Research work

Part I. Anti-HIV-1 integrase activity of compounds from *Eclipta prostrata*

1. Introduction

Eclipta prostrata Linn. (syn : *E. alba* Hassk., *E. erecta* Hassk.) is a plant in the Compositae family. It is a perennial herb that grows widely throughout tropical areas especially in Asia. In Thai traditional medicine, the leaf of this plant has been used for hair dying and treatment of skin diseases. The stem has been used as a blood tonic and for treatment of anaemia, tuberculosis, amoebiasis and asthma, whereas the root has been used as antibacterial agent, hepatoprotectant and tonic (Tungtrongjit, 1978; Wutthithamavet, 1997). It has been reported that *Eclipta prostrata* exhibits immunomodulatory effect on T-lymphocytes (Liu *et al.*, 2001), anti-inflammatory (Kobori *et al.*, 2004), antimicrobial (Wiart *et al.*, 2004) and hepatoprotective activities (Han *et al.*, 1998).

AIDS has been a major problem in Thailand since the late 1980s (58,000 dealths/year, 21,000 new HIV positives reported/year and 570,000 total HIV-positive patients todate). Specific drug treatment is expensive, and only a very small number of AIDS patients have access to the cocktail of modern antiviral agents. Therefore the majority of AIDS patients resort to using Thai traditional doctors, who prescribe a range of plant-based products. However, scientific studies supporting this use (efficacy, specificity, toxicity) have in most cases not yet been carried out.

A preliminary screening of Thai medicinal plants that are used as self-medication by AIDS patients revealed that the extract of *E. prostrata* exhibited high inhibitory activity against HIV-1 IN with an IC_{50} of 21.1 μg/mL (Tewtrakul *et al.*, 2006). In this study, we therefore report the isolation of active principles from *E. prostrata* and their HIV-1 PR and HIV-1 IN inhibitory activities.

2. Materials and Methods

Plant material

Whole plants of *Eclipta prostrata* were collected at the botanical garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand and identified by Assoc. Prof. Dr. Sanan Subhadhirasakul. The voucher specimen (No. SN 4412025) is deposited at Southern center of Traditional Medicine at Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Extraction and isolation

The dried whole plants (250.0 g) of *E. prostrata* were extracted sequentially with CH_2Cl_2 and MeOH (2 L x 2, 5 days with each solvent) at room temperature. The extracts were filtered and concentrated under reduced pressure to afford CH_2Cl_2 and MeOH crude extracts.

The CH_2Cl_2 extract (1.95 g) was subjected to quick column chromatography (QCC) over silica gel and eluted with a gradient of hexane : ethyl acetate (EtOAc) and EtOAc : MeOH to give four fractions (D1-D4). Fraction D2 (441.4 mg) was further purified by column chromatography (CC) with 5% EtOAc : hexane to yield **1** (10.0 mg), **2** (2.3 mg) and **3** (2.6 mg). Fraction D3 was separated by CC with 30% CH_2Cl_2 : hexane to afford **4** (4.6 mg).

The MeOH extract (1.0 g) was fractionated by CC with hexane and the polarity increased with CH_2Cl_2 and MeOH, respectively, to afford four fractions (M1-M4). Fraction M3 (12.5 mg) was further purified by preparative thin layer chromatography (preparative Silica TLC) with 5% MeOH : 95% CH_2Cl_2 to give **5** (5.4 mg). Fraction M4 (10.0 mg) was subjected by preparative silica TLC with 5% MeOH : 95% CH_2Cl_2 to afford **6** (6.2 mg). These compounds were identified by comparison of their spectroscopic data with those reported in the literatures (Das and Chakranarty, 1991; Ain and Sigh, 1988; Sashida *et al.*, 1983; Kosuge *et al.*, 1985).

Enzymes and chemicals

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

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 integrase enzyme was stored at -80 °C until use.

Assay of HIV-1 PR inhibitory activity

This assay was modified from the previously reported method (Tewtrakul *et al.*, 2003). 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 the ratio of 3 : 1. The substrate peptide, 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 2μL of substrate solution (2mg/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 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 11.33 and 9.47 min, respectively. The inhibitory activity on HIV-1 PR was calculated as follows : % inhibition = ($A_{control} - A_{sample}$) x 100/ $A_{control}$; whereas A is a relative peak area of the product hydrolysate. Acetyl pepstatin was used as a positive control.

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 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 was composed 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

Statistics

For statistical analysis, the results of anti-HIV-1 PR activity were expressed as mean ± S.D of three determinations, while anti-HIV-1 IN were as mean ± S.D of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. Dunnett's test was used versus control for calculation of statistical significance.

3. Results and Discussion

Four compounds belonging to terthiophene derivatives were isolated from the CH_2Cl_2 extract of the whole plants of *Eclipta prostrata*. They were found to be 5-hydroxymethyl-(2, 2′:5′, 2″)-terthienyl tiglate (**1**), 5-hydroxymethyl-(2, 2′: 5′, 2″)-terthienyl agelate (**2**), 5-hydroxymethyl-(2, 2′: 5′, 2″)-terthienyl acetate (**3**), ecliptal (**4**); whereas those of methanol fraction were orobol (**5**) and wedelolactone (**6**) (Figure 1). Of these compounds, wedelolactone (**6**) possessed the highest activity against HIV-1 IN with the IC_{50} value of 4.0 μM, followed by oroboi (**5**) (IC_{50} = 8.1 μM), while all isolated terthiophene compounds were apparently inactive (IC_{50} > 100 μM). Wedelolactone exhibited potent activity, comparable to that of the positive control, suramin (IC_{50} = 2.4 μM). In the case of HIV-1 PR inhibitory activity, **1** exhibited appreciable activity with the IC_{50} value of 58.3 μM, followed by **4** (IC_{50} = 83.3 μM) and **3** (IC_{50} = 93.7 μM), while compounds **2**, **5** and **6** were inactive. This result implies that wedelolactone (**6**)

and orobol (**5**) are selective inhibitors for HIV-1 IN, but not for HIV-1 PR; whereas inhibitory activity of the thiophene derivatives is selective for HIV-1 PR, but not for HIV-1 IN (Table 1, Figure 2 and Figure 3).

Several classes of photosensitizers including sulphur-containing compounds and some terthiophenes have been reported to possess anti-HIV-1 activities. In the present study, compound **1** (*cis*-form), a terthiophene derivative, displayed appreciable activity against HIV-1 PR (IC_{50} = 58.3 μM); whereas its isomer (**2**, *trans*-form) was inactive (IC_{50} > 100 μM).

Coumarins, such as calanolides and inophyllums, have been established as non-nucleotide specific inhibitors of HIV-1 reverse transcriptase (RT). Calanolides A and B isolated from *Calophyllum lanigerum* have been reported to be potent anti-HIV-1 agents (Cardellina *et al.*, 1995). Two coumarins isolated from *Glycyrrhiza glabra*, namely glycocoumarin and licopyranocoumarin, were reported to inhibit giant cell formation in HIV infected cell cultures (Hatano *et al.*, 1988). However, wedelolactone (**6**) which is also a coumarin derivative, has not previously been reported for anti-HIV-1 activity, as well as orobol (**5**), an isoflavone derivative.

In conclusion, among the isolated compounds from *E. prostrata*, wedelolactone (**6**) and orobol (**5**) are responsible for anti-HIV-1 IN activity; whereas 5-hydroxymethyl-(2, 2′: 5′, 2′′)-terthienyl tiglate (**1**), ecliptal (**4**) and 5-hydroxymethyl-(2, 2′: 5′, 2′′)-terthienyl acetate (**3**) act as HIV-1 PR inhibitors. These lead compounds could form the basis of future research in the search for potent selective and non-toxic anti-HIV molecules for the treatment of HIV patients worldwide. This finding also supports the use of *E. prostrata* in AIDS treatment which agrees with its traditional use for treatment of blood-related diseases.

Acknowledgements

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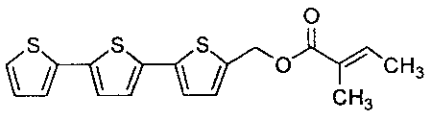
Table 1. Anti-HIV-1 PR and HIV-1 IN activities of compounds isolated from the whole plants of *Eclipta prostrata*

Compounds	IC_{50} (μM) ± S.D.	
	HIV-1 PR[a]	HIV-1 IN[b]
5-Hydroxymethyl-(2, 2′:5′, 2′′)-terthienyl tiglate (**1**)	58.3 ± 0.8	>100 (2.3 %)
5-Hydroxymethyl-(2, 2′: 5′, 2′′)-terthienyl agelate (**2**)	>100 (24.2 %)*	>100 (8.9 %)
5-Hydroxymethyl-(2, 2′: 5′, 2′′)-terthienyl acetate (**3**)	93.7 ± 0.8	>100 (4.0 %)
Ecliptal (**4**)	83.3 ± 1.6	>100 (11.2 %)
Orobol (**5**)	>100 (43.8 %)	8.1 ± 0.5
Wedelolactone (**6**)	>100 (32.7 %)	4.0 ± 0.2
Acetyl pepstatin (Positive control for HIV-1 PR)	3.4 ± 0.2	-
Suramin (Positive control for HIV-1 IN)	-	2.4 ± 0.1

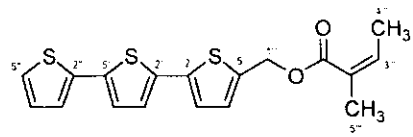
* Values in parenthesis are % inhibition at 100 μM.

[a] Each value represents the mean ± S.D of the three determinations.

[b] Each value represents the mean ± S.D of the four determinations.



5-Hydroxymethyl-(2,2':5',2")-terthienyl tiglate **(1)**



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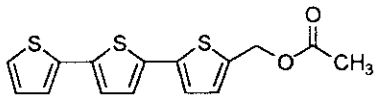
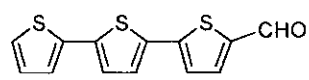
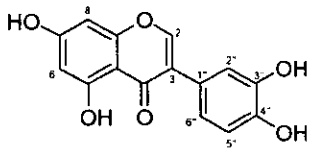


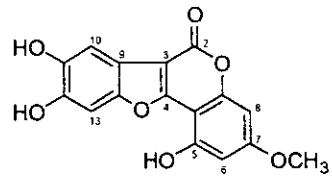
5-Hydroxymethyl-(2,2':5',2")-terthienyl acetate **(3)**



Ecliptal **(4)**



Orobol **(5)**



Wedelolactone **(6)**

Figure 1. Chemical structures of compounds isolated from the whole plants of *Eclipta prostrata*.

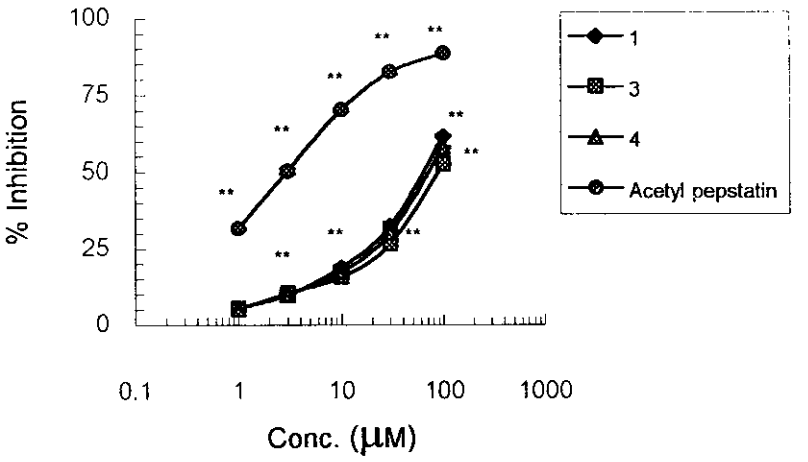


Figure 2. Dose-dependence of anti-HIV-1 PR activity of compounds **1**, **3**, **4** and acetyl pepstatin. Each value represents the mean ± S.D. of three determinations.

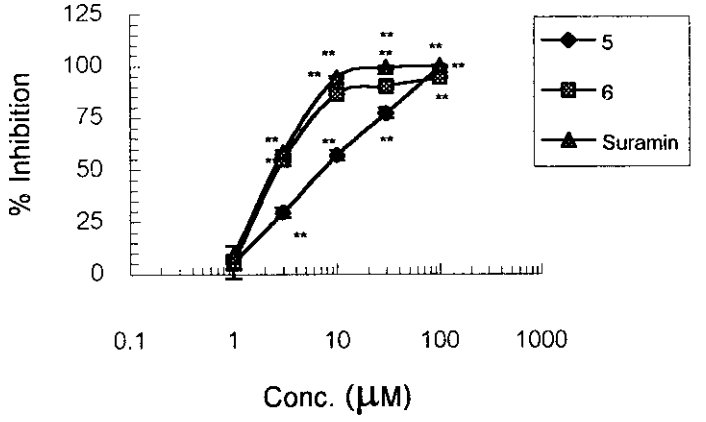


Figure 3. Dose-dependence of anti-HIV-1 IN activity of compounds **5**, **6** and suramin Each value represents the mean ± S.D. of four determinations.