รายงานวิจัยฉบับสมบูรณ์

โครงการ : ฤทธิ์ต้านการอักเสบและฤทธิ์ต้านเอนไซม์ HIV-1 integrase ของสารสำคัญจากแก่นฝางและใบสังกะโต้ง

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Research work

Acknowledgements

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บทคัดย่อ

สารสกัดจากฝางถูกนำมาใช้ในการศึกษาฤทธิ์ต้านการอักเสบ ต่อ NO, PGE_2 และ TNF-α ผลการทดสอบพบว่า brazilin (**8**) มีฤทธิ์ต้านการอักเสบต่อ NO ได้ดี ที่ IC_{50} = 10.3 μM ตามด้วย sappanchalcone (**2**, 31.0 μM นอกจากนี้ยังพบว่า brazilin (**8**) มีฤทธิ์ยับยั้งการสร้าง PGE_2 และ TNF-α ที่ IC_{50} เท่ากับ 12.6 และ 87.2 μM ตามลำดับ กลไกการออกฤทธิ์พบว่า brazilin (**8**) มีฤทธิ์ต้านการแสดงออกของ mRNA ของ iNOS, COX-2 และ TNF-α ในแบบ dose-dependent manner นอกจากนี้สารสกัดชั้น ethanol มีฤทธิ์เพิ่ม fibroblast proliferation, fibroblast migration และ collagen productionในขณะที่ brazilin (**8**) กระตุ้นเพียงfibroblast migration และยังพบว่าสารสกัดฝางไม่มีพิษต่อหนูแบบ acute toxicity

สารสำคัญจำนวน 9 สาร ได้ถูกแยกจากแก่นและรากของฝางเพื่อนำมาทดสอบฤทธิ์ต้านเอนไซม์ HIV-1 integrase (IN) ผลการทดลองพบว่า sappanchalcone (**2**) มีฤทธิ์ดีที่สุด (IC_{50} = 2.3 μM) ตามด้วย protosappanin A (**9**, IC_{50} = 12.6 μM). จาก Structure-activity relationships (SARs) ของสารจากฝางพบว่า vicinal hydroxyl moiety ของสาร **2** และ **9** มีผลต่อฤทธิ์ต้าน HIV-1 IN โดยการจับกับ amino acids Gln148 และ Thr66 ใน core domain ของ HIV- 1 IN

ใบสังกะโต้งได้ถูกนำมาทดสอบฤทธิ์ต้านภูมิแพ้และต้านการอักเสบ ผลการทดลองพบว่า 24-epi-piscidinol A (**5**) มีฤทธิ์ต้านภูมิแพ้ดีที่สุด (IC_{50} = 19.8 μM) ตามด้วย (-)-yangambin (**3**, IC_{50} = 33.8 μM), pyramidaglain A (**8**, IC_{50} = 37.1 μM), pachypodol (**2**, IC_{50} = 38.3 μM) และ pyramidaglain B (**9**, IC_{50} = 44.8 μM) ตามลำดับ สำหรับฤทธิ์ต้านการอักเสบ พบว่า 24-epi-piscidinol A (**5**)) มีฤทธิ์ดีที่สุด (IC_{50} = 24.0 μM) ตามด้วย pyramidaglain B (**9**, IC_{50} = 25.6 μM), pachypodol (**2**, IC_{50} = 34.5 μM) และ (-)-yangambin (**3**, IC_{50} = 37.4 μM) ตามลำดับ

จากสารที่แยกได้จากใบสังกะโต้ง พบว่าสาร **10** มีฤทธิ์ต้าน HIV-1 IN ดี โดยให้ค่า IC_{50} เท่ากับ 11.8 μg/mL จาก molecular docking พบว่า สาร **10** จับกับ amino acids Thr66, Asn155 และ Lys159 ของ HIV-1 IN และไม่พบความเป็นพิษในหนูของใบสังกะโต้งที่ ความเข้มข้น 2,000 mg/kg mice นอกจากนี้การศึกษานี้ยังเป็นการศึกษาฤทธิ์ต้าน HIV-1 IN ของใบสังกะโต้งเป็นครั้งแรก

Keywords: NO production; iNOS; COX-2; RAW264.7 cells; fibroblast L929 cells; anti-HIV-1 IN activity, molecular docking; *Caesalpinia sappan*; *Aglaia andamanica*

Abstract

Extracted compounds from *Caesalpinia sappan* L. were examined for the inhibitory activity against NO, PGE_2 and TNF-α productions and on associated transcription levels using RAW264.7 cells. They were also tested for their effects on wound healing using fibroblast L929 cells. Among the compounds tested, brazilin (**8**) was the most effective against LPS-induced NO production in RAW264.7 cells with an IC_{50} value of 10.3 μM, followed by sappanchalcone (**2**, 31.0 μM). Brazilin (**8**) also inhibited PGE_2 and TNF-α production with IC_{50} values of 12.6 and 87.2 μM, respectively. The anti-inflammatory mechanism of brazilin involved down regulation of the mRNA expressions of the iNOS, COX-2 and TNF-α genes in a dose-dependent manner. An ethanol (EtOH) extract of *Caesalpinia sappan* significantly increased fibroblast proliferation, fibroblast migration and collagen production, whereas brazilin (**8**) only stimulated fibroblast migration. In addition, the EtOH extract showed no acute toxicity in mice and it was therefore safe to make use of its potent anti-inflammatory and wound healing activities. Brazilin was mainly responsible for its anti-inflammatory effect through its ability to inhibit the production of NO, PGE_2 and TNF-α. This study supports the traditional use of *Caesalpinia sappan* for treatment of inflammatory-related diseases.

Caesalpinia sappan L. (Caesalpiniaceae) has been traditionally used as blood tonic, expectorant and astringent by boiling with water. Searching for HIV-1 integrase (HIV-1 IN) inhibitors from this plant is a promising approach. The EtOH extract of *Caesalpinia sappan* and its isolated compounds were tested for their anti-HIV-1 IN effect using the multiplate integration assay (MIA) and the active compounds were determined for their mechanisms by molecular docking technique. Extraction from the heartwoods and roots of *Caesalpinia sappan* led to the isolation of nine compounds. Among the compounds tested, sappanchalcone (**2**) displayed the strongest effect against HIV-1 IN with an IC_{50} value of 2.3 μM, followed by protosappanin A (**9**, IC_{50} = 12.6 μM). Structure-activity relationships (SARs) of compounds from *Caesalpinia sappan* were found, in which the vicinal hydroxyl moiety were essential for anti-HIV-1 IN effect of compounds **2** and **9** by binding with the amino acid residues Gln148 and Thr66 in the core domain of the HIV- 1 IN enzyme, respectively.

The leaves from *Aglaia andamanica* were determined for their anti-allergic and anti-inflammatory effects using RBL-2H3 and RAW264.7 cells, respectively. Among the isolated compounds, 24-epi-piscidinol A (**5**) exhibited the highest anti-allergic activity against β-hexosaminidase release with an IC_{50} value of 9.4 μg/mL, followed by pachypodol (**2**, IC_{50} = 13.2 μg/mL) and (-)-yangambin (**3**, IC_{50} = 15.1 μg/mL); whereas other compounds possessed moderate to mild effects (IC_{50} = 24.2->85.9 μg/mL). For anti-inflammatory activity, 24-epi-piscidinol A (**5**) possessed potent activity with an IC_{50} value of 11.4 μg/mL, followed by pachypodol (**2**, IC_{50} = 11.9 μg/mL), (-)-yangambin (**3**, IC_{50} = 16.7 μg/mL) and pyramidaglain B (**9**, IC_{50} = 17.8 μg/mL), respectively; whereas other compounds exhibited moderate to mild effects (IC_{50} = 24.1->46.1 μg/mL). These active compounds could be developed as anti-allergic and anti-inflammatory agents in the futures and this is the first report of *A. andamanica* for anti-allergic and anti-inflammatory activities.

The leaves and compounds (3-100 μg/mL) from *Aglaia andamanica* were determined for the anti-HIV-1 IN effect using the multiplate integration assay (MIA) by detection the absorbance of the final product, *p*-nitrophenol, at 405 nm. The molecular docking with the HIV-1 IN of the active compound *N*-methyl-trans-4-hydroxy-L-proline (**10**) was also studied. The Swiss albino mice were used for an acute toxicity test. Among the isolated compounds, **10** showed marked anti-HIV-1 IN effect with an IC$_{50}$ value of 11.8 μg/mL, whereas other compounds were inactive (IC$_{50}$ > 100 μg/mL). The molecular docking of compound **10** with an HIV-1 IN enzyme was also studied. The result revealed that this compound formed the hydrogen bonding with the Thr66, Asn155 and Lys159 of the HIV-1 IN binding site. The acute toxicity of the *Aglaia andamanica* extract was not observed at the dose 2,000 mg/kg mice. This is the first report of *Aglaia andamanica* for anti-HIV-1 IN activity.

Keywords: NO production; iNOS; COX-2; RAW264.7 cells; fibroblast L929 cells; anti-HIV-1 IN activity, molecular docking; *Caesalpinia sappan*; *Aglaia andamanica*

Executive summary

Anti-inflammatory and anti-HIV-1 integrase activities of *Caesalpinia sappan* heartwood and *Aglaia andamanica* leaves

Introduction

Nitric oxide (NO) is one of the inflammatory mediators that causes inflammation in several organs. This free radical has been implicated in pathological and physiological processes including vasodilation, non-specific host defense and acute or chronic inflammation. NO has a role in host defense mechanisms by damaging pathogenic DNA and as a regulatory molecule for homeostatic activities (Kou and Schroder, 1995). However, excessive production of this free radical is pathogenic to the host tissue itself, because NO can bind with other superoxide radicals and acts as a reactive radical that directly damages the function of normal cells (Moncada *et al*., 1991).

Wound healing processes comprise a complex series of events in which repair to the damaged tissue partially or completely depends on the severity of the wounding. This process can be characterized by three overlapping phases; an inflammatory phase (consisting of hemostasis and inflammation), a proliferative phase (consisting of granulation, contraction and epithelialization) and a remodeling phase in which structure are organized that have increased tensile strength (Wild *et al.*, 2010). The proliferation of fibroblast, fibroblast migration and collagen production are all involving in the wound healing processes.

HIV-1 integrase (IN) enzyme is a 32 kDa protein produced from the C-terminal portion of the pol-gene product which minimally functions as a dimer with 288 amino acids each. The catalytic core domain contains the invariant triad of acidic residues, the D,D-35E motif, comprising residues Asp64, Asp116, and Glu152 in the case of HIV-1 IN (Craigie, 2001). HIV-1 IN has become an interesting target since it has no human counterpart and only three inhibitors (raltegravir, elvitegravir, dolutegravir) are now clinically available.

Among 67.4 million Thai people, it is reported that 23,000 HIV patients died/year, 9,700 new HIV positives reported/year with 490,000 of total HIV-positive patients. Specific drug treatment is expensive, and only small number of AIDS patients have access to the cocktail of modern antiviral agents. Hence, some AIDS patients prefer to using Thai traditional healers, who prescribe a range of natural-based products. However, scientific research supporting this usage (efficacy, specificity, toxicity) has in most cases not yet been studied (Moncada et al., 1991).

Caesalpinia sappan L. is a plant that belongs to the Caesalpiniaceae family, a decoction with water of its heartwood has long been used in Thailand by traditional doctors to treat haemorrhoids, aphthous ulcer, epistaxis, stomatitis, pneumonia and acts as a blood tonic (Wutthithamavet, 1997).

Aglaia andamanica Hiern (Meliaceae) is a plant up to 30 m, distributed in South and Southeast Asia. The leaves have been used for treatment of headache. Some of benzofuran derivatives from *Aglaia* genus show interesting biological activities including antiviral, antileukemic and insecticidal activities.

In the present study, we have therefore investigated the inhibitory activity of these plants against inflammation. We also investigated the inhibitory activity of them against HIV-1 IN, and mechanism using molecular docking technique.

Material and Methods

Assay for NO, TNF-α and PGE_2 inhibitory effects from RAW264.7 cells

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

Assay on anti-HIV-1 integrase activity using multiplate integration assay (MIA)

The integration reaction was determined according to the method previously described (Tewtrakul et al., 2001). Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control. The % inhibition against HIV-1 IN was calculated as follows:

% Inhibition against HIV-1 IN = [(OD control - OD sample)/ OD control] x 100

Where OD = absorbance detected from each well

Results and discussions

Anti-inflammatory activity of compounds from *Caesalpinia sappan*

It seems that *Caesalpinia sappan* EtOH extract had potent anti-inflammatory activity by inhibiting the production of NO, PGE_2 and TNF-α and promoted a wound healing effect. The compound that was mainly responsible for anti-inflammatory activity was brazilin. Brazilin was found to down regulate the mRNA expressions of the iNOS, COX-2 and the TNF-α genes in a dose-dependent manner. The EtOH extract of *Caesalpinia sappan* significantly enhanced the wound healing effect in L929 fibroblasts by increasing cell proliferation and migration as well as their collagen production. This study therefore may support the traditional use of this plant for treatment of aphthous ulcer, stomatitis, epistaxis, haemorrhoids and pneumonia which are inflammatory-related diseases without demonstrating any acute toxicity in mice.

Anti-HIV-1 integrase activity of compounds from *Caesalpinia sappan* heartwood

The present study found that sappanchalcone (**2**) and protosappanin A (**9**) isolated from *C. sappan* exhibited good anti-HIV-1 IN activity. The molecular docking of sappanchalcone and protosappanin A revealed the interaction of these two compounds with some crucial amino acid residues (Thr66, His67, Gln148 and Lys159 for sappanchalcone; Thr66, Gln148 and Lys159 for protosappanin A) in the core domain of an IN enzyme. This is the first report of anti-HIV-1 IN activity of compounds from *C. sappan*.

Anti-allergic and anti-inflammatory compounds from *Aglaia andamanica* leaves

Aglaia andamanica possesses appreciable anti-allergic and anti-inflammatory activities. Pachypodol (**2**), (-)-yangambin (**3**), 24-epi-piscidinol A (**5**) and pyramidaglain B (**9**) are responsible for both anti-allergic and anti-inflammatory activities of this plant. The mechanisms for anti-allergic and anti-inflammatory effects of these compounds will be further investigated. This is the first report of *A. andamanica* and its compounds for anti-allergic and anti-inflammatory activities.

Anti-HIV-1 integrase effect of compounds from *Aglaia andamanica* leaves and molecular docking study with acute toxicity test in mice

A. andamanica possesses anti-HIV-1 IN activity and safety for use. *N*-methyl-trans-4-hydroxy-L-proline (**10**) isolated from this plant is responsible for anti-HIV-1 IN effect by forming the hydrogen bonding with the Thr 66, Asn155 and Lys 159 of the IN enzyme. Moreover, this is the first report of *A. andamanica* on acute toxicity in mice, anti-HIV-1 IN activity and the mechanism of *N*-methyl-trans-4-hydroxy-L-proline as anti-HIV-1 IN agent.

Acknowledgements

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References

Goldsby, RA., Kindt, T.J., Osborne, B.A, Kuby, J., 2002. Immunology. fifth edition. W.H. Freeman and Company, New York, p.40.

Kou, P.C., Schroder, R.A., 1995. The emerging multifacated roles of nitric oxide. Annals of Surgery. 221, 220-235.

Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmaceutical Reviews. 43, 109-142.

Banskota, A.H., Tezuka, Y., Nguyen, N.T., Awale, S., Nobukawa, T., Kadota, S., 2003. DPPH radical scavenging and nitric oxide inhibitory activities of the constituents from the wood of *Taxus yunnanensis*. Planta Medica 69, 500-505.

Research work

Part I. Anti-inflammatory activity of compounds from *Caesalpinia sappan*

Abstract

Extracted compounds from *Caesalpinia sappan* L. were examined for the inhibitory activity against NO, PGE_2 and TNF-α productions and on associated transcription levels using RAW264.7 cells. They were also tested for their effects on wound healing using fibroblast L929 cells. Among the compounds tested, brazilin (**8**) was the most effective against LPS-induced NO production in RAW264.7 cells with an IC_{50} value of 10.3 μM, followed by sappanchalcone (**2**, 31.0 μM). Brazilin (**8**) also inhibited PGE_2 and TNF-α production with IC_{50} values of 12.6 and 87.2 μM, respectively. The anti-inflammatory mechanism of brazilin involved down regulation of the mRNA expressions of the iNOS, COX-2 and TNF-α genes in a dose-dependent manner. An ethanol (EtOH) extract of *Caesalpinia sappan* significantly increased fibroblast proliferation, fibroblast migration and collagen production, whereas brazilin (**8**) only stimulated fibroblast migration. In addition, the EtOH extract showed no acute toxicity in mice and it was therefore safe to make use of its potent anti-inflammatory and wound healing activities. Brazilin was mainly responsible for its anti-inflammatory effect through its ability to inhibit the production of NO, PGE_2 and TNF-α. This study supports the traditional use of *Caesalpinia sappan* for treatment of inflammatory-related diseases.

Keywords: NO production; iNOS; COX-2; RAW264.7 cells; fibroblast L929 cells; *Caesalpinia sappan*

INTRODUCTION

Nitric oxide (NO) is one of the inflammatory mediators that causes inflammation in several organs. This free radical has been implicated in pathological and physiological processes including vasodilation, non-specific host defense and acute or chronic inflammation. NO has a role in host defense mechanisms by damaging pathogenic DNA and as a regulatory molecule for homeostatic activities

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Caesalpinia sappan L. is a plant that belongs to the Caesalpiniaceae family, a decoction with water of its heartwood has long been used in Thailand by traditional doctors to treat haemorrhoids, aphthous ulcer, epistaxis, stomatitis, pneumonia and acts as a blood tonic (Wutthithamavet, 1997). The wood of this plant is a component of a famous tooth paste and tooth powder (Vicco Vajradanti^{TM}) in India. The powerful astringent, haemostatic and healing effects of the wood can stop bleeding in the gums and is useful in the treatment of gum erosions, aphthous ulcers and stomatitis because of its strong healing property. Moreover, it is commonly used in several other Ayurvedic formulations (Badami *et al*., 2004). In the present study, we have therefore investigated the inhibitory activity of this plant against the productions of NO, PGE_2 and TNF-α and its healing effect as well as the mechanism of action on iNOS and COX-2 mRNA expressions to establish if its traditional uses can be supported.

MATERIALS AND METHODS

General experimental procedures

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using the 300 MHz Bruker FTNMR Ultra Shield^{TM} spectrometers (Bremen, Germany). Chemical shifts were recorded in parts per million (δ) in $CDCl_3$, acetone-d_6 and dimethyl sulfoxide (DMSO)-d_6 with tetramethylsilane (TMS) as an internal reference.

Cell cultures and reagents

The mouse fibroblast L929 cell line (Chinese Academy of Preventive Medical Sciences, Beijing, China) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA) and murine macrophage-like RAW264.7 cell line (Cell Lines Services) was cultured in RPMI medium (RPMI, Gibco®, Grand Island, NY, USA).

Lipopolysaccharide (LPS, from *Escherichai coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), indomethacin, L-nitroarginine (L-NA), caffeic acid phenethylester (CAPE) and phosphate buffer saline (PBS) were from Sigma. Fetal calf serum (FCS) was from Gibco. Penicillin-streptomycin was from Invitrogen. 96-well microplates were from Nunc. ELISA test kits were from R&D Systems, Inc., USA. Other chemicals were from Sigma.

Plant material and preparation of extract

Caesalpinia sappan L. was collected from Khonkaen province, Thailand in October 2005. Identification was made by Prof. Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University and a specimen (No. SC07) has been deposited at the Prince of Songkla University Herbarium.

Isolation of compounds from a *Caesalpinia sappan* extract

The air-dried roots (1.7 kg) of *Caesalpinia sappan* were extracted with CH_2Cl_2 and acetone, successively (each 2 x 2 L, for 5 days) at room temperature (25°C). The crude extracts were evaporated under reduced pressure to afford a brownish CH_2Cl_2 (20.1 g) and acetone (35.2 g) extract, respectively. The crude CH_2Cl_2 and acetone extracts gave similar TLC chromatograms (data not shown) so they were combined and further purified by silica gel quick column chromatography (QCC) using CH_2Cl_2 as eluent and an increasing polarity with acetone to give seven fractions (R1-R7). Fraction R2 (2.0 g) was further purified by silica gel (100 g) column chromatography (CC) with EtOAc-CH_2Cl_2 (1:5, v/v; 50 mL, each) to give five subfractions (R2a-R2e). Subfraction R2b (140.2 mg) was separated by silica gel (10 g) CC with acetone-CH_2Cl_2 (1:20, v/v; 25 mL, each) to give **1** (8.0 mg). Fraction R3 (2.7 g) was separated by silica gel (130 g) CC with acetone-hexane (2:5, v/v; 50 mL, each) to afford seven subfractions (R3a-R3g). Subfraction R3b (200.2 mg) was purified by CC with EtOAc-hexane (2:5, v/v; 50 mL, each) to give **7** (16.0 mg). Subfraction R3e (838.4 mg) was separated by reverse phase (RP)-18 silica gel CC with MeOH-H_2O (2:5, v/v, 25 mL, each) to give **8** (15.0 mg), **4** (6.0 mg) and **2** (34.0 mg).

Chopped-dried heartwoods (5.3 kg) of *Caesalpinia sappan* were extracted with CH_2Cl_2 (2 x 5 L, for 5 days) at room temperature (25°C) and evaporated under reduced pressure to afford a brownish CH_2Cl_2 extract (135.0 g). A portion of crude CH_2Cl_2 extract (55.2 g) was further purified by silica gel (1000 g) QCC using CH_2Cl_2 as eluent and increasing polarity with EtOAc and acetone to give seven fractions (H1-H7). Fraction H3 (1.3 g) was further purified by silica gel (60 g) CC with EtOAc-CH_2Cl_2 (3:17, v/v; 50 mL, each) to give eight subfractions (H3a-H3i). Subfraction H3e (164.3 mg) was separated by silica gel CC with EtOAc-hexane (2:5, v/v; 25 mL, each) and followed by RP-18 silica gel CC with MeOH-H_2O (2:5, v/v; 25 mL, each) to give **9** (15.0 mg) and **3** (8.0 mg). Fraction H6 (5.9 g) was separated by silica gel (300 g) QCC with acetone-CH_2Cl_2 (1:5, v/v; 100 mL, each) to give six subfractions (H6a-H6f). Subfraction H6b (483.2 mg) was purified by silica gel (25 g) CC with EtOAc-hexane (1:1, v/v; 25 mL, each) to give **6** (20.0 mg) and **5** (15.0 mg). The structures of all these isolated compounds were elucidated by comparison with the ^1H and ^{13}C-NMR spectrum of those in the previous literature (Yodsaoue *et al*., 2009).

Acute toxicity test of *Caesalpinia sappan* extract in mice

Male and female Swiss albino mice (30-40 g) were used in the experiment. The animals were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat-Yai, Songkhla, Thailand. All experimental protocols were approved by The Animal Ethic Committee, Prince of Songkla University (MOE 0521.11/241). Swiss albino mice were housed in standard environmental conditions with a 12 h light/dark cycle. They were provided *ad libitum* with standard rodent diet and water.

The 50% lethal dose (LD_{50}) of the EtOH extract of *Caesalpinia sappan* was estimated by the up-and-down method in mice (Bruce, 1985). The animals were fasted for 6 h prior to dosing. Doses were adjusted by a constant multiplicative factor (viz., 3.2) for this experiment. The dose for each successive animal was adjusted up or down depending on the previous outcome. The crude extract was dissolved in the cosolvent solution (propylene glycol: water = 1:1) and orally administered in a single dose by gavage using a stomach tube to both groups of male and female mice. Animal behavior was observed individually at least once during the first 30 min after administration, periodically during the first 8 h and daily thereafter, for a total of 7 days. The signs of toxicity were observed including tremor, convulsion, hyperactivity, sedation, grooming, loss of righting reflex, respiratory depression and coma.

Assay for NO inhibitory effect from RAW264.7 cells

The inhibitory effect on NO production by RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota *et al.*, 2003). Briefly, the RAW264.7 cells were cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/mL), streptomycin (100 μg/mL) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1 x 10^5 cells/well and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 100 μg/mL of LPS together with the test samples at various concentrations (3-100 μg/mL for crude extract and 3-100 μM for pure compounds) and was then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the MTT colorimetric method. Briefly, after 48 h incubation with the test samples, MTT solution (10 μl, 5 mg/mL in PBS) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. L-NA, CAPE and indomethacin were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1%). Inhibition (%) was calculated using the following equation and IC_{50} values were determined graphically (n = 4):

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

A-C : NO_2^- concentration (μM) [A : LPS (+), sample (-); B : LPS (+), sample(+); C : LPS (-), sample (-)].

Inhibitory effects on the production of LPS-induced PGE_2 and TNF-α from RAW264.7 cells

Briefly, the RAW264.7 cell line was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/mL), streptomycin (100 μg/mL) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1.0 x 10^5 cells/well and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 100 μg/mL of LPS together with the test samples at various concentrations (3-100 μM) and was then incubated for 48 h. The supernatant was transferred into a 96 well ELISA plate and then PGE_2 and TNF-α concentrations were determined using commercial ELISA kits. The test sample was dissolved in DMSO, and the solution was added to RPMI. The inhibition on PGE_2 and TNF-α releases was calculated and IC_{50} values were determined graphically.

Total RNA isolation and RT-PCR

In order to understand the mechanism of action on the release cytokine of brazilin (**8**), assays for mRNA expression of iNOS, COX-2 and TNF-α were carried out. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with samples in various concentrations (3, 10, 30, 100 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis using the first strand cDNA synthesis kit (Rever Tra Ace-α, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers for iNOS, COX-2 and TNF-α were used (forward primer for iNOS : 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-

GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGCTC-3′; forward primer for TNF-α: 5′-TCTGTCTACTGAACTTCGGG-3′ and its reverse primer: 5′-AGATAGCAAATCGGCTGACG-3′; forward primer for β-actin (an internal standard): 5´-TGTGATGGTGGGAATGGGTCAG-3´ and reverse primer: 5´-TTTGATGTCACGCACGATTTCC-3´.

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

Cell proliferation and viability assay using L929 fibroblasts

L929 fibroblasts were seeded at 2×10^4 cells/well into 96-well plate in DMEM containing 10% FBS. After 48 h, cells were exposed to different concentrations (1-100 µg/mL for crude extract or 1-100 µM for the pure compound) of test samples and were then incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. The MTT solution (10 µL, 5 mg/mL) was added directly to the medium in each well, and the plate was then incubated at 37°C for 4 h. All medium was then aspirated and replaced with isopropanol containing 0.04 N HCl, and the optical density at 570 nm was detected. The percentage of cell proliferation was calculated and compared to a negative control.

Migration assay of fibroblast L929 cells

The migration of fibroblast L929 cells was examined using a wound healing method as previously described by Balekar *et al*. (2012). Briefly, L929 cells (5×10^4 cells/mL) in DMEM containing 10% FBS were seeded into each well of a 24 well plate and incubated at 37°C with 5% CO_2. After the confluent monolayer of L929 cells was formed and a sterile pipette tip was used to generate two horizontal scratches (left and right) in each well. Any cellular debris was removed by washing with PBS and replaced with 1 mL of fresh medium in the absence or presence of test samples. Photographs were taken of two views on the left and right of each well at a 4 × magnification using a microphotograph (Olympus CK2, Japan) on day 0, then plates were incubated at 37°C with 5% CO_2 and photographs were taken at days 1 and 2. To determine the migration of L929 cells, the images were analyzed using computing software (ImageJ1.42q/Java1.6.0_10). The percentage of the closed area was measured and compared with the value obtained before treatment (day 0). An increase of the percentage of closed area indicated the cells had migrated.

Determination of collagen type-I production

The collagen productions was determined according to the method described by Balekar *et al*. (2012). Fibroblast L929 cells in DMEM containing 10% FBS were seeded at an initial concentration of 2×10^4 cells/mL in a 96 well plate. After 24 h, the culture medium was replaced with a fresh medium containing the test samples at various concentrations (1.5-12.5 µg/mL for crude extract or 1.5-12.5 µM for the pure compound) and was then incubated for 48 h at 37°C with 5% CO_2. Cells without a test sample served as negative controls. After 48 h of incubation, cells secreted soluble collagen type-I into the medium, the supernatant (100 µL) were collected. The total amount of soluble collagen type-I was assayed using the Sircol® Collagen Assay Kit (Bicolor Life Science Assays, Northern Ireland, UK). Briefly, 100 µL of supernatant was mixed with 1 mL of dye solution at room temperature for 30 min. Then the samples were centrifuged at 15,000 × g for 10 min to form a pellet of collagen. All the supernatant was then aspirated and the soluble collagen was dissolved in 1 mL of alkali reagent. Thereafter, the alkali solutions were transferred to a 96 well plate and the optical density at 540 nm was recorded. The amount of collagen was calculated based on a standard curve of soluble collagen (bovine skin collagen type-I standard from American disease free animals).

Statistics

For statistical analysis, the values are expressed as a mean value ± S.E.M of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by the Dunnett's test.

RESULTS AND DISCUSSION

Effect of an EtOH extract and brazilin on the production of NO, PGE_2 and TNF-α

A Thai medicinal plant, *Caesalpinia sappan*, locally known in Thai as Phang was examined for its inhibitory activities against the production of NO, PGE_2 and TNF-α. Nine compounds were isolated from its roots and heartwoods (Figure 1). Among the compounds tested, brazilin (**8**), possessed the highest activity against LPS-induced NO production in RAW264.7 cells with an IC_{50} value of 10.3 μM, followed by sappanchalcone (**2**, 31.0 μM), whereas **1** and **7** showed only moderate activities with IC_{50} values of 58.6 and 54.8 μM, respectively. The activity on the NO inhibition by brazilin (**8**, IC_{50} = 10.3 μM) was higher than that by indomethacin (an anti-inflammatory drug, IC_{50} = 46.5 μM) and L-NA (a nitric oxide synthase inhibitor, IC_{50} = 61.8 μM), and was comparable to that of CAPE (an NF-κB inhibitor, IC_{50} = 5.6 μM). The structure-activity relationships (SARs) of compounds from *Caesalpinia sappan* on the anti-NO production were as follow: 1) the vicinal hydroxyl group of chalcones at position 3 and 4 conferred higher activity, as shown in **2** (IC_{50} = 31.0 μM) versus **1** (IC_{50} = 58.6 μM); and 2) the homoisoflavonoid-bearing rigid structure had a stronger effect than the non-rigid ones, as observed in **8** (IC_{50} = 10.3 μM) versus **3-6** (IC_{50} > 100 μM). Brazilin (**8**) was also tested on the production of cytokine including PGE_2 and TNF-α. The result showed that brazilin exhibited appreciable anti-inflammatory activity against PGE_2 and TNF-α releases with IC_{50} values of 12.6 and 87.2 μM, respectively. This is the first report that brazilin can inhibit these two inflammatory mediators.

Effect of brazilin on iNOS, COX-2 and TNF-α mRNA expressions

The anti-inflammatory mechanism of brazilin was shown to involve mainly the down regulation of mRNA expressions of the iNOS and COX-2 genes, especially at concentrations of 10, 30 and 100 µM, and at concentrations of 30 and 100 µM for the TNF-α gene expression, respectively (Figure 2). Recently, extracts from *Caesalpinia sappan* have been reported to possess antibacterial (Lim *et al*., 2006), antifungal (Naranjan Reddy *et al*., 2003), anti-inflammatory (Min *et al*., 2012; Wu *et al*., 2011) and anti-complementary activities (Oh *et al*., 1998). However, from the previous report (Min *et al*., 2012), the anti-inflammatory effect of brazilin was only on the expression of iNOS mRNA but not on COX-2 mRNA. The effects of phenolic compounds isolated from *Caesalpinia sappan* heartwood have only been previously reported to nitric oxide production without any study on their gene expression (Cuong *et al*., 2012). An EtOH extract of *Caesalpinia sappan* was reported to inhibit iNOS and COX-2 mRNA in human chondrocytes and macrophages without any information on the isolated compounds (Wu *et al*., 2011). Thus, the present study has reported the anti-inflammatory effect of compounds isolated from *Caesalpinia sappan* heartwoods and roots on NO production and brazilin (**8**), the most active compound, was tested for its mechanism of action and shown to down regulate the expression of mRNA from iNOS, COX-2 and TNF-α in a dose-dependent manner.

Effect of an EtOH extract and brazilin on cell proliferation and viability using L929 fibroblasts

Fibroblast proliferation is an important process in wound healing for the regeneration of tissue. The proliferative effect and cytotoxicity of the EtOH extract and brazilin (**8**) were evaluated by the MTT assay. After 24 h of treatment with the EtOH extract and brazilin, the EtOH extract (1.5-25.0 µg/mL), and brazilin (1.5-25.0 µM) produced a cell viability of more than 80%. However, at any higher concentrations, cytotoxic effects were observed (Table 3). From our results, fibroblast cells had a better viability at the lower concentrations. Moreover, enhancement on the growth of the L929 fibroblasts was clearly observed after treatment with the EtOH extract at 6.25 µg/mL ($p < 0.05$) with a proliferation rate of 113 %, whereas brazilin (**8**) showed no cell proliferation when compared to that of the control group (Table 3). This result showed that the EtOH extract of *Caesalpinia sappan* enhanced fibroblast proliferation more than brazilin.

Effect of EtOH extract and brazilin on migration of L929 cells

In the present study, the effects of an EtOH extract and brazilin (**8**) were determined on the rate of L929 migration using the scratch assay. The scratch assay is a useful method for gaining an insight into the potential of an extract or compounds to repair injured dermis. This assay was used to study cell migration *in vitro* by creation of an artificial gap on a confluent cell monolayer with a pipette tip. The cellular proliferation and migration of fibroblast cells as each edge of the gaps moved towards each other and to close the scratch area was studied on days 0, 1 and 2. As shown in Figure 3, in the presence of EtOH extract (6.25 µg/mL), the migration of L929 fibroblasts increased significantly ($p < 0.01$) on both day 1 and day 2 by 65.2 and 100.0%, whereas those of the control group increased 39.0 and 64.6%, respectively. Brazilin (**8**) significantly increased the migration especially on day 2 by 75.9% (Table 4). Hence, the EtOH extract and brazilin produced a significantly enhanced migration effect when compared to those of the control group and the EtOH extract again showed a higher activity than brazilin.

Effect of EtOH extract and brazilin on collagen type-I production

Collagens are the most abundant family of protein in the body that provide strength to all tissues and they also have a crucial role in the wound healing process (Enoch and Leaper, 2008). Collagen type-I is a main structural component of the extracellular matrix, of skin and newly healed wounds. The study on the effect of EtOH extract and brazilin (**8**) on the type-I collagen production by fibroblasts was performed using the Sircol collagen assay kit. Collagen production by the L929 cells increased significantly ($p < 0.05$) after treatment with the EtOH extract at concentrations of 6.25 and 12.5 µg/mL with the production of collagen at 63.1 and 125.6 µg/mL, respectively, whereas that of the control was 48.5 µg/mL. However, no significant increase in the amount of collagen generated by fibroblasts after treatment with brazilin (Table 5). This result implied that the *Caesalpinia sappan* EtOH extract was better to use rather than the pure brazilin to increase collagen production.

Acute toxicity of EtOH extract of *Caesalpinia sappan*

For the test of acute toxicity, the oral LD_{50} of the EtOH extract of *Caesalpinia sappan* was found to be greater than 2,000 mg/kg of body weight in both male and female Swiss albino mice. At the end of study period (7 days), all animals survived and appeared to be active and healthy. During the period of observation during the day, the animals showed no significant sign of toxicity, adverse pharmacological effects or abnormal behavior. This result indicated that the *Caesalpinia sappan* EtOH extract showed no acute toxicity and was safe to use.

CONCLUSION

In conclusion, it seems that *Caesalpinia sappan* EtOH extract had potent anti-inflammatory activity by inhibiting the production of NO, PGE_2 and TNF-α and promoted a wound healing effect. The compound that was mainly responsible for anti-inflammatory activity was brazilin. Brazilin was found to down regulate the mRNA expressions of the iNOS, COX-2 and the TNF-α genes in a dose-dependent manner. The EtOH extract of *Caesalpinia sappan* significantly enhanced the wound healing effect in L929 fibroblasts by increasing cell proliferation and migration as well as their collagen production. This study therefore may support the traditional use of this plant for treatment of aphthous ulcer, stomatitis, epistaxis, haemorrhoids and pneumonia which are inflammatory-related diseases without demonstrating any acute toxicity in mice.

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Conflicts of interest

The authors report no conflicts of interest.

REFERENCES

Badami S, Moorkoth S, Suresh B. 2004. *Caesalpinia sappan*, a medicinal and dye yielding plant. *Natural Product Radiance* **3**: 75-82.

Balekar N, Katkam NG, Nakpheng T, Jehtae K, Srichana T. 2012. Evaluation of wound healing potential of *Wedelia trilobata* (L.) leaves. *Journal of Ethnopharmacology* **141**: 817-824.

Banskota AH, Tezuka Y, Nguyen NT, Awale S, Nobukawa T, Kadota S. 2003. DPPH radical scavenging and nitric oxide inhibitory activities of the constituents from the wood of *Taxus yunnanensis*. *Planta Medica* **69**: 500-505.

Bruce RD. 1985. An up-and-down procedure for acute toxicity. *Fundamental and Applied Toxicology* **5**: 151-157.

Cuong TD, Hung TM, Kim JC, Kim EH, Woo MH, Choi JS, Lee JH, Min BS. 2012. Phenolic compounds from *Caesalpinia sappan* heartwood and their anti-inflammatory activity. *Journal of Natural Products* **75** (12): 2069-2075.

Enoch S, Leaper DJ. 2008. Basic science of wound healing. *Surgery* **26**: 31-37.

Kou PC, Schroder RA. 1995. The emerging multifacated roles of nitric oxide. *Annals of Surgery* **221**: 220-235.

Lim MY, Jeon JH, Jeong EY, Lee CH, Lee HS. 2006. Antimicrobial activity of 5-hydroxy-1,4-naphthoquinone isolated from *Caesalpinia sappan* toward intestinal flora. *Food Chemistry* **100** (3): 1254-1258.

Min BS, Cuong TD, Hung TM, Min BK, Shin BS, Woo MH. 2012. Compounds from the heartwood of *Caesalpinia sappan* and their anti-inflammatory activity. *Bioorganic & Medicinal Chemistry Letters* **22** (24): 7436-7439.

Moncada S, Palmer RMJ, Higgs EA. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmaceutical Reviews* **43**: 109-142.

Naranjan Reddy VL, Ravikanth V, Jansi Lakshmi VVNS, Suryanarayan Murty U, Venkateswarlu Y. 2003. Inhibitory activity of homoisoflavonoids from *Caesalpinia sappan* against *Beuveria bassiana*. *Fitoterapia* **74** (6): 600-602.

Oh SY, Kim DS, Lee IS, Jung KY, Lee JJ, Lee HK. 1998. Anticomplementary activity of constituents from the heartwood of *Caesalpinia sappan*. *Planta Medica* **64** (5): 456-458.

Wild T, Rahbarnia A, Kellner M, Sobotka L, Eberlein T. 2010. Basics in nutrition and wound healing. *Nutrition* **26**: 862-866.

Wu SQ, Ptero M, Unger FM, Goldring MB, Phrutivorapongkul A, Chiari C, Kolb A, Viernstein H, Toegel S. 2011. Anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract in human chondrocytes and macrophages. *Journal of Ethnopharmacology* **138** (2): 364-372.

Wutthithamavet W. 1997. Thai Traditional Medicine, revised edition. Odean Store Press: Bangkok: 340.

Yodsaoue O, Cheenpracha S, Karalai C, Ponglimanont C, Tewtrakul S. 2009. Anti-allergic activity of principles from the roots and heartwood of *Caesalpinia sappan* on antigen-induced β-hexosaminidase release. *Phytotherapy Research* **23**: 1028-1031.

Table 1. Inhibition of NO production[a] by compounds **1-9** isolated from *Caesalpinia sappan*

Compounds	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
3-Deoxysappanchalcone (**1**)	0.0 ± 0.8	-	-2.6 ± 1.2	15.6 ± 0.6**	73.5 ± 1.3**	58.6
Sappanchalcone (**2**)	0.0 ± 0.8	-	14.0 ± 1.8**	36.8 ± 1.8**	99.4 ± 0.6**	31.0
3R-(3,4-Dihydroxybenzyl)-7-hydroxychroman-4-one (**3**)	0.0 ± 0.8	-	-	7.2 ± 1.4*	38.8 ± 1.4**	>100
Episappol (**4**)	0.0 ± 4.2	-	-	-	25.1 ± 0.7*	>100
4-O-Methylepisappol (**5**)	0.0 ± 4.2	-	-	-	16.7 ± 1.1*	>100
4-O-Methylsappanol (**6**)	0.0 ± 4.2	-	-	-	16.2 ± 1.1*	>100
4-(7-Hydroxy-2,2-dimethyl-9βH-1,3,5-trioxa-cyclopenta[α]naphthalen-3α-ylmethyl)-benzene-1,2-diol (**7**)	0.0 ± 2.5	-	3.9 ± 1.7	12.8 ± 1.3	77.9 ± 1.6**	54.8
Brazilin (**8**)	0.0 ± 2.5	12.5 ± 2.4	38.8 ± 1.7**	96.4 ± 0.8**	98.4 ± 0.7[b]**	10.3
Protosappanin A (**9**)	0.0 ± 2.5	-	-	-	17.8 ± 0.9	>100
L-Nitroarginine (L-NA)	0.0 ± 9.9	11.7 ± 4.6	20.2 ± 5.9	34.7 ± 1.8 *	71.6 ± 2.6**	61.8
Caffeic acid phenethylester (CAPE)	0.0 ± 9.9	30.7 ± 3.2	68.6 ± 3.4[b]**	98.7 ± 1.2[b]**	98.9 ± 2.1[b]**	5.6
Indomethacin	0.0 ± 2.0	-	12.3 ± 1.5*	31.3 ± 1.5**	74.0 ± 1.0**	46.5

[a] Each value is a mean value ± S.E.M. of four determinations. (-) = not determined.

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

[b] Cytotoxic effect was observed.

Table 2. Inhibitory effect[a] of brazilin (**8**) on PGE_2 and TNF-α production by RAW264.7 cells

Sample	IC_{50} (μM) against inflammatory mediators	
	PGE_2	TNF-α
Brazilin (**8**)	12.6 ± 0.9	87.2 ± 1.0
Indomethacin	0.4 ± 0.1	93.4 ± 1.2

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

Table 3. Effect of the EtOH extract of *Caesalpinia sappan* and brazilin on the viability of L929

Sample	%Viability of L929 cells at various concentrations							
	0	1.56	3.12	6.25	12.5	25.0	50.0	100.0
EtOH extract (µg/mL)	100.0 ± 2.3	102.9 ± 2.9	103.7 ± 2.0	113.0 ± 4.1*	105.0 ± 4.9	97.8 ± 3.1	78.5 ± 2.9*	37.7 ± 1.9*
Brazilin (µM)	100.0 ± 1.9	98.0 ± 1.0	94.9 ± 4.8	95.1 ± 1.1	93.9 ± 1.4	93.8 ± 2.9	67.9 ± 2.0*	19.2 ± 0.5*

Values are means ± S.E.M. (N=4). Significantly different from the control (0 µg/mL), *$p<0.05$

Table 4. Effect of the EtOH extract of *Caesalpinia sappan* and brazilin on the *in vitro* scratch assay using fibroblast L929 cells

Sample	Dose	Length between the scratch (µm)			%Migration rate of cells	
		Day 0	Day 1	Day 2	Day 1	Day 2
Control	-	620.7 ± 7.3	378.9 ± 9.0*	217.1 ± 10.3*	39.0 ± 1.4	64.6 ± 1.7
EtOH extract (µg/mL)	6.25	600.0 ± 8.7	200.0 ± 17.4*	0.0*	65.2 ± 3.0*	100.0*
Brazilin (µM)	6.25	574.4 ± 4.6	357.7 ± 5.4*	141.5 ± 4.5*	37.7 ± 0.9	75.9 ± 0.8*

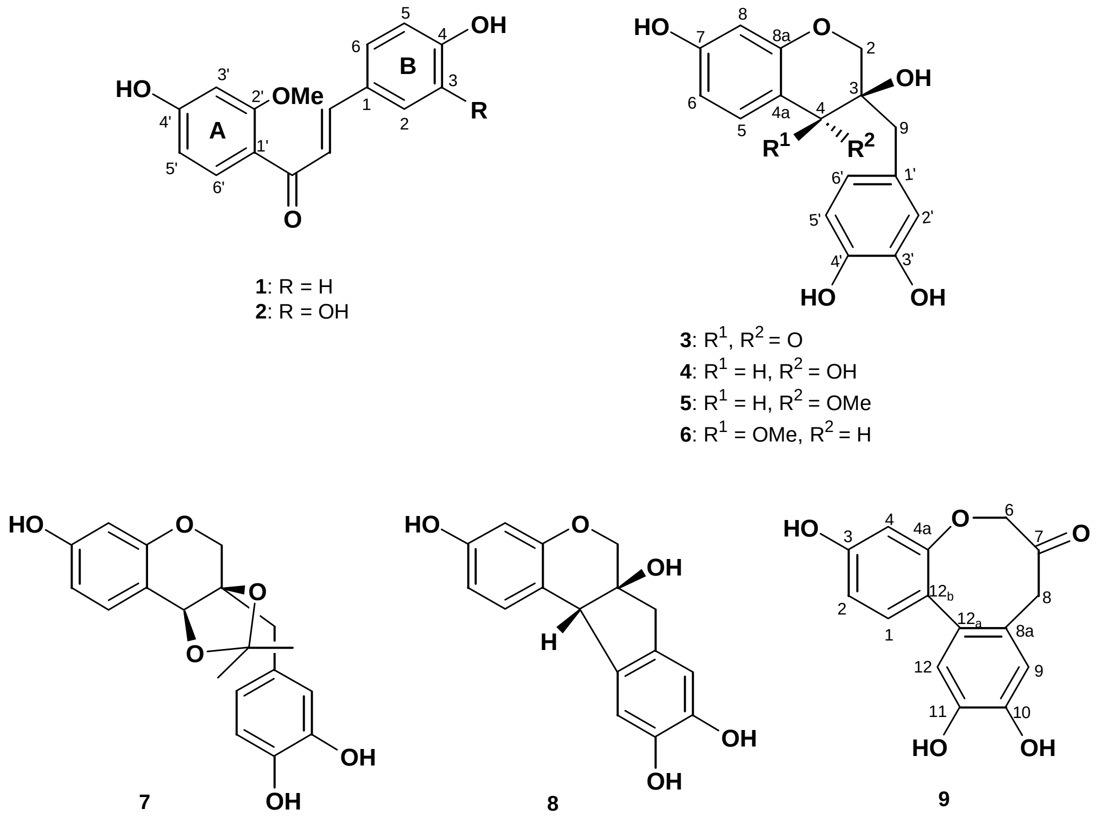
Values are represents means ± S.E.M. (N=4). Significantly different from the control, *p<0.01. (-) = not determined.

Table 5. Collagen type-I production by L929 cells when treated with the EtOH extract of *Caesalpinia sappan* and brazilin

Sample	Collagen production (µg/mL) at various concentrations				
	0	1.56	3.12	6.25	12.5
Control	48.5 ± 4.1	-	-	-	-
EtOH extract (µg/mL)		< 2.5	26.3 ± 3.2	63.1 ± 3.7*	125.6 ± 3.4*
Brazilin (µM)		< 2.5	< 2.5	14.0 ± 4.8	30.2 ± 5.5

Values are means ± S.E.M. (N=4). Significantly different from the control, *$p<0.05$

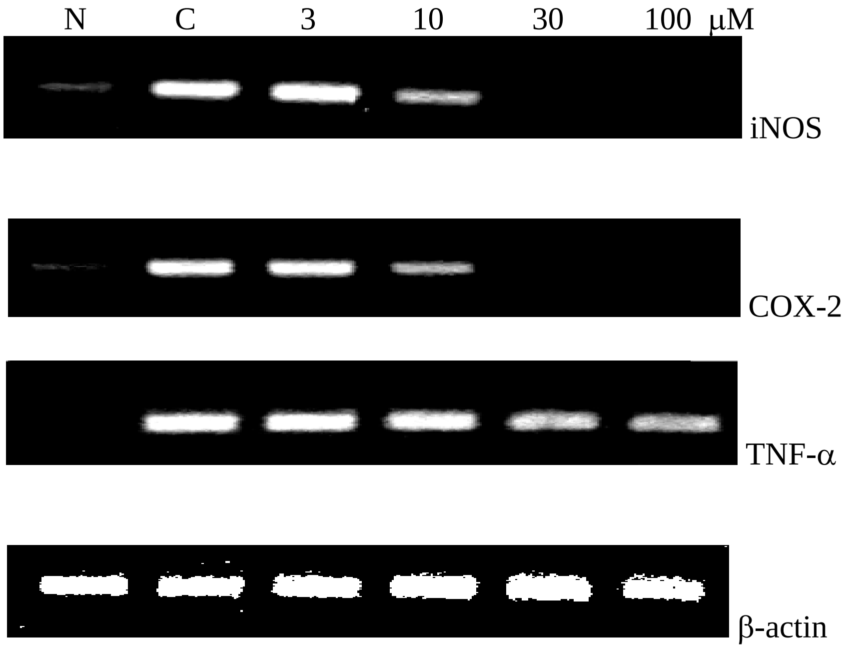
(-) = not determined.



1: R = H
2: R = OH

3: R^1, R^2 = O
4: R^1 = H, R^2 = OH
5: R^1 = H, R^2 = OMe
6: R^1 = OMe, R^2 = H

Figure 1. Structures of compounds **1-9** isolated from *Caesalpinia sappan* heartwood



mRNA expression of brazilin (**8**)

Figure 2. Effect of brazilin (**8**) at various concentrations (3, 10, 30, 100 μM) on the expression of mRNA from iNOS, COX-2 and TNF-α by LPS-induced NO, PGE_2 and TNF-α releases in RAW264.7 cells.

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

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

3-100 μM = LPS (+), sample (+)

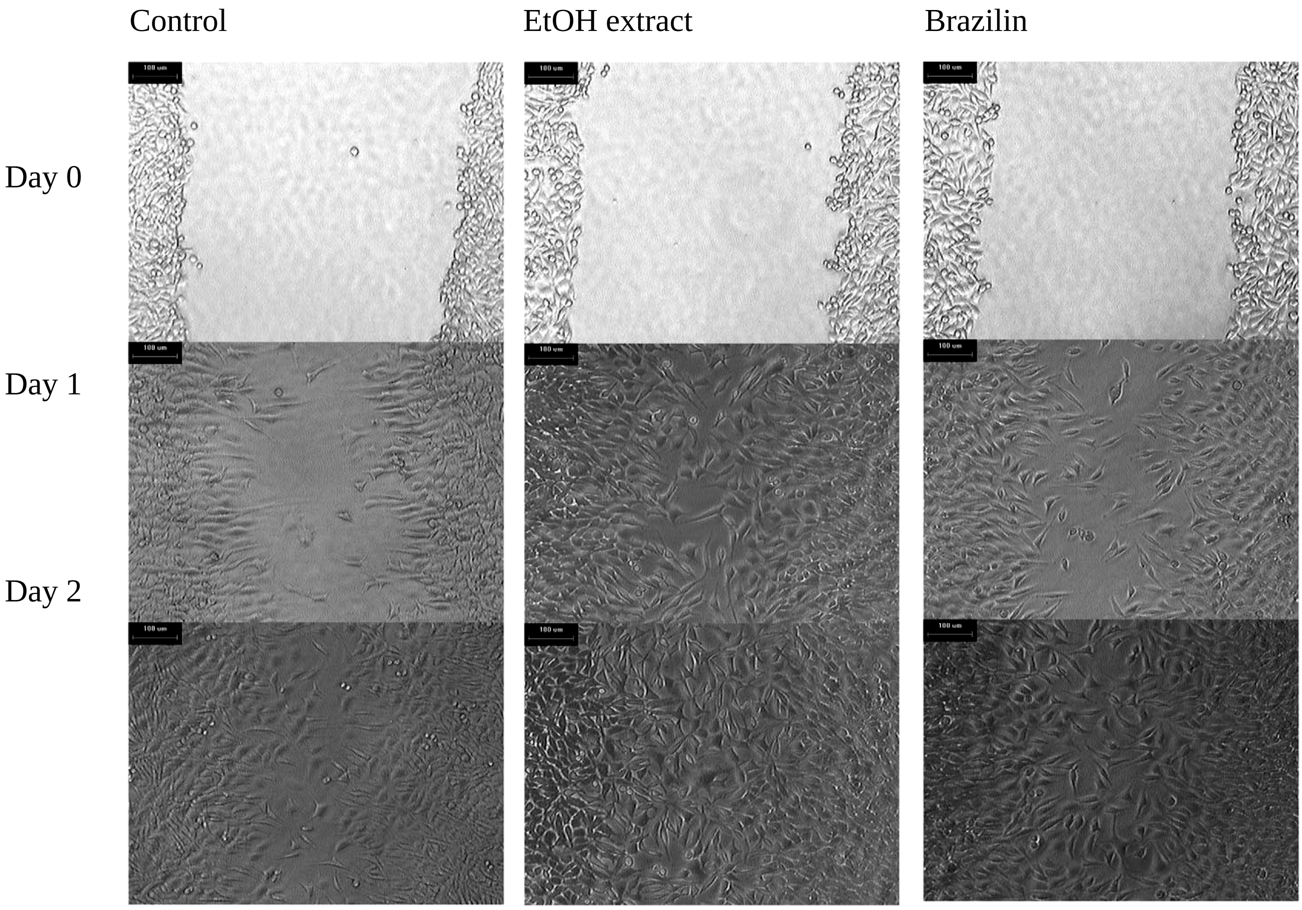


Figure 3. Effect of the EtOH extract of *Caesalpinia sappan* and brazilin on fibroblast L929 migration. Images were captured at day 0 and then treated with brazilin (6.25 µM), EtOH extract (6.25 µg/mL) and control without treatment. Another set of images were captured at day 1 and 2 after incubation. Quantitative analysis of the migration rate was quantified using computing software.

Part II. Anti-HIV-1 integrase activity of compounds from *Caesalpinia sappan*

Abstract

Caesalpinia sappan L. (Caesalpiniaceae) has been traditionally used as blood tonic, expectorant and astringent by boiling with water. Searching for HIV-1 integrase (HIV-1 IN) inhibitors from this plant is a promising approach. The EtOH extract of *Caesalpinia sappan* and its isolated compounds were tested for their anti-HIV-1 IN effect using the multiplate integration assay (MIA) and the active compounds were determined for their mechanisms by molecular docking technique. Extraction from the heartwoods and roots of *Caesalpinia sappan* led to the isolation of nine compounds. Among the compounds tested, sappanchalcone (**2**) displayed the strongest effect against HIV-1 IN with an IC_{50} value of 2.3 μM, followed by protosappanin A (**9**, IC_{50} = 12.6 μM). Structure-activity relationships (SARs) of compounds from *Caesalpinia sappan* were found, in which the vicinal hydroxyl moiety were essential for anti-HIV-1 IN effect of compounds **2** and **9** by binding with the amino acid residues Gln148 and Thr66 in the core domain of the HIV- 1 IN enzyme, respectively.

Keywords: Anti-HIV-1 IN activity, Molecular docking, *Caesalpinia sappan*, Caesalpiniaceae

INTRODUCTION

HIV-1 integrase (IN) enzyme is a 32 kDa protein produced from the C-terminal portion of the pol-gene product which minimally functions as a dimer with 288 amino acids each. The catalytic core domain contains the invariant triad of acidic residues, the D,D-35E motif, comprising residues Asp64, Asp116, and Glu152 in the case of HIV-1 IN (Craigie, 2001). HIV-1 IN has become an interesting target since it has no human counterpart and only three inhibitors (raltegravir, elvitegravir, dolutegravir) are now clinically available.

Among 67.4 million Thai people, it is reported that 23,000 HIV patients died/year, 9,700 new HIV positives reported/year with 490,000 of total HIV-positive patients. Specific drug treatment is expensive, and only small number of AIDS patients have access to the cocktail of modern antiviral agents. Hence, some AIDS patients prefer to using Thai traditional healers, who prescribe a range of natural-based products. However, scientific research supporting this usage (efficacy, specificity, toxicity) has in most cases not yet been studied (Moncada et al., 1991).

Caesalpinia sappan L. is the plant belonging to the Caesalpiniaceae family. Its heartwood has long been used as a blood tonic and anti-inflammation (Wutthithamavet, 1997). Recently, *C. sappan* has been reported to possess anti-inflammatory (Min et al., 2012), antibacterial (Lim et al., 2006), antifungal (Naranja et al., 2003) and anti-complementary activities (Oh et al., 1998). However, there has been no report for anti-HIV-1 IN activity of this plant. We therefore investigated the inhibitory activity of this plant against HIV-1 IN, structure-activity relationships (SARs) of compounds isolated from this plant and mechanism using molecular docking technique.

MATERIALS AND METHODS

Reagents

Recombinant HIV-1 IN was expressed in *Escherichia coli*, purified according to the method described in a previous publication (Jenkins et al., 1996), except that after removal of the His-tag with thrombin, chromatography on a Sephadex 200 column was the final purification step. The IN enzyme was stored at -80 °C until use.

Plant material and preparation of extract

Caesalpinia sappan L. was collected from Khonkaen province, Thailand in October 2005. Identification was made by Prof. Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University and a specimen (No. SC07) deposited at Prince of Songkla University Herbarium.

Isolation of compounds from *Caesalpinia sappan* extract

The air-dried roots (1.7 kg) of *Caesalpinia sappan* were extracted with CH_2Cl_2 and acetone, successively (each 2 x 2 L, for 5 days) at room temperature (25°C). The crude extracts were evaporated under reduced pressure to obtain a brownish CH_2Cl_2 (20.1 g) and acetone (35.2 g) extract, respectively. Crude CH_2Cl_2 and acetone extracts gave similar TLC chromatograms (data not shown), thus they were combined and further purified by silica gel quick column chromatography (QCC) using CH_2Cl_2 as eluent and increasing polarity with acetone to give seven fractions (R1-R7). Fraction R2 (2.0 g) was further purified by silica gel (100 g) column chromatography (CC) with EtOAc-CH_2Cl_2 (1:5, v/v; 50 ml, each) to give five subfractions (R2a-R2e). Subfraction R2b (140.2 mg) was separated by silica gel (10 g) CC with acetone-CH_2Cl_2 (1:20, v/v; 25 ml, each) to give **1** (8.0 mg). Fraction R3 (2.7 g) was separated by silica gel (130 g) CC with acetone-hexane (2:5, v/v; 50 ml, each) to afford seven subfractions (R3a-R3g). Subfraction R3b (200.2 mg) was purified by CC with EtOAc-hexane (2:5, v/v; 50 ml, each) to give **7** (16.0 mg). Subfraction R3e (838.4 mg) was separated by reverse phase (RP)-18 silica gel CC with MeOH-H_2O (2:5, v/v, 25 ml, each) to give **8** (15.0 mg), **4** (6.0 mg) and **2** (34.0 mg).

Chopped-dried heartwoods (5.3 kg) of *Caesalpinia sappan* were extracted with CH_2Cl_2 (2 x 5 L, for 5 days) at room temperature (25°C) and evaporated under reduced pressure to afford a brownish CH_2Cl_2 extract (135.0 g). A portion of crude CH_2Cl_2 extract (55.2 g) was further purified by silica gel (1000 g) QCC using CH_2Cl_2 as eluent and increasing polarity with EtOAc and acetone to give seven fractions (H1-H7). Fraction H3 (1.3 g) was further purified by silica gel (60 g) CC with EtOAc-CH_2Cl_2 (3:17, v/v; 50 ml, each) to give eight subfractions (H3a-H3i). Subfraction H3e (164.3 mg) was separated by silica gel CC with EtOAc-hexane (2:5, v/v; 25 ml, each) and followed by RP-18 silica gel CC with MeOH-H_2O (2:5, v/v; 25 ml, each) to give **9** (15.0 mg) and **3** (8.0 mg). Fraction H6 (5.9 g) was separated by silica gel (300

g) QCC with acetone-CH_2Cl_2 (1:5, v/v; 100 ml, each) to give six subfractions (H6a-H6f). Subfraction H6b (483.2 mg) was purified by silica gel (25 g) CC with EtOAc-hexane (1:1, v/v; 25 ml, each) to give **6** (20.0 mg) and **5** (15.0 mg). The structures of all compounds were identified by comparison with ^1H and ^{13}C-NMR spectrum of those in the previous report (Yodsaoue et al., 2009).

Assay for HIV-1 IN inhibitory activity

Oligonucleotide substrates

Oligonucleotides of target substrate (TS) DNA and long terminal repeat donor DNA (LTR-D) were purchased from QIAGEN Operon, USA and kept at -25°C before use. The sequence of biotinylated LTR donor DNA and its unlabelled complement were 5'-biotin-ACCCTTTTA GTCAGTGTGGAAAATCTCTAGCAGT-3' (LTR-D1) and 3'-GAAAATCAGTCACACCTTTTAGAGATCGTCA-5' (LTR-D2), respectively. The target substrate DNA (digoxigenin-labelled target DNA, TS-1) and its 3'-labelled complement were 5'-TGACCAAGGGCTAATTCACT-digoxigenin and digoxigenin-ACTGGTTCCCGATTAAGTGA-5' (TS-2), respectively.

Multiplate integration assay (MIA)

The integration reaction was determined according to the method previously described (Tewtrakul et al., 2001). 45 μL of a mixture, composed of 12 μL of IN buffer [containing 150 mM 3-(N-morpholino) propane sulfonic acid, pH 7.2 (MOPS), 75 mM $MnCl_2$, 5 mM dithiothritol (DTT), 25% glycerol and 500 μg/mL bovine serum albumin], 1 μL of 5 pmol/mL digoxigenin-labelled target DNA and 32 μL of sterilzed water, were added into each well of a 96-well plate. Subsequently, sample solution (6 μL) and 1/5 dilution of integrase enzyme (9 μL) was added to each well and incubated at 37°C for 80 min. The wells were washed with PBS 4 times, and 100 μL of 500 mU/mL alkaline phosphatase (AP) labelled anti-digoxigenin antibody was then added to all wells and incubated at 37°C for 1 h. The plate was washed again with washing buffer containing 0.05% Tween 20 in PBS 4 times and with PBS 4 times. Then, AP buffer (150 μL) containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ and 10 mM p-nitrophenyl phosphate was added to each well and incubated at 37°C for 1 h. Finally, the plate was measured with a microplate reader at a wavelength of 405 nm. A control consisted of a reaction mixture, 50% DMSO and an integrase enzyme, while a blank was buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA. 2Na), 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1 mM DTT and 4 M urea without the integrase enzyme. Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control. The % inhibition against HIV-1 IN was calculated as follows:

% Inhibition against HIV-1 IN = [(OD control - OD sample)/ OD control] x 100
Where OD = absorbance detected from each well

Molecular docking method

Ligand preparation

The three dimensional structure of all 9 compounds were constructed using the HyperChem professional 8.0 software. Subsequently, geometry optimization was carried out for each compound using the conjugated gradients algorithm at the semi-empirical PM3 level of theory. Finally, the Gasteiger charges were assigned to each compound using the AutoDockTools program.

HIV-1 IN preparation

The X-ray crystal structure of the core domain of HIV-1 IN in complexed with 5CITEP, its inhibitor, was obtained from the Protein Data Bank (PDB code 1QS4). Only Chain A which co-crystallized with 5-CITEP was selected, while chains B and C were deleted. All the water molecules and 5CITEP were removed while a magnesium ion at the active site was maintained. The missing residues in the loop at positions 141-144 were incorporated and polar hydrogen atoms were added to this chain (Vajragupta et al., 2005). The second magnesium ion was placed in the same relative position according to the two metal structure of the Prototype Foamy Virus integrase (PDB code 3OYA), a high structural homolog to HIV-1 IN (Krishnan & Engelman, 2012).

Molecular docking

Molecular docking was performed using the AutoDock 4.2 suite of program. A grid box with a dimension of 60 x 60 x 60 $Å^3$ and a grid spacing of 0.375 Å, which is large enough for a free rotation of a ligand, was built and placed at the location of the co-crystallized inhibitor (5CITEP) to cover the active site of HIV-1 IN.

The docking calculations were carried out using the Lamarckian genetic algorithm (LGA). The optimized docking parameters were set as follows: the number of GA run was 100, population size was 150, the maximum number of energy evaluations was increased to 2,500,000 per run and the maximum number of generation in the genetic algorithm was increased to 100,000. All other docking parameters were set at their default values.

A cluster analysis was applied to all 100 independent conformations for each compound, in which conformations with the root mean square deviation (RMSD) differ less than 2 Å were grouped together. The best docked conformations were the lowest binding energy and the greatest number of conformations in the cluster (Healy et al., 2009). Amino acid residues within 6 Å of the ligand were analyzed for H-bond interactions.

Statistical analysis

For statistical analysis, the values are expressed as a mean ± S.E.M of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

RESULTS AND DISCUSSION

Thai medicinal plant, *C. sappan*, was examined for its inhibitory effect against HIV-1 IN which led to the isolation of nine compounds from its heartwoods and roots (Figure 1).

Regarding anti-HIV-1 IN activity of this plant, it was found that sappanchalcone (**2**) possessed the strongest effect with an IC_{50} value of 2.3 μM, followed by protosappanin A (**9,** IC_{50} = 12.6 μM), whereas 3R-(3,4-dihydroxybenzyl)-7-hydroxychroman-4-one (**3**) and episappol (**4**) exhibited moderate effects with IC_{50} values of 36.7 and 33.0 μM, respectively (Table 1). The activity of sappanchalcone (**2**, IC_{50} = 2.3 μM) was comparable to that of suramin, an HIV-1 IN inhibitor (IC_{50} = 1.8 μM). The structure-activity relationships of *C. sappan* against HIV-1 IN activity are as follow: 1) the vicinal hydroxyl groups and methoxy moiety of chalcones were essential for the activity as shown in **2** (IC_{50} = 2.3 μM) versus **1** (IC_{50} >100 μM) and 2) homoisoflavonoid possessing vicinal hydroxyl group showed high activity as observed in **3** and **4** (IC_{50} = 36.7 and 33.0 μM, respectively). However, some homoisoflavonoids (**5-8**, IC_{50} > 100 μM) whose structures bearing vicinal hydroxyl group did not obey this rule. Thus, the stereochemistry of these homoisoflavonoids might affect their binding potential with an IN enzyme.

From molecular docking study, it was found that sappanchalcone (**2**) could interact with Thr66, His67, Gln148 and Lys159 in the core domain of IN enzyme with the binding energy at -5.42 kcal/mol (Figure 2, Table 2), resulting in high HIV-1 IN inhibitory activity. For protosappanin A (**9**), this compound bound to Thr66, Gln148 and Lys159 (binding energy = -5.05 kcal/mol) without binding with His67 (Figure 3, Table 2). The effect of sappanchalcone (**2,** IC_{50} = 2.3 μM) against HIV-1 IN then showed higher activity than that of protosappanin A (**9,** IC_{50} = 12.6 μM) since sappanchalcone could bind with one more amino acid residue in the IN core domain and had low binding energy than protosappanin A did.

Regarding anti-HIV-1 IN effect and molecular docking study of chalcone derivatives, it was found that sappanchalcone (**2**, IC_{50} = 2.3 μM) showed higher activity against IN enzyme than that of 3-deoxysappanchalcone (**1**, IC_{50} > 100 μM) (Table 1). These two compounds had comparable binding energy at -5.45 kcal/mol (compound **1**) and -5.42 kcal/mol (compound **2**). However, sappanchalcone could bind with four amino acid residues (Thr66, His67, Gln148, Lys159), whereas 3-deoxysappanchalcone interacted with only three amino acids (Thr66, Asp116, Lys159) (Table 2). Thus, sappanchalcone (**2**) may then have higher anti-HIV-1 IN effect than that of 3-deoxysappanchalcone (**1**).

For homoisoflavonoids (**3-8**), compounds **3** and **4** possessed good anti-HIV-1 IN activity with IC_{50} values of 36.7 and 33.0 μM, respectively; whereas compounds **5-8** were inactive (IC_{50} > 100 μM) (Table 1). From molecular docking data, **5-8** exhibited higher binding energy (-4.73, -4.70, -4.83, -4.84 kcal/mol, respectively) than those of **3** and **4** (-5.17, -4.97 kcal/mol, respectively). Therefore, it is resulting in lower anti-HIV-1 IN effect of **5-8** when compared with those of **3-4** (Table 2).

In conclusion, the present study found that sappanchalcone (**2**) and protosappanin A (**9**) isolated from *C. sappan* exhibited good anti-HIV-1 IN activity. The molecular docking of sappanchalcone and protosappanin A revealed the interaction of these two compounds with some crucial amino acid residues (Thr66, His67, Gln148 and Lys159 for sappanchalcone; Thr66, Gln148 and Lys159 for protosappanin A) in the core domain of an IN enzyme. This is the first report of anti-HIV-1 IN activity of compounds from *C. sappan*.

Acknowledgements

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Declaration of interest

The authors report no conflicts of interest.

REFERENCES

Craigie R. 2001. HIV-1 integrase, a brief overview from chemistry to therapeutics. *J Biol Chem* **276**: 23213-23216.

Healy EF, Sanders J, Peter J, King PJ, Robinson Jr WE. 2009. A docking study of L-chicoric acid with HIV-1 integrase. *J Mol Graphics Modell* **27**: 584-589.

Jenkins TM, Engelman A, Ghirlando R, Craigie R. 1996. A soluble active mutant of HIV-1 integrase. *J Biol Chem* **271**: 7712-7718.

Krishnan L, Engelman A. 2012. Retroviral integrase proteins and HIV-1 DNA integration. *J Biol Chem* **287**: 40858-40866.

Lim M-Y, Jeon J-H, Jeong E-Y, Lee, C-H, Lee H-S. 2006. Antimicrobial activity of 5-hydroxy-1,4-naphthoquinone isolated from *Caesalpinia sappan* toward intestinal flora. *Food Chem* **100**: 1254-1258.

Min BS, Cuong, TD, Hung TM, Min BK, Shin BS, Woo MH. 2012. Compounds from the heartwood of *Caesalpinia sappan* and their anti-inflammatory activity. *Bioorg & Med Chem Letts* **22**: 7436-7439.

Moncada S, Palmer RMJ, Higgs EA. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharm Rev* **43**: 109-142.

Naranjan Reddy VL, Ravikanth V, Jansi Lakshmi VVNS, Suryanarayan Murty U, Venkateswarlu Y. 2003. Inhibitory activity of homoisoflavonoids from *Caesalpinia sappan* against *Beuveria bassiana*. *Fitoterapia* **74**: 600-602.

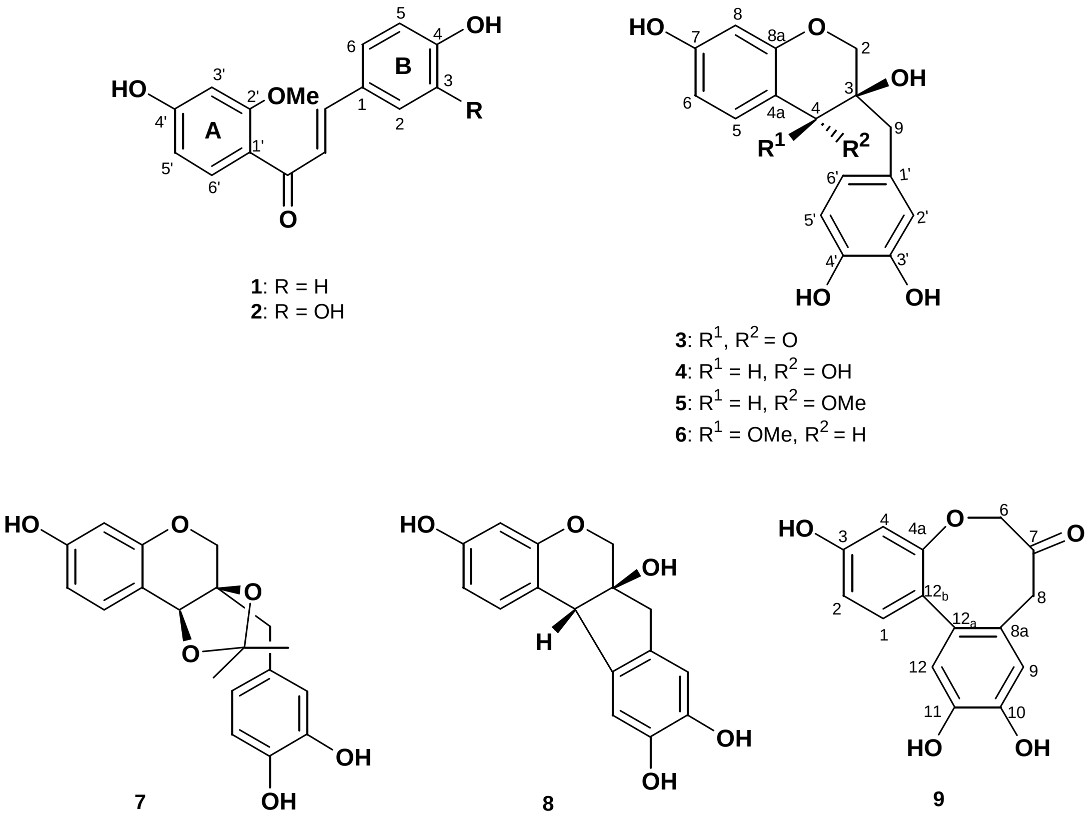
Oh SY, Kim DS, Lee IS, Jung KY, Lee JJ, Lee H-K. 1998. Anticomplementary activity of constituents from the heartwood of *Caesalpinia sappan*. *Planta Med* **64**: 456-458.

Tewtrakul S, Miyashiro H, Hattori M, Yoshinaga T, Fujiwara T, Tomimori T, Kizu H, Miyaichi Y. 2001. Inhibitory effects of flavonoids on human immunodeficiency virus type-1 integrase. *J Trad Med* **18**: 229-238.

Vajragupta O, Boonchoong P, Morrisc GM, Olsonc AJ. 2005. Active site binding modes of curcumin in HIV-1 protease and integrase. *Bioorg & Med Chem Letts* **15**: 3364-3368.

Wutthithamavet W. 1997. *Thai Traditional Medicine*, revised edition. Odean Store Press: Bangkok, 268.

Yodsaoue O, Cheenpracha S, Karalai C, Ponglimanont C, Tewtrakul S. 2009. Anti-allergic activity of principles from the roots and heartwood of *Caesalpinia sappan* on antigen-induced β-hexosaminidase release. *Phytother Res* **23**: 1028-1031.



1: R = H
2: R = OH

3: R^1, R^2 = O
4: R^1 = H, R^2 = OH
5: R^1 = H, R^2 = OMe
6: R^1 = OMe, R^2 = H

7

8

9

Figure 1. Structures of compounds from *Caesalpinia sappan* heartwood

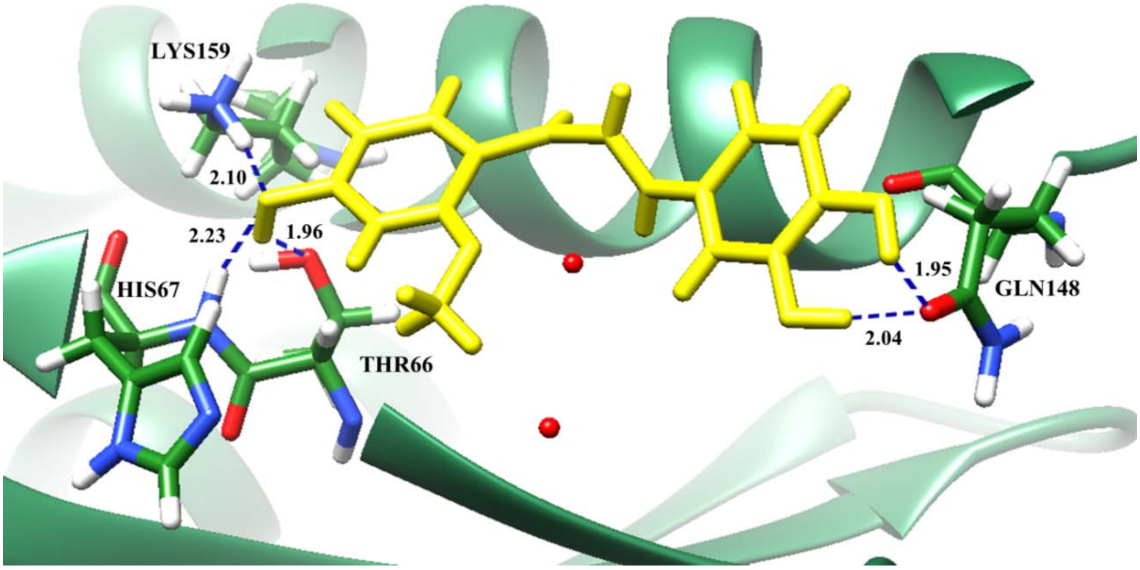


Figure 2

Molecular docking of sappanchalcone (**2**) with HIV-1 IN. The ribbon model shows the backbone of HIV-1 IN catalytic domain with all interacting amino acid residues shown as stick models and colored by element. Sappanchalcone is shown as yellow stick model. H-bond interactions are shown as blue dash lines and represent bond length in angstrom (Å). Mg^{2+}ions are shown as red balls.

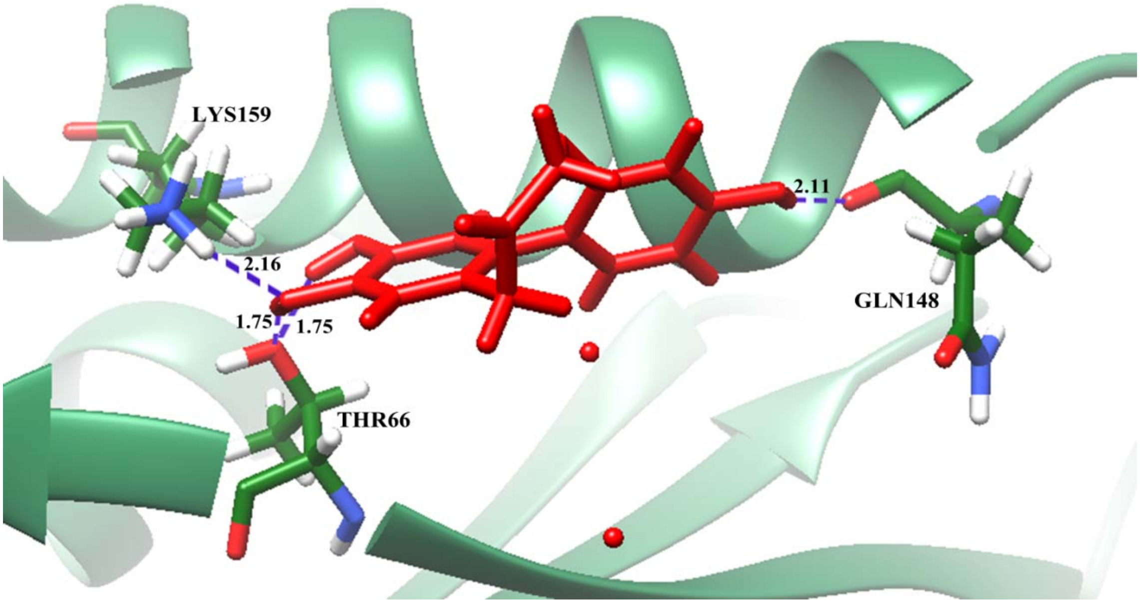


Figure 3

Molecular docking of protosappanin A (**9**) with HIV-1 IN. The ribbon model shows the backbone of HIV-1 IN catalytic domain with all interacting amino acid residues shown as stick models and colored by element. Protosappanin A is shown as red stick model. H-bond interactions are shown as blue dash lines and represent bond length in angstrom (Å). Mg^{2+} ions are shown as red balls.

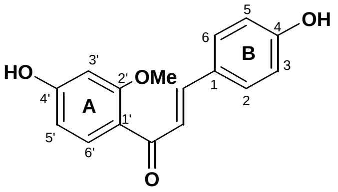
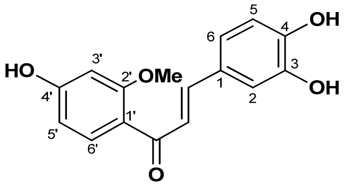
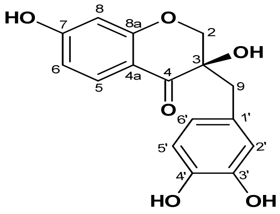
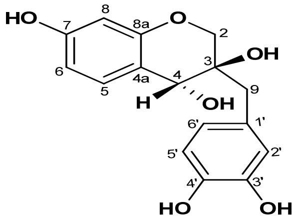
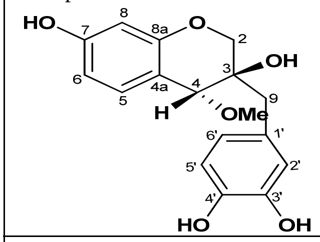
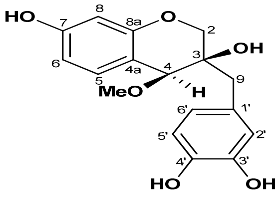
Table 1. Anti-HIV-1 IN activity of compounds **1-9** isolated from *Caesalpinia sappan* heartwood and roots

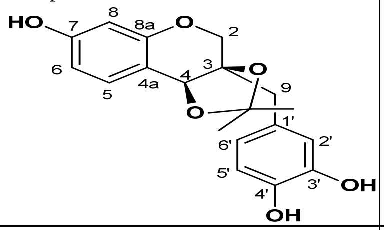
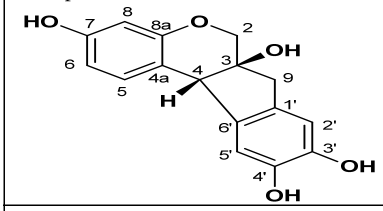
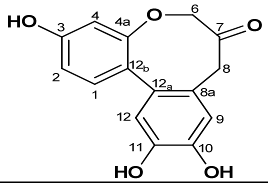
Compounds	% Inhibition at various concentrations (μM)						IC$_{50}$ (μM)
	0	1	3	10	30	100	
3-Deoxysappanchalcone (**1**)	0.0 ± 2.7	-	-	-	-	23.0 ± 3.6**	>100
Sappanchalcone (**2**)	0.0 ± 2.7	27.2 ± 2.5**	52.2 ± 3.7**	93.1 ± 0.8**	100.0 ± 0.1**	100.2 ± 0.3**	2.3
3R-(3,4-Dihydroxybenzyl)-7-hydroxychroman-4-one (**3**)	0.0 ± 2.7	-	-	5.5 ± 5.1	42.4 ± 2.3**	84.7 ± 1.2**	36.7
Episappol (**4**)	0.0 ± 2.7	-	-	1.3 ± 3.0	51.1 ± 5.8**	90.6 ± 0.6**	33.0
4-O-Methylepisappol (**5**)	0.0 ± 2.3	-	-	-	-	12.4 ± 6.4	>100
4-O-Methylsappanol (**6**)	0.0 ± 2.3	-	-	-	-	49.1 ± 4.6**	>100
4-(7-Hydroxy-2,2-dimethyl-9βH-1,3,5-trioxa-cyclopenta[α]naphthalen-3α-ylmethyl)-benzene-1,2-diol (**7**)	0.0 ± 2.3	-	-	-	-	29.1 ± 4.6*	>100
Brazilin (**8**)	0.0 ± 2.3	-	-	-	-	-80.3 ± 9.0	>100
Protosappanin A (**9**)	0.0 ± 1.8	-	10.5 ± 6.0	44.9 ± 3.8**	79.7 ± 2.7**	97.0 ± 0.4**	12.6
Suramin (Positive control)	0.0 ± 2.1	25.0 ± 2.3**	78.4 ± 1.9**	98.0 ± 0.9**	101.6 ± 0.5 **	-	1.8

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

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

Table 2. Molecular docking results of compounds **1-9** isolated from *Caesalpinia sappan*

Pure compounds	Anti-HIV1 IN activity (IC_{50} : µM)	Lowest binding energy (kcal/mol)	Amino acid residues	H-bond interactions	Distance (Å)
Compound **1** 	>100	-5.45	THR66 ASP116 LYS159	OG1---4-HO OD2---4'-HO HZ3---4-OH	1.66 1.80 2.38
Compound **2** 	2.3	-5.42	THR66 HIS67 GLN148 GLN148 LYS159	OG1---4'-HO HN---4'-OH OE1---3-HO OE1---4-HO HZ3---4'-OH	1.96 2.23 2.04 1.95 2.10
Compound **3** 	36.7	-5.17	THR66 THR66 GLN148	OG1---3'-HO OG1---4'-HO OE1---4-HO	1.76 1.79 2.07
Compound **4** 	33.0	-4.97	ASP64 GLN146 SER147 SER147	OD2---7-OH HE21---3'-OH O---3'-HO O---4'-HO	1.60 2.46 2.08 1.83
Compound **5** 	>100	-4.73	THR66 ASP116 ASP116 LYS159	OG1---7-HO OD2---3'-HO OD2---4'-HO HZ3---7-OH	1.54 1.57 2.45 1.97
Compound **6** 	>100	-4.70	THR66 THR66 ASP116 ASP116 LYS159	OG1---7-HO HG1---7-OH OD2---3'-HO OD2---4'-HO HZ3---7-OH	1.55 2.45 1.87 2.01 1.90

Compound **7** 	>100	-4.83	THR66 GLU152 ASN155 LYS159	OG1---7-HO OE1---3'-HO HD21---1-O HZ3---7-OH	1.55 1.97 1.94 2.02
Compound **8** 	>100	-4.84	CYS65 THR66 ASP116 LYS159	O---4'-HO OG1---3'-HO OD2---7-HO HZ3---3'-OH	2.05 1.93 1.89 1.97
Compound **9** 	12.6	-5.05	THR66 THR66 GLN148 LYS159	OG1---10-HO OG1---11-HO O---3-HO HZ3---10-OH	1.75 1.75 2.11 2.16

Part III. Anti-allergic and anti-inflammatory compounds from *Aglaia andamanica* leaves

Abstract

The leaves from *Aglaia andamanica* were determined for their anti-allergic and anti-inflammatory effects using RBL-2H3 and RAW264.7 cells, respectively. Among the isolated compounds, 24-epi-piscidinol A (**5**) exhibited the highest anti-allergic activity against β-hexosaminidase release with an IC_{50} value of 9.4 μg/mL, followed by pachypodol (**2**, IC_{50} = 13.2 μg/mL) and (-)-yangambin (**3**, IC_{50} = 15.1 μg/mL); whereas other compounds possessed moderate to mild effects (IC_{50} = 24.2->85.9 μg/mL). For anti-inflammatory activity, 24-epi-piscidinol A (**5**) possessed potent activity with an IC_{50} value of 11.4 μg/mL, followed by pachypodol (**2**, IC_{50} = 11.9 μg/mL), (-)-yangambin (**3**, IC_{50} = 16.7 μg/mL) and pyramidaglain B (**9**, IC_{50} = 17.8 μg/mL), respectively; whereas other compounds exhibited moderate to mild effects (IC_{50} = 24.1->46.1 μg/mL). These active compounds could be developed as anti-allergic and anti-inflammatory agents in the futures and this is the first report of *A. andamanica* for anti-allergic and anti-inflammatory activities.

Keywords: RBL-2H3 cells; RAW264.7 cells; *Aglaia andamanica*; Meliaceae

1. Introduction

Aglaia andamanica is a plant belonging to the Meliaceae family. This plant is up to 30 m, which is distributed in South and Southeast Asia. Its leaves are used for treatment of headache (Mabberley et al., 1995). Some of benzofuran derivatives from *Aglaia* genus show interesting biological effects including anti-viral (Joshi et al., 1987), anti-leukemic (Hayashi et al., 1982) and insecticidal activities (Ishibashi et al.,1993).

The allergy is an immune dysfunction, which is a serious health problem. Substances that cause allergic reaction are called allergens including pollen, dust mites, food, cosmetics and animal hairs. Hypersensitivity type I, an allergic reaction, is an IgE-mediated immune response, resulting in histamine secretion from mast cells and blood basophils. The histamine causes smooth muscle contraction, increased vascular permeability and vasodilation (Goldsby et al., 2002). Since, β-hexosaminidase is usually released along with histamine from mast cells or basophils, this enzyme is therefore used as the marker for mast cell degranulation in RBL-2H3 cell line (Cheong et al., 1998).

Nitric oxide (NO) is one of the inflammatory mediators that causes inflammation in several organs. This inorganic free radical has been implicated in physiological and pathological processes, such as vasodilation, non-specific host defense and acute or chronic inflammation. NO acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities (Kou & Schroder, 1995). However, excessive production of this free radical is pathogenic to the host tissue, because NO can bind with superoxide radicals and turn to be a reactive radical which directly damages the function of normal cells (Moncada et al., 1991).

Since the extract of *Aglaia andamanica* possessed potent anti-allergic and anti-inflamatory effects with IC_{50} values of 19.3 and 22.1 μg/mL, respectively. The compounds isolated from this plant were then tested for anti-allergic and anti-inflammatory activities.

2. Materials and Methods

2.1 Reagents

Minimum Essential Medium Eagle (MEM) and anti-DNP-IgE (Monoclonal anti-DNP) were purchased from Sigma; fetal calf serum (FCS) was from Gibco; dinitrophenylated bovine serum albumin was prepared as described previously (Tada & Okumura, 1971). Other chemicals were from Sigma. 24-well and 96-well plates were from Nunc.

Lipopolysaccharide (LPS, from *Salmonella enteritidis*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), L-nitroarginine (L-NA), caffeic acid phenethylester (CAPE), indomethacin and phosphate buffer saline (PBS) were purchased from Sigma. FCS was from Gibco. Penicillin-streptomycin was from Invitrogen. 96-well microplates were bought from Nunc. Other chemicals were from Sigma.

2.2 Plant material

Leaves of *A. andamanica* Hiern were collected in Thon-Nja-Chang National Park, Hat-Yai, Songkhla, Thailand in September 1995. A voucher specimen (JP9001) was identified by Dr. S. Vajrodaya, Department of Botany, Kasetsart University, Bangkok, Thailand and has been deposited in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla, Thailand.

2.3 Extraction and isolation of *Aglaia andamanica* leaves

The compounds (1–10) were isolated and identified by comparison of their physical and spectral data with those reported in the literatures (Puripattanavong et al., 2000; Greca et al., 1994; Vidari et al., 1971; Malan & Roux, 1979; Picker et al., 1973; Agrawal 1989; McChesney et al., 1997; Anjaneyulu et al., 1981; Marrien and Polonsky, 1971; Saifah et al., 1993; Figliuolo et al., 1987).

2.4 Anti-allergic activity assay

Inhibitory effects on antigen-induced β-hexosaminidase release from RBL-2H3 cells

Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells (purchased from ATCC) were evaluated by the following modified method (Matsuda et al., 2004). Briefly, RBL-2H3 cells were dispensed in 24-well plates at a concentration of 2×10^5 cells/well using MEM containing 10% FCS, penicillin (100 units/mL), streptomycin (100 unit/mL) and anti-DNP IgE (0.45 μg/mL), then incubated overnight at 37°C in 5% CO_2 for sensitization of the cells. The cells were washed twice with 500 μL of Siraganian buffer [119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM piperazine-*N*,*N′*-bis(2-ethanesulfonic acid) (PIPES), 0.1 % bovine serum albumin (BSA) and 40 mM NaOH, pH 7.2] and then incubated in 160 μL of Siraganian buffer for an additional 10 min at 37 °C. After that, 20 μL of test sample solution was added to each well and incubated for 10 min, followed by addition of 20 μL of antigen (DNP-BSA, final concentration is 10 μg/mL) at 37°C for 20 min to stimulate the cells to degranulate. The supernatant was transferred into a 96-well plate and incubated with 50 μL of substrate (1mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1 h. The reaction was stopped by adding 200 μL of stop solution (0.1 M

$Na_2CO_3/NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to Siraganian buffer (final DMSO concentration was 0.1 %). The inhibition (%) of the release of β-hexosaminidase by the test samples was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{Inhibition } \% = [1 - (T - B - N)/(C - N)] \times 100$$

Control (C): DNP-BSA (+), Test sample (–); Test (T) : DNP-BSA (+), Test sample (+); Blank (B) : DNP-BSA (–), Test sample (+); Normal (N) : DNP-BSA (–), Test sample (–)

2.5 Assay for NO inhibitory effect from RAW264.7 cells

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota et al., 2003). Briefly, the RAW264.7 cell line was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/mL), streptomycin (100 μg/mL) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 100 μg/mL of LPS together with the test samples at various concentrations (3-100 μg/mL for pure compounds) and was then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the MTT colorimetric method. Briefly, after 48 h incubation with the test samples, MTT solution was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. L-NA, CAPE and indomethacin were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1%). Inhibition (%) was calculated using the following equation and IC_{50} values were determined graphically (n = 4):

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

A-C : NO_2^- concentration (μM) [A : LPS (+), sample (-); B : LPS (+), sample (+); C: LPS (-), sample (-)].

2.6 Statistical analysis

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

3. Results and Discussion

The leaves of *Aglaia andamanica* were determined for their anti-allergic and anti-inflammatory effects using RBL-2H3 and RAW264.7 cells, respectively. Among the isolated compounds (Figure 1), 24-epi-piscidinol A (**5**) exhibited the highest anti-allergic activity against β-hexosaminidase release with an IC_{50} value of 9.4 μg/mL, followed by pachypodol (**2**, IC_{50} = 13.2 μg/mL) and (-)-yangambin (**3**, IC_{50} = 15.1 μg/mL); whereas other compounds possessed moderate to mild effects (IC_{50} = 24.2->85.9 μg/mL) (Table 1).

Compounds **5**, **2** and **3** exhibited higher anti-allergic effect than that of ketotifen fumarate, an anti-histamine drug (IC_{50} = 20.2 μg/mL).

For anti-inflammatory activity, 24-epi-piscidinol A (**5**) again possessed potent effect with an IC_{50} value of 11.4 μg/mL, followed by pachypodol (**2**, IC_{50} = 11.9 μg/mL), (-)-yangambin (**3**, IC_{50} = 16.7 μg/mL) and pyramidaglain B (**9**, IC_{50} = 17.8 μg/mL), respectively; whereas other compounds had moderate to mild effects (IC_{50} = 24.1-> 46.1 μg/mL) (Table 2). However, pachypodol (**2**) and **(-)-**yangambin (**3**) exhibited cytotoxicity (25-30%) at high concentration (100 μg/mL). Compounds **5**, **2**, **3** and **9** showed appreciable activity against NO production when compared to that of the positive controls, L-NA (NO synthase inhibitor, IC_{50} = 13.5 μg/mL) and indomethacin (non-steroidal anti-inflammatory drug, IC_{50} = 16.6 μg/mL) but less effective than CAPE, an NF-κB inhibitor (IC_{50} = 1.4 μg/mL).

Methoxyflavones isolated from the rhizomes of *Kaempferia parviflora* has been reported to have anti-allergic effect (Tewtrakul et al., 2008). This result is concurrent with our study that methoxyflavone, pachypodol (**2**), showed marked anti-allergic activity. Epimagnolin B isolated from *Magnolia fargesii*, whose structure is similar to (-)-yangambin, has been reported to have anti-inflammatory effect against NO and PGE_2 production (Kim et al., 2009). *Aglaia odorata* which contains diterpenoids and triterpenoids has shown anti-inflammatory activity toward NO and PGE_2 release (Yodsaoue et al., 2012). These results are accompanied with the present study that triterpenes and lignan derivatives derived from *A. andamanica* possessed anti-inflammatory effect.

In conclusion, the present study shows that *Aglaia andamanica* possesses appreciable anti-allergic and anti-inflammatory activities. Pachypodol (**2**), (-)-yangambin (**3**), 24-epi-piscidinol A (**5**) and pyramidaglain B (**9**) are responsible for both anti-allergic and anti-inflammatory activities of this plant. The mechanisms for anti-allergic and anti-inflammatory effects of these compounds will be further investigated. This is the first report of *A. andamanica* and its compounds for anti-allergic and anti-inflammatory activities.

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Declaration of interest

The authors report no conflict of interest.

References

Agrawal, P.K. 1989. Carbon-13 NMR of flavonoids, Elsevier Science Publishing Com. Inc., New York, 162-163.

Anjaneyulu, A.S.R. and Suryanarayana, M.V. 1981. Chemical examination of heartwood of Guazuma tomentosa Kunth. Indian Journal of Chemistry Section B. 20, 85-87.

Banskota, A.H., Tezuka, Y., Nguyen, N.T., Awale, S., Nobukawa, T. and Kadota, S. 2003. DPPH radical scavenging and nitric oxide inhibitory activities of the constituents from the wood of *Taxus yunnanensis*. Planta Medica. 69, 500-505.

Cheong, H., Choi, E.J., Yoo, G.S., Kim, K.M. and Ryu, S.Y. 1998. Desacetylmatricarin, an anti-allergic compound from *Taraxacum platycarpum*. Planta Medica 64, 577-578.

Figliuolo, R., Naylor, S., Wang, J. and Langenheim, J.H. 1987. Unusual nonprotein imino acid and its relationship to phenolic and nitrogenous compounds in Copaifera. Phytochemistry. 26, 3255-3259.

Goldsby, R.A., Kindt, T.J., Osborne, B.A. and Kuby, J. 2002. Immunology. fifth edition. W.H. Freeman and Company, New York, pp. 361-370.

Greca, M., Fiorentino, A., Monaco, P. and Previtera, L. 1994. Cycloartane triterpenes from *Juncus effusus*. Phytochemistry. 35, 1017-1022.

Hayashi, N., Lee, K.H., Hall, I.H., McPhail, A.T. and Huang, H.C. 1982. Structure and stereochemistry of (-)-odorinol, an antileukemic diamide from *Aglaia odorata*. Phytochemistry. 21, 2371-2373.

Ishibashi, F., Satasook, C., Isman, M.B. and Towers, G.H.N. 1993. Insecticidal 1*H*-cyclopentatetrahydro[*b*]benzofurans from *Aglaia odorata*. Phytochemistry. 32, 307-310.

Joshi, M.N., Chowdhury, B.L., Vishnoi, H.P., Shoeb, A. and Kapil, R.S. 1987. Antiviral activity of (+)-odorinol. Planta Medica. 53, 245-255.

Kim, J.Y., Lim, H.J., Lee, D.Y., Kim, J.S., Kim, D.H., Lee, H.J., Kim, H.D,. Jeon, R. and Ryu, J.H. 2009. In vitro anti-inflammatory activity of lignans isolated from *Magnolia fargesii.* Bioorganic and Medicinal Chemistry Letters. 19, 937-940.

Kou, P.C. and Scheroder, R.A. 1995. The emerging multifaceated roles of nitric oxide. Annals of Surgery. 221, 220-235.

Mabberley, D.J., Pannell, C.M. and Sing, A.M. 1995. Flora Malesiana ser. I. 12, 1-407.

Malan, E. and Roux, D.G. 1979. Flavonoids from *Distemonanthus benthamianus* Baillon. Methoxylated flavones and inter-relationships of benthamianin, a [2]benzopyrano[4,3-b][1]benzopyran. Journal of Chemical Society. Perkin Transection I. 11, 2696-2703.

Marrien, A. and Polonsky, J. 1971. The natural occurrence of melianodiol and its diacetate in *Samadera madagascariensis* (Simaroubaceae): Model experiment of melianodiol directed towards simarolide. Journal of Chemical Society and Chemical Communications. 6, 261-263.

McChesney, J.D., Dou, J., Sindela,r R.D., Goins, D.K., Walker, L.A. and Rogers, R.D. 1997. Tirucallane-type triterpenoids: NMR and X-ray diffraction analyses of 24-epi-piscidinol A and piscidinol A. Journal of Chemical Crystallography. 27, 283-290.

Matsuda, H., Tewtrakul, S., Morikawa, T., Nakamura, A. and Yoshikawa, M. 2004. Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the release of TNF-α and IL-4 in RBL-2H3 cells. Bioorganic and Medicinal Chemistry. 12, 5891-5898.

Moncada, S., Palmer, R.M.J. and Higgs, E.A. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmaceutical Review. 43, 109-142.

Picker, K., Ritchie, E. and Taylor, W.C. 1973. Constituents of Eupomatia species III. New eupomatenoid lignans from the leaves and wood of *Eupomatia laurina*. Australian Journal Chemistry. 26, 1111-1119.

Puripattanavong, J., Weber, S., Brecht, V. and Frahm A.W. 2000. Phytochemical Investigation of *Aglaia andamanica.* Planta Medica. 66, 740-745.

Saifah, E., Puripattanavong, J. Likhitwitayawuid, K., Cordell, G.A., Chai, H.Y. and Pezzuto, J.M. 1993. Bisamides from Aglaia species: Structure analysis and potential to reverse drug resistance with cultured cells. Journal of Natural Products. 56, 473-477.

Tada, T. and Okumura, K. 1971. Regulation of homocytotropic antibody formulation in the rat. I. Feed-back regulation by passive administered antibody. Journal of Immunology. 106, 1002-1011

Tewtrakul, S., Subhadhirasakul, S. and Kummee, S. 2008. Anti-allergic activity of compounds from *Kaempferia parviflora*. Journal of Ethnopharmacology. 116, 191-193.

Vidari, G., Finzi, P.V. and Bernardi, M.D. 1971. Zingiberaceae flavonols and quinones in stems of *Aframomum giganteum*. Phytochemistry. 10, 3335-3339.

Yodsaou, O., Sonprasit, J., Karalai, C., Ponglimanont, C., Tewtrakul, S. and Chantrapromma, S. 2012. Diterpenoids and triterepnoids with potential anti-inflammatory activity from the leaves of *Aglaia odorata.* Phytochemistry. 76, 83-91.

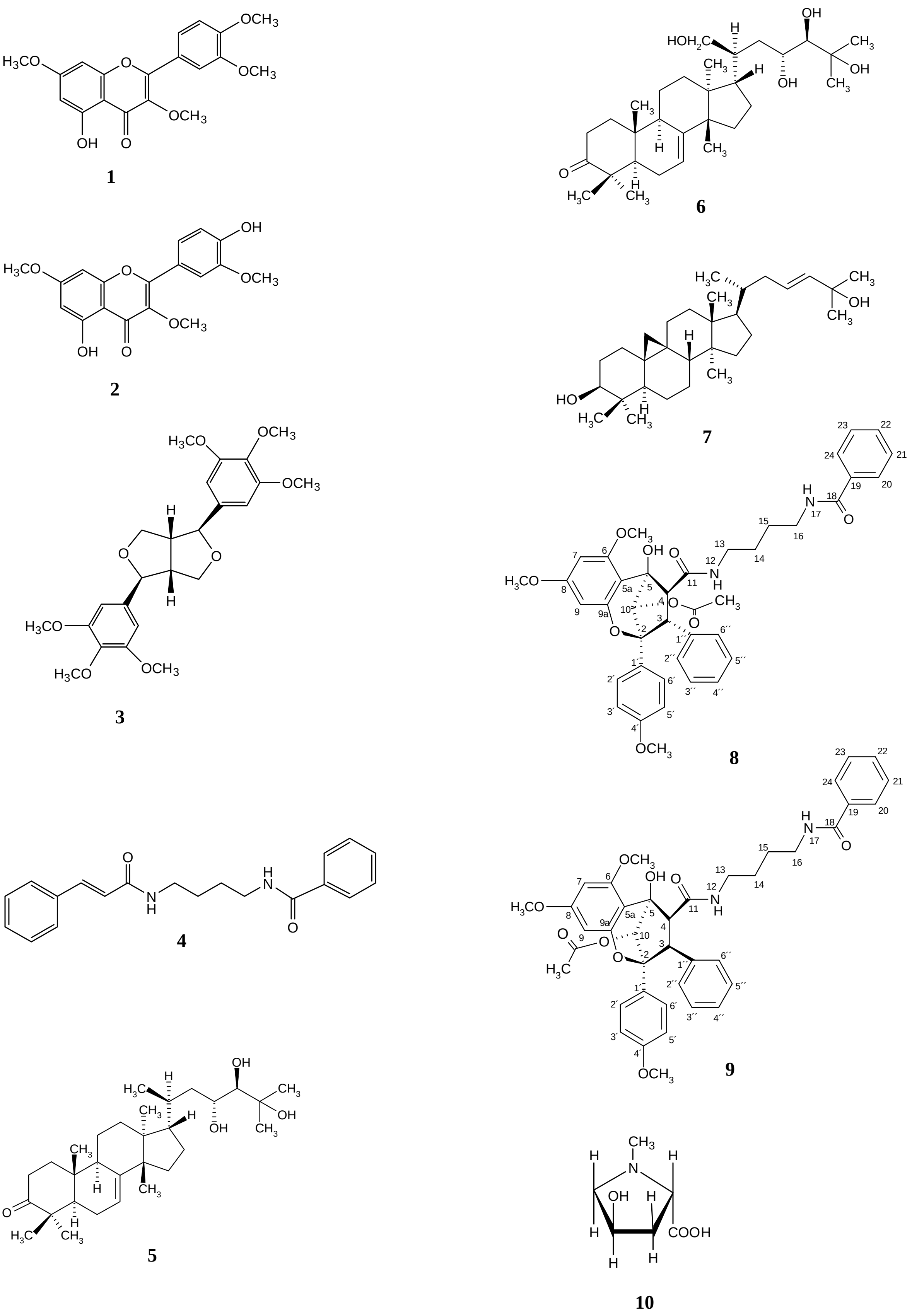

Figure 1. Structure of compounds from *Aglaia andamanica* leaves

Table 1. Anti-allergic activity of compounds **1-10** isolated from *Aglaia andamanica* leaves

Compound	IC_{50} (µM) [a]
Retusin (**1**)	67.5 ± 1.4
Pachypodol (**2**)	38.3 ± 1.1
(-)-Yangambin (**3**)	33.8 ± 1.5
Pyramidatine (**4**)	>100
24-epi-Piscidinol A (**5**)	19.8 ± 1.5
Aglaiodiol (**6**)	93.6 ± 1.8
Cycloart-23E-ene-3β-25 diol (**7**)	>100
Pyramidaglain A (**8**)	37.1 ± 1.0
Pyramidaglain B (**9**)	44.8 ± 1.8
N-Methyl-trans-4-hydroxy-L-proline (**10**)	>100
Ketotifen fumarate	47.5 ± 0.8

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

Table 2. Anti-NO production of compounds **1-10** isolated from *Aglaia andamanica* leaves

Compound	IC_{50} (μM) [a]
Retusin (**1**)	>100
Pachypodol (**2**)	34.5 ± 1.5
(-)-Yangambin (**3**)	37.4 ± 1.4
Pyramidatine (**4**)	>100
24-epi-Piscidinol A (**5**)	24.0 ± 1.1
Aglaiodiol (**6**)	>100
Cycloart-23E-ene-3β-25 diol (**7**)	54.5 ± 1.1
Pyramidaglain A (**8**)	54.2 ± 1.3
Pyramidaglain B (**9**)	25.6 ± 1.6
N-Methyl-trans-4-hydroxy-L-proline (**10**)	>100
L-Nitroarginine (L-NA)	61.8 ± 1.9
Caffeic acid phenethylester (CAPE)	5.2 ± 1.2
Indomethacin	46.5 ± 0.6

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

Part IV. Anti-HIV-1 integrase effect of compounds from *Aglaia andamanica* leaves and molecular docking study with acute toxicity test in mice

Abstract

Among the isolated compounds, **10** showed marked anti-HIV-1 IN effect with an IC_{50} value of 11.8 μg/mL, whereas other compounds were inactive (IC_{50} > 100 μg/mL). The molecular docking of compound **10** with an HIV-1 IN enzyme was also studied. The result revealed that this compound formed the hydrogen bonding with the Thr66, Asn155 and Lys159 of the HIV-1 IN binding site. The acute toxicity of the *Aglaia andamanica* extract was not observed at the dose 2,000 mg/kg mice. This is the first report of *Aglaia andamanica* for anti-HIV-1 IN activity.

Keywords: Multiplate integration assay; Meliaceae; proline derivative; up and down method

Introduction

Aglaia andamanica Hiern (Meliaceae) is a plant up to 30 m, distributed in South and Southeast Asia (Mabberley et al., 1995). The leaves have been used for treatment of headache (Mabberley et al., 1995). Some of benzofuran derivatives from *Aglaia* genus show interesting biological activities including antiviral (Joshi et al., 1987; Zhou et al., 2013), antileukemic (Hayashi et al., 1982) and insecticidal activities (Ishibashi et al., 1993).

Acquired immunodeficiency syndrome (AIDS) has been a serious problem in Thailand since the late 1980s. In Thailand, it is reported that among 490,000 of total HIV-positive patients, 23,000 HIV patients died/year with 9,700 new HIV positives reported/year (UNAIDS, 2012). Drug treatment of this disease is expensive, and a small number of AIDS patients have access to the cocktail of modern antiviral agents. HIV-1 IN has become a crucial target for AIDS treatment since there are only three HIV-1 IN inhibitors (raltegravir, elvitegravir, dolutegravir) now available in the market. Therefore the majority of AIDS patients prefer to use Thai traditional doctors, who prescribe a range of plant-based products. However, scientific studies supporting this use have in most cases not yet been carried out (Moncada et al., 1991). HIV-1 IN enzyme is a 32 kDa protein which functions as a dimer with 288 amino acids each. The integration process is composed of two steps which are 3´-processing and strand transfer reactions (Katz & Skalka, 1994; Kamarasa et al., 2006). The catalytic core domain contains the catalytic triad of acidic residues, the D, D-35E motif which are Asp64, Asp116, and Glu152 in the structure of HIV-1 IN (Craigie, 2001).

In our study, it was showed that *A. andamanica* extract possessed potent anti-HIV-1 IN effect with an IC_{50} value of 29.6 μg/mL. The compounds from this plant were then isolated and tested for this activity. Moreover, the study on the mechanism of an active compound using molecular docking with HIV-1 IN enzyme was also performed.

Materials and methods

Reagents

An HIV-1 IN recombinant was expressed in *Escherichia coli,* purified according to the method described in a previous literature (Jenkins et al., 1996), except that after removal of the His-tag with thrombin, chromatography on a Sephadex column was the final purification process. The IN enzyme was stored at -80 °C until use.

Plant material

Leaves of *A. andamanica* were collected in Thon-Nja-Chang National Park, Hat-Yai, Songkhla, Thailand in September 1995. A voucher specimen (JP9001) was identified by Dr. S. Vajrodaya, Department of Botany, Kasetsart University, Bangkok, Thailand and has been deposited in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla, Thailand.

The isolated compounds (**1**−**10**) were kept in 4 °C air-tight containers and were elucidated by comparison of their spectral data with those reported in the literature (Puripattanavong et al., 2000).

Acute toxicity test of *Aglaia andamanica* extract in mice

Male and female Swiss albino mice (30-40 g) were used in the experiment. The animals obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat-Yai, Songkhla, Thailand. All experimental protocol was approved by The Animal Ethic Committee, Prince of Songkla University (MOE 0521.11/241). Swiss albino mice were housed in standard environmental conditions with a 12 h light/dark cycle. They were provided *ad libitum* with standard rodent diet and water.

The 50% lethal dose (LD_{50}) of the MeOH extract of *A. andamanica* was estimated by the up and down method in mice (Bruce, 1985). The animals were fasted for 6 h prior to dosing. Doses were adjusted by a constant multiplicative factor (viz., 3.2) for this experiment. The dose for each successive animal was adjusted up or down depending on the previous outcome. The crude extract was dissolved in the cosolvent solution (propylene glycol: water = 1:1) and orally administered in a single dose by gavage using a stomach tube to both groups of male and female mice. Animal behaviors were observed individually at least once during the first 30 min after administration, periodically during the first 8 h and daily thereafter, for a total of 7 days. The signs of toxicity were observed including tremor, convulsion, hyperactivity, sedation, grooming, loss of righting reflex, respiratory depression and coma.

Assay for HIV-1 IN inhibitory activity

Oligonucleotide substrates

Oligonucleotides of long terminal repeat donor DNA (LTR-D) and target substrate (TS) DNA were purchased from QIAGEN Operon, USA and stored at -25°C before use. The sequence of biotinylated LTR donor DNA and its unlabelled complement were 5´-biotin-ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3´ (LTR-D1) and 3´-GAAAATCAGTCACACCTTTTAGAGATCGTCA-5´ (LTR-D2), respectively. Those of the target substrate DNA (digoxigenin-labelled target DNA, TS-1) and its 3´-labelled complement were 5´-TGACCAAGGGCTAATTCACT-digoxigenin and digoxigenin-ACTGGTTCCCGATTAAGTGA-5´ (TS-2), respectively.

Multiplate integration assay (MIA)

The integration reaction was evaluated according to the method previously described (Tewtrakul et al., 2001). A mixture (45 μL), composed of 12 μL of IN buffer [containing 150 mM 3-(N-morpholino) propane sulfonic acid, pH 7.2 (MOPS), 75 mM $MnCl_2$, 5 mM dithiothritol (DTT), 25% glycerol and 500 μg/mL bovine serum albumin], 1 μL of 5 pmol/mL digoxigenin-labelled target DNA and 32 μL of sterilzed water, were added into each well of a 96-well plate. Subsequently, 6 μL of sample solution and 9 μL of 1/5 dilution of integrase enzyme was added to each well and incubated at 37°C for 80 min. The wells were then washed with PBS 4 times, and 100 μL of 500 mU/mL alkaline phosphatase (AP) labelled anti-digoxigenin antibody was then added to all wells and incubated at 37°C for 1 h. The plate was washed again with washing buffer containing 0.05% Tween 20 in PBS 4 times and with PBS 4 times. Then, AP buffer (150 μL) containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ and 10 mM p-nitrophenyl phosphate was added to each well and incubated at 37°C for 1 h. Finally, the plate was measured with a microplate reader at a wavelength of 405 nm. A control consisted of a reaction mixture, 50% DMSO and an integrase enzyme, while a blank was buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA. 2Na), 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1 mM DTT and 4 M urea without the integrase enzyme. Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control. The % inhibition against HIV-1 IN was calculated as follows:

% Inhibition against HIV-1 IN = [(OD control - OD sample)/ OD control] x 100

Where OD = absorbance detected from each well

Molecular docking method

Ligand preparation

The three dimensional structure of active compound was constructed using the HyperChem professional 8.0 software (Gainesville, FL, USA). Subsequently, geometry optimization was carried out using the conjugated gradients algorithm at the semi-empirical PM3 level of theory. Finally, the Gasteiger charges were assigned using the AutoDockTools program (La Jolla, CA, USA).

HIV-1 IN preparation

The X-ray crystal structure of an HIV-1 IN core domain in complexed with 5CITEP, its inhibitor, was obtained from the Protein Data Bank (PDB code 1QS4). Only chain A which co-crystallized with 5CITEP was selected, while chains B and C were deleted. All the water molecules and 5CITEP were removed, while a magnesium ion at the active site was maintained. The missing residues in the loop at positions 141-144 were incorporated and polar hydrogen atoms were added to this chain (Vajragupta et al., 2005). The second magnesium ion was placed in the same relative position according to the two metal structure of the prototype foamy virus integrase (PDB code 3OYA), a high structural homolog to HIV-1 IN (Krishnan & Engelman, 2012).

Molecular docking

Molecular docking was performed with version 4.2. AutoDock program. Before docking, the grid boxes were prepared for IN structures using AutoGrid 4.0 (La Jolla, CA, USA). The center of the grid boxes were set on the location of the co-crystallized inhibitor. The grid dimensions were set to 60 x 60 x 60 Å in each dimension, with a grid spacing of 0.375 Å which is large enough for the free rotation of the ligand.

The docking calculations were carried out using the Lamarckian genetic algorithm (LGA). The optimized docking parameters were set as follows: the number of GA run was 100, population size was 150, the maximum number of energy evaluations was increased to 2,500,000 per run and the maximum number of generation in the genetic algorithm was increased to 100,000. All other docking parameters were set at their default.

A cluster analysis was used to group similar conformation, all 100 independent conformations for each compound with the root mean square deviation (RMSD) differ less than 2 Å were clustered together. The best docked conformations were the lowest binding energy and the greatest number of conformations in the cluster (Healy et al., 2009). Amino acid residues within 6 Å of the ligand in the IN active site were chosen for H-bond interactions analysis using the H-bond monitor in the DS Viewer Pro program (Roseau Valley, Dominica).

Statistical analysis

The results were expressed as mean ± S.E.M of four determinations at each concentration for each sample. The IC_{50} values were calculated using the Microsoft Excel program.

Results and discussion

The leaves and compounds from *A. andamanica* were determined for the anti-HIV-1 IN effect using the MIA assay (IC_{50} = 29.6 μg/mL). Among the isolated compounds (Figure 1), *N*-methyl-trans-4-hydroxy-L-proline (**10**) showed marked anti-HIV-1 IN effect with an IC_{50} value of 11.8 μg/mL, whereas other compounds were inactive (IC_{50} > 100 μg/mL). The positive control, suramin, had an IC_{50} value of 2.5 μg/mL (Table 1). Proline derivatives have been reported to possess antifungal, antiangiotensin converting enzyme (ACE) (Morais et al., 2013) and antiviral (Zhou et al., 2013) activities. It has been reported that methionine-proline anilides can inhibit the protease enzyme of dengue virus NS2B-NS3 (Zhou et al., 2013). Regarding the activity of *A. andamanica* leaves, it is reported that the leaves from this plant exhibited marked antiallergic and antiinflammatory effects using RBL-2H3 and RAW264.7 cells, respectively. Among the tested compounds, 24-epi-piscidinol A possessed potent for both antiallergic and antiinflammatory activities (Puripattanavong and Tewtrakul, 2015).

The molecular docking of compound **10** with an HIV-1 IN enzyme was also studied. The result revealed that the carboxylic and hydroxyl groups of this compound formed the hydrogen bonding with the Thr 66, Asn155 and Lys 159 of the IN enzyme with the binding energy at -4.50 kcal/mol (Table 2 and Figure 2). It has been reported that Thr66 is essential for 3´-processing, whereas Asn155 and Lys159 are important for strand transfer reaction (Dayam & Neamati, 2004). Thus this compound may have anti-HIV-1 IN effect by binding with the key amino acid residues of the HIV-1 IN binding site which finally interfere the binding between viral DNA and IN enzyme.

For the acute toxicity test, the oral LD_{50} of the MeOH extract of *A. andamanica* was not observed at the dose 2,000 mg/kg body weight of both male and female Swiss albino mice. At the end of study period (7 days), all animals survived and appeared active and healthy. During the day of observation period, the animals had no significant signs of toxicity, adverse pharmacological effects or abnormal behaviors. This result may indicate that *A. andamanica* extract has no acute toxicity and is safety to use.

In conclusion, the present study shows that *A. andamanica* possesses anti-HIV-1 IN activity and safety for use. *N*-methyl-trans-4-hydroxy-L-proline (**10**) isolated from this plant is responsible for anti-HIV-1 IN effect by forming the hydrogen bonding with the Thr 66, Asn155 and Lys 159 of the IN enzyme. Moreover, this is the first report of *A. andamanica* on acute toxicity in mice, anti-HIV-1 IN activity and the mechanism of *N*-methyl-trans-4-hydroxy-L-proline as anti-HIV-1 IN agent.

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Declaration of interest

The authors have no conflicts of interest. The authors are grateful to the Thailand Research Fund (TRF, RSA5680012) for financial support.

References

Bruce RD. (1985). An up-and-down procedure for acute toxicity. *Fund Appl Toxicol* 5: 151-157.

Camarasa MJ, Velazquez S, San-Felix A, Perez-Peez M.J, Gago F. (2006). Dimerization inhibitors of HIV-1 reverse transcriptase, protease and integrase: A single mode of inhibitor for the three HIV enzymes? *Antivir Res* 71: 260-267.

Craigie R. (2001). HIV-1 integrase, a brief overview from chemistry to therapeutics. *J Biol Chem* 276: 23213-23216.

Dayam R, Neamati N. (2004). Active site binding modes of the β-diketoacids: a multi-active site approach in HIV-1 integrase inhibitor design. *Bioorg Med Chem* 12: 6371-6381.

Hayashi N, Lee KH, Hall IH, McPhail AT, Huang HC. (1982). Structure and stereochemistry of (-)-odorinol, an antileukemic diamide from *Aglaia odorata*. *Phytochemistry* 21: 2371-2373.

Healy EF, Sanders J, Peter J, King PJ, Robinson Jr WE. (2009). A docking study of L-chicoric acid with HIV-1 integrase. *J Mol Graphics Modell* 27: 584-589.

Ishibashi F, Satasook C, Isman MB, Towers GHN. (1993). Insecticidal 1*H*-cyclopentatetrahydro[*b*]benzofurans from *Aglaia odorata*. *Phytochemistry* 32: 307-310.

Jenkins TM, Engelman A, Ghirlando R, Craigie R. (1996). A soluble active mutant of HIV-1 integrase. *J Biol Chem* 271: 7712-7718.

Joshi MN, Chowdhury BL, Vishnoi HP, Shoeb A, Kapil RS. (1987). Antiviral activity of (+)-odorinol. *Planta Med* 53: 245-255.

Katz RA, Skalka, AM. (1994). The retroviral enzymes. *Annual Rev Biochem* 63: 133-173.

Krishnan L, Engelman A. (2012). Retroviral integrase proteins and HIV-1 DNA integration. *J Biol Chem* 287: 40858-40866.

Mabberley DJ, Pannell CM, Sing AM. (1995). *Meliaceae. Flora Malesiana* series I. vol. 12 part 1. Rijksherbarium/Hortus Botanicus, Leiden University, Leiden, Netherlands.

Moncada S, Palmer RMJ, Higgs EA. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharm Review* 43: 109-142.

Morais KLP, Lanzer D, Miranda, JRR, Melo RL, Guerreiro JR, Santos RAS, Ulrich H, Lameu C. (2013). Proline rich-oligopeptides: Diverse mechanisms for anti-hypertensive action. *Peptides* 48: 124-133.

Puripattanavong J, Tewtrakul S. (2015). Antiallergic and antiinflammatory compounds from *Aglaia andamanica* leaves. *Songklanakarin J Sci Technol* 37: 37-41.

Puripattanavong J, Weber S, Brecht V, Frahm AW. (2000). Phytochemical investigation of *Aglaia andamanica. Planta Med* 66: 740-745.

Tewtrakul S, Miyashiro H, Hattori M, Yoshinaga T, Fujiwara T, Tomimori T, Kizu H, Miyaichi Y. (2001). Inhibitory effects of flavonoids on human immunodeficiency virus type-1 integrase. *J Trad Med* 18: 229-238.

UNAIDS. (2012). Global Report 2012, AIDS info.

Vajragupta O, Boonchoong P, Morrisc GM, Olsonc AJ. (2005). Active site binding modes of curcumin in HIV-1 protease and integrase. *Bioorg & Med Chem Letts* 15: 3364-3368.

Vidari G, Finzi PV, Bernardi MD. (1971). Zingiberaceae flavonols and quinones in stems of *Aframomum giganteum*. *Phytochemistry* 10: 3335-3339.

Zhou GC, Weng Z, Shao X, Liu F, Nie X, Liu J, Wang D, Wang C, Guo K. (2013). Discovery and SAR studies of methionine-proline anilides as dengue virus NS2B-NS3 protease inhibitors. *Bioorg & Med Chem Letts* 23: 6549-6554.

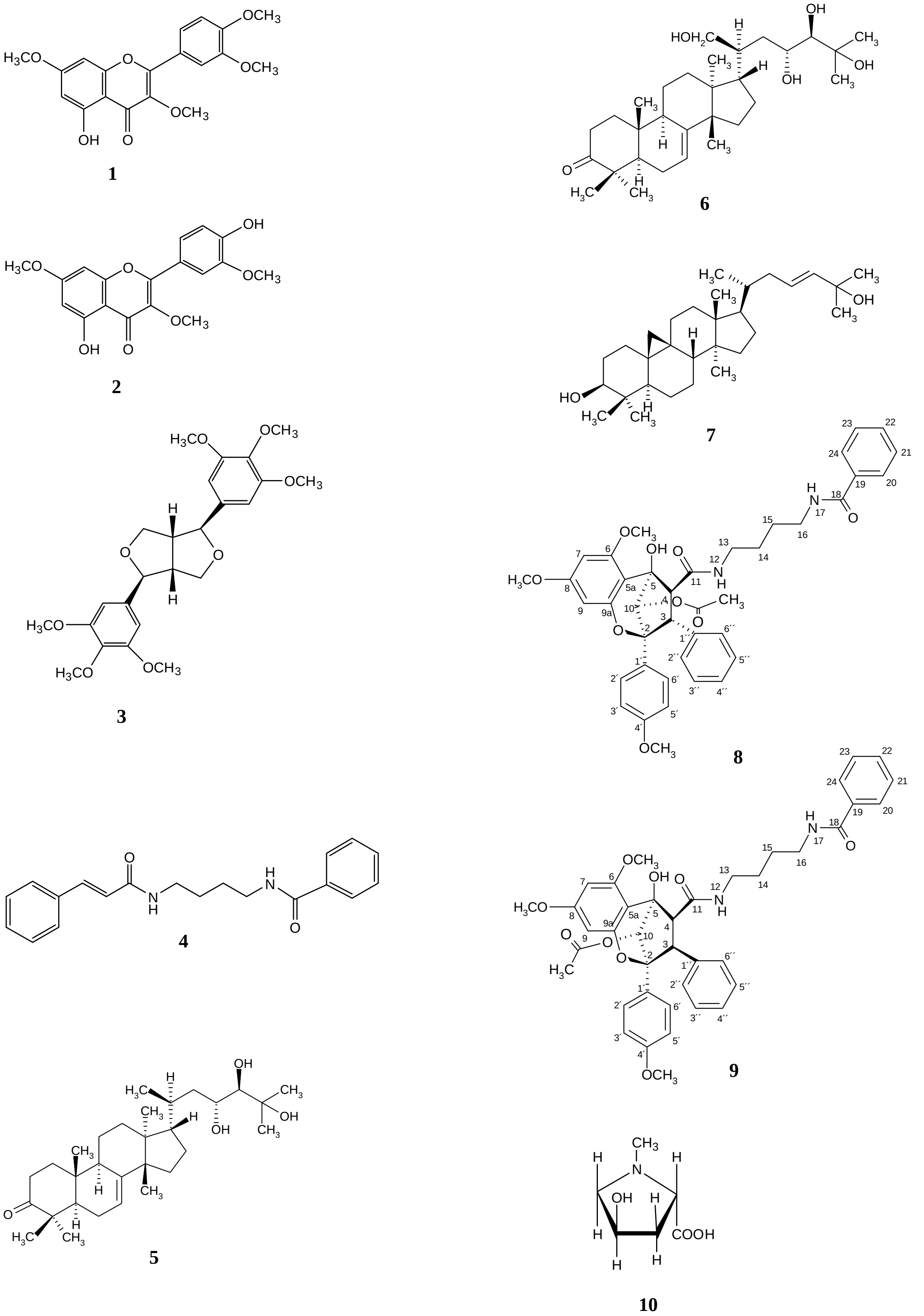


Figure 1 Structures of compounds from *Aglaia andamanica* leaves

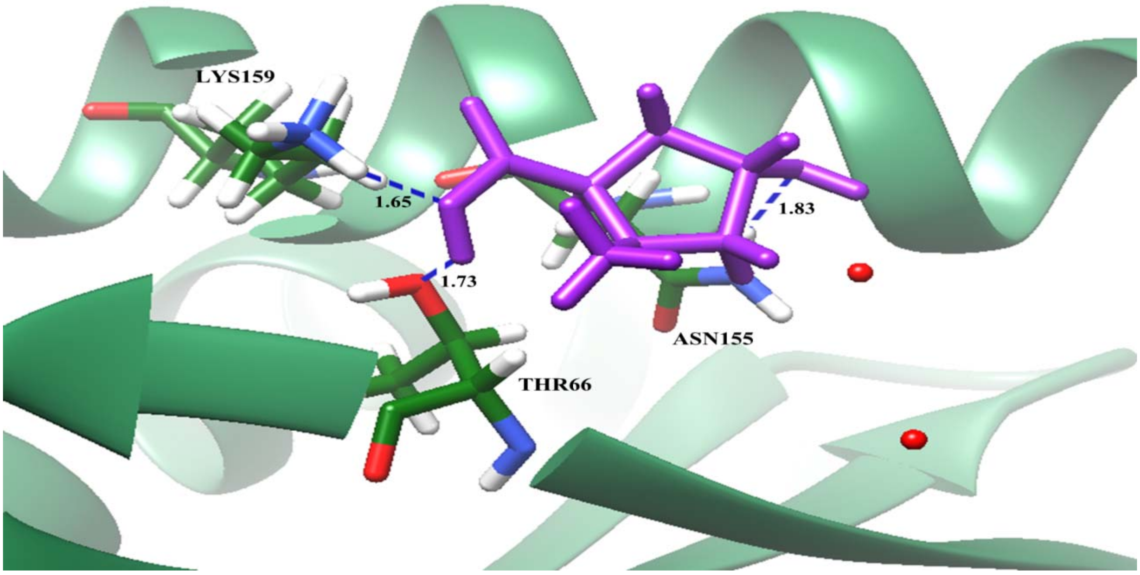


Figure 2

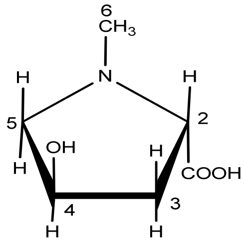
Molecular docking of *N*-methyl-trans-4-hydroxy-L-proline with HIV-1 IN. The ribbon model shows the backbone of HIV-1 IN catalytic domain with all interacting amino acid residues shown as stick models and colored by element. *N*-Methyl-trans-4-hydroxy-L-proline is shown as purple stick model. H-bond interactions are shown as blue dash lines and represent bond length in angstrom (Å). Mg^{2+} ions are shown as red balls.

Table 1. Anti-HIV-1 IN activity[a] of compounds **1-10** isolated from *Aglaia andamanica* leaves

Compound	IC_{50} (μg/mL)
Retusin (**1**)	>100
Pachypodol (**2**)	>100
(-)-Yangambin (**3**)	>100
Pyramidatine (**4**)	>100
24-epi-Piscidinol A (**5**)	>100
Aglaiodiol (**6**)	>100
Cycloart-23E-ene-3β-25 diol (**7**)	>100
Pyramidaglain A (**8**)	>100
Pyramidaglain B (**9**)	>100
N-Methyl-trans-4-hydroxy-L-proline (**10**)	11.8 ± 0.9
Suramin (Positive control)	2.5 ± 0.5

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

Table 2. Molecular docking study of *N*-Methyl-trans-4-hydroxy-L-proline (**10**)

Compound	Binding energy (kcal/mol)	Amino acid residues with H-bonding (distance : Å)			
		ASP64	THR66	ASN155	LYS159
N-Methyl-trans-4-hydroxy-L-proline 	-4.50 C = 1 F = 92	-	OG1---7-H (1.73)	HD21---4-O (1.83)	HZ3---7-O (1.65)

Outputs from this research work

1. Among the compounds tested, brazilin (**8**) was the most effective against LPS-induced NO production in RAW264.7 cells with an IC_{50} value of 10.3 μM, followed by sappanchalcone (**2**, 31.0 μM). Brazilin (**8**) also inhibited PGE_2 and TNF-α production with IC_{50} values of 12.6 and 87.2 μM, respectively. The anti-inflammatory mechanism of brazilin involved down regulation of the mRNA expressions of the iNOS, COX-2 and TNF-α genes in a dose-dependent manner.
2. The present study found that sappanchalcone (**2**) and protosappanin A (**9**) isolated from *C. sappan* exhibited good anti-HIV-1 IN activity. The molecular docking of sappanchalcone and protosappanin A revealed the interaction of these two compounds with some crucial amino acid residues (Thr66, His67, Gln148 and Lys159 for sappanchalcone; Thr66, Gln148 and Lys159 for protosappanin A) in the core domain of an IN enzyme. This is the first report of anti-HIV-1 IN activity of compounds from *C. sappan*.
3. *Aglaia andamanica* possesses appreciable anti-allergic and anti-inflammatory activities. Pachypodol (**2**), (-)-yangambin (**3**), 24-epi-piscidinol A (**5**) and pyramidaglain B (**9**) are responsible for both anti-allergic and anti-inflammatory activities of this plant. The mechanisms for anti-allergic and anti-inflammatory effects of these compounds will be further investigated. This is the first report of *A. andamanica* and its compounds for anti-allergic and anti-inflammatory activities.
4. *A. andamanica* possesses anti-HIV-1 IN activity and safety for use. *N*-methyl-trans-4-hydroxy-L-proline (**10**) isolated from this plant is responsible for anti-HIV-1 IN effect by forming the hydrogen bonding with the Thr 66, Asn155 and Lys 159 of the IN enzyme. Moreover, this is the first report of *A. andamanica* on acute toxicity in mice, anti-HIV-1 IN activity and the mechanism of *N*-methyl-trans-4-hydroxy-L-proline as anti-HIV-1 IN agent.

Publications from this research work

1. **Tewtrakul, S***., Tungcharoen, P., Sudsai, T., Karalai, C., Ponglimanont, C., Yodsaoue, O. 2015. Anti-inflammatory and wound healing effects of compounds from *Caesalpinia sappan* L. Phytotherapy Research. 29 (6), 850-856 (ISI, IF 2014 = 2.66).
2. **Tewtrakul, S***., Chaniad, P., Wattanapiromsakul, C., Pianwanit, S. 2015. Anti-HIV-1 integrase activity and moleculardocking study of compounds from *Caesalpinia sappan* L. Phytotherapy Research. 29 (5), 724-729 (ISI, IF 2014 = 2.66).
3. Puripattanavong, J., **Tewtrakul, S*.** 2015. Anti-allergic and anti-inflammatory compounds from *Aglaia andamanica* leaves. Songklanakarin Journal of Science and Technology. 37 (1), 37-41 (Scopus).
4. Puripattanavong, J., Tungcharoen, P., Chaniad, P., Pianwanit, S., **Tewtrakul, S***. 2016. Anti-HIV-1 integrase effect of compounds from *Aglaia andamanica* leaves and molecular docking study with acute toxicity in mice. Pharmaceutical Biology. 54 (4), 654-549 (ISI, IF 2014 = 1.241).

Appendix

PHYTOTHERAPY RESEARCH
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Anti-HIV-1 Integrase Activity and Molecular Docking Study of Compounds from *Caesalpinia sappan* L.

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Caesalpinia sappan **L. (Caesalpiniaceae) has been traditionally used as blood tonic, expectorant, and astringent by boiling with water. Searching for HIV-1 integrase (IN) inhibitors from this plant is a promising approach. The EtOH extract of *C. sappan* and its isolated compounds were tested for their anti-HIV-1 IN effect using the multiplate integration assay, and the active compounds were determined for their mechanisms by molecular docking technique. Extraction from the heartwoods and roots of *C. sappan* led to the isolation of nine compounds. Among the compounds tested, sappanchalcone (2) displayed the strongest effect against HIV-1 IN with an IC_{50} value of 2.3 μM followed by protosappanin A (9, $IC_{50} = 12.6\,\mu$M). Structure-activity relationships of compounds from *C. sappan* were found, in which the vicinal hydroxyl moiety were essential for anti-HIV-1 IN effect of compounds 2 and 9 by binding with the amino acid residues Gln148 and Thr66 in the core domain of the HIV- 1 IN enzyme, respectively. Copyright © 2015 John Wiley & Sons, Ltd.**

Keywords: anti-HIV-1 IN activity; molecular docking; *Caesalpinia sappan*; Caesalpiniaceae.

INTRODUCTION

HIV-1 integrase (IN) enzyme is a 32 kDa protein produced from the C-terminal portion of the pol-gene product that minimally functions as a dimer with 288 amino acids each. The catalytic core domain contains the invariant triad of acidic residues, the D,D-35E motif, comprising residues Asp64, Asp116, and Glu152 in the case of HIV-1 IN (Craigie, 2001). HIV-1 IN has become an interesting target because it has no human counterpart, and only three inhibitors (raltegravir, elvitegravir, and dolutegravir) are now clinically available.

Among 67.4 million Thai people, it is reported that 23,000 AIDS patients died/year, 9700 new AIDS-positive patients reported/year with 490,000 of total AIDS-positive patients. Specific drug treatment is expensive, and only a small number of AIDS patients have access to the cocktail of modern antiviral agents. Hence, some AIDS patients prefer on using Thai traditional healers, who prescribe a range of natural-based products. However, scientific research supporting this usage (efficacy, specificity, and toxicity) has in most cases not yet been studied (Moncada *et al.,* 1991).

Caesalpinia sappan L. is the plant belonging to the Caesalpiniaceae family. Its heartwood has long been used as blood tonic and anti-inflammation (Wutthithamavet, 1997). Recently, *C. sappan* has been reported to possess anti-inflammatory (Min *et al.,* 2012), antibacterial (Lim *et al.,* 2006), antifungal (Naranjan Reddy *et al.,* 2003), and anti-complementary activities (Oh *et al.,* 1998). However, there has been no report for anti-HIV-1 IN activity of this plant. We therefore investigated the inhibitory activity of this plant against HIV-1 IN, structure-activity relationships of compounds isolated from this plant and mechanism using molecular docking technique.

MATERIALS AND METHODS

Reagents. Recombinant HIV-1 IN was expressed in *Escherichia coli*, purified according to the method described in a previous publication (Jenkins *et al.,* 1996), except that after removal of the His-tag with thrombin, chromatography on a Sephadex 200 column was the final purification step. The IN enzyme was stored at −80 °C until use.

Plant material and preparation of extract. *Caesalpinia sappan* L. was collected from Khonkaen province, Thailand in October 2005. Identification was made by Professor Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University, and a specimen (No. SC07) was deposited at Prince of Songkla University Herbarium.

Isolation of compounds from *Caesalpinia sappan* extract. The air-dried roots (1.7 kg) of *C. sappan* were extracted with CH_2Cl_2 and acetone successively (each

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2×2 L, for 5 days) at room temperature (25 °C). The crude extracts were evaporated under reduced pressure to obtain a brownish CH_2Cl_2 (20.1 g) and acetone (35.2 g) extract, respectively. Crude CH_2Cl_2 and acetone extracts gave similar TLC chromatograms (data not shown); thus, they were combined and further purified by silica gel quick column chromatography (QCC) using CH_2Cl_2 as eluent and increasing polarity with acetone to give seven fractions (R1–R7). Fraction R2 (2.0 g) was further purified by silica gel (100 g) column chromatography (CC) with EtOAc-CH_2Cl_2 (1:5, v/v; 50 mL, each) to give five subfractions (R2a–R2e). Subfraction R2b (140.2 mg) was separated by silica gel (10 g) CC with acetone-CH_2Cl_2 (1:20, v/v; 25 mL, each) to give **1** (8.0 mg). Fraction R3 (2.7 g) was separated by silica gel (130 g) CC with acetone–hexane (2:5, v/v; 50 mL, each) to afford seven subfractions (R3a–R3g). Subfraction R3b (200.2 mg) was purified by CC with EtOAc-hexane (2:5, v/v; 50 mL, each) to give **7** (16.0 mg). Subfraction R3e (838.4 mg) was separated by reverse phase-18 silica gel CC with MeOH-H_2O (2:5, v/v, 25 mL, each) to give **8** (15.0 mg), **4** (6.0 mg), and **2** (34.0 mg).

Chopped–dried heartwoods (5.3 kg) of *C. sappan* were extracted with CH_2Cl_2 (2 × 5 L, for 5 days) at room temperature (25 °C) and evaporated under reduced pressure to afford a brownish CH_2Cl_2 extract (135.0 g). A portion of crude CH_2Cl_2 extract (55.2 g) was further purified by silica gel (1000 g) QCC using CH_2Cl_2 as eluent and increasing polarity with EtOAc and acetone to give seven fractions (H1–H7). Fraction H3 (1.3 g) was further purified by silica gel (60 g) CC with EtOAc-CH_2Cl_2 (3:17, v/v; 50 mL, each) to give eight subfractions (H3a–H3i). Subfraction H3e (164.3 mg) was separated by silica gel CC with EtOAc-hexane (2:5, v/v; 25 mL, each) and followed by reverse phase-18 silica gel CC with MeOH-H_2O (2:5, v/v; 25 mL, each) to give **9** (15.0 mg) and **3** (8.0 mg). Fraction H6 (5.9 g) was separated by silica gel (300 g) QCC with acetone-CH_2Cl_2 (1:5, v/v; 100 mL, each) to give six subfractions (H6a–H6f). Subfraction H6b (483.2 mg) was purified by silica gel (25 g) CC with EtOAc-hexane (1:1, v/v; 25 ml, each) to give **6** (20.0 mg) and **5** (15.0 mg). The structures of all compounds were identified by comparison with ^1H and ^{13}C-NMR spectra of those in the previous report (Yodsaoue *et al.,* 2009).

Assay for HIV-1 integrase inhibitory activity. *Oligonucleotide substrates.* Oligonucleotides of target substrate (TS) DNA and long terminal repeat donor DNA (LTR-D) were purchased from QIAGEN Operon, USA, and kept at −25 °C before use. The sequence of biotinylated LTR-D and its unlabeled complement were 5'-biotin-ACCCTTTTA GTCAGTGTGGAAAATCTCTAGCAGT-3' (LTR-D1) and 3'-GAAAATCAGTCACACCTTTTAGAGATCGT-CA-5' (LTR-D2), respectively. The target substrate DNA (digoxigenin-labeled target DNA, TS-1) and its 3'-labeled complement were 5'-TGACCAAGGGCTAATTCACT-digoxigenin and digoxigenin-ACTGGTTCCCGATTAAG-TGA-5' (TS-2), respectively.

Multiplate integration assay. The integration reaction was determined according to the method previously described (Tewtrakul *et al.,* 2001). The 45 μL of a mixture, composed of 12 μL of IN buffer [containing 150 mM 3-(N-morpholino) propane sulfonic acid, pH 7.2 (MOPS), 75 mM $MnCl_2$, 5 mM dithiothritol (DTT), 25% glycerol, and 500 μg/mL bovine serum albumin], 1 μL of 5 pmol/mL digoxigenin-labeled target DNA, and 32 μL of sterilized water were added into each well of a 96-well plate. Subsequently, sample solution (6 μL) and 1/5 dilution of IN enzyme (9 μL) were added to each well and were incubated at 37 °C for 80 min. The wells were washed with phosphate buffer saline (PBS) four times, and 100 μL of 500 mU/mL alkaline phosphatase (AP) labeled anti-digoxigenin antibody was then added to all wells and was incubated at 37 °C for 1 h. The plate was washed again with washing buffer containing 0.05% tween 20 in PBS four times and with PBS four times. Then, AP buffer (150 μL) containing 100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$, and 10 mM p-nitrophenyl phosphate was added to each well and was incubated at 37 °C for 1 h. Finally, the plate was measured with a microplate reader at a wavelength of 405 nm. A control consisted of a reaction mixture, 50% DMSO and an IN enzyme, while a blank was buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA 2Na), 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1 mM DTT, and 4 M urea without the IN enzyme. Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control. The % inhibition against HIV-1 IN was calculated as follows:

$$\begin{aligned}&\%\ \text{Inhibition against HIV}-1\ \text{IN}\\&\quad=[(\text{OD control}-\text{OD sample})/\text{OD control}]\times 100,\end{aligned}$$

where OD = absorbance detected from each well.

Molecular docking method. *Ligand preparation.* The 3D structure of all nine compounds were constructed using the HYPERCHEM PROFESSIONAL 8.0 software (Hypercube, Inc., Gainesville, FL, USA). Subsequently, geometry optimization was carried out for each compound using the conjugated gradients algorithm at the semi-empirical PM3 level of theory. Finally, the Gasteiger charges were assigned to each compound using the AUTODOCKTOOLS program.

HIV-1 integrase preparation. The x-ray crystal structure of the core domain of HIV-1 IN in complexed with 5CITEP, its inhibitor, was obtained from the Protein Data Bank (PDB code 1QS4). Only chain A that co-crystallized with 5-CITEP was selected, while chains B and C were deleted. All the water molecules and 5CITEP were removed, while a magnesium ion at the active site was maintained. The missing residues in the loop at positions 141–144 were incorporated, and polar hydrogen atoms were added to this chain (Vajragupta *et al.,* 2005). The second magnesium ion was placed in the same relative position according to the two-metal structure of the prototype foamy virus IN (PDB code 3OYA), a high structural homologue to HIV-1 IN (Krishnan and Engelman, 2012).

Molecular docking. Molecular docking was performed using the AUTODOCK 4.2 SUITE of program. A grid box with a dimension of 60 × 60 × 60 $Å^3$ and a grid spacing of 0.375 Å, which is large enough for a free rotation of a ligand, was built and placed at the location of the co-crystallized inhibitor (5CITEP) to cover the active site of HIV-1 IN.

The docking calculations were carried out using the Lamarckian genetic algorithm. The optimized docking parameters were set as follows: (i) the number of genetic algorithm run was 100; (ii) population size was 150; (iii) the maximum number of energy evaluations was increased to 2,500,000 per run; (iv) and the maximum number of generation in the genetic algorithm was increased to 100,000. All other docking parameters were set at their default values.

A cluster analysis was applied to all 100 independent conformations for each compound, in which conformations with the root mean square deviation differ less than 2 Å were grouped together. The best docked conformations were the lowest binding energy and the greatest number of conformations in the cluster (Healy *et al.,* 2009). Amino acid residues within 6 Å of the ligand were analyzed for H-bond interactions.

Statistical analysis. For statistical analysis, the values are expressed as a mean ± standard error of the mean. (SEM) of four determinations. The IC$_{50}$ values were calculated using the MICROSOFT EXCEL program. The statistical significance was calculated by a one-way analysis of variance followed by Dunnett's test.

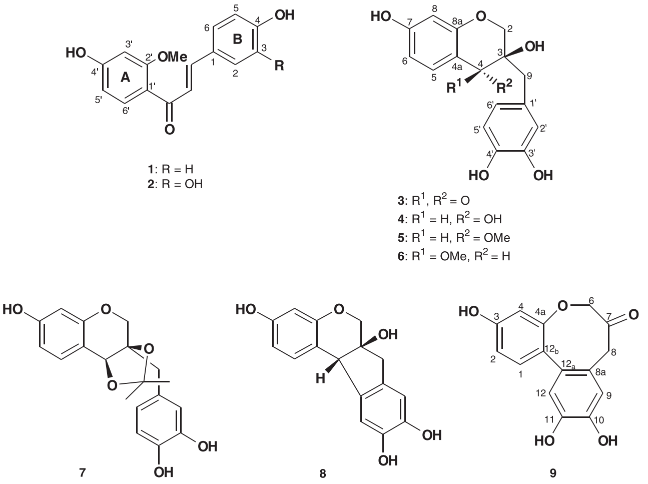


Figure 1. Compounds isolated from *Caesalpinia sappan* heartwood and roots.

RESULTS AND DISCUSSION

Thai medicinal plant, *C. sappan*, was examined for its inhibitory effect against HIV-1 IN that led to the isolation of nine compounds from its heartwoods and roots (Fig. 1).

Regarding anti-HIV-1 IN activity of this plant, it was found that sappanchalcone (**2**) possessed the strongest effect with an IC$_{50}$ value of 2.3 μM followed by protosappanin A (**9,** IC$_{50}$ = 12.6 μM), whereas 3R-(3,4-dihydroxybenzyl)-7-hydroxychroman-4-one (**3**) and episappol (**4**) exhibited moderate effects with IC$_{50}$ values of 36.7 and 33.0 μM, respectively (Table 1). The activity of sappanchalcone (**2**, IC$_{50}$ = 2.3 μM) was comparable to that of suramin, an HIV-1 IN inhibitor (IC$_{50}$ = 1.8 μM). The structure-activity relationships of *C. sappan* against HIV-1 IN activity are as follows: (i) the vicinal hydroxyl groups and methoxy moiety of chalcones were essential for the activity as shown in **2** (IC$_{50}$ = 2.3 μM) versus **1** (IC$_{50}$ > 100 μM) and (ii) homoisoflavonoid possessing vicinal hydroxyl group showed high activity as observed in **3** and **4** (IC$_{50}$ = 36.7 and 33.0 μM, respectively). However, some homoisoflavonoids (**5–8**, IC$_{50}$ > 100 μM) whose structures bearing vicinal hydroxyl group did not obey this rule. Thus, the stereochemistry of these homoisoflavonoids might affect their binding potential with an IN enzyme.

From molecular docking study, it was found that sappanchalcone (**2**) could interact with Thr66, His67, Gln148, and Lys159 in the core domain of IN enzyme with the binding energy at −5.42 kcal/mol (Fig. 2 and Table 2), resulting in high HIV-1 IN inhibitory activity. For protosappanin A (**9**), this compound bound to Thr66, Gln148, and Lys159 (binding energy = −5.05 kcal/mol) without binding with His67 (Fig. 3 and Table 2). The

Table 1. Anti-HIV-1 integrase (IN) activity of compounds 1–9 isolated from *Caesalpinia sappan* heartwood and roots

	% Inhibition at various concentrations (μM)						
Compounds	0	1	3	10	30	100	IC$_{50}$ (μM)
3-Deoxysappanchalcone (**1**)	0.0 ± 2.7	–	–	–	–	23.0 ± 3.6**	>100
Sappanchalcone (**2**)	0.0 ± 2.7	27.2 ± 2.5**	52.2 ± 3.7**	93.1 ± 0.8**	100.0 ± 0.1**	100.2 ± 0.3**	2.3
3R-(3,4-Dihydroxybenzyl)-7-hydroxychroman-4-one (**3**)	0.0 ± 2.7	–	–	5.5 ± 5.1	42.4 ± 2.3**	84.7 ± 1.2**	36.7
Episappol (**4**)	0.0 ± 2.7	–	–	1.3 ± 3.0	51.1 ± 5.8**	90.6 ± 0.6**	33.0
4-O-Methylepisappol (**5**)	0.0 ± 2.3	–	–	–	–	12.4 ± 6.4	>100
4-O-Methylsappanol (**6**)	0.0 ± 2.3	–	–	–	–	49.1 ± 4.6**	>100
4-(7-Hydroxy-2,2-dimethyl-9βH-1,3,5-trioxa-cyclopenta [α]naphthalen-3α-ylmethyl)-benzene-1,2-diol (**7**)	0.0 ± 2.3	–	–	–	–	29.1 ± 4.6*	>100
Brazilin (**8**)	0.0 ± 2.3	–	–	–	–	−80.3 ± 9.0	>100
Protosappanin A (**9**)	0.0 ± 1.8	–	10.5 ± 6.0	44.9 ± 3.8**	79.7 ± 2.7**	97.0 ± 0.4**	12.6
Suramin (Positive control)	0.0 ± 2.1	25.0 ± 2.3**	78.4 ± 1.9**	98.0 ± 0.9**	101.6 ± 0.5 **	–	1.8

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

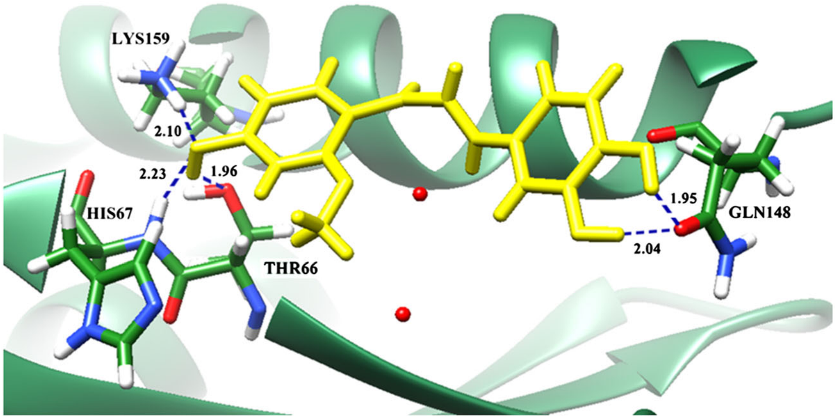
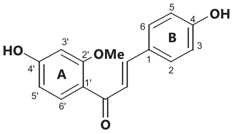
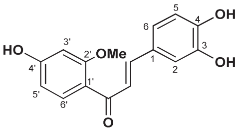
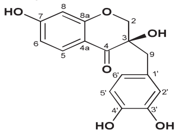
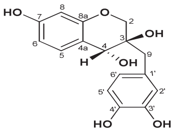
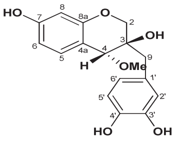


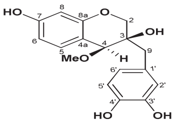
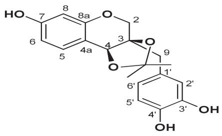
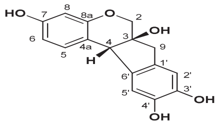
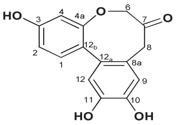
Figure 2. Molecular docking of sappanchalcone (**2**) with HIV-1 integrase (IN). The ribbon model shows the backbone of HIV-1 IN catalytic domain with all interacting amino acid residues shown as stick models and colored by element. Sappanchalcone is shown as a yellow stick model. H-bond interactions are shown as blue dash lines and represent bond length in angstrom (Å). Mg^{2+} ions are shown as red balls.

Table 2. Molecular docking results of compounds 1–9 isolated from *Caesalpinia sappan*

Pure compounds	Anti-HIV1 integrase (IN) activity (IC_{50}:μM)	Lowest binding energy (kcal/mol)	Amino acid residues	H-bond interactions	Distance (Å)
Compound **1** 	>100	−5.45	THR66	OG1---4-HO	1.66
			ASP116	OD2---4’-HO	1.80
			LYS159	HZ3---4-OH	2.38
Compound **2** 	2.3	−5.42	THR66	OG1---4’-HO	1.96
			HIS67	HN---4’-OH	2.23
			GLN148	OE1---3-HO	2.04
			GLN148	OE1---4-HO	1.95
			LYS159	HZ3---4’-OH	2.10
Compound **3** 	36.7	−5.17	THR66	OG1---3’-HO	1.76
			THR66	OG1---4’-HO	1.79
			GLN148	OE1---4-HO	2.07
Compound **4** 	33.0	−4.97	ASP64	OD2---7-OH	1.60
			GLN146	HE21---3’-OH	2.46
			SER147	O---3’-HO	2.08
			SER147	O---4’-HO	1.83
Compound **5** 	>100	−4.73	THR66	OG1---7-HO	1.54
			ASP116	OD2---3’-HO	1.57
			ASP116	OD2---4’-HO	2.45
			LYS159	HZ3---7-OH	1.97

(Continues)

Table 2. (Continued)

Pure compounds	Anti-HIV1 integrase (IN) activity (IC_{50}:μM)	Lowest binding energy (kcal/mol)	Amino acid residues	H-bond interactions	Distance (Å)
Compound **6** 	>100	−4.70	THR66	OG1---7-HO	1.55
			THR66	HG1---7-OH	2.45
			ASP116	OD2---3'-HO	1.87
			ASP116	OD2---4'-HO	2.01
			LYS159	HZ3---7-OH	1.90
Compound **7** 	>100	−4.83	THR66	OG1---7-HO	1.55
			GLU152	OE1---3'-HO	1.97
			ASN155	HD21---1-O	1.94
			LYS159	HZ3---7-OH	2.02
Compound **8** 	>100	−4.84	CYS65	O---4'-HO	2.05
			THR66	OG1---3'-HO	1.93
			ASP116	OD2---7-HO	1.89
			LYS159	HZ3---3'-OH	1.97
Compound **9** 	12.6	−5.05	THR66	OG1---10-HO	1.75
			THR66	OG1---11-HO	1.75
			GLN148	O---3-HO	2.11
			LYS159	HZ3---10-OH	2.16

effect of sappanchalcone (**2,** $IC_{50} = 2.3\,\mu M$) against HIV-1 IN then showed higher activity than that of protosappanin A (**9,** $IC_{50} = 12.6\,\mu M$) because sappanchalcone could bind with one more amino acid residue in the IN core domain and had low binding energy than protosappanin A did.

Regarding anti-HIV-1 IN effect and molecular docking study of chalcone derivatives, it was found that sappanchalcone (**2**, $IC_{50} = 2.3\,\mu M$) showed higher activity against IN enzyme than that of 3-deoxysappanchalcone (**1**, $IC_{50} > 100\,\mu M$) (Table 1). These two compounds had comparable binding energy at −5.45 kcal/mol (compound **1**) and −5.42 kcal/mol (compound **2**). However, sappanchalcone could bind with four amino acid residues (Thr66, His67, Gln148, and Lys159), whereas 3-deoxysappanchalcone interacted with only three amino acids (Thr66, Asp116, and Lys159) (Table 2). Thus, sappanchalcone (**2**) may

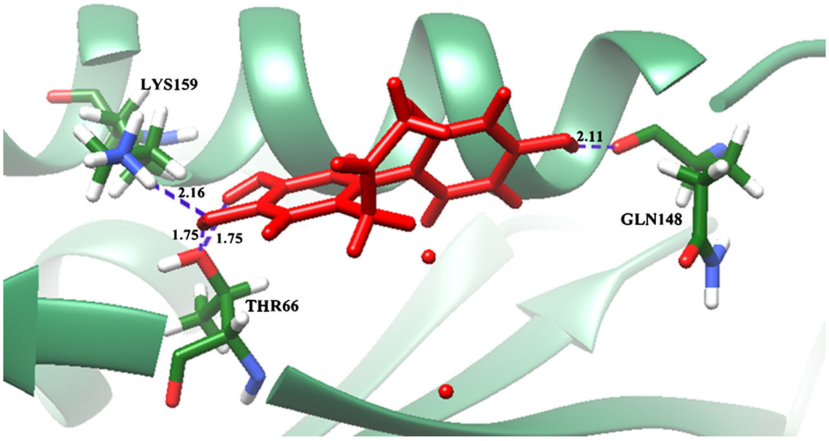


Figure 3. Molecular docking of protosappanin A (**9**) with HIV-1 integrase (IN). The ribbon model shows the backbone of HIV-1 IN catalytic domain with all interacting amino acid residues shown as stick models and colored by element. Protosappanin A is shown as a red stick model. H-bond interactions are shown as blue dash lines and represent bond length in angstrom (Å). Mg^{2+} ions are shown as red balls.

then have higher anti-HIV-1 IN effect than that of 3-deoxysappanchalcone (**1**).

For homoisoflavonoids (**3–8**), compounds **3** and **4** possessed good anti-HIV-1 IN activity with IC_{50} values of 36.7 and 33.0 μM, respectively whereas, compounds **5–8** were inactive ($IC_{50} > 100\,\mu M$) (Table 1). From molecular docking data, **5–8** exhibited higher binding energy (−4.73, −4.70, −4.83, and −4.84 kcal/mol, respectively) than those of **3** and **4** (−5.17 and −4.97 kcal/mol, respectively). Therefore, it is resulting in lower anti-HIV-1 IN effect of **5–8** when compared with those of **3–4** (Table 2).

In conclusion, the present study found that sappanchalcone (**2**) and protosappanin A (**9**) isolated from *C. sappan* exhibited good anti-HIV-1 IN activity. The molecular docking of sappanchalcone and protosappanin A revealed the interaction of these two compounds with some crucial amino acid residues (Thr66, His67, Gln148, and Lys159 for sappanchalcone; Thr66, Gln148, and Lys159 for protosappanin A) in the core domain of an IN enzyme. This is the first report of anti-HIV-1 IN activity of compounds from *C. sappan*.

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Conflict of Interest

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

REFERENCES

Craigie R. 2001. HIV-1 integrase, a brief overview from chemistry to therapeutics. *J Biol Chem* **276**: 23213–23216.

Healy EF, Sanders J, Peter J, King PJ, Robinson WE Jr. 2009. A docking study of L-chicoric acid with HIV-1 integrase. *J Mol Graph Model* **27**: 584–589.

Jenkins TM, Engelman A, Ghirlando R, Craigie R. 1996. A soluble active mutant of HIV-1 integrase. *J Biol Chem* **271**: 7712–7718.

Krishnan L, Engelman A. 2012. Retroviral integrase proteins and HIV-1 DNA integration. *J Biol Chem* **287**: 40858–40866.

Lim M-Y, Jeon J-H, Jeong E-Y, Lee C-H, Lee H-S. 2006. Antimicrobial activity of 5-hydroxy-1,4-naphthoquinone isolated from *Caesalpinia sappan* toward intestinal flora. *Food Chem* **100**: 1254–1258.

Min BS, Cuong TD, Hung TM, Min BK, Shin BS, Woo MH. 2012. Compounds from the heartwood of *Caesalpinia sappan* and their anti-inflammatory activity. *Bioorg Med Chem Lett* **22**: 7436–7439.

Moncada S, Palmer RMJ, Higgs EA. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharm Rev* **43**: 109–142.

Naranjan Reddy VL, Ravikanth V, Jansi Lakshmi VVNS, Suryanarayan Murty U, Venkateswarlu Y. 2003. Inhibitory activity of homoisoflavonoids from *Caesalpinia sappan* against *Beuveria bassiana*. *Fitoterapia* **74**: 600–602.

Oh SY, Kim DS, Lee IS, Jung KY, Lee JJ, Lee H-K. 1998. Anticomplementary activity of constituents from the heartwood of *Caesalpinia sappan*. *Planta Med* **64**: 456–458.

Tewtrakul S, Miyashiro H, Hattori M, *et al*. 2001. Inhibitory effects of flavonoids on human immunodeficiency virus type-1 integrase. *J Tradit Med* **18**: 229–238.

Vajragupta O, Boonchoong P, Morrisc GM, Olsonc AJ. 2005. Active site binding modes of curcumin in HIV-1 protease and integrase. *Bioorg Med Chem Lett* **15**: 3364–3368.

Wutthithamavet W. 1997. Thai Traditional Medicine, revised edn. Odean Store Press: Bangkok, 268 pp.

Yodsaoue O, Cheenpracha S, Karalai C, Ponglimanont C, Tewtrakul S. 2009. Anti-allergic activity of principles from the roots and heartwood of *Caesalpinia sappan* on antigen-induced β-hexosaminidase release. *Phytother Res* **23**: 1028–1031.

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Phytother. Res. **29**: 850–856 (2015)

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Antiinflammatory and Wound Healing Effects of *Caesalpinia sappan* L.

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Extracted compounds from *Caesalpinia sappan* L. were examined for the inhibitory activity against NO, PGE_2, and TNF-α productions and on associated transcription levels using RAW264.7 cells. They were also tested for their effects on wound healing using fibroblast L929 cells. Among the compounds tested, brazilin (8) was the most effective against lipopolysaccharide (LPS)-induced NO production in RAW264.7 cells with an IC_{50} value of 10.3 μM, followed by sappanchalcone (2, 31.0 μM). Brazilin (8) also inhibited PGE_2 and TNF-α production with IC_{50} values of 12.6 and 87.2 μM, respectively. The antiinflammatory mechanism of brazilin involved down regulation of the mRNA expressions of the iNOS, COX-2, and TNF-α genes in a dose-dependent manner. An ethanol (EtOH) extract of *C. sappan* significantly increased fibroblast proliferation, fibroblast migration, and collagen production, whereas brazilin (8) only stimulated fibroblast migration. In addition, the EtOH extract showed no acute toxicity in mice, and it was therefore safe to make use of its potent antiinflammatory and wound healing activities. Brazilin was mainly responsible for its antiinflammatory effect through its ability to inhibit the production of NO, PGE_2, and TNF-α. This study supports the traditional use of *C. sappan* for treatment of inflammatory-related diseases. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: NO production; iNOS; COX-2; RAW264.7 cells; fibroblast L929 cells; *Caesalpinia sappan*.

INTRODUCTION

Nitric oxide (NO) is one of the inflammatory mediators that causes inflammation in several organs. This free radical has been implicated in pathological and physiological processes including vasodilation, non-specific host defense, and acute or chronic inflammation. NO has a role in host defense mechanisms by damaging pathogenic DNA and as a regulatory molecule for homeostatic activities (Kou and Schroder, 1995). However, excessive production of this free radical is pathogenic to the host tissue itself, because NO can bind with other superoxide radicals and acts as a reactive radical that directly damages the function of normal cells (Moncada *et al.,* 1991).

Wound healing processes comprise a complex series of events in which repair to the damaged tissue partially or completely depends on the severity of the wounding. This process can be characterized by three overlapping phases; an inflammatory phase (consisting of hemostasis and inflammation), a proliferative phase (consisting of granulation, contraction, and epithelialization), and a remodeling phase in which structures are organized that have increased tensile strength (Wild *et al.,* 2010). The proliferation of fibroblast, fibroblast migration, and collagen production are all involved in the wound healing processes.

Caesalpinia sappan L. is a plant that belongs to the Caesalpiniaceae family, and a decoction with water of its heartwood has long been used in Thailand by traditional doctors to treat hemorrhoids, aphthous ulcer, epistaxis, stomatitis, and pneumonia and acts as a blood tonic (Wutthithamavet, 1997). The wood of this plant is a component of a famous tooth paste and tooth powder (Vicco Vajradanti™) in India. The powerful astringent, haemostatic, and healing effects of the wood can stop bleeding in the gums and are useful in the treatment of gum erosions, aphthous ulcers, and stomatitis because of its strong healing property. Moreover, it is commonly used in several other Ayurvedic formulations (Badami *et al.,* 2004). In the present study, we have therefore investigated the inhibitory activity of this plant against the productions of NO, PGE_2, and TNF-α and its healing effect as well as the mechanism of action on iNOS and COX-2 mRNA expressions to establish if its traditional uses can be supported.

MATERIALS AND METHODS

General experimental procedures. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using the 300 MHz Bruker fourier transform nuclear magnetic resonance (FTNMR) Ultra Shield™ spectrometers

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(Bremen, Germany). Chemical shifts were recorded in parts per million (δ) in $CDCl_3$, acetone-d_6, and dimethyl sulfoxide (DMSO)-d_6 with tetramethylsilane (TMS) as an internal reference.

Cell cultures and reagents. The mouse fibroblast L929 cell line (Chinese Academy of Preventive Medical Sciences, Beijing, China) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA), and murine macrophage-like RAW264.7 cell line (Cell Lines Services) was cultured in Rouswell Park Memorial Institute (RPMI) medium (RPMI, Gibco, Grand Island, NY, USA).

Lipopolysaccharide (LPS, from *Escherichai coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), indomethacin, L-nitroarginine (L-NA), caffeic acid phenethylester (CAPE), and phosphate buffer saline (PBS) were from Sigma. Fetal calf serum (FCS) was from Gibco. Penicillin-streptomycin was from Invitrogen. Ninety-six-well microplates were from Nunc. Enzyme-linked immunosorbent assay (ELISA) test kits were from R&D Systems, Inc., USA. Other chemicals were from Sigma.

Plant material and preparation of extract. *Caesalpinia sappan* L. was collected from Khonkaen province, Thailand, in October 2005. Identification was made by Prof. Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University, and a specimen (no. SC07) has been deposited at the Prince of Songkla University Herbarium.

Isolation of compounds from a *Caesalpinia sappan* extract. The air-dried roots (1.7 kg) of *C. sappan* were extracted with CH_2Cl_2 and acetone, successively (each 2 × 2 L, for 5 days) at room temperature (25 °C). The crude extracts were evaporated under reduced pressure to afford a brownish CH_2Cl_2 (20.1 g) and acetone (35.2 g) extract, respectively. The crude CH_2Cl_2 and acetone extracts gave similar thin layer chromatography (TLC) chromatograms (data not shown) so they were combined and further purified by silica gel quick column chromatography (QCC) using CH_2Cl_2 as eluent and an increasing polarity with acetone to give seven fractions (R1–R7). Fraction R2 (2.0 g) was further purified by silica gel (100 g) column chromatography (CC) with EtOAc-CH_2Cl_2 (1:5, v/v; 50 mL, each) to give five subfractions (R2a–R2e). Subfraction R2b (140.2 mg) was separated by silica gel (10 g) CC with acetone-CH_2Cl_2 (1:20, v/v; 25 mL, each) to give **1** (8.0 mg). Fraction R3 (2.7 g) was separated by silica gel (130 g) CC with acetone-hexane (2:5, v/v; 50 mL, each) to afford seven subfractions (R3a–R3g). Subfraction R3b (200.2 mg) was purified by CC with EtOAc-hexane (2:5, v/v; 50 mL, each) to give seven (16.0 mg). Subfraction R3e (838.4 mg) was separated by reverse phase-18 silica gel CC with MeOH–H_2O (2:5, v/v, 25 mL, each) to give **8** (15.0 mg), **4** (6.0 mg), and **2** (34.0 mg).

Chopped-dried heartwoods (5.3 kg) of *C. sappan* were extracted with CH_2Cl_2 (2 × 5 L, for 5 days) at room temperature (25 °C) and evaporated under reduced pressure to afford a brownish CH_2Cl_2 extract (135.0 g). A portion of crude CH_2Cl_2 extract (55.2 g) was further purified by silica gel (1000 g) QCC using CH_2Cl_2 as eluent and increasing polarity with EtOAc and acetone to give seven fractions (H1–H7). Fraction H3 (1.3 g) was further purified by silica gel (60 g) CC with EtOAc–CH_2Cl_2 (3:17, v/v; 50 mL, each) to give eight subfractions (H3a–H3i). Subfraction H3e (164.3 mg) was separated by silica gel CC with EtOAc-hexane (2:5, v/v; 25 mL, each) and followed by reverse phase-18 silica gel CC with MeOH-H_2O (2:5, v/v; 25 mL, each) to give **9** (15.0 mg) and **3** (8.0 mg). Fraction H6 (5.9 g) was separated by silica gel (300 g) QCC with acetone-CH_2Cl_2 (1:5, v/v; 100 mL, each) to give six subfractions (H6a–H6f). Subfraction H6b (483.2 mg) was purified by silica gel (25 g) CC with EtOAc-hexane (1:1, v/v; 25 mL, each) to give **6** (20.0 mg) and **5** (15.0 mg). The structures of all these isolated compounds were elucidated by comparison with the ^1H and ^{13}C-NMR spectrum of those in the previous literature (Yodsaoue *et al.,* 2009).

Acute toxicity test of *Caesalpinia sappan* extract in mice. Male and female Swiss albino mice (30–40 g) were used in the experiment. The animals were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat-Yai, Songkhla, Thailand. All experimental protocols were approved by The Animal Ethic Committee, Prince of Songkla University (MOE 0521.11/241). Swiss albino mice were housed in standard environmental conditions with a 12 h light/dark cycle. They were provided *ad libitum* with standard rodent diet and water.

The 50% lethal dose (LD_{50}) of the EtOH extract of *C. sappan* was estimated by the up-and-down method in mice (Bruce, 1985). The animals were fasted for 6 h prior to dosing. Doses were adjusted by a constant multiplicative factor (viz., 3.2) for this experiment. The dose for each successive animal was adjusted up or down depending on the previous outcome. The crude extract was dissolved in the cosolvent solution (propylene glycol: water = 1:1) and orally administered in a single dose by gavage using a stomach tube to both groups of male and female mice. Animal behavior was observed individually at least once during the first 30 min after administration, periodically during the first 8 h and daily thereafter, for a total of 7 days. The signs of toxicity were observed including tremor, convulsion, hyperactivity, sedation, grooming, loss of righting reflex, respiratory depression and coma.

Assay for NO inhibitory effect from RAW264.7 cells. The inhibitory effect on NO production by RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota *et al.,* 2003). Briefly, the RAW264.7 cells were cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/mL), streptomycin (100 µg/mL), and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with a fresh medium containing 100 µg/mL of LPS together with

Table 1. Inhibition of nitric oxide production[1] by compounds 1–9 isolated from *Caesalpinia sappan*

Compounds	% inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
3-Deoxysappanchalcone (**1**)	0.0 ± 0.8	-	−2.6 ± 1.2	15.6 ± 0.6**	73.5 ± 1.3**	58.6
Sappanchalcone (**2**)	0.0 ± 0.8	-	14.0 ± 1.8**	36.8 ± 1.8**	99.4 ± 0.6**	31.0
3R-(3,4-Dihydroxybenzyl)-7-hydroxychroman-4-one (**3**)	0.0 ± 0.8	-	-	7.2 ± 1.4*	38.8 ± 1.4**	>100
Episappol (**4**)	0.0 ± 4.2	-	-	-	25.1 ± 0.7*	>100
4-O-Methylepisappol (**5**)	0.0 ± 4.2	-	-	-	16.7 ± 1.1*	>100
4-O-Methylsappanol (**6**)	0.0 ± 4.2	-	-	-	16.2 ± 1.1*	>100
4-(7-Hydroxy-2,2-dimethyl-9βH-1,3,5-trioxa-cyclopenta[α]naphthalen-3α-ylmethyl)-benzene-1,2-diol (**7**)	0.0 ± 2.5	-	3.9 ± 1.7	12.8 ± 1.3	77.9 ± 1.6**	54.8
Brazilin (**8**)	0.0 ± 2.5	12.5 ± 2.4	38.8 ± 1.7**	96.4 ± 0.8**	98.4 ± 0.7[2]**	10.3
Protosappanin A (**9**)	0.0 ± 2.5	-	-	-	17.8 ± 0.9	>100
L-Nitroarginine	0.0 ± 9.9	11.7 ± 4.6	20.2 ± 5.9	34.7 ± 1.8 *	71.6 ± 2.6**	61.8
Caffeic acid phenethylester	0.0 ± 9.9	30.7 ± 3.2	68.6 ± 3.4[2]**	98.7 ± 1.2[2]**	98.9 ± 2.1[2]**	5.6
Indomethacin	0.0 ± 2.0	-	12.3 ± 1.5*	31.3 ± 1.5**	74.0 ± 1.0**	46.5

(-) = not determined.

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

[2] Cytotoxic effect was observed.

*$p < 0.05$.

**$p < 0.01$.

the test samples at various concentrations (3–100 μg/mL for crude extract and 3–100 μM for pure compounds) and was then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the MTT colorimetric method. Briefly, after 48 h incubation with the test samples, MTT solution (10 μL, 5 mg/mL in PBS) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. L-NA, CAPE and indomethacin were used as positive controls. The stock solution of each test sample was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to the medium RPMI (final DMSO is 1%). Inhibition (%) was calculated using the following equation, and IC_{50} values were determined graphically ($n = 4$):

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

A–*C*: NO_2^- concentration (μM) [*A*: LPS (+), sample (−); *B*: LPS (+), sample (+); *C*: LPS (−), sample (−)]. (Table 1)

Inhibitory effects on the production of LPS-induced PGE_2 and TNF-α from RAW264.7 cells. Briefly, the RAW264.7 cell line was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/mL), streptomycin (100 μg/mL), and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1.0×10^5 cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with a fresh medium containing 100 μg/mL of LPS together with the test samples at various concentrations (3–100 μM) and was then incubated for 48 h. The supernatant was transferred into a 96-well ELISA plate and then PGE_2 and TNF-α concentrations were determined using commercial ELISA kits. The test sample was dissolved in DMSO, and the solution was added to RPMI. The inhibition on PGE_2 and TNF-α releases was calculated, and IC_{50} values were determined graphically. (Table 2)

Total RNA isolation and Reverse transcription polymerase chain reaction (RT-PCR). In order to understand the mechanism of action on the release cytokine of brazilin

Table 2. Inhibitory effect[1] of brazilin (8) on PGE_2 and TNF-α production by RAW264.7 cells

Sample	IC_{50} (μM) against inflammatory mediators	
	PGE_2	TNF-α
Brazilin (**8**)	12.6 ± 0.9	87.2 ± 1.0
Indomethacin	0.4 ± 0.1	93.4 ± 1.2

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

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

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

Cell proliferation and viability assay using L929 fibroblasts. L929 fibroblasts were seeded at 2×10^4 cells/well into 96-well plate in is dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS). After 48 h, cells were exposed to different concentrations (1–100 μg/mL for crude extract or 1–100 μM for the pure compound) of test samples and were then incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. The MTT solution (10 μL, 5 mg/mL) was added directly to the medium in each well, and the plate was then incubated at 37 °C for 4 h. All medium was then aspirated and replaced with isopropanol containing 0.04 N HCl, and the optical density at 570 nm was detected. The percentage of cell proliferation was calculated and compared with a negative control.

Migration assay of fibroblast L929 cells. The migration of fibroblast L929 cells was examined using a wound healing method as previously described by Balekar *et al.* (2012). Briefly, L929 cells (5×10^4 cells/mL) in DMEM containing 10% FBS were seeded into each well of a 24 well plate and incubated at 37 °C with 5% CO_2. After the confluent monolayer of L929, cells were formed, and a sterile pipette tip was used to generate two horizontal scratches (left and right) in each well. Any cellular debris was removed by washing with PBS and replaced with 1 mL of fresh medium in the absence or presence of test samples. Photographs were taken of two views on the left and right of each well at a 4× magnification using a microphotograph (Olympus CK2, Japan) on day 0, then plates were incubated at 37 °C with 5% CO_2, and photographs were taken at days 1 and 2. To determine the migration of L929 cells, the images were analyzed using computing software (ImageJ1.42q/Java1.6.0_10). The percentage of the closed area was measured and compared with the value obtained before treatment (day 0). An increase of the percentage of closed area indicated the cells had migrated.

Determination of collagen type-I production. The collagen productions were determined according to the method described by Balekar *et al.* (2012). Fibroblast L929 cells in DMEM containing 10% FBS were seeded at an initial concentration of 2×10^4 cells/mL in a 96-well plate. After 24 h, the culture medium was replaced with a fresh medium containing the test samples at various concentrations (1.5–12.5 μg/mL for crude extract or 1.5–12.5 μM for the pure compound) and was then incubated for 48 h at 37 °C with 5% CO_2. Cells without a test sample served as negative controls. After 48 h of incubation, cells secreted soluble collagen type-I into the medium, the supernatant (100 μL) were collected. The total amount of soluble collagen type-I was assayed using the Sircol® Collagen Assay Kit (Bicolor Life Science Assays, Northern Ireland, UK). Briefly, 100 μL of supernatant was mixed with 1 mL of dye solution at room temperature for 30 min. Then the samples were centrifuged at 15,000 g for 10 min to form a pellet of collagen. All the supernatant was then aspirated, and the soluble collagen was dissolved in 1 mL of alkali reagent. Thereafter, the alkali solutions were transferred to a 96-well plate and the optical density at 540 nm was recorded. The amount of collagen was calculated based on a standard curve of soluble collagen (bovine skin collagen type-I standard from American disease-free animals).

Statistics. For statistical analysis, the values are expressed as a mean value ± standard error of the mean (S.E.M.) of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. The statistical significance was calculated by one-way analysis of variance, followed by the Dunnett's test.

RESULTS AND DISCUSSION

Effect of an EtOH extract and brazilin on the production of NO, PGE_2, and TNF-α

A Thai medicinal plant, *C. sappan*, locally known in Thai as Phang was examined for its inhibitory activities against the production of NO, PGE_2, and TNF-α. Nine

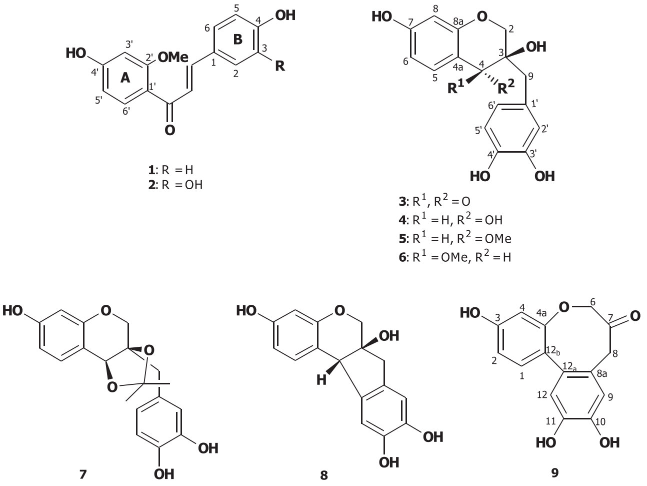

Figure 1. Structures of compounds **1–9** isolated from *Caesalpinia sappan* heartwood.

compounds were isolated from its roots and heartwoods (Fig. 1). Among the compounds tested, brazilin (**8**) possessed the highest activity against LPS-induced NO production in RAW264.7 cells with an IC_{50} value of 10.3 μM, followed by sappanchalcone (**2**, 31.0 μM), whereas **1** and **7** showed only moderate activities with IC_{50} values of 58.6 and 54.8 μM, respectively. The activity on the NO inhibition by brazilin (**8**, IC_{50} = 10.3 μM) was higher than that by indomethacin (an antiinflammatory drug, IC_{50} = 46.5 μM) and L-NA (a nitric oxide synthase inhibitor, IC_{50} = 61.8 μM) and was comparable with that of CAPE (an NF-κB inhibitor, IC_{50} = 5.6 μM). The structure–activity relationships of compounds from *C. sappan* on the anti-NO production were as follows: (i) the vicinal hydroxyl group of chalcones at position 3 and 4 conferred higher activity, as shown in **2** (IC_{50} = 31.0 μM) versus **1** (IC_{50} = 58.6 μM); and (ii) the homoisoflavonoid-bearing rigid structure had a stronger effect than the non-rigid structure, as observed in **8** (IC_{50} = 10.3 μM) versus **3–6** (IC_{50} > 100 μM). Brazilin (**8**) was also tested on the production of cytokine including PGE_2 and TNF-α. The result showed that brazilin exhibited appreciable antiinflammatory activity against PGE_2 and TNF-α releases with IC_{50} values of 12.6 and 87.2 μM, respectively. This is the first report that brazilin can inhibit these two inflammatory mediators.

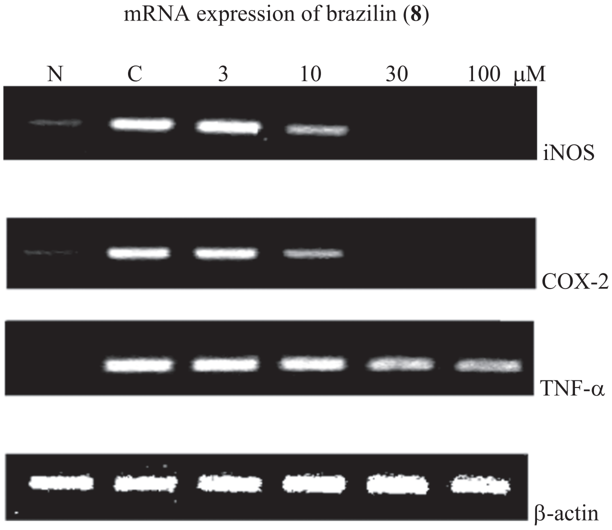

Figure 2. Effect of brazilin (**8**) at various concentrations (3, 10, 30, and 100 μM) on the expression of mRNA from iNOS, COX-2, and TNF-α by LPS-induced NO, PGE_2, and TNF-α releases in RAW264.7 cells. (*N*) = LPS (−), sample (−); (C) = LPS (+), sample (−); 3–100 μM = LPS (+), sample (+).

Effect of brazilin on iNOS, COX-2, and TNF-α mRNA expressions

The antiinflammatory mechanism of brazilin was shown to involve mainly the down regulation of mRNA expressions of the iNOS and COX-2 genes, especially at concentrations of 10, 30, and 100 μM, and at concentrations of 30 and 100 μM for the TNF-α gene expression, respectively (Fig. 2). Recently, extracts from *C. sappan* have been reported to possess antibacterial (Lim *et al.,* 2006), antifungal (Naranjan Reddy *et al.,* 2003), antiinflammatory (Min *et al.,* 2012; Wu *et al.,* 2011), and anti-complementary activities (Oh *et al.,* 1998). However, from the previous report (Min *et al.,* 2012), the antiinflammatory effect of brazilin was only on the expression of iNOS mRNA but not on COX-2 mRNA. The effects of phenolic compounds isolated from *C. sappan* heartwood have only been previously reported to nitric oxide production without any study on their gene expression (Cuong *et al.,* 2012). An EtOH extract of *C. sappan* was reported to inhibit iNOS and COX-2 mRNA in human chondrocytes and macrophages without any information on the isolated compounds (Wu *et al.,* 2011). Thus, the present study has reported the antiinflammatory effect of compounds isolated from *C. sappan* heartwoods and roots on NO production and brazilin (**8**), the most active compound, was tested for its mechanism of action and shown to down regulate the expression of mRNA from iNOS, COX-2, and TNF-α in a dose-dependent manner.

Effect of an EtOH extract and brazilin on cell proliferation and viability using L929 fibroblasts

Fibroblast proliferation is an important process in wound healing for the regeneration of tissue. The proliferative effect and cytotoxicity of the EtOH extract and brazilin (**8**) were evaluated by the MTT assay. After 24 h of treatment with the EtOH extract and brazilin, the EtOH extract (1.5–25.0 μg/mL), and brazilin (1.5–25.0 μM) produced a cell viability of more than 80%. However, at any higher concentrations, cytotoxic effects were observed (Table 3). From our results, fibroblast cells had a better viability at the lower concentrations. Moreover, enhancement on the growth of the L929 fibroblasts was clearly observed after treatment with the EtOH extract at 6.25 μg/mL ($p < 0.05$) with a proliferation rate of 113%, whereas brazilin (**8**) showed no cell proliferation when compared with that of the control group (Table 3). This result showed that the EtOH extract of *C. sappan* enhanced fibroblast proliferation more than brazilin.

Effect of EtOH extract and brazilin on migration of L929 cells

In the present study, the effects of an EtOH extract and brazilin (**8**) were determined on the rate of L929

Table 3. Effect of the EtOH extract of *Caesalpinia sappan* and brazilin on the viability of L929 cells

	% viability of L929 cells at various concentrations							
Sample	0	1.56	3.12	6.25	12.5	25.0	50.0	100.0
EtOH extract (μg/mL)	100.0 ± 2.3	102.9 ± 2.9	103.7 ± 2.0	113.0 ± 4.1*	105.0 ± 4.9	97.8 ± 3.1	78.5 ± 2.9*	37.7 ± 1.9*
Brazilin (μM)	100.0 ± 1.9	98.0 ± 1.0	94.9 ± 4.8	95.1 ± 1.1	93.9 ± 1.4	93.8 ± 2.9	67.9 ± 2.0*	19.2 ± 0.5*

Values are means ± S.E.M. ($N = 4$). Significantly different from the control (0 μg/mL).
*$p < 0.05$.

migration using the scratch assay. The scratch assay is a useful method for gaining an insight into the potential of an extract or compounds to repair injured dermis. This assay was used to study cell migration *in vitro* by creation of an artificial gap on a confluent cell monolayer with a pipette tip. The cellular proliferation and migration of fibroblast cells as each edge of the gaps moved toward each other and to close the scratch area was studied on days 0, 1, and 2. As shown in Fig. 3, in the presence of EtOH extract (6.25 μg/mL), the migration of L929 fibroblasts increased significantly ($p < 0.01$) on both days 1 and 2 by 65.2% and 100.0%, whereas those of the control group increased 39.0% and 64.6%, respectively. Brazilin (**8**) significantly increased the migration especially on day 2 by 75.9% (Table 4). Hence, the EtOH extract and brazilin produced a significantly enhanced migration effect when compared with those of the control group, and the EtOH extract again showed a higher activity than brazilin.

Effect of EtOH extract and brazilin on collagen type-I production

Collagens are the most abundant family of protein in the body that provide strength to all tissues, and they also

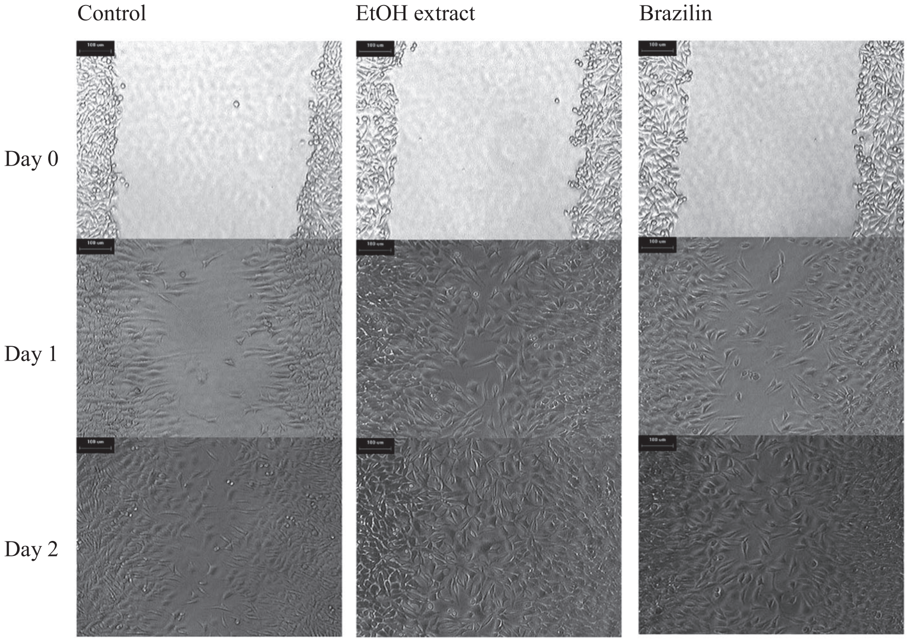

Figure 3. Effect of the EtOH extract of *Caesalpinia sappan* and brazilin on fibroblast L929 migration. Images were captured at day 0 and then treated with brazilin (6.25 μM), EtOH extract (6.25 μg/mL) and control without treatment. Another set of images were captured at days 1 and 2 after incubation. Quantitative analysis of the migration rate was quantified using computing software.

Table 4. Effect of the EtOH extract of *Caesalpinia sappan* and brazilin on the *in vitro* scratch assay using fibroblast L929 cells

		Length between the scratch (μm)			% migration rate of cells	
Sample	Dose	Day 0	Day 1	Day 2	Day 1	Day 2
Control	-	620.7 ± 7.3	378.9 ± 9.0*	217.1 ± 10.3*	39.0 ± 1.4	64.6 ± 1.7
EtOH extract (μg/mL)	6.25	600.0 ± 8.7	200.0 ± 17.4*	0.0*	65.2 ± 3.0*	100.0*
Brazilin (μM)	6.25	574.4 ± 4.6	357.7 ± 5.4*	141.5 ± 4.5*	37.7 ± 0.9	75.9 ± 0.8*

Values are represents means ± S.E.M. ($N = 4$). Significantly different from the control.
(-) = not determined.
*$p < 0.01$.

Table 5. Collagen type-I production by L929 cells when treated with the EtOH extract of *Caesalpinia sappan* and brazilin

	Collagen production (μg/mL) at various concentrations				
Sample	0	1.56	3.12	6.25	12.5
Control	48.5 ± 4.1	-	-	-	-
EtOH extract (μg/mL)		<2.5	26.3 ± 3.2	63.1 ± 3.7*	125.6 ± 3.4*
Brazilin (μM)		<2.5	<2.5	14.0 ± 4.8	30.2 ± 5.5

Values are means ± S.E.M. (*N* = 4). Significantly different from the control.
(-) = not determined.
*$p < 0.05$.

have a crucial role in the wound healing process (Enoch and Leaper, 2008). Collagen type-I is a main structural component of the extracellular matrix, of skin and newly healed wounds. The study on the effect of EtOH extract and brazilin (**8**) on the type-I collagen production by fibroblasts was performed using the Sircol collagen assay kit. Collagen production by the L929 cells increased significantly ($p < 0.05$) after treatment with the EtOH extract at concentrations of 6.25 and 12.5 μg/mL with the production of collagen at 63.1 and 125.6 μg/mL, respectively, whereas that of the control was 48.5 μg/mL. However, there is no significant increase in the amount of collagen generated by fibroblasts after treatment with brazilin (Table 5). This result implied that the *C. sappan* EtOH extract was better to use rather than the pure brazilin to increase collagen production.

Acute toxicity of EtOH extract of *Caesalpinia sappan*

For the test of acute toxicity, the oral LD_{50} of the EtOH extract of *C. sappan* was found to be greater than 2000 mg/kg of body weight in both male and female Swiss albino mice. At the end of study period (7 days), all animals survived and appeared to be active and healthy. During the period of observation during the day, the animals showed no significant sign of toxicity, adverse pharmacological effects, or abnormal behavior. This result indicated that the *C. sappan* EtOH extract showed no acute toxicity and was safe to use.

CONCLUSION

In conclusion, it seems that *C. sappan* EtOH extract had potent antiinflammatory activity by inhibiting the production of NO, PGE_2, and TNF-α and promoted a wound healing effect. The compound that was mainly responsible for antiinflammatory activity was brazilin. Brazilin was found to down regulate the mRNA expressions of the iNOS, COX-2, and the TNF-α genes in a dose-dependent manner. The EtOH extract of *C. sappan* significantly enhanced the wound healing effect in L929 fibroblasts by increasing cell proliferation and migration as well as their collagen production. This study therefore may support the traditional use of this plant for treatment of aphthous ulcer, stomatitis, epistaxis, hemorrhoids, and pneumonia, which are inflammatory-related diseases without demonstrating any acute toxicity in mice.

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Conflict of Interest

The authors report no conflicts of interest.

REFERENCES

Badami S, Moorkoth S, Suresh B. 2004. *Caesalpinia sappan*, a medicinal and dye yielding plant. *Nat Prod Radiance* **3**: 75–82.
Balekar N, Katkam NG, Nakpheng T, Jehtae K, Srichana T. 2012. Evaluation of wound healing potential of *Wedelia trilobata* (L.) leaves. *J Ethnopharmacol* **141**: 817–824.
Banskota AH, Tezuka Y, Nguyen NT, Awale S, Nobukawa T, Kadota S. 2003. DPPH radical scavenging and nitric oxide inhibitory activities of the constituents from the wood of *Taxus yunnanensis*. *Planta Med* **69**: 500–505.
Bruce RD. 1985. An up-and-down procedure for acute toxicity. *Fundam Appl Toxicol* **5**: 151–157.
Cuong TD, Hung TM, Kim JC, *et al*. 2012. Phenolic compounds from *Caesalpinia sappan* heartwood and their anti-inflammatory activity. *J Nat Prod* **75**(12): 2069–2075.
Enoch S, Leaper DJ. 2008. Basic science of wound healing. *Surgery* **26**: 31–37.
Kou PC, Schroder RA. 1995. The emerging multifacated roles of nitric oxide. *Ann Surg* **221**: 220–235.
Lim MY, Jeon JH, Jeong EY, Lee CH, Lee HS. 2006. Antimicrobial activity of 5-hydroxy-1,4-naphthoquinone isolated from *Caesalpinia sappan* toward intestinal flora. *Food Chem* **100**(3): 1254–1258.
Min BS, Cuong TD, Hung TM, Min BK, Shin BS, Woo MH. 2012. Compounds from the heartwood of *Caesalpinia sappan* and their anti-inflammatory activity. *Bioorg Med Chem Lett* **22** (24): 7436–7439.
Moncada S, Palmer RMJ, Higgs EA. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmaceut Rev* **43**: 109–142.
Naranjan Reddy VL, Ravikanth V, Jansi Lakshmi VVNS, Suryanarayan Murty U, Venkateswarlu Y. 2003. Inhibitory activity of homoisoflavonoids from *Caesalpinia sappan* against *Beuveria bassiana*. *Fitoterapia* **74**(6): 600–602.
Oh SY, Kim DS, Lee IS, Jung KY, Lee JJ, Lee HK. 1998. Anticomplementary activity of constituents from the heartwood of *Caesalpinia sappan*. *Planta Med* **64**(5): 456–458.
Wild T, Rahbarnia A, Kellner M, Sobotka L, Eberlein T. 2010. Basics in nutrition and wound healing. *Nutrition* **26**: 862–866.
Wu SQ, Ptero M, Unger FM, *et al*. 2011. Anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract in human chondrocytes and macrophages. *J Ethnopharmacol* **138**(2): 364–372.
Wutthithamavet W. 1997. Thai Traditional Medicine, revised edn. Odean Store Press: Bangkok; 340.
Yodsaoue O, Cheenpracha S, Karalai C, Ponglimanont C, Tewtrakul S. 2009. Anti-allergic activity of principles from the roots and heartwood of *Caesalpinia sappan* on antigen-induced β-hexosaminidase release. *Phytother Res* **23**: 1028–1031.

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Original Article

Anti-allergic and anti-inflammatory compounds from *Aglaia andamanica* leaves

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Abstract

The leaves from *Aglaia andamanica* were determined for their anti-allergic and anti-inflammatory effects using RBL-2H3 and RAW264.7 cells, respectively. Among the isolated compounds, 24-epi-piscidinol A (5) exhibited the highest anti-allergic activity against β-hexosaminidase release with an IC_{50} value of 19.8 μM, followed by (-)-yangambin (3, IC_{50} = 33.8 μM), pyramidaglain A (8, IC_{50}= 37.1 μM), pachypodol (2, IC_{50}= 38.3 μM) and pyramidaglain B (9, IC_{50}= 44.8 μM), respectively; whereas other compounds possessed moderate to mild effects (IC_{50} = 67.5->100 μM). For anti-inflammatory activity, 24-epi-piscidinol A (5) possessed potent activity with an IC_{50} value of 24.0 μM, followed by pyramidaglain B (9, IC_{50} = 25.6 μM), pachypodol (2, IC_{50} = 34.5 μM) and (-)-yangambin (3, IC_{50} = 37.4 μM), respectively; whereas other compounds exhibited moderate to mild activities (IC_{50} = 54.2->100 μM). These active compounds could be developed as anti-allergic and anti-inflammatory agents in the future.

Keywords: RBL-2H3 cells, RAW264.7 cells, *Aglaia andamanica*, Meliaceae

1. Introduction

Aglaia andamanica is a plant belonging to the Meliaceae family. This plant grows at a height of 30 m, and is distributed in South and Southeast Asia. Its leaves are used for treatment of headache (Mabberley *et al.*, 1995). Some of benzofuran derivatives from *Aglaia* genus show interesting biological effects including anti-viral (Joshi *et al.*, 1987), anti-leukemic (Hayashi *et al.*, 1982) and insecticidal activities (Ishibashi *et al.*, 1993).

The allergy is an immune dysfunction, which is a serious health problem. Substances that cause allergic reaction are called allergens including pollen, dust mites, food, cosmetics and animal hairs. Hypersensitivity type I, an allergic reaction, is an IgE-mediated immune response, resulting in histamine secretion from mast cells and blood basophils. The histamine causes smooth muscle contraction, increased vascular permeability and vasodilation (Goldsby *et al.*, 2002). Since, β-hexosaminidase is usually released along with histamine from mast cells or basophils, this enzyme is used as the marker for mast cell degranulation in RBL-2H3 cell line (Cheong *et al.*, 1998).

Nitric oxide (NO) is one of the inflammatory mediators that causes inflammation in several organs. This inorganic free radical has been implicated in physiological and pathological processes, such as vasodilation, non-specific host defense and acute or chronic inflammation. NO acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities (Kou & Schroder, 1995). However, excessive production of this free radical is pathogenic to the host tissue, because NO can bind with superoxide radicals and turn to be a reactive radical which directly

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damages the function of normal cells (Moncada *et al.*, 1991). Since the extract of *Aglaia andamanica* possessed potent anti-allergic and anti-inflamatory effects with IC_{50} values of 19.3 and 22.1 µg/mL, respectively, the compounds isolated from this plant were then tested for anti-allergic and anti-inflammatory activities.

2. Materials and Methods

2.1 Reagents

Minimum Essential Medium Eagle (MEM) and anti-DNP-IgE (Monoclonal anti-DNP) were purchased from Sigma; fetal calf serum (FCS) was from Gibco; dinitrophenylated bovine serum albumin was prepared as described previously (Tada & Okumura, 1971). Other chemicals were from Sigma. 24-well and 96-well plates were from Nunc.

Lipopolysaccharide (LPS, from *Salmonella enteritidis*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), L-nitroarginine (L-NA), caffeic acid phenethylester (CAPE), indomethacin and phosphate buffer saline (PBS) were purchased from Sigma. FCS was from Gibco. Penicillin-streptomycin was from Invitrogen. 96-well microplates were bought from Nunc. Other chemicals were from Sigma.

2.2 Plant material

Leaves of *Aglaia andamanica* Hiern were collected in Thon-Nga-Chang National Park, Hat-Yai, Songkhla, Thailand, in September 1995. A voucher specimen (JP9001) was identified by Dr. S. Vajrodaya, Department of Botany, Kasetsart University, Bangkok, Thailand, and has been deposited in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

2.3 Extraction and isolation of *Aglaia andamanica* leaves

The compounds (**1-10**) were isolated and identified by comparison of their physical and spectral data with those reported in the literatures (Puripattanavong *et al.*, 2000; Greca *et al.*, 1994; Vidari *et al.*, 1971; Malan & Roux, 1979; Picker *et al.*, 1973; Agrawal 1989; McChesney *et al.*, 1997; Anjaneyulu *et al.*, 1981; Marrien and Polonsky, 1971; Saifah *et al.*, 1993; Figliuolo *et al.*, 1987).

2.4 Anti-allergic activity assay

1) Inhibitory effects on antigen-induced β-hexosaminidase release from RBL-2H3 cells

Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells (purchased from ATCC) were evaluated by the following modified method (Matsuda *et al.*, 2004). Briefly, RBL-2H3 cells were dispensed in 24-well plates at a concentration of 2×10^5 cells/well using MEM containing 10% FCS, penicillin (100 units/mL), streptomycin (100 unit/mL) and anti-DNP IgE (0.45 µg/mL), then incubated overnight at 37°C in 5% CO_2 for sensitization of the cells. The cells were washed twice with 500 µL of Siraganian buffer [119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES), 0.1 % bovine serum albumin (BSA) and 40 mM NaOH, pH 7.2] and then incubated in 160 µL of Siraganian buffer for an additional 10 min at 37 °C. After that, 20 µL of test sample solution was added to each well and incubated for 10 min, followed by addition of 20 µL of antigen (DNP-BSA, final concentration is 10 µg/mL) at 37°C for 20 min to stimulate the cells to degranulate. The supernatant was transferred into a 96-well plate and incubated with 50 µL of substrate (1mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1 h. The reaction was stopped by adding 200 µL of stop solution (0.1 M $Na_2CO_3/NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to Siraganian buffer (final DMSO concentration was 0.1 %). The inhibition (%) of the release of β-hexosaminidase by the test samples was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{Inhibition } \% = [1-(T-B-N)/(C-N)] \times 100$$

Control (C): DNP-BSA (+), Test sample (-); Test (T) : DNP-BSA (+), Test sample (+); Blank (B) : DNP-BSA (-), Test sample (+); Normal (N) : DNP-BSA (-), Test sample (-)

2.5 Assay for NO inhibitory effect from RAW264.7 cells

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota *et al.*, 2003). Briefly, the RAW264.7 cell line was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/mL), streptomycin (100 µg/mL) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 100 µg/mL of LPS together with the test samples at various concentrations and was then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the MTT colorimetric method. Briefly, after 48 h incubation with the test samples, MTT solution was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm.

L-NA, CAPE and indomethacin were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1%). Inhibition (%) was calculated using the following equation and IC_{50} values were determined graphically (n = 4):

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

A-C : NO_2^- concentration (μM) [A : LPS (+), sample (-); B : LPS (+), sample (+); C: LPS (-), sample (-)].

2.6 Statistical analysis

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

3. Results and Discussion

The leaves of *Aglaia andamanica* were determined for their anti-allergic and anti-inflammatory effects using RBL-2H3 and RAW264.7 cells, respectively. Among the isolated compounds (Figure 1), 24-epi-piscidinol A (**5**) exhibited the highest anti-allergic activity against β-hexosaminidase release with an IC_{50} value of 19.8 μM, followed by (-)-yangambin (3, IC_{50} = 33.8 μM), pyramidaglain A (**8**, IC_{50} = 37.1 mM), pachypodol (**2**, IC_{50} = 38.3 μM) and pyramidaglain B (**9**, IC_{50} = 44.8 μM), respectively; whereas other compounds possessed moderate to mild effects (IC_{50} = 67.5->100 μM) (Table 1). Compounds **5**, **3**, **8**, **2** and **9** exhibited higher anti-allergic effect than that of ketotifen fumarate, an anti-histamine drug (IC_{50} = 47.5 μM).

For anti-inflammatory activity, 24-epi-piscidinol A (**5**) possessed potent activity with an IC_{50} value of 24.0 μM, followed by pyramidaglain B (**9**, IC_{50} = 25.6 μM), pachypodol (**2**, IC_{50} = 34.5 μM) and (-)-yangambin (**3**, IC_{50} = 37.4 μM), respectively; whereas other compounds exhibited moderate to mild effects (IC_{50} = 54.2->100 μM) (Table 2). However, pachypodol (**2**) and (-)-yangambin (**3**) exhibited cytotoxicity (25-30%) at high concentration (100 μM). Compounds **5**, **9**, **2** and **3** showed higher activity against NO production when compared to that of the positive controls, L-NA (NO synthase inhibitor, IC_{50} = 61.8 μM) and indomethacin (non-steroidal anti-inflammatory drug, IC_{50} = 46.5 μM) but less effective than CAPE, an NF-kB inhibitor (IC_{50} = 5.2 μM).

Methoxyflavones isolated from the rhizomes of *Kaempferia parviflora* have been reported to have anti-allergic effect (Tewtrakul *et al.*, 2008). This result is consistent with our study that methoxyflavone, pachypodol (**2**), showed marked anti-allergic activity. Epimagnolin B isolated from *Magnolia fargesii*, whose structure is similar to (-)-yangambin, has been reported to have anti-inflammatory effect against NO and PGE_2 production (Kim *et al.*, 2009). *Aglaia odorata* which contains diterpenoids and triterpenoids has shown anti-inflammatory activity toward NO and PGE_2 release (Yodsaoue *et al.*, 2012). It has been reported that compound isolated from *Kaempferia parviflora*, 5-hydroxy-3, 7, 3′, 4′-tetramethoxyflavone, exhibited marked activity against NO production (Tewtrakul *et al.*, 2008). These results are accompanied with the present study that flavonoids, triterpenes and lignan derivatives possess anti-inflammatory effect.

In conclusion, the present study shows that *Aglaia andamanica* possesses appreciable anti-allergic and anti-inflammatory activities. Pachypodol (**2**), (-)-yangambin (**3**), 24-epi-piscidinol A (**5**), pyramidaglain A (**8**) and pyramidaglain B (**9**) are responsible for both anti-allergic and anti-inflammatory activities of this plant. The mechanisms for anti-allergic

Table 1. Anti-allergic activity of compounds **1-10** isolated from *Aglaia andamanica* leaves

Compound	IC_{50} (μM)[a]
Retusin (**1**)	67.5 ± 1.4
Pachypodol (**2**)	38.3 ± 1.1
(-)-Yangambin (**3**)	33.8 ± 1.5
Pyramidatine (**4**)	>100
24-epi-Piscidinol A (**5**)	19.8 ± 1.5
Aglaiodiol (**6**)	93.6 ± 1.8
Cycloart-23E-ene-3β-25 diol (**7**)	>100
Pyramidaglain A (**8**)	37.1 ± 1.0
Pyramidaglain B (**9**)	44.8 ± 1.8
N-Methyl-trans-4-hydroxy-L-proline (**10**)	>100
Ketotifen fumarate	47.5 ± 0.8

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

Table 2. Anti-NO production of compounds **1-10** isolated from *Aglaia andamanica* leaves

Compound	IC_{50} (μM)[a]
Retusin (**1**)	>100
Pachypodol (**2**)	34.5 ± 1.5
(-)-Yangambin (**3**)	37.4 ± 1.4
Pyramidatine (**4**)	>100
24-epi-Piscidinol A (**5**)	24.0 ± 1.1
Aglaiodiol (**6**)	>100
Cycloart-23E-ene-3β-25 diol (**7**)	54.5 ± 1.1
Pyramidaglain A (**8**)	54.2 ± 1.3
Pyramidaglain B (**9**)	25.6 ± 1.6
N-Methyl-trans-4-hydroxy-L-proline (**10**)	>100
L-Nitroarginine (L-NA)	61.8 ± 1.9
Caffeic acid phenethylester (CAPE)	5.2 ± 1.2
Indomethacin	46.5 ± 0.6

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

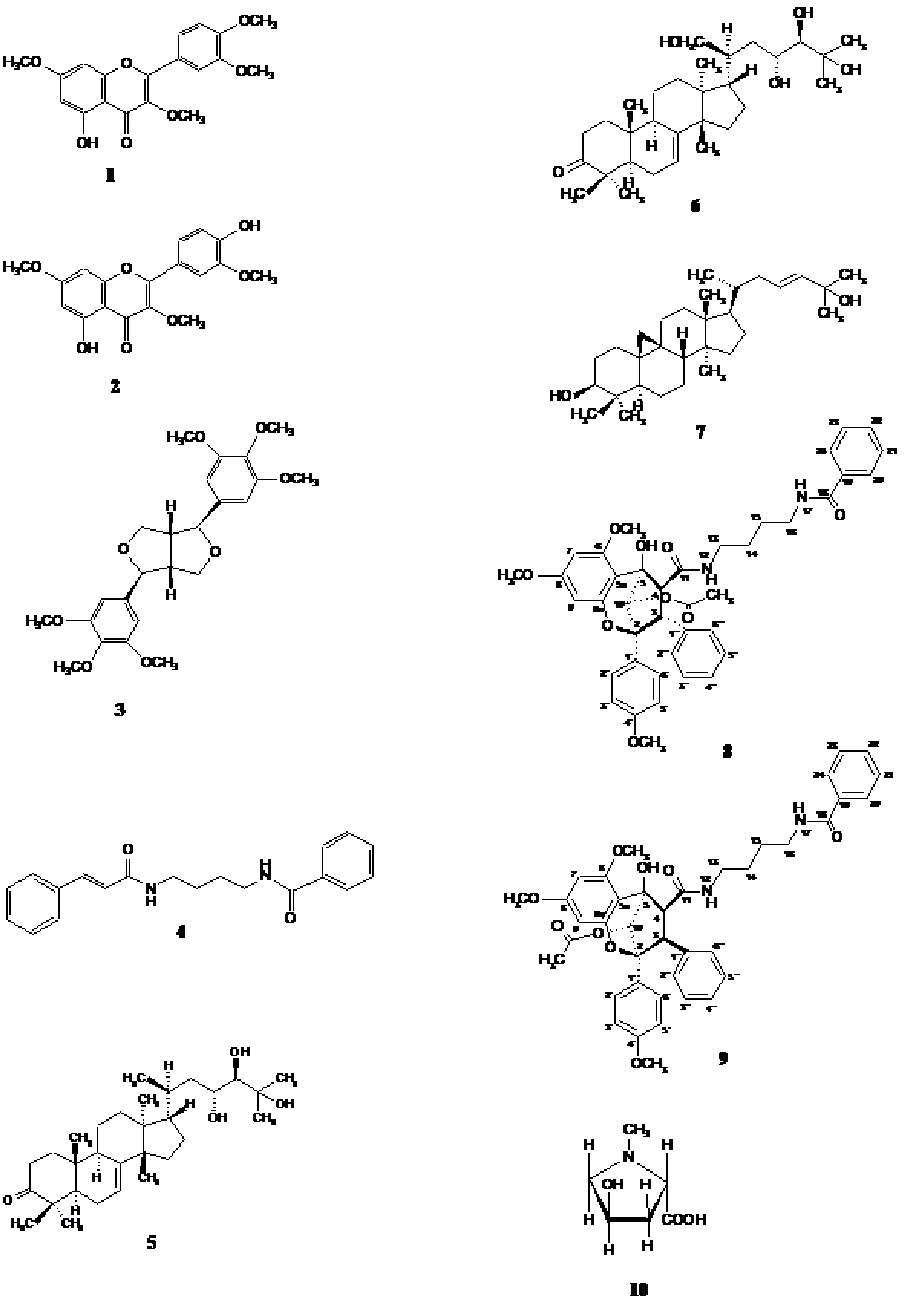

Figure 1. Structure of compounds from *Aglaia andamanica* leaves

and anti-inflammatory effects of these compounds will be further investigated. This is the first report of *Aglaia andamanica* and its compounds for anti-allergic and anti-inflammatory activities.

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References

Agrawal, P.K. 1989. Carbon-13 NMR of flavonoids, Elsevier Science Publishing Com. Inc., New York, U.S.A., 162-163.

Anjaneyulu, A.S.R. and Suryanarayana, M.V. 1981. Chemical examination of heartwood of *Guazuma tomentosa* Kunth. Indian Journal of Chemistry Section B. 20, 85-87.

Banskota, A.H., Tezuka, Y., Nguyen, N.T., Awale, S., Nobukawa, T. and Kadota, S. 2003. DPPH radical scavenging and nitric oxide inhibitory activities of the constituents from the wood of *Taxus yunnanensis*. Planta Medica. 69, 500-505.

Cheong, H., Choi, E.J., Yoo, G.S., Kim, K.M. and Ryu, S.Y. 1998. Desacetylmatricarin, an anti-allergic compound from *Taraxacum platycarpum*. Planta Medica 64, 577-578.

Figliuolo, R., Naylor, S., Wang, J. and Langenheim, J.H. 1987. Unusual nonprotein imino acid and its relationship to phenolic and nitrogenous compounds in Copaifera. Phytochemistry. 26, 3255-3259.

Goldsby, R.A., Kindt, T.J., Osborne, B.A. and Kuby, J. 2002. Immunology. fifth edition. W.H. Freeman and Company, New York, U.S.A., pp. 361-370.

Greca, M., Fiorentino, A., Monaco, P. and Previtera, L. 1994. Cycloartane triterpenes from *Juncus effusus*. Phytochemistry. 35, 1017-1022.

Hayashi, N., Lee, K.H., Hall, I.H., McPhail, A.T. and Huang, H.C. 1982. Structure and stereochemistry of (-)-odorinol, an antileukemic diamide from *Aglaia odorata*. Phytochemistry. 21, 2371-2373.

Ishibashi, F., Satasook, C., Isman, M.B. and Towers, G.H.N. 1993. Insecticidal 1*H*-cyclopentatetrahydro[*b*] benzofurans from *Aglaia odorata*. Phytochemistry. 32, 307-310.

Joshi, M.N., Chowdhury, B.L., Vishnoi, H.P., Shoeb, A. and Kapil, R.S. 1987. Antiviral activity of (+)-odorinol. Planta Medica. 53, 245-255.

Kim, J.Y., Lim, H.J., Lee, D.Y., Kim, J.S., Kim, D.H., Lee, H.J., Kim, H.D,. Jeon, R. and Ryu, J.H. 2009. In vitro anti-inflammatory activity of lignans isolated from *Magnolia fargesii.* Bioorganic and Medicinal Chemistry Letters. 19, 937-940.

Kou, P.C. and Scheroder, R.A. 1995. The emerging multi-faceated roles of nitric oxide. Annals of Surgery. 221, 220-235.

Mabberley, D.J., Pannell, C.M. and Sing, A.M. 1995. Flora Malesiana ser. I. 12, 1-407.

Malan, E. and Roux, D.G. 1979. Flavonoids from *Distemonanthus benthamianus* Baillon. Methoxylated flavones and inter-relationships of benthamianin, a [2]benzopyrano[4,3-b][1]benzopyran. Journal of Chemical Society. Perkin Transection I. 11, 2696-2703.

Marrien, A. and Polonsky, J. 1971. The natural occurrence of melianodiol and its diacetate in *Samadera madagascariensis* (Simaroubaceae): Model experiment of melianodiol directed towards simarolide. Journal of Chemical Society and Chemical Communications. 6, 261-263.

McChesney, J.D., Dou, J., Sindela,r R.D., Goins, D.K., Walker, L.A. and Rogers, R.D. 1997. Tirucallane-type triterpenoids: NMR and X-ray diffraction analyses of 24-epi-piscidinol A and piscidinol A. Journal of Chemical Crystallography. 27, 283-290.

Matsuda, H., Tewtrakul, S., Morikawa, T., Nakamura, A. and Yoshikawa, M. 2004. Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the release of TNF-α and IL-4 in RBL-2H3 cells. Bioorganic and Medicinal Chemistry. 12*,* 5891-5898.

Moncada, S., Palmer, R.M.J. and Higgs, E.A. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmaceutical Review. 43, 109-142.

Picker, K., Ritchie, E. and Taylor, W.C. 1973. Constituents of Eupomatia species III. New eupomatenoid lignans from the leaves and wood of *Eupomatia laurina*. Australian Journal Chemistry. 26, 1111-1119.

Puripattanavong, J., Weber, S., Brecht, V. and Frahm, A.W. 2000. Phytochemical Investigation of *Aglaia andamanica.* Planta Medica. 66, 740-745.

Saifah, E., Puripattanavong, J. Likhitwitayawuid, K., Cordell, G.A., Chai, H.Y. and Pezzuto, J.M. 1993. Bisamides from Aglaia species: Structure analysis and potential to reverse drug resistance with cultured cells. Journal of Natural Products. 56, 473-477.

Tada, T. and Okumura, K. 1971. Regulation of homocytotropic antibody formulation in the rat. I. Feed-back regulation by passive administered antibody. Journal of Immunology. 106, 1002-1011

Tewtrakul, S., Subhadhirasakul, S. and Kummee, S. 2008. Anti-allergic activity of compounds from *Kaempferia parviflora*. Journal of Ethnopharmacology. 116, 191-193.

Tewtrakul, S. and Subhadhirasakul, S. 2008. Effects of compounds from *Kaempferia parviflora* on nitric oxide, prostaglandin E_2 and tumor necrosis factor-alpha productions in RAW264.7 macrophage cells. Journal of Ethnopharmacology. 120, 81-84.

Vidari, G., Finzi, P.V. and Bernardi, M.D. 1971. Zingiberaceae flavonols and quinones in stems of *Aframomum giganteum.* Phytochemistry. 10, 3335-3339.

Yodsaou, O., Sonprasit, J., Karalai, C., Ponglimanont, C., Tewtrakul, S. and Chantrapromma, S. 2012. Diterpenoids and triterepnoids with potential anti-inflammatory activity from the leaves of *Aglaia odorata.* Phytochemistry. 76, 83-91.

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ORIGINAL ARTICLE

Anti-HIV-1 integrase effect of compounds from *Aglaia andamanica* leaves and molecular docking study with acute toxicity test in mice

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Abstract

Context: Acquired immunodeficiency syndrome (AIDS) is a serious health problem worldwide. It has been reported that *Aglaia andamanica* Hiern (Meliaceae) leaves possessed an antiviral effect. Therefore, a search of anti-HIV-1 integrase (HIV-1 IN) agents from *A. andamanica* is a promising target.
Objective: The objective of this study is to evaluate anti-HIV-1 IN activity of isolated compounds from *A. andamanica* using an *in vitro* assay and molecular docking study as well as testing acute toxicity in mice using the up and down method.
Materials and methods: The leaves and compounds (3–100 μg/mL) from *A. andamanica* were determined for the anti-HIV-1 IN effect using the multiplate integration assay (MIA) by detection the absorbance of the final product, *p*-nitrophenol, at 405 nm. The molecular docking with the HIV-1 IN of the active compound *N*-methyl-trans-4-hydroxy-L-proline (**10**) was also studied. The Swiss albino mice were used for an acute toxicity test.
Results and discussion: Among the isolated compounds, **10** showed marked anti-HIV-1 IN effect with an IC_{50} value of 11.8 μg/mL, whereas other compounds were inactive (IC_{50} value > 100 μg/mL). The molecular docking of compound **10** with an HIV-1 IN enzyme was also studied. The result revealed that this compound formed the hydrogen bonding with the Thr66, Asn155, and Lys159 of the HIV-1 IN binding site. The acute toxicity of the *A. andamanica* extract was not observed at the dose 2000 mg/kg mice. This is the first report of *A. andamanica* for anti-HIV-1 IN activity.

Keywords

Meliaceae, multiplate integration assay, proline derivative, up and down method

History

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Introduction

Aglaia andamanica Hiern (Meliaceae) is a plant up to 30 m, distributed in South and Southeast Asia (Mabberley et al., 1995). The leaves have been used for treatment of headache (Mabberley et al., 1995). Some of benzofuran derivatives from *Aglaia* genus show interesting biological activities including antiviral (Joshi et al., 1987; Zhou et al., 2013), antileukemic (Hayashi et al., 1982), and insecticidal activities (Ishibashi et al., 1993).

Acquired immunodeficiency syndrome (AIDS) has been a serious problem in Thailand since the late 1980s. In Thailand, it is reported that among 490 000 of total HIV-positive patients, 23 000 HIV patients died/year with 9700 new HIV positives reported/year (UNAIDS, 2012). Drug treatment of this disease is expensive, and a small number of AIDS patients have access to the cocktail of modern antiviral agents. HIV-1 IN has become a crucial target for AIDS treatment since there are only three HIV-1 IN inhibitors (raltegravir, elvitegravir, and dolutegravir) now available in the market. Therefore, the majority of AIDS patients prefer to use Thai traditional doctors, who prescribe a range of plant-based products. However, scientific studies supporting this use have in most cases not yet been carried out (Moncada et al., 1991). HIV-1 IN enzyme is a 32 kDa protein which functions as a dimer with 288 amino acids each. The integration process is composed of two steps which are 3′-processing and strand transfer reactions (Camarasa et al., 2006; Katz & Skalka, 1994). The catalytic core domain contains the catalytic triad of acidic residues, the D, D-35E motif which are Asp64, Asp116, and Glu152 in the structure of HIV-1 IN (Craigie, 2001).

In our study, it was shown that *A. andamanica* extract possessed potent anti-HIV-1 IN effect with an IC_{50} value of 29.6 μg/mL. The compounds from this plant were then isolated and tested for this activity. Moreover, the study on

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the mechanism of an active compound using molecular docking with HIV-1 IN enzyme was also performed.

Materials and methods

Reagents

An HIV-1 IN recombinant was expressed in *Escherichia coli*, purified according to the method described in a previous literature (Jenkins et al., 1996), except that after removal of the His-tag with thrombin, chromatography on a Sephadex column was the final purification process. The IN enzyme was stored at −80 °C until use.

Plant material

Leaves of *A. andamanica* were collected in Thon-Nja-Chang National Park, Hat-Yai, Songkhla, Thailand, in September 1995. A voucher specimen (JP9001) was identified by Dr. S. Vajrodaya, Department of Botany, Kasetsart University, Bangkok, Thailand, and has been deposited in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla, Thailand.

The isolated compounds (**1**–**10**) were kept in 4 °C air-tight containers and were elucidated by comparison of their spectral data with those reported in the literature (Puripattanavong et al., 2000).

Acute toxicity test of *A. andamanica* extract in mice

Male and female Swiss albino mice (30–40 g) were used in the experiment. The animals obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat-Yai, Songkhla, Thailand. All experimental protocol was approved by The Animal Ethic Committee, Prince of Songkla University (MOE 0521.11/241). Swiss albino mice were housed in standard environmental conditions with a 12 h light/dark cycle. They were provided *ad libitum* with standard rodent diet and water.

The 50% lethal dose (LD_{50}) of the MeOH extract of *A. andamanica* was estimated by the up and down method in mice (Bruce, 1985). The animals were fasted for 6 h prior to dosing. Doses were adjusted by a constant multiplicative factor (namely 3.2) for this experiment. The dose for each successive animal was adjusted up or down depending on the previous outcome. The crude extract was dissolved in the cosolvent solution (propylene glycol:water = 1:1) and orally administered in a single dose by gavage using a stomach tube to both groups of male and female mice. Animal behaviors were observed individually at least once during the first 30 min after administration, periodically during the first 8 h and daily thereafter, for a total of 7 d. The signs of toxicity were observed including tremor, convulsion, hyperactivity, sedation, grooming, loss of righting reflex, respiratory depression, and coma.

Assay for HIV-1 IN inhibitory activity

Oligonucleotide substrates

Oligonucleotides of long terminal repeat donor DNA (LTR-D) and target substrate (TS) DNA were purchased from QIAGEN Operon (Alameda, CA) and stored at −25 °C before use. The sequence of biotinylated LTR donor DNA and its unlabelled complement were 5′-biotin-ACCCTTTTAGTC AGTGTGGAAAATCTCTAGCAGT-3′ (LTR-D1) and 3′-GA AAATCAGTCACACCTTTTAGAGATCGTCA-5′ (LTR-D2), respectively. Those of the target substrate DNA (digoxigenin-labelled target DNA, TS-1) and its 3′-labelled complement was 5′-TGACCAAGGGCTAATTCACT-digoxigenin and digoxigenin-ACTGGTTCCCGATTAAGTGA-5′ (TS-2), respectively.

Multiplate integration assay (MIA)

The integration reaction was evaluated according to the method previously described (Tewtrakul et al., 2001). A mixture (45 μL), composed of 12 μL of IN buffer [containing 150 mM 3-(*N*-morpholino) propane sulfonic acid, pH 7.2 (MOPS), 75 mM $MnCl_2$, 5 mM dithiothritol (DTT), 25% glycerol and 500 μg/mL bovine serum albumin], 1 μL of 5 pmol/mL digoxigenin-labelled target DNA, and 32 μL of sterilzed water, were added into each well of a 96-well plate. Subsequently, 6 μL of sample solution and 9 μL of 1/5 dilution of integrase enzyme was added to each well and incubated at 37 °C for 80 min. The wells were then washed with PBS four times, and 100 μL of 500 mU/mL alkaline phosphatase (AP)-labelled anti-digoxigenin antibody was then added to all wells and incubated at 37 °C for 1 h. The plate was washed again with washing buffer containing 0.05% Tween 20 in PBS four times and with PBS four times. Then, AP buffer (150 μL) containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$, and 10 mM *p*-nitrophenyl phosphate was added to each well and incubated at 37 °C for 1 h. Finally, the plate was measured with a microplate reader at a wavelength of 405 nm. A control consisted of a reaction mixture, 50% DMSO and an integrase enzyme, while a blank was buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA. 2Na), 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1 mM DTT, and 4 M urea without the integrase enzyme. Suramin, a polyanionic HIV-1 IN inhibitor, was used as a positive control. The % inhibition against HIV-1 IN was calculated as follows:

$$\%\ \text{Inhibition against HIV} - 1\ \text{IN}$$
$$= \left[\frac{(\text{OD control} - \text{OD sample})}{\text{OD control}}\right] \times 100$$

where OD is the absorbance detected from each well.

Molecular docking method

Ligand preparation

The three-dimensional structure of active compound was constructed using the HyperChem professional 8.0 software (HyperChem Inc., Gainesville, FL). Subsequently, geometry optimization was carried out using the conjugated gradients algorithm at the semi-empirical PM3 level of theory. Finally, the Gasteiger charges were assigned using the AutoDockTools program (Scripps Research Institute, La Jolla, CA).

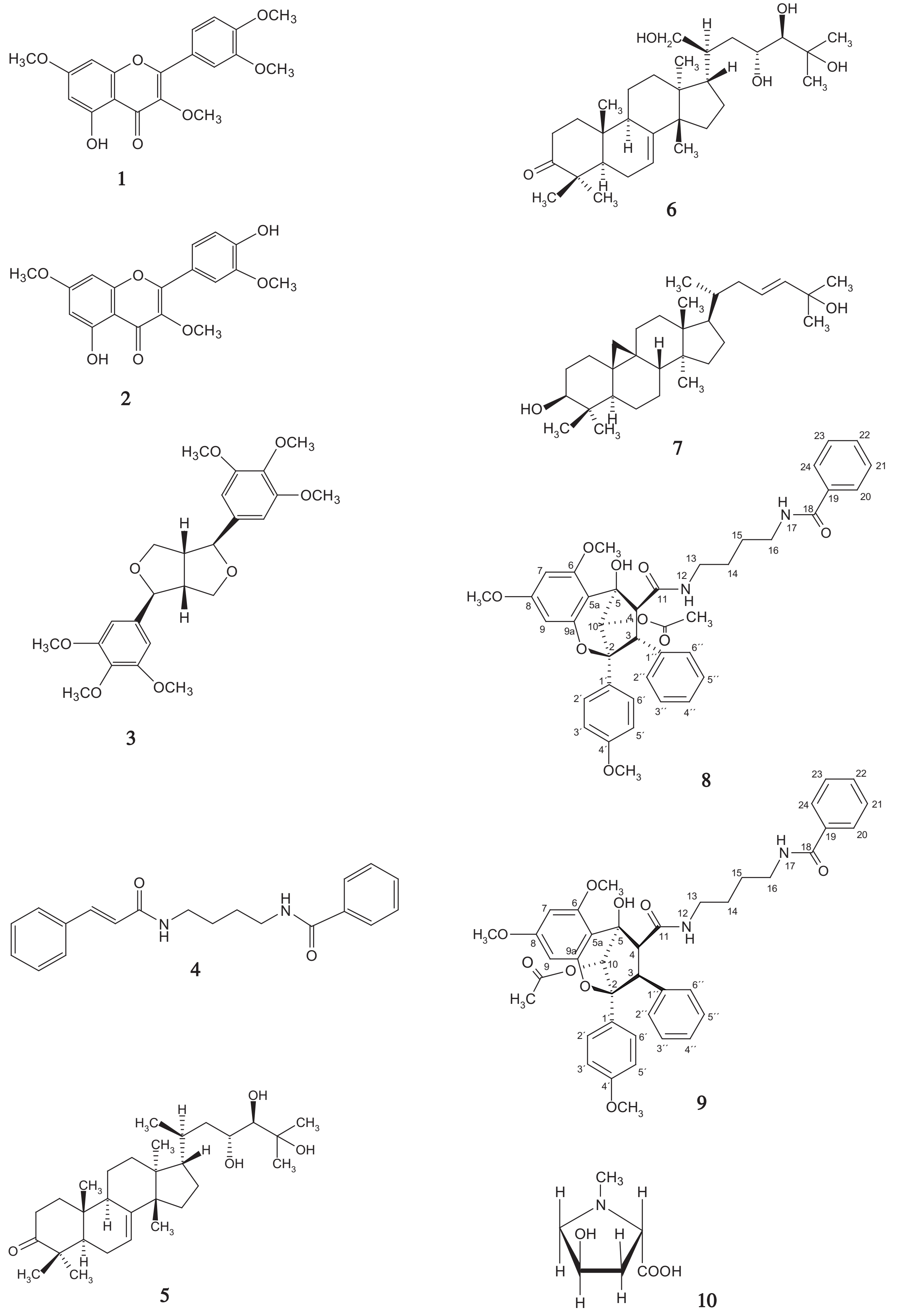


Figure 1. Structures of compounds from *Aglaia andamanica* leaves.

HIV-1 IN preparation

The X-ray crystal structure of an HIV-1 IN core domain in complexed with 5CITEP, its inhibitor, was obtained from the Protein Data Bank (PDB code 1QS4). Only chain A which co-crystallized with 5CITEP was selected, while chains B and C were deleted. All the water molecules and 5CITEP were removed, while a magnesium ion at the active site was maintained. The missing residues in the loop at positions 141–144 were incorporated and polar hydrogen atoms were added to this chain (Vajragupta et al., 2005). The second magnesium ion was placed in the same relative position according to the two metal structure of the prototype foamy virus integrase (PDB code 3OYA), a high structural homolog to HIV-1 IN (Krishnan & Engelman, 2012).

Molecular docking

Molecular docking was performed with version 4.2. AutoDock program (Scripps Research Institute, La Jolla, CA). Before docking, the grid boxes were prepared for IN structures using AutoGrid 4.0 (Scripps Research Institute, La Jolla, CA). The center of the grid boxes was set on the location of the co-crystallized inhibitor. The grid dimensions were set to 60 × 60 × 60 Å in each dimension, with a grid spacing of 0.375 Å which is large enough for the free rotation of the ligand.

The docking calculations were carried out using the Lamarckian genetic algorithm (LGA). The optimized docking parameters were set as follows: the number of GA run was 100, the population size was 150, the maximum number of energy evaluations was increased to 2 500 000 per run and the maximum number of generation in the genetic algorithm was increased to 100 000. All other docking parameters were set at their default.

A cluster analysis was used to group similar conformation; all 100 independent conformations for each compound with the root mean square deviation (RMSD) differ less than 2 Å were clustered together. The best docked conformations were the lowest binding energy and the greatest number of conformations in the cluster (Healy et al., 2009). Amino acid residues within 6 Å of the ligand in the IN active site were chosen for H-bond interactions analysis using the H-bond monitor in the DS Viewer Pro program (Roseau Valley, Dominica).

Statistical analysis

The results were expressed as mean ± SEM of four determinations at each concentration for each sample. The IC_{50} values were calculated using the Microsoft Excel program.

Results and discussion

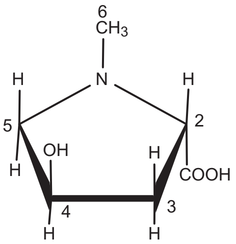
The leaves and compounds from *A. andamanica* were determined for the anti-HIV-1 IN effect using the MIA assay (IC_{50} value = 29.6 μg/mL). Among the isolated compounds (Figure 1), *N*-methyl-*trans*-4-hydroxy-L-proline (**10**) showed marked anti-HIV-1 IN effect with an IC_{50} value of 11.8 μg/mL, whereas other compounds were inactive (IC_{50} value > 100 μg/mL). The positive control, suramin, had an IC_{50} value of 2.5 μg/mL (Table 1). Proline derivatives have been reported to possess antifungal, antiangiotensin-converting enzyme (ACE) (Morais et al., 2013) and antiviral (Zhou et al., 2013) activities. It has been reported that methionine–proline anilides can inhibit the protease enzyme of dengue virus NS2B-NS3 (Zhou et al., 2013). Regarding the activity of *A. andamanica* leaves, it is reported that the leaves from this plant exhibited marked antiallergic and antiinflammatory effects using RBL-2H3 and RAW264.7 cells, respectively. Among the tested compounds, 24-epi-piscidinol A possessed

Table 1. Anti-HIV-1 IN activity[a] of compounds **1–10** isolated from *Aglaia andamanica* leaves.

Compound	IC_{50} value (μg/mL)
Retusin (**1**)	>100
Pachypodol (**2**)	>100
(−)-Yangambin (**3**)	>100
Pyramidatine (**4**)	>100
24-*epi*-Piscidinol A (**5**)	>100
Aglaiodiol (**6**)	>100
Cycloart-23E-ene-3β-25 diol (**7**)	>100
Pyramidaglain A (**8**)	>100
Pyramidaglain B (**9**)	>100
N-Methyl-*trans*-4-hydroxy-L-proline (**10**)	11.8 ± 0.9
Suramin (positive control)	2.5 ± 0.5

[a] Each value represents mean ± SEM of four determinations.

Table 2. Molecular docking study of *N*-methyl-*trans*-4-hydroxy-L-proline (**10**).

		Amino acid residues with H-bonding (distance: Å)			
Compound	Binding energy (kcal/mol)	ASP64	THR66	ASN155	LYS159
N-Methyl-*trans*-4-hydroxy-L-proline  	−4.50 C = 1 F = 92	–	OG1-7-H (1.73)	HD21-4-O (1.83)	HZ3-7-O (1.65)

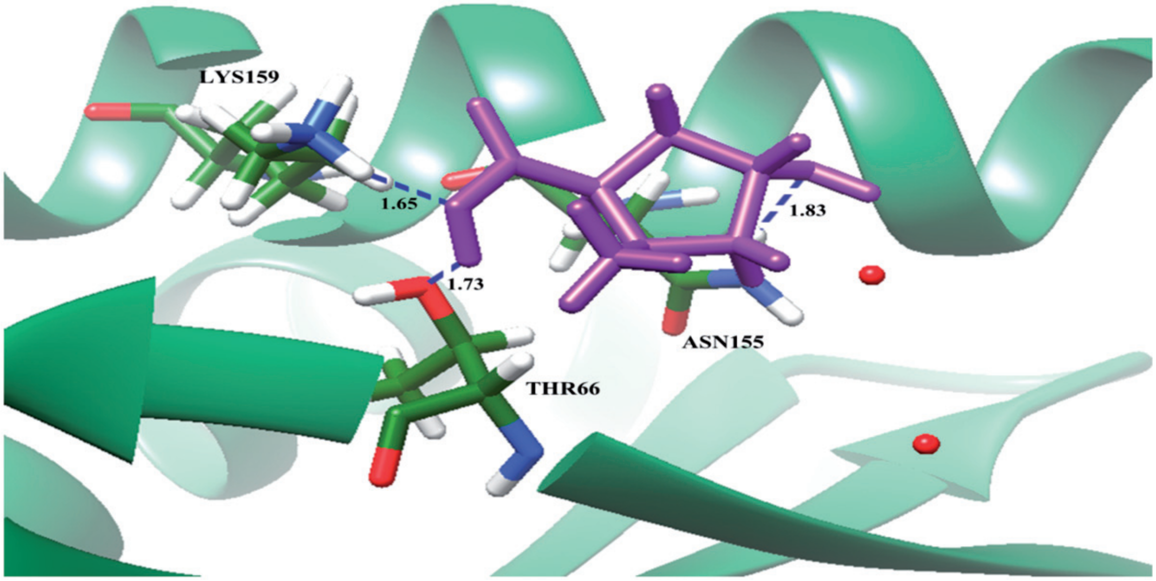


Figure 2. Molecular docking of *N*-methyl-*trans*-4-hydroxy-L-proline with HIV-1 IN. The ribbon model shows the backbone of HIV-1 IN catalytic domain with all interacting amino acid residues shown as stick models and colored by element. *N*-Methyl-*trans*-4-hydroxy-L-proline is shown as purple stick model. H-bond interactions are shown as blue dash lines and represent bond length in angstrom (Å). Mg^{2+} ions are shown as red balls.

potent for both antiallergic and antiinflammatory activities (Puripattanavong & Tewtrakul, 2015).

The molecular docking of compound **10** with an HIV-1 IN enzyme was also studied. The result revealed that the carboxylic and hydroxyl groups of this compound formed the hydrogen bonding with the Thr 66, Asn155, and Lys 159 of the IN enzyme with the binding energy at −4.50 kcal/mol (Table 2 and Figure 2). It has been reported that Thr66 is essential for 3′-processing, whereas Asn155 and Lys159 are important for strand transfer reaction (Dayam & Neamati, 2004). Thus this compound may have anti-HIV-1 IN effect by binding with the key amino acid residues of the HIV-1 IN-binding site which finally interfere the binding between viral DNA and IN enzyme.

For the acute toxicity test, the oral LD_{50} value of the MeOH extract of *A. andamanica* was not observed at the dose 2000 mg/kg body weight of both male and female Swiss albino mice. At the end of study period (7 d), all animals survived and appeared active and healthy. During the day of observation period, the animals had no significant signs of toxicity, adverse pharmacological effects or abnormal behaviors. This result may indicate that *A. andamanica* extract has no acute toxicity and is safety to use.

In conclusion, the present study shows that *A. andamanica* possesses anti-HIV-1 IN activity and safety for use. *N*-methyl-*trans*-4-hydroxy-L-proline (**10**) isolated from this plant is responsible for anti-HIV-1 IN effect by forming the hydrogen bonding with the Thr 66, Asn155, and Lys 159 of the IN enzyme. Moreover, this is the first report of *A. andamanica* on acute toxicity in mice, anti-HIV-1 IN activity, and the mechanism of *N*-methyl-*trans*-4-hydroxy-L-proline as anti-HIV-1 IN agent.

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Declaration of interest

The authors have no conflicts of interest. The authors are grateful to the Thailand Research Fund (TRF, RSA5680012) for financial support.

References

Bruce RD. (1985). An up-and-down procedure for acute toxicity. *Fund Appl Toxicol* 5:151–7.

Camarasa MJ, Velazquez S, San-Felix A, et al. (2006). Dimerization inhibitors of HIV-1 reverse transcriptase, protease and integrase: A single mode of inhibitor for the three HIV enzymes? *Antivir Res* 71: 260–7.

Craigie R. (2001). HIV-1 integrase, a brief overview from chemistry to therapeutics. *J Biol Chem* 276:23213–16.

Dayam R, Neamati N. (2004). Active site binding modes of the β-diketoacids: A multi-active site approach in HIV-1 integrase inhibitor design. *Bioorg Med Chem* 12:6371–81.

Hayashi N, Lee KH, Hall IH, et al. (1982). Structure and stereochemistry of (−)-odorinol, an antileukemic diamide from *Aglaia odorata*. *Phytochemistry* 21:2371–3.

Healy EF, Sanders J, Peter J, et al. (2009). A docking study of L-chicoric acid with HIV-1 integrase. *J Mol Graphics Model* 27:584–9.

Ishibashi F, Satasook C, Isman MB, Towers GHN. (1993). Insecticidal 1*H*-cyclopentatetrahydro[*b*]benzofurans from *Aglaia odorata*. *Phytochemistry* 32:307–10.

Jenkins TM, Engelman A, Ghirlando R, Craigie R. (1996). A soluble active mutant of HIV-1 integrase. *J Biol Chem* 271:7712–18.

Joshi MN, Chowdhury BL, Vishnoi HP, et al. (1987). Antiviral activity of (+)-odorinol. *Planta Med* 53:245–55.

Katz RA, Skalka, AM. (1994). The retroviral enzymes. *Annu Rev Biochem* 63:133–73.

Krishnan L, Engelman A. (2012). Retroviral integrase proteins and HIV-1 DNA integration. *J Biol Chem* 287:40858–66.

Mabberley DJ, Pannell CM, Sing AM. (1995). Meliaceae. Flora Malesiana series I, vol. 12 Part 1. Leiden (Netherlands): Rijksherbarium/Hortus Botanicus, Leiden University.

Moncada S, Palmer RMJ, Higgs EA. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharm Rev* 43:109–42.

Morais KLP, Lanzer D, Miranda, JRR, et al. (2013). Proline rich-oligopeptides: Diverse mechanisms for anti-hypertensive action. *Peptides* 48:124–33.

Puripattanavong J, Tewtrakul S. (2015). Antiallergic and antiinflammatory compounds from *Aglaia andamanica* leaves. *Songklanakarin J Sci Technol* 37:37–41.

Puripattanavong J, Weber S, Brecht V, Frahm AW. (2000). Phytochemical investigation of *Aglaia andamanica*. *Planta Med* 66:740–5.

Tewtrakul S, Miyashiro H, Hattori M, et al. (2001). Inhibitory effects of flavonoids on human immunodeficiency virus type-1 integrase. *J Trad Med* 18:229–38.

UNAIDS. (2012). Global Report 2012, AIDS info.

Vajragupta O, Boonchoong P, Morrisc GM, Olsonc AJ. (2005). Active site binding modes of curcumin in HIV-1 protease and integrase. *Bioorg Med Chem Lett* 15:3364–8.

Zhou GC, Weng Z, Shao X, et al. (2013). Discovery and SAR studies of methionine–proline anilides as dengue virus NS2B-NS3 protease inhibitors. *Bioorg Med Chem Lett* 23:6549–54.