JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Virucidal, antiviral and immunomodulatory activities of β-escin and *Aesculus hippocastanum* extract

Flavia M. Michelini[a,b], Laura E. Alché[a,b] and Carlos A. Bueno[a,b]

[a]Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Biológica, Laboratorio de Virología, and [b]CONICET – Universidad de Buenos Aires, Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN), Buenos Aires, Argentina

Keywords
Aesculus hippocastanum; antiviral; immunomodulatory; medicinal plants; β-escin

Correspondence
Carlos A. Bueno, CONICET – Universidad de Buenos Aires, Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN), C-1428GBA Buenos Aires, Argentina.
Email: cbueno@qb.fcen.uba.ar

Received April 20, 2018
Accepted August 4, 2018

doi: 10.1111/jphp.13002

Abstract

Objectives β-Escin, one of the constituents of *Aesculus hippocastanum* L. (Hippocastanaceae) seed extract (AH), inhibits NF-κB activation, which plays an important role in HSV-1 replication. The aim was to examine the antiherpetic activity of β-escin and AH, as well as their effect on the activation of NF-κB and AP-1 and cytokine secretion in epithelial cells and macrophages.

Methods Cell viability was evaluated using MTT assay, and antiviral and virucidal activity was determined by plaque assay. The effect on NF-κB and AP-1 signalling pathways activation was determined by a luciferase reporter assay, and cytokine production was measured by ELISA.

Key findings β-Escin and AH had virucidal and anti-HSV-1 activities, and the antiviral activity was discovered for other enveloped viruses (VSV and Dengue). Moreover, β-escin and AH significantly reduced NF-κB and AP-1 activation and cytokine production in macrophages stimulated with HSV-1 and TLRs ligands. However, an enhanced activation of these pathways and an increase in the levels of pro-inflammatory cytokines in β-escin and AH-treated HSV-1-infected epithelial cells were found.

Conclusions This study demonstrates virucidal and broad-spectrum antiviral activities for β escin and AH. Besides, β-escin and AH modulate cytokine production depending on the stimuli (viral or non-viral) and the cell type under study.

Introduction

HSV-1 is a leading cause of corneal disease and blindness in humans. HSV-1-induced ocular disease occurs as a result of a primary infection in the corneal epithelium, and then, cells like macrophages intervene in clearing the virus from the infected eye and in the development of the immunologically driven herpetic stromal keratitis (HSK).[1–3] Besides, conjunctival cells are also involved in amplifying the inflammatory processes in the eye.[4] It has been reported that HSV-1 induces a strong activation of NF-κB and AP-1 to promote viral replication, prevents virus-induced apoptosis and mediates the immune response to the invading pathogen.[5]

The current standard of care for HSK includes antivirals, such as nucleoside analogues, to inactivate and prevent further viral replication, and corticosteroids to combat the immunopathological component of stromal disease.[6] However, corticosteroids have adverse side effects, and there is concern that they prolong viral shedding.[6] In most HSV-related diseases, the prevalence rates of acyclovir (ACV) resistance are much higher among immunocompromised patients. However, there have been several reports of ACV^R strains causing HSK in immunocompetent patients.[7,8] Thus, considering the rise of the resistant viruses against the current antivirals and the adverse effect of corticoids,[6] there is a need to search for new potential anti-HSV agents that act with different mechanisms. In this sense, the finding of novel compounds that inhibit both viral multiplication and the immunopathology triggered by the virus is an interesting approach that is being considered.[9,10]

Medicinal plants have been used to treat viral infections for decades. In fact, in the last years, plant extracts and herbal compounds have been investigated for their anti-HSV and immune regulatory properties.[11,12]

β-Escin is one of the main bioactive constituents of *Aesculus hippocastanum* L. (Hippocastanaceae) seed extract (AH), commonly known as horse chestnut. Both β-escin and the seed extract are well-reported for beneficial role in clinical therapy because of its anti-oedematous, anti-inflammatory and antioxidative effects.[13,14] Regarding the therapeutic significance and popularity of β-escin, in the United States and Europe remains one of the best-selling herbal products.[15] With respect to its mechanism of anti-inflammatory action, it has been reported that β-escin inhibits NF-κB activation in LPS-treated mice and in several tumour cell types, macrophages and endothelial cells *in vitro*.[15–18]

Considering that β-escin inhibits NF-κB activation in different conditions, which plays an important role in HSV-1 replication, the aim of this study was to examine the antiherpetic activity of β-escin and AH in ocular epithelial cells, the target of HSV-1 multiplication in HSK, as well as their effect on the activation of NF-κB and AP-1 in infected cells. Besides, we also evaluated the modulating effect of β-escin and AH on the production of different cytokines in HSV-1-infected ocular epithelial cells and macrophages.

Material and Methods

Herbal extract and chemical compound

β-Escin and AH used in the experiments were obtained as a lyophilized powder in a 10-mg vial kindly provided by the pharmaceutical company Spedrog Caillon S.A., Buenos Aires, Argentina. β-Escin was originally obtained from Indena S.p.A., Milan, Italy (Batch N° 31259/M2), with a purity of 98.3%, as stated by the manufacturer. The identification and purity were also analysed by Spedrog Callion S.A. by HPLC and comply the specifications of the Argentinian Pharmacopoeia. AH was originally purchased from Martin Bauer Group (Finzelberg GmbH & Co), Andernach, Germany (Batch N° 13013823). This herbal extract was produced according to the German Pharmacopoeia (Deutsches Arzneibuch – DAB). Moreover, the identity (TLC) and purity (HPLC) were also analysed by Spedrog Callion S.A. and comply the specifications of the Argentinian Pharmacopoeia.

The β-escin and AH used in this report were approved by Food, Drug and Medical technology Administration (ANMAT) in Argentina to be administered in humans.

Reagents

LPS (TLR4 ligand) from Escherichia coli serotype 055:B5 was obtained from Sigma. Pam2CSK4 (TLR2/TLR6 ligand), poly (I:C) (TLR 3 ligand), imiquimod (TLR7 ligand) and ODN2395 (TLR9 ligand) were purchased from InvivoGen. Acyclovir (ACV) and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich.

Cells and viruses

Human corneal-limbal epithelial (HCLE) cells were kindly provided by Dr Ilene K. Gipson and Dr. Pablo Argüeso (The Schepens Eye Research Institute, Harvard Medical School, Boston, USA) and grown in GIBCO keratinocyte serum-free medium. The human IOBA-NHC cell line (normal human conjunctival epithelial cell line) and the human A549 cell line (human lung carcinoma cell line) were grown in DMEM/F12 supplemented with 10% inactivated foetal bovine serum (FBS) (DMEM/F12, 10%), Murine macrophage cell line J774A.1 was kindly provided by Dr. Osvaldo Zabal (INTA – Castelar, Buenos Aires) and grown in RPMI 1640 medium supplemented with 10% FBS. Vero cells were grown in MEM supplemented with 10% FBS. The KOS strain was chosen as HSV-1 wild-type reference and B2006 and Field are HSV-1 thymidine kinase-deficient (TK−) strains of HSV-1. All HSV-1 strains, VSV, adenovirus 5 and dengue virus type 2 were used and propagated at low multiplicity of infection (moi).

Cytotoxicity assay

Cell viability was determined using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) according to the manufacturer's instructions. The cytotoxic concentration 50 (CC_{50}) was calculated as the concentration of compounds required to reduce cell viability by 50% relative to untreated cells.

Antiviral activity

Cells grown in 96-well plates were infected (moi=1). After 1-h adsorption at 37°C, the inoculum was removed and medium containing the compounds was added, in triplicate. The plates were incubated at 37°C until 24 h p.i. After cell disruption by three cycles of freezing and thawing, supernatants were harvested and pooled. Virus yields were titrated by plaque assay, and the effective concentration 50 (EC_{50}) was calculated as the concentration of compounds required to reduce viral yields by 50% relative to the untreated virus control.

Virucidal effect

HSV-1 KOS (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 Vero cells.

Time-of-addition and time-of-removal assays

For pre-infection assays, cells were treated with the compound during 2 h at 37°C, washed with PBS and then infected with HSV-1 (moi=1). For co-infection, cells were simultaneously treated with HSV-1 and the compound of interest. After 1-h adsorption at 37°C, the virus–drug mixture was removed and compound-free medium was added. For postinfection (p.i.) assays, cells were infected with HSV-1 for 1 h at 37°C and then treated with the tested compound at 0 and 7 h p.i. For time-of-removal assay, cells were infected with HSV-1, and after 1 h of incubation at 37°C, the inoculum was discarded and the compound was added. Drug was removed at 7 h p.i., and then, cells were washed with PBS and compound-free medium was added. A control culture that was infected but not treated (CV) was simultaneously performed. Total virus yields were always determined by plaque assay at 24 h p.i.

Extracellular and intracellular virus yields

Cells were infected with HSV-1 KOS at moi of 10 PFU/cell for 1 h at 37°C and then treated with the compound of interest at 0 h p.i. After 15 h of incubation, supernatants were harvested. Fresh medium was added and after cell disruption by three cycles of freezing and thawing. Supernatants were then pooled, and intracellular virus was harvested. Extracellular and intracellular virus yields were determined by plaque assay.

Cytokine determination

Mouse TNF-α and IL-6 were quantified by commercial ELISA sets (BD OptEIATM, Becton Dickinson) according to the manufacturer's instructions.

Transfections

Transfection assays with Lipofectamine 2000 reagent (Invitrogen) were performed according to the manufacturer's instructions. The NF-κB-LUC reporter vector and RSV-β-gal plasmid were kindly provided by Dr. Susana Silberstein (Universidad de Buenos Aires, Argentina). The Ap-1-LUC reporter vector was kindly provided by Prof. Dr. Thomas F. Schulz (Medizinische Hochschule Hannover, Germany).

Statistical analysis

CC_{50} and EC_{50} were calculated from dose–response curves using the software GraphPad Prism 4.0. All assays were carried out in triplicate. Statistically significant differences were evaluated by one-way ANOVA followed by a Tukey's multiple comparison test. P-value <0.05 was considered significant.

Results

Antiviral activity of β-escin and AH against HSV-1 in HCLE and NHC cells

HSV-1 establishes infection in the epithelial layer of the cornea. Then, it spreads to conjunctival cells leading to viral dissemination in the eye.[1–3] Thus, we decided to investigate the anti-HSV-1 effect of β-escin and AH in human corneal cells (HCLE) and human conjunctival cells (NHC), where HSV-1 multiplies reaching similar viral titres.[19–21] When HCLE and NHC cells were infected with HSV-1 KOS and then treated with different concentrations of β-escin and AH, a dose-dependent inhibition of viral yields was observed (Table 1). With respect to β-escin, an EC_{50} value of 1.5 and 2.4 μg/ml was calculated in HCLE and NHC cells, respectively, whereas AH exhibited an EC_{50} value of 9.4 and 10 μg/ml. Moreover, the positive control ACV also exhibited an antiviral effect against HSV-1 KOS in both cell lines. Besides, β-escin and AH have no cytotoxic effect at all concentrations tested (100–0.1 μg/ml). The next step was to evaluate the ability of β-escin and AH to inhibit the propagation of HSV-1 ACV-resistant strains, B2006 and field in HCLE and NHC cells, where these strains multiply reaching similar viral titres. First, we verified that both strains are resistant to ACV in these cell lines and conditions. As expected, the increase in EC_{50} values was high enough to be considered resistant according to the commonly accepted criteria.[22] Noteworthy, β-escin and AH inhibited the propagation of B2006 and field strains in HCLE and NHC cells with similar EC_{50} values compared to the values obtained against KOS strain (Table 1).

Virucidal activity of β-escin and AH against HSV-1

Virucidal assays were performed to rule out the possibility that the antiviral action against HSV-1 was caused by direct inactivation of the released virus. Suspensions of HSV-1 virus were incubated with different concentrations of β-escin, AH and the positive control SDS[23] for 30, 60 and 120 min at 37°C, followed by titration of the remaining infectivity in Vero cells. Results showed an inactivating effect for β-escin, AH and SDS which was time- and concentration-dependent (Table 2). Besides, the concentrations of β-escin and AH needed to inactivate HSV particles during 120 min (EC_{50} of 15.9 and 58.5 μg/ml for β-escin and AH, respectively) were higher than those observed for antiviral activity.

Table 1 Antiviral activity of β-escin and AH against wild-type and ACV-resistant HSV-1 strains. EC_{50} was calculated by nonlinear regression

	EC_{50} μg/ml)								
	HCLE			NHC			Vero		
Compound	KOS	Field	B2006	KOS	Field	B2006	KOS	Field	B2006
β-Escin	1.5 ± 0.2	2.7 ± 0.3	2.2 ± 0.7	2.4 ± 0.8	3.5 ± 2.2	2.5 ± 0.5	1.9 ± 0.7	2.4 ± 0.6	2.3 ± 0.4
AH	9.4 ± 0.1	9.9 ± 0.2	10.1 ± 0.6	10 ± 1.2	11.1 ± 1.4	10.2 ± 1.2	9.8 ± 1.3	10.5 ± 3.4	10.3 ± 0.9
ACV	0.9 ± 0.2	37.8 ± 0.4	38.1 ± 1.8	0.7 ± 0.3	36.7 ± 2.2	38.9 ± 4.2	1.7 ± 0.7	62.8 ± 5.5	61.4 ± 6.1

EC_{50}, effective concentration 50; ACV, acyclovir; AH, *Aesculus hippocastanum* L. seed extract.

Table 2 Virucidal activity of β-escin and AH against HSV-1 KOS. EC_{50} was calculated by nonlinear regression

	EC_{50}		
Compound	30 min	60 min	120 min
β-Escin	47.2 μg/ml ± 5.3	25.3 μg/ml ± 3.5	15.9 μg/ml ± 1.3
AH	85.1 μg/ml ± 8.8	65.9 μg/ml ± 8.4	58.5 μg/ml ± 6.9
SDS	$1 \times 10^{-4}\% \pm 1 \times 10^{-5}$	$1 \times 10^{-4}\% \pm 1 \times 10^{-5}$	$2.5 \times 10^{-4}\% \pm 1 \times 10^{-5}$

EC_{50}, effective concentration 50; SDS, sodium dodecyl sulfate; AH, *Aesculus hippocastanum* L. seed extract.

Influence of the duration of treatment with β-escin and AH on HSV- 1 infectivity

To further characterize the inhibitory action of β-escin and AH, a time-of-addition experiment was performed. HCLE and NHC cells were exposed to β-escin and AH pre-infection, during or postinfection with HSV-1 KOS and virus yields were determined at 24 h p.i.

To study solely the ability of β-escin and AH to interfere with some intracellular events during the replication cycle of HSV-1 independently of their virucidal properties, we used concentrations of β-escin and AH that did not inactivate viral particles during 120 min of exposition but prevented viral replication (5 μg/ml of β-escin and 20 μg/ml of AH). When β-escin and AH were added before or during HSV-1 inoculation (moi= 1), no significant inhibition of viral multiplication was detected. However, HSV-1 virus yields significantly decreased when β-escin and AH were added after infection (Figure 1a). Then, we decided to make a time-of-addition/time-of-removal assay at 7 h p.i. to evaluate whether compounds exert their antiherpetic effect in the later stages of the virus replication cycle. Results showed that β-escin and AH were able to inhibit infectious particle formation even when both were added at 7 h p.i. Furthermore, when β-escin and AH were removed at 7 h p.i., there was no inhibition of viral replication (Figure 1b), suggesting that β-escin and AH affected the later stages of the viral cycle. To broaden this initial result, we determined the amount of cell-associated infectious particles as well as virus yield in the supernatants of treated cells. Hence, Vero cells were infected with HSV-1 KOS (moi = 10) for 1 h at 37°C and then treated or not with β-escin (5 μg/ml) and AH (20 μg/ml). After 15 h of incubation, extracellular and intracellular virus yields were determined by plaque assay. The formation of intracellular mature virus and extracellular enveloped virus was reduced to the same level in β-escin and AH-treated cells with respect to CV (Figure 1c). These data demonstrated that, besides the direct inactivation on viral particles, β-escin and AH inhibited viral replication by affecting a later stage of the viral cycle, which would not be related to the inhibition of virus egress.

Antiviral activity of β-escin and AH against adenovirus, Dengue virus type 2 and VSV

It has been reported that β-escin inhibits Sars-CoV replication.[24] Thus, to investigate whether we were dealing with broad-spectrum antivirals, we evaluated the antiviral activity of β-escin and AH against an enveloped negative-stranded RNA virus (VSV), an enveloped positive-stranded RNA virus (Dengue virus type 2), and a non-enveloped DNA virus (adenovirus 5). When Vero cells were infected with VSV (moi=1) and dengue virus type 2 (moi=1) during 24 h, and then treated with 10 μg/ml of β-escin and 40 μg/ml of AH, the multiplication of both virus was significantly inhibited. In contrast, when A549 cells were infected with adenovirus 5 (moi=1) and incubated with the same concentrations of β-escin and AH, adenovirus plaque formation remained unaffected by β-escin and AH (Figure 2). Thus, β-escin and AH would exert their antiviral effect only on enveloped viruses.

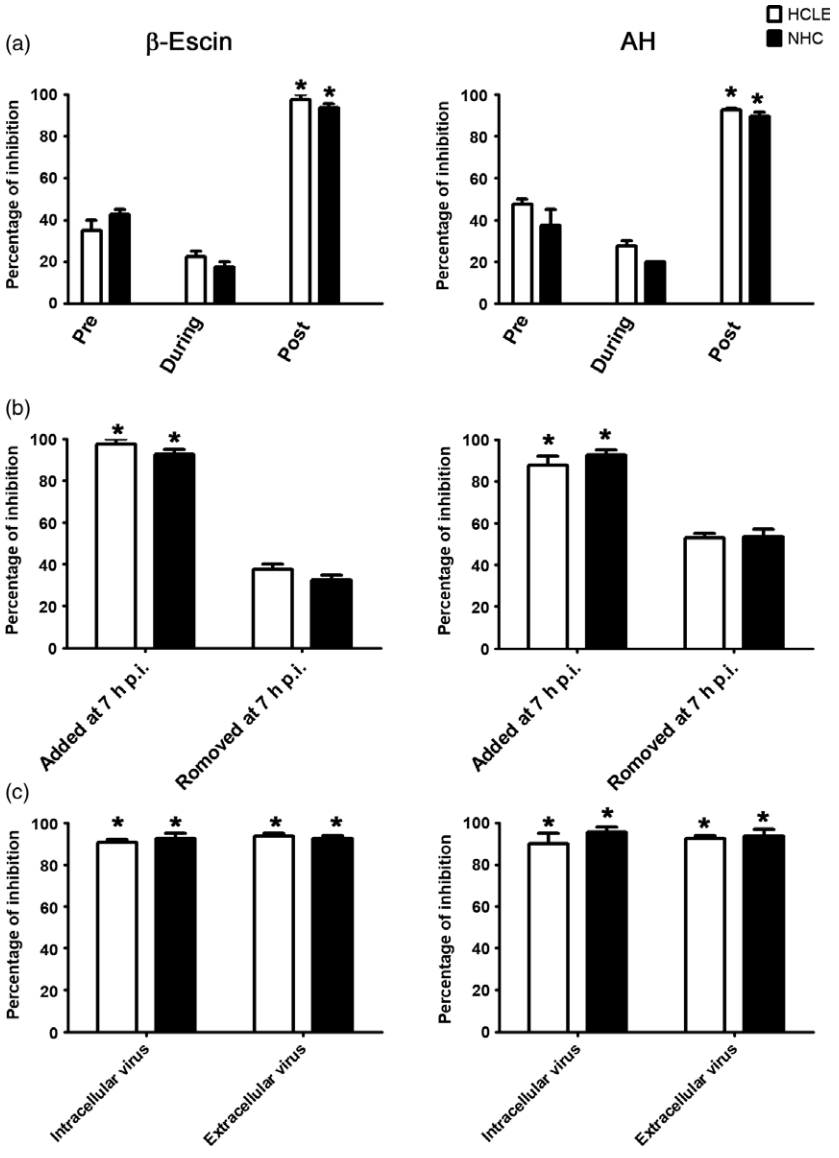

Figure 1 Influence of time of treatment with β-escin and AH on HSV-1 infectivity. (a) HCLE and NHC cells were exposed or not to β-escin (5 μg/ml) and AH (20 μg/ml) before, during and after infection with HSV-1 KOS. (b) For time-of-addition/time-of-removal assays, infected cells were treated with β-escin (5 μg/ml) and AH (20 μg/ml) immediately after adsorption and removed at 7 h p.i. or treated with β-escin (5 μg/ml) and AH (20 μg/ml) at 7 h p.i. up to 24 h p.i. Total virus yields were always determined by plaque assay in Vero cells at 24 h p.i. (c) Vero cells were infected with HSV-1 strain KOS (moi = 10) for 1 h at 37°C and then treated or not with β-escin (5 μg/ml) and AH (20 μg/ml). After 15 h of incubation, extracellular and intracellular virus yields were determined by plaque assay. *Significantly different from CV (*P*-value <0.05).

NF-κB and AP-1 activation is modulated by β-escin and AH after HSV-1 infection in epithelial cells

NF-κB and AP-1 activation following HSV infection is necessary for viral replication.[5] Besides, the downregulation of NF-κB activation by β-escin has been described in different conditions.[15–18] Therefore, we decided to investigate whether β-escin and AH could exert their antiviral action through the inhibition of HSV-1-induced NF-κB and AP-1 activation. Thus, NHC and HCLE cells were transfected with a NF-κB-LUC and AP-1 LUC reporter vector and β-galactosidase control plasmid and, 24 h later, infected with HSV-1 (moi=1) and treated with β-escin (5 μg/ml) and AH (20 μg/ml) during 24 h. We found that β-escin and AH did not induce NF-κB and AP-1 activation in uninfected cells. We verified that HSV-1 induced NF-κB and AP-1 activation, and, surprisingly, the activation induced

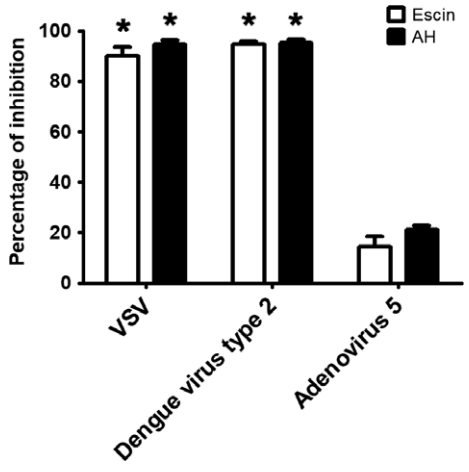

Figure 2 Antiviral activity of β-escin and AH against VSV, dengue virus type 2 and adenovirus 5. Vero cells were infected with VSV (moi=1) and dengue virus type 2 (moi=1), and A549 cells were infected with adenovirus 5 and then treated with 10 μg/ml of β-escin and 40 μg/ml of AH during 24 h. Total virus yields were always determined by plaque assay in Vero cells. *Significantly different from CV (*P*-value <0.05).

by HSV-1 was strongly enhanced by β-escin and AH (Figure 3a and 3b).

Considering that NF-κB and AP-1 have been shown to upregulate the expression of antiviral type I interferons in other systems,[5] and that β-escin and AH upregulated NF-κB and AP-1 activation in HSV-1 epithelial-infected cells, we studied whether the antiviral activity of β-escin and AH was related to interferon. When type I interferon-deficient Vero cells were infected with HSV-1 KOS, B2006 and field strains, and then treated with both compounds, β-escin and AH exhibited a similar antiviral activity, suggesting that it was independent of type I interferon effect (Table 1).

Modulation of cytokine production by β-escin and AH in infected epithelial cells

It has been already reported that HSV-1 is able to activate NF-κB and AP-1 concomitant with the expression of pro-inflammatory cytokines.[5] Besides, HCLE cells fail to produce TNF-α, whereas NHC cells are weak TNF-α producer.[20,25] Thus, to assess the effect of β-escin and AH on cytokine production, we measured IL-6 secretion. Supernatants harvested from HCLE and NHC cells infected with HSV-1 (moi=1) treated or not with β-escin (5 μg/ml) and AH (20 μg/ml) were used to quantify IL-6 by ELISA.

No significant differences between IL-6 release from untreated and β-escin and AH-treated cells were detected. As expected, IL-6 production was higher in HSV-1-infected cells than in uninfected cells.[20,25,26] Nevertheless, β-escin and AH significantly increased IL-6 production when added to HSV-1-infected cells (Figure 3c). In summary, β-escin and AH enhanced the activation of the NF-κB and AP-1 signalling pathways, and consequently the release of IL-6, in HSV-1-infected epithelial cells of ocular origin.

NF-κB and AP-1 activation and cytokine production are inhibited by β-escin and AH after TLRs ligand stimulation in epithelial cells

Toll-like receptors (TLRs) are innate immune sensors implicated in the control of infection since they trigger several cellular responses, including NF-κB and AP-1 activation and cytokine secretion. Several TLRs, especially TLRs 2, 3, 4, 7 and 9, are involved in the early recognition of HSV components.[27,28] To investigate whether the modulation of the NF-κB and AP-1 signalling pathway induced by β-escin and AH could be triggered solely by TLRs interaction, we analysed the effect of β-escin and AH in ocular epithelial cells stimulated by TLRs ligands.

NHC and HCLE cells were transfected with the NF-κB-LUC reporter vector and, 24 h later, stimulated with different TLRs ligands and treated with β-escin (5 μg/ml) and AH (20 μg/ml) during 8 h. Only TLR2/6 and TLR3 ligands induced a strong NF-κB and AP-1 activation in NHC and HCLE cells. Interestingly, we observed that β-escin and AH inhibited NF-κB and AP-1 activation induced by these TLRs ligands (Figure 4a and 4b).

Moreover, when NHC and HCLE cells were stimulated with TLR2/6 and TLR3 ligands and treated with β-escin and AH for 8 h, the two ligands were able to induce IL-6 in HCLE and NHC cells, while it was significantly inhibited by β-escin and AH (Figure 4c).

NF-κB and AP-1 activation is inhibited by β-escin and AH after HSV-1 infection and TLRs ligand stimulation in macrophages

Macrophages play a crucial role as one of the dominant cell infiltrates in the infected cornea.[3] Since β-escin inhibits NF-κB activation in macrophages stimulated with LPS,[16] and that we observed that β-escin and AH modulated NF-κB and AP-1 activation in ocular epithelial cells, we evaluated the effect of β-escin and AH in J774A.1 cells infected with HSV-1.

First, we investigated the antiviral action of β-escin and AH in J774A.1 cells, finding that they inhibited HSV-1 multiplication with an EC_{50} value of 5.4 and 16.1 μg/ml, respectively, without cytotoxic effect.

Then, J774A.1 cells were transfected with the NF-κB-LUC reporter vector and, 24 h later, infected with HSV-1 and treated with β-escin (5 μg/ml) and AH (20 μg/ml) for 8 h. We found that β-escin and AH did not induce NF-κB and AP-1 activation in uninfected cells. We verified that

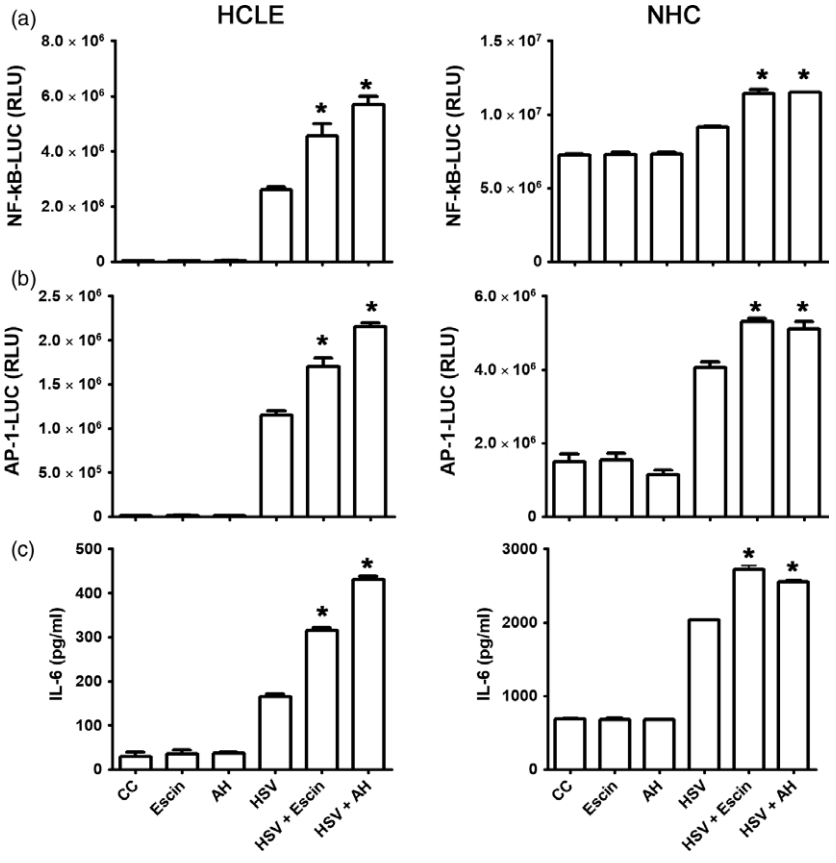

Figure 3 Effect of β-escin and AH on NF-κB and AP-1 activation and cytokine production in HSV-1-infected epithelial cells. HCLE and NHC cells were infected with HSV-1 (moi=1) and treated or not with β-escin (5 μg/ml) and AH (20 μg/ml) during 24 h. (a and b) Luciferase activity was measured in cell extracts, and each value was normalized to β-galactosidase activity in relative luciferase units (RLUs). (c) IL-6 was determined by ELISA. CC: cell control (unstimulated cells); *Significantly different from HSV-1-infected cells (*P*-value <0.05).

HSV-1 induced a strong NF-κB and AP-1 activation as previously reported,[9] and that, interestingly, both pathways were strongly inhibited by β-escin and AH in HSV-1 in J774A.1 HSV-1-infected cells (Figure 5a). We next determined whether the NF-κB and AP-1 pathways were affected by β-escin (5 μg/ml) and AH (20 μg/ml) in macrophages stimulated with different TLRs ligands during 8 h. Stimulation with TLRs ligands activated NF-κB and AP-1 pathways, whereas β-escin and AH prevented TLRs induced NF-κB and AP-1 activation (Figure 6a).

β-escin and AH abrogates cytokine secretion from HSV-1-infected and TLRs stimulated J774A.1 cells

Next, we investigated whether β-escin and AH affected IL-6 and TNF-α secretion in HSV-1 and TLRs stimulated macrophages.

J774A.1 cells were infected with HSV-1 during 24 h or stimulated with different TLRs ligands for 8 h and were treated or not with β-escin (5 μg/ml) and AH (20 μg/ml). No significant differences between IL-6 and TNF-α release from untreated and treated uninfected cells were detected (Figure 5b). IL-6 and TNF-α production in TLRs ligands stimulated or HSV-1-infected J774A.1 cells was significantly higher than in unstimulated cells. When μ-escin and AH were added to HSV-1 or TLRs ligand stimulated cells, secretion of IL-6 and TNF-α was significantly reduced (Figures 5b and 6b).

In conclusion, β-escin and AH decreased TNF-α and IL-6 secretion in J774A.1 cells infected with HSV-1 or stimulated with TLRs ligands (Figures 5b and 6b), which, in turn, could be associated with the inhibition of the NF-κB and AP-1 signalling pathways (Figures 5a and 6a) in J774A.1 cells infected with HSV-1 or stimulated with TLRs ligands.

Discussion

Although ethnopharmacological research provides evidence for the broad use of β-escin and AH to treat numerous diverse

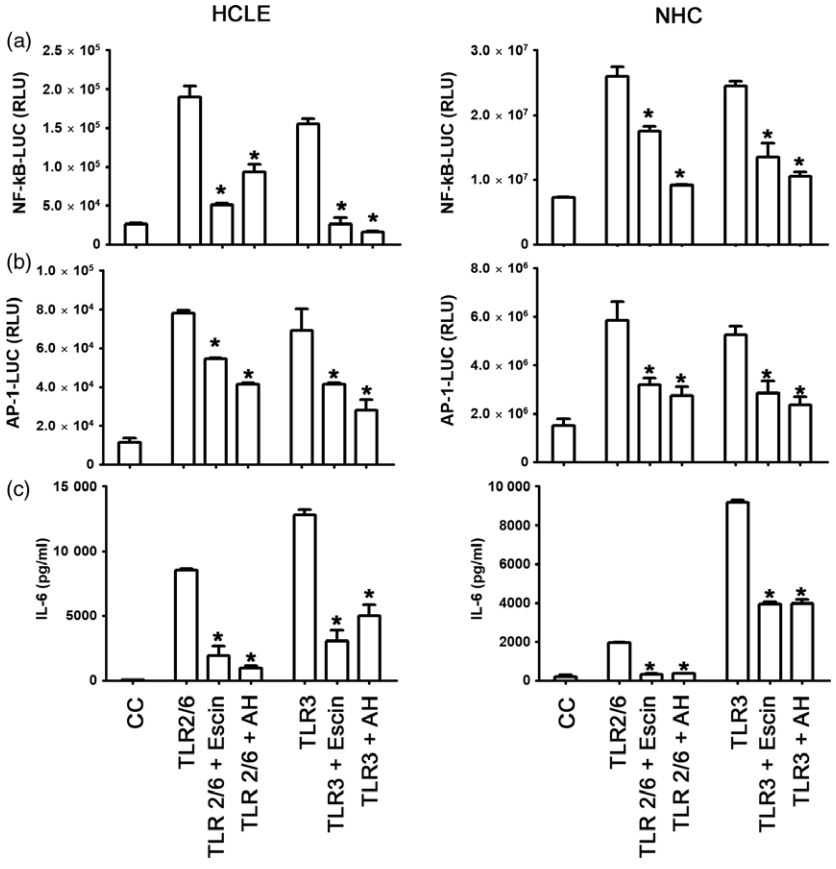

Figure 4 Effect of β-escin and AH on NF-κB and AP-1 activation and cytokine production in TLRs stimulated epithelial cells. HCLE and NHC cells were stimulated with TLR2/6 (100 ng/ml) and TLR3 (10 μg/ml) ligands and treated or not with β-escin (5 μg/ml) and AH (20 μg/ml) for 8 h. (a and b) Luciferase activity was measured in cell extracts, and each value was normalized to β-galactosidase activity in relative luciferase units (RLUs). (c) IL-6 was determined by ELISA. CC: cell control (unstimulated cells); *Significantly different from TLRs stimulated cells (*P*-value <0.05).

disorders, their current use is restricted mainly to venotonic and venoprotective indications due to their anti-inflammatory and anti-oedematous properties. Indeed, randomized controlled trials confirmed the effectiveness of β-escin and AH for the treatment of chronic venous insufficiency.[13,14]

In the present study, we have shown for the first time that β-escin and AH have virucidal and antiviral activities, as well as pro-inflammatory effects unknown till now.

No previous reports account for β-escin and AH biological properties against viruses, except for the inhibitory activity of escin against the enveloped Sars-CoV detected by means of a screening assay.[24] We have found that β-escin and AH exerted a virucidal activity at concentrations higher than those needed to restrain HSV-1 replication, which broaden their biological action against viruses (Tables 1 and 2). Particularly, β-escin and AH anti-HSV-1 effect was observed irrespective of the cellular substrate, since both inhibited viral multiplication in epithelial cells from ocular origin, Vero cells and macrophages, with similar values of EC_{50} (Table 1).

The requirement of NF-κB and AP-1 signalling pathways for HSV-1 replication has been already demonstrated.[5] Even though β-escin and AH have shown anti-HSV-1 activity in both macrophages and ocular epithelial cells (Table 1), we observed that NF-κB and AP-1 pathways were inhibited in β-escin and AH-treated HSV-1-infected macrophages, whereas an enhanced activation of these pathways in treated HSV-1-infected ocular epithelial cells was found (Figures 3a, 3b and 5a). Therefore, the anti-HSV-1 activity of β-escin and AH would not be ascribed to the modulation of these signalling cascades in ocular epithelial cells.

Besides, the antiviral activity was discovered for other enveloped viruses in addition to HSV-1, such as VSV and dengue, but neither β-escin nor AH was able to inhibit a non-enveloped virus replication, such as adenovirus (Figure 2). Therefore, considering the well-known ability of β-escin to interfere with cell membranes,[29] and that cholesterol homeostasis is critical for enveloped viruses replication,[30] we speculate that the virucidal as well as the

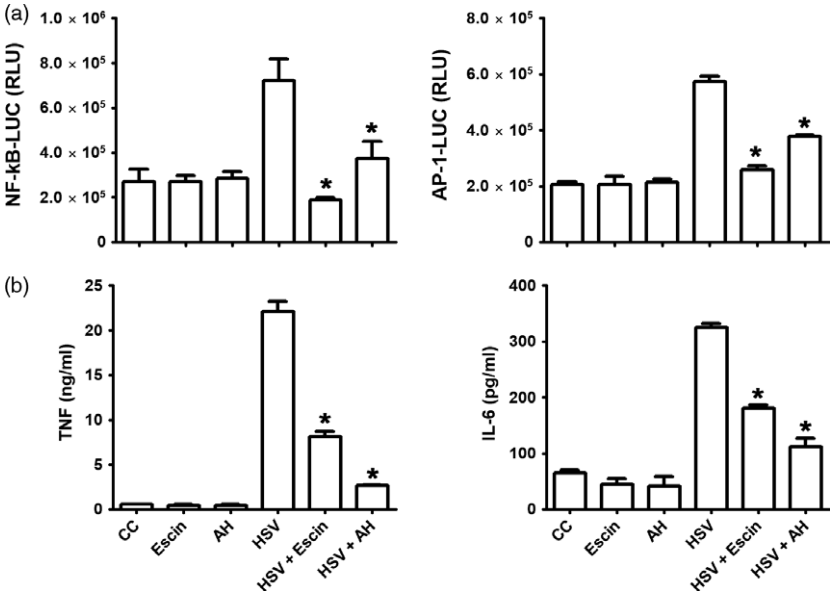

Figure 5 Effect of β-escin and AH on NF-κB and AP-1 activation and cytokine production in HSV-1-infected macrophages. J774A.1 cells were infected with HSV-1 (moi=1) and treated or not with β-escin (5 μg/ml) and AH (20 μg/ml) during 24 h. (a) Luciferase activity was measured in cell extracts, and each value was normalized to β-galactosidase activity in relative luciferase units (RLUs). (b) IL-6 and TNF-α were determined by ELISA. CC: cell control (unstimulated cells); *Significantly different from HSV-infected cells (*P*-value <0.05).

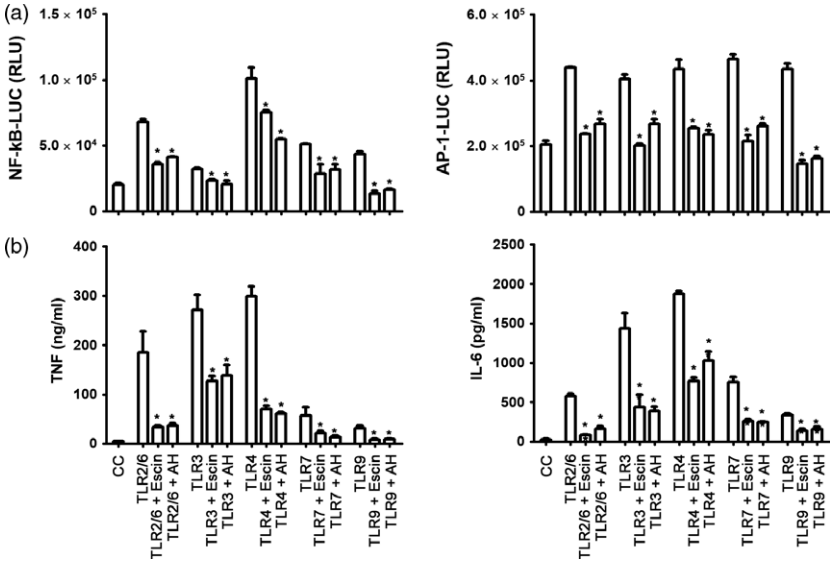

Figure 6 Effect of β-escin and AH on NF-κB and AP-1 activation and cytokine production in TLRs stimulated macrophages. J774A.1 cells were stimulated with TLR2/6 (100 ng/ml), TLR3 (10 μg/ml), TLR4 (100 ng/ml), TLR7 (10 μg/ml) and TLR9 (5 μg/ml) ligands and treated or not with β-escin (5 μg/ml) and AH (20 μg/ml) for 8 h. (a) Luciferase activity was measured in cell extracts, and each value was normalized to β-galactosidase activity in relative luciferase units (RLUs). (b) IL-6 and TNF-α were determined by ELISA. CC: cell control (unstimulated cells); *Significantly different from TLRs stimulated cells (*P*-value <0.05).

antiviral activity of β-escin and AH could be at least partially attributed to their perturbation of membrane integrity. In fact, with respect to the antiviral activity, we observed that it was related to a late event during viral multiplication, distinct from HSV-1 egress (Figure 1). Thus, β-escin and AH interference with membranes could induce a disruption of the viral envelope inside the host cell, finally affecting its acquisition by the viral particle.

β-Escin-induced perturbation in cholesterol homeostasis is considered the triggering event in a cascade of cellular responses, leading to many of its pharmacological properties. It is proposed that the anti-inflammatory mechanism of β-escin involves disturbances in cholesterol homeostasis followed by decreased NF-κB activation in endothelial cells.[15] Moreover, it has been reported that β-escin inhibits NF-κB signalling pathway in macrophages stimulated with LPS, a TLR4 ligand.[16]

Here, we found that β-escin and AH not only inhibited AP-1 signalling in macrophages stimulated with LPS, but also significantly reduced NF-κB and AP-1 activation in macrophages stimulated with HSV-1 and other TLRs ligands (Figures 5a and 6a). Thus, β-escin and AH blocked the activation of NF-κB and AP-1 pathways in macrophages stimulated with viral and non-viral stimuli, suggesting an immunosuppressive action over inflammatory cells.

Importantly, the modulation of the NF-κB and AP-1 pathways induced by β-escin and AH in both epithelial cells and macrophages was in accordance with the pattern of cytokine secretion obtained (Figures 3, 4, 5 and 6).

To the best of our knowledge, for the first time, it is reported that β-escin and AH might reduce or promote cytokine release by inhibiting or activating NF-κB and AP-1 pathways depending on the stimuli and the cell type under study. In addition, β-escin and AH did not modulate the NF-κB pathway specifically, because they also modulate the AP-1 signalling pathway. In fact, previously it was reported that β-escin inhibits the activation of P38 and ERK in endothelial cells, and one nuclear target of these MAP kinase signalling pathways is the transcription factor AP-1.[31,32] After HSV-1 infection, the innate immune system reacts by activating antiviral effectors, including type I IFNs, natural killer cells, pro-inflammatory cytokines and chemokines to control the infection. In turn, natural products can stimulate immune responses to inhibit HSV infection.[11,12]

Even though the antiviral activity of β-escin and AH do not seem to be mediated by IFN, their pro-inflammatory effect in ocular epithelial-infected cells could be helpful to elicit the innate immune response to eliminate HSV-1 from the site of infection.[1–3,27,28] Thus, the balance between the antiviral activity of β-escin and AH against wild-type and ACV-resistant HSV populations observed in corneal and conjunctival cells, and the increase in pro-inflammatory cytokine levels, might solve the progress of HSV infection.

On the other hand, angiogenesis represents a major step in HSK pathogenesis.[2,26] Taken into account that β-escin and AH display anti-angiogenic activity *in vitro* and *in vivo* conditions,[15] together with their virucidal, antiviral and immunomodulatory activities reported here, we believe that β-escin and AH would become a promising therapy to control the progress of HSK.

Further *in vivo* studies will be needed to determine the potential therapeutic effects and the clinical relevance of escin and AH to treat HSV infection-associated diseases and other viral diseases.

Conclusion

This study demonstrates a novel virucidal and broad-spectrum antiviral activities for β-escin and AH, which could be connected with the well-known ability of β-escin and AH to interfere with cell membranes and cholesterol homeostasis. Besides, β-escin and AH might modulate cytokine production depending on the stimuli (viral or non-viral) and the cell type under study. Thus, our data provide evidence for novel therapeutic potentials of β-escin and AH beyond current vascular indications.

Declarations

Acknowledgements

We thank Guillermo Assad Ferek for their technical assistance. We are indebted to Spedrog Callion which supplied the vials of β-escin and AH. This work was supported by Grants from ANPCYT (PICT N° 2014-3331 and PICT 2013-2281), CONICET (PIP 20120100538) and UBA (20020130100584BA). Dr. L.E. Alché, Dr. F.M. Michelini and Dr. C.A. Bueno are research members of CONICET.

Conflict of interest

The Authors declare that they have no conflict of interest to disclose.

References

1. Azher TN *et al.* Understanding the role of chemokines and cytokines in experimental models of herpes simplex keratitis. *J Immunol Res* 2017; 2017: 1–5.
2. Biswas PS, Rouse BT. Early events in HSV keratitis-setting the stage for a blinding disease. *Microbes Infect* 2005; 7: 799–810.
3. Lee DH, Ghiasi H. Roles of M1 and M2 macrophages in herpes simplex virus 1 infectivity. *J Virol* 2017; 12: 91.
4. Kase S *et al.* Activation of nuclear factor-kappa B in the conjunctiva with the epithelial scraping of the mouse cornea and human epidemic keratoconjunctivitis. *Br J Ophthalmol* 2004; 88: 947–949.
5. Panday A *et al.* Transcription factor NF-κB: an update on intervention strategies. *Arch Immunol Ther Exp (Warsz)* 2016; 2016: 463–483.

6. Austin A *et al.* Update on the management of infectious keratitis. *Ophthalmology* 2017; 124: 1678–1689.
7. Duan R *et al.* Acyclovir-resistant corneal HSV-1 isolates from patients with herpetic keratitis. *J Infect Dis* 2008; 198: 659–663.
8. Rousseau A *et al.* Recurrent herpetic keratitis despite antiviral prophylaxis: a virological and pharmacological study. *Antiviral Res* 2017; 146: 205–212.
9. Bueno CA *et al.* Natural and semisynthetic diterpenoids with antiviral and immunomodulatory activities block the ERK signaling pathway. *Med Microbiol Immunol* 2015; 204: 575–584.
10. Wei P *et al.* Anti-inflammatory and antiviral activities of cynanversicoside A and cynanversicoside C isolated from *Cynanchun paniculatum* in influenza A virus-infected mice pulmonary microvascular endothelial cells. *Phytomedicine* 2017; 1: 18–25.
11. Hassan ST *et al.* Bioactive natural products with anti-herpes simplex virus properties. *J Pharm Pharmacol* 2015; 67: 1325–1336.
12. Son M *et al.* Bioactive activities of natural products against herpesvirus infection. *J Microbiol* 2013; 51: 545–551.
13. Sirtori CR. Aescin: pharmacology, pharmacokinetics and therapeutic profile. *Pharmacol Res* 2001; 44: 183–193.
14. Pittler MH, Ernst E. Horse chestnut seed extract for chronic venous insufficiency. *Cochrane Database Syst Rev* 2012; 11: CD003230.
15. Domanski D *et al.* Molecular mechanism for cellular response to β-escin and its therapeutic implications. *PLoS ONE* 2016; 11: e0164365.
16. Cheng Y *et al.* Escin increases the survival rate of LPS-induced septic mice through inhibition of HMGB1 release from macrophages. *Cell Physiol Biochem* 2015; 36: 1577–1586.
17. Liu S *et al.* Escin inhibits lipopolysaccharide-induced inflammation in human periodontal ligament cells. *Mol Med Rep* 2012; 6: 1150–1154.
18. Xin W *et al.* Escin exerts synergistic anti-inflammatory effects with low doses of glucocorticoids *in vivo* and *in vitro*. *Phytomedicine* 2011; 18: 272–277.
19. Barquero AA *et al.* 1-Cinnamoyl-3,11-dihydroxymeliacarpin is a natural bioactive compound with antiviral and nuclear factor-kappaB modulating properties. *Biochem Biophys Res Commun* 2006; 344: 955–962.
20. Bueno CA *et al.* A natural tetranortriterpenoid with immunomodulating properties as a potential anti-HSV agent. *Virus Res* 2009; 141: 47–54.
21. Michelini FM *et al.* Synthetic stigmastanes with dual antiherpetic and immunomodulating activities inhibit ERK and Akt signaling pathways without binding to glucocorticoid receptors. *Biochim Biophys Acta* 2016; 1860: 129–139.
22. Safrin S *et al.* A rapid screen test for *in vitro* susceptibility of clinical herpes simplex virus isolates. *J Infect Dis* 1994; 169: 879–882.
23. Howett MK *et al.* A broad-spectrum microbicide with virucidal activity against sexually transmitted viruses. *Antimicrob Agents Chemother* 1999; 43: 314–321.
24. Wu CY *et al.* Small molecules targeting severe acute respiratory syndrome human coronavirus. *Proc Natl Acad Sci U S A* 2004; 101: 10012–10017.
25. Michelini FM *et al.* The *in vitro* immunomodulatory activity of a synthetic brassinosteroid analogue would account for the improvement of herpetic stromal keratitis in mice. *J Steroid Biochem Mol Biol* 2008; 108: 164–170.
26. Bueno CA *et al.* A natural antiviral and immunomodulatory compound with antiangiogenic properties. *Microvasc Res* 2012; 84: 235–241.
27. Johnson AC *et al.* Activation of toll-like receptor (TLR)2, TLR4, and TLR9 in the mammalian cornea induces MyD88-dependent corneal inflammation. *Invest Ophthalmol Vis Sci* 2005; 46: 589–595.
28. Sarangi PP *et al.* Innate recognition network driving herpes simplex virus-induced corneal immunopathology: role of the toll pathway in early inflammatory events in stromal keratitis. *J Virol* 2007; 81: 11128–11138.
29. Bottger S, Melzig MF. The influence of saponins on cell membrane cholesterol. *Bioorg Med Chem* 2013; 21: 7118–7124.
30. Ye J *et al.* Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci U S A* 2003; 100: 15865–15870.
31. Whitmarsh AJ *et al.* Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med (Berl)* 1996; 74: 589–607.
32. Wang XH *et al.* Effect of beta-escin sodium on endothelial cells proliferation, migration and apoptosis. *Vascul Pharmacol* 2008; 49: 158–165.