82 (3H, *s*, H-31'), 0.81 (3H, *s*, H-18), 0.79 (3H, *s*, H-31") . ^{13}C-NMR (400 MHz, $CDCl_3$): δ 172.1 (C16,C=O), 173.7 (C1', C=O), 173.4 (C1ð, C=O), 147.9 (C8,C), 143.1 (C14,CH), 135.7 (C12,CH), 129.2 (C13,C), 121.3 (C11,CH), 109.2 (C17, CH_2), 79.7 (C3,CH), 69.5 (C15, CH_2), 64.7 (C19, CH_2), 61.7 (C9, CH), 54.8 (C5, CH), 41.4 (C4,C), 38.7 (C10, C), 38.3 (C7, CH_2), 36.7 (C1, CH_2), 24.2 (C6, CH_2), 24.0 (C2, CH_2), 22.7 (C20, CH_3), 15.2 (C18, CH_3), 14.1 (C16¢,16ð, CH_3), ESI *m/z*: 882.75.

Antipyretic activity

The antipyretic effect of **1** and its derivatives on the fever test were expressed as mean ± S.D. (see Table I) for changes of T_R from basal T_R of the animals in each group. All tested compounds reduced the rectal temperature. However, as shown in Fig. 1, it appeared that, at doses of 4 mg/kg only **3** and **5** – but not **1**, **2** and **4** – significantly ($p < 0.05$) reduced rats' rectal temperature when compared to 5% DMSO (control). The antipyretic effects of 4 mg/kg of **3** and **5** were comparable to the effect of 1.25 mmole/kg or 189 mg/kg paracetamol (see Fig. 2).

Analgesic effect

The hot plate test for the screening of analgesic effect: It was found that **1**, **2**, **3**, **4** and **5** could

Table I. Antipyretic effect of the andrographolide and andrographolide derivatives (4 mg/kg, i.p.), in baker yeast-induced fever test

Treatment	T_{R-1} change	T_{R-2} change	T_{R-3} change	T_{R-4} change	T_{R-5} change	T_{R-6} change	T_{R-7} change	T_{R-8} change
Control 5% DMSO	0.70 ± 0.60	0.84 ± 0.73	1.24 ± 0.53	1.56 ± 0.68	1.64 ± 0.69	1.88 ± 0.60	2.20 ± 1.01	2.50 ± 0.66
Paracetamol 1.25mmol/kg	0.64 ± 0.71	0.22 ± 1.00	-0.54 ± 0.88*	-1.06 ± 0.71*	0.12 ± 1.39*	0.30 ± 1.27	0.46 ± 0.92*	0.66 ± 0.54*
1	0.22 ± 0.41	0.02 ± 0.70	0.06 ± 0.67	-0.14 ± 0.36*	0.36 ± 0.49	0.94 ± 0.51	0.96 ± 0.49	1.34 ± 0.82
2	-0.16 ± 0.53	0.14 ± 0.63	0.24 ± 0.54	0.48 ± 0.30	0.28 ± 0.56	0.74 ± 0.67	0.80 ± 0.87	1.28 ± 0.26
3	-0.33 ± 0.60	-0.60 ± 0.88	-0.15 ± 0.78	-0.05 ± 1.06*	-0.18 ± 0.32*	0.27 ± 0.46*	0.45 ± 0.39*	0.80 ± 0.63*
4	-0.22 ± 0.55	-0.24 ± 0.91	0.42 ± 0.81	0.48 ± 0.88	0.76 ± 0.50	0.46 ± 0.99	0.92 ± 1.03	0.86 ± 0.65
5	-0.26 ± 0.64	-0.04 ± 0.53	-0.14 ± 083	0.08 ± 0.33*	0.10 ± 0.67*	0.38 ± 0.83	0.86 ± 0.68	0.90 ± 0.75*

Results were expressed as mean ± S.D. for the changes of the T_R from basal T_R of the animal in each group.
*$p < 0.05$ when compared to the control group.

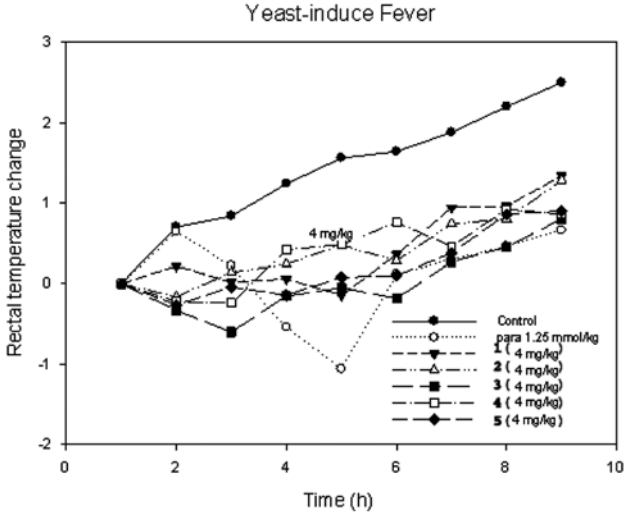


Fig. 1. Antipyretic effect of the andrographolide and andrographolide derivatives (4 mg/Kg^{-1} dose) in baker yeast-induce fever test. Rats were injected intraperitoneally with 5% DMSO, 125 mmole/Kg paracetamol or 4 mg/kg of **1, 2**, **3**, **4** or **5**. Results were expressed as mean ± S.D. for the changes of the T_R from basal T_R of the animal in each group. *$p < 0.05$ when compared to the control group.

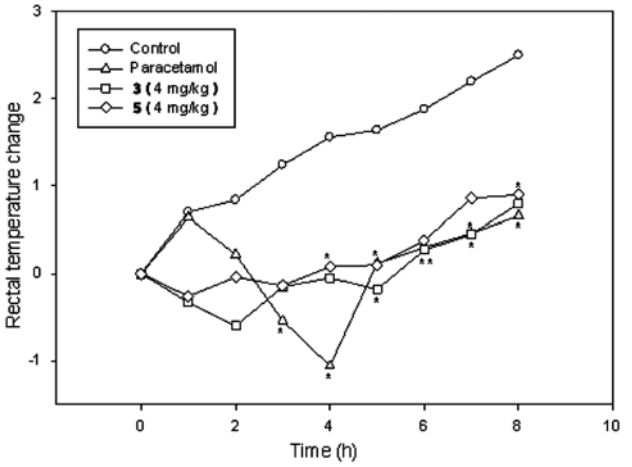


Fig. 2. Antipyretic effect of the **1**, **3** and **5**, at 4 mg/kg dose, in Baker yeast-induce fever test

Table II. Analgesic effect of compounds **1**, **2**, **3**, **4** and **5** at the doses of (4 mg/kg) in the hot plate test

Treatment	Thermal threshold
Control 5% DMSO	4.94 ± 0.05
1	8.19 ± 0.07*
2	9.45 ± 0.43*
3	8.43 ± 0.70*
4	8.80 ± 0.70*
5	8.30 ± 0.53*

Results were expressed as mean ± S.D. for reaction time.
*$p < 0.05$ when compared to the control group.

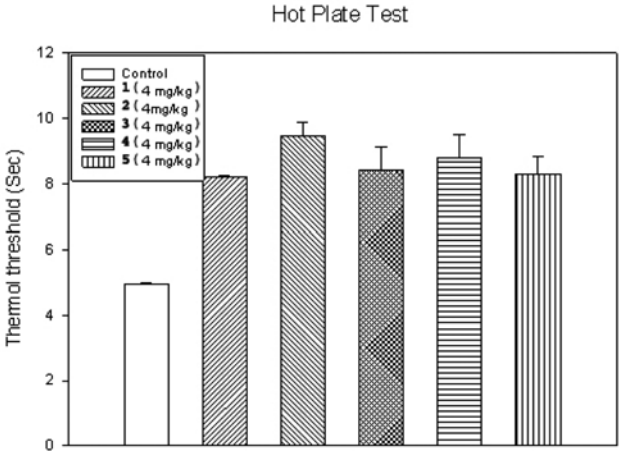


Fig. 3. Analgesic effect of the andrographolide and its derivatives in the hot plate test. Mice were injected intraperitoneally with either 5% DMSO or 4 mg/kg of **1**, **2**, **3**, **4** or **5**. Results were expressed as mean ± S.D. for reaction time. *$p < 0.05$ when compared to the control group.

increase the thermal threshold significantly ($p < 0.05$) when compared to 5% DMSO. Compounds **2**, **3** and **5** showed the most potent analgesic effect (see Table II and Fig. 3). The semisynthesis of **3** and **5** were selected to study the dose-effect relationship, as they also showed good effect on antipyretic activity.

Table III. Dose-effect relationship of analgesic test of 3 and 5 in the hot plate test

Compounds mg/kg	Treatment	Thermal threshold
	Control 5% DMSO	5.09 ± 0.62
3	0.5	5.90 ± 1.12
	1	6.84 ± 0.45*
	4	8.43 ± 1.57*
	8	7.37 ± 0.80
5	0.5	5.54 ± 0.65
	1	7.80 ± 1.22
	4	8.30 ± 1.19*
	8	6.41 ± 0.80*

Results were expressed as mean ± S.D. for reaction time (s).
*$p < 0.05$ when compared to the control group.

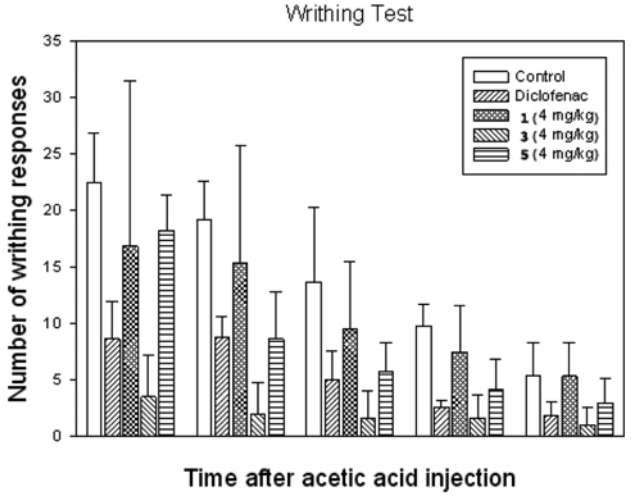


Fig. 4. Analgesic effect in the Writhing test. Mice were injected intraperitoneally with either 5% DMSO, 20 mg/kg diclofenac or 4 mg/kg of **1**, **3** and **5**. At 5, 15, 25, 35, 45 minutes after 1% acetic acid injection, number of Writhing responses observed as mean ± S.D. in 5 minute-interval each. *$p < 0.05$ when compared to the control group.

Dose-effect relationship of analgesic test: As shown in Table III, **3**, with dosages of 4 and 8 mg/kg, and **5**, with dosages of 1 and 4 mg/kg, could increase the thermal threshold significantly ($p < 0.05$) when compared to 5% DMSO. The analgesic effects of 4 mg/kg of **3** and **5** were the most potent in comparison to other doses (0.5 and 1, 8 mg/kg).

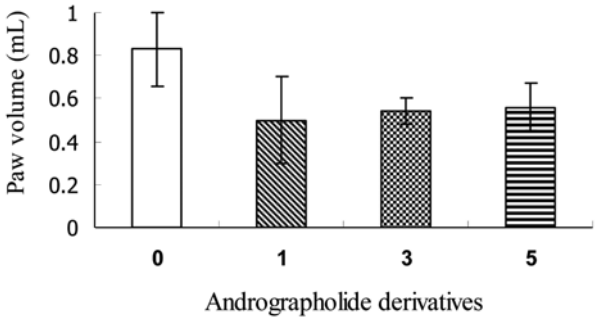


Fig. 5. Anti-inflammation effect in the carrageenan-induced paw edema test. Rats were received intraperitoneal injection with 5% DMSO or 4 mg/kg of **1**, **3** or **5**. Rat's paw volume changes, hour 3, were expressed as mean ± S.D.

Writhing test

The writhing test was used for analgesic effects of **1**, **3** and **5**. The results are shown as mean ± S.D. in Table IV and Fig. 4. It appears that diclofenac and **3** significantly ($p < 0.05$) reduced the writhing response at 5, 15, 25, 35 and 45 min, whereas **5** significantly ($p < 0.05$) reduced the writhing response at 25 and 35 min when compared to 5% DMSO. On the contrary, **1** did not reduce the writhing response when compared to 5% DMSO.

Anti-inflammation effect: carrageenan-induced paw edema test

It was shown that **1**, **3** and **5** at 4 mg/kg significantly reduced paw volume when compared to 5% DMSO, as shown in Table 5 and Fig. 5.

Acute toxicity test

Animal behavioral changes were observed for 6 hours, and mortality rate was observed at 24 h after injection. No behavioral changes were noted for either

Table IV. Analgesic effect of the tested substances in the Writhing test

Treatment	minutes				
	5-10	15-20	25-30	35-40	45-50
5% DMSO	22.5 ± 4.37	19.17 ± 3.43	13.71 ± 6.53	9.83 ± 1.84	5.33 ± 2.94
Diclofenac	8.67 ± 3.327*	8.83 ± 1.84*	5.00 ± 2.55*	2.60 ± 0.55*	1.80 ± 1.30*
1	16.83 ± 14.65	15.33 ± 10.39	9.50 ± 5.96	7.50 ± 4.09	5.33 ± 3.01
3	3.5 ± 3.728*	2.00 ± 2.76*	1.67 ± 2.42*	1.67 ± 2.07*	1.00 ± 1.55*
5	18.20 ± 3.11	8.67 ± 4.18	5.71 ± 2.56*	4.14 ± 2.67*	3.00 ± 2.16

Mice were injected intraperitoneally with either 5% DMSO, 20 mg/kg diclofenac or 4 mg/kg of 1, 3 and 5. At 5, 15, 25, 35, 45 min after 1% acetic acid injection, number of writhing responses observed as mean ± S.D. in 5 min each.
*$p < 0.05$ when compared to the control group.

Table V. Anti-inflammation effect in the carrageenan-induced paw edema test

Treatment	Changes of paw volume
Control 5% DMSO	0.83 ± 0.17
1	0.50 ± 0.20*
3	0.54 ± 0.06*
5	0.56 ± 0.11*

Rats were given an intraperitoneal injection with 5% DMSO or 4 mg/kg of **1**, **3** or **5**. Rat's paw volume changes, hour 3, were expressed as mean ± S.D.
*$p < 0.05$ when compared to the control group.

compound after 6 h of observation, and at 24 h after injection, animals in all groups had survived.

DISCUSSION AND CONCLUSIONS

As **1** has the reputation as "king of the bitter", "Nonbitter Andrographolide" project was initiated. Derivatives at various functional groups of **1** were made to encounter the bitterness as well as wider delivery systems can be performed. Acetic and palmitic acids were selected to represent short chain and long chain fatty acids, respectively. To minimized the animal used in experiments, only compounds selected for further development for drug delivery were tested for their original biological activity and compared to their parent compounds.

In this study, the effects of the parent compound **1** were compared with the effects of its derivatives. All five compounds showed antipyretic activity at doses of 4 mg/kg. However, only **3** and **5** showed significant results. The 4 mg/kg doses of **3** and **5** were equivalent to 3.59 and 1.73 mg/kg of **1**. It had been reported earlier that **1**, with oral doses of 100 and 300 mg/kg, produced a significant antipyretic effect after 3 h administration of brewer's yeast-induced fever in rats Madave et al. (1995). In addition, doses of 180 or 360 mg/kg of **1** were also found to relieve fever in humans by the third day after administration (Thamlikitkul et al., 1991). It is interesting to note that the doses used in those studies were much higher than the doses used in our study. It might be possible that at 4 mg/kg, the plasma level of **1** could not reach the minimum effective concentration for antipyretic effect. In addition, **1** is 55% bound to plasma protein (Panossian et al., 2000), thus limiting the amount of free drug that can enter the central nervous system. The binding site of **1** on plasma protein has not yet been investigated. In contrast, **3** and **5** – the two semisynthetic derivatives of **1** whose 3- and 19-OHs were occupied with isopropylidinyl and dipalmitoyl groups, respectively – showed antipyretic activity comparable to paracetamol. This would suggest that one possibility is that the molecular parts of **1** which bind to protein are most probably these two hydroxyl groups.

Regarding analgesic effect (the hot plate test), all compounds showed significant activity compared to the control. Compounds **3** and **5** were further investigated compared to **1**, their parent compound, by the writhing test, and for anti-inflammatory effect by the carrageenan-induced paw edema test. It was found that **3** and **5**, but not **1**, showed an effect on writhing, whereas for the anti-inflammatory effect, compounds **1**, **3** and **5** all significantly reduced paw volume.

Madav et al. (1995) reported that 300 mg/kg of **1**, administered orally, had significant analgesic activity on acetic-induced writhing in mice and on the Randall-Selitto test in rats, but without any effect on the hot plate test in mice. Oral administration of **1** at 30, 100 and 300 mg/kg also showed anti-inflammatory activity in different models in rats (Madave et al., 1996). The different pattern of analgesic effects of andrographolide on hot plate and writhing tests in early reports versus the results of this study is not very agreeable. In the writhing test, doses of **1** used by Madave et al. (1995) were about 75% higher than the present study. Interestingly, in the hot plate test, 4 mg/kg administered intraperitoneally exhibited an analgesic effect, whereas the former study, 300 mg/kg administered orally did not. The different routes of administration between these experiments could contribute to this discrepancy. It has been shown that first-pass metabolism or presystemic biotransformation reactions can happen throughout the gastrointestinal and hepatic systems after oral administration of substances. These reactions might cause a reduction of active drug levels in systemic circulation. Moreover, the bioavailability of oral administration might be decreased by incomplete absorption, while intraperitoneal administration is not affected. It is known that the hot plate test represents the central action of test drugs. Previous studies have suggested that treatment with **1** at a daily dose equal to 4 mg/kg significantly reduced an inflammatory demyelinating disease of the central nervous system, autoimmune encephalitis, by inhibiting T cells in mice; this finding also supports our result (Iruretagoyena et al., 2005).

Varying doses of **3** and **5** characteristically showed a relatively all-or-none response; and a plateau effect was reached at a dose of 4 mg/kg. It is also interesting that 1 may have many molecular targets in biological systems, such as iNOS, kinase proteins, etc. Increasing the doses of these compounds might reduce the selectivity of the effects, and probably results in the

contradictory effects that have so often been reported in regard to certain neuromodulators (Marsh et al., 1999). In addition, Panossian et al. (2000) have shown that the bioavailability of **1** decreased fourfold when a 10-times-higher dose was used. Accordingly, increasing the dose of the substance does not increase plasma level in a first-order manner.

Although the exact mechanisms of **1** and its derivatives in regulating analgesic, antipyretic and anti-inflammatory effects remain unknown, it has recently been reported that **1** can inhibit NF-kB binding to DNA, thus reducing the expression of pro-inflammatory proteins such as cyclooxygenase-2 (COX-2) (Shen et al., 2002). COX-2 is an inducible enzyme and plays an important role in pathological processes such as inflammation (Xia et al., 2004). COX-2 catalyzes the biosynthesis of prostaglandins from arachinodic acid. The prostaglandins are also involved in the pathogenesis of fever, pain and inflammation. The most important of these is prostaglandin E_2 (PGE_2). PGE_2 is the ultimate mediator of the fever response, and the set-point temperature of the body will remain elevated until PGE_2 is no longer present. In regard to pain, PGE_2 induces hyperalgesia because it makes the skin hypersensitive to pain stimuli. In the case of inflammation, PGE_2 causes vasodilation and increases the permeability of post-capillary veins, thus potentiating edema formation (Kumar et al., 2005).

If **1** is the initial pharmacophore, **3** and **4** were prodrugs of **1**, and **5** was a prodrug of **2**. The polarity sequence of these compounds, estimated from their structures, are **1** > **4** > **2** > **3** > **5**. From this study, **3** and **5** were found to have superior activities to **1**. As has been mentioned earlier, **1** is high protein-bound, rapidly metabolized, and excreted mainly as metabolites in urine and feces; thus the two prodrugs might be less affected by these processes. Besides that, the lesser polarity would enhance the adsorptivity of these derivatives to target cells. The protein-binding effect and biotransformation of **1** would suggest that these semisynthesized compounds were less affected by these activities, other than the increased adsorptivity. This resulted in a very high potency of **3** and **5** for these pharmacological activities.

Based on the results of this study, **3** and **5** are the most potent bipartate prodrugs. Their 13- and 19-hydroxyl groups were substituted with isopropylidene as a six-membered ring **3**, and two groups of palmitoyl **5**. Doses of 4 mg/kg of **3** and **5** were equivalent to 3.59 and 1.73 mg/kg of **1**, respectively. From the results it was found that the crucial functional groups of **1** depended highly upon the polarity at 3-, and 19-OHs. Decreasing the polarity either by ether or ester linkages is not the main criteria.

As mentioned previously, if **1** is the active pharmacophore for all activity, enzymatic cleavage of the ether and ester linkages in the biological system would be primarily responsible for catalyzing these two compounds into parent compounds, which would then be conjugated by sulfate or glucuronic acid. These substitutions, either ester or ether hinder the metabolism of **1**, and thus survive to interact with the active site. Another benefit of these findings is that a smaller dose of 1 is sufficient for these activities.

In conclusion, the improved activity from lower doses (equivalent to **1**) of **3** and **5** is considered to result from a combination of increased lipophilicity, better tissue penetration, and delay in metabolism and protein binding.

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