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RESEARCH ARTICLE

Hypericum hircinum L. components as new single-molecule inhibitors of both HIV-1 reverse transcriptase-associated DNA polymerase and ribonuclease H activities

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Combination antiretroviral therapy has forever changed the outcome of AIDS, transforming it from an untreatable, fatal syndrome into a manageable chronic condition. However, the search for new therapeutic targets is always on, because of the issue of mutation in the virus determining resistance to all drugs in use. Esposito *et al.* describe the activities of compounds that they tested against RNase H, a viral enzyme that has not been fully exploited as a target for antiretrovirals. Their results will be relevant to the development of new drugs to treat HIV infection.

Keywords
HIV; dual inhibitors; betulinic acid; RT; RNase H.

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Abstract

Among HIV-1 reverse transcriptase (RT)-associated functions, DNA polymerase and Ribonuclease H (RNase H) are both essential for HIV replication and excellent targets for drug development. While all RT inhibitors approved for therapy target the DNA polymerase activity, there is the pressing need for new RT inhibitors possibly targeting the RNase H function. In the last 20 years, many natural substances have shown antiviral activity against HIV-1, but only a few against the RNase H function. In this study, we have tested the ethanolic extracts obtained by the *Hypericum hircinum* L. (*Hypericaceae*) growing in Sardinia (Italy) on the HIV-1 RT-associated RNase H function and found that they have inhibitory effects. Active extracts were fractionated up to obtain the main components that have been isolated, tested, and identified to be betulinic acid, shikimic acid, chlorogenic acid, quercetin, 5,7,3′,5′-tetrahydroxyflavanone, and 5,7,3′,5′-tetrahydroxyflavanone 7-O-glucoside. Betulinic acid and 5,7,3′,5′-tetrahydroxyflavanone 7-O-glucoside were active on both RT-associated activities, and betulinic acid was also active on HIV-1 mutant RTs resistant to efavirenz. Overall, our results suggest that some of these compounds inhibit the HIV-1 RT binding to an allosteric site previously described for other natural compounds and are potential leads for further drug development of a single molecules having dual inhibitory activity.

Introduction

The human immunodeficiency virus type 1 (HIV-1) epidemic is still a worldwide health issue despite the availability of more than 20 drugs currently approved for treatment (Mehellou & De Clercq, 2010). In particular, the selection of drug-resistant viral strains and the toxic side effects due to the chronic drug administration lead to the need of developing new inhibitors with novel mechanism of action and effective on HIV drug-resistant strains (Esposito *et al.*, 2012a, b). The viral protein reverse transcriptase (RT), responsible for the retrotranscription of the viral RNA genome into dsDNA, is probably the most widely explored HIV-1 drug target. During reverse transcription, RT accomplishes several enzymatic functions such as RNA- and DNA-dependent DNA polymerization, degradation of the RNA strand of the RNA/DNA hybrids (termed 'ribonuclease H (RNase H) activity'), strand transfer, and strand displacement synthesis (Esposito *et al.*, 2012a). In particular, the RT-associated RNase H function is essential for viral

replication, but until now, it has been under-explored as pharmaceutical target, and no clinically approved RT-targeted drug actually inhibits the RNase H function (Tramontano, 2006; Tramontano & Di Santo, 2010; Distinto *et al.*, 2013).

The prophylactic and therapeutic action of natural extracts on viral diseases has been known since ancient times. In the last years, many reports have been published describing the antiviral activity exerted by plant components such as, for example, anthraquinones, which show antiviral effects on human cytomegalovirus (Barnard *et al.*, 1992), hepatitis B virus (Shuangsuo *et al.*, 2006), Epstein–Barr virus activation (Koyama *et al.*, 2006), and HIV-1 RT activity (Kharlamova *et al.*, 2009; Esposito *et al.*, 2011, 2012b; Tramontano *et al.*, 2011). Recently, a few studies have been published on *Hypericum hircinum* L., an evergreen shrub belonging to the *Hypericaceae* family, widely used in Lucanian folk medicine for the treatment of cough (Pieroni *et al.*, 2004) and in Sardinian medicine for its antiseptic properties and burns treatment (Ballero *et al.*, 1997). It has been previously reported that *Hypericum hircinum* leaves contain chlorogenic acid, rutin, hyperoside, isoquercitrin, quercetin, quercetron, mangiferin, hyperforin, hypericin, and biapigenin and that the *H. hircinum* essential oil shows antimicrobial, antioxidant, and antiproliferative activities (Pistelli *et al.*, 2000; Chimenti *et al.*, 2006; Cecchini *et al.*, 2007; Sagratini *et al.*, 2008; Maggi *et al.*, 2010; Quassinti *et al.*, 2012). The *H. hircinum* ethanolic extract free radical scavenging and protective effects on doxorubicin-induced cardiotoxicity in rats have recently been demonstrated (Shah *et al.*, 2012). In addition, the *H. hircinum* flavone component quercetin has been reported to inhibit HIV-1 Integrase (IN), the viral enzyme that catalyzes the HIV dsDNA integration into the cell genome (Fesen *et al.*, 1993; Vandegraaff *et al.*, 2001). Notably, HIV-1 IN and RNase H belong to the same family of polynucleotide transferases, and in fact, compounds designed to target IN have been shown to inhibit also the HIV-1 RNase H function (Tramontano *et al.*, 2005; Esposito *et al.*, 2012a).

In the present study, to identify new chemical structures that may be used as anti-HIV-1 RNase H lead compound for further drug development, we evaluated the *H. hircinum* L. ethanol extracts from its aerial part on the HIV-1 RT-associated RNase H activity and isolated the main active chemical constituents. Results show that components never reported for this plant are able to inhibit both HIV-1 RT-associated functions and represent an interesting lead for new single-molecule dual HIV inhibitors.

Materials and methods

Plant material and sample preparation

Aerial parts of plant were collected in Jerzu (Sardinia, Italy). A voucher specimen (Herbarium CAG 232) has been retained in the General Herbarium of the botanical garden of the University of Cagliari. Leaves were dried and ground with electrical grinder, and milled material (400 g) was consecutively extracted three times using hydro alcoholic solvent (6 L) at various percentages for 48 h at room temperature. Extract I was carried out with 96% ethanol; extract II and extract III were carried out using 80% ethanol. The extracts were collected separately, and alcohol was eliminated at reduced pressure at 50 °C; the resulting water suspension was lyophilized. All extracts were stored in dark and refrigerated at 0–4 °C until their fractionation by silica gel column chromatography, and the different fractions were then assayed.

Chemical analysis

NMR spectra were recorded on Varian Mercury 300 MHz instrument using $CDCl_3$, CD_3OD, or D_2O as deuterated solvents, and the chemical shifts were expressed in ppm from TMS. MS spectra were performed on a Q-TOF MICRO spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source that operated in the negative or positive ion mode. The flow rate of sample infusion was 10 μL min^{-1} with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters.

Separation of active compounds

Extracts were chromatographed on silica gel column using a mixture of chloroform/methanol at various percentages, increasing the polarity of the eluting mixture during the chromatography (95:5, 9:1, 8:2, 7:3, 6:4) or *n*-butanol saturated with water, as eluting solvents. The separation was monitored by TLC, and the compounds detection was performed using a suitable spray reagents (2N H_2SO_4, 3% aqueous $FeCl_3$) followed by heating at 120 °C. Fractions were collected together on the basis of chromatographic homogeneity; generally, they revealed mixtures of various substances, where the presence of triterpenoids, waxes, flavonoids and polyphenolic compounds, shikimic acid, glycosides, and sugars was demonstrated by means of spectroscopic methods (NMR, MS). Fractions that resulted more active on biological tests, independently by the original extract were monitored by TLC and NMR spectra. Fractions that showed a very high similarity were collected together and rechromatographed to separate the single or the main components. Pure isolated compounds were as follows: betulinic acid, shikimic acid, chlorogenic acid, quercetin, 5,7,3′,5′-tetrahydroxyflavanone, 5,7,3′,5′-tetrahydroxyflavanone-7-O-glucoside. Compounds that were present in quantities lower than 5% w/w of the examined fractions were not considered in this purification procedure.

Betulinic acid: ^{1}H NMR, $CDCl_3$, 300 MHz: δ 0.77 (s, H25); 0.83 (s, H24); 0.94 (s, H26); 0.97 (s, H23-H27); 1.66 (s, H30); 3.17 (m, H3); 4.57 (bs, H29b); 4.70 (bs, H29a). ^{13}C NMR, $CDCl_3$, 75 MHz: δ 14.6 (C27), 15.3 (C24), 15.9 (C26), 16.0 (C25), 18.3 (C6), 19.3 (C30), 21.0 (C11), 25.6 (C12), 27.9 (C2), 28.1 (C23), 29.6 (C21), 30.6 (C15), 32.1 (C16), 34.0 (C7), 36.1 (C22), 37.2 (C10), 38.3 (C13), 38.7 (C1), 38.8 (C4), 40.7 (C8), 42.4 (C14), 46.9 (C18), 49.2 (C19), 50.5 (C9), 55.3 (C5), 56.1 (C17), 79.0 (C3), 109.6

(C29), 150.3 (C20), 180.4 (C28). ESI-MS: *m/z* 455.74 $[M-H]^-$ (Peng *et al.*, 1998).

Shikimic acid: ^{1}H NMR, CD_3OD, 300 MHz: δ 2.18 (1H, m, H6b), 2.71 (1H, m, H6a), 3.66 (1H, m, H4), 3.96 (1H, m, H5), 4.36 (1H, bs, H3), 6.78 (1H, bs, H2). 13C NMR, 75 MHz, CD_3OD: 31.9 (C6), 67.4 (C5), 68.5 (C4), 72.9 (C3), 131.3 (C1), 138.3 (C2), 170.6 (C7). ESI-MS *m/z* 173.04 $[M-H]^-$; *m/z* 174.98 $[M+H]^+$; *m/z* 197.08 $[M+Na]^+$ (Zhang *et al.*, 2004).

Chlorogenic acid: ^{1}H NMR, D_2O, 300 MHz: δ 1.84 (2H, m, H2a-H2b), 1.98 (2H, m, H6a-H6b), 3.86 (1H, overlapped with other signals, H4), 4.04 (1H, m, H3), 5.17 (1H, m, H5), 5.93 (1H, d *J* 16.0 Hz, Hβ), 6.67 (1H, d *J* 8.2 Hz, H5′), 6.87 (1H, dd *J* 8.2 and 1.8 Hz, H6′), 7.01 (1H, d *J* 1.8 Hz, H2′), 7.18 (1H, d *J* 16.0 Hz, Hα). ^{13}C NMR, D_2O, 75 MHz: δ 38.2 (C8), 71.7 (C6), 72.8 (C10), 74.4 (C2), 75.9 (C5′), 114.2 (C1′), 115.0 (C6′), 116.0 (C3), 122.7 (C2), 126.0 (C3′), 129.2 (C4′), 144.2 (C9), 146.1 (C5), 147.1 (C7), 168.9 (C = O, caffeic acid), 180 (C = O, quinic acid). ESI-MS: *m/z* 352.85 $[M-H]^-$; *m/z* 377 $[M+Na]^+$ (Wei *et al.*, 2010).

Quercetin: ^{1}H NMR, CD_3OD, 300 MHz: δ 6.20 (1H, d *J* 1.8 Hz, H6), 6.41 (1H, d *J* 1.8 Hz, H8), 6.87 (1H, d *J* 7.2 Hz, H5′), 7.66 (1H, dd *J* 7.2 and 2.0 Hz, H6′), 7.67 (1H, d *J* 2.0, H2′). ^{13}C NMR, CD_3OD, 75 MHz: δ 94.9 (C8), 100.0 (C6), 100.5 (C10), 116.3 (C2), 117.2 (C5′), 122.8 (C1′), 123.5 (C6′), 133.5 (C3), 146.1 (C2), 146.2 (C3′), 150.3 (C4′), 158.5 (C9), 159.9 (C5), 166.3 (C7), 178.9 (C4). ESI-MS: m/z 301.03 $[M-H]^-$ (Miyazawa & Hisama, 2003).

5,7,3′,5′-tetrahydroxyflavanone: ^{1}H NMR, CD_3OD, 300 MHz: δ 2.69 (1H, dd *J* 17.1 and 3.0 Hz, H3b), 3.07 (1H, dd *J* 17.1 and 12.7 Hz, H3a), 5.28 (1H, dd *J* 12.7 and 2.8 Hz, H2), 5.88 (1H, d *J* 2.1 Hz, H6), 5.90 (1H, d *J* 2.1 Hz, H8), 6.78 (2H, d *J* 1 Hz, H2′-H6′), 6.91 (1H, bs, H4′). ^{13}C NMR, CD_3OD, 75 MHz: δ 44.1 (C3), 80.5 (C2), 96.2 (C8), 97.1 (C6), 103.4 (C10), 114.7 (C6′), 116.3 (C2′), 119.3 (C1′), 131.8 (C4′), 146.5 (C5′), 146.9 (C3′), 164.9 (C9), 165.5 (C5), 168.4 (C7), 192.7 (C4). ESI-MS: *m/z* 287.08 $[M-H]^-$; 289.0 $[M+H]^+$; 310.9 $[M+Na]^+$ (Zheng *et al.*, 2008).

5,7,3′,5′-tetrahydroxyflavanone-7-O-glucoside: ^{1}H NMR, CD_3OD, 300 MHz: δ 2.74 (1H, dd *J* 17.2 and 2.9 Hz, H3b), 3.13 (1H, dd *J* 17.2 and 12.7, H3a), 4.97 (1H, d *J* 7.1 Hz, H1″), 5.32 (1H, dd *J* 12.7 and 2.8 Hz, H2), 6.18 (1H, d *J* 2.1, H6), 6.21 (1H, d *J* 2.1 Hz, H8), 6.79 (2H, bs, H2′-H6′), 6.92 (1H, bs, H4′). ^{13}C NMR, CD_3OD, 75 MHz: δ 44.4 (C3), 62.4 (C6″), 71.2 (C4″), 74.7 (C2″), 77.8 (C3″), 78.3 (C5″), 80.7 (C2), 97.0 (C8), 98.0 (C6), 99.9 (C10), 101.3 (C1″), 114.8 (C6′), 116.3 (C2′), 119.4 (C1′), 131.6 (C4′), 146.6 (C5′), 147.0 (C3′), 164.6 (C9), 165.0 (C5), 167.1 (C7), 198.5 (C4). ESI-MS: *m/z* 449.16 $[M-H]^-$; 473.17 $[M+Na]^+$ (Chen *et al.*, 2009).

Protein expression and purification

The recombinant HIV-1 RT gene was subcloned into the p6HRT_prot plasmid, and protein was expressed in *E. coli* strain M15 (Tramontano & Cheng, 1992; Mellors *et al.*, 1993). Bacteria cells were grown up to an $OD_{600\ nm}$ of 0.8 and induced with 1.7 mM IPTG for 5 h. HIV-1 RT purification was carried out as described (Suchaud *et al.*, 2012). Briefly, cell pellets were resuspended in lyses buffer (20 mM Hepes, pH 7.5; 0.5 M NaCl; 5 mM β-mercaptoethanol; 5 mM imidazole; 0.4 mg mL^{-1} lysozyme), incubated on ice for 20 min, sonicated, and centrifuged at 30 000 ***g*** for 1 h. The supernatant was applied to a His-binding resin column and washed thoroughly with wash buffer (20 mM Hepes, pH 7.5; 0.3 M NaCl; 5 mM β-mercaptoethanol; 60 mM imidazole; 10% glycerol). RT was eluted by imidazole gradient, and the enzyme-containing fractions were pooled and dialyzed, and aliquots were stored at −80 °C.

RNase H polymerase-independent cleavage assay

The HIV-1 RT-associated RNase H activity was measured in 100 μL reaction volume containing 50 mM Tris–HCl, pH 7.8; 6 mM $MgCl_2$; 1 mM dithiothreitol (DTT); 80 mM KCl; hybrid RNA/DNA (5′-GTTTTCTTTTCCCCCCTGAC-3′-fluorescein, 5′-CAAAAGAAAAGGGGGGACUG-3′-Dabcyl); and 3.8 nM RT. The reaction mixture was incubated for 1 h at 37 °C, the reaction was stopped by the addition of EDTA, and products were measured with a Victor 3 (Perkin) at 490/528 nm (Distinto *et al.*, 2012).

DNA polymerase assay

The HIV-1 RT-associated RNA-dependent DNA polymerase (RDDP) activity was measured using the Invitrogen EnzCheck Reverse Transcriptase Assay Kit, in 50 μL volume containing 60 mM Tris–HCl, pH 8.1; 8 mM $MgCl_2$; 60 mM KCl; 13 mM DTT; 100 μM dTTP; 2 nM HIV-1 RT; and poly (A)-oligo(dT). The reaction mixture was incubated for 30 min at 37 °C. The enzymatic reaction was stopped by the addition of EDTA, and products were measured with a Victor 3 (Perkin) at 502/523 nm following PicoGreen addition.

Evaluation of $MgCl_2$ chelation

Compounds RDS 1643, quercetin, 5,7,3′,5′-tetrahydroxyflavanone, 5,7,3′,5′-tetrahydroxyflavanone-7-O-glucoside, shikimic acid, and betulinic acid were solubilized in 1 mL of 10% ethanol and 10 mM Tris–HCl, pH 7.8. The UV-Vis spectrum was recorded, from 250 to 600 nm, before and after the addition of 6 mM $MgCl_2$, and a third spectrum was recorded after the addition of 30 mM EDTA, pH 8.0.

Cells

The African green monkey kidney epithelial Vero 76 cell line, the human adenocarcinomic alveolar epithelial A549 cell line, the human Hepatocellular carcinoma Hep G2 cell line, the HeLa cell clone (that expresses human CD4, CCR5, and CXCR4 and contains HIV-1 Tat-regulated reporter genes), TZM-bl cell line, and the human T-lymphoid Jurkat cell line were from American Type Culture Collection (ATCC). Cell lines were grown in DMEM or in RPMI medium containing 10% fetal bovine serum (FBS) and 1% kanamycin sulfate. Cell cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

Cytotoxicity assays

For cytotoxicity assays, cell lines were seeded in 96-well plates (Spectra Plate, PerkinElmer) at an initial density of 10^5 cells mL^{-1} (100 μL volume) in medium containing 10% FBS and 1% kanamycin sulfate, in the absence or the presence of serial dilutions of test compounds. Plates were incubated for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. Cell viability was determined adding Presto-Blue™ Cell Viability Reagent (Invitrogen). After 1-h incubation at 37 °C, the relative fluorescence was measured with a Victor^3 (PerkinElmer).

Results and discussion

Within the great variety of the 350 endemic plants growing in Sardinia (an Italian island), the *H. hircinum* L. (*Hypericaceae*) has been reported to have antiseptic properties (Ballero *et al.*, 1997) and may have some constituents, structurally similar to some already identified (Fesen *et al.*, 1993; Vandegraaff *et al.*, 2001), potentially able to inhibit the HIV-1 RT-associated RNase H function. To verify this hypothesis, we obtained three ethanolic extracts from the Sardinian *H. hircinum* L. and tested them on the HIV-1 RT-associated RNase H function in *in vitro* biochemical assays (Table 1). All extracts (EI–EIII), differing in ethanol concentration and timing of maceration, inhibited the HIV-1 RNase H functions with IC_{50} values ranging from 5 to 8 μg mL^{-1} and were further fractionated. Six fractions (EI1–EI6) were obtained from extract I and were evaluated for their anti-RNase H activity (Table 2). Fractions EI1–EI4 showed IC_{50} values between 8 and 25 μg mL^{-1}, while fractions EI5–EI6 were inactive. The main components of fractions EI1–EI6 were isolated by column chromatography and preparative TLC, and their structures were identified by UV-, ^{1}H-, and ^{13}C-NMR spectroscopy (Table 2). Major components of these fractions were betulinic acid, shikimic acid, and 5, 7, 3′, 5′-tetrahydroxyflavanone 7-O-glucoside (Fig. 1). Other components were also present, even though they were not identified. In fact, while shikimic acid was the major component of fractions EI3–EI6, fractions EI3 and EI4 inhibited the RNase H activity, whereas fractions EI5 and EI6 were inactive, suggesting that some other active compounds might be present. Six fractions were also obtained from extract II (EII1–EII6) and were evaluated for their anti-RNase H activity (Table 2). All fractions inhibited the RNase H function showing IC_{50} values between 4 and 21 μg mL^{-1}, and their content was analyzed (Table 2).

Table 1 Effect of *Hypericum hircinum* L. extracts on the HIV-1 RT-associated RNase H function

Extract	IC_{50} (μg mL^{-1})*
EI	7 ± 1
EII	8 ± 3
EIII	5 ± 1

*Extract concentration required to inhibit the HIV-1 RT-associated RNase H activity by 50%.

Table 2 Effect of *Hypericum hircinum* L. fractions on the HIV-1 RT-associated RNase H function

Fraction	IC_{50} (μg mL^{-1})*	Main components
Extract I		
EI1	9 ± 2	Waxes, betulinic acid
EI2	10 ± 3	betulinic acid, shikimic acid, 5,7,3′,5′-tetrahydroxyflavanone 7-O-glucoside
EI3	25 ± 4	Shikimic acid
EI4	8 ± 2	Shikimic acid
EI5	> 100 (71%)†	Shikimic acid
EI6	> 100 (68%)	Shikimic acid, carbohydrates
Extract II		
EII1	4 ± 1	Waxes, betulinic acid, 5,7,3′, 5′-tetrahydroxyiflavanone
EII2	4 ± 1	Betulinic acid, 5,7,3′, 5′-tetrahydroxyflavanone 7-O-glucoside
EII3	5 ± 2	5,7,3′,5′-tetrahydroxyflavanone 7-O-glucoside
EII4	8 ± 2	Shikimic acid, quercetin, 5,7,3′, 5′-tetrahydroxyflavanone 7-O-glucoside
EII5	7 ± 3	Complex mixture, shikimic acid, quercetin, 5,7,3′, 5′-tetrahydroxyflavanone 7-O-glucoside
EII6	21 ± 3	Chlorogenic acid
Extract III		
EIII1	90 ± 4	–
EIII2	27 ± 2	Waxes, betulinic acid
EIII3	26 ± 3	Complex mixture, waxes, betulinic acid, flavonoids
EIII4	> 100 (55%)	–
EIII5	9 ± 2	5,7,3′,5′-tetrahydroxyflavanone
EIII6	> 100 (76%)	–
EIII7	23 ± 3	Complex mixture, betulinic acid, 5,7,3′, 5′-tetrahydroxyflavanone
EIII8	6 ± 1	Complex mixture, flavonoids

*Extract concentration required to inhibit the HIV-1 RT-associated RNase H activity by 50%.

†Percentage of control activity measured in the presence of 100 μg mL^{-1} extract concentration.

Major components of EII fractions were betulinic acid, shikimic acid, quercetin, 5,7,3′,5′-tetrahydroxyflavanone, 5,7,3′,5′-tetrahydroxyflavanone 7-O-glucoside, and chlorogenic acid (Fig. 1). Finally, 8 fractions (EIII1–EIII8) were obtained from extract III and were evaluated for their anti-RNase H activity (Table 2). Fractions EIII2, EIII3, EIII5, EII7, and EIII8 inhibited the HIV-1 RNase H activity with IC_{50} values ranging from 6 to 27 μg mL^{-1}, and their content was analyzed (Table 2). In this case, the major components were betulinic acid, 5,7,3′,5′-tetrahydroxyflavanone, and 5,7,3′,5′-tetrahydroxyflavanone 7-O-glucoside.

To verify whether the main single identified components of the *H. hircinum* L. extracts were effectively able to inhibit the RNase H activity, quercetin, shikimic acid, 5,7,3′,5′-tetrahydroxyflavanone, 5,7,3′,5′-tetrahydroxyflavanone 7-O-glucoside, and betulinic acid were assayed on the HIV-1

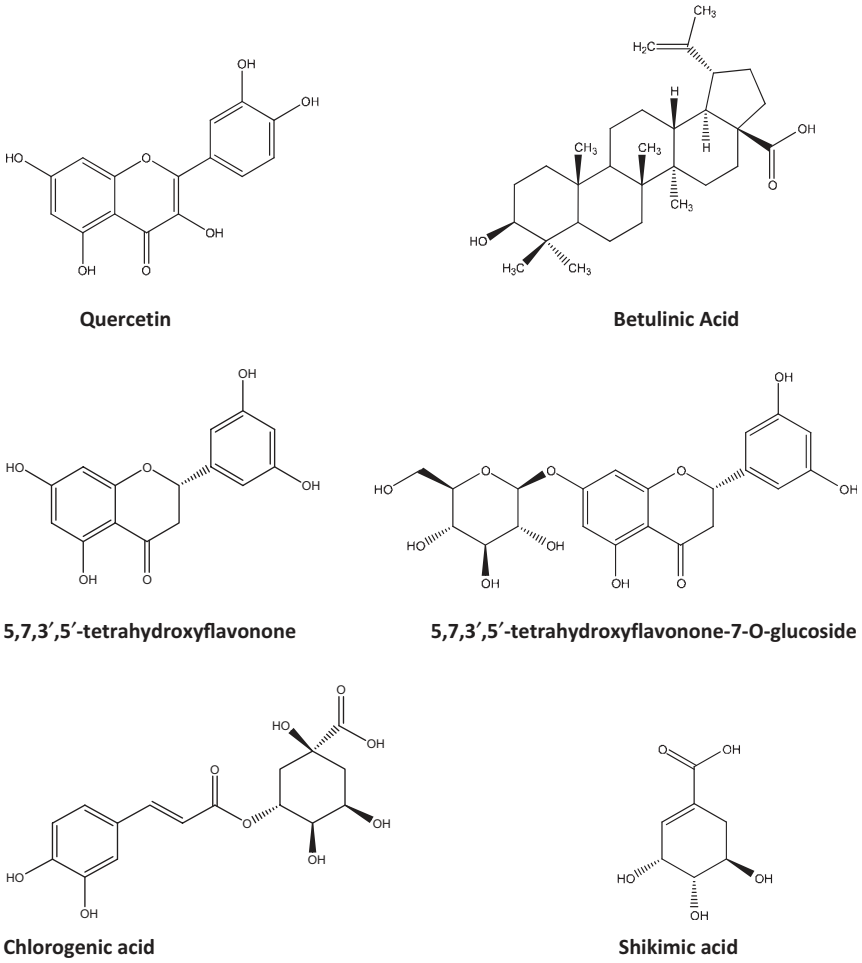

Fig. 1 Structures of the major components of the *Hypericum hircinum* L. extracts.

RT-associated RNase H activity and, to assess their inhibition specificity, also on the HIV-1 RT-associated RDDP activity (Table 3). RDS 1643 (Tramontano *et al.*, 2005), efavirenz, and K-49 (Esposito *et al.*, 2011) were used as positive controls. Quercetin has been previously reported to inhibit the HIV-1 IN (Fesen *et al.*, 1993; Vandegraaff *et al.*, 2001), and it was also able to block both HIV-1 RT-associated functions (Table 3). Similarly to quercetin, the newly identified flavanone 7-O-glucoside was able to inhibit both RT functions, even though at higher concentration (Table 3). Differently, the flavanone compound lacking the glucoside molecule was able to inhibit only the HIV-1 RNase H activity. Given that compounds such as hydrazone and anthraquinone derivatives, which have been reported to inhibit both HIV-1 RT-associated functions, have been shown to bind to a site close to the non-nucleoside RT inhibitors (NNRTI) binding pocket (Himmel *et al.*, 2006; Esposito *et al.*, 2011), it is possible to hypothesize that also these flavanone derivatives may bind to this site. This hypothesis is consistent with the report that small hydrazone derivatives inhibit the sole RNase H function, while bulkier hydrazone analogs inhibit both RT functions (Himmel *et al.*, 2006). Interestingly, shikimic acid was not able to inhibit the HIV-1 RNase H function, while it inhibited very slightly the HIV-1 RDDP function (Table 3). Therefore, even though shikimic acid was the major component of fractions EI3 and EI4, it was not responsible for their anti-RNase H activity. Finally, betulinic acid inhibited both RT-associated activities in the low micromolar range (Table 3, Fig. 2). Notably, betulinic acid is a member of the triterpene family that has been reported to inhibit HIV-1 replication by intervening at different steps of the virus life cycle such as entry, reverse transcription, integration, and viral maturation (Fujioka *et al.*, 1994; Mayaux *et al.*, 1994; Hashimoto *et al.*, 1997; Aiken & Chen, 2005; Dorr *et al.*, 2011; Lan *et al.*, 2011). In particular, betulinic and dihydrobetulinic acids were used to obtain potent antiviral derivatives such as the known antimaturation derivative 3-O-(3′,3′-dimethylsuccinyl)-betulinic acid (Kashiwada *et al.*, 1996; Li *et al.*, 2006; Yu *et al.*, 2006). It is interesting to note that conflicting reports were previously published describing the betulinic acid effect on the HIV-1 RT. Some authors reported that betulinic acid is not able to inhibit HIV-1 RT (Evers *et al.*, 1996; Kashiwada *et al.*, 1996; Hashimoto *et al.*, 1997; Wang *et al.*, 2008), while others reported that it inhibits the HIV-1 RT-associated RDDP activity (Reutrakul *et al.*, 2006). Our results confirm that

Table 3 HIV-1 RT inhibition by the major components of the *Hypericum hircinum* L. extracts

Compound	IC_{50} (μM)		CC_{50} (μM)*	EC_{50} (μM)†
	RNase H‡	RDDP§	TZM-bl	HIV
Quercetin	4.5 ± 0.5	21 ± 2	7.2 ± 0.3	> 7
Flavanone 7-O-glucoside	33 ± 3	80 ± 3	> 100	> 20
Flavanone	21 ± 2	> 100	92 ± 3	> 20
Shikimic acid	> 100	85 ± 18	> 100	> 20
Betulinic acid	2.0 ± 0.2	0.5 ± 0.1	24 ± 1	> 20
RDS 1643	8.1 ± 2.2	–	> 100	–
Efavirenz	–	0.013 ± 0.004	–	–
K-49	12 ± 3	11 ± 2	66 ± 2	–

*Compound concentration required to reduce by 50% TZM-bl cell viability.
†Compound concentration required to inhibit early phases of HIV-1 replication by 50%.
‡Compound concentration required to inhibit HIV-1 RT-associated RNase H activity by 50%.
§Compound concentration required to reduce the HIV-1 RT-associated RDDP activity by 50%.

betulinic acid has, indeed, an anti-RT effect (Table 3, Fig. 2). Hence, we asked whether betulinic acid might bind to the anthraquinone and hydrazone site. Given that most anthraquinone derivatives are active against the NNRTI-resistant K103N and Y181C RTs (Esposito *et al.*, 2011), we evaluated the betulinic acid effect on these mutant RTs using efavirenz as positive control (Table 4). Results showed that betulinic acid potency of inhibition on the RDDP function was reduced only by three- and twofold when assayed on the K103N and Y181C RTs, respectively, while its potency of inhibition on the RNase H function was reduced by threefold when tested against the Y181C RT,

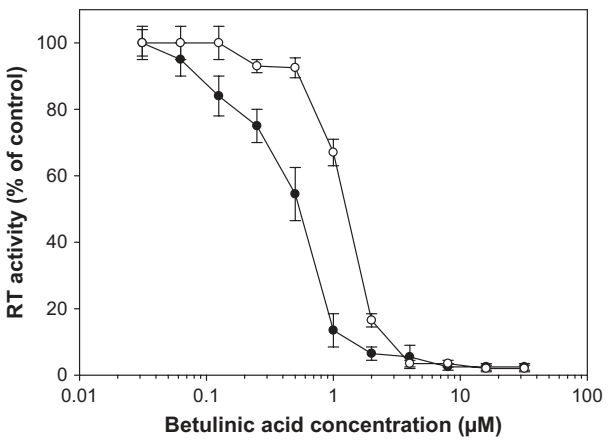

Fig. 2 Inhibition of HIV-1 RT-associated functions by betulinic acid. Inhibition curves of the RDDP (full circles) and RNase H (empty circles) functions. Reactions were carried out as described in Materials and methods. Data represent mean values from three independent determinations.

Table 4 Effects of betulinic acid on mutant HIV-1 RTs

Compound	IC_{50} (μM)			
	RDDP*		RNase H†	
	K103N RT	Y181C RT	K103N RT	Y181C RT
Betulinic acid	1.4 ± 0.2	1.1 ± 0.6	3.5 ± 0.7	7.5 ± 0.7
Efavirenz	0.68 ± 0.016	0.40 ± 0.008	–	–

*Compound concentration required to inhibit the HIV-1 RT-associated RDDP activity by 50%.
†Compound concentration required to inhibit the HIV-1 RT-associated RNase H activity by 50%.

and it was not reduced when assayed against the K103N RT (Table 4). These results are consistent with the hypothesis that betulinic acid may bind to the anthraquinone and hydrazone site.

To further characterize the mode of action of the identified natural components and to verify whether they may interact with the RNase H active site by chelating the magnesium ions, similarly to what has been shown for the diketo acid derivatives (Tramontano *et al.*, 2005), we measured their absorbance profile in the absence/presence of $MgCl_2$, as well as after the addition of EDTA (Fig. 3). We used the diketo acid derivative RDS 1643 as positive control (Tramontano *et al.*, 2005), showing that its capability to chelate Mg^{2+} is associated with a shift in its maximum of absorbance UV-Vis spectra, from 395.5 to 418 nm, and that this shift is modified after EDTA addition (Fig. 3a). Hence, we recorded the UV-Vis spectra for all the identified derivatives and found that quercetin spectra maximum of absorbance was shifted from 380 to 397 nm, in the presence of $MgCl_2$, and that this shift was completely reversed after EDTA addition (Fig. 3b). Differently, after the addition of Mg^{2+}, both flavanone derivatives showed only an increase in their maximum of absorbance intensity, and such increase was not reversible by the addition of EDTA, suggesting that the characteristics of a coordination complex are not present in these cases and nonspecific interactions with magnesium cannot be excluded (Fig. 3c and d). In addition, we observed that betulinic and shikimic acids do not absorb in the UV-Vis spectra and do not form visible coordination complexes with Mg^{2+} or Co^{2+} that is reported to eventually form colorful complexes (data not shown). Therefore, while we could not completely exclude their interaction with Mg^{2+}, these results, together with the analysis of their chemical structures, suggest that they are probably not able to chelate magnesium ions. Overall, these data support the possibility that quercetin may inhibit both RT-associated functions and IN activities by coordinating the Mg^{2+} ions in the three enzymes' active sites.

It has been reported that betulinic acid derivatives inhibit viral replication in cell culture blocking viral entry and maturation (Aiken & Chen, 2005). However, at the best of our knowledge, it is not clear at which level of the virus life cycle, the betulinic acid exerts its inhibition. Therefore, we

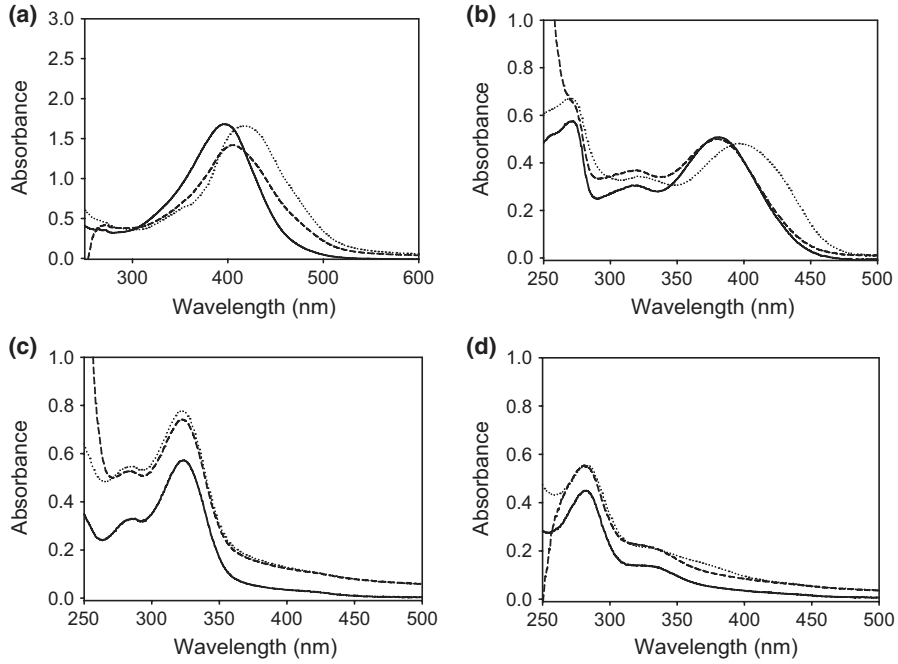

Fig. 3 Chelation of Mg^{2+} by the major components of the *Hypericum hircinum* L. extracts. UV–Vis spectrum of compounds was measured alone (unbroken line), in the presence of 6 mM $MgCl_2$ (dotted line) and in presence of 6 mM $MgCl_2$ and EDTA 30 mM (broken line). UV-Vis spectrum of RDS 1643 (a), quercetin (b), 5,7,3′,5′-tetrahydroxyflavanone (c), and 5,7,3′,5′-tetrahydroxyflavanone-7-O-glucoside (d) is reported.

Table 5 Cytotoxic effects of *Hypericum hircinum* L. components

	CC_{50} (μM)*			
Compound	Vero 76	A549	Hep G2	Jurkat
Betulinic acid	29 ± 6	17 ± 6	38 ± 2	> 50
Shikimic acid	> 100	> 100	> 100	> 50

*Compound concentration required to reduce by 50% cell viability.

asked whether its antiviral effect could be ascribed to the inhibition of the early steps of the virus cycle. To this end, we used an *env* complementation assay that measures the efficiency of the early events in a single round of infection (Helseth *et al.*, 1990). In this assay, an *env*-defective provirus encoding the bacterial chloramphenicol acetyltransferase gene was complemented in *trans* by the envelope glycoprotein derived from the laboratory-adapted T-cell-tropic strain HXBc2, which uses CXCR-4 as a coreceptor (Feng *et al.*, 1996). Results showed that betulinic acid was not able to inhibit the early events of the infection process (Table 3) and that it is cytotoxic in different cells lines at a concentration around 20 μM (Table 5). Similarly, also the other *H. hircinum* L. components quercetin, shikimic acid, 5,7,3′,5′-tetrahydroxyflavanone, and 5,7,3′,5′-tetrahydroxyflavanone 7-O-glucoside were assayed for their cytotoxicity and antiviral effects. While none of them was toxic in Jurkat cells at 50 μM concentration, they were not able to inhibit the HIV replication (Table 3 and data not shown).

In conclusion, we showed that some *H. hircinum* L. single components are able to inhibit the HIV-1 RT-associated functions in the low micromolar range. The fractionation of the extract allowed to identify among the active constituents of the *H. hircinum* L. extracts betulinic acid and two flavanone derivatives, both newly identified in *H. hircinum* L. In particular, betulinic acid is able to inhibit both HIV-1 RT-associated functions of wild-type and mutants RTs, even though it is not able to inhibit the reverse transcription in cell culture. Overall, because betulinic acid derivatives are currently developed to inhibit viral entry and maturation, more studies should be performed in designing new betulinic acid derivatives acting on different targets to obtain dual HIV inhibitors.

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