Sennoside A, derived from the Traditional Chinese Medicine plant *Rheum L.*, is a new dual HIV-1 inhibitor effective on HIV-1 replication

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ABSTRACT

Background: Despite the availability of effective antiretroviral therapies, drugs for HIV-1 treatment with new mode of action are still needed. An innovative approach is aimed to identify dual HIV-1 inhibitors, small molecules that can inhibit two viral functions at the same time. Rhubarb, originated from *Rheum palmatum* and *R. officinale*, is one of the earliest and most commonly used medicinal plants in Traditional Chinese Medicine (TCM) practice. We wanted to explore TCM for the identification of new chemical scaffolds with dual action abilities against HIV-1.

Methods: *R. palmatum* and *R. officinale* extracts along with their main single isolated constituents anthraquinone derivatives were tested on both HIV-1 Reverse Transcriptase (RT)-associated DNA Polymerase (RDDP) and Ribonuclease H (RNase H) activities in biochemical assays. Active compounds were then assayed for their effects on HIV-1 mutated RTs, integrase (IN) and viral replication.

Results: Both *R. palmatum* and *R. officinale* extracts inhibited the HIV-1 RT-associated RNase H activity. Among the isolated constituents, Sennoside A and B were effective on both RDDP and RNase H RT-associated functions in biochemical assays. Sennoside A was less potent when tested on K103N, Y181C, Y188L, N474A and Q475A mutated RTs, suggesting the involvement of two RT binding sites for its antiviral activity. Sennoside A affected also HIV-1

IN activity *in vitro* and HIV-1 replication in cell-based assays. Viral DNA production and time of addition studies showed that Sennoside A targets the HIV-1 reverse transcription process.

Conclusion: Sennoside A is a new scaffold for the development of HIV-1 dual RT inhibitors.

Keywords: Sennoside A, HIV-1, Reverse Transcriptase, antiviral activity, plant extracts, dual inhibitor.

Abbreviations: DKA, diketo acid; EC_{50}, HIV-1 replication by 50%; HAART, Highly Active Anti-Retroviral Therapy; HIV-1, Human Immunodeficiency Virus type 1; IN, integrase; INIs, HIV-1 IN inhibitors; NNRTI, Non-Nucleoside RT Inhibitor; NRTIs, Nucleoside RT Inhibitors; PI, Protease Inhibitor; qPCR, quantitative real-time PCR; RDDP, Reverse Transcriptase (RT)-associated DNA Polymerase; RNase H, Ribonuclease H; $TCID_{50}$50%/ml, Tissue Culture Infective Doses; TCM, Traditional Chinese Medicine.

1. INTRODUCTION

The Human Immunodeficiency Virus type 1 (HIV-1) encoded Reverse Transcriptase (RT) is responsible for the reverse transcription process of the viral genome, a fundamental step in the HIV-1 replication cycle and a still attractive target for the identification of new antiretroviral inhibitors (Esposito et al. 2012; Corona et al. 2013; Distinto et al. 2013; Esposito and Tramontano 2013). The HIV-1 RT is a multifunctional protein endowed of two main enzymatic functions: an RNA-Dependent DNA Polymerase (RDDP) activity, which accounts for the formation of the RNA:DNA intermediate, and a Ribonuclease H (RNase H) activity, involved in the hydrolytic cleavage of the RNA strand of the RNA:DNA hybrid (Esposito et al. 2012). Currently, for the > 30 million of people worldwide infected by HIV-1 no vaccine is available, but an effective antiretroviral therapy has been developed, termed Highly Active Anti-Retroviral Therapy (HAART). HAART first line treatment is usually composed by a combination of two Nucleoside RT Inhibitors (NRTIs) to which a Non-Nucleoside RT Inhibitor (NNRTI) or a Protease Inhibitor (PI) is added. HIV-1 IN inhibitors (INIs) are expected to retain efficacy against HIV-1 strains resistant to other antiretroviral drugs, and represent a valid second-line therapy. The acute and chronic drug toxicities and the selection of drug resistant strains, in particular to RTIs, represent a valid reason to identify new antiviral agents. Indeed, the development of drugs with new mode of action, such as single molecules that may act on two different target enzymes or two

94 different catalytic functions, would reduce the number of administered drugs,
95 their chronic toxicity and the chance of selecting drug resistant viruses
96 (Distinto et al. 2013; Esposito and Tramontano 2013) .
97 In the last 20 years, several research efforts have been focused on exploiting
98 natural products as scaffolds for the development of potential antiviral agents
99 (Li and Vederas 2009; Cos et al. 2008; Yu et al. 2007; Bicchi et al. 2009; Xu et
100 al. 2015), although no natural compound able to block HIV-1 replication has
101 ever entered clinical trials. In China, Traditional Chinese Medicine (TCM) is the
102 major ancient therapeutic system and its herbal component is the most
103 important one. Radix et Rhizoma Rhei (Da Huang in Chinese), usually
104 originated from *Rheum palmatum* and *R. officinale*, is one of the earliest and
105 also the most commonly used medicinal plant in TCM practice (Beijing:
106 Chinese Pharmaceutical Science and Technology Publishing House, 2010.
107 The earliest record of Rhubarb use appears to come from the Shen Nong
108 Materia Medica (A. D. 102-200) of E. Han Dynasty and it was reported to be
109 adopted to treat constipation and dysentery. Based on the modern
110 investigations, the new application of Rhubarb involves cure of chronic renal
111 failure, protection of damaged liver, and anti-aging effects (Xu et al. 2015; Lu
112 et al. 2014; Xie and Sang 2014). *Rheum* (Polygonaceae) includes more than
113 60 species distributed worldwide, with 39 species in the Western and Northern
114 part of China, is one of the most important medicinal resources in China and
115 one of the main plant source containing natural anthraquinone compounds

(Xiao et al. 1980). Among the anthraquinones reported to be present in Rhubarb (Ye et al. 2007), Sennosides (Sennoside A and Sennoside B) are considered to be the purgative components (Xiao et al. 1980; Zwaving 1972), Rhein and Physcion possess antitumor and anti-inflammatory properties (Zwaving 1972; He et al. 2011), with the latter presenting also hepatoprotective, antifungal and anticancer activities (He et al. 2011). Aloe-Emodin, Emodin and Chrysophanol have been reported to decrease the levels of serum total bilirubin, showing partial protective effects on cholestatic liver injury (Zhao et al. 2009). Glucosyl gallates (Maldonado et al. 2011), Naphthalenes (Tsuboi et al. 1977), and Catechins (Sill et al. 1974) exhibited potential antioxidant and anticancer properties. A number of polyphenol derivatives, including, for instance, anthraquinones, have been reported to interfere with the life cycle of different viruses, among which encephalomyocarditis virus in mice (Barnard et al. 1992), human cytomegalovirus (Barnard et al. 1992; Bamard et al. 1995), poliovirus (Semple et al. 2011) and hepatitis B virus (Dang et al. 2006), as well as to inhibit the catalytic activity of HCV NS5B Polymerase (Tramontano et al. 2011) and HIV-1 RT (Tramontano et al. 2011; Higuchi et al. 1991; Esposito et al. 2012; Esposito et al. 2011; Kharlamova et al. 2009) and integrase (IN) functions in biochemical assays (Tintori et al. 2015; Esposito et al. 2015). In particular, a number of anthraquinone derivatives were reported to affect both HIV-1 RDDP and RNase H RT-associated functions in biochemical assays, but were not

138 active on viral replication in cell-based assays (Tramontano et al. 2011;
139 Higuchi et al. 1991; Esposito et al. 2012; Esposito et al. 2011; Kharlamova et
140 al. 2009). Hence, we wanted to evaluate the effects of the chemical
141 components originated from the Rheum Chinese plants to inhibit the HIV-1
142 RT-associated and IN activities and to interfere with the HIV-1 life cycle in
143 order to identify new scaffold inhibiting multiple HIV-1 targets.
144

145 ## 2. MATERIALS AND METHODS

146 **2.1. Plant materials and reagents.** From June 2007 to September 2007, *R.*
147 *palmatum* and *R. officinale* were collected from Gansu and Sichuan province
148 respectively in China by one of the authors, and were naturally dried at room
149 temperature. The raw materials were authenticated by Prof. Anren Li (Institute
150 of Medicinal Plant Development, Beijing, China). The voucher specimens were
151 deposited in the Laboratory of Pharmaphylogeny, Institute of Medicinal Plant
152 Development (Beijing, China). The reference compounds Aloe-Emodin, Rhein,
153 Emodin, Chrysophanol, Physcion were purchased from the National Institute
154 for Control of Pharmaceuticals and Biological Products (Beijing, China), and
155 Sennoside A and Sennoside B from the Mansite Pharmaceutical Co. Ltd.
156 (Chengdu, China). HPLC grade acetonitrile and methanol were purchased
157 from Burdick & Jackson (Muskegon, MI, USA). Deionized water was prepared
158 by Milli-Q system (Millipore, Bedford, MA, USA). Phosphoric acid and

159 methanol were of analytical grade purchased from Beijing Beihai Fine
160 Chemicals Co. Ltd. (Beijing, China).

161 **2.2 Preparation of sample solutions.** The dried roots of plant materials were
162 grounded by an electric mill into powder (40 mesh). Individual samples (~200
163 mg) were accurately weighed, then mixed with 30 ml methanol/water (30 ml,
164 80:20, v/v) and sonicated for 60 min at room temperature. The extract solution
165 was cooled down to room temperature, and appropriate amount of the solution
166 was added to the original weight. The final solution was filtered through 0.22
167 μm membrane and then injected 10 μl for each HPLC analysis (Fugure S1).

168 **2.3 Protein expression and purification.** HIV-1 RT gene subcloned into the
169 p6HRT_prot plasmid was kindly provided by Stuart Le Grice (NCI, Frederick,
170 Maryland, USA). Protein expression and purification was performed in M15
171 *Escherichia coli* cells as described (Esposito et al. 2012). Full-length IN and
172 LEDGF proteins were expressed in BL21 *E. coli* (DE3) (Tintori et al. 2015;
173 Esposito et al. 2015). HIV-1 K103N, Y108C and Y188L RT mutants were
174 produced by site-directed mutagenesis using the Stratagene kit (Agilent
175 Technologies, Milano, Italy), according to manufacturer's indication as
176 described (Corona et al. 2014).

177 **2.4 RNase H polymerase-independent cleavage assay**. The HIV-1
178 RT-associated RNase H activity was measured as previously described
179 (Esposito et al. 2013).

2.5 RDDP assay. The HIV-1 RT-associated RDDP activity was measured using the Enz-Check Reverse Transcriptase Assay Kit (Life technologies, Carlsbad, California, USA), as previously described (Corona et al. 2014). The Yonetani-Theorell analysis was performed as previously reported (Esposito et al. 2011).

2.6 Homogeneous Time Resolved Fluorescence (HTRF) LEDGF dependent and independent assay. The IN LEDGF/p75 dependent assay allowed to measure the inhibition of the 3' processing and strand transfer IN reactions in the presence of recombinant LEDGF/p75 protein, as previously described (Tintori et al. 2015; Esposito et al. 2015).

2.7 Cell line cultures. The human T-lymphoblastoid Jurkat cell line, clone E6-1, and the human embryonic kidney 293T cell line were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI 1640 or DMEM (GIBCO, Life Technologies, Monza, Italy), respectively, supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in 5% CO_2 humidified atmosphere.

2.8 Plasmids and Viruses. HIV-1 stock was produced by transient transfection of Jurkat cells with the pSVC21 plasmid containing the infectious HXBc2 molecular clone of HIV-1 (Ratner et al. 1985) by the DEAE-dextran method as described previously (Cullen 1987) and stored at −80 °C until use. Viral titer was measured as 50% Tissue Culture Infective Doses ($TCID_{50}$)/ml

on C8166 cells by the Reed and Muench end point dilution method (Reed and Muench 1983).

Recombinant HIV-1 HXBc2 CAT virus was produced by co-transfection of 293T cells with the pSVC21 $Vpr^{+}Vpu^{+}Nef^{+}\Delta env$-CAT plasmid along with the pSVIIIenv plasmid (J. Sodroski, Harvard University, Boston), with the $CaPO_4$ method. The pSVC2.1 $Vpr^{+}Vpu^{+}Nef^{+}\Delta env$ CAT plasmid (Sartori et al. 2011) was generated from the pSVC2.1 plasmid (J. Sodroski, Harvard University, Boston) which contains the complete proviral genome of the HIV-1 HXBc2 (vif^{+}-vpu^{-}-vpr^{-}-nef^{-}) (Ratner et al. 1985), by inserting the Vpu, Vpr and Nef encoding sequences and deleting the BglII-BglII env region. The chloramphenicol acetiltransferase (CAT) reporter gene was inserted in the pSVC2.1 $Vpr^{+}Vpu^{+}Nef^{+}\Delta env$ vector with resulting inactivation of the rev gene. The pSVIIIenv plasmid expresses the HIV-1 laboratory-adapted T-cell-tropic strain HXBc2 envelope glycoprotein along with Rev. Recombinant HIV-1 virions expressing CAT were collected 48 hours post-transfection and filtered (0.45-µm-pore-size filter). Viral titer was determined in terms of Reverse Transcriptase (RT) activity (Rho et al. 1981).

2.9 HIV-1 *env* complementation assay. Jurkat cells (1×10^6) were incubated with 30,000 ^3H cpm RT units of recombinant CAT reporter virus at 37°C in the absence or presence of different amount of compounds (5, 10 and 20 µM). Cells were lysed 4 days after infection and CAT activity was determined. Compound concentration required to inhibit early phases of HIV-1 replication

223 by 50% (EC_{50}) was calculated by nonlinear regression analysis with Sigma
224 Plot (Jandel Scientific). Only results within a linear range (HIV-1 LTR-driven
225 reporter CAT gene expression, i.e. conversion of chloramphenicol to acetyl
226 chloramphenicol above 50%) were elaborated.
227 **2.10 Cytotoxicity assay.** The cytotoxicity of the compounds on the Jurkat cell
228 line was assessed using the MTT method (Mosmann 1993).
229 **2.11 Quantitative Real Time PCR Analysis of HIV-1 DNA.** Jurkat cells (1 x
230 10^6) were infected with 30,000 ^{3}H cpm RT units of HIV-1 HXBc2 CAT virus in
231 the absence or presence of drugs (Raltegravir was used at 0.5 μM, Efavirenz at
232 0.1 μM, Sennoside A at 20 μM, Sennoside B at 20 μM and RDS1760 at 25 μM).
233 Total intracellular DNA was purified from treated and untreated cells 16 hours
234 post-infection with DNeasy blood and tissue kit (Qiagen, Limburg Netherlands),
235 according to the manufacturer's instructions, and treated with 20 U/ml of DpnI
236 for 2 hours at 37°C to remove contaminating plasmid DNA (Munir et al. 2013).
237 The early (negative strand strong stop) and late (gag) reverse transcription
238 products and the total HIV-1 DNA were measured using quantitative real-time
239 PCR (qPCR), as previously described (Yu et al. 2008; Doitsh et al. 2010;
240 Cheney and McKnight 2010). Integrated HIV-1 DNA was detected by nested
241 Alu-gag PCR, as reported (Yu et al. 2008; Liszewski et al. 2009), with some
242 modifications. Pre-amplification reaction was performed using AmpliTaq Gold
243 DNA Polymerase (Applied Biosystems, Monza, Italy) and consisted of one
244 cycle of denaturation (10 min 95°C), 39 cycles of amplification (15 sec 94 °C,

30 sec 50 °C, 8 min 72 °C) and one cycle at 72 °C for 7 min. Quantitative PCR reactions were performed using TaqMan universal PCR master mix (Applied Biosystems ,Monza, Italy). The PCR consisted of one cycle of denaturation (10 min at 95°C) followed by 50 cycles of amplification (15 s at 95°C, 1 min at 60°C) in an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Monza, Italy).

2.12 Time of Addition. Jurkat cells were infected with HIV-1 IIIB at a multiplicity of infection (M.O.I.) of 0.5 $TCID_{50}$/cell. After 1 hour of incubation at 37 °C, the cultures were washed twice and 10^5 cells were maintained in the absence or presence of drugs. Test and reference compounds were added at different time points after infection (0, 1, 2, 3, 4, 5, 6, 7, 8 hours). Virus production in the supernatant of infected cells was determined by p24 assay at 31 hours post-infection, according to manufacter's instructions (Innotest®HIV Antigen mAb, Fujirebio, Ghent, Belgium). Raltegravir was used at 1 μM, Efavirenz at 1.5 μM, Zidovudine at 0.4 μM, and Sennoside A at 250 μM.

3. RESULTS AND DISCUSSION

3.1 Effect of extracts from Rheum Chinese species and of their chemical components of both HIV-1 RT-associated functions. With the aim of identifying new scaffolds able to inhibit both HIV-1 RT-associated functions to be developed as novel antiviral drugs (Distinto et al. 2013; Distinto et al. 2013), we thought that TCM could be a valid source of compound biodiversity. Hence

we tested extracts from *R. palmatum* and *R. officinale*, firstly for their ability to inhibit the HIV-1 RNase H activity, for which no drugs are currently available (Corona et al. 2013). Results showed that extracts from both plants potently inhibited this enzyme function with IC_{50} values of 0.9 and 0.25 μg/ml, respectively (Table S1).

Subsequently, single constituents of the extracts were purified by HPLC and seven phenolic components were found to be their major bioactive constituents (Table S1). Specifically, these components included anthraquinone derivatives such as Emodin, Crysophanol, Aloe-Emodin, Physcion, Rhein and Sennoside (both Sennoside A and Sennoside B). Hence, we investigated their ability to inhibit both HIV-1 RT-associated RNase H and RDDP functions using the RNase H selective diketo acid (DKA) derivative DS1759 (Corona et al. 2014) and the NNRTI Efavirenz as controls (Table 1). Physcion and Emodin were not significantly active on both HIV-1 RT-associated RDDP and RNase H activities (as the maximum concentration tested they inhibited slightly less than 50% of enzyme activity), while Aloe-Emodine and Crysophanol inhibited both HIV-1 RT-associated activities with IC_{50} values around 21-26 μM (Table 1). Differently, Rhein inhibited weakly the HIV-1 RT-associated RNase H function, but not its RDDP activity. Noteworthy, the most active components were Sennoside A and Sennoside B, that inhibited both HIV-1 RT-associated functions with IC_{50} values in the 2-5 μM range.

289 **3.2 Evaluation of the effects of Sennoside A and B on mutated RT.** Since
290 Sennosides A and B were identified as novel dual functions RTIs, we wanted
291 to compare them to known NNRTIs or RNase H inhibitors by using a series of
292 previously described HIV-1 RT mutants. Thus, we firstly tested the effect of
293 Sennoside A and B on three known single RT mutants involved in NNRTI
294 resistance such as K103N, Y181C and Y188L RTs, using Efavirenz as positive
295 control (Table 2). Results showed a significant difference between Sennoside
296 A and B. In fact, in the presence of these mutations Sennoside A showed a 4-
297 to 15-fold decrease in its potency of inhibition. On the contrary, Sennoside B
298 exhibited a 2- to 4-fold decrease in the inhibitory activity on K103N and Y188L
299 RTs, and displayed an IC_{50} value on Y181C RT similar to the one obtained on
300 wild type RT (Table 2). Next, we tested the effects of Sennoside A and B on
301 the RNase H activity of RTs bearing mutation in the 474 and 475 amino acid
302 residues (N474A and Q475A RTs) that are highly conserved and were recently
303 reported to be involved in the selective binding of the DKA derivative RDS1759
304 (Kharlamova et al. 2009; Corona et al. 2014; Corona et al. 2014). Results
305 indicated that, compared to wild type RT, the inhibitory activity of both
306 compounds were affected by the presence of the two mutations, leading to a
307 decrease in their efficacy by 5- to 12-fold (Table 2). Thus, in the case of RNase
308 H mutants, Sennoside A and B displayed a similar reduction in the inhibitory
309 activity.

310 Subsequently, considering the results obtained with Sennoside A on the
311 NNRTI resistant RT mutants, we wanted to better characterize its behavior in
312 the presence of Efavirenz in order to explore the possibility that the two
313 compounds may interact with the same RT binding site. Therefore we tested
314 the effect of increasing concentrations of Sennoside A and Efavirenz on the
315 RT-associated RDDP activity and analyzed the data by a Yonetani-Theorell
316 plot (Yonetani 1982) (Fig. 1). Such analysis can reveal whether simultaneous
317 binding (or inhibition) of the HIV-1 RT enzyme by the two compounds is
318 occurring or not. Results showed that Sennoside A and Efavirenz are
319 kinetically mutually exclusive, suggesting that either they bind to the same site
320 or the binding of one compound prevents the binding of the second one.
321 Taken together, this first set of data indicated that Sennoside A and B inhibit
322 both RT-associated functions. In particular, Sennoside A showed a reduced
323 ability to affect the RDDP activity of K103N, Y181C and Y188L RT mutants,
324 similarly to Efavirenz, and kinetic analysis indicated that Sennoside A and
325 Efavirenz are kinetically mutually exclusive. Overall, these results suggest that
326 Sennoside A binding might involve the NNRTIs binding pocket, and thus inhibit
327 the RT-associated RDDP function binding to this pocket. However, it is well
328 known that Efavirenz and other NNRTIs increase RT-associated RNase H
329 activity (Palanappian et al. 1995; Radzio and Sluis-Cremer 2008), while our
330 biochemical studies showed that Sennoside A inhibits this function. The
331 observation that Sennoside A is less effective in inhibiting the RNase H activity

in the presence of N474A and Q475A mutations, that were previously reported to strongly affect DKA derivatives binding and inhibitory activity (Corona et al. 2014), suggests that Sennoside A could also recognize a second site that might be localized nearby or within RNase H domain. Overall, the binding of Sennoside A to the first site, in the polymerase domain, would lead to the inhibition of the HIV-1 RT-associated RDDP function, while the binding to the second site, in the RNase H domain, would lead to the inhibition of the HIV-1 RNase H function.

3.3 Evaluation of the effects of Sennoside A and B on HIV-1 IN activity. Since a number of DKA derivatives inhibiting the RNase H activity by binding to the RNase H domain were reported to inhibit the HIV-1 IN activity (Esposito et al. 2012; Esposito et al. 2011; Tintori et al. 2015; Esposito et al. 2015; Tramontano et al. 2005; Costi et al. 2013; Costi et al. 2014; Cuzzuculi Crucitti et al. 2014), we asked whether Sennoside A and B might affect also the IN catalytic function. Thus, we tested their ability to inhibit the HIV-1 IN strand transfer reaction in the presence of the LEDGF/p75 cellular cofactor, using Raltegravir as positive control (Table 2). Data showed that Sennoside A inhibited the HIV-1 IN strand transfer activity with an IC_{50} value of 3.8 μM, while Sennoside B was 23-fold less potent.

3.4 Pharmacophoric differences between Sennoside A and B. Sennoside A and B were shown to differently affect HIV-1 wild type and mutant RTs as well as IN strand transfer catalytic function. Hence, in order to investigate the

different behavior of the two isomers A (Threo) and B (Erythro), we calculated their global minimum energy conformation (Table S2). Sennoside A and B are optical stereoisomers and therefore they do not differ for their chemical properties; however their pharmacophoric features are diverse for their spatial orientation (Fig. 2). These overall sterical differences can explain the different performance of the two Sennoside stereoisomers. In this respect, considering the large molecular surface of both Sennoside molecules, the different localization of wide hydrophobic areas in the two stereoisomers can dramatically affect the potential establishment of hydrophobic bonds and therefore influence the biological activity.

3.5 Characterization of the mechanism of action of Sennoside A and B in cell-based assays. Given their ability to inhibit both the HIV-1 RT and IN functions in biochemical assays, we wanted to evaluate the effect of Sennoside A and B on the early phases of the HIV-1 replication. Thus, we selected a transient trans-complementation assay to assess the replicative potential of HIV-1 in a single round of infection using a lymphoblastoid T cell line as target (Helseth et al. 1990; Parolin et al. 2003). In this assay, an env-defective HIV-1 HXBc2 $Vpr^{+}Vpu^{+}Nef^{+}\Delta env$ provirus encoding the bacterial CAT gene was complemented by the envelope glycoprotein from the HXBc2 laboratory-adapted T-tropic virus. The level of CAT expression in the cell lysates allowed us to examine early events in the infection process (Fig. 3). Results showed that Sennoside A inhibited HIV-1 replication with an EC_{50}

value around 9 μM in the absence of cytotoxicity, as determined by MTT assay, while Sennoside B was inactive at the highest tested concentration. In addition Crysophanol, even though it was able of inhibiting both HIV-1 RT-associated activities in biochemical assay (Table 1), did not exert any effect on HIV-1 replication.

In order to identify the viral process targeted by Sennoside A, we examined its effect on both reverse transcription and integration process by quantitative real time PCR (qPCR) analyzing viral cDNA intermediates in Jurkat infected cells treated with the drug. The primers and probe set used were selected in order to monitor sequential steps within the reverse transcription process, corresponding to generation of negative strong-stop DNA (early) and DNA strand elongation (late), and to quantify viral DNA that have successfully completed the integration process (Fig. 4A). Moreover, we assessed total viral DNA synthesis, including reverse transcription products that have completed the second strand transfer, as well as linear and circular form of viral DNA and integrated DNA (Fig 4A). Compounds were added at the time of infection and DNA collected at 16 hours post-infection. Efavirenz, Raltegravir and the DKA derivative RDS1760 (Corona et al. 2014) were used as positive controls (Fig. 4B). Results showed that negative strong stop DNA synthesis was only slightly inhibited by Sennoside A at 20 μM concentration (Fig. 4B). In contrast, late reverse transcription products generated after the first strand transfer decrease in the presence of Sennoside A, but not in the presence of

Sennoside B. The reduction of total viral DNA synthesis further confirmed this result.

Based on these observations, Sennoside A seemed to exhibit a mechanism of action similar to the one displayed by Efavirenz, affecting the RT activity during or soon after the first template exchange. Integration assay with an Alu-gag qPCR revealed that treatment with Sennoside A reduced also viral integration. Next, in order to gain further insights into the mechanism of action of Sennoside A, we performed a time of addition study in which compounds were added at the time of infection and every hour up to 8 hours after infection, using Efavirenz, Zidovudine and Raltegravir as controls (Fig. 5). Jurkat cells were infected with HIV-1 (Pauwels et al. 2011) and compounds were added with time lags of up to 8 hours after infection. According to the target of drug action or to the stage of interaction, addition of compound could be delayed for a specific number of hours without loss of antiviral activity. Results showed that Sennoside A profile of inhibition was similar to the one observed for the nucleoside analog Zidovudine (Fig. 5). These data confirm RT as a target of interaction of Sennoside A. On the other hand, our experimental setting cannot exclude that Sennoside A might have a slight effect also on HIV-1 IN.

4. CONCLUSIONS

Starting from the TCM heredity and knowledge, we successfully identified in the Rhubarb the chemical component Sennoside A that is able to inhibit in the

low micromolar range both HIV-1 RT-associated activities, IN function and virus replication. Mode of action studies performed in cell cultures, demonstrated that Sennoside A targets the HIV-1 reverse transcription process, while it seems to be unable to significantly affect IN activity. Overall, Sennoside A represents a novel attractive scaffold of dual function RTI that deserves further investigations by means of chemical modification, in search of new dual enzyme derivatives active on both HIV-1 RT and IN.

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Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

REFERENCES

Bamard, D.L., Fairbaim, D.W., O'Neill, K.L., et al. 1995. Anti-human cytomegalovirus activity and toxicity of sulfonated anthraquinones and anthraquinone derivatives. Antiviral Res. 28, 317-329.

443 Barnard, D.L., Huffman, J.H., Morris, J.L.B., et al. 1992. Evaluation of antiviral
444 activity of anthraquinones, anthrones and anthraquinone derivatives against
445 human cytomegalovirus. Antiviral Res. 17, 63-77.
446 Bicchi, C., Rubiolo, P., Ballero, M., et al. 2009. HIV-1-Inhibiting Activity of the
447 Essential Oil of Ridolfia segetum and Oenanthe crocata. Planta Med. 75, 1-5.
448 Cheney, K.M., McKnight, Á., 2010. Interferon-alpha mediates restriction of
449 human immunodeficiency virus type-1 replication in primary human
450 macrophages at an early stage of replication. PLoS One. 5, e13521.
451 Chinese Pharmacopoeia Commission. Pharmacopoeia of People's Republic of
452 China[S].Beijing: Chinese Pharmaceutical Science and Technology Publishing
453 House, 2010.
454 Corona, A., Di Leva, F.S., Thierry, S., et al. 2014. Identification of highly
455 conserved residues involved in the inhibition of the HIV-1 ribonuclease H
456 function by diketoacid derivatives. Antimicrob Agents Chemother.
457 58,6101-6110.
458 Corona, A., Masaoka, T., Tocco, G., et al. 2013. Active site and allosteric
459 inhibitors of the ribonuclease H activity of HIV reverse transcriptase. Future
460 Med. Chem. 5, 2127-2139.
461 Corona, A., Schneider, A., Schweimer, K., et al. 2014. Inhibition of foamy
462 virus reverse transcriptase by human immunodeficiency virus type 1
463 ribonuclease H inhibitors. Antimicrob Agents Chemother. 58, 4086-4093.

Cos, P., Maes, L., Vlietinck, A., Pieters, L., 2008. Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection - an update (1998 - 2007). Planta Med. 74,1323–1337.

Costi, R., Métifiot, M., Chung, S., et al. 2014. Basic quinolinonyl diketo acid derivatives as inhibitors of HIV integrase and their activity against RNase H function of reverse transcriptase. J Med Chem. 57, 3223-3234.

Costi, R., Métifiot, M., Esposito, F., et al. 2013. 6-(1-Benzyl-1H-pyrrol-2-yl)-2,4-dioxo-5-hexenoic acids as dual inhibitors of recombinant HIV-1 integrase and ribonuclease H, synthesized by a parallel synthesis approach. J Med Chem. 56, 8588-8859.

Cullen, B.R., 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. Methods Enzymol. 152,684-704.

Cuzzucoli Crucitti, G., Métifiot, M., Pescatori, L., et al. 2014. Structure-activity relationship of pyrrolyl diketo acid derivatives as dual Inhibitors of HIV-1 integrase and reverse transcriptase ribonuclease H domain. J Med Chem. 58,1915-1928.

Dang, S., Zhang, Z., Chen, Y., et al. 2006. Inhibition of the replication of hepatitis B virus in vitro by emodin. Med Sci Monit. 12, 302-306.

Distinto, S., Esposito, F., Kirchmair, J., et al. 2012. Identification of HIV-1 reverse transcriptase dual inhibitors by a combined shape-, 2D-fingerprint- and pharmacophore-based virtual screening approach. Eur J Med Chem. 50, 216-229.

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Distinto, S., Maccioni, E., Meleddu, R., et al. 2013. Molecular aspects of the RT/drug interactions. Perspective of dual Inhibitors. Curr Pharm Design. 19, 1850-1859.

Doitsh, G., Cavrois, M., Lassen, K.G., et al. 2010. Abortive HIV infection mediates CD4 T cell depletion and inflammation in human lymphoid tissue. Cell. 143, 789-801.

Esposito, F., Corona, A., Tramontano, E., 2012. HIV-1 reverse transcriptase still remains a new drug target: structure, function, classical inhibitors, and new inhibitors with innovative mechanisms of actions. Mol Biol Int. doi: 10.1155/2012/586401.

Esposito, F., Corona, A., Zinzula, L., et al. 2012. New Anthraquinone Derivatives as Inhibitors of the HIV-1 Reverse Transcriptase-Associated Ribonuclease H Function. Chemotherapy. 58, 299-307.

Esposito, F., Kharlamova, T., Distinto, S., et al. 2011. Alizarine derivatives as new dual inhibitors of the HIV-1 reverse transcriptase-associated DNA polymerase and RNase H activities effective also on the RNase H activity of non-nucleoside resistant reverse transcriptases. FEBS J. 278, 1444-1457.

Esposito, F., Sanna, C., Del Vecchio, C., et al. 2013. Hypericum hircinum L. components as new single-molecule inhibitors of both HIV-1 reverse transcriptase-associated DNA polymerase and ribonuclease H activities. Pathog Dis. 68, 116-124.

507 Esposito, F., Tintori, C., Martini, R., et al. 2015. Kuwanon-L as a new allosteric
508 HIV-1 integrase inhibitor: molecular modeling and biological evaluation.
509 ChemBioChem. DOI: 10.1002/cbic.201500385.
510 Esposito, F., Tramontano E., 2013. Past and Future. Current drugs targeting
511 HIV-1 integrase and reverse transcriptase-associated ribonuclease H activity:
512 single and dual active site inhibitors. Antiv Chem and Chemother. 23, 129-144.
513 He, Z.H., Zhou, R, He, M.F., et al. 2011. Anti-angiogenic effect and
514 mechanism of rhein from Rhizoma Rhei. Phytomedicine. 18, 470-478.
515 Helseth, E., Kowalski, M., Gabuzda, D., et al. 1990. Rapid complementation
516 assays measuring replicative potential of human immunodeficiency virus type
517 1 envelope glycoprotein mutants. J Virol. 64, 2416-2420.
518 Higuchi, H., Mori, K., Kato, A., et al. 1991. Antiretroviral activities of
519 anthraquinones and their inhibitory effects on reverse transcriptase. Antiviral
520 Res. 15,205-216.
521 Kharlamova, T., Esposito, F., Zinzula, L., et al. 2009. Inhibition of HIV-1
522 ribonuclease H activity by novel frangula-emodine derivatives. Med Chem. 5,
523 398-410.
524 Li, J., Vederas, J.C., 2009. Drug discovery and natural products: end of an era
525 or an endless frontier? Science 325,161-165.
526 Liszewski, M.K., Yu, J.J., O'Doherty, U., 2009. Detecting HIV-1 integration by
527 repetitive-sampling Alu-gag PCR. Methods. 47, 254-60.

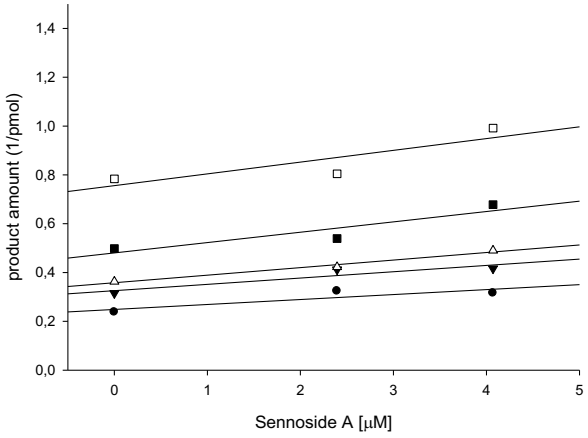
528 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data
529 using real time quantitative PCR and the 2-ΔΔCt method. Methods. 25,
530 402-408.
531 Lu, L., Li, H.Q., Fu, D.L., et al. 2014. Rhubarb root and rhizome-based Chinese
532 herbal prescriptions for acute ischemic stroke: a systematic review and
533 meta-analysis. Complement Ther Med. 22, 1060-1070.
534 Maldonado, O.S., Lucas, R., Comelles, F., et al. 2011. Synthesis and
535 characterization of phenolic antioxidants with surfactant properties: glucosyl-
536 and glucuronosyl alkyl gallates. Tetrahedron. 67,7268-7279.
537 Mosmann, T., 1993. Rapid colorimetric assay for cell growth and survival.
538 Application to proliferation and cytotoxicity. J Immunol Methods. 65, 55-63.
539 Munir, S., Thierry, S., Subra, F., et al. 2013. Quantitative analysis of the
540 time-course of viral DNA forms during the HIV-1 life cycle. Retrovirology.
541 10,87.
542 Palanappian, C., Fay, P.G., Bambara, R.A., 1995. Nevirapine alters the
543 cleavage specificity of ribonuclease H of human immunodeficiency virus 1
544 reverse transcriptase. J Biol Chem. 270, 4861-4869.
545 Parolin, C., Gatto, B., Del Vecchio, C., et al. 2003. New anti-human
546 immunodeficiency virus type 1 6-aminoquinolones: mechanism of action.
547 Antimicrob Agents Chemother. 47, 889-896.

548 Pauwels, R., De Clercq, E., Pannecouque, C., 2011. A time-of-drug addiction
549 approach to target identification of antiviral compounds. Nat Protoc. 6,
550 925-933.
551 Radzio, J., Sluis-Cremer, N., 2008. Efavirenz accelerates HIV-1 reverse
552 transcriptase ribonuclease H cleavage, leading to diminished zidovudine
553 excision. Mol Pharmacol. 73, 601-606.
554 Ratner, L., Haseltine, W., Patarca, R., et al. 1985. Complete nucleotide
555 sequence of the AIDS virus, HTLV-III. Nature. 313, 277-284.
556 Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent
557 endpoints. Am J Hyg. 27, 493-497.
558 Rho, H.M., Poiesz, B., Ruscetti, F.W., et al. 1981. Characterization of the
559 reverse transcriptase from a new retrovirus (HTLV) produced by a human
560 cutaneous T-cell lymphoma cell line. Virology. 112, 355-360.
561 Sartori, E., Calistri, A., Salata, C., et al. 2011. Herpes simplex virus type 2
562 infection increases human immunodeficiency virus type 1 entry into human
563 primary macrophages. Virol J. 8, 166.
564 Semple, S.J., Pyke, S.M., Reynolds, G.D., et al. 2011. In vitro antiviral activity
565 of the anthraquinone chrysophanic acid against poliovirus. Antiviral Res. 49,
566 169-178.
567 Sill, A.D., Andrews, E.R., Sweet, F.W., et al. 1974. Bis-basic-substituted
568 polycyclic aromatic compounds. A new class of antiviral agents. Bis-basic

569 ethers of anthraquinone and bisalkamine esters of anthraquinonedicarboxylic
570 acids. J Med Chem. 17, 965-968.
571 Tintori, C., Esposito, F., Morreale, F., et al. 2015. Investigation on the sucrose
572 binding pocket of HIV-1 Integrase by molecular dynamics and synergy
573 experiments. Bioorg Med Chem Lett. 25, 3013-3016.
574 Tramontano, E., Esposito, F., Badas, R., et al. 2005.
575 6-[1-(4-Fluorophenyl)methyl-1H-pyrrol-2-yl)] -2,4-dioxo-5-hexenoic acid ethyl
576 ester a novel diketo acid derivative which selectively inhibits the HIV-1 viral
577 replication in cell culture and the ribonuclease H activity in vitro. Antiv.
578 Research. 65,117-124.
579 Tramontano, E., Kharlamova, T., Zinzula, L., et al. 2011. Effects of new
580 quinizarin derivatives on both HCV NS5B RNA polymerase and HIV-1 reverse
581 transcriptase associated ribonuclease H activities. Journal of Chemoth.
582 23,273-276.
583 Tsuboi, K.K., Edmunds, N.H., Kwong, L.K., 1977. Selective inhibition of
584 pyrimidine biosynthesis and effect on proliferative growth of colonic cancer
585 cells. Cancer Res. 37, 3080-3087.
586 Xiao, P.G., Chen BZ, Wang LW, et al. 1980. A preliminary study of the
587 correlation between phylogeny, chemical constituents and therapeutic effects
588 of Rheum species. Acta Pharmaceutica Sinica.Yao Xue Xue Bao. 15: 33-39.

589 Xie, H.C., Shang, J., 2014. Study on the extraction process of total
590 anthraquinones in Radix et Rhizoma Rhei and their antilipemic effects. Afr J
591 Tradit Complement Altern Med. 11,358-362.
592 Xu, L., Grandi, N., Del Vecchio, C., et al. 2015. From the traditional Chinese
593 medicine plant Schisandra chinensis new scaffolds effective on HIV-1 reverse
594 transcriptase resistant to non-nucleoside inhibitors. J Microbiol. 53,287-293.
595 Ye, M, Han, J, Chen, H., et al. 2007. Analysis of phenolic compounds in
596 rhubarbs using liquid chromatography coupled with electrospray ionization
597 mass spectrometry. J Am Soc Mass Spectrom. 18, 82-91.
598 Yonetani ,T., 1982. The Yonetani-Theorell graphical method for examining
599 overlapping subsites of enzyme active centers. Methods Enzymol. 87,
600 500-509.
601 Yu, D., Morris-Natschke, S.L., Lee, K.H., 2007. New developments in natural
602 products-based anti-AIDS research. Natural Med Res. 1, 108-132.
603 Yu, J.J., Wu, T.L., Liszewski, M.K., et al. 2008. A more precise HIV integration
604 assay designed to detect small differences finds lower levels of integrated
605 DNA in HAART treated patients. Virology. 379, 78-86.
606 Zhao, Y.L., Wang, J.B., Zhou, G.D., et al. 2009. Investigations of free
607 anthraquinones from rhubarb against alpha-naphthylisothiocyanate-induced
608 cholestatic liver injury in rats. Basic Clin Pharmacol Toxicol. 104, 463-469.
609 Zwaving, J. H., 1972. The sennoside content of Rheum palmatum. Planta
610 Medica. 21,254-262.

Figure 1. Yonetani–Theorell plot of the combination of Sennoside A and Efavirenz on the HIV-1 RT RNA-dependent DNA polymerase activity.



HIV-1 RT was incubated in the presence of Sennoside A alone (●) or in the presence of different Efavirenz concentrations: 6 nM (▼), 9 nM (Δ), 13 nM (■), 20 nM (□). In Y axis it was represented the reverse of the product formation.

Figure 2.

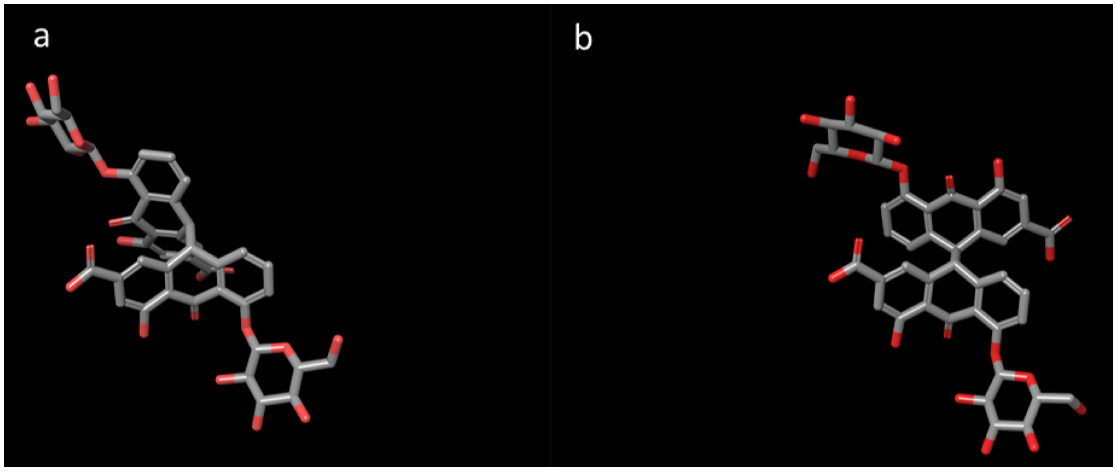
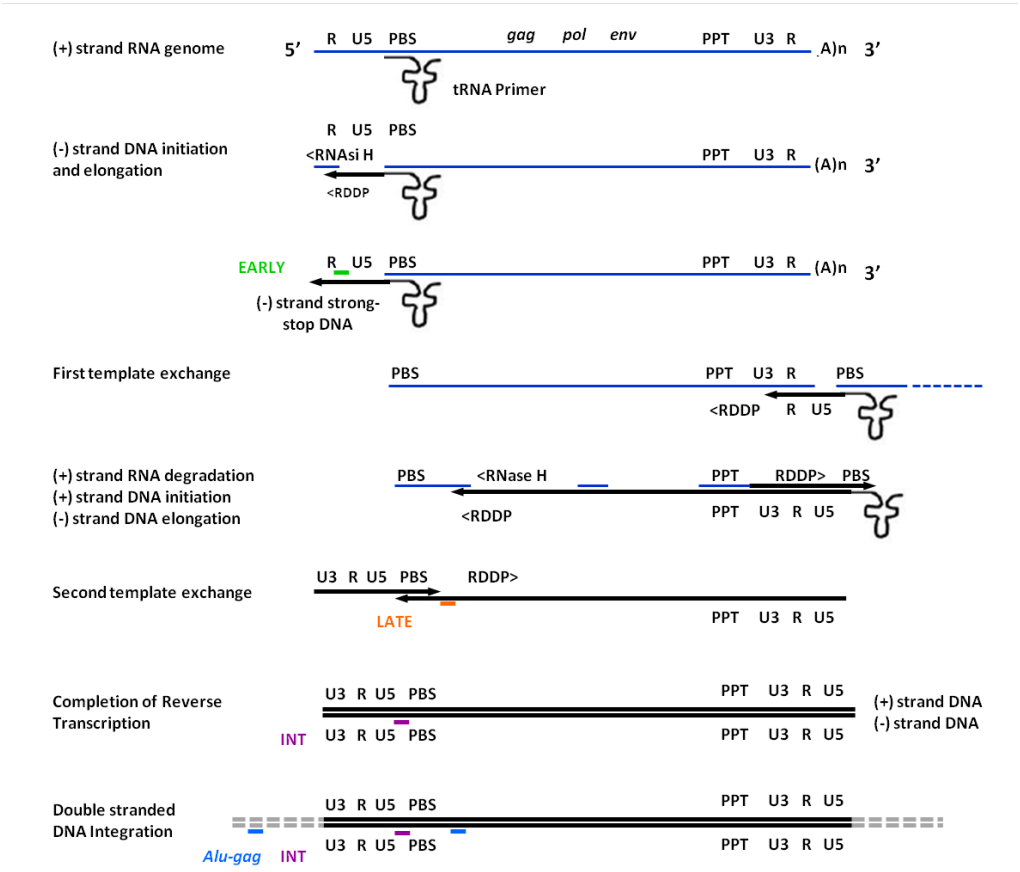


Figure 3. Effect of Sennoside A and B on HIV-1 replication.

Figure 4. Quantitative determination of HIV-1 reverse transcription and integration products in the presence of Sennoside A and B. Quantitative Real Time PCR analysis was used to measure the amount of HIV-1 early and late reverse transcription products, integrated and total DNA in the presence of test and reference compounds. **A.** Schematic

representation of primer set positions within reverse transcription and integration intermediates. **B**. Total intracellular DNA was extracted from Jurkat infected cells 16 hours post infection. Test and reference compounds were added at the time of infection (Efavirenz 0.1 µM, Raltegravir 0.5 µM, Sennoside A 20 µM, Sennoside B 20 µM, RDS1760 25 µM). The level of each viral DNA products was calculated as fold change relative to infected untreated cells using ΔΔCt method (Livak and Schmittgen 2001). The same positive control (no drug) was used for all analyses, thereby allowing the effect of Sennoside A and B on reverse transcription and integration processes to be evaluated. β-globin housekeeping gene was used as endogenous control. Values are means of duplicate determinations.

A



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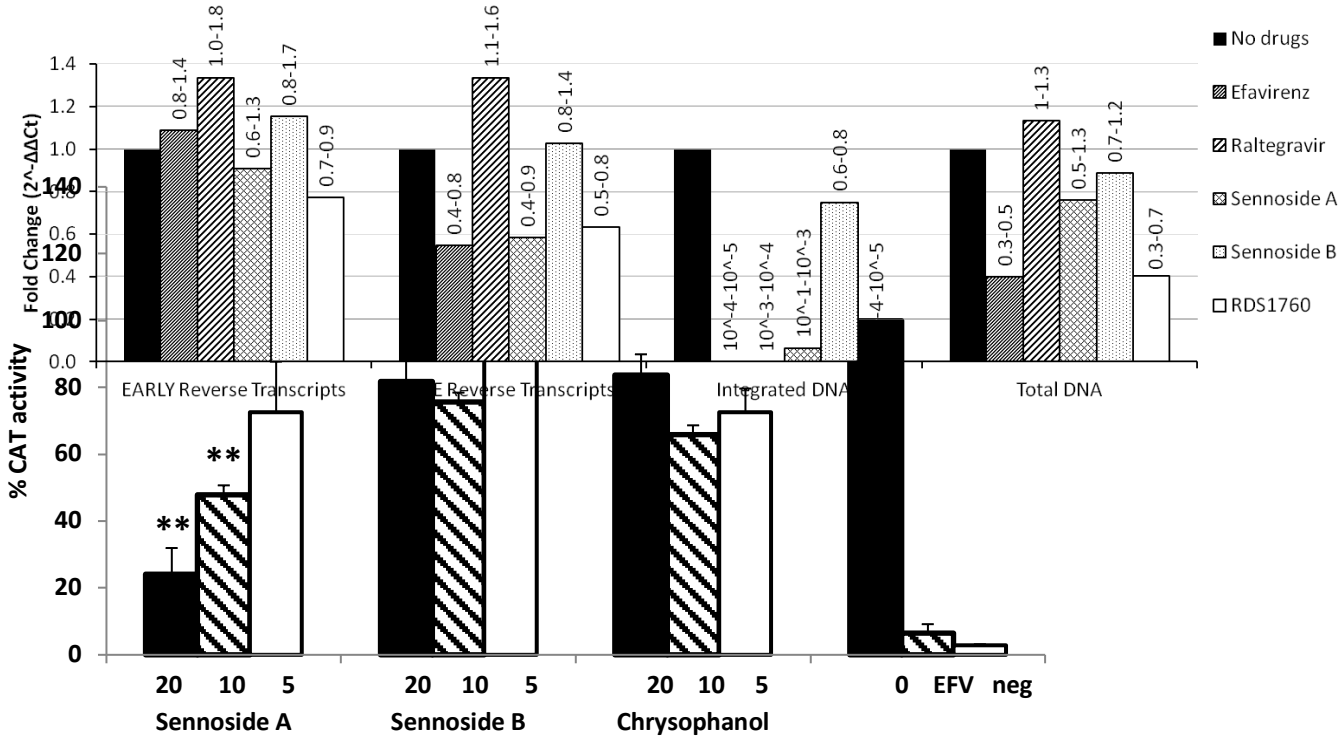
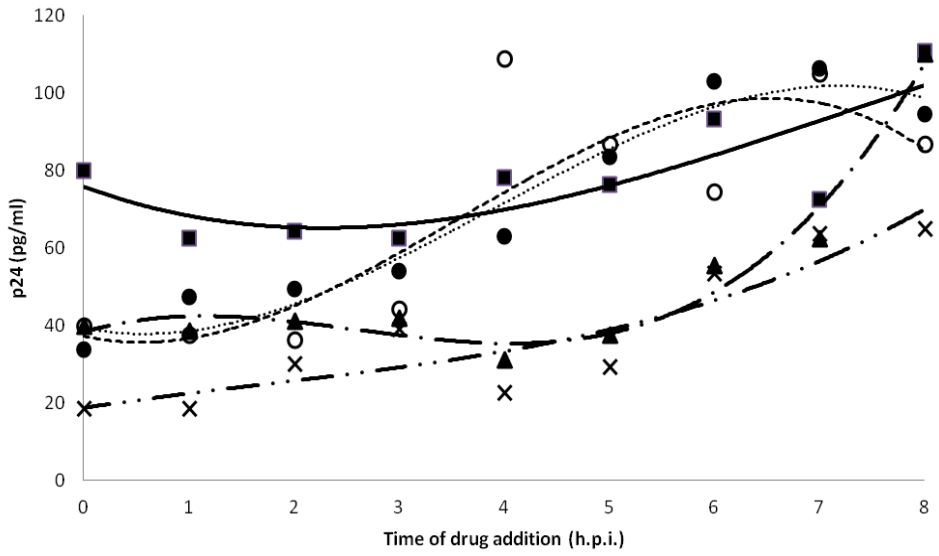


CAT activity was determined in Jurkat cells infected with HIV-1 recombinant CAT virus pseudotyped with the envelope glycoprotein from the HXBc2 laboratory-adapted T-tropic virus, in the absence (0) or presence of 20 µM, 10 µM and 5 µM of selected compounds 72 hours post infection. The results are reported as percent of conversion of [^{14}C]chloramphenicol to acetylated forms relative to the positive control (0). neg, negative

control; EFV, efavirenz. The means of two independent experiments and standard deviations are presented. ** indicates p=0.01.

Figure 5. Time of addition. Jurkat cells were infected with HIV-1 and the test compounds were added at different time points after infection. Virus production was determined by p24 Ag production in the supernatant at 31 hours post infection (h.p.i.). No drugs (positive control, ■); Raltegravir, 1 µM, (×); Efavirenz, 1.5 µM (▲); Zidovudine, 0.4 µM (●); Sennoside A, 250 µM (o). This graph is representative of data from 2 independent experiments.



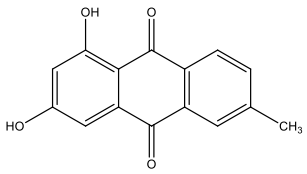
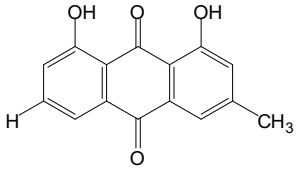
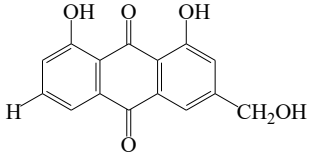
695 **Table 1. Description of Species, collecting place, origin and components of Rheum**
696 **Chinese and their effects on HIV-1 RNase H.**
697

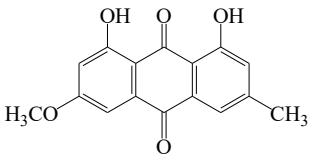
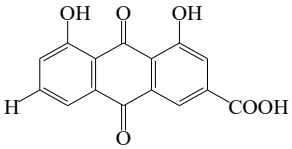
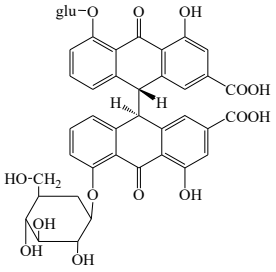
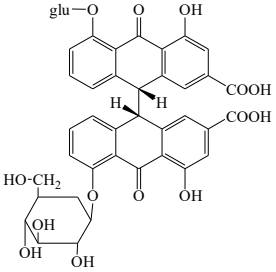
Species	Place of collection	Origin	Aloe Emodin (mg/g)	Rhein (mg/g)	Emodin (mg/g)	Chrysophanol (mg/g)	Physcion (mg/g)	Sennoside A (mg/g)	Sennoside B (mg/g)	H RN[a] (μg
R. palmatum (D-13)	Tianzhu, Gansu	Wild	0.023	-	0.046	0.055	0.102	0.864	0.489	
R. officinale (D-15)	Songpan, Sichuan	Wild	0.05	0.039	0.033	0.107	0.16	0.162	0.037	

698 [a]Extract concentration required to inhibit the HIV-1 RT-associated RNase H activity by 50%

699

700 **Table 2. Effect of Rheum single components on the HIV-1 RT-associated**
701 **activities.**

Compound	Chemical structure	[a]IC_{50} (μM)	
		RNase H	RDDP
Emodin		>100 (59%)[b]	>100 (51%)
Chrysophanol		25.0 ± 4.0	26.5 ± 2.5
Aloe-Emodin		23.0 ± 5.0	21.0 ± 4.0

Physcion		> 100 (53%)	>100 (57%)
Rhein		60 ± 12	>100 (62%)
Sennoside A		1.9 ± 0.3	5.3 ± 0.1
Sennoside B		2.1 ± 0.2	2.3 ± 0.4
Efavirenz		> 50[c]	0.025 ± 0.0005[c]
RDS1759		7.4 ± 0.2[c]	> 50[c]

[a]Compound concentration required to reduce HIV-1 RT-associated RNase H and RT-associated RNA Dependant DNA Polymerase activities by 50%.

[b]Percentage of control activity in the presence of 100 µM concentration of compound.

[c]Data were expressed in µM.