

Effects of diterpenes from latex of *Euphorbia lactea* and *Euphorbia laurifolia* on human immunodeficiency virus type 1 reactivation

Liliana Avila^a, Moises Perez^b, Gonzalo Sanchez-Duffhues^b, Rosario Hernández-Galán^c, Eduardo Muñoz^b, Fabio Cabezas^d, Winston Quiñones^a, Fernando Torres^a, Fernando Echeverri^{a,*}

^aGrupo de Química Orgánica de Productos Naturales, Instituto de Química, Universidad de Antioquia, Medellín, Colombia

^bDepartamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Córdoba, Spain

^cDepartamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, Cádiz, Spain

^dDepartamento de Química, Facultad de Ciencias, Universidad del Cauca, Popayán, Colombia

ARTICLE INFO

Article history:

Received 9 August 2009

Received in revised form 1 October 2009

Available online 10 November 2009

Keywords:

Euphorbiaceae

E. laurifolia

E. lactea

HIV

Latency

Reactivation therapy

ABSTRACT

The persistence of latent HIV-infected cellular reservoirs represents the major hurdle to virus eradication in patients treated with highly active antiretroviral therapy, referred to as HAART. HIV-1 reservoirs are long-lived resting CD4⁺ memory cells containing the virus latently integrated. Since the HIV-1 reservoirs are not targeted by HAART, reactivation therapy has been suggested to purge viral latency. Bioassay-guided study of an ethyl acetate extract of *Euphorbia laurifolia* afforded two isomeric diterpenes that showed differential activity over HIV-1 reactivation. A previously reported compound was isolated too from *Euphorbia lactea*. This compound showed a potent HIV-1 reactivating effect. Bioassays results showed that HIV-1 reactivation activity is influenced by distinct structural characteristics.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

The Euphorbiaceae family includes nearly 8000 species, most of which are characterized by latex production. Some species are important as medicinal and nutritional plants, whereas others are sources of oil and biofuels; nearly 43 species of plants from the *Euphorbia* genus are distributed in Colombia and many are used in traditional medicine (Murillo, 2004). Additionally, about 400 diterpenoids have been isolated from plants of the *Euphorbia* genus (Lu et al., 2008) with a wide spectrum of biological activities such as skin and eye irritants (Ahmed et al., 1999), apoptotic (Blanco-Molina et al., 2001), cytotoxic (Vigone et al., 2005) and cell proliferation induction (Touraine et al., 1977) properties. Non-tumor-promoter diterpenes such as prostratin (12-deoxyphorbol 13-acetate) have anti-HIV-1 activity (Gulakowski et al., 1997).

Although the actual HAART is undoubtedly a life-saving therapy for millions of AIDS patients, the persistence of latent HIV-infected cellular reservoirs represents the major hurdle to virus eradication with HAART, since latently infected cells remain a permanent source of viral reactivation. As a result, a sudden rebound of the virus load after interruption of HAART is generally observed (Blankson et al., 2002; Persaud et al., 2003), and eradication of viral

reservoirs should be a new goal for HIV-1 therapeutics (Richman et al., 2009). In this sense, it has been suggested that reactivation of the latent reservoirs could allow effective targeting and possible eradication of the virus (Richman et al., 2009). It is thought that viral reactivation would result in lytic cell death of CD4⁺ T-cells because of either the cytopathic effect of the virus or through recognition of infected cells by the immune system. In addition, viral reactivation in the presence of HAART would prevent new infection events.

Viral reactivation therapy is a potential therapeutic option to purge the viral reservoirs and should not induce polyclonal T-cell activation (Williams and Greene, 2007). Natural or synthetic protein kinase C (PKC) agonists lacking tumor-promoter activity clearly fulfil this criterion and this has opened new avenues for the possible treatment of HIV-1 latency. Thus, a host of small molecules, including prostratin (Kulkosky et al., 2001), ingenols (Warrilow et al., 2006) and 1,2-diacylglycerol analogs (Hamer et al., 2003), has been suggested as agents to reactivate HIV and eradicate the pool of latently HIV-infected CD4⁺ T-cells (Kulkosky and Bray, 2006). Prostratin and other non-tumorigenic PKC agonists reactivate HIV-1 latency in “vitro” by activating NF-κB through a PKC-dependent pathway (Trushin et al., 2005; Marquez et al., 2008).

In this paper, we report the activity of extracts and chromatographic fractions of *Euphorbia laurifolia* and *Euphorbia lactea* latex,

* Corresponding author. Tel.: +57 42196595; fax: +57 42196565.

E-mail address: echeverri@quimbaya.udea.edu.co (F. Echeverri).

and the isolation and structural elucidation of three diterpenes from those plants that antagonized HIV-1 latency in the Jurkat-LAT-GFP cells.

2. Results and discussion

2.1. Structural elucidation

An *E. laurifolia* ethyl acetate extract was fractionated using a Si gel column eluted with increasing mixtures of hexane:ethyl acetate; 20 fractions of 30 mL were obtained. Fraction 12 was selected on the basis of preliminary bioassays on activation of HIV latency and the presence of diterpenes, according to its proton NMR spectroscopic profile. Therefore, this fraction was separated by preparative TLC with successive elutions (3×) in hexane–acetone 3:1 (v/v). From this purification process, two diterpenes were obtained, **1** and **2**.

Compound **1** was obtained as a resinous solid whose HRMS exhibited an ion at m/z 488.2774 which is in agreement with the molecular formula $C_{28}H_{40}O_7$, from which nine degrees of unsaturation were deduced. The 1H NMR spectrum of **1** (600 MHz) (Table 1) showed signals for the presence of two acetoxy groups [δ 2.07 (s) and δ 2.06 (s)] and an isobutyrate group [2.58 (septet, $J = 7.0$ Hz, 1.17 and 1.18 (d, $J = 7.0$ Hz)]. Further, its 1H NMR spectrum showed resonances for two olefinic protons [δ 6.58 (dd, $J = 11.5$ and 1.2 Hz) and δ 5.50 (dq, $J = 10.7$ and 7.5 Hz)],

two vinylic methyl groups at δ 1.42 (d, $J = 1.3$ Hz) and 1.83 ppm (d, $J = 1.2$ Hz) and two signals for protons attached to carbon oxygenated atoms at δ 4.97 (dt, $J = 11.3$ and 3.6 Hz) and 4.78 ppm (dd, $J = 7.5$ and 5.4 Hz).

Analysis of 1H – 1H -COSY, HMQC and HMBC spectra provided evidence for two spin-systems X and Y (Fig. 1). Fragment X was deduced through a correlation observed in the 1H – 1H -COSY spectrum between the *doublet* assignable to the vinyl proton at δ 6.58 ppm (H-12) and the doublet of doublets at δ 1.60 ppm (H-11), which has a coupling with the *dd* at δ 1.24 ppm (H-9). Additionally, the last signal displayed a coupling with the *dt* at δ 4.97 (H-8) ppm, which in turn exhibited two other couplings with methylene protons at δ 1.94 (H-7 α) and at δ 2.63 (H-7 β). Finally, the same vinylic proton at δ 6.58 displayed a weak correlation with the doublet at 1.83 ppm (Me-20).

Two long-range correlations observed in the HMBC experiment for the second olefinic proton at δ 5.50 ppm (H-5) permit us to established fragment Y. The first one was weak, indicating an allylic coupling with the methyl group at δ_H 1.42 ppm (Me-17). A strong coupling with a methine proton (H-4) at δ_H 2.80 ppm was also observed. Another sequence of correlations was observed from H-4 to H-3 (4.78 ppm), from H-3 to H-2 (2.26 ppm), and from H-2 to the C-1 methylene protons and the secondary methyl group at δ_H 1.06 ppm. Fragments X and Y (Fig. 1) account for a total of 15 carbons.

Table 1
NMR spectroscopic data for compounds 1–3 (600 MHz for 1H NMR and 125 MHz for ^{13}C NMR) in $CDCl_3$.

Position	Compound 1			Compound 2			Compound 3	
	δ 1H (J in Hz)	δ ^{13}C	NOE	δ 1H (J in Hz)	δ ^{13}C	NOE	δ 1H (J in Hz)	δ ^{13}C
1 α	2.17 dd (14.6, 7.1)	40.83, t		3.53 dd (14.1, 8.3)	40.21, t	H-1 β , H-2	2.81, dd (15.0, 8.5)	31.72, t
1 β	2.61 dd (14.6, 9.0)			1.47 dd (14.1, 12.5)			1.67, dd, (15.0, 1.5)	
2	2.26 (m)	38.12, d		2.15 m	38.78, d	H-3, H-4, H1 α , Me-16	2.57, m	29.72, d
3	4.78 dd (7.5, 5.4)	82.03, d		5.22 t (3.5)	80.58, d	H-2, H-4		77.66, d
4	2.80 dd (10.5, 7.5)	48.19, d		2.48 dd (10.6, 3.5)	51.48, d		5.19, d (9.5)	71.41, s
5	5.50 dq (10.5, 7.5)	122.27, d	H-8, H-12, H-7 β	5.56 ddq (10.9, 1.3, 1.6)	122.41, d		5.77, sbr	116.55, d
6		137.52, s			137.51, s			141.36, s
7 β	2.63 (m)	43.27, t		2.63 m	43.22, t		4.28, sbr	76.38, d
7 α	1.94 dd (12.5, 11.3)			1.94 dd (12.5, 11.2)				
8	4.97 dt (11.3, 3.6)	74.83, d	H-5, H-7 β , H-12, H-18	4.94 dt ($J = 10.9, 3.8$)	74.98, d	H-5, H-7 β , H-12, H-18	4.55, dd (10.5, 1.5)	74.35, d
9	1.24 dd (11.3, 8.6)	36.48, d		1.24*	36.44, d		1.42, dd (10.0, 10.5)	23.64, d
10		24.88, s			25.07, s		1.09, dd (10.5, 9.0)	19.10, s
11	1.60 dd (11.5, 8.6)	30.00, d		1.59 dd (11.5, 8.3)	29.15, d		4.86, dd (10.5, 3.9)	30.95, d
12	6.58 dd (11.5, 1.2)	144.33, d		6.66 dd (11.5, 1.3)	144.64, d		2.91, dq, (6.0, 3.9)	70.80, d
13		133.80, s			133.59, s			43.29, d
14		195.22, s			195.28, s			207.59, s
15		95.51, s			94.97, s		0.89, d, (7.5)	73.73, s
16	1.07 d (6.9)	16.45, q		0.95 d (6.7)	16.48, q	H-3, H-2, H-1 β	2.06, sbr	16.96, q
17	1.42 d (1.3)	20.31, q		1.44 d (1.6)	20.55, q		1.05, s	17.44, q
18 (β)	1.06 s	19.38, q		1.05 s	19.46, q		0.82, s	20.09, q
19 (α)	1.19 s	29.15, q		1.19 s	29.14, q		1.04, d (8.0)	16.36, q
20	1.83 d (1.2)	12.56, q	H-11, H-17	1.83 d (1.3)	12.62, q	H-11, H-17	2.10, s	13.31, q
8-Ac ₂	2.06 s	171.22, s		2.07 s	170.48, s		2.09, s	12-OAc
		21.80, q			21.65, q			170.42, s
								21.00, q
15-Ac ₁	2.07 s	171.35, s		2.03 s	170.18, s		6.83, qq (7.0, 1.0)	3-OAc
		21.80, q			21.75, q		1.80, dq, (6.0, 1.0)	170.62, s
							1.83, dq, (1.0, 1.0)	20.66, q
COCH(CH ₃) ₂	2.58 sep (7.0)	34.65, d		2.65 m **	34.60, d		2.81, dd, (15.0, 8.5)	8-O ⁺ Tig
							1.67, dd, (15.0, 1.5)	167.42, s
								2' 128.37, q
								3' 138.16, d
								4' 12.01, q
								5' 14.68, q
COCH(CH ₃) ₂	1.17 and 1.18 d (7.0)	175.26, s		1.25 and 1.28 d (7.0)	176.83, s			
		19.72, q			19.74, q			
		20.08, q			20.55, q			

* Signal overlapped to $-(CH_3)_2$.

** Signal overlapped to H-7.

The dimethylcyclopropane moiety was deduced from long-range correlations involving the two methyl signals at δ_{H} 1.19 and 1.06 ppm, H-11 1.60 ppm and the quaternary carbon at δ_{C} 24.9 ppm (C-10).

The connectivities of these partial structures were established by the long-range C–H correlations found in the HMBC. In this spectrum, C–H correlation between the carbonyl group signal at δ_{C} 195.22 ppm, the signal at δ_{H} 1.83 ppm corresponding to the methyl group on C-13 (Me-20) and the methine resonance at δ_{H} 2.80 ppm (H-4) were observed. Further correlations between Me-17 (δ 1.44, d) and C-7 (δ_{C} 43.27 ppm) and between a quaternary oxygenated carbon at 95.51 ppm (s) with H-1 and the correlation above between H-1, H-2, H-3 and H-4 allowed the establishment of a lathyrane framework.

The location of the substituent was confirmed in a similar way. The isobutyrate group was located on H-3 through the ^3J and ^2J correlations to the carbonyl group (175.26 ppm) from the *dd* at δ_{H} 4.78 ppm (H-3), the methine group at δ_{H} 2.58 ppm and the methyl group at δ_{H} 1.17 ppm. In consequence, the other two substituents corresponding to two acetoxyl group, were located on C-8, according to the long-range coupling between a carbonyl group at δ_{C} 171.22 ppm with signal at δ_{H} 4.97 ppm (H-8), and by deduction on C-15. With regard to the above data, the structure of compound **1** was established as depicted in Fig. 2. Although the basic skeleton was reported before, these substituents are reported for the first time.

Compound **2** was an amorphous white solid whose molecular formula was assigned as $\text{C}_{28}\text{H}_{40}\text{O}_7$ according to HRMS. Its ^1H and ^{13}C NMR spectra were similar to those of **1**. The main differences were a downfield chemical shift of the signal at 5.22 ppm (t, $J = 7.0$, H-3) in **2**, and the presence of a *dd* at δ 3.53 ($J = 8.3$ and 14.1 Hz), corresponding to a methylene proton, whose geminal proton was at δ_{H} 1.47 ppm. In the methyl region, nine signals were observed: δ 0.94 ($J = 7.2$ Hz, Me-16), 1.05 (s, Me-19), 1.19 (s, Me-18), 1.25 (d, $J = 7.0$ Hz), 1.28 (d, $J = 6.7$ Hz), 1.44 (d, $J = 1.4$ Hz,

Me-17), 1.82 (d, $J = 1.0$, Me-20), 2.03 (s), 2.07 ppm (s). Two acetates and an isobutyrate were also detected.

HMBC long-range correlations were observed between an α,β -unsaturated carbonyl group at δ_{C} 195.28 ppm (C-14), a vinylic methyl group at δ_{H} 1.82 ppm and two methylene protons at δ_{H} 3.50 and 1.46 ppm (C-1), which is unusually downfield shifted when compared to compound **1**. The isobutyrate carbonyl group at δ_{C} 176.83 ppm showed a ^3J correlation with the t at δ_{H} 5.10 ppm, confirming that **2** is an isomer of **1** at C-2. Modifications in the stereochemistry of methyl group caused changes in the chemical shifts of H-4, H-3 and the methylene group located in C-1.

The relative configuration of both compounds was deduced from coupling constant values, NOEs and the ROESY spectrum assuming an orientation for H-4 α on biogenetic grounds (Duarte et al., 2007; Appendino et al., 1999; Ferreira et al., 2002). The coupling constants observed between protons H-2, H-3 and H-4 ($J_{2-3} = J_{3-4} = 3.5$ Hz in compound **2** and $J_{3-2} = 5.4$ Hz and $J_{3-4} = 7.5$ Hz in compound **1**) indicate they are only isomeric at C-2. The ROESY experiment for compound **1** showed a cross-peak between H-3, H-4 and Me-16 indicated an α -configuration for the methyl on C-2 and beta for the isobutyrate attached in C-3. NOE experiments displayed interactions of H-3 with H-2 and H-4 in compound **2**, indicating that the C-2 methyl group was α and the isobutyrate at C-3 was β . Correlations in the ROESY spectra of both compounds showed that H-5, H-8 and H-12 lie on the β face of the molecule, while Me-17, Me-20 and H-11 lies on the α face. Assuming an α -orientation for H-11, characteristic for lathyrane-type diterpenes, we optimized the geometry and got low energy conformers for both possible diastereoisomers on C-8 (ChemBio 3D Ultra 7.5), noticing that the observed cross-peak in the ROESY were only possible with an α -relative disposition of substituent on C-8. In these molecules, the stereochemistry is very important since both compounds possess differential activity against HIV latency reactivation.

Additionally, the *E. lactea* ethyl acetate extract was fractionated by column chromatography as for *E. laurifolia*. Twenty fractions were collected and monitored through TLC and NMR spectroscopic analyses for the presence of diterpenes; fraction 12 was the richest in this type of compounds, on the basis of the presence of several methyl groups and signals for a cyclopropyl moiety in its ^1H NMR spectra. Additionally, a high latency reactivation activity was observed. Purification by preparative TLC using hexane:acetone 9:1 as eluent yielded an ingol diterpene **3** (Fig. 2), which was isolated as a resinous material. The structure of compound **3** was identified by physical and spectroscopic data measurement ($[\alpha]_{\text{D}}^{20}$, ^1H NMR, ^{13}C NMR, 2D-NMR and MS) and by comparing the data obtained with published values, such as 3,12-di-O-acetyl-8-O-tigloylingol (**3**), a triester previously isolated from the same source, whose stereochemistry was confirmed by ROESY and X-ray diffraction (Upadhyay and Hecker, 1975; Ahmed et al., 1999).

2.2. Biological activity

2.2.1. Effects of extracts and fractions from latex of *E. laurifolia* and *E. lactea* on HIV-1 reactivation from latency

The effect of extracts and fractions from latex of *E. laurifolia* (LEL) and *E. lactea* (LEC) on HIV-1 reactivation in Jurkat-LAT-GFP cells was evaluated (Fig. 3). Jurkat-LAT-GFP cells contain a single, full-length integrated HIV provirus in which GFP has been substituted for Nef. Similar to latently infected CD4^+ T-cells, the HIV-1 genome is inhibited at the transcriptional level and viral reactivation can be measured by cytometric detection of GFP epifluorescence (Marquez et al., 2008). Thus, Jurkat-LAT-GFP cells were treated for 12 h with 1 $\mu\text{g}/\text{ml}$ of either the extract LEL or each of the seven fractions obtained from the crude extracts. We found that LEL potentially reactivated HIV-1 from latency (93.7% of GFP⁺

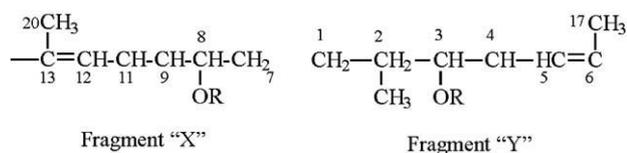


Fig. 1. Partial structure of compound **1**.

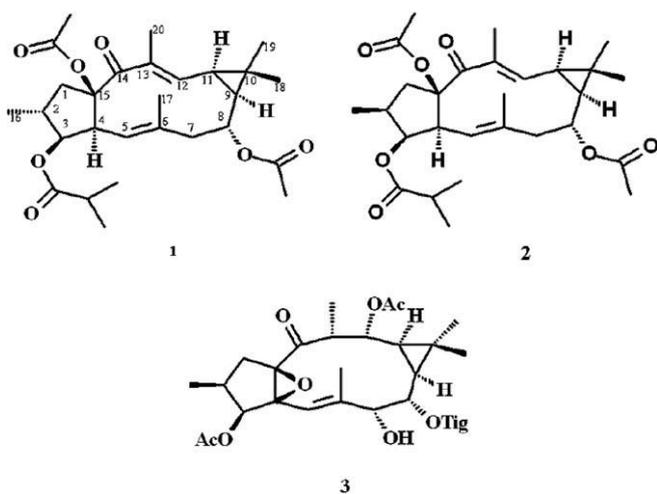


Fig. 2. Diterpenes isolated from *E. laurifolia* (**1** and **2**) and *E. lactea* (**3**).

cells), and that this activity was retained mainly in fractions F12, F15 and F20 (Fig. 3A). The extract from *E. lactea* (LEC) was also a potent antagonist of HIV-1 latency (82.3% of GFP⁺ cells) and this activity was retained in fraction 12 (F12) (Fig. 3B). We shown in Fig. 3 that they were able to antagonize HIV-1 latency very efficiently. As a positive control, we used PMA (phorbol-12-myristate-13-acetate), a potent phorbol ester with PKC agonistic activity. The effect of the extracts and fractions on Jurkat-LAT-GFP was dose-dependent reaching maximum activation at the concentration of 1 $\mu\text{g/ml}$.

2.2.2. Effect of compounds 1, 2 and 3 on HIV reactivation

Subsequently, we investigated the biological activity of the two isolated compounds from F12 of LEC extracts (**1** and **3**) and the isolated compound from F12 or LEL extracts (**3**). Compound **3** was able to reactivate HIV-1 latency in a concentration dependent manner (data not shown) with an EC_{50} of 0.5 $\mu\text{g/ml}$ and reaching maximal GFP induction at 5 $\mu\text{g/ml}$ (84.3%). In contrast, compounds **1** and **2**, even at concentrations of 25 $\mu\text{g/ml}$, were significantly less active to antagonize HIV-1 latency (33% and 0%, respectively) than the original fraction (F12 from *E. laurifolia*) from which they were isolated (Fig. 4). The lost of activity is probably due to either decomposition of active substances or perhaps to a synergistic ef-

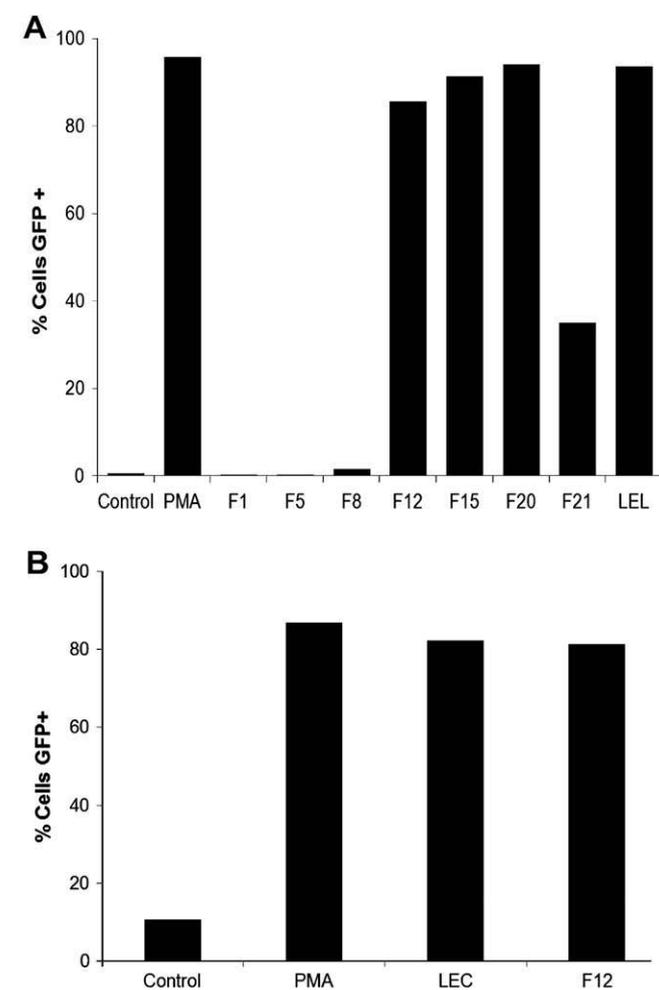


Fig. 3. Effect of extracts from *E. laurifolia* and *E. lactea* and chromatographic fractions (F) on HIV-1 reactivation. (A) Jurkat-LAT-GFP cells were stimulated with either PMA (50 ng/ml), or extract LEL or fractions F1, F5, F8, F12, F15, F20 and F21 for 12 h and the expression of GFP determined by flow cytometry. (B) Effect of extract LEC and F12 fraction on HIV reactivation. The results show the percentage of GFP⁺ cell and represent the mean of three independent experiments.

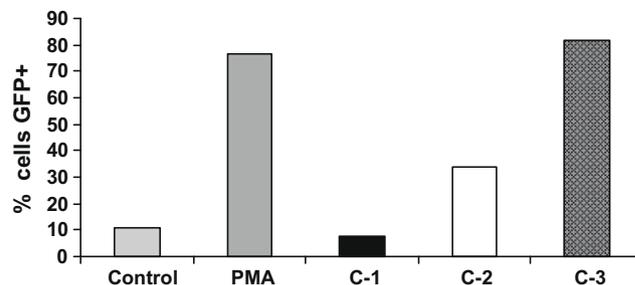


Fig. 4. Effect of isolated compounds on HIV-1 reactivation. Jurkat-LAT-GFP cells were stimulated with PMA and compounds **1–3** (C-1, C-2 and C-3) for 6 h and later analyzed by flow cytometry. The results show the percentage of GFP⁺ cells.

fect with all molecules in the chromatographic fractions. The decomposition of some diterpenes from Euphorbiaceae during fractionation and isolation procedures has been reported previously (Keeler and Tu, 1991).

2.2.3. Compound 3 reactivates HIV-1 latency through a PKC-dependent pathway

Protein kinase C (PKC) belongs to serine/threonine kinases family and plays a central role in signals transduction in response to extracellular stimulus carried out by diacylglycerol (DAG). The PKC isozymes are differently involved in regulation of cell proliferation, differentiation, cell survival, apoptosis and carcinogenesis (Kheifets and Mochly-Rosen, 2007). Natural compounds such as phorbol esters and ingenols isolated from Euphorbiaceae family are known to bind the C1 domain of PKC leading to their activation (Kazanietz, 2002; Hritz et al., 2004). Due to the structural similarity between compound **3** and phorbol esters such as PMA and prostratin, we studied the role of PKC on compound **3**-induced HIV-1 reactivation. Thus, Jurkat-LAT-GFP cells were pretreated with the chemical inhibitors G_06976 (classical PKCs inhibitor) and G_06850 (classical and novel PKCs inhibitor) for 30 min, then the cells were stimulated with increasing concentrations of compound **3** for 6 h and finally the percentage of GFP positive cells were determined by flow cytometry. The results showed that GFP expression induced by compound **3** was strongly inhibited by G_06976 and G_06850 (Fig. 5), and this result strongly suggests that compound **3** reactivates HIV-1 through a PKC-dependent pathway.

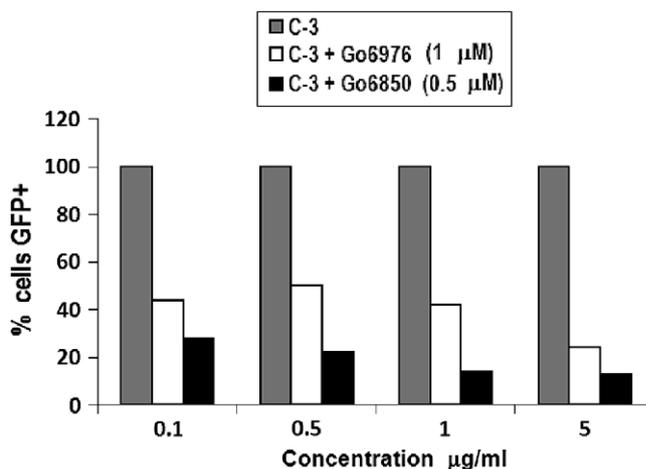


Fig. 5. Effect of PKC inhibitors G_06976 and G_06850 on Jurkat-LAT-GFP cells stimulated with compound **3**. The cells were pretreated with the indicated inhibitors for 30 min at the indicated concentration and then stimulated with compound **3** for 6 h. Percentage of GFP⁺ cells was measured by flow cytometry. The results are the percentage of activation compared to treated cells in absence of chemical inhibitors (100% of activation).

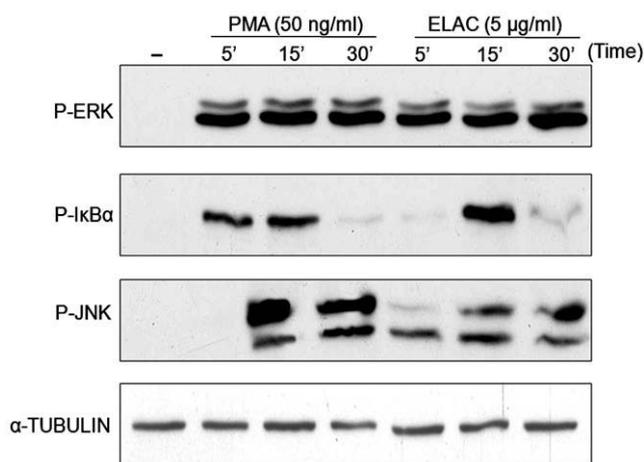


Fig. 6. Effects of compound **3** on IκB, JNK and ERK phosphorylation. Jurkat-LAT-GFP cells were stimulated for 5, 15 and 30 min. IκB, JNK and ERK phosphorylation and α-tubulin levels were analyzed by western blot using specific antibodies.

2.2.4. Effect of compound **3** on signaling pathways

Several groups have independently demonstrated that different PKC agonists reactivate HIV-1 latency by activating a PKC-dependent NF-κB pathway (Williams et al., 2004; Trushin et al., 2005; Marquez et al., 2008). To study the biochemical pathways activated by compound **3**, we incubated Jurkat-LAT-GFP with either PMA (positive control) or compound **3** (5 μg/ml) for 5, 15 and 30 min, total proteins extracted and the phosphorylation of both IκBα, and the MAPKs (JNK and ERK) was analyzed by western blot using specific antibodies. In Fig. 6, it is shown that both PMA and compound **3** clearly activated these pathways with a similar kinetic. IκBα phosphorylation is the hallmark of canonical pathway of the NF-κB activation and JNK and ERK activation is also involved in HIV-1 transcriptional activity. Altogether our results demonstrated that compound **3** antagonize HIV-1 latency by activating a PKC–NF-κB–MAPKs signaling pathways similarly to the pathways described for other naturally occurring PKC activators (Marquez et al., 2008).

3. Conclusion

The existence of HIV-1 reservoirs resistant to HAART has increased the interest in searching molecules with ability to activate latent provirus and use them in combination with current antiretroviral therapy. PKC agonists lacking tumor-promoter activity such as prostratin have been suggested as pharmacological candidates to purge HIV-1. Prostratin, isolated from *Homalanthus nutans*, is a very promising compound for the treatment of HIV-1 reservoir that has recently received renewed attention because its synthesis starting from phorbol has been reported. However, the suitability of this compound to be used in humans is unknown since preclinical testing is still underway. Moreover, relatively high concentrations were required to activate the provirus, and therefore it has been suggested that prostratin could have negative secondary effects to long term treatments (Williams et al., 2004).

In this work, three diterpenes were isolated; compounds **1** and **2**, isolated from *E. laurifolia* are reported for the first time. Compound **3**, isolated from *E. lactea*, was identified as 3,12-di-*O*-acetyl-8-*O*-tigloyl-ingol and was previously reported by Upadhyay and Hecker; this compound effectively promoted HIV-1 replication. Compound **1** showed a moderate activity, whereas the isomeric compound **2** was practically inactive. It seems the stereochemistry of the side-chain and then the conformational facts between cyclopentyl and the parent (basic) skeleton are responsible for this biological effect.

Additionally, the mechanism of action of compound **3** seems to involve the PKC pathway because its effect was antagonized by

PKC inhibitors G_o6976 and G_o6850; similar results are reported by Marquez et al. (2008), where G_o6976 and G_o6850 were able to inhibit GFP expression in Jurkat-LAT-GFP cells stimulated with prostratin and phorbol 13-stereate. In the same way, compound **3** induces IκBα phosphorylation and its subsequent degradation, showing that the activation can be induced by NFκB in a similar way to prostratin (Williams et al., 2004). Finally, a JNK and ERK phosphorylation were detected, probably indicating the participation of other transcription factors such as AP-1 that can contribute with NF-κB to reactivate HIV-1 from latency (Warrilow et al., 2006; Williams et al., 2004).

Although compound **3** is closely related to another compound which displayed similar action on HIV -1 (Daoubi et al., 2007), ingol-type compounds as **1** and **2** are reported for the first time with this activity and triggers a reexamination of the structural requirements postulated before in prostratin and phorbol to promote cell proliferation or HIV latent reactivation (Marquez et al., 2008). Moreover compound **3** did not show any cytotoxicity at the concentrations and times investigated suggesting that it is endowed with a high selectivity index (data not shown).

In conclusion, some fractions and pure compounds isolated from *E. lactea* and *E. laurifolia* promote the latent HIV-1 replication. The wide chemical diversity of diterpene from Euphorbiaceae species and their biological effects can be useful to understanding the cellular process involved in HIV-1 latency and development of new drugs for AIDS treatment.

4. Experimental

4.1. General

The solvent used for latex extraction and fractionation was freshly distilled. TLC plates Si 60 were performed on Merk kieselgel F₂₅₄, and for preparative chromatography Analtech Silica gel GF plaques were used. All compounds were detected by spraying with 10% H₂SO₄, followed by heating. For column chromatography (CC) silica gel 60H (Merk) and Sephadex LH 20 (Sigma) were used. The UV spectra were recorded on a Perkin Elmer Lambda 12 spectrophotometer, the IR spectra were recorded on a Nicolet Avatar 330 FT-IR spectrophotometer. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker AMX 300 and Varian 600 MHz NMR spectrometers, operating at 300 and 600 MHz for ¹H and 75 and 150 MHz for ¹³C, respectively. MS were recorded on an Agilent LCTOF-APCI spectrometer. Flow cytometry was measured in a Coulter (Hialeah, FL). Immunodetection of specific proteins was carried out with primary antibodies using an ECL system (GE Healthcare). Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA, USA).

4.2. Cell lines and reagents

Jurkat-LAT-GFP cells were grown at 37 °C and 5% CO₂ in supplemented RPMI 1640 medium (Cambrex Co., Barcelona) containing 10% heat-inactivated fetal bovine serum, 2mM glutamine, penicillin (50 U/ml) and streptomycin (50 mg/ml). The G_o6976 and G_o6850 inhibitors were from Calbiochem (EMD Biosciences, Inc., Darmstadt, Germany). The antibody anti-phospho-IκBα (92465) was from Cell Signaling (MA, US), mAb anti-tubulin was purchased from Sigma Co. (St. Louis, MO, USA), anti-phospho-ERK (sc-7383) was from Santa Cruz Biotechnology (CA, US) and anti-phospho-JNK (9255S) was from New England Biolabs (Hitchin, UK).

4.3. Plant material

Latex from *E. laurifolia* was collected in March of 2007 in Cauca, Colombia and latex from *E. lactea* was collected in Tolima, Colom-

bia, in June of 2007. Latex was obtained making cut on stalk and collecting milky secretion. A voucher of *E. laurifolia* was deposited in the Universidad del Cauca Herbarium (Colombia) with the code CAUP fl22168 and a voucher of *E. lactea* was deposited in the Universidad del Tolima Herbarium (Colombia) with the code TOLI2367.

4.4. Extraction and isolation of compounds

Latex from *E. laurifolia* and *E. lactea* (100 ml each one) were extracted with EtOAc, and then filtered and evaporated to produce 3.5 and 4.0 g of crude extract, respectively. The laths were individually fractionated using silica gel 60 CC, eluted with hexane:EtOAc (95:5 v/v). Hundred fractions were obtained for each extract and those were combined in 21 fractions for *E. laurifolia* and 20 fractions for *E. lactea* according to TLC profiles. Fractions 12 (120 mg) and 15 (150 mg) from *E. laurifolia* and fraction 12 from *E. lactea* (25 mg) were further fractionated by preparative TLC. From *E. laurifolia*, fraction 12 compound **1** (6 mg) and **2** (8 mg) were isolated using hexane:acetone (1.7:0.3 v/v) as eluent, and from fraction 12 (*E. lactea*) compound **3** (10 mg) was isolated using as eluent hexane:acetone (9:1 v/v), using fraction 15 (*E. laurifolia*), it was not possible to isolate any compound due to their lability.

4.5. Biological assays

4.5.1. Determination of HIV-1 reactivation in a cellular model of HIV-1 latency

Jurkat-LAT-GFP is a Jurkat derived clone latently infected with a recombinant virus containing the GFP gene driven by the HIV-LTR promoter (Marquez et al., 2008). The cells were stimulated with either phorbol-12-myristate-13-acetate (PMA, 1 µg/ml) or compounds **1**, **2** and **3** for 6 or 12 h and the GFP expression was analyzed by flow cytometry in an EPIC XL flow cytometry. Ten thousand gated events were collected per sample and the fluorescence pattern was determined.

4.5.2. Western blot

Jurkat-LAT-GFP cells (10^6 cells/ml) were stimulated with compound **3** for 5, 15 and 30 min, then cells were washed with PBS and proteins extracted in 50 ml of lysis buffer (20 mM HEPES pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na₃VO₄, 5 mM NaF, 1 mM DTT, leupeptin 1 mg/ml, pepstatin 0.5 mg/ml, aprotinin 0.5 mg/ml, and 1 mM PMSF) containing 0.5% NP-40. Protein concentration was determined by the Bradford assay and 30 mg of proteins were boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V) for 1 h. Blots were blocked in TBS solution containing 0.1% Tween 20 and 5% non-fat dry milk overnight at 4 °C and immune detection of specific proteins was carried out with primary antibodies using an ECL system.

Acknowledgments

Author thanks COLCIENCIAS (Colombia) grant 1115-12-17832, and Universidad de Antioquia for financial support, and to L.A. Gonzalez for material collection. We also appreciate support from CYTED-Spain, the Alfa Program of the European Community (EULADIV Project AML/B7-311/97/0666/II-0375-FA) and Junta de Andalucía (R.H.G., P07-FQM-02925). E.M., M.P., and G.S-D were supported by the Spanish RIS Network "Red de Investigación en SIDA" (RD06/0006/0028).

References

- Ahmed, A., Couladis, M., Mahmoud, A., De Adams, A., Mabry, T., 1999. Ingot diterpene ester from the latex of *Euphorbia lactea*. *Fitoterapia* 70, 140–143.
- Appendino, G., Belloro, E., Tron, G.C., Jakupovic, J., Ballero, M., 1999. Diterpenoids from *Euphorbia pithyusa* subsp. Cupanii. *J. Nat. Prod.* 62, 1399–1404.
- Blanco-Molina, M., Tron, G., Macho, A., Lucena, C., Calzado, M., Muñoz, E., Appendino, G., 2001. Ingenol esters induce apoptosis in Jurkat cells through an AP-1 and NF-UB independent pathway. *Chem. Biol.* 8, 767–778.
- Blankson, J.N., Gallant, J.E., Quinn, T.C., Bartlett, J.G., Siliciano, R.F., 2002. Loss of HIV-1-specific immunity during treatment interruption in 2 chronically infected patients. *JAMA* 288, 162–164.
- Daoubi, M., Marquez, N., Mazoir, N., Benharref, A., Hernandez-Galan, R., Muñoz, E., Collado, I., 2007. Isolation of new phenylacetylglucosyl derivatives that reactivate HIV-1 latency and a novel spirotriterpenoid from *Euphorbia officinarum* latex. *Bioorg. Med. Chem.* 15, 4577–4584.
- Duarte, N., Varga, A., Cherepnev, G., Radics, R., Molnar, J., Ferreira, M.-J.U., 2007. Apoptosis induction of P-glycoprotein mediated multidrug resistance by new macrocyclic lathyrane-type diterpenes. *Bioorg. Med. Chem.* 15, 546–554.
- Ferreira, A.M.V.D., Carvalho, L.H.M., Carvalho, M.J.M., Sequiera, M.M., Silva, A.M.S., 2002. Jatrophone and lathyrane diterpenoids from *Euphorbia hyberna* L. *Phytochemistry* 61, 373–377.
- Gulakowski, R.J., McMahon, J.B., Buckheit Jr., R.W., Gustafson, K.R., Boyd, M.R., 1997. Antireplicative and anticytopathic activities of prostratin, a non-tumor-promoting phorbol ester, against human immunodeficiency virus (HIV). *Antiviral Res.* 33, 87–97.
- Hamer, D.H., Bocklandt, S., McHugh, L., Chun, T.W., Blumberg, P.M., Sigano, D.M., Marquez, V.E., 2003. Rational design of drugs that induce human immunodeficiency virus replication. *J. Virol.* 77, 10227–10236.
- Hritz, J., Ulicny, J., Laaksonen, A., Jancura, D., Miskovsky, P., 2004. Molecular interaction model for the C1B domain of protein kinase C-γ in the complex with its activator phorbol-12-myristate-13-acetate in water solution and lipid bilayer. *J. Med. Chem.* 47, 6547–6555.
- Kazanietz, M., 2002. Novel "nonkinase" phorbol ester receptors: the C1 domain connection. *Mol. Pharmacol.* 61, 759–767.
- Keeler, R., Tu, A. (Eds.), 1991. *Toxicology of Plant and Fungal Compounds*. CRC Press, New York, p. 665.
- Kheifets, V., Mochly-Rosen, D., 2007. Insight into intra- and inter-molecular interactions of PKC: design of specific modulators of kinase function. *Pharmacol. Res.* 55, 467–476.
- Kulkosky, J., Bray, S., 2006. HAART-persistent HIV-1 latent reservoirs: their origin, mechanisms of stability and potential strategies for eradication. *Curr. HIV Res.* 4, 199–208.
- Kulkosky, J., Culnan, D.M., Roman, J., Dornadula, G., Schnell, M., Boyd, M.R., Pomerantz, R.J., 2001. Prostratin: activation of latent HIV-1 expression suggests a potential inductive adjuvant therapy for HAART. *Blood* 98, 3006–3015.
- Lu, Z., Yang, M., Zhang, J., Chen, G., Huang, H., Guan, S., Ma, C., Liu, X., Guo, D., 2008. Ingenane diterpenoids from *Euphorbia esula*. *Phytochemistry* 69, 812–819.
- Marquez, N., Calzado, M., Sanchez-Duffhues, G., Perez, M., Minassi, A., Pagani, A., Appendino, G., Diaz, L., Muñoz-Fernandez, M.A., Muñoz, E., 2008. Differential effects of phorbol-13-monoesters on human immunodeficiency virus reactivation. *Biochem. Pharmacol.* 75, 1370–1380.
- Murillo, J., 2004. Las Euphorbiaceae de Colombia. *Biota Colombiana* 5, 183–200.
- Persaud, D., Zhou, Y., Siliciano, J.M., Siliciano, R.F., 2003. Latency in human immunodeficiency virus type 1 infection: no easy answers. *J. Virol.* 77, 1659–1665.
- Richman, D.D., Margolis, D.M., Delaney, M., Greene, W.C., Hazuda, D., Pomerantz, R.J., 2009. The challenge of finding a cure for HIV infection. *Science* 323, 1304–1307.
- Touraine, J., Hadden, J., Touraine, F., Hadden, E., Estensen, R., Good, R., 1977. Phorbol myristate acetate: a mitogen selective for a T-lymphocyte subpopulation. *J. Exp. Med.* 145, 460–465.
- Trushin, S.A., Bren, G.D., Asin, S., Pennington, K.N., Paya, C.V., Badley, A.D., 2005. Human immunodeficiency virus reactivation by phorbol esters or T-cell receptor ligation requires both PKCα and PKCθeta. *J. Virol.* 79, 9821–9830.
- Upadhyay, R., Hecker, E., 1975. Diterpene esters of the irritant and cocarcinogenic latex of *Euphorbia lactea*. *Phytochemistry* 14, 2514–2515.
- Vigone, A., Tron, G.C., Surico, D., Baj, G., Appendino, G., Surico, N., 2005. Ingenol derivatives inhibit proliferation and induce apoptosis in breast cancer cell lines. *Eur. J. Gynaecol. Oncol.* 26, 526–530.
- Warrilow, D., Gardner, J., Darnell, G., Suhrbier, A., Harrich, D., 2006. HIV type 1 inhibition by protein kinase C modulatory compounds. *AIDS Res. Hum. Retroviruses* 22, 854–864.
- Williams, S.A., Greene, W.C., 2007. Regulation of HIV-1 latency by T-cell activation. *Cytokine* 39, 63–74.
- Williams, S., Chen, L., Kwon, H., Fenard, D., Bisgrove, D., Verdin, E., Greene, W., 2004. Prostratin antagonizes HIV latency by activating NF-κB. *J. Biol. Chem.* 279, 42008–42017.