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Antiherpes activity of glucoevatromonoside, a cardenolide isolated from a Brazilian cultivar of *Digitalis lanata*

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ABSTRACT

Cardiac glycosides, known ligands of the sodium pump, are widely used in the treatment of heart failure, such as digoxin and digitoxin. Besides this important activity, other biological activities, such as the antiviral activity, have been described for this group. HSV are responsible for many infections of oral, ocular and genital regions. Treatment with nucleoside analogs such as acyclovir is effective in most cases; however drug-resistance may arise due to prolonged treatment mainly in immunocompromised individuals. In this study, an antiherpes screening was performed with 65 cardenolide derivatives obtained from different sources, and one natural cardenolide, glucoevatromonoside, inhibited HSV-1 and HSV-2 replication at very low concentrations. This cardenolide showed viral inhibitory effects if added up to 12 h p.i. and these effects appear to take place by the inhibition of viral proteins synthesis (ICP27, U_L42, gB, gD), the blockage of virus release and the reduction of viral cell-to-cell spread. This compound also showed synergistic antiviral effects with acyclovir and anti-Na^+K^+ATPase activity, suggesting that cellular electrochemical gradient alterations might be involved in the mechanism of viral inhibition. These results suggest that cardenolides might be promising for future antiviral drug design.

1. Introduction

Herpes Simplex Virus types 1 and 2 (HSV-1; HSV-2) are alphaherpesviruses with double-stranded DNA packed in an icosahedral capsid and a lipidic envelope formed by various glycoproteins. They replicate by three rounds of transcription, resulting in α (immediate early) proteins that mainly regulate viral replication, such as ICP27; β (early) proteins that synthesize and package DNA, such as U_L42; and γ (late) proteins, most of which are virion proteins, like gB and gD. Inhibition of any of the former stages blocks HSV replication and therefore are potential targets for antiviral therapy (Roizman et al., 2007). The vast majority of the currently licensed drugs for the systemic and/or topical treatment of herpesvirus infections share the same target, the viral DNA polymerase, and the nucleoside analogs are the most widely used drugs for the treatment of such infections (De Clercq, 2009). The development of new antiviral molecules derived from acyclovir increases the selection pressure risk of resistant strains (Danve-Szatanek et al., 2004) that have been observed *in vivo* since the first large therapeutic trials (McLaren et al., 1985). Therefore, the search for new antiviral agents, especially those with different mechanisms of action, is a crucial goal (Butler, 2008).

Cardiac glycosides belong to a group of naturally derived compounds that bind to and inhibit Na^+K^+ATPase (Lingrel et al., 1997). Members of this group have been traditionally used for the treatment of heart failure and atrial arrhythmia, such as digoxin, digitoxin and ouabain (Rahimtoola and Tak, 1996). Recently, other important applications have been suggested for these compounds related to their potential anticancer (Prassas and Diamandis, 2008) and antiviral activity (Dodson et al., 2007; Hartley et al., 2006; Hoffmann et al., 2008; Su et al., 2008).

In this report, we screened 65 cardenolide derivatives obtained from plants, by synthesis or by fungi biotransformation, for anti HSV-1 and HSV-2 activity. Among them, glucoevatromonoside (Fig. 1), isolated from a Brazilian cultivar of *Digitalis lanata* (Braga et al., 1996) was chosen for its lower IC_{50} against HSV to further elucidate its mechanism of action.

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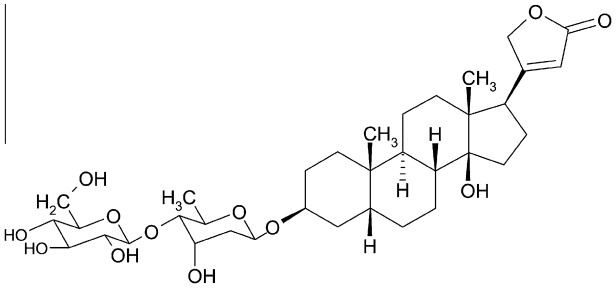

Fig. 1. Chemical structure of glucoevatromonoside (GEV).

2. Materials and methods

2.1. Compounds, cells and viruses

The 65 tested cardenolide derivatives were obtained from plants (Braga et al., 1996; Braga et al., 1997), by synthesis (Extrasynthèse, Genay, France; Merck, Darmstadt, Germany; Boehringer, Mannheim, Germany; Carl Roth, Karlsruhe, Germany), or by fungi biotransformation (Pádua et al., 2005; Pádua et al., 2007). Acyclovir, digoxin, dextran sulfate and furosemide were obtained from Sigma (St. Louis, MO, USA). All compounds were dissolved in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany), not exceeding the minimum non cytotoxic concentration of 1% DMSO and were further diluted in culture medium prior its use.

Vero (ATCC: CCL 81) and GMK-AH1 (Department of Clinical Virology, University of Göteborg, Sweden) cells were grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 100 U/mL penicillin G, 100 μg/mL streptomycin and 25 μg/mL amphotericin B (Cultilab) and maintained at 37 °C in a humidified 5% CO_2.

HSV-1 [KOS and 29R (acyclovir-resistant) strains] (Faculty of Pharmacy, University of Rennes, France), and HSV-2 [333 strain (Department of Clinical Virology, Göteborg University, Sweden)] were propagated in Vero and GMK AH1 cells, respectively. Viral stocks were stored at −80°C and titrated based on plaque forming units (PFU) count by plaque assay as previously described (Burleson et al., 1992).

2.2. Screening for antiherpes compounds

Firstly, cytotoxicity was determined by MTT assay (Mosmann, 1983). Briefly, confluent Vero or GMK cells were exposed to different concentrations of compounds for 72 or 48 h, respectively, and after incubation, the 50% cytotoxic concentration (CC_{50}) of each one was calculated as the concentration that reduces cell viability by 50%, when compared to the untreated control.

After that, to screen antiherpes activity, a plaque reduction assay was performed following the general procedures described by Silva et al. (2010). Cell monolayers were infected with approximately 100 PFU of each virus for 1 h at 37 °C and then were overlaid with MEM containing 1.5% carboxymethylcellulose (CMC; Sigma) either with the presence or absence of different concentrations of the compounds. After 48 h (HSV-2) or 72 h (HSV-1) of incubation at 37 °C, cells were fixed and stained with naphthol blue–black (Sigma), and plaques were counted. The IC_{50} of each compound was calculated as the concentration that inhibited 50% of viral plaque formation, when compared to untreated controls. Acyclovir was used as positive control. The selectivity index ($SI = CC_{50}/IC_{50}$) was calculated for each tested compound.

2.3. Assays for anti-HSV-1 (KOS strain) activity

To investigate the potency of the detected antiherpes activity, an yield reduction assay was performed as previously described by Hussein et al. (2008). Vero cell monolayers were infected with HSV-1 at three different MOI (0.004, 0.04 and 0.4) for 1 h at 37 °C. Cells were washed, different concentrations of glucoevatromonoside were added, and the plates incubated during 72 h at 37 °C. After, culture supernatants were harvested and virus titers were calculated by plaque reduction assay as previously described.

The virucidal assay was conducted as described by Ekblad et al. (2006), where the mixtures of serial two-fold dilutions of glucoevatromonoside and 4×10^4 PFU of HSV-1 in serum free MEM were co-incubated for 15 min at 37 °C prior to the dilution of these mixtures to non-inhibitory concentrations of this compound (1:100). The residual infectivity was determined by viral plaque reduction assay as described above.

The pretreatment (Bettega et al., 2004) was performed with Vero cell monolayers, which were pretreated with different concentrations of glucoevatromonoside for 3 h at 37 °C prior virus infection. After washing, cells were infected with 100 PFU of HSV-1 for 1 h at 37 °C. The infected cells were washed, overlaid with MEM containing 1.5% CMC, incubated for 72 h, and treated as described earlier for plaque reduction assay. For the simultaneous treatment (Onozato et al., 2009), 100 PFU of HSV-1 and different concentrations of glucoevatromonoside were added concomitantly to Vero cells for 1 h at 37 °C. After washing, cells were overlaid with MEM containing 1.5% CMC, incubated for 72 h, and treated as described earlier for plaque reduction assay.

The attachment and penetration assays followed the procedures also described by Silva et al. (2010). Briefly, for attachment assay, confluent Vero cells prechilled at 4 °C for 1 h were infected with 100 PFU of HSV-1, treated with different concentrations of glucoevatromonoside, and incubated at 4 °C for 2 h. Unabsorbed viruses were removed by washing with cold PBS, and cells were covered with overlay medium, and treated as formerly described for plaque reduction assay. For penetration assay, 100 PFU of HSV-1 were adsorbed for 2 h at 4 °C on confluent Vero cells prechilled at 4 °C for 1 h, and after incubated at 37 °C for 5 min to allow virus penetration. Following, cells were treated with different concentrations of glucoevatromonoside, and incubated for 1 h at 37 °C. Unpenetrated viruses were inactivated with warm citrate-buffer (pH 3.0) for 1 min. Cells were washed with PBS, and treated as described above for plaque reduction assay. For attachment and penetration assays, the dextran sulfate (Sigma) was used as a positive control (Aguilar et al., 2007).

The time-of-addition and removal assays were performed as previously described by Su et al. (2008) and Zhen et al. (2006), with minor modifications. For the time-of-addition assays, Vero cell monolayers were infected with 100 PFU of HSV-1 and incubated at 37 C for 1 h. Different concentrations of glucoevatromonoside were added to the cells at intervals of 3, 6, 9, 12, 18 and 24 h post-infection (p.i.). After 72 h of incubation, this assay followed the procedures described earlier for plaque reduction assay. In the assessment of time-of-removal assays, cells were infected 100 PFU of HSV-1 and incubated at 37 °C for 1 h, and different concentrations of glucoevatromonoside were added. At the intervals of 3, 6, 9, 12, 18 and 24 h p.i., the medium containing the glucoevatromonoside was removed, cells were washed with PBS and only MEM was added into the wells. After 72 h of incubation, this assay followed the procedures described earlier for plaque reduction assay.

For the viral plaque size reduction assay, different concentrations of glucoevatromonoside were added to Vero cells 1 h after their infection with 100 PFU of HSV-1, and the plates were incubated during the entire period of plaques development. Images of

20 viral plaques formed in the absence (viral control) and presence of each concentration of glucoevatromonoside were captured using a cooled digital camera attached to an Olympus BX41 microscope (Olympus America Inc., Pennsylvania, PA). The area of each plaque was determined by using the Image J 1.43u version software (NIH, Bethesda, MD) (Silva et al., 2010).

The virus release assay followed the procedures described by Su et al. (2008), with minor modifications. Confluent Vero cells were infected with HSV-1, at MOI 0.4 for 1 h. After, cell monolayers were washed and different concentrations of glucoevatromonoside were added to the cells for 24 h at 37 °C. After, the supernatants and cell pellets were collected separately, and the pellets were frozen and thawed three times before virus titration by plaque reduction assay.

2.4. Polymerase chain reaction (PCR)

Genomic DNA and total RNA of Vero cells infected with HSV-1 were extracted using Brazol (LGC Biotecnologia, Cotia, SP, Brazil), following the manufacture's instructions. DNase treatment was performed on the eluted RNA to avoid residual DNA contamination. Eight hundred nanograms of the eluted RNA was subjected to reverse transcription by a commercial kit (Superscript™ III; Invitrogen, Carlsbad, CA, USA), following the manufacture's instructions, and then subjected to PCR amplification using the primers pairs for U_L54 and U_L13 as described Kleymann et al. (2002) and Tal-Singer et al. (1997) and for U_L52 and ACTB (β-actin) as described by Su et al. (2008). The PCR reaction was carried out in a final volume of 25 μl containing 20 mM Tris–HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.2 μM of each specific primer, 2.5 U of GoTaq DNA polymerase (Promega, Madison, WI, USA), and genomic DNA or cDNA. The PCR program for U_L52, U_L13 and U_L54 and ACTB consists of denaturation at 94 °C for 5 min and 30 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, and polymerization at 72 °C for 40 s, followed by a final extension at 72 °C for 10 min. The expected sizes for U_L54, U_L13, U_L52 and ACTB are 283, 600, 259 and 314 bp, respectively. Five-microliter aliquots of the PCR products were resolved on a 1.5% agarose gel.

2.5. Western blotting analysis

Vero cell monolayers were infected with HSV-1 at MOI 0.2 for 1 h. Next, residual viruses were removed with PBS and cells received different treatments for 18 h. Then, cells were trypsinized and lysed with lysis buffer [0.125 M Tris–HCl (pH 7.4), 30% glycerol, 100 μg/ml phenylmethylsulfonyl fluoride, 2% sodium dodecyl sulfate and 5% β-mercaptoethanol]. Cell lysates were clarified by centrifugation, and proteins were denatured by boiling, and equivalent amounts of protein (5 μg) were separated on 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and blocked with 5% non-fat milk in blotting buffer [25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20]. All membrane washing steps were performed using this blotting buffer. The membranes were incubated for 90 min with the following primary antibodies: goat monoclonal antibody against ICP27 protein (1:700 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal antibody against U_L42 protein (1:5000 dilution) (Millipore); mouse monoclonal antibody against gD (1:5000 dilution) (Santa Cruz Biotechnology), mouse monoclonal antibody against gB (1:5000 dilution) (Millipore); rabbit monoclonal antibody against β-actin (1:5000 dilution) (Millipore). After washing, the membranes were incubated with the respective secondary antibodies for 1 h. The immunoblots were developed and detected using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA), according to the manufacture's instructions.

2.6. Evaluation of potential synergistic effects of glucoevatromonoside plus acyclovir

Potential synergistic effects of glucoevatromonoside with acyclovir were evaluated by plaque reduction assay, as described by Chou (2006). Therefore, each drug by itself or in combination was tested at an equipotency ratio based on its corresponding IC_{50} value. The interaction degree between glucoevatromonoside and acyclovir was calculated through combination index (CI) equation, based on the median-effect principle of the mass-action law, using Calcusyn software (version 2.1, Biosoft). According to the CI theorem, CI values <1, =1, and >1 indicate synergism, additive effect, and antagonism, respectively.

2.7. Anti-ATPase assay

This assay followed the procedures described earlier (Hu et al., 2009). Adenosine 5′-triphosphatase (NKATPase) activity assay was determined using a Quantichrom ATPase/GTPase assay kit (Bioassay, Hayward, CA, USA), according to the manufacture's instructions.

3. Results and discussion

3.1. Screening for antiherpes compounds

The initial screening of 65 cardenolide derivatives for anti-HSV activity was performed only with HSV-1(KOS strain) using a plaque reduction assay. Following the same strategy proposed by Su et al. (2008), we decided that compounds showing IC_{50} values $\leqslant 1$ μM would be chosen to proceed another screening for anti-HSV-1 (29R strain) and anti-HSV-2 (333 strain) activity. Among the 65 tested compounds, 16 were found to possess antiherpes activity with IC_{50} values $\leqslant 1$ μM, and a natural compound, glucoevatromonoside, isolated from a Brazilian cultivar of *Digitalis lanata* (Braga et al., 1996) was chosen for further evaluation of its mode of action (Table 1) due to its high SI and lower IC_{50}, when compared to acyclovir, and also because there was enough quantity to perform all designed assays necessary for this purpose. Digoxigenin showed an $IC_{50} \geqslant 1$ μM, but it was tested in the second screening because it is the aglycone of some tested cardenolide derivatives and showed antiherpes effects in a previous work (Su et al., 2008). As shown in Table 1, HSV-1 (29R strain, resistant to acyclovir) was highly sensitive to the treatment with the tested cardenolides, which implicates that the targets of these compounds are probably different from those of acyclovir. Hence, they might represent a novel group of drugs with distinct antiviral mechanism from those of conventional drugs.

3.2. Mechanism of antiherpes activity of glucoevatromonoside

Firstly, in order to compare the potency of glucoevatromonoside antiherpes activity, at different MOI, a yield reduction assay was conducted. As shown in Fig. 2, both concentrations of glucoevatromonoside were able to inhibit HSV-1 replication at all tested conditions, even at the MOI 0.4 which is thousand times higher than that used in the initial screening. At the higher tested MOI, the glucoevatromonoside at 0.26 μM, which is two times higher than its IC_{50} value, showed a reduction of 5.1 Log, in comparison with viral controls. Remarkably, this reduction was higher than that obtained with acyclovir (2.5 Log) at the same conditions Since this antiviral potency is not commonly observed for other antiviral agents, this

Table 1
Anti-HSV activity of cardenolide derivatives.

Compound (origin)	CC_{50} (μM)		HSV-1 (KOS strain)		HSV-1 (29R strain)		HSV-2 (333 strain)	
	VERO cells	GMK AH1 cells	IC_{50} (μM)	SI	IC_{50} (μM)	SI	IC_{50} (μM)	SI
Digitoxigenin monodigitoxoside (P)	6.60 ± 0.28	>250	0.14 ± 0.00	47	0.11 ± 0.01	60	0.03 ± 0.00	>8.333
Digitoxigenin bisdigitoxoside (P)	10.05 ± 2.33	>250	0.12 ± 0.01	84	0.10 ± 0.01	100	0.09 ± 0.01	>2.777
Glucoevatromonoside (P)	273.95 ± 46.46	>250	0.13 ± 0.01	2.107	0.06 ± 0.01	4.566	0.04 ± 0.00	>6.250
Digitoxin (P)	5.67 ± 0.22	>25	0.34 ± 0.06	17	0.32 ± 002	18	0.20 ± 0.02	>125
β-acetyldigitoxin (P)	10.55 ± 0.49	>250	0.36 ± 0.01	29	0.34 ± 0.01	31	0.50 ± 0.01	>500
Lanatoside A (P)	19.00 ± 0.28	>250	0.54 ± 0.00	35	0.39 ± 0.04	49	0.24 ± 0.00	>1.042
Glucogitoroside (P)	148.50 ± 6.79	>250	0.83 ± 0.01	179	0.60 ± 0.01	247	0.45 ± 0.02	>555
Digoxigenin bisdigitoxoside (P)	174.25 ± 24.68	284.65 ± 16.62	0.27 ± 0.01	645	0.22 ± 0.01	792	0.23 ± 0.02	1.238
Deacetyl lanatoside C (P)	22.15 ± 0.21	>250	0.94 ± 0.05	23	0.44 ± 0.03	50	0.54 ± 0.00	>463
Digoxigenin (P)	>250	>250	3.50 ± 0.49	>71	3.56 ± 0.79	>70	1.22 ± 0.01	>205
Digoxin (S)	5.57 ± 0.40	>25	0.66 ± 0.01	8	0.18 ± 0.01	31	0.65 ± 0.01	>38
K strophantoside (S)	>250	>250	0.33 ± 0.03	>757	0.50 ± 0.03	>500	0.34 ± 0.04	>735
α-methyldigitoxin (S)	88.50 ± 10.69	>250	0.12 ± 0.20	737	0.25 ± 0.05	354	0.05 ± 0.02	>5.000
β-methyldigitoxin (S)	132.30 ± 8.14	>250	0.14 ± 0.02	945	0.26 ± 0.01	509	0.09 ± 0.01	>2.777
Convalatoxin (S)	201.90 ± 11.46	>250	0.02 ± 0.00	10.095	0.03 ± 0.00	6.730	0.02 ± 0.00	>12.500
Helveticoside (S)	>250	>250	0.08 ± 0.00	>3.125	0.11 ± 0.01	>2.273	0.04 ± 0.00	>6.250
Cymarin (S)	>250	>250	0.14 ± 0.02	>1.786	0.13 ± 0.01	>1.923	0.11 ± 0.01	>2.273
ACV	>2.000	>2.000	3.45 ± 0.42	>580	NA	NA	8.60 ± 1.96	>232

Values represent the mean ± standard deviations of three or more independent experiments; CC_{50} = concentration that showed 50% cytotoxic effects in VERO or GMK AH1 cells; IC_{50} = concentration that inhibited 50% of HSV replication; IS = CC_{50}/IC_{50}; P = plant origin; S = synthetic origin; NA = no activity.

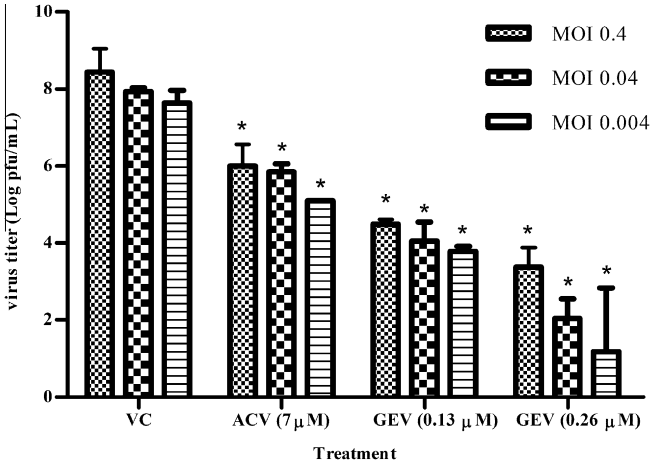

Fig. 2. Anti-HSV-1 (KOS strain) activity of glucoevatromonoside (GEV, 0.13 and 0.26 μM) at three different MOI values. Acyclovir (ACV, 7 μM) was used as control. Results represent the mean ± standard deviation of three independent experiments. (∗) Indicate significant statistical differences ($p < 0.05$), ANOVA/Dunnett tests were carried out for each treatment in different MOI and compared to their respective virus controls (VC).

compound holds a clear advantage over them (Talarico and Damonte, 2007).

A strategy for the treatment of HSV infections is the topical application of drugs that can permanently inactivate the viral particles (Ekblad et al., 2010). To elucidate if glucoevatromonoside presented virus-inactivating activity, a virucidal assay was performed, where infectious particles of HSV-1 were put in contact with different concentrations of glucoevatromonoside prior to be titrated by a plaque reduction assay. This treatment was not able to inhibit HSV-1 replication, even at a concentration 80 times higher (10 μM) than its IC_{50} (0.13 μM). Therefore, the anti-HSV activity of this compound was not exerted directly on HSV-1 particles before they have entered into the cells confirming the findings previously described for other cardenolides (Hartley et al., 2006; Nagai et al., 1972; Su et al., 2008).

To explore the effects of glucoevatromonoside directly on the host cells, a pretreatment assay was performed. This strategy has not shown to inhibit HSV-1 replication, suggesting that this compound did not present prophylactic effect *in vitro*. Next, HSV-1 and glucoevatromonoside were added to Vero cells simultaneously to investigate if it could interfere with the early stages of viral infection. This strategy has also not shown inhibit HSV replication suggesting that viral adsorption and penetration were not affected. To confirm these findings, viral attachment and penetration were individually investigated, and the results attested that glucoevatromonoside indeed did not affect these early stages, even when tested at 2 μM – 16 times higher than its IC_{50} (0.13 μM) – corroborating our results obtained during the simultaneous treatment and those by other authors (Dodson et al., 2007; Su et al., 2008). Fig. 3 shows a summary of these results.

In order to detect in which stages of HSV replication cycle the glucoevatromonoside could be acting, time-of-addition and removal assays were performed. As shown in Fig. 4, the anti-HSV-1 activity of glucoevatromonoside was preserved when added up to 12 h p.i. decreasing thereafter. Concordantly, when glucoevatromonoside was removed the activity significantly reduced up to12 h p.i. These data suggested that glucoevatromonoside should be added up to 12 h p.i. to affect the HSV replication.

Since glucoevatromonoside inhibited HSV-1 at the first 12 h of its replication cycle, after viral attachment and penetration into the cells, the viral transcription was investigated through RT-PCR to determine if this process was impaired by this cardenolide affecting or not the HSV-1 gene expression. For the post-infection treatment, Vero cells were infected for 1 h, and then treated with glucoevatromonoside, acyclovir or a combination of both, during 6 and 12 h p.i. Fig. 5 shows the mRNA levels of U_L54, U_L52 and U_L13 HSV genes, which are α, β and γ genes, respectively. The treatments with glucoevatromonoside (0.13 μM), acyclovir (5 μM) or a combination of both during 12 h p.i. were not able to reduce the mRNA expression of the tested genes, when compared to viral control, indicating that glucoevatromonoside and acyclovir do not inhibit the transcription process. The same results were obtained when the treatments were performed at 6 h p.i. (data not shown). Nevertheless, Su et al. (2008) demonstrated that U_L52 (β) and U_L13 (γ) mRNA levels were inhibited by digitoxin. The β-actin (Fig. 5D), was used as an internal standard, and the expression level of its mRNA was not affected.

Since the glucoevatromonoside did not inhibit mRNA expression, the next step of HSV replication to be evaluated was the pro-

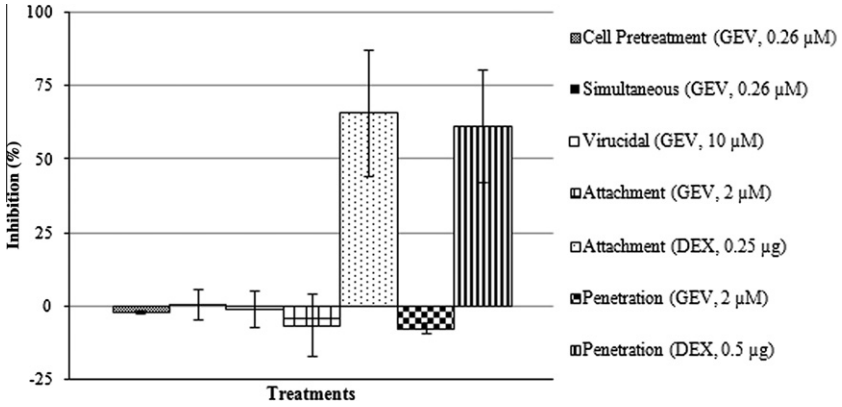

Fig. 3. Effects of glucoevatromonoside (GEV) at different stages of HSV-1 (KOS strain) infection by plaque reduction assay. Dextran sulfate (DEX) was used as a positive control to the attachment and penetration assays. The values represent the mean ± standard deviation of three independent experiments.

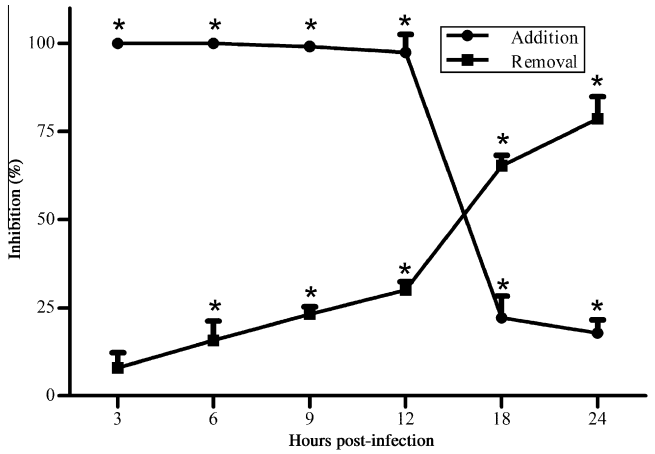

Fig. 4. Effects of delayed addition and early removal of glucoevatromonoside (GEV) on HSV-1 (KOS strain, MOI 0.0004) replication determined by plaque reduction assay. Results are plotted as% of viral inhibition in comparison to the untreated controls. The values represent the mean ± standard deviation of three independent experiments. (∗) Indicate significant statistical differences between the tested sample and virus control ($p < 0.01$). ANOVA/Dunnett tests were carried out as appropriate.

tein synthesis. Likewise glucoevatromonoside, acyclovir, furosemide + potassium chloride (KCl) as well as their combinations were also tested. The relevance of intracellular K^+ to the viral replication has already been reported (Hartley et al., 2006; Hartley et al., 1993; Nagai et al., 1972). Furosemide is a loop diuretic also known as an inhibitor of $Na^+K^+Cl^-$ cotransport activity (NKCC), which prevents the entry of K^+ into the cells (Russel, 2000), and has also shown antiherpes activity (Hartley et al., 2006). Thus, furosemide was investigated in order to check if it was able to reduce the viral protein levels. Likewise, the supplementation of K^+ by adding KCl to the culture medium was also tested to confirm if this ion was important for the viral inhibition caused by glucoevatromonoside. Fig. 6 shows the effects of these treatments on some HSV-1 proteins and on β-actin that is use as an internal standard. As shown in Fig. 6, β-actin cell protein was expressed in all treatments; consequently, the tested drugs were not cytotoxic.

The individual treatments with glucoevatromonoside (lane 5) and acyclovir (lane 3) reduced the levels of all tested viral proteins, when compared to virus control. The glucoevatromonoside completely inhibited all viral protein synthesis, whereas acyclovir was able to inhibit completely only the U_L42 and the gB proteins expression. The treatment with furosemide (lane 8) did not reduce the levels of any viral protein, when compared to viral control indicating that this drug could not affect this stage of HSV-1 replication or that the tested concentration was insufficient to induce protein synthesis inhibition.

When the treatment was performed with glucoevatromonoside + acyclovir (lane 4) or glucoevatromonoside + furosemide (lane 9), a complete inhibition of protein levels was also detected, as well as when glucoevatromonoside (lane 5) was tested alone. Therefore, it was not possible to verify synergistic effects between glucoevatromonoside and acyclovir or glucoevatromonoside and furosemide.

However, the inhibition caused by glucoevatromonoside on HSV protein levels could indicate that the Na^+K^+ATPase has been inhibited for this compound, as it is a cardenolide and its inhibition ability is well established. This inhibition could reduce the K^+ concentration, and the HSV replication will not occur as usual. Thus, ability of the glucoevatromonoside to inhibit Na^+K^+ATPase activity was also investigated to confirm these data, as demonstrated by the results below.

If the glucoevatromonoside inhibit the Na^+K^+ATPase, a reversion of the viral inhibition by cells exposure to culture medium containing an increased amount of K^+ would be expected. Therefore, MEM was modified to contain 27 mM of potassium (5 times more than in the usual MEM = 5.4 mM). When Vero cells were infected and remained in this modified MEM (lane 6), the HSV replication occurred characteristically suggesting that the K^+ supplementation did not cause any alterations to the host cells, and consequently to the virus replication. In the same way, when the infected cells were treated with glucoevatromonoside and remained in the modified medium (lane 7), the viral protein levels were not inhibited. Furthermore, the treatment with glucoevatromonoside at 0.26 µM (IC_{100}) using the plaque reduction assay performed with K^+ supplementation reestablished 22% of the viral replication capacity (data not shown), so these results indicated that the ion K^+ is required to HSV-1 replication confirming the results obtained by Nagai et al. (1972).

Still striving to elucidate the mechanism of antiherpes activity of glucoevatromonoside, its ability to interfere on virus release was investigated through the determination of intracellular and extracellular HSV-1 titers. Digitoxin, an inhibitor of this stage (Su et al., 2008), was used as a positive control. Every glucoevatromonoside tested concentrations significantly ($p < 0.0001$) reduced the extracellular and intracellular virus titers confirming the inhibition of this step of virus replication (data not shown). For example, the lower tested concentration (0.065 µM) reduced the extracellular and intracellular virus titers more than 99.99% (a reduction of 4.8 Log) and 90% (a reduction of 1.6 Log), respectively.

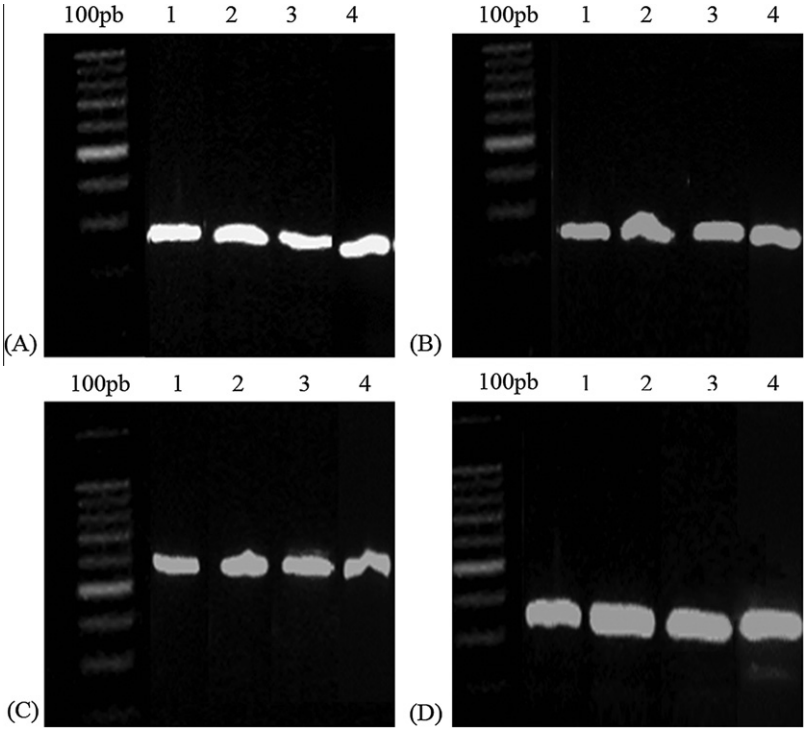

Fig. 5. Effects of post-infection treatment (12 h) with glucoevatromonoside (GEV), acyclovir (ACV), and a combination of both on HSV-1 (KOS strain) expression of the following genes: U_L54 (283 pb) (**A**), U_L52 (259 pb) (**B**), U_L13(600 pb) (**C**) and β-actin (315 pb) (**D**) by RT-PCR. Columns: **1**- treatment with GEV (0.13 μM) plus ACV (5 μM); **2**- Virus control (no treatment); **3**- treatment with ACV (5 μM); **4**- treatment with GEV (0.13 μM).

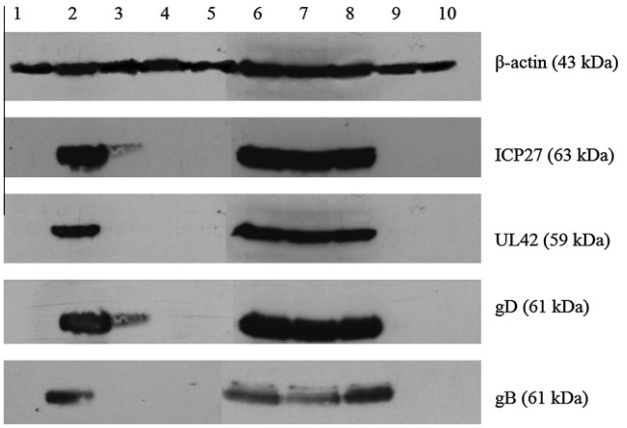

Fig. 6. Effects of glucoevatromonoside (GEV), acyclovir (ACV), furosemide (FUR), potassium chloride (KCl) and their combinations on HSV-1 (KOS strain) protein expression; proteins were extracted and analyzed by Western Blotting. Columns: **1**- cell control; **2**- virus control; **3**- treatment with ACV (5 μM); **4**- treatment with GEV (0.13 μM) + ACV (5 μM); **5**- treatment with GEV (0.13 μM); **6**- Virus control cultured with medium supplemented with KCl; **7**- treatment with GEV cultured with medium supplemented with KCl; **8**- treatment with FUR (500 μM); **9**- treatment with GEV (0.13 μM) + FUR (500 μM); **10**- Cell control cultured with medium supplemented with KCl.

In the same way, the percentages of virus release in the presence of different concentrations of glucoevatromonoside and digitoxin were calculated. These compounds inhibited HSV-1 release in a concentration-dependent manner. However, the glucoevatromonoside at its $IC_{50}/2$ (0.065 μM) was more effective (99.7% of viral release inhibition) than the digitoxin at its $IC_{50}/2$ (0.17 μM. (76% of viral release inhibition).

It is well known that HSV infect epidermis and mucosae cells, and a rapid viral cell-to-cell spread is very important to the establishment of productive primary or recurrent infections in humans (Nyberg et al., 2004). The effect of different concentrations (0.015–0.25 μM) of glucoevatromonoside on HSV-1 cell-to-cell spread was evaluated through a viral plaque size reduction assay, and the results showed a significant ($p < 0.001$) reduction in the areas of formed viral plaques (from 56% to 98%), when compared to those formed in viral control (data not shown). This effect could be a consequence of the inhibition of viral release.

Table 1 demonstrate the highly antiherpetic activity against acyclovir-resistant strain, and this fact suggests that the targets of cardenolides are probably different from those of acyclovir. So, the potential synergistic effects between glucoevatromonoside and acyclovir were tested at different concentrations (Table 2). The results shown CI values <1 indicating synergism between these compounds. In the same way, Hartley et al. (2006) were able to demonstrate synergism between digoxin and furosemide and improvement of anti-adenovirus and anti-cytomegalovirus activity. These findings corroborate the potential antiherpetic activity of glucoevatromonoside and support its use either alone or in combination with acyclovir for the treatment of herpes infections.

Glucoevatromonoside is a natural cardiac glycoside, although its capacity of Na^+K^+ATPase inhibition has not been reported yet. Therefore, an anti-ATPase assay was performed to assess this potential activity. Digoxigenin, digitoxin and digitoxin were used as positive controls (Pullen et al., 2004), and digitoxose was used as a negative control. All tested cardenolides inhibited the Na^+K^+ATPase activity, and Table 3 shows the values of IC_{50}.

The Na^+K^+ATPase inhibition would justify the inhibition of virus release if the energy used by this process was obtained from this system (Nagai et al., 1972). Hence, the inhibition of viral protein synthesis caused by glucoevatromonoside could be explained by the reduction of K^+ concentration into the cells, which is a consequence of the inhibition of this enzyme, since it is known that several enzymes, including those related to viral protein synthesis,

Table 2
Synergistic effects of combination of glucoevatromonoside (GEV) with acyclovir (ACV) on anti-HSV activity.

Compounds combination ratio	Sample concentration (μM)		Mean percentage of inhibition (%)	Experimental CI values	Description (Graded symbols)
	GEV	ACV	GEV + ACV		
$2 \times IC_{50}$	0.260	6.900	100.0	0.779	Moderate synergism (+ +)
$1 \times IC_{50}$	0.130	3.450	92.2	0.710	Moderate synergism (+ +)
$0.5 \times IC_{50}$	0.065	1.725	66.0	0.657	Synergism (+ + +)

CI: Combination Index, a quantitative measure calculated by Calcusyn Software. This index quantifies the interaction between the tested compounds as described by Chou (2006). The CI value from 0.10 to 0.30 means strong synergism, 0.30 to 0.70 means synergism, 0.70 to 0.85 means moderate synergism, and 0.85 to 0.90 means slight synergism. The values represent the mean of two independent experiments.

Table 3
Inhibition of Na^+K^+ATPase by tested cardenolides.

Compound	IC_{50} (μM)
Digitoxigenin	0.34 ± 0.03
Digitoxin	0.34 ± 0.01
Digoxigenin	1.04 ± 0.19
Gluevatromonoside	0.67 ± 0.16
Digitoxose	NA

NA: no activity. IC_{50} = concentration that inhibited 50% of Na^+K^+ATPase activity. The values represent the mean ± standard deviation of two independent experiments.

require K^+ for its activation (Di Cera, 2006). Due to the depletion of K^+, it seems that the inhibition of viral macromolecules by this cardenolide was not complete, because its antiviral activity was reversed when the K^+ concentration was restored. Hence, we believe that the antiviral activity of glucoevatromonoside could be a consequence of its primary action on the cellular electrochemical gradient causing no damage to the host cells (Hartley et al., 1993), and leading to a secondary action, which is the inhibition of viral replication. Accordingly, it acts discretely modifying the distribution and concentration of K^+ intracellular ion, and also affecting the synthesis of essential co-factors in the viral replication.

As it is well known, cardenolides have a long story of therapeutic applications and are frequently associated to systemic toxicity, but recent *in vitro* and *in vivo* toxicological results, and epidemiological data support new roles for such drugs in the treatment of several diseases, including cancer, neurological diseases and some viral infections (Prassas and Diamandis, 2008).

4. Conclusions

Taken together, the obtained results showed that glucoevatromonoside presents inhibitory effects of HSV-1 replication that seems to occur by the inhibition of viral protein synthesis (ICP27, U_L42, gB and gD), the blockage of virus release, and the reduction of viral cell-to-cell spread. This compound also showed anti-Na^+K^+ATPase activity, suggesting that cellular electrochemical gradient alterations might be involved in the mechanism of viral inhibition. These results alongside with those previously obtained by other authors suggest that this group of natural compounds might be promising for future antiviral drug design.

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