In vitro Inhibitory Effect of Lanostane Triterpenoids of *Kadsura coccinea* on the Human Immunodeficiency Virus Type-1 Protease

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Human immunodeficiency virus type-1 is the causative pathogen of acquired immunodeficiency syndrome and its protease is one of the primary targets for human immunodeficiency virus/acquired immune deficiency syndrome therapy. In this study, two *seco*-lanostane triterpenoids, 3,4-*seco*-9βH-lanost-4(28), 7,24-trien-3-oic acid and 24(E)-3,4-*seco*-9βH-lanost-4(28),7,24-trien-3,26-dioic acid isolated from the roots of *Kadsura coccinea*, were found to significantly inhibit human immunodeficiency virus-1 protease, with IC_{50} values of 1.0±0.03 and 0.05±0.009 µM, respectively. Neither compound was toxic to human embryonic kidney 293T cells at concentrations effective against human immunodeficiency virus-1 protease. Our findings indicate that these triterpenoids are potential candidates for development of antihuman immunodeficiency virus/acquired immune deficiency syndrome drugs.

Key words: HIV-1 protease inhibitor, *Kadsura coccinea*, *seco*-coccinic acid F, 24(E)-3,4-*seco*-9βH-lanost-4(28),7,24-trien-3,26-dioic acid, *seco*-lanostane triterpenoid

The protease (EC 3.4.23.16) encoded by the human immunodeficiency virus type 1 (HIV-1) genome plays a crucial role in the life cycle of the virus. Inhibition of the HIV-1 protease leads to the formation of immature non-infectious virions; therefore, the enzyme has become an important target in HIV drug development[1]. HIV-1 protease inhibitors developed to this point are substrate-based and compete with the natural substrate for the active site of the enzyme. Most are peptide mimetic compounds. However, it was reported that these compounds have low bioavailability when administered orally and are too expensive

to serve as a feasible treatment option for patients living in developing countries[2]. Several side effects associated with these drugs have been described, such as fat accumulation, kidney stone formation, and cutaneous effects. More importantly, a high mutation rate of the virus has been reported, which would result in the generation of new resistant strains of the virus[3]. To stop the rapid spread of the HIV epidemic, innovative combinations of drugs with diverse antiHIV mechanisms must be effectively used. Potential HIV-1 protease inhibitors obtained from natural sources are fast becoming a promising strategy for the development of new treatments[2,4].

Kadsura coccinea (Lem.) AC Smith is an evergreen member of the family Schisandraceae. The genus *Kadsura* is closely related to *Schisandra* and many of its species are extensively used as substitutes for *Schisandra* in Chinese medicinal formulations in Taiwan, Japan, and Vietnam[5,6]. Extracts of this plant have been used as tonics, decongestants, and digestive agents in Vietnamese traditional medicine. The roots are also used to treat chronic enteritis, acute gastritis, and rheumatic pain in the bones[7]. A number of triterpenoids, particularly of the lanostane type, were isolated from the roots of *K. coccinea* and shown to possess various biological activities such as inhibition of human leukaemia cell growth[8] and protective effects against haemocyte necrosis[7].

Wei *et al*.[9] reported the presence *seco*-triterpenoids with ring A from *Stauntonia obovatifoliola* Hayata subspecies. Intermediates have potent antiHIV-1 protease activity. The results of the study indicated that the 2,3-*seco*-2,3-dioc acid moiety in ring A of these triterpenoids is an important pharmacophore in the design and synthesis of HIV-1 protease inhibitors because some triterpenoids derivatives with a 3-*O*-acidic acyl group also exhibit strong antiHIV activity. It is of interest to us to examine the antiHIV activity of other *seco*-triterpenoids. In a previous study, two *seco*-lanostane-type triterpenoids, 3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3-oic acid (*seco*-coccinic acid F) and 24(E)-3,4-*seco*-9*β*H-lanost-4(28), 7,24-trien-3,26-dioic acid (fig. 1), were isolated from the ethanol (EtOH) extract of the roots of *K. coccinea*[6]. In this study, we evaluated the inhibitory effects of these compounds on HIV-1 protease. Maslinic acid (oleanane triterpenoid), ursolic acid (ursane triterpenoid), and fetal calf serum were purchased from Sigma-Aldrich (USA). Human embryonic kidney (HEK 293T) cells were purchased from Corning, USA. Recombinant HIV-1 protease was prepared in our laboratory according to a previously reported procedure[10]. The fluorogenic substrate for HIV-1 protease (QXL 520-GABA-SFNFPQITK-HiLyte Flour 488-NH_2, designed based on the specific hydrolysis sequence in the Gag-Pol protein substrate of HIV-1 protease) was synthesized and purified by high-performance liquid chromatography (HPLC: to 95 % purity by AnaSpec, USA). Other reagents and solvents used were of analytical grade.

The roots of *K. coccinea* were collected in the TrangDinh district, Lang Son province in September 2009, identified, sliced, dehumidified at 50° to dryness, and stored as a voucher specimen no. VDL.NR01.2009 in the herbarium (a dry room, temperature <25°, insects and microbial avoided) of the National Institute of Medicinal Materials, Vietnam.

Two lanostane-type triterpenoids, 3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3-oic acid (*seco*-coccinic acid F, $C_{30}H_{48}O_2$, molecular weight of 440, fig. 1) and 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid ($C_{30}H_{46}O_2$, molecular weight of 470, fig. 1), were isolated from the EtOH extract of the roots of *K. coccinea* according to the procedure described previously by Nguyen *et al*.[6]. Briefly, roots of *K. coccinea* (2 kg) were cut into small pieces and macerated three times with EtOH 80 % (v/v). Evaporation of the solvent under reduced pressure from the extract yielded the EtOH extract (150 g), which was then dissolved and suspended in H_2O (500 ml), then partitioned in turn with *n*-hexane, ethyl acetate (EtOAc), and butanol (BuOH). The hexane extract (64 g) was subjected to silica gel column chromatography, eluted with hexane/EtOAc (100:1, 90:1, 80:1, 10:1, 1:1, 0:1, 500 ml each) to yield fractions I-V. Fraction III (12.6 g) was rechromatographed on a silica gel column and eluted with a gradient solvent system of hexane/EtOAc (8/1, 6/1, 4/1 and 1/2, 1000 ml

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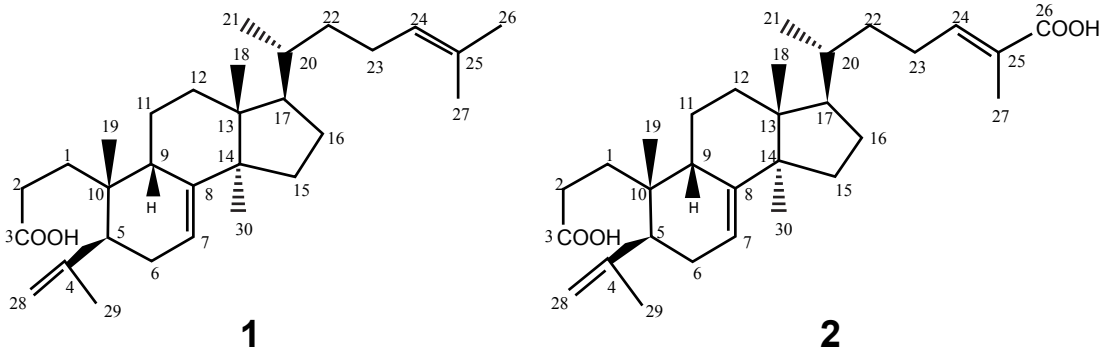

Fig. 1: Chemical structures of the triterpenoids isolated from *K. coccinea*
Chemical structures of 1. *seco*-coccinic acid F and 2. 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid isolated from *K. coccinea*

each) to obtain 3 fractions (III.1-III.3): III.1 (hexane/EtOAc 8/1, 6/1), III.2 (hexane/EtOAc 4/1), and III.3 (hexane/EtOAc 1/2). Fraction III.2 was repeatedly applied to a silica gel column and eluted with hexane/EtOAc (4:1) to obtain compound 1 (*seco*-coccinic acid F, 125 mg). Fraction IV (10.8 g) was repeatedly applied to a silica gel column and eluted with a gradient solvent system of hexane/EtOAc (4:1, 2:1, 1:1, 0:1; 1000 ml each) to obtain 4 fractions (IV.1-IV.4): IV.1 (hexane/EtOAc, 4:1), IV.2 (hexane/EtOAc, 2:1), IV.3 (hexane/EtOAc, 1:1), and IV.4 (hexane/EtOAc, 0:1). Fraction IV.3 appeared as the major mark on the chromatogram and was treated with methanol (MeOH) to give compound 2 (24(E)-3,4-seco-9βH-lanost-4(28),7,24-trien-3,26-dioic acid, 50 mg). Compounds 1 and 2 had a purity of ≥ 93 %, as indicated by HPLC.

Seco-coccinic acid F (3,4-*seco*-9*β*H-lanost-4(28), 7,24-trien-3-oic acid, compound 1) was an amorphous powder, melting point 160-163°. UV (MeOH) λ_{max}: 201 nm. IR ν_{max} (cm^{-1}): 2968, 1721, 1651, 1455, 1276, 1114. APCI-MS (*m/z*) 441 $[M+H]^+$ ($C_{30}H_{48}O_2$); ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) in dimethyl sulfoxide (DMSO; Table 1).

24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid (compound 2) was amorphous powder, melting point 234-236°; UV (MeOH) λ_{max}: 202 nm; IR ν_{max} (cm^{-1}): 3464; 2956; 1690; 1653; 1505; 1289; 1072; APCI-MS (*m/z*) 471 $[M+H]^+$ ($C_{30}H_{46}O_2$); ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) in DMSO (Table 1).

HIV-1 protease activity assay was performed using a fluorogenic substrate (QXL 520-GABA-SFNFPQITK-HiLyte Flour 488-NH_2). The assay mixture (30 µl) contained HIV-1 protease assay buffer (100 mM sodium acetate buffer, pH 4.7, containing 1 M NaCl, 1 mM ethylene diaminetetraacetic acid, 1 mM dithiothreitol, 5 % DMSO, 1 mg/ml bovine serum albumin, 150 ng HIV-1 protease, and 2 µM fluorogenic substrate). The assays were performed at 37° and the start of the reaction was recorded as the time at which the substrate was added into the mixture. The reaction mixture was excited at 490 nm and emitted at 525 nm using a Nanodrop 3300 fluorospectrometer (Thermo Fisher Scientific, USA).

For the HIV-1 protease inhibitory activity assay, the test compound (dissolved in 5 % DMSO) was preincubated with HIV-1 protease at different concentrations (0-10 µM *seco*-coccinic acid F and 0-1 µM 24(*E*)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic) for 5 min prior to the addition of the substrate. Based on residual activity ($(\Delta_{experimental} \times 100\%)/\Delta_{control}$, where Δ was the change in emission at 525 nm per second of HIV-1 protease activity), the inhibitory activity of the compound was calculated as a percent of the residual activity of the control, in which inhibitor was absent (100 %).

A tetrazolium salt (MTS) assay was performed to determine the toxicity of two lanostane triterpenoids against HEK 293T cells. HEK 293T cells were maintained in culture flasks and grown at 37° in a CO_2 incubator (5 %), as monolayers in Dulbecco's modified Eagle's medium (Corning) supplemented with 10 % (v/v) fetal calf serum and buffered with 3.7 g/l $NaHCO_3$. Cells were inoculated into a flat-bottomed clear 96-well microplate (Corning, USA) at a density of 1×10^4 cells/well in 180 µl of culture medium. The cultures were incubated for 24 h, then treated for

TABLE 1: DATA FOR ^{1}H- (500 MHz) AND ^{13}C-NMR (125 MHz) SPECTRA OF COMPOUNDS 1 AND 2

Position	Compound 1		Compound 2	
	seco-coccinic acid F		24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid	
	δ_H (m, *J* in *Hz*)	δ_C	δ_H (m, *J* in *Hz*)	δ_C
1	*	28.6	*	28.7
2	*	28.3	*	29.7
3	-	174.8	-	175.6
4	-	149.3	-	149.5
5	*	44.7	*	44.7
6	*	28.8	*	28.9
7	5.30 (1H, m)	117.1	5.29 (1H, m)	117.1
8	-	146.2	-	146.2
9	*	38.4	*	38.4
10	-	35.7	-	35.8
11	*	17.9	*	18
12	*	33.3	*	33.4
13	-	43.1	-	43.1
14	-	51	-	50.9
15	*	33.5	*	33.5
16	*	27.5	*	27.4
17	*	52.2	*	52.2
18	0.73 (3H, s)	21.1	0.74 (3H, s)	21.1
19	0.81 (3H, s)	23.4	0.80 (3H, s)	23.5
20	*	35.1	*	35.2
21	0.88 (3H, d, 6.0)	17.9	0.90 (3H, d, 6.5)	17.8
22	*	35.5	*	34.4
23	*	24.2	*	24.8
24	5.09 (1H, t, 7.0)	124.7	6.54 (1H, t, 7.0)	138.9
25	-	130	-	129.3
26	1.63 (3H, s)	24.9	-	-
27	1.58 (3H, s)	17.1	1.77 (3H, s)	12.2
28	4.84 (1H, br s) 4.78 (1H, br s)	111.3	4.83 (1H, br s) 4.77 (1H, br s)	111.2
29	1.77 (3H, s)	25.1	1.72 (3H, s)	24.9
30	1.00 (3H, s)	27	1.01 (3H, s)	27

Spectra were recorded in DMSO; '*' overlap signals; s- singlet; br s- broad singlet; d- doublet; t- triplet and m- multiplet

48 h with different concentrations of test compounds (0-125 µM *seco*-coccinic acid F and 0-37.5 µM 24(*E*)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic). Each concentration was tested in triplicate at 37° under 5 % CO_2. Thereafter, 20 µl of MTS solution (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega, USA) was added to each well and the cells were incubated for an additional 2.5 h at 37° and 5 % CO_2. The plate was gently shaken and absorbance at 490 nm was measured using a Model 680 Microplate reader (Bio-Rad, USA). All drug doses were tested using DMSO as the control in triplicate.

Data in all the figures are the mean±standard error of 3 determinations. *Seco*-coccinic acid F (3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3-oic acid (compound 1) and 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3, 26-dioic acid (compound 2) were isolated from the EtOH extracts of the roots of *K. coccinea*. The compounds had purity of ≥93 %, as determined by HPLC and were confirmed by UV, IR, MS, and NMR analysis (Table 1) before testing the inhibitory activity of HIV-1 protease.

In this study, we found that these two *seco*-lanostane triterpenoids strongly inhibited HIV-1 protease, with IC_{50} values of 1.0±0.03 µM (fig. 2a) and 0.05± 0.009 µM (fig. 2b), respectively. As references, two other triterpenoids, maslinic acid (oleanane triterpenoid) and ursolic acid (ursane triterpenoid) that are known inhibitors of HIV-1 protease were tested. They yielded IC_{50} values of 2.6±0.5 µM (fig. 2c) and 4±0.5 µM (fig. 2d), respectively, under the same experimental conditions.

Maslinic acid and ursolic acid are pentacyclic triterpenoids found in a variety of natural sources[11,12]

and are known non-peptide HIV-1 protease inhibitors, with IC_{50} values reported in the range of 2.5-8 μM[4,13]. However, their potency against HIV-1 protease was much lower than that of the two *seco*-lanostane triterpenoids evaluated in this study. The difference in the structures of the triterpenoids might have caused this discrepancy. The carboxylic moiety at the 3' position and 4,4,14-trimethyl cholestan frame seems to have conferred higher inhibitory activity to the *seco*-lanostane triterpenoids (IC_{50}, 0.05-1 μM) than that conferred by the hydroxy group to maslinic and ursolic acids (IC_{50}, 2.6-4 μM). This result was consistent with the observation that the polar-function group of the C-3 side chain of triterpenoids played an important role in interacting with HIV-1 protease[4].

To determine the mechanism by which *seco*-coccinic acid F and 24(E)-3,4-*seco*-9*β*H-lanost-4(28), 7,24-trien-3,26-dioic acid inhibited HIV-1 protease, the changes in the Michaelis-Menten constant (K_m) and the maximum reaction velocity (V_{max}) at different substrate concentrations were measured in the absence and presence of the inhibitors, using the Lineweaver-Burk Eqn. *Seco*-coccinic acid F at a final concentrations of 1 and 2 μM was added to the reaction mixtures containing HIV-1 protease either prior to or after the addition of substrate. Similarly, 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid was added in the same manner at concentrations of 0.025 and 0.075 μM. As shown in fig. 3, the V_{max} and K_m values in the absence of the *seco*-coccinic acid F or 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid were 43.09 RFU/s and 7.71 μM, respectively, and they were higher than values observed in the presence of the inhibitors. The inhibition patterns remained the same whether the inhibitors were added before or after the substrates (fig. 3). Altogether, the data indicated that *seco*-coccinic acid F and 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid did not bind HIV-1 protease at its active site and the compounds likely inhibited the enzyme in an uncompetitive manner.

Seco-coccinic acid F was isolated for the first time from the roots of *K. coccinea* and exhibited anticancer activity by inhibiting the growth of human leukemia HL-60 cells[7,13], whereas 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid was isolated from *Abies koreana* and exhibited weak activity against

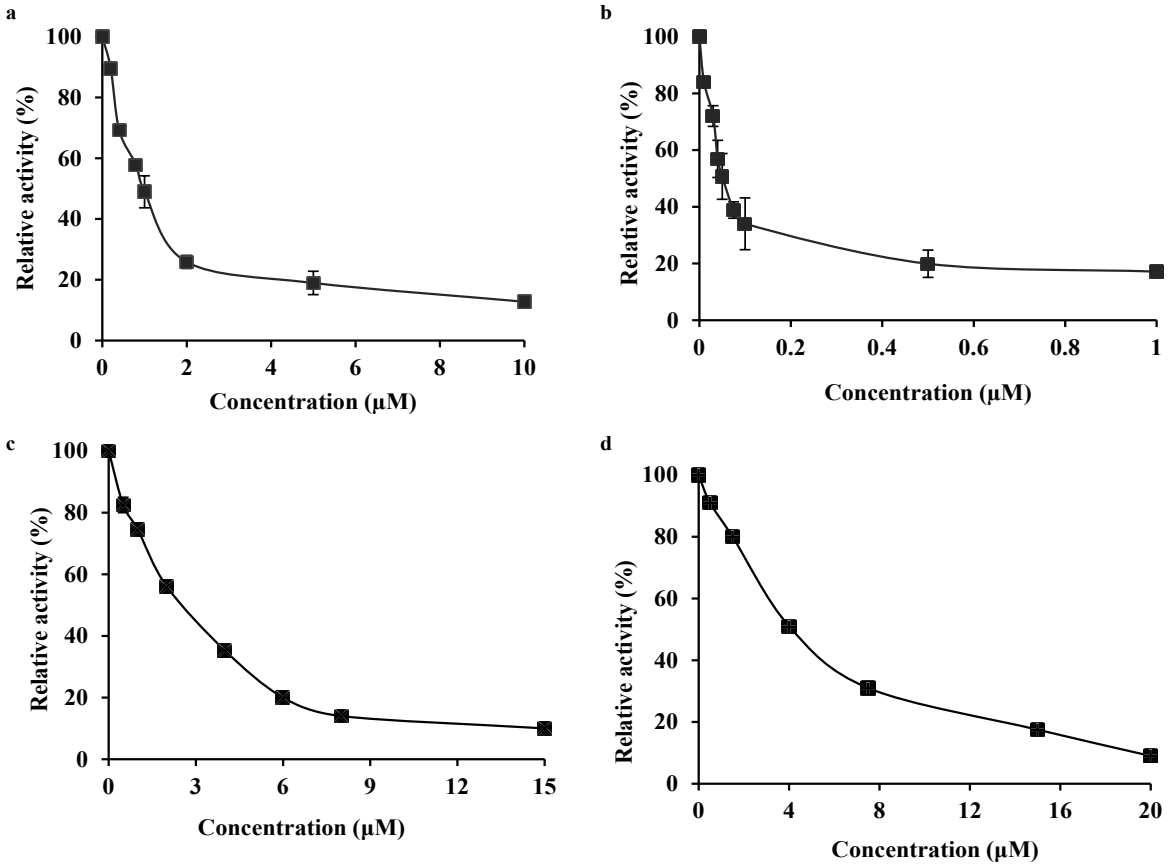

Fig. 2: Inhibitory effect of triterpenoids on HIV-1 protease
Inhibitory effect of a. *seco*-coccinic acid F, b. 24(*E*)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid, c. maslinic acid and d. ursolic acid on HIV-1 protease

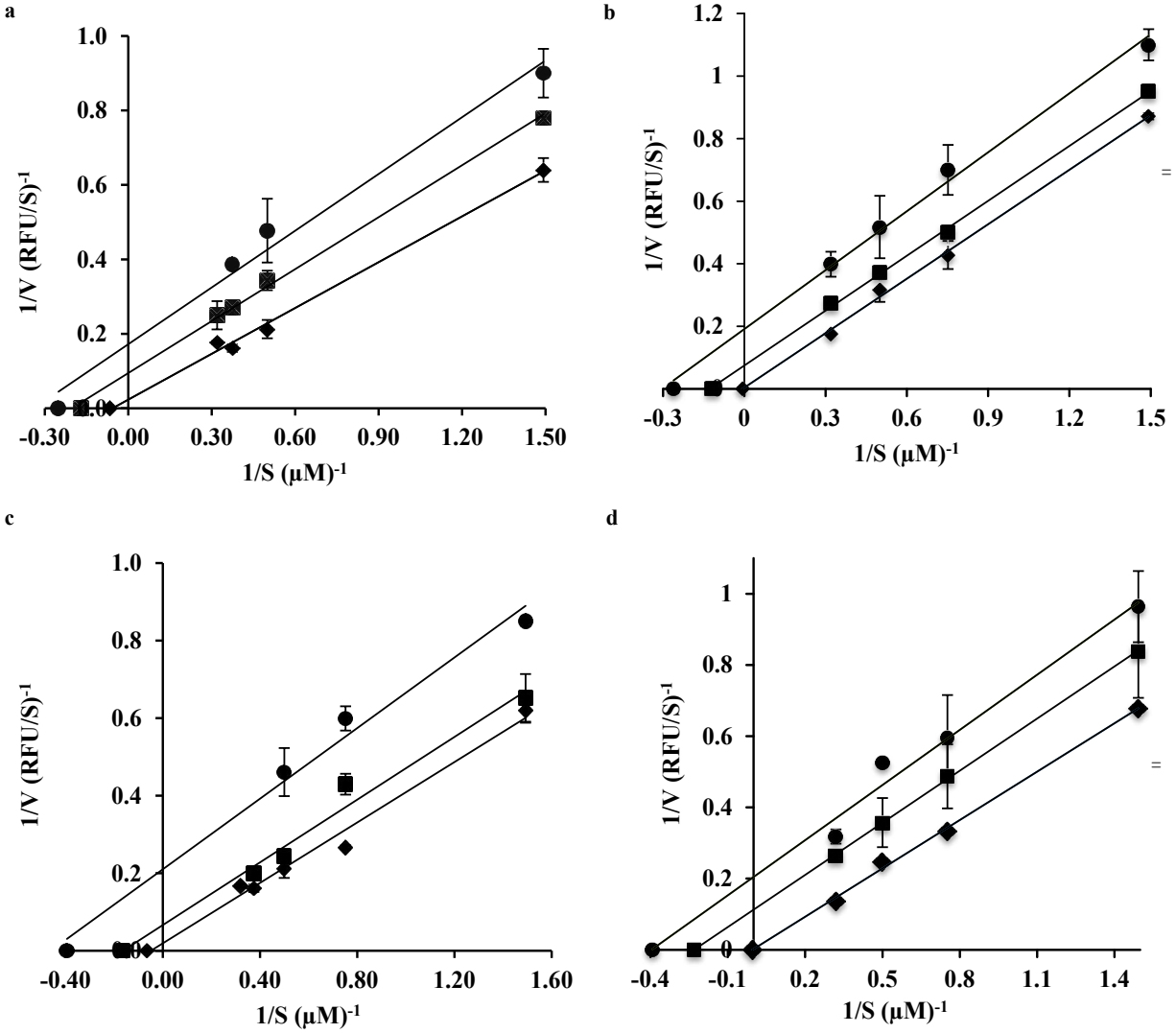

Fig. 3: Lineweaver-Burk plots for kinetic analysis of HIV-1 protease inhibitory activity

Lineweaver-Burk plots of HIV-1 protease inhibitory activity by (a, b) *seco*-coccinic acid F and (c, d) 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid. Symbols (◊), (■), (●) represent *seco*-coccinic acid F at concentrations of 0, 1, and 2 µM, respectively, added into the assay mixture (a) after and (b) before substrate as well as 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid at concentration of 0, 0.025, and 0.075 µM, respectively, added into the assay mixture (c) after and (d) before substrate

human tumor cell lines[14]. Several previous studies reported that many compounds isolated from the *Kadsura* genus of plants were active against HIV. For example, binankadsurin A, isolated from *K. angustifolia*, exhibited potent antiHIV activity, with an EC_{50} value of 3.86 µM[15]; kadsulignan N, isolated from *K. coccinea*, exhibited strong activity against HIV *in vitro*, with an IC_{50} value of 0.0119 µM; and schizarin E, isolated from *K. matsudai*, demonstrated a strong inhibitory effect against HIV replication in H9 lymphocytes, with an IC_{50} value of 2.08 µg/ml[16]. Many lanostane triterpenoids were isolated from *K. coccinea*[5,8,13], but this is the first study to evaluate the inhibitory activity of *seco*-coccinic acid F and 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid against HIV-1 protease.

The toxicity of *seco*-coccinic acid F and 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid against HEK 293T cells was determined at different concentrations, starting from 0.5 µM to the maximum concentration of each compound soluble in DMSO (125 µM in case of *seco*-coccinic acid F and 37.5 µM in case of 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid). Intrinsic cytotoxicity was represented by the concentration leading to 50 % cell death (IC_{50}), as determined by MTS assay. The results indicated that *seco*-coccinic acid F killed HEK 293T cells, with an IC_{50} value of 40 µM (fig. 4a), whereas at the same concentration, 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid did not exhibit any effect on the cells (fig. 4b). As mentioned

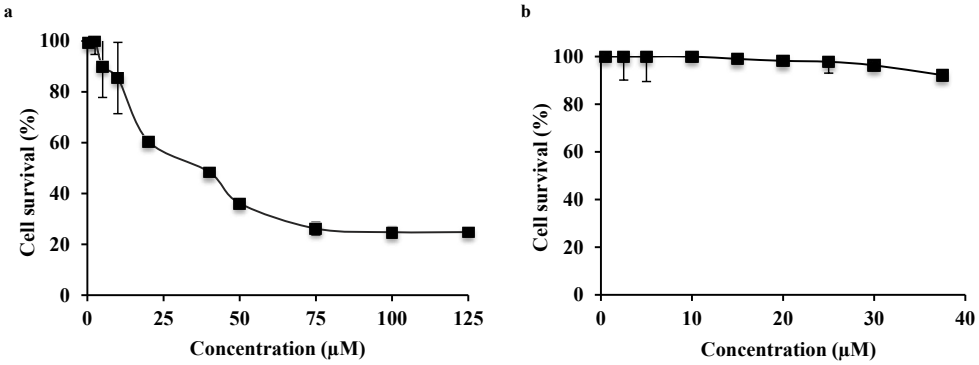

Fig. 4: Toxicity against HEK 293T cells
Toxicity of (a) *seco*-coccinic acid F and (b) 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid against HEK 293T cells

above, the two *seco*-lanostane triterpenoids investigated were potent HIV-1 protease inhibitors with IC_{50} values of 1.0±0.03 and 0.05±0.009 µM, respectively. These concentrations were much lower than the concentrations at which they showed toxicity against HEK 293T cells. Consequently, our data indicated that *K. coccinea*, a traditional medicinal plant used in Vietnam, and these *seco*-lanostane triterpenoids were potential candidates for development of antiHIV/AIDS drugs.

This study indicated that two *seco*-lanostane triterpenoids (*seco*-coccinic acid F and 24(E)-3,4-*seco*-9*β*H-lanost-4(28),7,24-trien-3,26-dioic acid) isolated from the roots of *K. coccinea* significantly inhibited HIV-1 protease and showed no toxicity against HEK 293T cells at concentrations effective against HIV-1 protease. These findings support the potential development of these compounds as drugs for the treatment of HIV/AIDS.

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Conflict of interest

The authors declare no conflicts of interest.

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