Antiviral Effect of Natural and Semisynthetic Diterpenoids against Adenovirus Infection *in vitro*

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ABSTRACT

The emergence and re-emergence of viruses has highlighted the need to develop new broad-spectrum antivirals to mitigate human infections. Pursuing our search for new bioactive plant-derived molecules, we study several diterpene derivatives synthesized from jatropholones A and B and carnosic acid isolated from *Jatropha isabellei* and *Rosmarinus officinalis*, respectively. Here, we investigate the antiviral effect of the diterpenes against human adenovirus (HAdV-5) that causes several infections for which there is no approved antiviral therapy yet. Ten compounds are evaluated and none of them present cytotoxicity in A549 cells. Only compounds 2, 5 and 9 inhibit HAdV-5 replication in a concentration-dependent manner, without virucidal activity, whereas the antiviral action takes place after virus internalization. The expression of viral proteins E1A and Hexon is strongly inhibited by compounds 2 and 5 and, in a lesser degree, by compound 9. Since compounds 2, 5 and 9 prevent ERK activation, they might exert their antiviral action by interfering in the host cell functions required for virus replication. Besides, the compounds have an anti-inflammatory profile since they significantly inhibit the levels of IL-6 and IL-8 produced by THP-1 cells infected with HAdV-5 or with an adenoviral vector. In conclusion, diterpenes 2, 5 and 9 not only exert antiviral activity against adenovirus but also are able to restrain pro-inflammatory cytokines induced by the virus.

Introduction

Human adenoviruses (HAdV) produce highly prevalent infections that cause a broad range of clinical conditions [1]. In immunocompetent individuals, the infection is self-limited; however, HAdV infections could be a major problem in immunocompromised patients causing high mortality, especially in pediatric recipients of allogeneic hematopoietic stem cell transplant [2–5]. HAdV belonging to species C mostly produces diseases in the respiratory tract and severe infections of the conjunctiva, which can

ABBREVIATION

ACV	acyclovir
CPE	cytopathic effect
ERK	extracellular signal-regulated kinase
HAdV-5	human adenovirus type 5
HAdV-V	human adenovirus vector
HSV-1	herpes simplex type 1
m.o.i.	multiplicity of infection
PFU	plate forming unit
TLR	Toll-like receptor

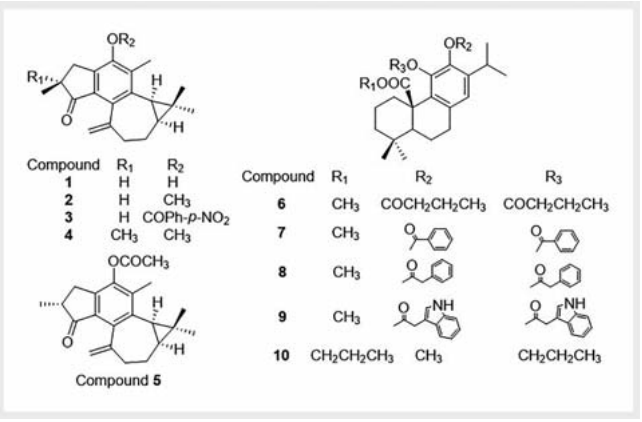
result in keratoconjunctivitis [6–8]. Besides, HAdV-5 is one of the adenoviruses most associated with human disease [9].

The first line of defense against HAdV is innate immunity, and the response exerted is associated with severe acute manifestations, which also plays a role in acute toxicity attributable to HAdV vectors [10]. The diversity of cytokines and chemokines generated plays a major role in the pathogenesis of tissue damage [11]. Considering that there are no approved specific antivirals against this virus, new therapeutics are required to control adenovirus infection.

Plants are an endless source of new drugs. The chemical diversity and broad spectrum of antiviral activity of natural products make them ideal candidates for new therapeutics [12]. We have reported that meliacine (MA), a principle present in partially purified leaf extracts of *Melia azedarach* L., has a broad-spectrum antiviral activity, and its bioassay-guided purification has led to the isolation of the tetranortriterpenoid 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM), which hinders herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) multiplication and exhibits immunomodulatory properties *in vitro* and antiangiogenic activities both *in vitro* and *in vivo* [13–22].

Pursuing our search for new bioactive plant-derived molecules, we studied the anti-herpetic and immunomodulatory activities of several diterpenes. The jatropholones A and B isolated from the rhizomes of *Jatropha isabellei* Müll. Arg. (Euphorbiaceae) and their derivatives described by Pertino et al. [23–25], together with carnosic acid isolated from the leaves of *Rosmarinus officinalis* L. (Lamiaceae), were used to synthesize different derivatives [26]. In a previous study [27], three diterpenes, namely jatropholone derivatives 2 and 5 and the carnosic acid derivative 9 (▶ **Fig. 1**), hindered both HSV-1 multiplication and HSV-1- and TLR ligand-induced inflammatory response *in vitro*. The compounds 2, 5 and 9 were effective to restrain the multiplication of TK− strains of HSV-1 resistant to acyclovir (ACV), which indicated that they would have a different mechanism of action from that of ACV [27].

Infections caused by HAdV are treated with broad-spectrum antivirals like Ribavirin, as well as cidofovir and brincidofovir, which are associated with significant toxicity to the kidney and gastrointestinal tract [2, 28]. Due to the importance of finding new therapies against the virus, the antiviral activity of various natural and synthetic compounds has been reported against HAdV [29–33].



▶ **Fig. 1** Structure of the jatropholone and carnosic acid derivatives.

Considering the previous results obtained, the present study aims to assess the antiviral and anti-inflammatory activities of natural and semisynthetic jatropholone and carnosic acid derivatives against HAdV-5.

Results

The antiviral activity of jatropholone A (1), the semisynthetic jatropholones (2–5) and the carnosic acid derivatives (6–10) against HAdV-5 infection was evaluated. The structure of the compounds is summarized in ▶ **Fig. 1**. First, the cytotoxicity of the compounds in the A549 cells was determined. For that purpose, A549 cells were treated with the compounds (6.25–400 µM), and after incubation at 37 °C for 24 h, an MTT assay was performed. The compounds showed CC_{50} values from 247 µM to > 400 µM and were considered not cytotoxic (▶ **Table 1**).

Dose-response studies of the compounds were performed using virus yield-reduction assay. Briefly, A549 cells were inoculated with HAdV-5 at a multiplicity of infection (m.o.i.) of 1. After adsorption, non-bound virions were removed by washing with PBS, then different concentrations of compounds were added and, at 24 h p.i., titers of infectious virus were determined by a standard plaque assay. The percentage of virus inhibition was determined and EC_{50} values were calculated from the curves (▶ **Fig. 2a**). Only compounds 2, 5 and 9 were able to inhibit HAdV-5 in a concentration-dependent way with EC_{50} values of 50 µM, 37.2 µM and 50.2 µM, respectively (▶ **Table 1**). Under the same experimental conditions, the EC_{50} value of Ribavirin was 20 µM.

To further test the potential effect of the compounds to inhibit HAdV-5 cytopathic effect (CPE), confluent A549 cells were inoculated with 0.1 PFU/cell of HAdV-5. After virus adsorption, the infected cells were incubated either with maintenance medium (control) or with serial dilutions of the compounds at 37 °C until 56 h p.i., when 100 % of cell death was observed in untreated infected control cells. The inhibition of HAdV-5-CPE exerted by the compounds was high, reaching 99.9 % inhibition at low concentrations. The EC_{50} values were 6.25 µM, 4.10 µM, 1.10 µM and 10 µM for compounds 2, 5, 9 and Ribavirin, respectively. None of the other compounds inhibited the CPE. Notwithstanding, the assay

▶ **Table 1** Screening of jatropholones and carnosic acid derivatives: cytopathic effect and cytotoxicity assays

Compound	Cytotoxicity (CC_{50}, μM)		HAdV-5 Inhibition (EC_{50}, μM)		SI	HAdV-V Inhibition (EC_{50}, μM)
	A549 cells	AD293 cells	m.o.i. = 0.1	m.o.i. = 1		
1	320	nd	> 200	> 200	nd	nd
2	383.8	> 400	6.25	50	7.7	6
3	302	nd	> 200	> 200	nd	nd
4	> 400	nd	> 200	> 200	nd	nd
5	> 400	> 400	4.10	37.2	> 10.8	7
6	> 400	nd	> 200	> 200	nd	nd
7	> 400	nd	> 200	> 200	nd	nd
8	350	nd	> 200	> 200	nd	nd
9	247	358	1.10	50.2	4.9	15
10	303	nd	> 200	> 200	nd	nd
Ribavirin	> 1000	> 1000	10	25	> 40	73

CC_{50}: 50% Cytotoxic Concentration; EC_{50}: 50% Effective Concentration; SI: Selective Indice (CC_{50}/EC_{50}); nd: not determined

was performed during 56 h; no cytotoxic effect was observed at the concentrations evaluated (▶ **Fig. 2b**).

To characterize the inhibitory effect of the compounds, different assays were performed. First, to establish whether the compounds produced a direct effect on the viral particle, a virucidal assay was achieved. For that purpose, 100 μM of compounds 2, 5 and 9 were incubated with HAdV-5 for 30, 60 and 120 min at 37 °C and then titrated by plaque assay. The titers obtained were similar to the HAdV-5 untreated control, suggesting that the compounds have no virucidal activity (▶ **Fig. 2c**).

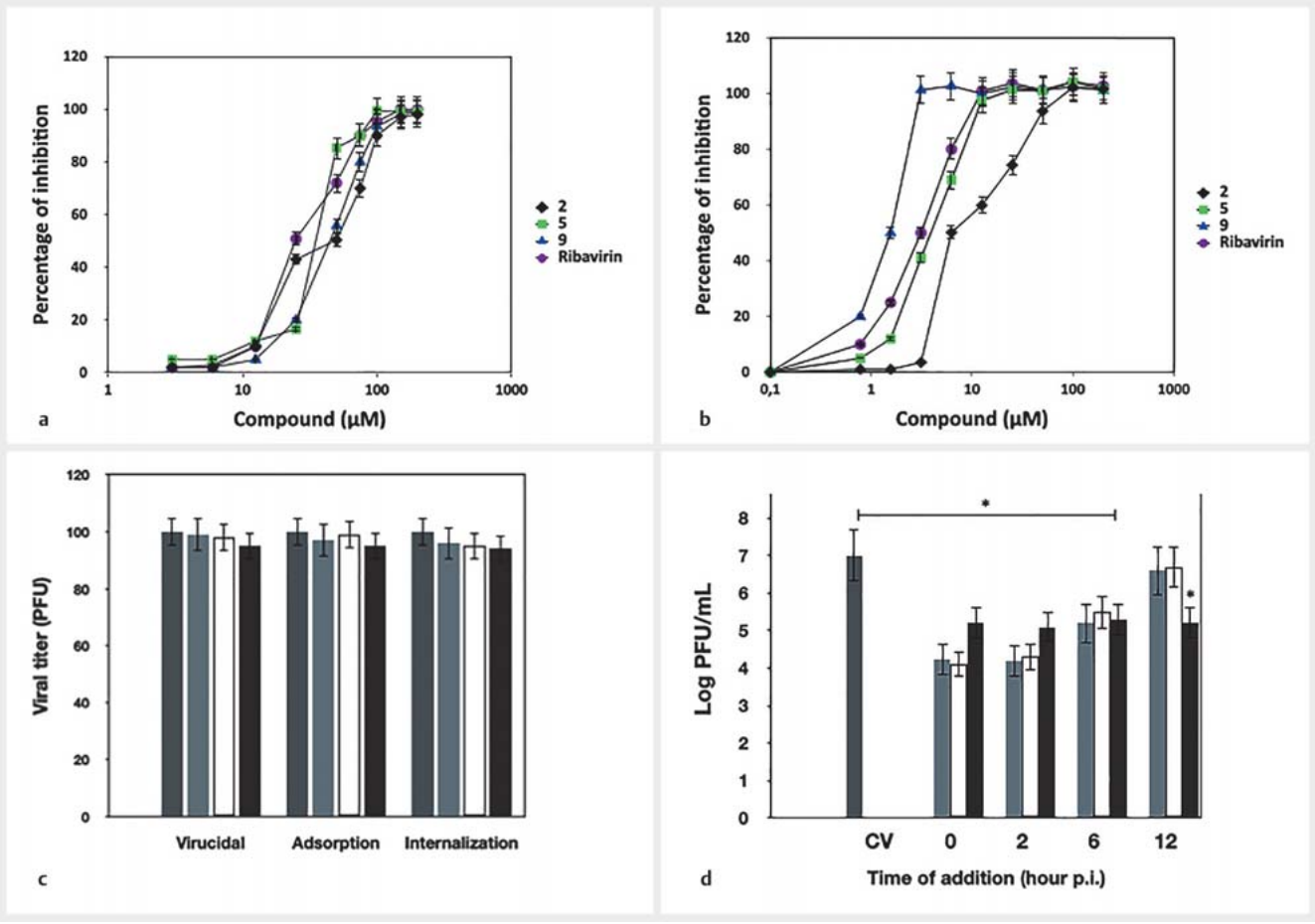
Next, we determined if compounds 2, 5 and 9 restricted HAdV-5 virus adsorption. HAdV-5 was incubated together with the compounds in A549 cells (m.o.i. = 1) for 1 h at 4 °C to let the virus adsorb to the host cell membrane without being internalized. After that, cells were washed to eliminate the compounds and the non-adsorbed virus, overlaid with a medium containing 0.7% methylcellulose and incubated at 37 °C for 5 days. We found no significant differences between treated and untreated cells. Hence, compounds 2, 5 and 9 did not interfere with virus adsorption to the cells (▶ **Fig. 2c**).

To study if the compounds affected HAdV-5 internalization to the cells, A549 cells grown in 24-well plates were infected with HAdV-5 (100 PFU). After adsorption, cells were treated with compounds 2, 5 and 9 for 1 h at 37 °C. Non-internalized viral particles were inactivated with citrate buffer (pH 3) and cells were washed, overlaid with a medium containing 0.7% methylcellulose and incubated at 37 °C for 5 days. We observed no reduction in virus titers in treated-infected cells. Therefore, compounds 2, 5 and 9 did not inhibit HAdV-5 internalization, either (▶ **Fig. 2c**).

To further characterize the inhibitory action of compounds 2, 5 and 9, a time of addition experiment was carried out. For that purpose, 100 μM of compounds 2, 5 and 9 was added to HAdV-5-infected A549 cells at different times after infection and, at 24 h p.i., infectivity was determined. All compounds presented a two-log inhibition of viral yield when added between 0 and 6 h p.i. ($p < 0.05$). Only compound 9 was able to reduce HAdV-5 infectivity when added at 12 h p.i. ($p < 0.05$) (▶ **Fig. 2d**).

To investigate the effect of the compounds on viral propagation, an immunofluorescence assay was performed. A549 cells grown in coverslips were infected with HAdV-5 at an m.o.i. of 1 and, after adsorption, treated or not with 100 μM of compounds 2, 5 or 9 until 24 h p.i. After that time, the IFI staining was achieved using antibodies against E1A and Hexon HAdV proteins. As can be seen in ▶ **Fig. 3a**, compounds 2 and 5 completely inhibited the expression of both viral proteins, while compound 9 inhibited E1A and Hexon expression in 41.9% and 35.9%, respectively ($p < 0.05$). On the other hand, Ribavirin also completely restrained E1A expression, though only a 70% inhibition was achieved for Hexon protein. To confirm these findings, the expression of viral proteins E1A and Hexon was determined by Western blotting. A549 cells were infected with HAdV-5 at an m.o.i. of 1 and, after adsorption, treated or not with 100 μM of compounds 2, 5 or 9 until 24 h p.i., when cells were processed. ▶ **Figs. 3c** and **d** show that E1A expression was diminished by all the compounds, but Hexon expression was only inhibited by compounds 2 and 5. These results are in accordance with those obtained by the immunofluorescence assay (▶ **Fig. 3a**).

Then, we evaluated the antiviral activity of the compounds against a replicative deficient adenovirus serotype 5 (HAdV-V) in which the e1A gene has been deleted, replaced by a *β*-galactosidase gene and propagated in AD293 cells that constitutively express E1A. For that purpose, we tested the effect of the compounds to inhibit HAdV-V CPE on confluent AD293 cells that were inoculated with 0.1 PFU/cell of HAdV-V. After virus adsorption, infected cells were incubated either with maintenance medium (control) or with serial dilutions of the compounds at 37 °C until 56 h p.i., when 100% of cell death was observed in virus control. All compounds inhibited HAdV-V replication in a dose-dependent



▶ **Fig. 2** Antiviral activity of compounds 2, 5 and 9 in A549 cells. **a** HAdV-5 infected cells (m. o. i. = 1) were treated with different concentrations of compounds 2, 5 and 9. After 24 h, cultures were frozen and thawed, followed by centrifugation at 10 000 xg for 10 min, and titers of infectious virus in supernatants were determined by plaque assay. **b** HAdV-5 infected cells (m. o. i. = 0.1) were treated with different concentrations of 2, 5 and 9. After 56 h, viral CPE was evaluated. **c** Virucidal effect, treatments during adsorption and internalization of HAdV-5 were performed as described in Material and Methods. **d** A549 cell monolayers were infected with HAdV-5 (m. o. i. = 1) and incubated for 1 h at 37 °C. The inoculum was discarded, and cells were further incubated with fresh medium or treated with 100 µM of compounds, at different times p. i. (0, 2, 6 and 12 h. p. i.). At 24 h. p. i., virus yields were collected and titrated by plaque assay in A549 cells. CV (dark grey), compounds: 2 (light grey), 5 (white), 9 (black). Data are expressed as the mean ± SD of three separate experiments. * significantly different ($p < 0.05$)

way, and without cytotoxic effect, meaning that the inhibition observed is not restored by the presence of E1A (▶ **Table 1**). The EC_{50} values obtained were similar to those obtained with HAdV-5: 6 µM, 7 µM and 15 µM for compounds 2, 5 and 9, respectively. However, the EC_{50} was significantly increased for Ribavirin, rising from 10 µM in HAdV-infected cells to 73 µM in HAdV-V-infected ones.

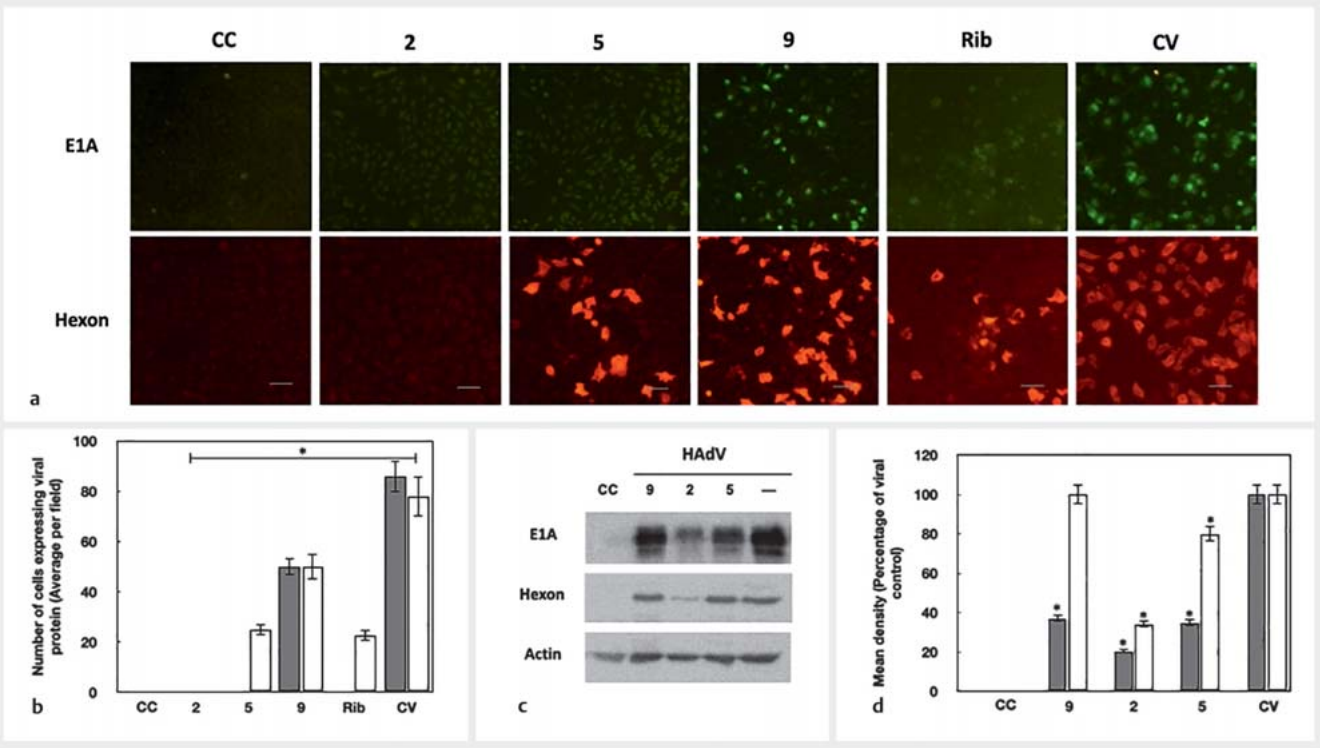
Next, we evaluated the effect of the compounds on cytokines production. For that purpose, THP-1 cells were infected or not with HAdV-5 and HAdV-V and treated with 100 µM of compounds 2, 5 and 9 during 24 h. At that time, the level of IL-6 and IL-8 was determined by an ELISA. No significant differences between IL-6 and IL-8 release from non-treated and treated cells were detected in non-infected cells (▶ **Fig. 4**). Interestingly, we found that secretion of IL-6 and IL-8 was significantly reduced when any of the compounds was added to HAdV-5-infected cells ($p < 0.05$) (▶ **Figs. 4 a** and **c**), even though infective virus was not detected in none of the cultures (data not shown). As expected, HAdV-V induced higher levels of IL-6 and IL-8 cytokines than HAdV-5, but all the compounds were able to reduce them (▶ **Figs. 4 b** and **d**).

Discussion

Human adenoviruses are an important cause of infections in both immunocompetent and immunocompromised individuals. Indeed, in May 2022, adenovirus was associated with reports of severe acute hepatitis of unknown cause in previously healthy children: at least 450 probable cases had been reported worldwide, with 31 requiring liver transplantation [34].

Since there are no approved specific antivirals against this virus, new approaches are necessary.

In this work, we evaluated the antiviral activity of 10 plant-derived molecules against HAdV-5. We found that all of them presented low cytotoxicity in A549 cells despite the fact that some



▶ **Fig. 3** Effect of compounds 2, 5 and 9 on HAdV-5 protein expression in A549 infected cells. **a** A549 cells were infected with HAdV-5 (m. o. i. = 1) and treated with 100 µM of compounds 2, 5, 9 and Ribavirin or not (CV). At 24 h p. i., E1A and Hexon were localized by IFI staining. Magnification: 10× (Scale bars, 50 µm). **b** The number of cells expressing E1A and Hexon proteins from a) were determined. E1A (light grey), Hexon (white). **c** A549 cells were infected with HAdV-5 (m. o. i. = 1) and treated or not with 100 µM of compounds 2, 5 or 9 for 24 h. Cells were lysed and subjected to SDS-PAGE, followed by immunoblotting with antibodies against E1A and Hexon. **d** Mean density, percentage of viral control. E1A (light grey), Hexon (white). Data are expressed as the mean ± SD. * significantly different ($p < 0.05$)

of them were used for more than 56 h. These results were in accordance with already reported findings that showed that these compounds were not cytotoxic for other cell lines [23–27, 35].

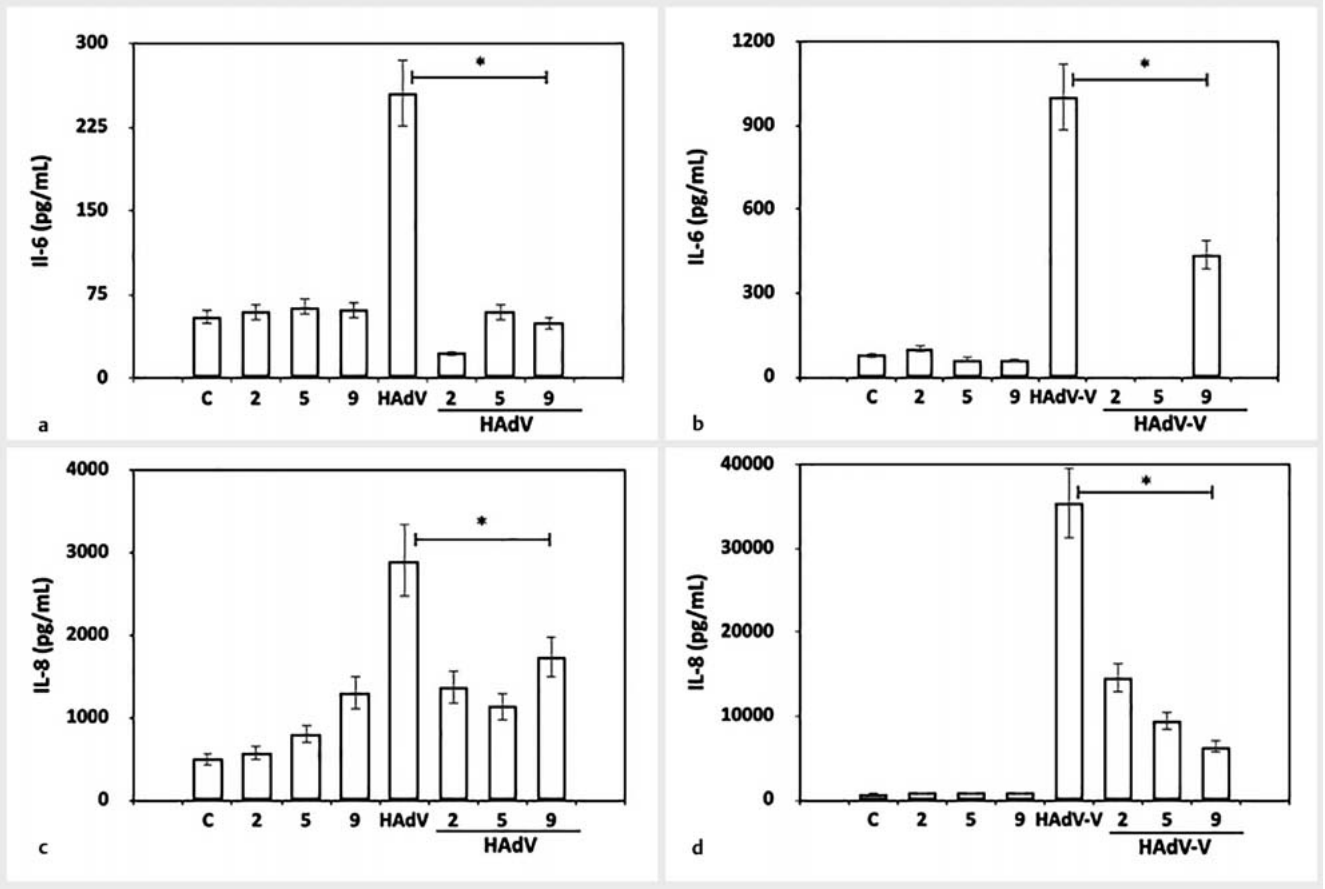
Three of these molecules, namely compounds 2, 5 and 9, inhibited adenovirus replication significantly in a concentration-dependent manner, reaching a decrease of more than two logarithms in viral titers at the highest concentrations tested (▶ **Fig. 2**). Diterpenes 2, 5 and 9 not only exerted antiviral activity against adenovirus in A549 cells but also inhibited herpesvirus multiplication in Vero cells [27]. Besides, none of the molecules showed virucidal activity, and we were able to verify that the antiviral action took place after virus internalization. Whereas the inhibition exerted by compounds 2 and 5 was maximum at early stages of viral replication (▶ **Fig. 2 d**), compound 9 was able to sustain the inhibition even when it was added at 12 h p. i. (▶ **Fig. 2 d**). The temporarily effect of the compounds correlated with the inhibition of viral proteins expression seen by immunofluorescence and Western blot assays (▶ **Figs. 3 a** and **d**). The expression of protein E1A, the first viral protein produced *de novo* during HAdV infection, was strongly inhibited by 2 and 5 and in a modest way by 9. Similar results were obtained with the late protein Hexon (▶ **Figs. 3 a** and **d**).

These results indicate that compounds might exert their inhibitory effect between the virus internalization and virus protein expression phases, and they would behave as potential broad-spectrum anti-HAdV since E1A expression is highly dependent on host factors.

Previous studies have proven that adenovirus induces ERK phosphorylation initially upon infection, independently of virus replication [36], and pharmacologic inhibition of ERK phosphorylation reduced HAdV-5 recovery. The block of cellular MEK/ERK signaling affected virus DNA replication and mRNA levels only weakly, but strongly reduced the amount of viral proteins [37].

In a preceding work, we have demonstrated that compounds 2, 5 and 9 prevent ERK activation induced by herpes virus replication, which results in virus yield inhibition [27]. Therefore, compounds 2, 5 and 9 might exert their antiviral action by interfering in the host cell functions required for virus replication. Hence, we cannot discard that these compounds have presented an antiviral action as a consequence of the inhibition of the ERK pathway. On the other hand, diterpenes also inhibited the replication of the adenoviral vector in cells that express E1A constitutively, and therefore, the inhibition carried out by the three molecules is not restored by E1A (▶ **Table 1**).

Interestingly, when we evaluated the effect of the compounds on the immune response triggered by the adenovirus, we found that all of them had an anti-inflammatory profile since they significantly inhibited the levels of IL-6 and IL-8 produced by THP-1 cells



▶ **Fig. 4** Effect of compounds 2, 5 and 9 on cytokine production. THP-1 cells were infected with HAdV-5 (m. o. i. = 1) for 24 h (**a** and **c**) or infected with HAdV-V (m. o. i.= 1) (**b** and **d**) and treated with culture medium (C) or with 100 µM of compounds 2, 5 or 9. IL-8 and IL-6 were determined by ELISA. Data are expressed as the mean ± SD of three separate experiments. * significantly different ($p < 0.05$)

(▶ **Figs. 4 a** and **c**). This inhibitory effect was also seen when THP-1 cells were infected with the adenoviral vector and treated with the compounds (▶ **Figs. 4 b** and **d**). These results are concordant with previous findings since the compounds were able to restrain the TNF-alpha and IL-6 levels in J774A.1 macrophages infected with herpes simplex virus type 1 [27].

Considering that compounds 2, 5 and 9 were able to block ERK phosphorylation in murine macrophages [27], that ERK activation is necessary for IL-8 induction, and the inhibition of ERK is sufficient to block IL-8 induction by HAdV in tissue [38], we hypothesize that the anti-inflammatory effect observed is due to a block of its activation.

In recent years, and due to the emergence and re-emergence of different viruses, the search for broad-spectrum antivirals has become essential [39]. The most promising candidates are inhibitors of intracellular signaling cascades that are essential for virus replication [40], making these diterpenes a good treatment option.

Euphorbiaceae diterpenes, mainly related to the jatrophane skeleton and derived by different rearrangements, are relevant in the search for new bioactive products [41]. Antiviral effect has been described for several derivatives [42]. Jatrophone has been shown to display antiviral activity against RSV-induced respiratory infection [43]. The pharmacophoric requirements for anti-MDR activity of membrane protein P-gp in the jatrophane group of diterpenes was proposed [42]. However, much more derivatives are needed for a similar comparison with the jatropholones reported in this work. The most active jatropholones in this study were the compounds 2 and 5, with EC_{50} of 6 and 7 µM and SI of 7.7 and > 10.8, respectively. Both compounds showed a single methyl group at C-2, with different stereochemistry and either a methoxy or acetate at C-14, being less polar than the corresponding natural product with the free phenolic hydroxyl.

Some abietane diterpenes have shown antiviral activity, including ferruginol and carnosic acid. Carnosic acid inhibits HIV-1 virus and HIV-1 protease and shows the effect of human respiratory syncytial virus [44]. The diindolacetate 9 was the most active from our carnosic acid derivatives, with an EC_{50} value of 15 µM toward HAdV-V. Under the same experimental conditions, the EC_{50} value of the reference compound Ribavirin was 73 µM.

Here, we have presented three molecules derived from nature with both antiviral and anti-inflammatory properties. Diterpenes 2, 5 and 9 not only exerted antiviral activity against adenovirus in A549 cells but were also able to restrain pro-inflammatory cyto-

kines induced by HAdV-5 and HAdV-V in human macrophagic cells. The finding of compounds 2, 5 and 9 exerting both antiviral and anti-inflammatory effects deserves further studies. They would be an interesting option to mitigate, for example, ocular infections caused by HSV and HAdV, whose clinical complications are due to a strong inflammation response elicited by viral infection.

Materials and Methods

Compounds

The jatropholones A and B were isolated from the rhizomes of *J. isabellei*, and the derivatives were synthesized as described in the literature [23–25]. Carnosic acid was isolated from the leaves of *R. officinalis* and used to synthesize the derivatives [26, 35]. Voucher herbarium specimens have been deposited at the University of Talca Herbarium under Schmeda N°1594 and Pertino 001/2007 for *J. isabellei* and *R. officinalis*, respectively. The plants were identified by Dr. Patricio Peñailillo, University of Talca Herbarium. Jatropholones A (compound **1**) and B were isolated from the ethyl acetate extract of *J. isabellei* rhizomes by silica gel column chromatography followed by purification of the fraction pool eluting with petroleum ether (PE):ethyl acetate (EtOAc) (90 : 10–70 : 30) using Sephadex LH-20 permeation with methanol. The mixture of jatropholones A and B was resolved by column chromatography on silica gel using PE : EtOAc (90 : 10). Jatropholone A methyl ester (compound **2**) was prepared by treating **1** in dimethylformamide (DMF) with a stoichiometric amount of NaH and then adding CH_3I. Jatropholone A *p*-nitrophenyl ester (compound **3**) was synthesized by treating **1** with 4-nitrobenzoyl chloride in DMF. 2-methyljatropholone A methyl ether (**4**) was obtained by treating jatropholone A in DMF with NaH in excess and then adding CH_3I. Jatropholone B acetate (compound **5**) was prepared by acetylation of jatropholone B with acetic anhydride/pyridine. The reaction mixtures were worked up as previously described [23–25]. Carnosic acid was isolated from the leaves of *R. officinalis* [26, 35]. The air-dried powdered leaves were extracted under reflux with PE and EtOAc. The combined extracts were resuspended in hot MeOH, left at − 20 °C overnight, filtered to remove waxes and nonpolar compounds and submitted to column chromatography on silica gel. Elution with PE : EtOAc (60 : 40–30 : 70) afforded a carnosic-acid-enriched pool. Permeation on Sephadex LH-20 with MeOH allowed the obtaining of pure carnosic acid and 12-*O*-methylcarnosic acid.

Carnosic acid was methylated with CH_2N_2 to afford the methyl ester. The derivatives **6**–**9** were prepared by esterification of carnosic acid methyl ester with butyric acid (compound **6**), benzoic acid (compound **7**), phenylacetic acid (compound **8**) and indoleacetic acid (compound **9**) using dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) in dry CH_2Cl_2 at room temperature. Treatment of the natural compound 12-*O*-methylcarnosic acid with NaH and propyl iodide in DMF afforded compound **10**. The purity of all derivatives was over 98%, as assessed by ^{1}HNMR spectroscopy.

The compounds were solubilized in DMSO and then diluted in culture medium. The final concentration of DMSO was less than 0.1%.

Cell lines and virus

A549 cells and THP1 were obtained from ATCC. A549 cells were cultured with Eagle's minimal essential medium supplemented with 5% fetal bovine serum (MEM 5%) and 50 µg/ml gentamicin. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% inactivated FBS (RPMI 10%) and 50 µg/ml gentamicin.

HAdV-5 is a wild-type adenovirus that was kindly provided by Dr. Barrero (Laboratorio de Virología, Hospital Gutierrez, Buenos Aires, Argentina) and was propagated in A549 cells. The replicative-deficient adenovirus serotype 5 (HAdV-V), in which the e1A gene has been deleted, was kindly provided by Dr. Podhajcer (Laboratorio de Terapia Molecular y Celular, Fundación Leloir, Buenos Aires, Argentina) and was propagated in AD293 cells that constitutively express viral E1A.

Cytotoxicity assay

Cell viability was determined by MTT assay. A549 cells, treated with twofold serial dilution of compounds (from 6.25 to 400 µM) for 24 h, were incubated for 2 h with culture medium containing 0.5 mg/mL of MTT. Then, the formazan salts produced were dissolved with ethanol, and the absorbance was measured at 570 nm. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration required to reduce cell viability by 50%. All experiments were performed in triplicate.

Antiviral assay

A549 cells were inoculated with HAdV-5 (m. o.i = 1); after adsorption, non-bound virions were removed by washing with PBS and different concentrations of compounds were added. Twenty-four h p. i., cultures were frozen and thawed, followed by 10 minutes of centrifugation at 10 000 xg, and titers of infectious virus in supernatants were determined by a standard plaque assay. Briefly, A549 cells monolayers were incubated for viral adsorption with the sample dilutions for 1 h at 37 °C and then were overlaid with MEM supplemented with 0.7% of methyl cellulose. After 5 days at 37 °C, cells were fixed and stained with crystal violet. Viral plaques were counted, and the number of PFU per milliliter was calculated. The effective concentration 50% (EC_{50}) was defined as the concentration of compound that caused a 50% reduction in viral yields, with respect to untreated virus control.

Cytopathic effect assay

Cells seeded at a density of 10^5 cells/well, grown in 96-well plates during 24 h, were infected or not with HAdV-5 at an m. o. i. of 0.1 PFU/cell. After 1 h adsorption at 37 °C, the medium containing or not containing the compounds was added in triplicate. The plates were incubated at 37 °C until 56 h post-infection (p. i.), when 100% of cell death was observed in virus control. Then, cells were fixed with 10% formalin for 15 min at room temperature, washed once with distilled water and stained with 0.05% crystal violet over 30 min. Afterward, cells were washed once and eluted with a solution of 50% ethanol and 0.1% acetic acid in water. The absorbance was measured on a Euro-genetics MPR-A 4i microplate reader using a test wavelength of 590 nm. Results were analyzed as the percentage of absorbance of treated and infected cells compared with control (untreated/uninfected) cells. We consid-

ered the untreated/uninfected control cells as 100 % of cell survival.

Virucidal effect

HAdV-5 (10^7 PFU) was diluted in culture medium containing or not each compound and incubated for 30, 60 and 120 min at 37 °C. Aliquots were diluted to a non-inhibitory drug concentration and titrated by plaque assay on A549 cells.

Adsorption and penetration assay

A549 cells grown in 24-well plates were inoculated with 100 PFU of HAdV and adsorbed for 1 h at 4 °C with or without the compounds. To quantify adsorbed virus, cells were washed twice with cold PBS, overlaid with medium containing 0.7 % methylcellulose and incubated at 37 °C for 5 days.

To determine internalized virus, cells were incubated at 37 °C to maximize virus penetration after viral adsorption at 4 °C for 1 h, with or without the compounds. At 120 min, monolayers were washed twice with PBS and treated for 1 min with citrate buffer (pH 3). To quantify internalized virus, cells were washed twice with cold PBS, overlaid with medium containing 0.7 % methylcellulose and incubated at 37 °C for 5 days until plaque formation.

Time of addition assay

Compounds were added to confluent monolayers of A549 cells infected with HAdV-5 at an m. o. i. of 1, at 0, 2, 6 and 12 h after infection. Cells were further incubated at 37 °C till 24 h p. i. After that, cultures were frozen and thawed, followed by centrifugation at low speed (10 000 g), and titers of infectious virus in supernatants were obtained by plaque assay in A549 cells.

Indirect immunofluorescence assay (IFI)

Cells grown on glass coverslips, infected with HAdV-5 and treated with the compounds during 24 h, were fixed with methanol for 10 min at − 20 °C. After three washes with PBS, coverslips were inverted on a drop of primary antibody for 30 min at 37 °C, then returned to culture dishes and subjected to three additional washes with PBS. Afterward, cells were incubated with secondary antibody for 30 min at 37 °C. Finally, coverslips were rinsed, mounted and photographed with an Olympus BX51 microscope with epifluorescence optics.

Western blot analysis

Whole extracts of cells infected with HAdV-5 and treated with the compounds for 24 h were loaded on 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes for 60 min at 75 mA. Membranes were blocked in PBS containing 5 % nonfat milk overnight and then incubated with diluted primary antibodies overnight at 4 °C. After washing, membranes were incubated with diluted peroxidase-conjugated antibodies for 1.5 h at 37 °C. The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, PerkinElmer). The bands were quantified using Image J for Windows.

Cytokine determination

Cells were frozen and thawed, and then, supernatants were harvested and centrifuged at 10 000 xg for 10 min, and cytokines were quantified by ELISA in triplicate. Human IL-8 and IL-6 were quantified by commercial ELISA sets (BD OptEIATM, Becton–Dickinson), according to the manufacturer's instructions.

Statistical analysis

CC_{50} and EC_{50} values were calculated from dose–response curves using the software GraphPad Prism 6.01. All assays were carried out in triplicate. Statistically significant differences were evaluated by unpaired *t*-test or one-way ANOVA followed by a Tukey's multiple comparison test.

Contributors' Statement

LEA and EP designed the study. EP and JEB planned and conducted the experiments. EP performed the data analysis and prepared the original manuscript. MP and GS-H prepared all compounds. LEA, MP, GS-H and EP revised the manuscript. All authors agreed with the final version.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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