ผลงานอาจารย์

HIV-1 Protease Inhibitory Substances from *Cassia garrettiana*

(สารที่มีฤทธิ์ต้านเอนไซม์ HIV-1 protease ของต้นแสมสาร)

Contents

Abstract

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HIV-1 Protease Inhibitory Substances from *Cassia garrettiana*

Cassia garrettiana Craib, a Thai medicinal plant locally known as Samae-sarn, was investigated for its active constituents against HIV-1 protease (HIV-1 PR). Bioassay-guided fractionation of the heart wood of this plant led to the isolation of a stilbene derivative (**1**, piceatannol) and an anthraquinone derivative (**2**, chrysophanol). Piceatannol exhibited appreciable inhibitory effect against HIV-1 PR with an IC_{50} value of 25.4 μg/ml, whereas that of chrysophanol was 73.5 μg/ml. In addition, other two stilbenoids together with three anthraquinone derivatives were also investigated for their anti-HIV-1 PR activities. The result indicated that resveratrol possessed anti-HIV-1 PR activity with an IC_{50} value of 85.0 μg/ml, whereas other stilbenoid (oxyresveratrol) and anthraquinone derivatives (emodin, aloe-emodin, rhein) were inactive ($IC_{50} > 100$ μg/ml).

Keywords : HIV-1 protease; inhibitory substances; *Cassia garrettiana*

บทคัดย่อ

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สารที่มีฤทธิ์ต้านเอนไซม์ HIV-1 protease ของต้นแสมสาร (*Cassia garrettiana*)

การศึกษาฤทธิ์ต้านเอนไซม์ HIV-1 protease จากแก่นต้นแสมสาร โดยการทำ bioassay-guided fractionation สามารถแยกสารได้ 2 ชนิด คือ piceatannol (**1**) ซึ่งเป็นสารในกลุ่ม stilbene และ chrysophanol (**2**) ซึ่งเป็นสารในกลุ่ม anthraquinone จากการศึกษาฤทธิ์ต้านเอนไซม์ดังกล่าว พบว่า piceatannol ให้ค่า IC_{50} เท่ากับ 25.4 μg/ml ในขณะที่ chrysophanol มีค่า IC_{50} เท่ากับ 73.5 μg/ml นอกจากนี้ยังมีการศึกษาฤทธิ์ต้านเอนไซม์ HIV-1 PR ของอนุพันธ์ stilbenes และ อนุพันธ์ anthraquinones อื่นๆ ผลการวิจัยพบว่า resveratrol มีฤทธิ์ยับยั้งเอนไซม์ HIV-1 PR ที่ IC_{50} เท่ากับ 85.0 μg/ml ในขณะที่อนุพันธ์ stilbene (oxyresveratrol) และอนุพันธ์ anthraquinones อื่นๆ (emodin, aloe-emodin, rhein)ไม่มีฤทธิ์ดังกล่าว (IC_{50} > 100 μg/ml)

Introduction

Cassia garrettiana Craib, locally known in Thai as Samae-sarn, is one of the plants in the Caesalpiniaceae family. In Thai traditional medicine, the heart wood of this plant has been used to cure feminine disease and as blood tonic for women. *C. garrettiana* has been reported to show many biological activities such as anticancer (Kimura *et al.*, 2000), antifungal (Inamori *et al.*, 1984), acid secretion inhibitor (Murakami *et al.*, 1992), anti-allergy (Inamori *et al.*, 1991) and anti-hypertension activities (Inamori *et al.*, 1984).

Previously, we reported anti-HIV-1 PR activities of Thai medicinal plants in the Caesalpiniaceae family (Tewtrakul *et al.*, 2003). It was found that the ethanolic extract of *C. garrettiana* showed appreciable activity against HIV-1 PR (IC_{50} = 32 μg/ml). The present study is therefore aimed to investigate HIV-1 PR inhibitory activity of compounds isolated from *C. garrettiana*, as well as their related compounds.

Materials and methods

Plant materials and preparation of an extract

The heart wood of *C. garrettiana* was bought from the Thai traditional drug store in Hat-Yai, Songkhla, Thailand. The specimen was identified by Assoc. Prof. Dr. Sanan Subhadhirasakul, Dept. of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences. The voucher specimen (No. SKP 2060307) is kept at the Southern Center of Thai Medicinal Plants, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

The dried heart wood of *C. garrettiana* (3 kg) was crushed and extracted with ethanol (EtOH) 6 L x 6. The crude EtOH extract (214 g) was obtained and fractionated further into four fractions of *n*-hexane (10.7 g), chloroform (4.2 g), ethyl acetate (113.9 g) and water fractions (25.6 g), respectively. These fractions were tested against HIV-1 PR activity and the isolation of active principles from the chloroform fraction was then carried out using the preparative HPLC (Apollo C18, 5 μ, 250 mm x 22 mm).

The chloroform fraction (2 g), was further isolated over silica gel column using chloroform and methanol in the ratio of 9:1, 8:2, 7:3, 6:4 and 1:1, respectively and three subfractions of fr.A (0.10 g), fr. B (1.3 g) and fr. C (0.61 g) were obtained. The inhibitions at 100 μg/ml of these three subfractions against HIV-1 PR were found to be 6.7%, 90.1% and 93.3%, respectively. Fr. B was then purified using preparative HPLC with a mobile phase of 100% MeOH at flow rate 3 ml/min, to give compounds **1** (piceatannol, 35 mg) and **2** (chrysophanol, 25 mg), whereas that from fr. C was compound **1** (50 mg). The condition of the preparative HPLC for fr.C was acetonitrile and 0.2%TFA (in water) in the ratio of 45 : 55 at flow rate of 3 ml/min.

The structures of piceatannol (**1**) and chrysophanol (**2**) were elucidated on the basis of spectroscopic methods comparing to those reported in the literature (Inamori *et al.*, 1991).

Enzymes and chemicals

Recombinant HIV-1 PR, substrate peptides and acetyl pepstatin, were purchased from Sigma Chemical Co., St. Louis, USA.

Resveratrol, emodin, aloe-emodin and rhein were obtained from Sigma chemical Co., Ltd., whereas oxyresveratrol was isolated from the extract of *Artrocarpus lakoocha* heart wood.

Assay of HIV-1 protease inhibitory activity

This assay was modified from the previously reported method (Tewtrakul *et al.*, 2003). Briefly, 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 the ratio of 3 : 1. The substrate peptides, Arg-Val-Nle-(*p*NO_2-Phe)-Glu-Ala-Nle-NH_2, was diluted with a buffer solution of 50 mM sodium acetate (pH 5.0). Two microliters 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 hr. A control reaction was performed under the same condition but without the plant extract. The reaction was stopped by heating the reaction mixture at 90 °C for 1 min. Subsequently, 20 µl of sterilile water was added and an aliquot of 10 µl was analyzed by HPLC using RP-18 column (4.6 x 150 mm I.D., Supelco 516 C-18-DB 5 µm, USA). Ten microlitres of the reaction mixture was injected to 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*-NO_2-Phe-bearing hydrolysate were 11.332 and 9.470 min, respectively. The inhibitory activity on HIV-1 PR was calculated as follows : % inhibition = $(A_{control} - A_{sample}) \times 100 / A_{control}$; whereas A is

a relative peak area of the product hydrolysate. Acetyl pepstatin was used as a positive control.

Statistics

The results of anti-HIV-1 PR activity were expressed as mean ± S.D of three determinations. The IC_{50} values were calculated using the Microsoft Excel program.

Results and discussion

The *n*-hexane, chloroform, ethyl-acetate and water fractions of *C. garettiana* were tested against HIV-1 PR activity. The result indicated that chloroform fraction possessed the most potent inhibitory activity with an inhibition of 95.2% at 100 μg/ml, followed by water fraction (94.6%), ethyl acetate fraction (56.0%) and hexane fraction (0.4%), respectively (Table 1).

Table 1. HIV-1 PR inhibitory effects of EtOH extract and fractions derived from the heart wood of *C. garrettiana*

Sample	**% inhibition at 100 μg/ml**
EtOH extract	72.0 ± 1.2
n-Hexane	-0.4 ± 0.3
Chloroform	95.2 ± 0.8
Ethyl acetate	56.0 ± 1.5
Water	94.6 ± 2.0
Acetyl pepstatin (positive control)	98.4 ± 0.2

Compounds **1** and **2**, together with their related compounds (**3-7**) were tested against HIV-1 PR. The result (Table 2) showed that compound **1** (piceatannol) possessed marked activity with an IC_{50} value of 25.4 μg/ml, followed by **2** (chrysophanol, IC_{50} = 73.5 μg/ml) and **4** (resveratrol, IC_{50} = 85.0 μg/ml), whereas others (**3**, **5**, **6**, and **7**) were inactive (IC_{50} > 100 μg/ml). Acetyl pepstatin was used as a positive control, its IC_{50} value was found to be 2.2 μg/ml. The structures of compounds **1-7** are shown in Figure 1.

Table 2. HIV-1 PR inhibitory activities of compounds **1-7** isolated from *C. garrettiana*

Compounds	**IC_{50} (μg/ml)**
Piceatannol (**1**)	25.4
Chrysophanol (**2**)	73.5
Oxyresveratrol (**3**)	>100
Resveratrol (**4**)	85.0
Emodin (**5**)	>100
Aloe-emodin (**6**)	>100
Rhein (**7**)	>100
Acetyl pepstatin (positive control)	2.2

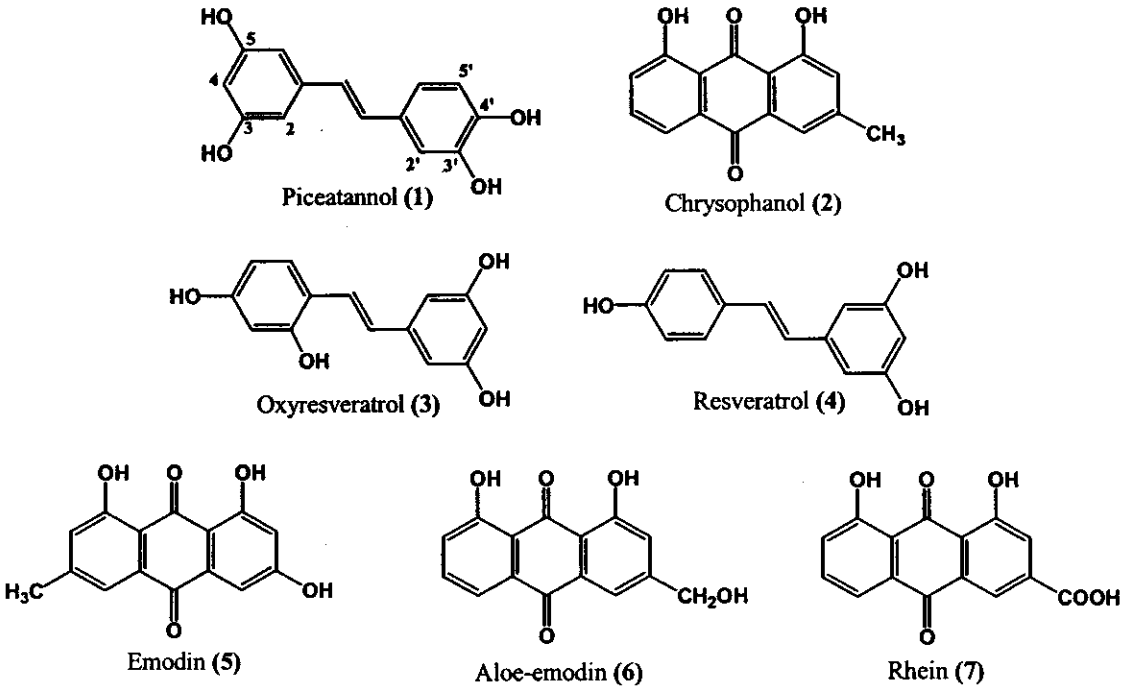

Figure 1. Chemical structures of compounds **1** and **2**, and their related compounds (**3-7**)

Regarding of biological activities, piceatannol, the main component in the chloroform fraction of *C. garrettiana*, was reported to show anti-allergic activity in RBL-2H3 cell line (Inamori, *et al.*, 1991), anti-inflammatory activity (Matsuda *et al.*, 2000) and coronary vasodilatory activity (Inamori, *et al.*, 1984). Chrysophanol and its derivatives, emodin, aloe-emodin and rhein, were found to be laxative agents by induction chloride uptake into the intestinal lumen (Dewick *et al.*, 2000) and exhibited anticancer activity (Fenig *et al.*, 2004; Cha *et al.*, 2005).

As a result, it may be concluded that piceatannol and chrysophanol containing in the heart wood of *C. garrettiana* are responsible for anti-HIV-1 PR activity. In addition, this is the first time to report inhibitory effect against HIV-1 PR of *C. garrettiana* heartwood.

Acknowledgements

The authors thank Prince of Songkla University for the financial support and thank the Faculty of Pharmaceutical Sciences for laboratory facilities.

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