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Antiviral activity of seed extract from *Citrus bergamia* towards human retroviruses

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ABSTRACT

The effects of an extract from *Citrus bergamia* (BSext) and those of two products purified from the same extract, that is, nomilin and limonin, and reference compounds, towards HTLV-1 have been reported. Moreover, they were also compared with those obtained towards HIV-1. Results showed that the efficacy of both BSext and limonin in inhibiting HTLV-1 as well as HIV-1 expression in infected cells, as evaluated by comparable quantitative assays, was close to that of the effective, reference compounds, respectively. The protective effect of BSext and of the purified products was associated with the inhibition of both HTLV-1 and HIV-1 RT activities in conceptually similar, cell-free assays. The cytotoxicity of the assayed compounds of natural origin was substantially less pronounced than that of the reference compounds, thus showing a favourable selectivity index for the novel BSext product.

1. Introduction

Before the evidence that HIV-1 was the aetiological agent of AIDS, the first retrovirus discovered as associated to human diseases has been the oncogenic, delta retrovirus human T-cell leukaemia/lymphoma virus type 1 (HTLV-1), spread in endemic areas, such as Japan, North and South America, where about 5% of the estimated 20 million HTLV-1 infected people develop HTLV-1-associated diseases, such as adult T-cell leukaemia (ATL), human myelopathy/tropical spastic paraparesis (HAM/TPS) or other minor inflammatory diseases.[1,2] Although HIV-1 and HTLV-1 share the characteristic to be CD4+ lymphotropic, their biological, pathological and therapeutic features show important differences. Moreover, while an immense body of basic studies on anti-HIV drugs has been accumulated during the past 25 years,[3,4] only a limited number of antiretroviral compounds have been actually tested towards HTLV-1 infection in vitro.[5] In addition, except one case,[6] the screening of anti-HTLV-1 compounds was restricted to compounds already developed and selected as anti-HIV-1 agents. Thus, there is a general need for increasing the spectrum of molecules potentially able to control the infections caused by HTLV-1. Besides, it is worth noting that, despite the first description of an effective antiretroviral agent that dates back 25 years, a comparative evaluation of anti-HIV-1 and anti-HTLV-1 activities of compounds of interest within the same study and by means of similar methodological approaches has not yet been reported in the literature. Thus, no reliable data exist on the different sensitivities of HTLV-1 and HIV-1, or their respective RT, to compounds of interest, in vitro. In the past years several plant-derived natural compounds have been screened as anti-HIV-1 agents in order to find lead compounds with novel structures or mechanisms of action.[7,8] In particular, several triterpenes including betulinic acid derivatives have been found to exhibit some anti-HIV-1 activity.[9–14] Interestingly, the antiviral activities of limonoids that are modified triterpenoids derived from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton have been recently described.[15] The preparation of limonoids requires a multistep and laborious synthetic process. Thus, plant extracts can be pursued as suitable alternative sources of limonoids. Moreover, in case the crude

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natural plant extracts prove to be sufficiently effective as antiretrovirals, they can be produced at a very low cost extending their potential usage to resource-poor countries.

In the present study we have extended the results of plant-derived natural compounds, and particularly of triterpenoids, as antiretroviral agents, for the first time to HTLV-1. To this purpose, the antiretroviral activity of a crude extract produced from the seeds of bergamot (*Citrus bergamia*), a typical fruit grown wildly in Calabria (Italy), has been directly evaluated towards HTLV-1 in vitro infection of peripheral blood mononuclear cells from healthy donors. In addition two components of the crude bergamot seed extract (hereafter referred to as BSext), that is, limonin and nomilin, already known to exert an anti-HIV-1 activity, have been isolated from the seed extract and biologically evaluated towards HTLV-1 by comparison with the total crude extract.

Moreover, to get information on mechanisms involved in their antiretroviral activity, the effects of the different products on HTLV-1 RT enzymatic activity have been assayed. In addition, results obtained on HTLV-1 were also compared with those on HIV-1 by means of similarly structured assays. The results indicate that the examined products are actually endowed with an appreciable antiretroviral activity and that mechanisms involved may include the inhibition of viral RT activity. No appreciable cytotoxic effect by the assayed products was detected.

2. Results

2.1. Extraction and isolation

The methanolic extract from *C. bergamia* (BSext) was obtained from the bergamot seed by trituration and maceration in methanol. From the same extract, nomilin and limonin were isolated by medium pressure liquid chromatography (MPLC) on silica gel column using a mixture of chloroform/methanol. The ^{1}H NMR and mass spectra of nomilin and limonin were totally superimposable with that of commercial standards. LC/MS/MS analysis of the BSext crude extract, according to literature data,[16] revealed the presence of limonin, nomilin, ichangensin, ichangin, obacunone, deacetylnomilin, methyl isoobacunoate, methyl isoobacunoate diosphenol, methyl deacetylnominilate and calamin (Fig. 1).

2.2. Evaluation of the inhibitory activity of BSext, limonin and nomilin on HTLV-1 and HIV-1 infection in vitro

The activity of BSext, limonin from BSext and nomilin from BSext towards HTLV-1 in vitro was assayed. The concentrations to be used for the novel and the reference compounds were chosen on the basis of preliminary dose-effect experiments and previous experience. Moreover, in order to get reliable information also on the sensitivity of HIV-1 to the same compounds, inhibition of infection was assayed by the detection of viral expression using methods comparable, as much as possible, for both HTLV-1 and HIV-1 infections. For HTLV-1, infection was detected after 3 weeks in culture post infection (pi), while for HIV-1, infection was detected after 5 days in culture pi. In the first round of experiments, infection was evaluated through conventional RT-PCR analysis. The results showed that BSext and nomilin completely inhibited HTLV-1 tax/rex expression at 10 μg/ml (see Fig. S1 in Supplementary data). Limonin was able to inhibit HTLV-1 tax/rex expression also at the concentration of 5 μg/ml. AZT and 3TC exerted an anti-HTLV-1 minimal 100% inhibitory activity, as detected by this assay, at concentrations corresponding to 0.27 μg/ml and 22.93 μg/ml, respectively. These results confirmed the very low natural sensitivity of HTLV-1 to 3TC. In parallel, HIV-1 gag expression was completely inhibited by 5 μg/ml and 1 μg/ml BSext and limonin, respectively (see Fig. S2 in Supplementary data). Nomilin, conversely, inhibited gag expression only at the concentrations of 10 μg/ml. Reference compounds showed a minimal 100% inhibitory activity on gag expression at concentrations corresponding to 0.27 μg/ml for AZT and to 0.23 μg/ml for 3TC, while NEV (concentration range 1.3, 0.27, 0.13, 0.03 μg/ml) showed a minimal 100% inhibitory activity at 0.27 μg/ml (data not shown). Thus, interestingly, this first round of experiments showed that the natural compounds exhibited an inhibitory effect on HTLV-1 viral gene expression at concentrations presumably not very far from that of the reference compounds as well as from that capable to inhibit HIV-1 gene expression in a similar assay. Then, in order to obtain real quantitative information on the inhibitory activity of the compounds of interest, in successive experiments HTLV-1 gene expression was evaluated through quantitative-real-time RT-PCR (RQ-PCR) analysis in cells subjected to experimental infections and treatment in the same conditions described above. Table 1 shows the results of RQ-PCR expressed as mean '$2^{-\Delta\Delta Ct}$' values of infection inhibitory concentration 50% ± standard deviation (RQ-IC_{50} ± SD), calculated on the three '$2^{-\Delta\Delta Ct}$' values obtained in three different experiments, each performed in triplicate, that gave similar results. Limonin exhibited a RQ-IC_{50} value of 1.07 ± 0.09, being about three times more efficacious than BSext (3.15 ± 0.65) and nomilin (3.98 ± 0.71), respectively, in inhibiting HTLV-1 tax/rex expression. Moreover, limonin was less efficacious than AZT. Finally, all BSext, limonin and nomilin confirmed to be much more effective than 3TC towards HTLV-1, also by this assay. When, in parallel, the effects of the compounds of interest were quantitatively assessed on HIV-1 expression, limonin exhibited an RQ-IC_{50} that was similar to that detected for HTLV-1 infection and about 13 times more efficacious in inhibiting gag expression than nomilin (13.00 ± 1.37). Limonin was also about four times less efficacious than AZT or 3TC and about 10 times less efficacious than NEV in inhibiting HIV-1 expression following infection. In addition, interestingly, fully quantitative assays confirmed that the efficacy of BSext in inhibiting HIV-1 expression in infected cells (1.00 ± 0.05) was superimposable to that of limonin, that is, not far from that of AZT, 3TC and NEV.

2.3. Inhibitory effects of BSext, limonin and nomilin on HTLV-1 and HIV-1 RT activity

To investigate the mechanism by which BSext, limonin and nomilin exert their anti-HTLV-1 activity, their ability to inhibit the RT activity of HTLV-1, by means of a cell-free assay, was studied.[17] To provide semi-quantitative information of the RT inhibitory effect, the assay was performed at a low number of amplification cycles (20 cycles), in the presence or in the absence of 10, 8, 6, 4, 2, 1 ng/ml for BSext or nomilin, and of 1, 0.8, 0.6, 0.4, 0.2, 0.1 ng/ml for limonin. Regarding to the non-nucleoside reference compound NEV, it was used at concentration ranges from 26.6 to 266.3 ng/ml due to its presumed poor activity on HTLV-1 RT. The results of the RT activity inhibition assay are expressed as mean values of the three concentrations of the products that were able to inhibit the RT activity by 50% (RT inhibitory concentration 50%, $RTIC_{50}$) ± SD obtained in three independent experiments, as calculated from densitometry analysis values of the bands. Table 2 shows the results of the HTLV-1 RT inhibitory activity exerted by BSext in comparison with that of the purified limonin and nomilin fractions. The $RTIC_{50}$ values of limonin and nomilin were about 13 and 1.5 times lower, respectively, in comparison with that of BSext. Moreover, by comparing the $RTIC_{50}$ value of the limonoid products with that of the reference compounds, we infer that the ability of limonin to directly inhibit HTLV-1 RT activity was more than 1000 times higher than that of NEV, showing the latter for the first time its inability to inhibit HTLV-1 RT activity. Moreover, it was similar or only slightly lower than that

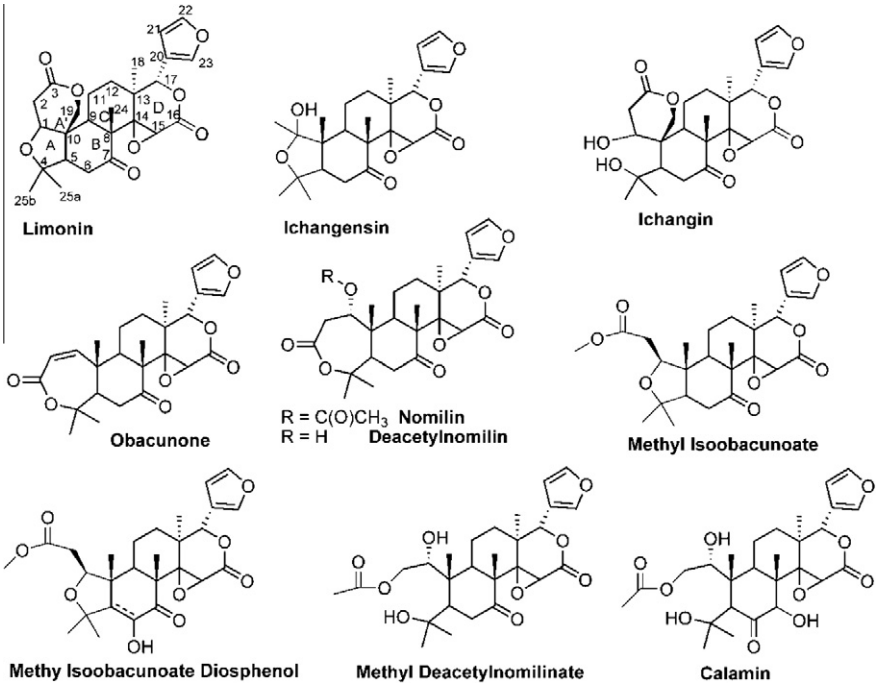

Figure 1. Limonoids detected in bergamot seed extract.

Table 1
Quantitative evaluation of HTLV-1 and HIV-1 infections

	RQIC$_{50}$ ± SD[a] (μg/ml)	
	HTLV-1	HIV-1
AZT	0.16 ± 0.00	0.26 ± 0.01
3TC	14.24 ± 0.19	0.19 ± 0.01
NEV	ND	0.09 ± 0.02
BSext	3.15 ± 0.65	1.00 ± 0.05
Limonin	1.07 ± 0.09	0.92 ± 0.09
Nomilin	3.98 ± 0.71	13.00 ± 1.37

[a] RQIC$_{50}$ = drug concentration inhibiting 50% $2^{-\Delta\Delta Ct}$ values of real time PCR.

Table 2
Inhibitory activity of BSext, limonin and nomilin on HTLV-1 and HIV-1 RT enzymatic activities

	RTIC$_{50}$ ± SD[a] (ng/ml)	
	HTLV-1-vl[b]	HIV-1-vl[c]
NEV	>266.30	0.11 ± 0.05
BSext	3.95 ± 0.21	26.36 ± 2.69
Limonin	0.31 ± 0.01	2.11 ± 0.03
Nomilin	2.60 ± 0.06	3.56 ± 0.12

[a] RTIC$_{50}$ = concentration inhibiting 50% HTLV-1 and HIV-1 RT activities.
[b] Virion lysate (vl) from HTLV-1 chronically infected cells as RT source.
[c] Virion lysate (vl) from HIV-1 chronically infected cells as RT source.

of the pre-activated AZT, based on results previously published by us.[6,17] For comparison, in parallel we also studied the direct effects of our products of interest also on HIV-1 RT activity, by means of an assay conceptually similar to that previously set up for HTLV-1 RT. Limonin and nomilin were 12 and 7.4 times more effective than BSext, respectively, in inhibiting HIV-1 RT. By comparing the activity of limonoids with that of the reference compound, we observe that limonin (RTIC$_{50}$ = 2.11 ng/ml) was effective in inhibiting HIV-1 RT at levels that were about 20 times lower than that of NEV (RTIC$_{50}$ = 0.10 ng/ml). Moreover, these data show also that limonin and BSext were about seven times more efficacious in inhibiting HTLV-1 RT than HIV-1 RT, as detected in our assays. Conversely, nomilin was quite equally effective in inhibiting HIV-1 RT and HTLV-1 RT activities. Finally, when a commercially available purified HIV-1 RT was utilized as a source of RT for our assay in place of the HIV-1 virion lysate, results were very similar to those obtained using the latter preparation (Table 3).

2.4. Toxicity of BSext, limonin and nomilin

In order to assess the cytotoxic potential of BSext, limonin and nomilin, their effects on the metabolic activity of uninfected cells was evaluated by means of an MTS assay. Particularly, lymphoid MOLT-3 cells and PHA stimulated PBMCs were treated in vitro with seven different concentrations of the products, ranging from 1 ng/ml to 1 mg/ml. After 24 h, the inhibition of oxidative burst was assayed. Results reported in Table 4 are represented as the product concentrations able to inhibit the metabolic activity of MOLT-3 cells by 50% (metabolic activity cytotoxic concentration 50%, in MOLT-3 MmaCC$_{50}$ ± SD) and in PBMC (metabolic activity cytotoxic concentration 50%, ±SD in PBMC, PmaCC$_{50}$). The results are the means of three maCC$_{50}$ values obtained in three independent MTS experiments. Data indicate that BSext and nomilin were four times less effective than limonin in inhibiting the oxygen burst in MOLT-3 cells. The comparison of the cytotoxicity of the products of interest with that of AZT and 3TC and NEV, by means of this assay, showed that AZT was at least 230 times more toxic

Table 3
Inhibitory activity of BSext, limonin and nomilin on enzymatic activity of commercial purified HIV-1 RT

	RTIC$_{50}$ ± SD[a] (ng/ml) HIV-1 RT
NEV	0.09 ± 0.00
BSext	39.30 ± 6.12
Limonin	4.23 ± 0.31
Nomilin	3.42 ± 0.52

[a] RTIC$_{50}$ = compound concentration inhibiting 50% HTLV-1 and HIV-1 RT activities.

Table 4
Evaluation of cytotoxic effects of BSext, limonin and nomilin

	MOLT-3 $maCC_{50}$ ± SD[a] (μg/ml)	PBMC $maCC_{50}$ ± SD (μg/ml)
AZT	3.24 ± 0.45	31.20 ± 5.04
3TC	5.94 ± 0.32	52.97 ± 5.31
NEV	20.06 ± 1.32	40.88 ± 12.09
BSext	783.47 ± 67.12	861.50 ± 111.00
Limonin	201.56 ± 33.21	451.91 ± 65.00
Nomilin	755.73 ± 39.87	691.56 ± 115.9

[a] maCC50 is the metabolic activity cytotoxic inhibitory concentration 50%, evaluated in MOLT-3 and PHA stimulated PBMC, respectively, by MTS assay.

than BSext and nomilin as well as 62 times more toxic than limonin. Similarly, 3TC was about 130 times more toxic than BSext and nomilin, while 33 times more toxic than limonin. Conversely NEV was 10 times more toxic than limonin, and about 40 times more toxic than BSext and nomilin. The MTS assay on stimulated PBMC showed that these cells were less sensitive to the cytotoxic effect of both natural and control compounds except to nomilin. Actually, BSext was two times less toxic than limonin, and 28, 16 and 20 times less toxic than AZT, 3TC and NEV, respectively. Therefore, the products under study were endowed with a remarkable lower level of cytotoxicity with respect to the known, antiretroviral reference compounds assayed. Moreover, these results clearly demonstrate that the effects of both BSext, limonin and nomilin versus both HTLV-1 and HIV-1 infection were owed to their real antiviral effects rather than to a possible cytotoxic potential. Actually, the concentration of BSext able to induce a cytotoxic effect was about 860 times higher than that endowed with an antiviral activity towards HIV-1 infection, while limonin was cytotoxic at a concentration 450 times higher in comparison with that exerting an antiviral efficacy towards the same infectious agent.

3. Discussion

The present study demonstrates for the first time that a seed extract from *C. bergamia* in which limonin and nomilin, two limonoids already described as weak HIV-1 protease inhibitors, are present in a total approximately amount of 5.5%, exerts by itself an incontrovertible antiretroviral activity towards HTLV-1 and that this effect is associated to its capability to inhibit HTLV-1 RT enzymatic activity. Moreover, here we report the first comparative evaluation of the inhibitory activities of the new potential or consolidated antiretroviral agents, towards both HTLV-1 and HIV-1 human retroviruses.

In fact, BSext was about 20 times less effective than AZT in inhibiting HTLV-1 viral expression. At the same time, BSext was actually found to inhibit gag expression in HIV-1 infected cells showing an efficiency four times lower than that of AZT and 10 times lower than that of NEV, respectively, as evaluated using a method of viral gene detection comparable with those utilized for HTLV-1. Meanwhile, BSext resulted about 28 times less cytotoxic than AZT and about 20 times less cytotoxic than NEV in PBMC, thus showing in any case a more favourable selectivity index. Regarding the examined purified components of BSext, limonin was practically as efficient as the whole extract in protecting from HTLV-1 as well as from HIV-1 infections. At the same time, it was more effective than nomilin in inhibiting both HTLV-1 tax/rex and HIV-1 gag expression in infected cells. However, the lower antiretroviral activity of nomilin with respect to limonin was compensated by its lower toxicity. The exact mechanism by which these compounds exert their anti-retroviral action has not been fully addressed. However, we have investigated one of the potential mechanisms involved. To this purpose, we analysed the effects of the examined products directly on HTLV-1 and, for comparison, HIV-1 RT enzymatic activities using cell-free, virus-free assays. The results showed that all the natural compounds assayed are endowed with an RT inhibitory activity. Limonin was the most efficacious natural product in directly inhibiting both HTLV-1 and HIV-1 RT, in comparison with nomilin and BSext. Nevertheless, limonin was less effective than the reference compound in inhibiting HIV-1 RT. Meanwhile, HTLV-1 RT activity was apparently more sensitive with respect to HIV-1 RT to inhibition by limonin and BSext. However, although the two methods for assaying the HTLV-1 RT and the HIV-1 RT activity and for preparing the occurring products have been set up according to identical criteria, usage of non-purified extracts as a source of human retrovirus RT could introduce uncontrolled variables in the systems that might bias the comparative analysis of the results obtained from the two assays. Nevertheless, a possible different sensitivity of HTLV-1 RT to an examined product when compared to HIV-1 RT, as evidenced for the first time in this study, is not surprising. The tertiary structures of HIV-1 RT and HTLV-1 RT seem to show a remarkable similarity[18,19] but the two retroviral enzymes share only 20% of amino acid sequence identity. Relevant to this, our previous studies in vitro demonstrated that HTLV-1 shows a different sensitivity to nucleoside inhibitors with respect to HIV-1.[20] Regarding mechanisms involved in RT inhibition, the natural compounds we assayed are not nucleoside reverse transcriptase inhibitors (NRTIs). However, they could still bind at the polymerase active site.[21] This could account for their capacity to inhibit both HTLV-1 and HIV-1 RT. In addition, although our data show that BSext and its assayed components exhibited an apparent preferential inhibitory activity for HTLV-1 RT with respect to HIV-1 RT, this did not result in a corresponding higher capability of the same product to prevent HTLV-1 infection rather than HIV-1 infection in vitro. In addition, the higher capacity of limonin with respect to BSext in inhibiting RT activity from HIV-1 was not paralleled by a higher capability of the former product to prevent HIV-1 infection. Thus, altogether these results suggest that the antiretroviral activity exerted by BSext might only partly directly depend on inhibition of the RT activity. In fact, although triterpenes have been shown by years to exert inhibitory effects on HIV-1 RT,[9] also different mechanisms of antiviral activity have been recently described for compounds belonging to this family. Interestingly, recent data have shown that glycyrrhizin, a triterpen compound, inhibited influenza A virus uptake by interfering with cellular membrane structure.[22] It has been also suggested that inhibition of HIV-1 infection upon treatment with glycyrrhizin was due to a suppression of the fluidity of the plasma membrane, leading to inhibition of virus fusion.[23] In addition other authors have shown that a structural related triptenoid, oleanolic acid inhibited in vitro HIV protease activity.[24] Moreover, the betulinic acid, whose structure closely resembles that of limonoids, has been shown to act as a HIV-1 maturation inhibitor.[25] Furthermore, its derivative, bevirimat, was launched in phase IIb clinical trials.[14] Regarding to limonin and nomilin, these compounds have been already reported to inhibit HIV replication in both PBMC and monocytic/macrophagic cells.[26] Indeed, in this study limonin and nomilin showed 30 and 3 times lower activity, respectively, when compared to our results. This might be ascribed to different experimental conditions including protocols of infection and treatment (at the time of infection, as in our experiments, or after infection, as in the mentioned experiments). Another important aspect to address with respect to the antiretroviral activity of BSext is whether components other than limonin and nomilin could contribute to its efficacy. Regarding this point we must consider that limonin and nomilin are present in the extract in an approximate amount of 5.5%, and that usage of the same compounds as purified products did not consistently lead to an higher, but rather in some cases to a lower, antiretroviral activity (see Table 1). This allows us to speculate that also other

components of BSext, including the minor limonoid components (4.5%) may be involved in the antiretroviral activity. Further investigation is necessary to clarify this aspect. In addition, we must note that, using our assays, we found a high discrepancy in the anti-HIV $RQIC_{50}$ (inhibition of virus expression) value in comparison with the anti-HIV $RTIC_{50}$ (inhibition of RT enzymatic activity) value of the compounds. Interestingly, however, the magnitude of discrepancy we found for HIV-1 in this study is very similar to that we found for HTLV-1 in this and previous[17,31,32] studies with different compounds, using similar assays. Considering their low in vitro toxicity these natural compounds deserve further studies to compare their activity to that of antiretroviral drugs currently available.

4. Conclusions

Our study clearly demonstrates that a low-cost product of natural origin, consisting in a crude extract from bergamot seed, exerts a potent antiretroviral activity towards HTLV-1 infection in vitro. Moreover, these results were comparable with those obtained towards HIV-1, using similar assays. Indeed, based on its very poor cytotoxic effects, the antiretroviral potential of BSext seems even higher than that of AZT, 3TC or NEV. This aspect further encourages us to search for improving the specific antiretroviral activity of the natural compounds we identified and to investigate the possibility of their therapeutic application.

5. Experimental

5.1. Materials

Bergamot seeds were obtained from *C. bergamia* Risso, a typical fruit of the Reggio Calabria province in southern Italy. As reference compounds, the nucleoside reverse transcriptase inhibitors AZT (Sigma, St. Louis, USA), 3TC (Wellcome Research Labs., Beckenam, Kent, UK) and the non-nucleoside reverse transcriptase inhibitor nevirapine (NEV; Boehringer Ingelheim GmbH, Ingelheim, Germany) were utilized.

5.2. Extraction and isolation

An amount of 12 g of dried seeds obtained from fruit sample (10 kg) has been triturated and submitted to maceration with methanol (3 extractions of 48 h) and the extracts were filtered and evaporated in vacuum. The residue (2.7 g) was divided into two portions: the first portion (700 mg) was directly subjected to biological screening, while the second portion (2 g) was fractionated by medium pressure liquid chromatography (MPLC) on silica gel column using a mixture of chloroform/methanol from 1:0 to 0:1 as an eluent, to obtain three different fractions. The first and the third eluted fractions were discarded, since the ^{1}H NMR analysis showed the only presence of lipids and carbohydrate components, respectively. The second eluted fraction was further purified by MPLC, using chloroform/methanol 98:2 as an eluent, to obtain nomilin (40 mg) and limonin (70 mg), whose ^{1}H NMR and mass spectra were totally superimposable with that of commercial standards. Moreover, we observed that the purity of our products was higher than that of the commercially available compounds. LC/MS/MS analysis of the BSext crude extract, according to literature data[16] revealed the presence of limonin, nomilin, ichangensin, ichangin, obacunone, deacetylnomilin, methyl isoobacunoate, methyl isoobacunoate diosphenol, methyl deacetylnominilate and calamin. MPLC separation, ^{1}H NMR and LC/MS/MS data revealed that limonoids represent almost 10% of the extract and that limonin and nomilin are present in the extract in an amount of 3.5% and 2.0%, respectively.

5.3. Bioassay methods

5.3.1. Cell culture, HTLV-1 and HIV-1 infections and treatment

The chronically HTLV-1-infected human cell lines MT-2[27] the chronically HIV-infected cell line H9/HIV-1_{IIIB} (ATCC number CRL-8543) and MOLT-3 cells were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), glutamine and penicillin-streptomycin (complete medium, CM; Gibco-Invitrogen, Paisley, Scotland, United Kingdom). Peripheral blood mononuclear cells (PBMC) from healthy adult donors seronegative for HIV and hepatitis B and C viruses were separated using Ficoll-Hypaque density gradient (Cederlane, Hornby, Ontario, Canada) and were then washed twice in D-PBS w/o calcium magnesium (Euroclone, Italy). HTLV-1 infection of PBMC was performed as previously described by us.[21] For HIV-1 infection, PBMC were stimulated with 2 μg/ml of phytohemagglutinin (PHA) (Sigma Chemical Co., Bornem, Belgium) for O/N and then infected with twice clarified (at 1000×*g* for 10 min) supernatant from H9/HIV-1_{IIIB} culture.[28] The PHA stimulated PBMC were infected with 20 ng/ml of p24 for 2 h in CM, then PBMCs were washed with D-PBS and medium was replaced with CM plus IL2 20 U/ml. This amount of p24 was used on the basis of preliminary experiments showing that at this p24 concentration, 100% of PBMCs resulted infected, as observed by flow cytometry analysis. The compound's ability to protect PBMC from HTLV-1 infection was assayed by adding the compounds to the cultures at the onset of infection and at 3, 7, and 10 days post infection (pi), at half concentration, while for HIV infection experiments, compounds were added 4 h before infection and re-added daily at half of the initial concentration used. Given that BSext, limonin and nomilin were dissolved in methanol and that the higher final concentration of the vehicle in treated cultures was 0.5%, infected control cultures were set up in the presence of 0.5% methanol, as a vehicle.

5.3.2. Detection of infection by quantitative-real time RT-PCR analysis of HTLV-1 and HIV-1 gene expression

To detect HTLV-1 infection in PBMC, 3 weeks pi, cells from cultures subjected to various treatments were harvested and centrifuged on a density gradient to eliminate debris and dead cells. For the detection of HIV-1 infection, cells were harvested 5 days pi and processed as described above. RNA isolation was performed using an Eurogold total rna kit (Euroclone), according to the manufacturer's instructions. To adopt a comparable method for detection of HTLV-1 and HIV-1 viral RNA expression, quantitative-real time RT-PCR (RQ-PCR) analysis was utlized. For RQ-PCR, 0.25 μg of total RNA from each sample was reverse transcribed in a total volume of 20 μl using the high-capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Amplification of specific PCR products was detected using the iQ SYBR Green Supermix (Bio-Rad, CA, USA). The RQ-PCR was performed in triplicate in a total reaction volume of 25 μl containing 1X Sybr green RQ-PCR Mix, 150 nM forward and reverse primers, and 200 ng cDNA as a template. Samples were heated for 10 min at 95 °C and were subjected to 40 cycles of PCR amplification, each cycle consisting of 15 s at 95 °C and 60 s at 60 °C. For the detection of the HTLV-1 Tax/Rex region expression, RPXPR1 and RPX4 primers were used[29] while for HIV expression primer pairs specific for the gag region were used (forward 5'-TGCTATGTCAGTTCCCCTTGGTTCTCT-3', reverse 5'-AGTTGGAGGACATCAAGCAGCCATGCAAAT-3').[30] Within each experiment, no-template control was used to verify any contamination. In addition the glucuronidase beta (GUSB) housekeeping gene (NM_000181; forward primer, 5'-CAGTTCCCTCCAGCTTCAATG-3', and reverse primer, 5'-ACCCAGCCGACAAAATGC-3') used as a reference gene was run in parallel. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of

unspecific products and primer dimers. Quantification was performed using the Ct comparative method. Relative gene mRNA levels were calculated as follows: $2^{-[\Delta Ct(\text{sample}) - \Delta Ct\ (\text{calibrator})]} = 2^{-\Delta\Delta Ct}$, where ΔC_t (sample) = [C_t(*tax/gag* gene) − C_t(GUSB)] represents the difference, in threshold cycle number, between genes and GUSB. RQ-PCR experiments were run on RT CFX96 Real-Time PCR Detection System (Bio-Rad). All primers were purchased from Primm (Milano Italy).

5.3.3. HTLV-1 and HIV-1 reverse transcriptase inhibition assay

The capacity of BSext, limonin, nomilin to inhibit HTLV-1 or HIV-1 RT enzymatic activities was investigated by means of conceptually similar, cell-free, RT inhibition assays, set up by us using crude RT preparations from HTLV-1 and HIV-1 viral lysates, according to a method already validated and utilized by us for HTLV-1 in previous studies.[6,17,31] Consistent with the preparation of HTLV-1 RT, as a source of HIV-1 RT a viral lysate was prepared from 1 ml of supernatant from the HIV-1 chronically infected cell line H9/HIV-1_{IIIB}. Supernatants were clarified twice at 1000×*g* for 8 min, and then ultracentrifuged at 30,000×*g* for 1 h at 4 °C. The pellet was resuspended in 50 μl of lysing buffer containing Tris–HCl 50 mM pH 7.4, NaCl 150 mM, NP 40 0.5% (Sigma). The amount of RT in the viral lysates was indirectly estimated by measuring the amount of p19 and p24 in the supernatants from MT2 and H9/HIV-1_{IIIB} cells, respectively, through an antigen capture assay (Zeptometrix, Buffalo, NY). RNA template was specifically reverse transcribed by 10 μl of the HTLV-1 or HIV-1 RT preparations, equivalent to a standardized amount of 32 pg of p19, for HTLV-1, or to 25 pg of p24, for HIV-1, respectively. The other phases of the assays were performed as previously described.[6] For comparison, a commercially available, purified HIV-1 RT (Invitrogen, Carlsbed, CA) was also utilized. Neither the compounds of interest nor the non-nucleoside reference compound NEV was subjected to any pre-activation procedure.

5.3.4. Determination of cytotoxicity of the compounds

The cytotoxicity of the compounds on uninfected MOLT-3 cells or PBMC was evaluated by MTS assay. Inhibition of cell metabolic activity was detected through formazan product formation, using a commercial colorimetric kit (MTS [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt]) (Cell Titer 96 Aqueous One Solution; Promega). The assay was performed by seeding 1×10^4 MOLT-3 cells and 1×10^5 PBMC, stimulated with PHA 5 μg/ml (Sigma), in 100 μl in the presence or absence of the different compounds at seven different concentrations ranging from 1 ng/ml to 1 mg/ml for BSext, limonin and nomilin and ranging from 0.3 ng/ml to 0.3 mg/ml for AZT and NEV and from 0.23 ng/ml to 0.23 mg/ml for 3TC, respectively, in RPMI medium supplemented with 5% FBS. Twenty microlitres of 'Cell Titer 96 Aqueous One Solution' reagent was added directly to culture wells at the end of the culture period (3 days for PBMC and 1 day for MOLT-3 cells) and samples were incubated for 1 h. Successively the absorbance was read at 490 nm.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.01.024.

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