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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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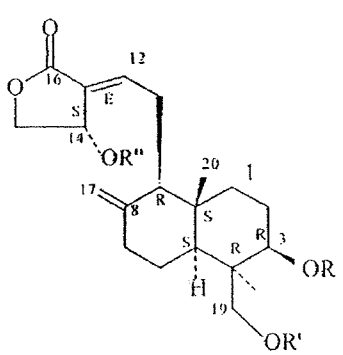
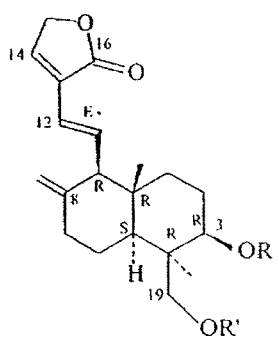
induced writhing in mice, as well as in the Randall-Selitto test in rats at 300 mg/kg dose. For antipyretic effect, oral doses of 100 and 300 mg/kg produced significant ($p < 0.05$) activity in brewer's yeast-induced pyrexia in rats. Significant antiulcerogenic activity was also found at doses of 100 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}. ^{1}H 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 198-200°C , lit 203-204°C (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 194-196°C (lit 194-196°C, 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 168.29°C (onset 163.65, endset 170.41°C, lit 168-170°C, 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 57-61°C, 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}.^1H-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 (C1∂, 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¢,16∂, 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.

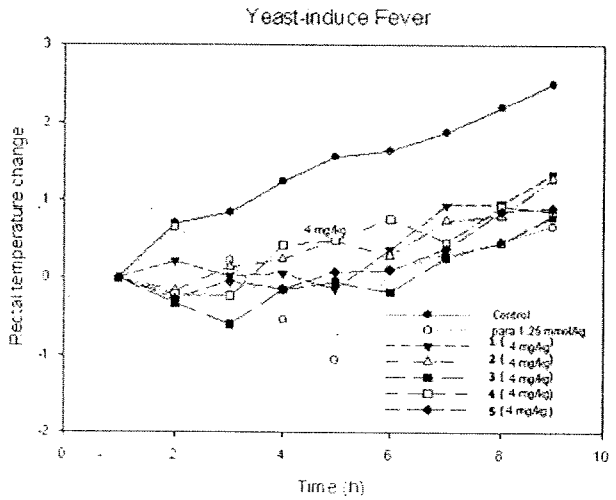


Fig. 1. Antipyretic effect of the andrographolide and andrographolide derivatives (4 mg/Kg^{-1} dose) in baker yeast-induce fever test. Rats were injected intraperitoneally with 5% DMSO, 125 mmole/Kg paracetamol or 4 mg/kg of **1**, **2**, **3**, **4** or **5**. Results were expressed as mean ± S.D. 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.

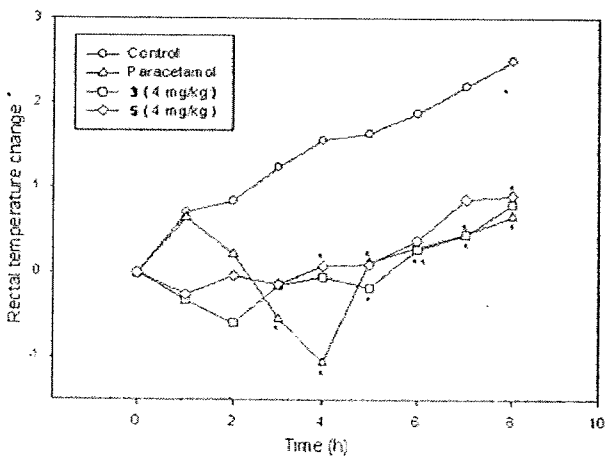


Fig. 2. Antipyretic effect of the **1**, **3** and **5**, at 4 mg/kg dose, in Baker yeast-induce fever test

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.

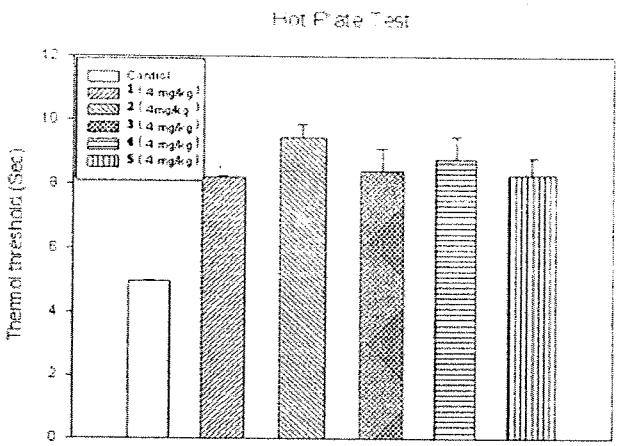


Fig. 3. Analgesic effect of the andrographolide and its derivatives in the hot plate test. Mice were injected intraperitoneally with either 5% DMSO or 4 mg/kg of **1**, **2**, **3**, **4** or **5**. Results were expressed as mean ± S.D. for reaction time. *$p < 0.05$ when compared to the control group.

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.

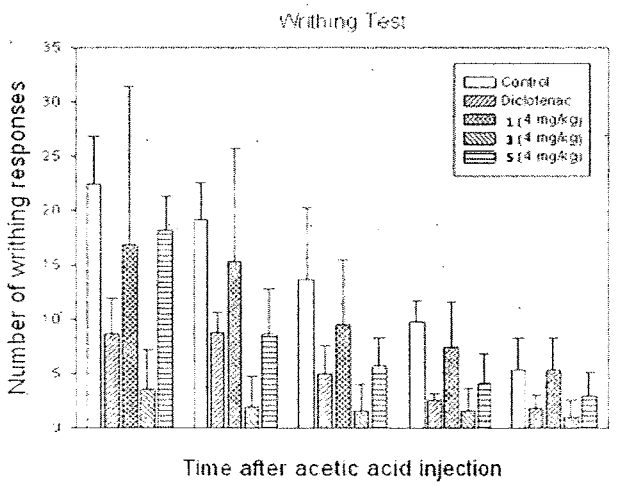


Fig. 4. Analgesic effect in the Writhing test. Mice were injected intraperitoneally with either 5% DMSO, 20 mg/kg diclofenac or 4 mg/kg of **1**, **3** and **5**. At 5, 15, 25, 35, 45 minutes after 1% acetic acid injection, number of Writhing responses observed as mean ± S.D. in 5 minute-interval each.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

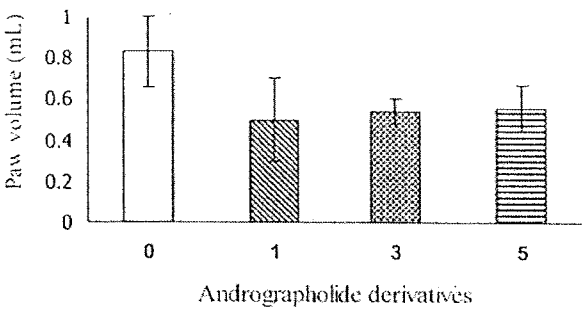


Fig. 5. Anti-inflammation effect in the carrageenan-induced paw edema test. Rats were received intraperitoneal injection with 5% DMSO or 4 mg/kg of **1**, **3** or **5**. Rat's paw volume changes, hour 3, were expressed as mean ± S.D.

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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Effects of andrographolide on sexual functions, vascular reactivity and serum testosterone level in rodents

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ABSTRACT

In this study, effects of andrographolide from *Andrographis paniculata* on sexual functions, vascular reactivity and serum testosterone level in experimental animals were observed. The suspension of andrographolide in 5% DMSO was administered orally at the dose of 50 mg/kg to male ICR mice. The female mice involved in mating were made receptive by hormonal treatment. The mating behaviors, mounting latency and mounting frequency, were determined and compared with the standard reference drug sildenafil citrate. Administration of andrographolide significantly decreased the mounting latency at 120 and 180 min and increased mounting frequency at 180 min after treatment. In endothelium-intact rat aortic strips, norepineprine-induced contraction was reduced by preincubation with andrographolide. Administration of 50 mg/kg andrographolide orally to male mice once daily for 2, 4, 6 or 8 weeks had no significant effects on sperm morphology and motility. Interestingly, at week 4, serum testosterone level in mice treated with andrographolide was significantly increased when compared to the control. Thus, the effects of andrographolide on vascular response to norepinephrine and testosterone level observed in this study might be contributed to the sexual enhancing properties observed.

1. Introduction

Andrographis paniculata (Burm. F.) Wall. Ex Nees is one of a plant belongs to family *Acanthaceae*. It is one of the important herbal medicines which have been used for centuries in Asia to treat several diseases such as gastro-intestinal tract and upper respiratory infections, fever and herpes. Indian Pharmacopoeia describes that it is a predominant constituent of at least 26 Ayurvedic formulations. In traditional Chinese medicine, *A. paniculata* is considered as the herb possessing an important "cold property" useful to treat the heat of body in fever, and to dispel toxins from the body. In Scandinavian countries, it is commonly used to prevent and treat common cold (Mishra et al., 2007). The fresh and dried leaves as well as the juice of the whole plant are widely used in China, India, Thailand and other Southeastern Asian countries, it is also known as King of Bitters (English), Mahatikta (Sanskrit), Kiryato (Gujarati), Mahatita (Hindi), Kalmegh (Bengali) or Fah Talai Jone (Thai) (Li et al., 2007). Andrographolide, a major component of *A. paniculata*, has been reported to have multiple pharmacological properties, such as antipyretic (Pongnaratorn et al., 2007), anti-inflammatory (Shen et al., 2002), anti-allergic (Xia et al., 2004), anti-platelet aggregation (Amroyan et al., 1999), antiviral (Wiart et al., 2005), anti-HIV (Reddy et al., 2005), antithrombotic (Thisoda et al., 2006), and antidiabetic activities (Reyes-Balaguer et al., 2005; Yu et al., 2008). It also has immunostimulatory (Xu et al., 2007), hepatoprotective (Singha et al., 2007), and anticancer activities by inhibition of cell cycle progression (Shi et al., 2008). Accordingly, the compound has been widely used for the treatment of fever, cold, inflammation, diarrhea and other infectious diseases.

Several animal studies showed that *A. paniculata* may have contraceptive or anti-fertility effects following long-term treatment at high doses (20 mg/rat) (Akbarsha et al., 1990). On the other hand, there was a large degree of discrepancy in the results, with some studies demonstrating no untoward effects even at the 1000 mg/kg dose (Burgos et al., 1997). Moreover, in a phase I clinical study, no significant negative effect of *A. paniculata* (fixed combination "Kan Jang®") on male semen quality and fertility could be observed, and rather, the comment by the principally of volunteers was feeling of enhanced sexual potency during masturbation (Mkrtchyan et al., 2005).

Since, quite a few scientifically studies had been reported for the effects of andrographolide on sexual functions, in this study andrographolide was tested for their effects on sexual behaviors, vascular reactivity, sperm quantity and motility, and serum testosterone level in male mice.

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2. Materials and methods

2.1. Preparation of andrographolide from A. paniculata

Andrographolide was isolated from dried powdered *A. paniculata* purchased from an Ubonratchathani Agriculturer Community, the plant's voucher (No. ISB 003) deposited at the Faculty of Pharmaceutical Sciences, Khon Kaen University, had been compared to the authentic plant, DMSc Herbarium No. 821. The methanolic extract of *A. paniculata* leave was separated on a column chromatography, using methylene chloride and methanol as gradient eluting solvents (Fujita et al., 1984). It was purified by recrystallization in methanol. The structure was identified by using proton and carbon 13 Nuclear Magnetic Resonance (NMR, Varian® Mercury 400), Mass Spectrophotometry (LCMS, Bruker Daltonics, Billerica, USA), infrared absorption (IR, Perkin–Elmer® Series 1600, KBr Disc). Andrographolide was white or colourless crystals (CH_2Cl_2), melting point 225–227 °C uncorrected, lit 218–221 °C; Rf 0.35, silica gel 60F254 (conformed to the andrographolide purchased from Aldrich), 10% methanol in CH_2Cl_2.

IR (KBr) ν_{max} cm^{-1}: 3399 with shoulder at 3322 (broad and s), 3152 (w), 2932 (m), 2849, 1732 (very s), 1675(m), 1221, 1033.

δ ppm ^1H *NMR (*CD_3OD*, 400 MHz,):* 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).

δ ppm ^{13}C *NMR (400 MHz,* CD_3OD*):* 172.7 (C, C-16), 149.4 (CH, C-12), 148.9 (C, C-8), 129.8 (C, C-13), 109.2 (CH_2, C-17), 80.9 (CH, C-3), 76.2 (CH_2, C-15), 66.7 (CH, C-14), 65.0 (CH_2, C-19), 57.4 (CH, C-9), 56.3 (CH, C-5), 43.7 (C, C-4), 40.0 (C, C-10), 39.0 (CH_2, C-7), 38.1 (CH_2, C-1), 29.0 (CH_2, C-2), 25.73 (CH_2, C-11), 25.2 (CH_2, C-6), 23.4 (CH_3, C-18), 15.5 (CH_3, C-20).

The percentage yield of andrographolide was 2.3.

2.2. Animals

Experiments were conducted using ICR mice and Sprague-Dawley rats obtained from the Animal House, Faculty of Medicine, Khon Kaen University, Thailand. The animals were housed in group 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 h. The experiments were approved by the Animal Ethic Committee, Khon Kaen University, Khon Kaen, Thailand No. 0514.1.12.2/13.

2.3. Sexual behavior test

To examine the effects of andrographolide on sexual behaviors in mice, mating behavior test (Helmrick and Reiser, 2000; Tajuddin et al., 2004) was performed. The healthy and naïve female mice used in this study were artificially brought into estrous by subcutaneous injections of β-estradiol-3-benzoate in corn oil (10 μg/mouse) 48 h before experiment, then, received a subcutaneous injection of progesterone in corn oil (500 μg/mouse) 4 h prior to the test. Male mice were treated orally with either 5% DMSO, 50 mg/kg andrographolide in 5% DMSO or 5 mg/kg sildenafil citrate. At various time after treatment, sexual behavior test was made by introducing estrous female mouse into the cage of treated male mouse. The observation period was 10 min. Sexual behaviors were determined by mounting latency [ML, time elapsed from introduction of male with female to the first mount (male approaches a female and assumes a copulatory position)] and mounting frequency [MF, number of mounts].

2.4. Rat aortic strips

Male Sprague-Dawley rat was killed and opened the chest, then, thoracic aorta was rapidly removed, gently cleaned taking care not to damage the endothelium and transferred to a dish containing Krebs' solution. After removal of loose connective tissue, cut spirally about 2 mm-wide and 2 cm long. Each rat provided two strips of thoracic aorta smooth muscle that were studied separately. Aortic strips were placed in a 25 ml organ bath containing Krebs solution (in mM: 119NaCl, 4.7KCl, 2.5$CaCl_2$, 1$MgCl_2$, 25$NaHCO_3$, 1.2K_2HPO_4, 11 Glucose). One end of the strip was connected to tissue holder and the other end connected to Grass FT03 force displacement transducer. The bathing solution was gassed with 95–5% CO_2 at 37 °C. The tissue was equilibrated for 30 min under a resting tension of 1 g. During this time, Krebs solution was replaced every 15 min with fresh solution. Tension was recorded using Grass polygraph recorder. Norepinephrine (0.15 ng/ml, final concentration in the bath) was used to induce the strip contraction. The change of strip tension was recorded for 10 min, after which the preparations were rinsed two times and allowed to recover. The effect of andrographolide (0.2 mg/ml) on norepinephrine contraction was done by preincubated the strip with andrographolide for 10 min before adding norepinephrine.

2.5. Effect of andrographolide on serum testosterone level

Mice were orally administered with either 5% DMSO or 50 mg/kg andrographolide once daily. By the end of weeks 2, 4, 6 and 8 of treatment, mice were anesthetized with ether. Blood was collected and serum was separated for the measurement of serum testosterone level by the DSL-4000 ACTIVE® Testosterone Coated-Tube Radioimmunoassay Kits (Diagnostic Systems Laboratories, Texas, USA). Cauda epididymis were removed and used for the experiment in Section 2.6.

2.6. Effect of andrographolide on sperm count, and sperm motility

Cauda epididymis were cut and homogenized in 5 ml saline (NaCl 0.9%). Homogenates were kept refrigerated at 4 °C for 24 h to allow sperm be released from the walls. Then, 5 ml of eosin (2%) was added and vortexed. One milliliter of this mixture was diluted with 2 ml eosin (2%) and a sample was placed in a hemocytometer chamber and sperm heads were counted. The contralateral epididymis was cut at the cauda position and cut by scissors to release sperm. A drop of fluid was taken and diluted with saline. The sample was observed under a light microscope at 40×. Approximately 100 spermatozoa (both motile and non-motile spermatozoa) were counted.

2.7. Statistical methods

The data were expressed as mean ± SE and one-way analysis of variance (ANOVA) and supplementary Fisher LSD test for pair wise comparison were tested to determine for any significant difference at $p < 0.05$.

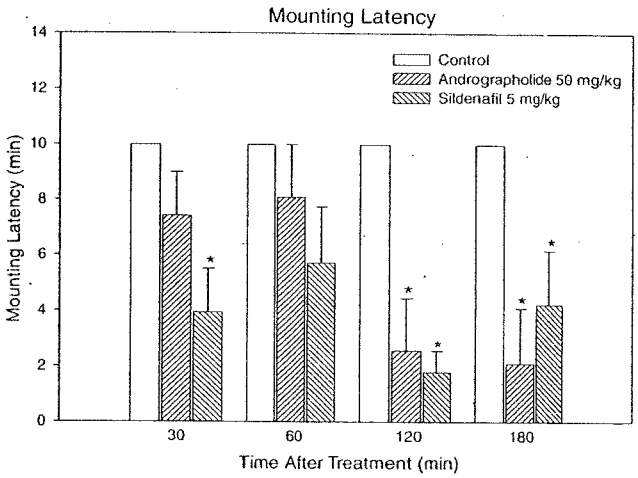

Fig. 1. Effect of andrographolide on mounting latency of male mice. Male mice were orally fed with either 5% DMSO (control), 50 mg/kg andrographolide or 5 mg/kg sildenafil. At various time after treatment, mounting latency was recorded after introducing estrous female mouse into the cage of treated male mouse. There were 5–6 animals in each group. *p value <0.05 when compared to the control at the same time point.

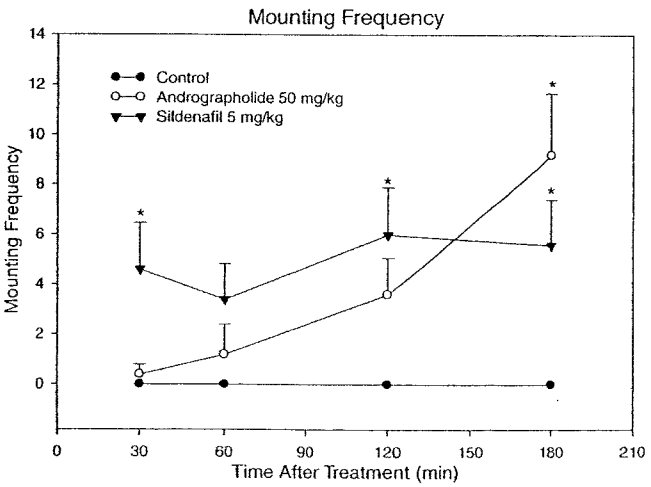

Fig. 2. Effect of andrographolide on mounting frequency of male mice. Treatment protocol was the same as mentioned in Fig. 1 legend.

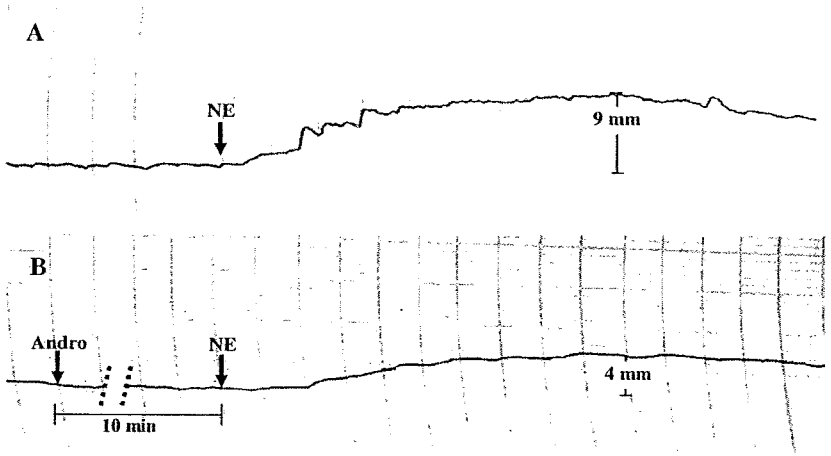

Fig. 3. Original tracing of experiments that demonstrate the effects of 0.15 ng/ml norepinephrine (NE) on rat aortic strip: (A) without and (B) with the 10 min preincubation of 0.2 mg/ml andrographolide (Andro).

3. Results

3.1. Andrographolide increased sexual behavior in male mice

Administration of 50 mg/kg andrographolide orally showed a significant aphrodisiac effect in male mice especially at 180 min after treatment. A significant decrease in mounting latency and a significant increase in mounting frequency could be clearly seen at 120 and 180 min after treatment. Although the effects of sildenafil on both mounting latency and frequency could be significantly observed as early as 30 min after treatment, the effects of andrographolide were comparable with sildenafil citrate, at 120 and 180 min after treatment (Figs. 1 and 2).

3.2. Andrographolide inhibited norepinephrine-induced contraction of rat aortic strips

Norepinephrine at 0.15 ng/ml could rapidly contract endothelium-intact rat aortic strip and increase aortic strip tension as shown in Fig. 3. Adding andrographolide (0.2 mg/ml) had no obvious effect on aortic strip tension (data not shown). However, preincubation of the aortic strip with andrographolide for 10 min before adding norepinephrine resulted in a significant reduction of norepinephrine effect on aortic strip tension (Fig. 4).

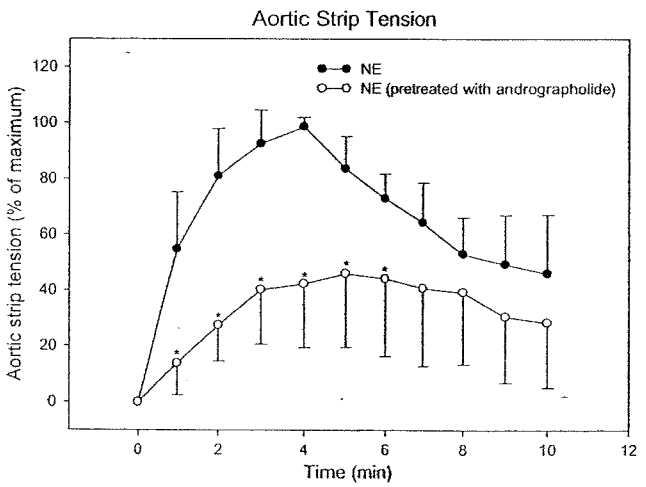

Fig. 4. Effect of 0.2 mg/ml andrographolide pretreatment on aortic strip tension treated with 0.15 ng/ml norepinephrine (NE). *p value <0.05 when compared to NE alone at the same time point.

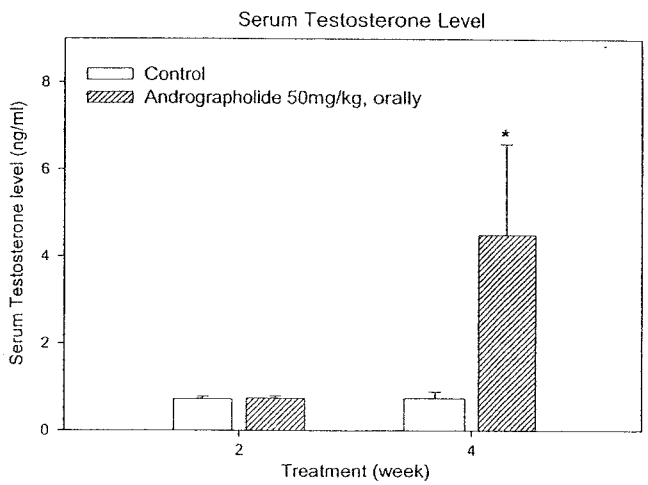

Fig. 5. Effect of andrographolide on serum testosterone level of male mice. *p value <0.05 when compared to the control at the same time point.

3.3. Effect of andrographolide on serum testosterone level

Male mice received andrographolide (50 mg/kg/day, orally) for 2 weeks had a comparable level of plasma testosterone with mice treated with 5% DMSO. Interestingly, at 4 weeks of treatment, mice treated with andrographolide had a significant higher level of plasma testosterone when compared to the control (Fig. 5). However, at weeks 6 and 8 of treatment, serum testosterone level of mice treated with andrographolide declined back to normal level.

3.4. Effect of andrographolide on sperm count, and sperm motility

Either at 2, 4, 6 or 8 weeks of treatment with andrographolide (50 mg/kg/day, orally), no effect on sperm count and/or sperm motility could be seen when compared to the control. Nevertheless, a tendency of a decrease in sperm number at 8 weeks of the treatment was seen (data not shown).

4. Discussion

This study showed that given andrographolide orally in male mice decreased mounting latency and increased mounting frequency. A significant change in mounting latency and frequency

was observed at 2 and 3 h after treatment suggested onset/peak of the effects was 120–180 min comparable to 5 mg/kg sildenafil and in accordance with the time to maximum plasma level in human (Wangboonskul et al., 2006).

Through the mechanism involved with blood vessels causing smooth muscle relaxation and increase blood flow into the penis, many plants and drug such as sildenafil citrate (Viagra®) are used to treat erectile dysfunction. Sildenafil acts by inhibiting cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type-5 (PDE-5) and produce a local release of NO (Gumus et al., 2004). Many plants used as traditional medicines for treating impotence have been reported to produce vasodilatation. Some of medicinal plants, such as *Hiptage benghalensis* and *Betula alnoides*, are the great sources of PDE inhibitors (Temkitthawon et al., 2008) and ginseng increased NO releasing (Nocerino et al., 2000). In this study, pretreatment with andrographolide (0.2 mg/ml) for 10 min could reduce the vasoconstrictive effect of norepinephrine on rat aortic strips and probably acting through α-adrenoceptors (Zhang and Tan, 1997). It might be the case that andrographolide increased sexual behaviors by producing the relaxation of blood vessel through the blockade of α-adrenergic receptor. Lukacs and colleagues reported that, in patients with benign prostatic hyperplasia (BPH) who received alfuzosin (α_1 receptor antagonist), self-perceived sense of sexual satisfaction was significantly improved from baseline (McVary, 2005; Lukacs et al., 1996; van Moorselaar et al., 2005). In addition, andrographolide was also reported to selectively block voltage-operated calcium channels in vas deferens smooth muscle (Burgos et al., 2000). The effect on calcium channels might contribute to the vasodilatation effect of andrographolide.

Chronic daily treatment of andrographolide showed a prominent increase in serum testosterone level at week 4 of the treatment. However, with continuing of the treatment, serum testosterone level returned to normal at weeks 6 and 8. It could be the case that, increasing testosterone level via an unknown mechanism seen at week 4 of the treatment may further cause negative feedback on testosterone synthesis and bring back the level to normal concentration. Although some reports showed that the extract of *A. paniculata*, at doses up to 1000 mg/kg in male rats for 60 days and more (Burgos et al., 1997; Allan et al., 2009), could not produce any significantly effect on plasma concentration of testosterone, it should be noted that, in this study high testosterone level was observed at week 4 (28 days), but not at week 8 (56 days).

The effects of andrographolide on sperm have been reported earlier either negative (Akbarsha et al., 1990; Akbarsha and Murugaian, 2000), positive (Burgos et al., 1997; Mkrtchyan et al., 2005) or no effects (Allan et al., 2009). In this study, no toxicity of andrographolide (50 mg/kg) treatment for up to 8 weeks on number and motility of sperm could be observed.

In summary, the results of this study established that, the aphrodisiac-like effect of andrographolide is comparable to sildenafil. The acute aphrodisiac response might be attributed to the effect on blood vessel. While the effect on sex hormone after using andrographolide or *A. paniculata* in patients with impaired testosterone level might be able to bring back hormone level to normal and help to repair the decreased libido and decreased mental and physical sexual activities.

Conflict of interest

The authors declare that there are no conflicts of interest.

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High-performance liquid chromatographic method for andrographolide analogues used for anti-herpes simplex virus type-1 agents

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Abstract: A reversed phase high-performance liquid chromatography (HPLC) method was established for quantitative determination of anti-*herpes simplex* virus type-1 (HSV-1) agents, andrographolide analogues, namely androgra -pholide (1), 14-deoxy-11,12-didehydroandrogra-pholide (2), 14-deoxyandrographolide (3) and 3,19-isopropylideneandrographolide (4). The system consisted of a Hypersil® BDS C-18 (250x4.6 mm, 5μm) column flowed with (70:30) acetronitrile/water at 1 mL.min^{-1}. The UV-detector was set at 200 nm for (1)-(3) and 223 nm for (4). Validation parameters, were evaluated according to the ICH guideline. The linearity of standard curves over concentration ranges are as followed: 18.6-223.5 μg.mL^{-1}, r^2=1; 13.0-157.5 μg.mL^{-1}, r^2= 0.9995; 16.0-192.0 μg.mL^{-1}, r^2= 0.9998, and 12.5-150 μg.mL^{-1}, r^2=0.9999 for (1), (2), (3) and (4), respectively. The precision and reproducibility parameters of all compounds were in the ranges of 0.06-0.08 and 0.57-3.01%, respectively and the accuracies of various concentrations of (1) to (4) were 92.5-102.3%, 92.6-105.5%, 91.5-102.0% and 101.7-107.1%, respectively. The limits of quantitation (LOQ) were 4.7, 4.2, 4.2, and 5.2 μg.mL^{-1} for (1) to (4), respectively.

Introduction

Andrographolide is a diterpene lactone and a major consituent of *Andrographis paniculata* (Burm.f.) Nees. (Acanthaceae), a well-recognized medicinal plant in Thailand. Wiart et al. (2005) reported anti-HSV-1 effect of neoandrographolide, andrographolide and 14-deoxy-11,12-didehydroandrographolide [1]. In our investigation, andrographolide analogues both natural and synthetic analogues were comparatively studied for the anti-HSV-1 effect [2]. The correlation between the structures and anti-HSV-1 activity were established with good trend for further drug development. In this study a HPLC method was developed for quantitative determination of andrographolide (1) and its analogues, 14-deoxy-11,12-didehydroandrographolide (2), 14-deoxyandrographolide (3) and 3,19-isopropylideneandrographolide (4) (Fig. 1), so that further investigation of relevant properties can be determined.

The method was validated according to the ICH guideline for the following parameters: linearity, precision, % recovery and limit of quantification (LOQ).

Materials and Methods

Standard, reagents, and apparatus

Extracting solvents, namely hexane, dichloromethane, ethyl acetate and methanol, are of analytical grade and HPLC solvents were obtained from Merck (Darmstadt, Germany). HPLC analysis was performed using an Agilent Technologies Series 1200 system equipped with an automatic injector, a quaternary pump, a vacuum degasser, and a photodiode array UV-visible detector.

Andrographolide (1) and its analogues, 14-deoxy-11,12-didehydroandrographolide (2), 14-deoxyandro -grapholide (3) were isolated from *Andrographis paniculata* purchased from Agriculturer Community in Ubon Ratchathani. Compound (4) was prepared as described by Jada and Nanduri [3,4]. All compounds were confirmed by spectroscopic data as described in literatures [3-5].

HPLC analysis

Preparation of stock solutions: 1.0 mg.mL^{-1} of each compound in acetronitrile was prepared separately as stock solutions. Various concentrations of each compound were made by dilution each of stock solution with acetronitrile to give a concentration range of 50-80 μg.mL^{-1}.

Stationary phase: A Hypersil® BDS C-18 column (250x4.6 mm, 5 μm particle size) was used and the column temperature was maintained at 25 °C.

Mobile phase: a 70:30 mixture of acetronitrile and water with the flow rate of 1 mL.min^{-1}

Detection wavelengths: 200 nm for (1)-(3) and at 223 nm for (4).

Procedure: A 20 μL of each sample was injected separately.

Validation of HPLC method

The system suitability of HPLC method was evaluated with validation parameters included linearity, precision, accuracy and limit of quantification (LOQ) according to the ICH guidelines. The intra- and inter-day replications were carried out

to determine the reproducibility and precision, respectively. The accuracy was evaluated by standard addition method. Known amounts of each of the standard was added into corresponding test solutions, The added amount was determined by the method and compared to the actual amount and calculated as a % recovery.

Results and Discussion

Characterization of four analogues

The structures of andrographolide analogues, (1)-(4), see Fig. 1, were isolated and identified by comparison of their NMR, MS, IR spectroscopic data with literature values [3,4,5]. Natural analogues (1)-(3) were obtained 2.3, 0.8 and 0.1% yields, respectively and 85% 3,19-isopropylideneandrographolide was yielded from the synthesis.

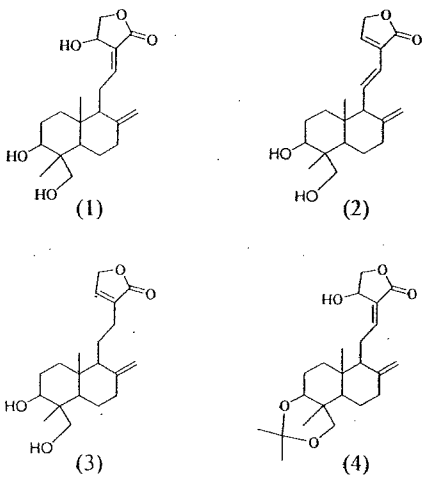

Figure 1. Chemical structures of andrographolide (1), 14-deoxy-11,12-didehydroandrographolide (2) and 14-deoxyandrographolide (3) and 3,19-isopropylidene-andrographolide (4).

HPLC method for analysis

The chromatograms obtained for HPLC analyses of (1) to (4) at various wavelengths are showed in Fig 2A to 2E. Their retention times were 2.81, 3.36, 3.39 and 4.84 min for (1), (2), (3) and (4), respectively. Since the structures of (2) and (3) are closely related, their elutions were not resolved from each other. Therefore, they were not able to be determined simultaneously. By the way, three components, (1), (2) and (4), or (1), (3) and (4) can be determined simultaneously if required. The highest sensitivity of (1)-(4) were at the wavelength of 200 nm (Fig 2A- 2E). However, (4) was determined at 223 nm to avoid some interference when the method was applied to determine the content in sample.

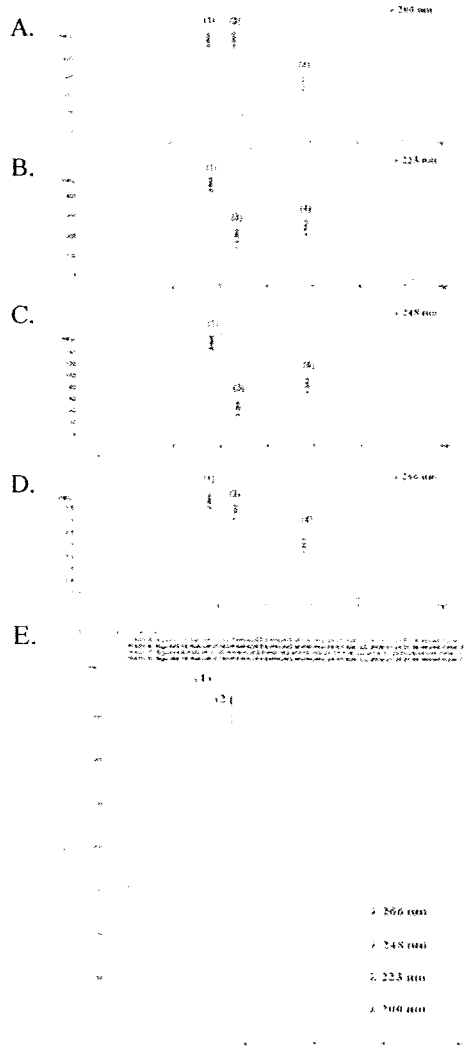

Figure 2. Chromatograms of (1), (3) and (4) at various wavelengths; A. 200 nm, B. 223 nm, C. 248 nm and D. 266 nm; and E. 3D chromatogram of (2) at various wave lengths.

Validation method

Although in each triple combination, each component does not interfere to each other. They were validated individually. Their validation parameters were shown in Table 1.

To assess the precision of the method, the inter-day and intra-day variations were preformed six times on the same day and six times a day for three consecutive days. The relative standard variation was less than 0.08% for the intra-day and less than 3.01% for the inter-day, as shown in Table 2. The accuracy was evaluated by recovery studies. The experiment was performed by adding and extra amount of each compound to three standard concentrations. The mean recoveries were 98.7, 100.3, 96.7 and 104.7 % for (1) to (4), respectively (Table 2).

Table 1. Linearity and LOQ values of (1)-(4).

Compounds	Linearity ranges ($\mu g.mL^{-1}$)	Linearity Equations	R^2	R_t (min)	LOQ ($\mu g.mL^{-1}$)
(1)	18.6-223.5	$y = 25189x$-38.763	1.0000	2.81	4.72
(2)	13.0-157.5	$y = 33521x$-44.753	0.9995	3.36	4.24
(3)	16.0-192.0	$y = 33205x$-41.832	0.9998	3.39	4.18
(4)	12.5-150.0	$y = 24051x$-47.659	0.9999	4.84	5.21

Table 2. Recoveries, intra-day, inter-day variations of (1)-(4).

Compounds	Recovery (N=3)				Precision (N=6)	
	Amount Comp ($\mu g.mL^{-1}$)	Amount added ($\mu g.mL^{-1}$)	Recovery (%)	Mean ± S.D. (%)	Intra-day (R.S.D.)	Inter-day (R.S.D.)
(1)	18.6	74.5	92.47			
	74.5	74.5	102.33	98.66±5.39	0.06	0.57
	223.5	74.5	101.18			
(2)	13.0	52.5	92.62			
	52.5	52.5	105.49	100.27±6.77	0.08	3.01
	157.5	52.5	102.69			
(3)	16.0	64.0	91.49			
	64.0	64.0	102.00	96.69±5.25	0.06	1.76
	192.0	64.0	96.59			
(4)	12.5	50.0	107.12			
	50.0	50.0	101.71	104.69±2.75	0.07	0.74
	150.00	50.0	105.24			

Conclusions

In conclusion, a method for quantitative determination of andrographolide and its analogues was established by using a reversed phase high pressure liquid chromatography. Satisfactory validation parameters were obtained and it was applicable to analyse these compounds in some high content of ionic matrix.

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Date: 16-07-2010
To: "Chantana Aromdee" chaaro@kku.ac.th
From: "Mi-Ock Lee" eic.pskor@gmail.com
Subject: ARPR: Your manuscript entitled Effect of the derivatives of Andrographolide on the morphology of B. subtilis

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Ref.: Ms. No. ARPR-D-09-00230R1
Effect of the derivatives of Andrographolide on the morphology of B. subtilis
Archives of Pharmacal Research

Dear Ms Aromdee,

I am pleased to tell you that your work has now been accepted for publication in Archives of Pharmacal Research.

It was accepted on 16-07-2010.

Thank you for submitting your work to this journal.

With kind regards

Young-Pyo Jang, Ph.D., Associate Editor
Sang-Geon Kim, Editor in Chief
Mi-Ock Lee, Editor

Archives of Pharmacal Research

The manuscript has been properly revised according to reviewers' commnets and ready for the publication on this journal

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Editorial Manager(tm) for Archives of Pharmacal Research
Manuscript Draft

Manuscript Number: ARPR-D-09-00230R1

Title: Effect of the derivatives of Andrographolide on the morphology of B. subtilis

Article Type: Original Article

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Abstract: Abstract
Andrographis paniculata has been reported to have antiviral, antipyretic and anticancer activities, and andrographolide, an ent-labdane diterpene, is an active constituent in this plant. In this study, andrographolide (1) and its natural derivative 14-deoxy-11,12-didehydroandrographolide (2) and 5 other semisynthetic derivatives were tested for their activity against Gram-positive and Gram-negative bacteria and Candida albicans. Only derivatives bearing a 14-acetyl group showed activity, and this activity was only against Gram-positive bacteria. 14-Acetylandrographolide showed the highest potency against Bacillus subtilis; the other 14-acetylandrographolides with additional substitution at the 3- and 19-hydroxyl groups showed lower activity against Gram-positive bacteria. The morphology of B. subtilis after being treated with 14-acetylandrographolide was investigated with TEM. This is the first report on 14-acetylandrographolide's quantified antibacterial activity, and the crucial functional group of this ent-labdane that plays an important role in perturbing the morphogenesis of B. subtilis leading to cell death.

Key words: acetylated andrographolides, antibacterial, B. subtilis, morphological change

Response to Reviewers: Response to reviewer:
Thank you so much for your kind response and reflecting the error and some ambiguous phrases of the manuscript. Right now we added and altered some words so that the statements will be clearer to understand.

Reviewer1:
1. Abstract: we edited as shown in this document.
2. We have our manuscript totally edited by The American Journal Expert (UK English).
Reviewer 2: Error corrected.

Chantana

Abstract

Andrographis paniculata has been reported to have antiviral, antipyretic and anticancer activities, and andrographolide, an *ent*-labdanediterpene, is an active constituent in this plant. In this study, andrographolide (1) and its natural derivative 14-deoxy-11,12-didehydroandrographolide (2) and 5 other semisynthetic derivatives were tested for their activity against Gram-positive and Gram-negative bacteria and *Candida albicans*. Only derivatives bearing a 14-acetyl group showed activity, and this activity was only against Gram-positive bacteria. 14-Acetylandrographolide showed the highest potency against *Bacillus subtilis*; the other 14-acetylandrographolides with additional substitution at the 3- and 19-hydroxyl groups showed lower activity against Gram-positive bacteria. The morphology of *B. subtilis* after being treated with 14-acetylandrographolide was investigated with TEM. This is the first report on 14-acetylandrographolide's quantified antibacterial activity, and the crucial functional group of this *ent*-labdane that plays an important role in perturbing the morphogenesis of *B. subtilis* leading to cell death.

Key words: acetylated andrographolides, antibacterial, *B. subtilis,* morphological change

1. Introduction

Andrographis paniculata (Burm. f.) Wall. ex. Nees (Acanthaceae) is a traditional medicine widely used in Asian countries for protozoacidal, antihepatotoxic, anti-HIV, immunostimulant, anticancer, hypoglycemic and hypotensive activities (Nanduri *et al.*, 2004). Andrographolide is an *ent*-labdane diterpene, a bicyclic hydrocarbon with a γ-lactone. Andrographolide (1) and its 22 natural derivatives isolated from *A. paniculata* were qualitatively evaluated for their antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, *Candida albicans*, *C. sake* and *Aspergillus niger* (Shen *et al.*, 2006). Andrograpanin, 14-deoxy-11,12-didehydroandrographolide, 14-deoxy-12-hydroxyandrographolide and isoandrographolide at a concentration of 10 μg/ml were found to inhibit *B. subtilis* with clear inhibition zones of 7-8 mm. The other compounds were inactive against all of the organisms tested.

Fan *et al.*, (2006) investigated the activity of acetylandrographolide on some bacteria, mildews, yeasts and phytopathogens. They found that acetylandrographolide was a stronger antimicrobial agent than (1) against a majority of the tested microbes, and particularly on *Bacillus subtilis*, but the extract could not inhibit or kill all *S. aureus*.

Some other bicyclic diterpenoids with or without a γ-lactone ring were found to have activity against Gram-positive bacteria (Habibi *et al.*, 2000; Hanson, 2005). Until now, it has been found that all bacteria sensitive to labdane diterpenes were Gram-positive (Shen *et al.*, 2006; Fan *et al.*, 2006; Habibi *et al.*, 2000; Hanson, 2005).

In this work, two natural compounds, andrographolide (1) and 14-deoxy-11,12-didehydroandrographolide (2), as well as the semisynthetic derivatives 3,19-isopropylideneandrographolide (3), 14-acetyl-3,19-isopropylideneandrographolide (4),

14-acetylandrographolide (5), 3,14,19-triacetylandrographolide (6) and 14-deoxy-11,12-didehydro-3,19-diacetylandrographolide (7), see Figure 1, were tested for antimicrobial activity against *S. aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 90028. Compound(s) showing the strongest antimicrobial activity were selected for examination of their effect on the microbial cellular morphology by transmission electron microscopy. Thus, postulation of the mode or site of action of the *ent*-labdanes could be established, and the functional groups that play important roles in the activity could be recognised. Utilisation of this structure activity relationship (SAR) could be applied in the future development of antimicrobial compounds against other related microorganisms for therapeutic or other purposes.

2. Materials and Methods

Andrographolide and 14-deoxy-11,12-didehydroandrographolide were isolated from dried and powdered *A. paniculata* purchased from an Ubon Ratchathani community agricultural agent. The plant's voucher number (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. The source of this material was ascertained to contain very high andrographolide content (Aromdee *et al.*, 2005). 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, MA, USA). Melting temperatures were determined on a melting point apparatus (Electrothermal Engineering Ltd. IA9900 Series no. 9808). LCMS samples were introduced by flow injection and electrospray ionisation for positive mass and detected

with a TOF detector from 50 m/z to 3000 m/z. Spectroscopic data of known compounds were compared with published data.

All andrographolide and andrographolide derivatives were detected by a thin-layer chromatography (TLC) system using silica gel GF_{254} precoated plates (Merck KGaA, Germany) as a stationary phase, and mixtures of methanol in dichloromethane as mobile phases. The plates were examined under ultraviolet (UV) light at 366 nm, and the existence of the intact γ-lactone was confirmed by spraying with the Kedde reagent. Reaction mixtures were partitioned in water and dichloromethane. The dichloromethane phase was dried with anhydrous sodium sulphate and chromatographed on a silica column eluted with mixtures of hexane and ethyl acetate.

2.1. Isolation and semisynthesis of andrographolide derivatives

Isolation of andrographolide (1) and 14-deoxy-11,12-didehydroandrographolide (2)

Dried powder of *A. paniculata* was macerated in methanol for 3 d. The extract was evaporated to dryness (11.1% yield) and chromatographed on a column using dichloromethane and 1-5% methanol in dichloromethane as eluents (see Scheme 1). Eluates were continuously collected in 100 ml fractions and monitored by a TLC system using 10% methanol in dichloromethane as a mobile phase. Compound (1) was eluted in fractions 59-72; the yield was 4% – white or colourless crystals (CH_2Cl_2), mp 225-227 °C, lit 218-221 °C (Fujita *et al.*, 1984).

Compound (2) was eluted in fractions 42-50; the yield was 0.5% – white or colourless crystals (CH_2Cl_2), mp 198-200 °C, lit 203-204 °C (Fujita *et al.*, 1984).

3,19-Isopropylideneandrographolide (3)

As described by Nanduri *et al.* (2004), (3) was prepared from the reaction of (1) and dimethoxypropane using pyridinium *p*-toluenesulfonate as a catalyst. Compound (3) was obtained in 80% yield – a crystalline powder from hexane and ethyl acetate (1:1); mp 194-196 °C.

14-acetyl-3,19-isopropylideneandrographolide (4)

Compound (3) (0.1934 g, 0.495 mmole) in dichloromethane was stirred with acetic anhydride (2.5 ml, 26.4 mmole) and 2 mg of DMAP for 1 h at room temperature. Compound (4) and compound (5) were obtained in 30% and 60% yields, respectively. (4) – amorphous powder (CH_2Cl_2), hygroscopic, mp 58-59 °C.

14-acetyl andrographolide (5)

Compound (5) was obtained as described in the semisynthesis of (4). (5) – white powder (CH_2Cl_2); mp 168.29 °C (onset at 163.65, end at 170.41 °C, lit 168-170 °C (Jada *et al.*, 2007).

3,14,19-triacetyl andrographolide (6)

Andrographolide (1) (0.5368 g, 1.53 mmole) in DMSO and dichloromethane was reacted with 2.5 ml (26.4 mmole) of acetic anhydride and 5 mg of DMAP for 1 h at room temperature. The yield of (6) was 95% – crystalline powder (CH_2Cl_2), mp 110-111 °C, lit 110-111 °C (Jada *et al.*, 2007).

14-deoxy-11,12-didehydro-3,19-diacetyl andrographolide (7)

Andrographolide (1) (0.5004 g, 1.43 mmole) was mixed with acetic anhydride (0.33 ml, 3.23 mmole) and 5.0 ml of pyridine for 4 h at room temperature. Compound (7) was obtained in 50% yield – amorphous powder (CH_2Cl_2), mp 107-109 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.15 (1H, *s*, H-12), 6.91 (1H, *dd*, *J*=10.1 and 15.6 Hz, H-12), 6.12 (1H, *d*, *J*=15.8 Hz, H-14), 4.80 (2H, *s*, H-15), 4.79 (1H, *s*, 17a), 4.59 (1H, *t*, *J*=8.4 and 7.9 Hz, H-3), 4.55 (1H, *s*, H-17b), 4.37 (1H, *d*, *J*=11.4 Hz, H-19a), 4.14 (1H, *d*, *J*=11.9 Hz, H-19b), 2.03 (6H, 2Ac), 1.02 (3H, *s*, H-20), 0.89 (3H, *s*, H-18). ^{13}C-NMR ($CDCl_3$) δ: 172.2 (C, C-16), 170.9 (C, Ac), 170.6(C, Ac), 147.9 (C, C-8), 143.2 (CH, C-12), 135.6 (CH, C-11), 129.2 (C, C-13), 121.4 (CH, C-14), 109.3 (CH_2, C-17), 80.0 (CH, C-3), 69.6 (CH_2, C-15), 64.8 (CH_2, C-19), 61.7 (CH, C-9), 54.8 (CH, C5), 41.1 (C, C-4), 38.6 (C, C-10), 38.3 (CH_2, C-7), 36.7 (CH_2, C-1), 24.1 (CH_2, C-2), 23.9 (CH_2, C-6), 22.7 (CH_3, C-18), 21.1 (CH_3, 2Ac), 15.2 (CH_3, C-20). IR (KBr) cm^{-1}: 2933 (s), 1749, 1729(s), 1247(s), 1037cm^{-1}. ESI *m/z*: 439.2054 $(M+Na)^+$.

2.2 Antimicrobial assays

Determination of antibacterial activity

Broth microdilution was used to determine antibacterial activities of compounds, as described in the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) M7-A4 method (National Committee for Clinical Laboratory Standards, 1997). The test bacteria were *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. Each bacterial strain suspension was prepared and diluted in Mueller-Hinton broth to obtain 10^6 CFU/ml. A 50 µl

inoculum was dispensed into each well containing 50 µl of test compound. All compounds were tested in duplicate at a final concentration of 2 mM.

After incubation at 37 °C for 24 h, a 20 µl aliquot of *p*-iodonitrotetrazolium (INT) solution (1 mg/ml) was added into each well. The assay plates were further incubated for 1 h. A violet colour developed in the well indicated growth of the test organism. No change in colour indicated no growth, and thus antibacterial activity of the test compound.

Compounds showing antibacterial activity were further tested for the minimum inhibitory concentrations (MICs) over a final concentration range of 3.9 µM to 2000 µM. Ampicillin was used to compare the potency of the active compounds. MIC was defined as the lowest concentration that inhibited the growth of test bacteria. The solution from each well that showed no growth was further inoculated onto Mueller-Hinton agar and incubated at 37 °C for 24 h to determine the minimum bactericidal concentration (MBC). MBC was defined as the lowest concentration that killed test bacteria.

Determination of activity against C. albicans ATCC 90028

Anti-*C. albicans* activities of andrographolide and 14-acetylandrographolide were determined by the broth microdilution method as described in CLSI M27-A2 (National Committee for Clinical Laboratory Standards, 2002). *C. albicans* ATCC 90028 suspension was prepared and diluted in RPMI-1640 medium to yield $1x10^3$ - $5x10^3$ CFU/ml. A 100 µl inoculum was dispensed into each well containing 100 µl of test compound.

All compounds were tested in duplicate at a final concentration of 2 mM. Sample solutions were prepared immediately before use. After incubation at 37 °C for 24 h, 20 µL of *p*-iodonitrotetrazolium (INT) solution (1 mg/ml) was added into each well. The assay plate was further incubated for 24 h.

Transmission electron microscopy

B. subtilis cells were grown in tryptic soy broth at 37 °C for 2-3 h and further diluted in Mueller-Hinton broth to yield 10^6 CFU/ml. The 14-acetylandrographolide was added at a final concentration of 125 µM (4x MIC), and cells were harvested after 4 h of incubation in an incubator shaker at 37 °C and 200 rpm. Untreated cells were grown and harvested under the same conditions. Bacterial pellets were washed in sterile normal saline solution and centrifuged at 1000 x *g* for 10 min. Cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C overnight. After centrifugation, the pellets were washed three times with buffer only. The washed cell pellets were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4 °C. They were dehydrated in a graded ethanol series. After dehydration, the specimens were treated with propylene oxide for 20 min (2 times), an equal mixture of propylene oxide and resin for 1 h, a 1:2 mixture overnight, and finally with resin only overnight. They were embedded in resin by polymerisation at 45 °C for 2 d and 60 °C for 2 d. The embedded cells were sectioned with a diamond knife on an ultramicrotome. Thin sections were mounted on copper grids and stained with alcoholic saturated uranyl acetate and lead citrate. The stained sections were examined with a JEM 2100 transmission electron microscope (JEOL Ltd.) at 120 kV.

3. Results

Antimicrobial activity

All compounds tested showed no detectable inhibitory activity against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 or *C. albicans* ATCC 90028. Some derivatives of andrographolide, however, displayed substantial activity against Gram-positive bacteria *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212 and *S. aureus* ATCC 25923 (Table 1). Among the andrographolide derivatives tested in this study, 14-acetylandrographolide (5) was the most active compound against *B. subtilis* and *S. aureus*, with both MIC and MBC values of 31.25 and 62.5 μM, respectively. It was also relatively active against *E. faecalis* (MIC, MBC = 125 μM), slightly less active than 3,14,19-triacetyl andrographolide (6), which could inhibit and kill *E. faecalis* at 62.5 μM. However, (6) was less active against *S. aureus* and *B. subtilis*, having MIC and MBC values of 125 and 250 μM, respectively. Compared with (5) and (6), 14-acetyl-3,19-isopropylideneandrographolide (4) exhibited weaker antibacterial activity. It was the least active compound against *S. aureus* and *E. faecalis* (MIC, MBC = 250 and 1,000 μM, respectively). However, it was more active against *B. subtilis* (MIC, MBC = 62.5 μM) than compound (6), but not as good as (5). The natural compounds andrographolide (1), 14-deoxy-11,12-didehydroandrographolide (2) and the semi-synthetic derivatives 3,19-isopropylideneandrographolide (3) and 14-deoxy-11,12-didehydro-3,19-diacetyl andrographolide (7) showed no activity against the three Gram-positive bacteria tested.

Transmission electron microscopy

Since (5) demonstrated the highest activity against *B. subtilis*, it was selected for further study of the morphological changes it induced using TEM. Treating *B. subtilis* with compound (5) at 4 times the minimum inhibitory concentration (125 μM) for 4 h resulted in the formation of large cytoplasmic aggregates, cell elongation with abnormal cell septation, cytoplasmic disintegration, and finally cell lysis (Figure 2).

4. Discussion

Andrographolide (1) showed no activity against the tested bacteria and *C. albicans*. Acetylation of the 14-OH group rendered it active against *B. subtilis*, *S. aureus* and *E. faecalis* (see compound (5), Table 1). Interestingly, compound (5) was more potent than ampicillin at killing *B. subtilis*. Further acetylation of the remaining two hydroxyl groups (3-OH, 19-OH) improved the activity against *E. faecalis*, but slightly weakened the activity against *S. aureus* and, to a greater extent, the activity against *B. subtilis* (see compound (6), Table 1). Thus, differential acetylation of andrographolide could create antibacterial activity in an organism-selective manner. The three andrographolide derivatives (4), (5) and (6) acted on Gram-positive bacteria with MIC values equal to the MBC values, which suggested that their activities were bactericidal. It would be interesting to further investigate the 3-acetyl, 19-acetyl, 3,19-diacetyl, 3,14-diacetyl and 14,19-diacetyl derivatives of andrographolide, which has not been done in this study.

Modification of andrographolide by introducing the isopropylidene group to the 3-OH and 19-OH did not impart any antimicrobial activity to the molecule (see compound (3) Table 1). Addition of isopropylidene groups at the 3-OH and 19-OH of compound (5) rendered the molecule less active against all three Gram-positive bacteria (see

compound (4), Table 1), indicating the negative effect of having isopropylidene at these positions.

14-Deoxy-11,12-didehydroandrographolide (2) exhibited no antimicrobial activity. Acetylation at position 14, which had been shown to be essential for the antibacterial activity of andrographolide, was not applicable since (2) is devoid of the 14-OH. Acetylation at the remaining 3-OH and 19-OH groups created no antimicrobial activity.

Previously, several antibacterial compounds from natural products had been reported to cause cell elongation or filamentation in *B. subtilis* (Leon *et al.*, 2005; Leon and Moujir, 2008; Beuria *et al.*, 2005; Jaiswal *et al.*, 2007). Treating *B. subtilis* with a series of 6-oxophenolic triterpenoids (zeylasteral, demethylzeylasteral and zeylasterone) isolated from *Maytenus blepharodes* Lundell resulted in abnormally long cells (Leon *et al.*, 2005; Leon and Moujir, 2008) and multiseptate filaments (Leon *et al.*, 2005). The compounds were found to damage the cytoplasmic membrane and compromise cell wall synthesis, leading to loss of cytoplasmic material. Sanguinarine, a benzophenanthridine alkaloid derived from the rhizomes of *Sanguinaria canadensis*, blocked cytokinesis and induced filamentation in *B. subtilis* 168 by inhibiting Z-ring formation. However, nucleoid segregation and the cell membranes of treated bacteria were not affected by this compound (Beuria *et al.*, 2005). More recently, totarol, a naturally occurring diterpenoid phenol extracted from *Podocarpus totara*, was found to induce filamentation in *B. subtilis* 168 without affecting the cell membrane. It perturbed the assembly dynamics of *Mycobacterium tuberculosis* FtsZ (*Mtb*FtsZ) protofilaments in the Z-ring and potently suppressed the GTPase activity of *Mtb*FtsZ (Jaiswal *et al.*, 2007). Curcumin, a dietary polyphenolic compound isolated from the rhizomes of *Curcuma longa*, also induced filamentation in *B. subtilis* 168 without perturbing cell

membrane structure. It inhibited FtsZ protofilament assembly and increased the GTPase activity of FtsZ (Rai *et al.*, 2008). Nisin, a small cationic lanthionine antibiotic produced by *Lactococcus lactis*, was reported to cause abnormal morphogenesis in *B. subtilis* (Hyde *et al.*, 2006). At lethal doses, nisin retarded bacterial cell elongation by interfering with cell wall synthesis, and accelerated cell division, leading to cell length reduction and the formation of double or multiple septa in the midcell division region.

In contrast to the above reported antibacterials, treatment of *B. subtilis* with compound (5) caused cell elongation with normal cell diameters compared to control untreated cells (Figures 2A-2E). A noticeable septation near the cell poles instead of the normal midcell division site was observed (Figures 2C-2E). This suggested that compound (5) neither inhibits cell wall synthesis nor directly affects FtsZ assembly, but rather interferes with factors that regulate the position of normal septum formation. It is known that bacterial cell division depends on the FtsZ protein, which self-assembles into a membrane-associated ring structure that establishes the location of the nascent division site (Margolin, 2005). Division site selection in *B. subtilis* is controlled by a division inhibitor, MinCD, which prevents FtsZ assembly. The MinC and MinD proteins of *B. subtilis* are tethered to the cell poles by another protein, DivIVA, which binds strongly to the cell poles. In this way, MinCD-DivIVA prevents aberrant polar division of the cell (Marston *et al.*, 1998). One possible explanation of polar septation observed in compound-(5)-treated *B. subtilis* is that the compound may interfere with the DivIVA protein. Inhibition of DivIVA might cause delocalisation of MinCD from the pole regions, leading to polar division and suppression of midcell septation.

Cytoplasmic membranes and cell walls of the treated cells were apparently intact until the late stages, suggesting that they might not be the primary antibacterial targets of

compound (5). Subsequently, the affected cells lost their cytoplasmic contents, but the large aggregates formed from the action of compound (5) remained within ghost cells surrounded by an almost intact cell wall (Figure 2F).

4. Conclusion

This study demonstrated that acetylation of andrographolide could create organism-selective antibacterial activity. The active derivatives were found to be bactericidal and appeared to have mechanism of action distinct from antibacterial drugs presently in clinical use. Our finding allows more understanding of the structure-activity relationship of this *ent*-labdane, which could be useful for the future development of new antibacterial agents.

Acknowledgements

We are grateful to the Department of Chemistry, Faculty of Science, Khon Kaen University, for acquiring NMR spectra. This work was financially supported by the Thailand Research Fund.

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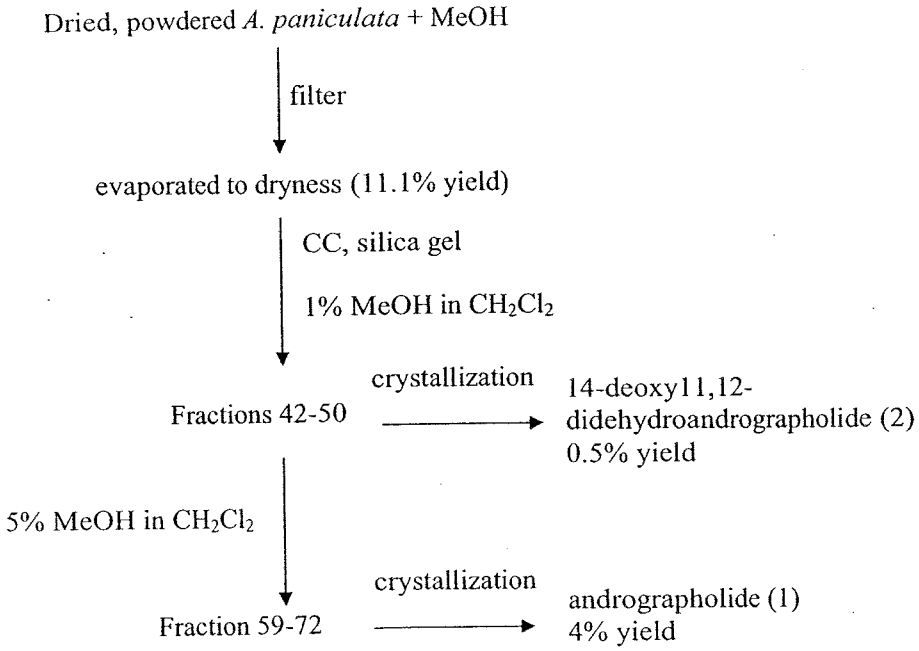


Scheme 1 Separation of andrographolide (1) and 14-deoxy, 11, 12-didehydroandrographolide (2).

List of Figures

Number of Figures = 2

Figure 1 Structure of andrographolide and its derivatives.

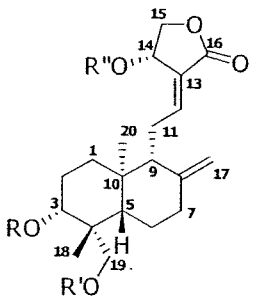
Figure 2 Transmission electron micrographs showing effects of compound (5) on morphogenesis of *B. subtilis* ATCC6633. Untreated cells (A). Cells after treatment with compound (4) at 125 μM (4 x MIC) for 4 h (B-F). Abnormal cytoplasmic aggregates (B, C, E, F, arrow). Elongated cells (B-E). Abnormal polar septation (C-E).

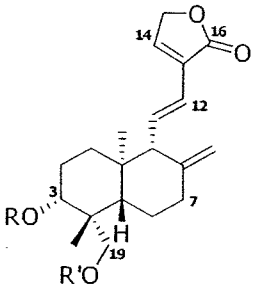
Table 1 Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of andrographolide and its acetyl derivatives against Gram-positive bacteria.

Compound	MIC, MBC (μM)		
	B. subtilis	*E. faecalis*	*S. aureus*
(1)	I	I	I
(2)	I	I	I
(3)	I	I	I
(4)	62.5, 62.5	1000, 1000	250, 250
(5)	31.25, 31.25	125, 125	62.5, 62.5
(6)	250, 250	62.5, 62.5	125, 125
(7)	I	I	I
Ampicillin	80, 80	5, 5	0.31, 0.31

I = Inactive

Ampicillin was used to compare the potency of active compounds.





Andrographolide and derivatives

(1) R, R', R" = H

(3) Isopropylidene R, R' = *i*-Pr, R" = H

(4) R, R' = *i*-Pr, R" = Ac

(5) R, R' = H, R" = Ac

(6) R, R', R" = Ac

14-Deoxy11,12-didehydroandrographolide

(2) R, R' = H

(7) R, R' = Ac

Figure 1 Structure of andrographolide and its derivatives.

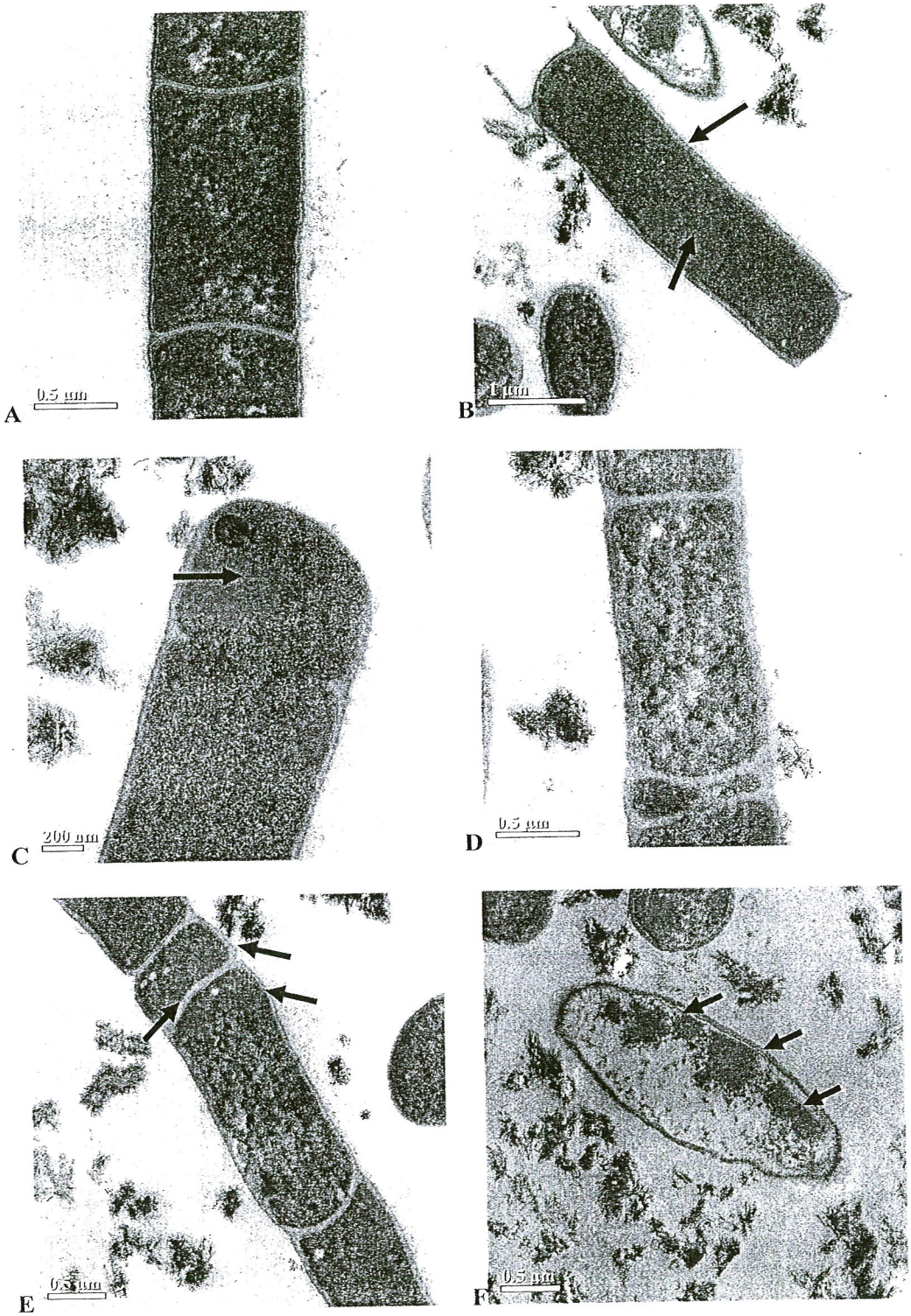

A
0.5 μm
B
1 μm
C
200 nm
D
0.5 μm
E
0.5 μm
F
0.5 μm

Figure 2 Transmission electron micrographs showing effects of compound (5) on the morphogenesis of *B. subtilis* ATCC6633. Untreated cells (A). Cells after treatment with compound (4) at 125 μM (4 x MIC) for 4 h (B-F). Abnormal cytoplasmic aggregates (B, C, E, F, arrow). Elongated cells (B-E). Abnormal polar septation (C-E).

แบบ สป/สผ/อสป/003-ก

สำหรับเจ้าหน้าที่

คำขอที่ .....

รับวันที่ .....

คำขอแก้ไขเพิ่มเติมคำขอรับสิทธิบัตร/อนุสิทธิบัตร

~~คำขอรับสิทธิบัตร/อนุสิทธิบัตร~~ เลขที่ .....1001000837.....

วันยื่นคำขอ ..... 27 พฤษภาคม 2553 .....

ชื่อที่แสดงถึงการประดิษฐ์/~~การออกแบบผลิตภัณฑ์~~ ...อนุพันธ์แอนโดรกราโฟไลด์ การสังเคราะห์และการใช้สารเหล่านั้น.....

ชื่อผู้ขอรับสิทธิบัตร/~~อนุสิทธิบัตร~~ .....มหาวิทยาลัยขอนแก่น .....

ข้อ 1. ข้าพเจ้า ..... นางจิราภรณ์ เหลืองไพรินทร์..... อยู่บ้านเลขที่ .....123.....ถนน.. มิตรภาพ.....สำนักงานบริหารจัดการทรัพย์สินทางปัญญา..... มหาวิทยาลัยขอนแก่น..... ตำบล/แขวง .... ในเมือง.....อำเภอ/เขต .....เมือง..... จังหวัด .....ขอนแก่น ..... โทรศัพท์ .....0-4336-4409 ... ซึ่งเป็นผู้~~ขอรับสิทธิบัตร/อนุสิทธิบัตรหรือ~~ตัวแทนของผู้ขอรับสิทธิบัตร/~~อนุสิทธิบัตร~~ที่ระบุข้างต้น ขอแก้ไขเพิ่มเติมคำขอรับสิทธิบัตร/~~อนุสิทธิบัตร~~ดังกล่าว ดังมีรายละเอียดตามที่แนบมาพร้อมนี้

ข้อ 2.ข้าพเจ้าขอยืนยันว่าการแก้ไขเพิ่มเติมนี้เป็นไปตามมาตรา 20 แห่งพระราชบัญญัติสิทธิบัตร พ.ศ. 2522 กล่าวคือ ไม่เป็นการเพิ่มเติมสาระสำคัญของการประดิษฐ์หรือการออกแบบผลิตภัณฑ์

วันที่ ...30... เดือน ...พฤษภาคม... พ.ศ. ...2553.....

ลายมือชื่อ .....

(..... นางจิราภรณ์ เหลืองไพรินทร์.....)

หมายเหตุ ให้ขีดข้อความที่ไม่ใช้ออก

คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☑ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☐ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ 2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ 2535 และ พระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ 2542

สำหรับเจ้าหน้าที่

วันรับคำขอ	เลขที่คำขอ
วันยื่นคำขอ	

สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ

ใช้กับแบบผลิตภัณฑ์
ประเภทผลิตภัณฑ์

วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร

ลายมือชื่อเจ้าหน้าที่

1.ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์
อนุพันธ์แอนโดรกราโฟไลด์ การสังเคราะห์ และ การใช้สารเหล่านั้น

2.คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่
ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร และที่อยู่ (เลขที่ ถนน ประเทศ)
1. มหาวิทยาลัยขอนแก่น
สำนักงานบริหารจัดการทรัพย์สินทางปัญญา ชั้น 3 อาคารสถาบันวิจัยและพัฒนา มหาวิทยาลัยขอนแก่น อ. เมือง จ. ขอนแก่น 40002
2. สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
เลขที่ 979/17-21 ชั้น 14 อาคาร เอส เอ็ม ทาวเวอร์
ถนนพหลโยธิน แขวงสามเสนใน เขตพญาไท กรุงเทพมหานคร 10400

3.1 สัญชาติ ไทย
3.2 โทรศัพท์ 0-4320-2222-41
3.3 โทรสาร -
3.4 อีเมล์ -

4.สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5.ตัวแทน(ถ้ามี)/ที่อยู่ (เลขที่ ถนน จังหวัด รหัสไปรษณีย์)
นางจิราภรณ์ เหลืองไพรินทร์
สำนักงานบริหารจัดการทรัพย์สินทางปัญญา
ชั้น 3 อาคารสถาบันวิจัยและพัฒนา มหาวิทยาลัยขอนแก่น
อ. เมือง จ. ขอนแก่น ประเทศไทย 40002

5.1 ตัวแทนเลขที่ 2217
5.2 โทรศัพท์ 0-4336-4409
5.3 โทรสาร 0-4336-4409
5.4 อีเมล์ ip@kku.ac.th

6.ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)
6.1 รองศาสตราจารย์ ฉันทนา อารมย์ดี คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น จ. ขอนแก่น 40002
6.2 รองศาสตราจารย์ จินตนา สัตยาศัย คณะแพทย์ศาสตร์ มหาวิทยาลัยขอนแก่น จ. ขอนแก่น 40002

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร เลขที่ วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียด

8.การยื่นคำขอนอกราชอาณาจักร

วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย

☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9.การแสดงการประดิษฐ์ หรือการออกแบบผลิตภัณฑ์ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด

วันแสดง วันเปิดงานแสดง ผู้จัด

10.การประดิษฐ์เกี่ยวกับจุลชีพ

10.1 เลขทะเบียนฝากเก็บ	10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ

11.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา

☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่นๆ

12.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้หลังจากวันที่ เดือน พ.ศ

☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา

13.คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย

ก. แบบพิมพ์คำขอ 2 หน้า

ข. รายละเอียดการประดิษฐ์ หรือคำพรรณนาแบบผลิตภัณฑ์ 9 หน้า

ค. ข้อถือสิทธิ 2 หน้า

ง. รูปเขียน 11 รูป 8 หน้า

จ. ภาพแสดงแบบผลิตภัณฑ์

☐ รูปเขียน รูป หน้า

☐ ภาพถ่าย รูป หน้า

ฉ. บทสรุปการประดิษฐ์ 1 หน้า

14.เอกสารประกอบคำขอ

☐ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์

☐ หนังสือมอบอำนาจ

☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ

☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย

☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ

☐ เอกสารอื่น ๆ

15. ข้าพเจ้าขอรับรองว่า

☑ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/ อนุสิทธิบัตรมาก่อน

☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก.....

16.ลายมือชื่อ (☐ ผู้ขอรับสิทธิบัตร / อนุสิทธิบัตร; ☑ ตัวแทน)

(นางจิราภรณ์ เหลืองไพรินทร์)

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

หนังสือสัญญาโอนสิทธิขอรับสิทธิบัตร/อนุสิทธิบัตร

เขียนที่ มหาวิทยาลัยขอนแก่น
123 ถ. มิตรภาพ ต.ในเมือง อ. เมือง
จ. ขอนแก่น 40002

วันที่ 30 พฤษภาคม 2553

สัญญาระหว่างผู้โอน 1.รองศาสตราจารย์ ฉันทนา อารมย์ดี ที่อยู่ คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น และ 2. รองศาสตราจารย์ จินตนา สัตยาศัย ที่อยู่คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ต.ในเมือง อ. เมือง จ. ขอนแก่น รหัสไปรษณีย์ 40002 และผู้รับโอน คือ สำนักงานกองทุนสนับสนุนการวิจัย ในนาม ศาสตราจารย์ ดร. สวัสดิ์ ตันตระรัตน์ ผู้อำนวยการสำนักงานกองทุนสนับสนุนการวิจัย ที่อยู่ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) เลขที่ 979/17-21 ชั้น 14 อาคาร เอส เอ็ม ทาวเวอร์ ถนนพหลโยธิน แขวงสามเสนใน เขตพญาไท กรุงเทพมหานคร 10400 โดยสัญญานี้ ผู้โอนซึ่งเป็นผู้ประดิษฐ์ "อนุพันธ์แอนโดรกราโฟไลด์ การสังเคราะห์ และการใช้สารเหล่านั้น" ขอโอนสิทธิในการประดิษฐ์ดังกล่าว ซึ่งรวมถึงสิทธิขอรับสิทธิบัตร/อนุสิทธิบัตร และสิทธิอื่นๆ ที่เกี่ยวข้องให้แก่ผู้รับโอน เพื่อเป็นพยานหลักฐานแห่งการนี้ ผู้โอนและผู้รับโอนได้ลงลายมือชื่อไว้ข้างล่างนี้

(ลงชื่อ) ..... ผู้โอน
(รองศาสตราจารย์ ฉันทนา อารมย์ดี)

(ลงชื่อ) ..... ผู้โอน
(รองศาสตราจารย์ จินตนา สัตยาศัย)

ลงชื่อ) ..... ผู้รับโอน
(ศาสตราจารย์ ดร. สวัสดิ์ ตันตระรัตน์)

(ลงชื่อ).....พยาน
(นางสาวนฤมล เบญจปัก)

(ลงชื่อ).....พยาน
(นายเศกสิทธิ์ พรหมพฤฒา)

หนังสือมอบอำนาจ

มหาวิทยาลัยขอนแก่น

อ.เมือง จ. ขอนแก่น 40002

วันที่ 30 พฤษภาคม 2553

โดยหนังสือฉบับนี้ ข้าพเจ้า สำนักงานกองทุนสนับสนุนการวิจัย โดย ศาสตราจารย์ ดร. สวัสดิ์ ตันตระรัตน์ ผู้อำนวยการสำนักงานกองทุนสนับสนุนการวิจัย ที่อยู่ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) เลขที่ 979/17-21 ชั้น 14 อาคาร เอส เอ็ม ทาวเวอร์ ถนนพหลโยธิน แขวงสามเสนใน เขตพญาไท กรุงเทพมหานคร 10400 ขอมอบอำนาจและแต่งตั้งให้ นางจิราภรณ์ เหลืองไพรินทร์ อยู่บ้านเลขที่ 123 สำนักงานบริหารจัดการทรัพย์สินทางปัญญา มหาวิทยาลัยขอนแก่น ต.ในเมือง อ. เมือง จ.ขอนแก่น 40002 เป็นตัวแทนและผู้รับมอบอำนาจของข้าพเจ้ามีอำนาจในการยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรและดำเนินการจดทะเบียนสำหรับสิทธิบัตร/อนุสิทธิบัตร จำนวน 4 เรื่อง คือ

1. ยาเม็ดเมทริกซ์บรรจุสารประกอบเชิงซ้อนนิโคติน-เคลย์
2. อนุพันธุ์แอนโดรกราโฟไลด์ การสังเคราะห์ และการใช้สารเหล่านั้น
3. ยาฉีดเมลาโทนิน
4. เฮดกิมบอลแอสเซมบลี้ (Head Gimbal Assembly) ที่ลดผลกระทบจากการรบกวนทางแม่เหล็กไฟฟ้า

โดยให้ตัวแทนดังกล่าวมีสิทธิลงชื่อในเอกสารทั้งมวลในนามของข้าพเจ้าแทนข้าพเจ้า แก้ไขเปลี่ยนแปลงเอกสารและเอกสารอื่นๆ ที่เกี่ยวข้อง รวมทั้งการอุทธรณ์ต่างๆ ด้วย

ข้าพเจ้าขอรับผิดชอบในการที่ผู้รับมอบอำนาจได้กระทำไปตามหนังสือมอบอำนาจ ที่เสมือนว่าข้าพเจ้าได้กระทำด้วยตนเองทั้งสิ้น

เพื่อเป็นหลักฐาน ข้าพเจ้าได้ลงลายมือชื่อไว้เป็นสำคัญต่อหน้าพยาน

รับรองสำเนาถูกต้อง

(ลงชื่อ).....ผู้มอบอำนาจ

(ศาสตราจารย์ ดร. สวัสดิ์ ตันตระรัตน์)

(ลงชื่อ).....ผู้รับมอบอำนาจ

(นางจิราภรณ์ เหลืองไพรินทร์)

(ลงชื่อ).....พยาน

(นางสาวนฤมล เบญจปัก)

(ลงชื่อ).....พยาน

(นายเศกสิทธิ์ พรหมพฤฒา)