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Anti-HIV-1 Protease Activity of Compounds from *Cassia garrettiana*

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Abstract

For the purpose of discovering anti-HIV-1 agents from Thai plant preparations with a history of use in Thai traditional medicine as agents assisting health longevity, aqueous and ethanol extracts from 24 Thai plants were screened for their inhibitory activities against HIV-1 protease (PR) using an anti-HIV-1 PR assay. Five extracts (10 %), 3 ethanol and 2 water, from only 4 plants, *Cassia garrettiana, Dioscorea bulbifera, Albizia procera*, and *Areca catechu*, produced extracts with IC_{50} values of < 45 µg/ml. Thirty five extracts (90 %) had no detectable activity, with IC_{50} values >100 µg/ml. The ethanol extracts of *Cassia garrettiana* (heartwood), *Areca catechu*, and *Dioscorea bulbifera* were the most potent, with IC_{50} values of 15.6, 24.9, and 44.4 µg/ml, respectively. For the water extracts, the most potent activity was found in *Albizia procera* (bark) and *Cassia garrettiana* (heartwood), with IC_{50} values of 22.6 and 26.8 µg/ml, respectively. Pure compounds from ethanol extracts of *Cassia garrettiana* (heartwood) were separated to give 5 compounds, chrysophanol (**1**), piceatannol (**2**), aloe-emodin (**3**), emodin (**4**), and cassigarol E (**5**). Of the tested samples, piceatannol (**2**) showed the highest anti-HIV-1 PR activity (IC_{50} = 48.29 µM), whereas the % inhibition (mean±S.E.M., n=3) produced by 100 µM of cassigarol E (**5**), aloe-emodin (**3**), chrysophanol (**1**), and emodin (**4**) were only 27.64±3.8, 27.20±0.7, 26.66±0.6, and 4.52±1.9, respectively.

Keywords: Anti-HIV-1 protease activity, longevity preparations, *Cassia garrettiana*, Thai plants

Introduction

Cassia garrettiana Craib, locally known in Thai as Samae-sarn, is a plant in the Caesalpiniaceae family. In Thai traditional medicine, the heartwood of this plant has been used as emmenagogue and blood tonic for women [1]. Moreover, *C. garrettiana* heartwood has been used to treat Herpes zoster, leukemia, constipation, and nematodes infestation [2]. *C. garrettiana* has been reported to show many biological activities, such as anti-cancer [3] and anti-HIV-1 integrase [4].

Acquired immunodeficiency syndrome (AIDS) has spread to many countries and is now a worldwide public health problem. AIDS develops from infection from a retrovirus called human immunodeficiency virus, or "HIV". HIV leads to immunosuppression, which allows opportunistic pathogens to cause disease and death in AIDS patients from such conditions as tuberculosis and pneumonia. Half a million people around the world died in the past year because of co-infection with *Mycobacterium tuberculosis* and HIV [5].

Three enzymes that are essential for the HIV-1 life cycle are HIV-1 protease (PR), reverse transcriptase (RT), and integrase (IN). Thai medicine health "longevity preparations" have been recommended by Thai national health physicians for improving the quality of life and health. In the present study, 24 Thai plants that are described as longevity preparations were investigated for their HIV-

1 PR inhibitory activity. Since the anti-HIV-PR activity of these plants has not been previously well studied, it will be of interest to establish if any of these Thai plant preparations could assist against HIV-1 infections, perhaps by inhibiting HIV-1 PR activity. Any such effects could lead to the development of new natural anti-HIV-PR agents.

Materials and methods

Plant materials

The 24 Thai plants were collected in 2010 at the Suan Ya Thai Thongnoppakhun herbal garden in Chonburi province, and were identified by the Thai traditional doctor Mr. Sraupsin Thongnoppakhun. The *Cassia garrettiana* voucher specimen number is SKP 034030701. The *Cassia garrettiana* heartwood sample was kept at the Herbarium of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Preparation of plant extracts

Twenty grams of each dried plant were extracted twice with water and ethanol separately (150 ml each) under reflux for 1 h. Each extract was dried under reduced pressure and then re-dissolved in 50 % DMSO for bioassay. Sample solutions of these extracts were prepared in the concentration range of 3 - 100 μg/ml.

Isolation of compounds from *Cassia garrettiana* extract

The dried powder of *C. garrettiana* heartwood (2.1 kg) was extracted 3 times with ethanol (34 l) at room temperature. The ethanol extract (124.4 g) was successively partitioned to obtain hexane (8.5 g), chloroform (0.7 g), ethyl acetate (47.2 g), and water fractions (68.0 g) respectively. The ethyl acetate fraction (10.0 g) was separated by silica gel column chromatography using 10 % methanol in $CHCl_3$ to afford 15 fractions (F1 - F15). Fraction F2 (40.0 mg) was purified by column chromatography on sephadex LH-20 using 100 % methanol to give chrysophanol (**1**) (orange solid, 11.3 mg, 0.113 % w/w). Fraction F3 (2.0 g) was purified by column chromatography on silica gel using 10 % methanol in chloroform to give subfractions (F3/1a - F3/7a). Subfraction F3/2a (120.0 mg) was purified by column chromatography on silica gel using 10 % methanol in chloroform to give piceatannol (**2**) (white crystal, 50.3 mg, 0.503 % w/w). Fraction F3/5a (600.0 mg) was purified by column chromatography on silica gel using 20 % methanol in chloroform to give cassigarol E (**5**) (pale yellow solid, 207.5 mg, 2.075 % w/w). Fraction F3/7a (60.0 mg) was purified by sephadex LH-20 using 100 % methanol to give aloe-emodin (**3**) (yellow solid, 2.0 mg, 0.020 % w/w) and emodin (**4**) (yellow solid, 2.3 mg, 0.023 % w/w), respectively (**Figure 2**).

The structures of compounds **1-5** were elucidated using spectroscopic techniques and compared with reported spectral data [6-10].

Assay of HIV-1 protease inhibitory activity

This assay was modified from a previously reported method [1]. In brief, the recombinant HIV-1 PR solution was diluted with a buffer composed of a solution containing 50 mM of sodium acetate (pH 5.0), 1 mM ethylenediamine disodium (EDTA.2Na), and 2 mM 2-mercaptoethanol (2-ME), and mixed with glycerol in a ratio of 3:1. The substrate peptide, Arg-Val-Nle-(*p*NO2-Phe)-Glu-Ala-Nle-NH2, was diluted with a buffer solution of 50 mM sodium acetate (pH 5.0). Two microlitres of plant extract and 4 μl of HIV-1 PR solution (0.025 mg/ml) were added to a solution containing 2 μl of 50 mM buffer solution (pH 5.0) and 2 μl of substrate solution (2 mg/ml), and the reaction mixture (10 μl) was incubated at 37 °C for 1 h. A control reaction was performed under the same conditions but without the plant extract. The reaction was stopped by heating the reaction mixture at 90 °C for 1 min. Subsequently, 20 μl of sterile water was added and an aliquot of 10 μl was analyzed by HPLC using an RP-18 column (4.6×150 mm i.d., Supelco 516 C-18-DB 5 μm, USA). Ten microlitres of the reaction mixture was injected into the column and gradiently eluted with acetonitrile (15 - 40 %) and 0.2 % trifluoroacetic acid (TFA) in water, at a flow rate of 1.0 ml/min. The elution profile was monitored at 280 nm. The retention times of the

substrate and *p*-NO2-Phe-bearing hydrolysate were 11.33 and 9.47 min, respectively. The inhibitory activity on HIV-1 PR was calculated as follows:

% inhibition (A control – A sample) × 100/A control

where A is a relative peak area of the product hydrolysate. Acetyl pepstatin was used as a positive control. It bound to the HIV-1 protease active sites.

Statistics

For statistical analysis, the values were expressed as a mean ± S.E.M of 3 determinations. The IC_{50} values were calculated using the Microsoft Excel program.

Results and discussion

Aqueous and ethanol extracts of the Thai plants *Caesalpinia sappan*, *Bauhinia strychnifolia*, *Cassia garrettiana*, *Cassia timoriensis*, *Cryptolepis buchanani*, *Betula alnoides*, *Anamirta cocculus*, *Derris scandens*, *Piper chaba*, *Spilanthes acmella*, *Albizia procera*, *Fagraea fragrans*, *Ficus foveolata*, *Piper nigrum*, *Diospyros rhodocalyx*, *Morinda elliptica*, *Artocarpus heterophyllus*, *Areca catechu*, *Stephania pierrei*, *Dioscorea bulbifera*, *Zingiber ottensii*, *Piper ribesioides*, *Cyperus rotundus*, and *Blumea balsamifera* were screened for their inhibitory activities against HIV-1 PR using the anti-HIV-1 PR assay (**Table 1**).

Table 1 IC_{50} values of aqueous- and ethanolic extracts of Thai health longevity plants against HIV-1 PR activity.

Botanical name	Family	Part used	Yield (% w/w)	Extract	IC_{50} (µg/ml)
1.พิงถ่อน			20.1	Ethanol	>100
Albizia procera Benth	Mimosaceae	bark	25.1	Water	22.6
2.โคคลาน			4.8	Ethanol	>100
Anamirta cocculus L.	Menispermaceae	wood	6.8	Water	>100
3.หมาก			9.0	Ethanol	24.9
Areca catechu L.	Palmaceae	fruit	14.2	Water	>100
4.ขนุน			3.4	Ethanol	>100
Artocarpus heterophyllus Lam	Moraceae	heartwood	2.9	Water	>100
5.ย่านางแดง			19.6	Ethanol	>100
Bauhinia strychnifolia Craib	Fabaceae	vine	13.1	Water	>100
6.กำลังเสือโคร่ง			3.6	Ethanol	>100
Betula alnoides Buch.-Ham	Betulaceae	wood	6.4	Water	>100
7.หนาด			12.6	Ethanol	>100
Blumea balsamifera L.	Compositae	leaf	18.3	Water	>100
8.ฝาง			17.0	Ethanol	>100
Caesalpinia sappan L.	Caesalpiniaceae	heartwood	11.2	Water	>100
9.แสมสาร			7.3	Ethanol	15.6
Cassia garrettiana Craib	Caesalpiniaceae	heartwood	11.6	Water	26.8

Botanical name	Family	Part used	Yield (% w/w)	Extract	IC_{50} (µg/ml)
10.ขี้เหล็กเลือด *Cassia timoriensis* DC	Caesalpiniaceae	wood	4.2 6.21	Ethanol Water	>100 >100
11.เอ็นอ่อน *Cryptolepis buchanani* Roem & Schult	Asclepiadaceae	vine	6.0 8.0	Ethanol Water	>100 >100
12.แห้วหมู *Cyperus rotundus* L.	Cyperaceae	rhizome	1.9 10.5	Ethanol Water	>100 >100
13.เถาวัลย์เปรียง *Derris scandens* Benth	Papilionaceae	vine	5.3 11.2	Ethanol Water	>100 >100
14.กลิ้งกลางดง *Dioscorea bulbifera* L	Menispermaceae	tuber	5.6 3.8	Ethanol Water	44.4 >100
15.ตะโกนา *Diospyros rhodocalyx* Kurz	Ebenaceae	bark	16.2 7.3	Ethanol Water	>100 >100
16.กรันเกรา *Fagraea fragrans* Roxb	Gentianaceae	leaf	20.6 26.0	Ethanol Water	>100 >100
17.ม้ากระทืบโรง *Ficus foveolata* Wall	Moraceae	vine	6.7 16.1	Ethanol Water	>100 >100
18.ยอป่า *Morinda elliptica* Ridl	Rubiaceae	bark	0.5 8.0	Ethanol Water	>100 >100
19.ดีปลี *Piper chaba* Hunt	Piperaceae	fruit	10.1 6.2	Ethanol Water	>100 >100
20.พริกไทย *Piper nigrum* L.	Piperaceae	fruit	9.9 20.3	Ethanol Water	>100 >100
21.สะค้าน *Piper ribesioides* Wall	Piperaceae	vine	5.3 11.1	Ethanol Water	>100 >100
22.ผักคราดหัวแหวน *Spilanthes acmella* L.	Compositae	flower	13.8 44.5	Ethanol Water	>100 >100
23.สบู่เลือด *Stephania pierrei* Diels	Menispermaceae	tuber	17.1 24.0	Ethanol Water	>100 >100
24.ไพลดำ *Zingiber ottensii* Valeton	Zingiberaceae	rhizome	8.2 18.3	Ethanol Water	>100 >100
Acetyl pepstatin (positive control)	-		-	-	2.2

Table 2 % inhibition and IC_{50} values of isolated compounds from fraction HIV-1 PR activity.

Sample	% Inhibition at various concentrations (μM)				
	3	**10**	**30**	**100**	**IC_{50} (μM)**
1) Sample Chrysophanol Mean±SEM	-	-	-	26.66±0.6	>100
2) Sample Piceatannol Mean±SEM	-	9.16±0.18	17.51±0.3	81.38±1.4	48.29
3) Sample Aloe-emodin Mean±SEM	-	-	-	27.20±0.7	>100
4) Sample Emodin Mean±SEM	-	-	-	4.52±1.9	>100
5) Sample Cassigarol E Mean±SEM	-	-	-	27.64±3.8	>100
6) Sample Acetyl pepstatin (positive control) Mean±SEM	-	51.1±0.6	71.1±0.5	88.6±0.2	8.9

The ethanol extracts of *Cassia garrettiana* (heartwood) were the most potent, with IC_{50} values of 15.6 μg/ml, followed by *Areca catechu* (fruit), IC_{50} = 24.9 μg/ml, and *Dioscorea bulbifera* (tuber), IC_{50} = 44.4 μg/ml. For the water extract, the greatest inhibition of activity was from *Albizia procera* (bark), IC_{50} = 22.6 μg/ml, followed by *Cassia garrettiana* (heartwood), IC_{50} = 26.8 μg/ml. Other plant extracts possessed no activity, with IC_{50} values >100 μg/ml (**Table 1** and **Figure 1**). These results are consistent with previous findings. It has been shown that extracts from *Cassia garrettiana* have anti-fungal [11], anti-tumor, and anti-metastatic effects [12]. *Areca catechu* extracts had anti-fungal [13], anti-radical [14] analgesic, anti-inflammatory and anti-oxidant properties [15]. Extracts from *Dioscorea bulbifera* had an anti-cancer effect [16], and *Albizia procera* had anti-inflammatory [17] and anti-cancer activities [18]. It seemed that most of these have been used previously as anti-inflammatory agents, anti-cancer agents, and perhaps anti-oxidative agents, characteristics that might have some relevance to AIDS patients. The ethanol extracts of *Cassia garrettiana, Areca catechu*, and *Dioscorea bulbifera* exhibited marked HIV-1 PR inhibitory activity. Since the ethanol extract of *C. gerrettiana* was the most potent, it was further separated to give 5 pure compounds, which were stilbene derivatives piceatannol (**2**), cassigarol E (**5**), and anthraquinone derivatives chrysophanol (**1**), aloe-emodin (**3**), and emodin (**4**). Of the tested samples, piceatannol (**2**) showed the highest anti-HIV-1 PR activity (IC_{50} = 48.2 μM), followed by cassigarol E (**5**), aloe-emodin (**3**), chrysophanol (**1**), and emodin (**4**), with % inhibitions of 27.64±3.8, 27.20±0.7, 26.66±0.6, and 4.52±1.9 at concentrations of 100 μM, respectively (**Table 2** and **Figures 1** and **2**). For the stilbene, it was found that piceatannol (**2**) was active against HIV-1 PR, but the dimer of this compound cassigarol E (5) could decrease the effect against HIV-1 PR. In this prospective study, the discovery that small-molecule ligands can modulate catalytic activities has an enormous therapeutic effect [23]. The structural activity relationships of hydroxyanthraquinones (**Figure 3**) for anti-HIV-1 PR activity with % inhibitions of (mean±S.E.M., n=3) produced by 100 μM found that chrysophanol (**1**), aloe-emodin (**3**), and emodin **(4)** bearing the 1- and 8-OH groups were essential for anti-HIV-1 PR activity. The CH_3 and CH_2OH groups substituted at position 6 chrysophanol (**1**) showing a % inhibition of 26.66±0.6 μM, and aloe-emodin (**3**) showing a % inhibition of 27.20±0.7 μM increased the activity, whereas CH_3 substitution at position 3 and OH substitution at position 6 emodin (**4**) showing **a** % inhibition of 4.52±1.9 μM decreased the activity (**Table 2**). Piceatannol has been reported to show anti-cancer [19], anti-viral [20], anti-HIV-1 IN [4], anti-oxidant [21], and anti-inflammatory effects [22]. For cassigarol E, this study is the first report of the chemical constituents and biological activities of *C. garrettiana* for anti-HIV-1 PR activity. Moreover, anti-breast cancer [3] and anti-HIV-1 integrase activities [4] have been reported.

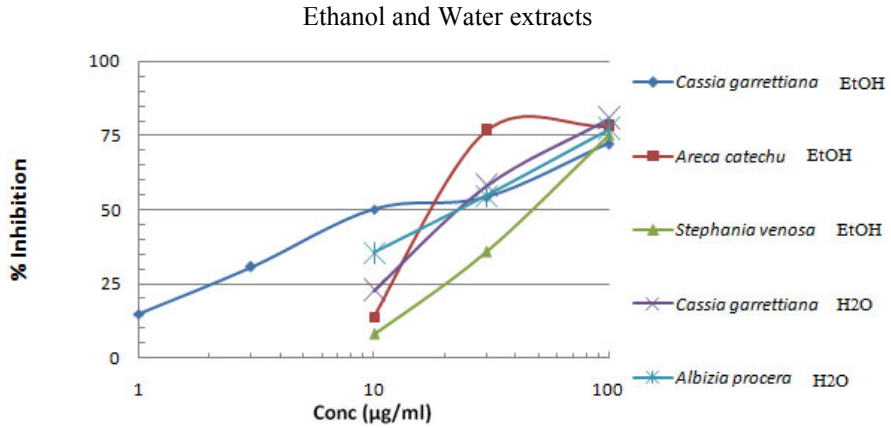
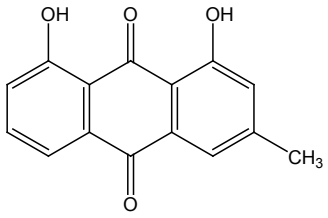
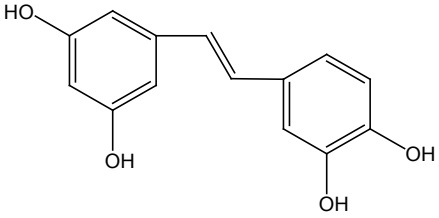


Figure 1 Dose-response curves of the ethanol extract, with IC_{50} range of 15.6 - 44.4 µg/ml, and water extract, with IC_{50} range of 22.6 - 26.8 µg/ml, on anti-HIV-1 PR activity in Thai health longevity plants.



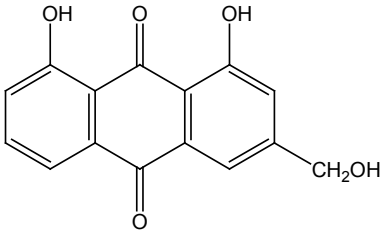
Compound 1

Chrysophanol



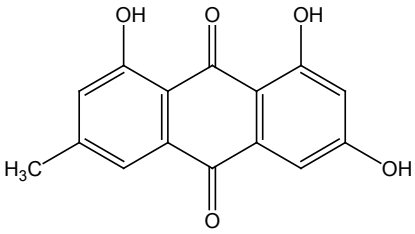
Compound 2

Piceatannol



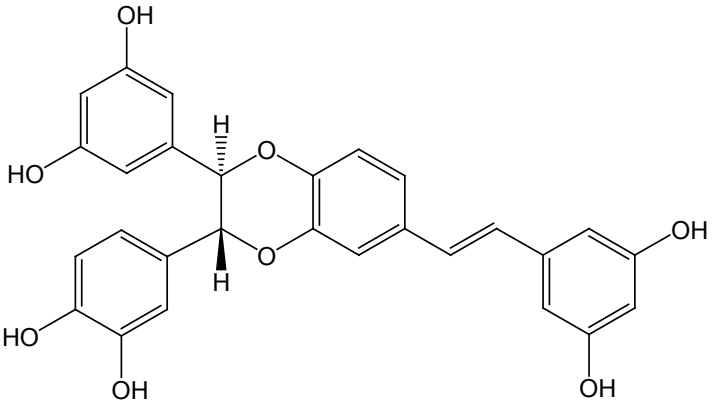
Compound 3

Aloe-emodin



Compound 4

Emodin



Compound 5

Cassigarol E

Figure 2 Structures of compounds **1-5** isolated from *Cassia garrettiana* heartwood.

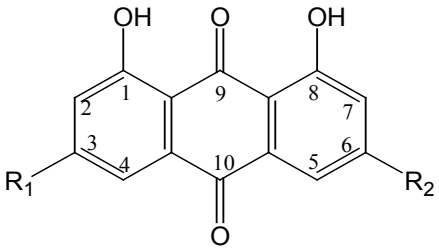


Figure 3 The chemical structure of hydroxyanthraquinone.

Conclusions

The aqueous and ethanol extracts of 24 Thai plants were screened for their inhibitory activities against HIV-1 PR using the anti-HIV-1 PR assay. The ethanol extract of *C. gerrettiana* was the most potent, with IC_{50} values of 15.6 µg/ml. It was further separated to give 5 pure compounds, of which piceatannol (**2**) showed the highest anti-HIV-1 PR activity (IC_{50} = 48.29 µM), followed by cassigarol E (**5**), aloe-emodin (**3**), chrysophanol (**1**), and emodin (**4**); % inhibition (mean±S.E.M., n=3) produced by 100 µM of cassigarol E (**5**), aloe-emodin (**3**), chrysophanol (**1**), and emodin (**4**) were only 27.64±3.8, 27.20±0.7, 26.66±0.6 and 4.52±1.9, respectively. For cassigarol E, this study is the first report of the chemical constituents and biological activities of *C. garrettiana* for anti-HIV-1 PR activity. It is suggested that these plants could be useful for the further study of AIDS treatment.

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