

Accepted Manuscript

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PII: S0166-3542(18)30638-7

DOI: <https://doi.org/10.1016/j.antiviral.2019.01.018>

Reference: AVR 4463

To appear in: *Antiviral Research*

Received Date: 19 October 2018

Revised Date: 3 January 2019

Accepted Date: 28 January 2019

Please cite this article as: Salinas, F.M., Vázquez, L., Gentilini, Marí.Virginia., O´Donohoe, A., Regueira, E., Nabaes Jodar, M.S., Viegas, M., Michelini, F.M., Hermida, G., Alché, L.E., Bueno, C.A., *Aesculus hippocastanum* L. seed extract shows virucidal and antiviral activities against respiratory syncytial virus (RSV) and reduces lung inflammation *in vivo*, *Antiviral Research* (2019), doi: <https://doi.org/10.1016/j.antiviral.2019.01.018>.

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Aesculus hippocastanum* L. seed extract shows virucidal and antiviral activities against respiratory syncytial virus (RSV) and reduces lung inflammation *in vivo

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Abstract

Respiratory syncytial virus (RSV) is a leading cause of lower respiratory tract disease and bronchiolitis in children worldwide. No vaccine or specific, effective treatment is currently available. β -escin is one of the main bioactive constituents of *Aesculus hippocastanum* L. (Hippocastanaceae) seed extract (AH), and both β -escin and AH have demonstrated a beneficial role in clinical therapy because of their anti-edematous, anti-inflammatory and antioxidative effects. Besides, we have reported that β -escin and AH show virucidal, antiviral and immunomodulatory activities against the enveloped viruses HSV-1, VSV and Dengue virus *in vitro*. In this study, we demonstrate that β -escin and AH have virucidal and antiviral activities against RSV, as well as NF- κ B, AP-1 and cytokine modulating activities in RSV infected epithelial and macrophage cell lines *in vitro*. Besides, in a murine model of pulmonary RSV infection, AH treatment improves the course of acute disease, evidenced by decreased weight loss, reduced RSV lung titers, and attenuated airway inflammation. In contrast, even though β -escin showed, similarly to AH, antiviral and immunomodulatory properties *in vitro*, it neither reduces viral titers nor attenuates lung injury *in vivo*. Thus, our data demonstrate that AH restrains RSV disease through antiviral and immunomodulatory effect.

Keywords: Respiratory syncytial virus (RSV); Antiviral; Immunomodulatory; Medicinal Plants; *Aesculus hippocastanum*; β -escin

1. Introduction

The human respiratory syncytial virus (RSV) infection is a leading cause of acute respiratory tract infections in early childhood (Hall et al, 2013). The infection is frequently associated with bronchiolitis and pneumonia. RSV bronchiolitis is potentially life-threatening and requires admission to hospital (Piedimonte and Perez, 2014). Moreover, children experiencing severe or recurrent bronchiolitis have an increased risk for recurrent wheeze and asthma (Sigurs et al, 2010; Krishnamoorthy et al, 2012). The World Health Organization estimated that RSV is responsible for over 33 million new episodes of acute lower respiratory infection in children younger than 5 years (Nair et al, 2010). RSV causes significant mortality in the developing world, resulting in an estimated 200,000 annual deaths in young children globally, in addition to major morbidity (33.8 million episodes worldwide annually) (Nair et al, 2010). Besides, RSV is a leading cause of morbidity and mortality in elderly and immunocompromised individuals (Kwon et al, 2017).

The transmission of RSV is difficult to prevent since it is easily transmitted by close contact and by unprotected coughing and sneezing. Although practically all children will have had an infection by the age of two (Dawson-Caswell and Muncie et al 2011), recurrent infection is very common since the virus has developed an arsenal of strategies to modulate host immune response and antiviral immunity (Christiaansen et al 2015; Larranaga et al 2009). The virus has the ability to modulate cytokine and chemokine signalling networks, such as NF- κ B and AP-1 signalling pathways, interfere with immune cell function and antibody response (Dey et al, 2011; Christiaansen et al 2015; Li et al, 2016). Despite the prevalence of RSV bronchiolitis, there is no vaccine available and, apart from supportive measures, there is no specific effective treatment (Tregoning and Schwarze, 2010) since routine use of bronchodilators or antiviral ribavirin has been proven to be of no significant benefit (Turner et al 2014).

In the last years, plant extracts and herbal compounds have been investigated for their antiviral and immunomodulatory properties (Hassan et al. 2015; Bueno et al, 2015; Michelini et al, 2018; Shi et al, 2016). β -escin is one of the main bioactive constituents of *Aesculus hippocastanum* L. (Hippocastanaceae) seed extract (AH), and both β -escin and AH have demonstrated a beneficial role in clinical therapy because of their anti-edematous, anti-inflammatory and antioxidative effects (Sirtori, 2001; Pittler and Ernst. 2012). In fact, in the United States and Europe, they are one of the best-selling herbal products (Domanski et al. 2016). With respect to their mechanism of action, we and others have reported that β -escin and AH modulate NF- κ B and AP-1 activation in different cell types and conditions *in vitro* (Cheng et al. 2015; Domanski et al. 2016; Liu et al. 2012; Michelini et al, 2018). Moreover, we have demonstrated virucidal and broad spectrum antiviral activities for β -escin and AH against the enveloped viruses HSV-1, VSV and Dengue (Michelini et al, 2018).

Thus, considering that NF- κ B and AP-1 activation play an important role in RSV replication (Dey et al, 2011; Li et al, 2016), the aim of the present study was to examine the antiviral activity of β -escin and AH against RSV in epithelial cells, as well as their effect on the activation of the NF- κ B and AP-1 signalling pathways and on the production of different cytokines in RSV infected epithelial and macrophage cell lines. Finally, we studied their activity against RSV infection in a murine model of pulmonary infection.

2. Materials and Methods

2.1 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 Callion 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 analyzed by Spedrog Callion S.A. by HPLC, and complies 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). AH contains about 3-6% of escin (according to DAB, not less than 3%, expressed as anhydrous escin and calculated with reference to the dried drug). Moreover, the identity (Thin-layer Chromatography (TLC)) and purity (High performance liquid chromatography (HPLC)) were also analyzed by Spedrog Callion S.A., and complies 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.

AH was dissolved in DMEM/F12, and β -escin was dissolved in dimethylsulfoxide (DMSO) and diluted with DMEM/F12. The maximum concentration of DMSO used (1%) exhibited no toxicity under *in vitro* and *in vivo* conditions.

2.2 Cells and viruses

The human HEp-2 cell line (human epidermoid cancer cell line) and the human A549 cell line (human lung carcinoma cell line) were grown in DMEM/F12 supplemented with 10% inactivated fetal bovine serum (FBS) (DMEM/F12, 10%), Murine macrophage cell line J774A.1 was kindly provided by Dr. Osvaldo Zabal (INTA–Castelar, Buenos Aires, Argentina) and grown in RPMI 1640 medium supplemented with 10% FBS. Vero cells were grown in MEM supplemented with 10% FBS. Human RSV strains A2 and line 19 were kindly provided by Dr. Laura Talarico (INFANT–Buenos Aires, Argentina). Working stocks of RSV were prepared as previously described (Caidi et al, 2016). Briefly,

semiconfluent monolayers of HEp-2 cells were infected with RSV strains line 19 and A2 (multiplicity of infection (moi)=0.2) and were incubated 3–4 days, monitoring the development of CPE daily, until CPE \geq 80% of cell monolayer, but still intact and attached to flask bottom. Then, supernatant was removed and 5 mL of cold 25% (w/v) sterile sucrose was added. Then the flask was transferred to $-80\text{ }^{\circ}\text{C}$, being sure that cell surface is covered with sucrose solution while in the freezer. After three cycles of freezing and thawing, lysates were transferred to sterile 50 mL conical tubes. Cellular debris was removed by centrifugation at $500 \times g$ and $4\text{ }^{\circ}\text{C}$ for 10 min, and supernatants were aliquoted and stored at -80°C until use. Sucrose in concentrations at 25% has a stabilizing effect and reduces loss of infectivity of this very labile virus. Virus titration was performed in Vero cells by plaque assay.

2.3 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, that were incubated with medium alone.

2.4 Antiviral activity

Cells grown in 24-well plates were infected with a moi of 1. After 1 h adsorption at $37\text{ }^{\circ}\text{C}$, the inoculum was removed and medium containing the compounds was added, in triplicate. The plates were incubated at $37\text{ }^{\circ}\text{C}$ until 24 h p.i. After cell disruption by freezing and thawing, supernatants were titrated by plaque assay in Vero cells, 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, that were incubated with medium alone.

2.5 Virucidal effect

RSV line 19 and A2 (10^7 PFU) were diluted in culture medium containing or not each compound and incubated for 120 min at 37 °C. Aliquots were diluted to a non-inhibitory drug concentration and titrated by plaque assay on Vero cells.

2.6 Time-of-addition assays

For pre-infection assays, cells were treated with the compound during 2 h at 37°C, washed with PBS and then infected with RSV A2 (moi=1). For co-infection, cells were simultaneously infected with RSV A2 and treated with the compound of interest. After 1 h adsorption at 37°C, the virus-drug mixture was removed, washed and compound free medium was added. For post-infection (p.i.) assays, cells were infected with RSV for 1 h at 37°C and then treated with the tested compound at 0, 2, 4, 6, 8, 12 and 16 h after infection. A control culture that was infected but not treated (CV) was simultaneously performed. Cells were further incubated at 37 °C till 24 h p.i., and after cell disruption by freezing and thawing, supernatants were titrated by plaque assay in Vero cells.

2.7 RSV qRT-PCR assay.

At the indicated time points p.i./compound incubation, total RNAs were extracted from supernatants, after freezing and thawing the infected cells, with QIAamp® Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The first-strand cDNA synthesis was performed with SuperScript III Reverse Transcriptase (SSIII) (Thermo Fisher Scientific) following manufacturer's instructions and a primer which recognizes the M gene of the negative-strand RSV RNA genome. Then a quantitative PCR

(qPCR) with primers and probe targeting the M gene previously reported (Kim et al, 2011) was performed with SensiFAST™ Probe No-ROX Kit (Bioline) following manufacturer's instructions. RNase P DNA was used as a reference gene and was amplified with the corresponding gene specific primers and probe and the qPCR was performed with SensiFAST™ Probe No-ROX Kit (Bioline). Average viral RNA Cq values were normalized to the average Cq values of RNase P and $\Delta\Delta C_t$ based fold-change calculations were set relative to untreated-virus infected cells at 6 h p.i.

2.8 Cytokine determination

Mouse TNF- α and IL-6, and Human IL-6 and IL-8 were quantified by commercial ELISA sets (BD OptEIA™, Becton–Dickinson) according to the manufacturer's instructions.

2.9 Transfections

Transfection assays with Lipofectamine 2000 reagent (Invitrogen) were performed according to the manufacturer's instructions. The NF- κ B-LUC reporter vector and RSV- β -gal plasmid, coding for the bacterial β -galactosidase gene under the control of the viral RSV promoter, 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). Reporter quantitation with Luciferase Assay System E1500 (Promega) was performed according to the manufacturer's instructions.

2.10 Pulmonary infection in mouse model.

Animal studies were approved by the Comisión Institucional de Cuidado y Uso de Animales de Laboratorio (CICUAL) of the Facultad de Ciencias Exactas y Naturales,

Universidad de Buenos Aires, Argentina. Female Balb/C mice were purchased from Facultad de Veterinaria, Universidad de Buenos Aires, Argentina. The animals were housed in an Animal Facility Biosafety Level 3 (ABSL-3) (UOCCB, ANLIS-Malbrán, Buenos Aires, Argentina) in individually ventilated cages and fed with food and water ad libitum for at least 1 week before experimental use at 6–8 week of age. Mice received 50 μ l RSV line 19 and A2 (5×10^6 PFU) or 25% (w/v) sucrose by intranasal (i.n.) delivery under light general anesthesia (isoflurane) (6 per group). Infectious dose was chosen based on references that previously characterize line 19 and A2 strains of RSV in the mouse model (Shi et al, 2016; Rudd et al, 2016). A total of 10 mg/kg of AH or 1 mg/kg of β -escin or a vehicle (control group), was given 1 h p.i. intraperitoneally (i.p.). Mice further received a daily dose of 10 mg/kg of AH or 1mg/kg of β -escin until day 4 p.i. Body weights were monitored daily, and groups of mice were culled on day 4 or 8 p.i. Lungs from day 4 were removed, weighed, and used for titration of infectious virus. Briefly, snap frozen lungs were homogenized on ice using glass Dounce homogenizers. Tissue debris was pelleted by centrifugation at 4°C for 10 min at 300g and supernatants were immediately serially diluted in FBS free medium and titrated by plaque assay in Vero cells. The right lungs from day 8 were used for gene expression studies, and in parallel, the left lung was submerged in Bouin solution for fixation for 24 hr and subsequent histological sectioning and staining.

2.11 Cytokines qRT-PCR.

RNA from mouse lung tissue was isolated using TRIzol reagent (Life Technologies) and reverse transcribed with ImProm-II™ Reverse Transcription System A3800 (Promega), according to the manufacturer's instructions. Quantitative PCR was performed on the Bio-Rad iQ5 real-time PCR system using FastStart SYBR green Master Mix reagent (Roche). β -actin was used as an internal control for normalization. The data were

analyzed using the $2^{-\Delta\Delta C_t}$ formula. The sequences of the primers for mouse gene expression are listed (forward and reverse):

TNF- α : F: CAGGCGGTGCCTATGTCTC; R: CGATCACCCCGAAGTTCAGTAG

PrimerBank ID: 133892368c1(<https://pga.mgh.harvard.edu/primerbank/>)

IL-6: F:TAGTCCTTCTACCCCAATTTCC; R: TTGGTCCTTAGCCACTCCTTC

PrimerBank ID: 13624311a1 (<https://pga.mgh.harvard.edu/primerbank/>)

IL-4: F: GGTCTCAACCCCGAGCTAGT; R: GCCGATGATCTCTCTCAAGTGAT

PrimerBank ID: 10946584a1 (<https://pga.mgh.harvard.edu/primerbank/>)

IFN- γ : F: ATGAACGCTACACACTGCATC; R: CCATCCTTTTGCCAGTTCCTC

PrimerBank ID: 33468859a1(<https://pga.mgh.harvard.edu/primerbank/>)

IL-17A: F: TTAACTCCCTTGCGCAAAA; R: CTTTCCCTCCGCATTGACAC

PrimerBank ID: 6754324a1 (<https://pga.mgh.harvard.edu/primerbank/>)

β -actin: F: GTGACGTTGACATCCGTAAAGA; R: GCCGGACTCATCGTACTCC

PrimerBank ID 145966868c1(<https://pga.mgh.harvard.edu/primerbank/>)

2.12 Statistical analysis

CC₅₀ and EC₅₀ were calculated from dose–response curves using the software GraphPad Prism 4.0. Statistical significance was assessed either using a one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test, or a two-way ANOVA with Bonferroni's post test where appropriate. p-Value <0.05 was considered significant.

3. Results:

3.1 Antiviral and virucidal activities of β -escin and AH against RSV

The laboratory RSV strain A2 is important in the field as a reference strain and it has been used for studies including both *in vitro* and *in vivo* modeling of disease responses. Besides, RSV strain line 19 has been used to establish an animal model of severe RSV-induced disease, with significant airway hyperreactivity (AHR) and mucus overproduction (Woolums et al, 2011). In order to assess the antiviral activity of β -escin and AH against RSV, HEp-2, A549, and Vero cells were infected with both RSV strains line 19 and A2 (moi=1) and treated with different concentrations of β -escin and AH during 24 h (100-0.1 μ g/ml), and the selectivity index (SI), the relationship between CC_{50} and EC_{50} values, was calculated. β -escin and AH significantly reduced infectivity of both strains of RSV in a concentration-dependent manner ($p<0.05$) (Table 1 and 2, and Supplementary Figure 1). Besides, β -escin and AH have no cytotoxic effect at all concentrations tested ($CC_{50}>100$ μ g/ml).

Next, we evaluated whether the antiviral action against RSV was due to a direct inactivation of the released virus. Suspensions of RSV line 19 and A2 were incubated with different concentrations of β -escin and AH (100-0.1 μ g/ml) for 120 min at 37°C, followed by titration of the remaining infectivity in Vero cells. Results showed an inactivating effect for β -escin and AH against both strains of RSV (Table 3 and Supplementary Figure 1).

These data demonstrate that β -escin and AH have virucidal and antiviral activities against RSV line 19 and A2 infection *in vitro*.

3.2 Influence of the duration of treatment with β -escin and AH on RSV infectivity and on RSV RNA synthesis

To further characterize β -escin and AH inhibitory action, HEp-2 and A549 cells were exposed to both drugs before, during or after infection with RSV A2. Virus yields were quantified at 24 h p.i.

When β -escin (5 $\mu\text{g/ml}$) and AH (25 $\mu\text{g/ml}$) were added before or during RSV inoculation (moi= 1), no significant inhibition of viral multiplication was detected. However, RSV A2 virus yields significantly decreased when β -escin and AH were added after infection in HEp-2 and A549 cells (Fig 1A).

Then, we decided to make a time of addition assay at different times after infection. Results showed that β -escin (5 $\mu\text{g/ml}$) and AH (25 $\mu\text{g/ml}$) were able to inhibit infectious particle formation even when both were added at 8 h p.i. (Fig 1B). At later times, none of the compounds restrained virus infectivity in HEp-2 and A549 cells (Fig 1B).

Even though β -escin and AH showed virucidal properties (Table 3), they were not active when added during inoculation of virus particles, and, likewise, inhibition of infectious virus particle production is lost when these compounds were added at late times p.i. Besides, the concentrations of β -escin and AH needed to inactivate RSV particles were higher than those observed for antiviral activity. Thus, in order to analyze whether the dominant antiviral activity occurs intracellularly on RNA replication rather than on the virus particle itself, we examined if viral RNA synthesis was affected by β -escin and AH. Intracellular RNA synthesis of RSV A2 was analyzed by qRT-PCR at different time points p.i. in the presence of β -escin and AH. The amounts of viral RNA in untreated and treated HEp-2 cells were calculated in comparison to the content of viral RNA in untreated infected cells at 6 h p.i., defined as 1. As seen in Figure 1C, the time course of RSV RNA synthesis in control infected cells was in accordance with previous studies (Borg et al, 2003; Challa et al, 2015) with increasing levels of intracellular RNA from earlier to later times. At 6 h and 12 p.i., the content of viral RNA in cells infected with RSV and treated with β -escin and AH was similar to that in untreated infected cells. By contrast, the relative contents of viral RNA in cells infected and treated with β -escin and AH decreased with time, and the maximal difference between control infected cells with β -escin and AH treated infected

cells was observed at 18 h and 24 h p.i., when the peak in RNA synthesis was detected for untreated cells (Figure 1C). These results suggested that β -escin and AH impair viral RNA synthesis during RSV infection.

3.3 NF- κ B and AP-1 activation is modulated by β -escin and AH after RSV infection in epithelial and macrophage cell lines.

NF- κ B activation following RSV infection is necessary for viral replication (Masaki et al, 2011), and both NF- κ B and AP-1 activation are required for RSV-induced cytokine production (Dey et al, 2011; Masaki et al, 2011; Li et al, 2016; Tian et al, 2018). Besides, the downregulation of NF- κ B activation by β -escin has been described in different cell types and conditions (Cheng et al. 2015; Domanski et al. 2016; Liu et al. 2012; Michelini et al, 2018). Previously, we showed that β -escin and AH modulate NF- κ B and AP-1 activation in epithelial and macrophage cell lines infected with HSV-1 and stimulated with Toll Like Receptors (TLRs) ligands (Michelini et al, 2018). Therefore, we decided to investigate whether β -escin and AH could modulate RSV induced NF- κ B and AP-1 activation in epithelial and macrophage cell lines.

HEp-2, A549 and J774A.1 cells were transfected with a NF- κ B-LUC and AP-1 LUC reporter vector and β -galactosidase control plasmid and, 24 h later, infected with RSV line 19 and A2 (moi=1) and treated with β -escin (5 μ g/ml) and AH (25 μ g/ml) during 24 h. We verified that RSV line 19 and A2 induced NF- κ B and AP-1 activation, and, interestingly, both NF- κ B and AP-1 signalling pathways were strongly inhibited by β -escin and AH in HEp-2, A549 and J774A.1 cells (Fig 2).

3.4 Modulation of cytokine production by β -escin and AH in infected epithelial and macrophage cell lines

It has been already reported that RSV is able to activate NF- κ B and AP-1 concomitant with the expression of pro-inflammatory cytokines, including IL-6 and TNF- α and chemokines such as IL-8, that contribute to inflammation and the pathology of the infection (Dey et al, 2011; Masaki et al, 2011; Li et al, 2016). To assess the biological relevance of the inhibition of the activation of NF- κ B and AP-1 pathways provoked by β -escin and AH on cytokine production, we measured their effect on IL-6 and IL-8 secretion in infected epithelial cells, and IL-6 and TNF- α in infected macrophage cell line. Supernatants harvested from HEp-2, A549 and J774A.1 cells infected with RSV line 19 and A2 strains (moi=1) and treated or not with β -escin (5 μ g/ml) and AH (20 μ g/ml) were used to quantify IL-6, IL-8 and TNF- α by ELISA.

As expected, IL-6, IL-8 and TNF- α production were higher in RSV line 19 and A2 infected cells than in uninfected ones (Dey et al, 2011; Masaki et al, 2011; Li et al, 2016). Besides, β -escin and AH significantly reduced IL-6, IL-8 and TNF- α production when added to RSV HEp-2, A549 and J774A.1 infected cells (Fig 3). In summary, β -escin and AH proved to reduce the release of IL-6, IL-8 and TNF- α in RSV-infected epithelial and macrophage cell lines, probably as a consequence of the inhibition of the NF- κ B and AP-1 signalling pathways.

3.5 In vivo evaluation of β -escin and AH antiviral effect

Having demonstrated that β -escin and AH had antiviral and immunomodulatory properties against RSV line 19 and A2 *in vitro*, the question whether these properties were functional *in vivo* was addressed. For that purpose, a well-characterized model of murine pulmonary RSV infection was used (Lukacs et al, 2006; Stokes et al, 2011; Rudd et al, 2016; Woolums et al, 2011). Considering that in a mice model of allergic airway inflammation in the lung, i.p. administration of β -escin exerts a beneficial effects on airway

and lung inflammation by reducing infiltration of inflammatory cells into lung tissue and the release of asthma-associated cytokines, and in view of the close association between asthma and severe RSV infection (Lindner et al, 2010; Markus et al, 2014; Piedimont and Perez, 2014; Saravia et al, 2015), we decided to conduct the experiments with i.p. administration of β -escin and AH. In uninfected mice, an i.p. dose of 1 mg/kg of β -escin and 10 mg/kg of AH had no effect on general health and behavior, as previously reported (Sirtori et al, 2001; EMA, 2009; Pittler and Ernst, 2012). Thus, 1h after RSV line 19 and A2 infection by nasal instillation, mice were treated with i.p. administration of either 1 mg/kg of β -escin or 10 mg/kg of AH. Then, mice were subsequently treated with daily administration with AH or β -escin until day 4 p.i. Mice injected i.p. with PBS were used as untreated controls. Weight loss was assessed daily, viral load was determined on days 4, and pulmonary gene transcription and histopathology was assessed on day 8.

Weight loss is a quantitative measure of RSV illness severity in the BALB/c mouse model (Rudd et al, 2016; Stokes et al, 2011). Line 19 and A2 RSV-infected mice treated with β -escin showed an early weight reduction on day 2 p.i., (Fig 4A and B), comparable to the weight reduction observed for untreated RSV-infected mice (Stokes et al, 2011; Rudd et al, 2016). In contrast, RSV line 19 and A2 infected mice treated with AH not only had significantly less weight loss on day 2 p.i., but also over the entire course of the experiment when compared to untreated infected controls (Fig 4A and B).

RSV line 19 and A2 titers in the lung were significantly lower in AH-treated mice compared to untreated animals at day 4 (the time point of peak viral load in this model) (Fig 4C) (Stokes et al, 2011; Rudd et al, 2016). No difference between β -escin-treated mice and untreated mice was observed regarding line 19 and A2 RSV titers (Fig 4C).

A number of cytokines have previously been outlined to be important in the severity of the pathophysiology and the induction of AHR and mucus (Woolums et al, 2011). Thus,

to examine the magnitude of host response to infection, we evaluated cytokines gene expression by qPCR (Fig 5). RSV line 19 and A2 untreated and infected mice had a significant induction of transcript levels of pro-inflammatory cytokines TNF- α and IL-6, Th2-type cytokine IL-4, Th1-type cytokine IFN- γ , and Th17-type cytokine IL-17A, in the lungs with respect to uninfected animals. Interestingly, infected mice treated with AH had significantly lower levels of all these cytokines than those observed in control infected animals. On the contrary, infected animals treated with β -escin did not show any significant difference compared to RSV line 19 and A2 infected mice (Fig. 5).

As shown in Fig. 6, RSV line 19 and A2 infection recapitulated previously reported abnormal histology of lung sections (Rudd et al, 2016). H&E staining showed alveolar walls, and alveolar spaces filled with moderate to severe inflammatory infiltrates of cells in infected and mock-treated group. The lung sections from AH treated group were more closely resembled to that of the uninfected controls. Thus, AH reduced lung inflammation and inflammatory cell infiltration. On the contrary, lungs sections from β -escin treated mice show similar histopathology injury compared to infected controls (Fig 6).

Taken together, these results, demonstrated that AH restrained RSV infection in the lung, as well as ameliorated pro-inflammatory response and lung injury.

4. Discussion

RSV is a leading cause of lower respiratory tract disease and bronchiolitis in children worldwide. Despite decades of effort, there is no a safe and efficacious RSV vaccine or antiviral treatment (Turner et al, 2014). Herbal medicines have demonstrated therapeutic efficacy for symptoms of viral infection and inflammation although the underlining mechanisms are not clear. Particularly, in the case of β -escin and AH, although

research provides evidence for their broad use to treat numerous diverse disorders, their current clinical use is restricted mainly to venotonic and venoprotective indications due to their anti-inflammatory and anti-edematous properties. Indeed, randomized controlled trials confirmed the effectiveness of β -escin and AH for the treatment of chronic venous insufficiency (Sirtori et al, 2001; EMA, 2009; Pittler et al. 2012).

Previously, we have reported that β -escin and AH show virucidal and antiviral activities against the enveloped viruses HSV-1, VSV and Dengue virus (Michelini et al, 2018). In this study, we identified β -escin and AH as active herbal medicines against RSV *in vitro*. Airway epithelial cells are the major target of RSV infection in the lung, and importantly, β -escin and AH restrict line 19 and A2 RSV infection in epithelial cells in several aspects, including the inactivation of the viral particles and, more crucially, the inhibition of intracellular replication when added after infection. Indeed, one of the dominant process affected by β -escin and AH appears to be RSV RNA synthesis.

Besides, β -escin and AH inhibit NF- κ B and AP-1 signalling pathways induced by RSV, and consequently, they reduce pro-inflammatory cytokines production in epithelial and macrophage cell lines infected with RSV *in vitro*. Considering that NF- κ B activation plays an important role in RSV replication (Masaki et al, 2011), the inhibition of NF- κ B pathway in RSV-infected cells treated with β -escin and AH could account for the antiviral activity observed. Alternatively, the reduction of NF- κ B and AP-1 activation and cytokines production in β -escin and AH-treated and RSV-infected cells could be due to the antiviral activity of these compounds. However, it is well known the downregulation of NF- κ B activation by β -escin in different cell types and phisiopatological conditions in absence of infection (Cheng et al. 2015; Domanski et al. 2016; Liu et al. 2012; Michelini et al, 2018). In fact, it is proposed that β -escin decreases NF- κ B activation as a result of perturbations in cholesterol homeostasis, which is considered the triggering event in a cascade of cellular

responses leading to cytoskeletal disarrangements, with an impact in NF- κ B signal transduction (Domanski et al, 2016). In this sense, we have previously reported that β -escin and AH block the activation of NF- κ B and AP-1 pathways and reduce cytokine production in ocular epithelial cells and macrophages stimulated with non-viral stimuli, such as TLRs ligands (Michelini et al, 2018). In addition, we have also verified that β -escin and AH ablate the induction of NF- κ B and AP-1 and cytokine production in HEp-2 and A549 cells stimulated with TLRs ligands, independently of a viral infection (Supplementary Figure 2). Moreover, RSV does not multiply in J774A.1 cells in our experimental settings. Thus, the ablation of NF- κ B and AP1- induction and cytokine production upon AH and β -escin treatment of RSV infected J774A.1 cells, could not be due to the inhibition of viral infection.

RSV activation of the NF- κ B and AP-1 pathways is one of the main factors involved in viral pathogenesis *in vivo* (Li et al, 2016; Tian et al, 2018). The host senses RSV invasion through pattern recognition receptors, leading to the activation of NF- κ B and AP-1 signalling pathways, increased cytokine production, inflammatory cell influx, and subsequently mucus production, AHR and reduced lung function (Li et al, 2016; Tian et al, 2018). Thus, blockade of these signalling pathways involved in immunopathology during RSV infections may be an effective therapeutic strategy with a low risk of emergence of viral resistance. In this paper, in mice infected with RSV line 19 and A2 and treated with AH, there was not only a reduction of the viral titers, but also a reduction of the lung injury. In addition, there was a significant decrease in cytokine gene expression in AH treated infected mice, consistent with results from histological studies. Thus, considering that AH blocked NF- κ B and AP-1 signalling pathways *in vitro* and its well known immunomodulatory activity in different *in vivo* models (Cheng et al. 2015; Domanski et al. 2016; Liu et al. 2012; Xin et al, 2011), it is likely that AH would exhibit a protective effect

against pulmonary RSV infection not only by blocking viral infection processes but also by modulating the immune response.

In contrast, even though β -escin showed, similarly to AH, antiviral and immunomodulatory properties *in vitro*, it neither reduces viral titers nor attenuates lung injury *in vivo*, in the dose and route of administration tested. Since β -escin is one of the main constituent of AH together with others (AH contains 3-6% of escin), and that the dose of 10 mg/kg of AH contains less than 1 mg/kg of β -escin, the antiviral and immunomodulatory effects observed in AH treated infected mice would be ascribed to a synergic effect of some components present in the extract. In fact, it is reported that the therapeutically active constituent in AH is not known with certainty, and that the data in support of β -escin as the active substance of AH in chronic venous insufficiency is very weak (EMA, 2009).

Conclusion

This study demonstrates that β -escin and AH have virucidal and antiviral activities against RSV, as well as NF- κ B, AP-1 and cytokine modulating activities in RSV infected cells *in vitro*. Besides, in an *in vivo* model of RSV, AH treatment improves the course of acute disease, evidenced by decreased weight loss, reduced RSV lung titers, and attenuated airway inflammation. Thus, our data demonstrate that AH restrains RSV disease through antiviral and immunomodulatory effect. In this paper, we have shown that AH is highly effective if applied by i.p.administration. In view of a clinical application in humans in the future, further application routes, such as oral and intranasal, should be examined.

5. **Acknowledgements.** We thank Guillermo Assad Ferek for their technical assistance. We thank Dr. Laura Talarico (INFANT–Buenos Aires, Argentina) for providing RSV strains A2 and line 19. We are indebted to Spedrog Callion which supplied the vials of β -escin and AH. The authors are deeply grateful to the staff of the UOCCB, ANLIS-Malbrán, Buenos Aires, for their expert technical assistance in the ABSL-3 laboratory. This work was supported by Grants from ANPCYT (PICT N° 2014-3331 and PICT 2013-2281), CONICET (PIP 20120100538) and UBA (20020130100584BA).

6. **Declarations of interest:** none

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Tables

Table 1. EC₅₀ of β -escin and AH against RSV line 19 and A2

Compound	EC ₅₀ (μ g/ml)					
	HEp-2		A549		Vero	
	line 19	A2	line 19	A2	line 19	A2
β -escin	1.6 \pm 0.3	1.4 \pm 0.7	2.4 \pm 0.8	1.8 \pm 0.2	1.5 \pm 0.5	2.6 \pm 0.23
AH	13.3 \pm 0.4	12.7 \pm 1.2	14 \pm 1.2	17 \pm 3.4	7.5 \pm 0.6	9.8 \pm 2.7

EC₅₀: Effective Concentration 50. EC₅₀ were calculated by nonlinear regression. Data represent mean \pm SD for n = 3 independent experiments, performed in triplicate.

AH: *Aesculus hippocastanum* L. seed extract

Table 2. SI of β -escin and AH against RSV line 19 and A2

Compound	SI (CC ₅₀ /CE ₅₀)					
	HEp-2		A549		Vero	
	line 19	A2	line 19	A2	line 19	A2
β -escin	62.5	71.4	41.6	55.5	66.7	38.4
AH	7.5	7.9	7.1	5.9	13.3	10.2

SI: selectivity indices (ratio CC₅₀/CE₅₀)

AH: *Aesculus hippocastanum* L. seed extract

Table 3. Virucidal activity of β -escin and AH against RSV line 19 and A2

Compound	EC ₅₀ (μ g/ml)	
	line 19	A2
β -escin	14.5 \pm 2.3	15.1 \pm 3.1
AH	68.5 \pm 5.8	65.9 \pm 8.7

EC₅₀: Effective Concentration 50. EC₅₀ were calculated by nonlinear regression. Data represent mean \pm SD for n = 3 independent experiments, performed in triplicate.

AH: *Aesculus hippocastanum* L. seed extract

Figure captions

Figure 1. Influence of time of treatment with β -escin and AH on RSV infectivity and on RSV RNA synthesis. (A) For pre-infection assays, HEp-2 and A549 cells were exposed or not to β -escin (5 μ g/ml) and AH (25 μ g/ml) during 2 h, washed with PBS, and then infected with RSV A2 (moi=1) during 24 h. For co-infection, cells were simultaneously infected with RSV A2 (moi=1) and treated with the compound of interest. After 1 h adsorption, the virus-drug mixture was removed, washed with PBS, and compound free medium was added during 24 h. For p.i. assays, cells were infected with RSV A2 (moi=1) for 1 h, and then treated with the tested compound for 24 h. (B) HEp-2 and A549 cells infected with RSV A2 (moi=1) were treated or not (control) with β -escin (5 μ g/ml) and AH (25 μ g/ml) at 0, 2, 4, 6, 8, 12 and 16 h p.i. Total virus yields were determined by plaque

assay in Vero cells at 24 h p.i and plotted as the percentage of inhibition with respect to untreated–infected control (CV). (C) HEp-2 cells were infected with RSV A2 (moi=1) and treated or not (control) with β -escin (5 μ g/ml) and AH (25 μ g/ml). Total cellular RNA was extracted at the indicated times p.i. and viral RNA was quantified by qRT-PCR. The amounts of viral RNA in untreated and treated HEp-2 cells were calculated in comparison to the content of viral RNA in untreated infected cells at 6 h p.i., defined as 1. Data represent mean \pm SD for n = 3 independent experiments, performed in duplicate. *Significantly different from CV (p-value <0.05).

Figure 2. Effect of β -escin and AH on NF- κ B and AP-1 activation in RSV infected cells. J774A.1 (A), A549 (B) and HEp-2 (C) cells were infected with RSV line 19 and A2 (moi=1) and treated or not with β -escin (5 μ g/ml) and AH (25 μ g/ml) during 24 h. Luciferase activity was measured in cell extracts, and each value was normalized to β -galactosidase activity in relative luciferase units (RLUs). CC: cell control (unstimulated cells). Data represent mean \pm SD for n = 3 independent experiments, performed in duplicate. * Significantly different from RSV infected cells (p-value <0.05).

Figure 3. Effect of β -escin and AH on and cytokine production in RSV infected cells. J774A.1 (A), A549 (B) and HEp-2 (C) cells were infected with RSV line 19 and A2 (moi=1) and treated or not with β -escin (5 μ g/ml) and AH (25 μ g/ml) during 24 h. IL-6, IL-8 and TNF- α was determined by ELISA. CC: cell control (unstimulated cells). Data represent mean \pm SD for n = 3 independent experiments, performed in triplicate * Significantly different from RSV infected cells (p-value <0.05).

Figure 4. *In vivo* evaluation of β -escin and AH antiviral effect. Female Balb/c mice were infected with RSV line 19 and A2 (5×10^6 PFU) by intranasal instillation, concomitant with 10 mg/kg of AH or 1 mg/kg of β -escin by i.p. injection on day 0. On days 1–4, all mice received further inoculations of β -escin or AH i.p. (A-B) Weight was monitored and assessed as a percentage of starting weight. Day 0 refers to time right before inoculation. * at day 2, values for AH-treated infected mice were significantly higher than infected mice (p-value <0.05). (C) The animals were killed on day 4 and the lungs were used for titration of infectious virus. Data show mean \pm SD from n = 6 mice/condition. *Significantly different from RSV infected mice (p-value <0.05).

Figure 5. Cytokines gene expression determined by real-time PCR. Pulmonary cytokines expression was assessed on day 8 p.i. and the data were analyzed using the $2^{-\Delta\Delta Ct}$ formula. Actin was used as an internal for determination of gene expression. Data show mean \pm SD from n = 3 mice/condition. *Significantly different from RSV infected mice (p-value <0.05).

Figure 6. Lung histology. Light micrographic images of pulmonary histology of H&E-stained lungs collected at day 8 p.i., shown at original magnification x100, representative of n = 3/condition.

Supplementary Figure 1. Dose-dependent response of β -escin and AH on RSV replication and inactivation. (A) and (B), antiviral activity of different concentrations of β -escin and AH against RSV strains A2 and line 19 in HEp-2 and A549 cells, respectively. (C) virucidal activity of different concentrations of β -escin and AH against RSV strains A2

and line 19. Data represent mean \pm SD for $n = 3$ independent experiments, performed in triplicate.

Supplementary Figure 2. Effect of β -escin and AH on NF- κ B and AP-1 activation and cytokine production in TLRs stimulated epithelial cells. HEP-2 and A549 cells were stimulated with Toll Like Receptor (TLR)2/6 (100 ng/ml) and TLR3 (10 μ g/ml) ligands and treated or not with β -escin (5 μ g/ml) and AH (25 μ g/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 and D) IL-6 and IL-8 was determined by ELISA. CC: cell control (unstimulated cells). Data represent mean \pm SD for $n = 3$ independent experiments, performed in duplicate. *Significantly different from TLRs stimulated cells (p-value <0.05).

Figure 1

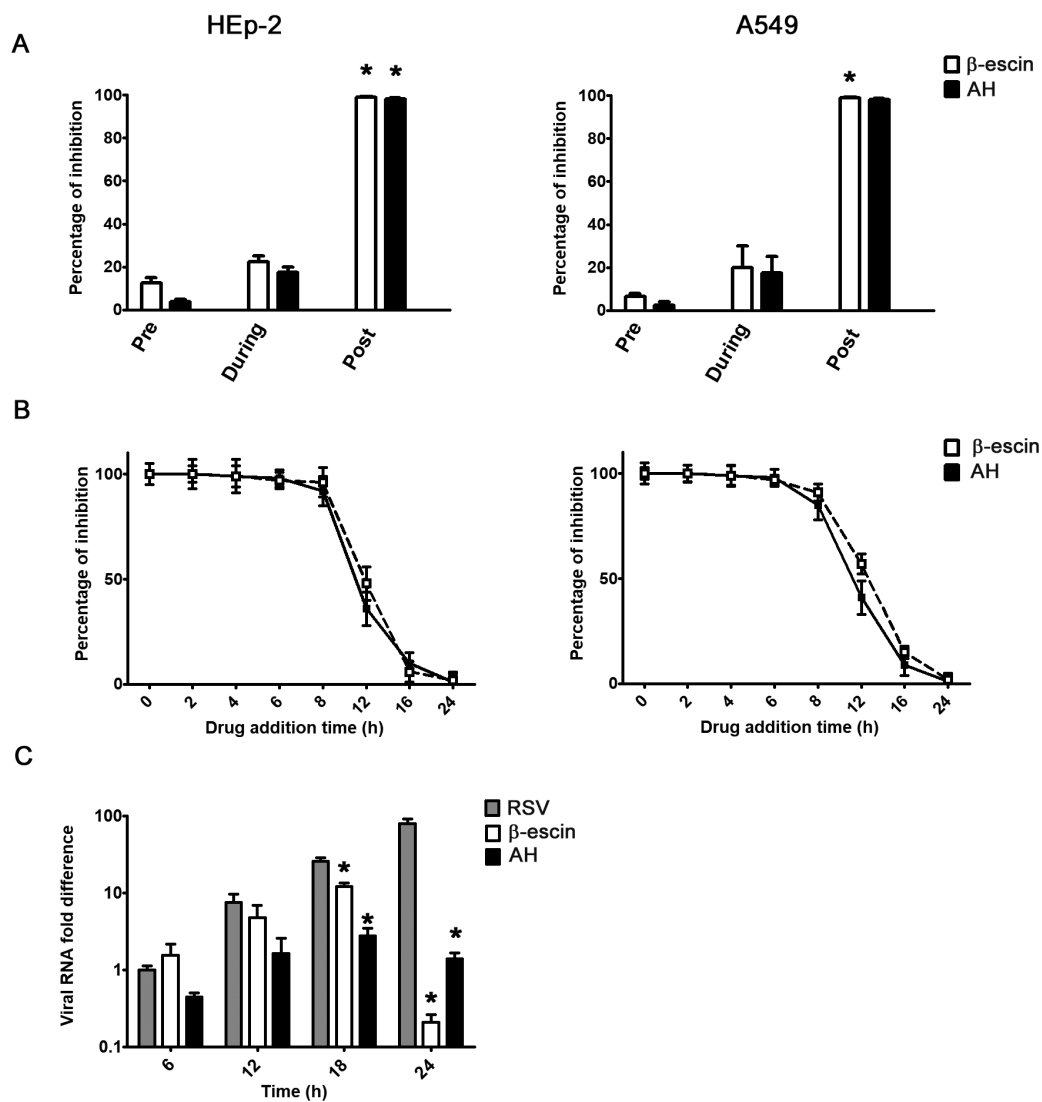


Figure 2

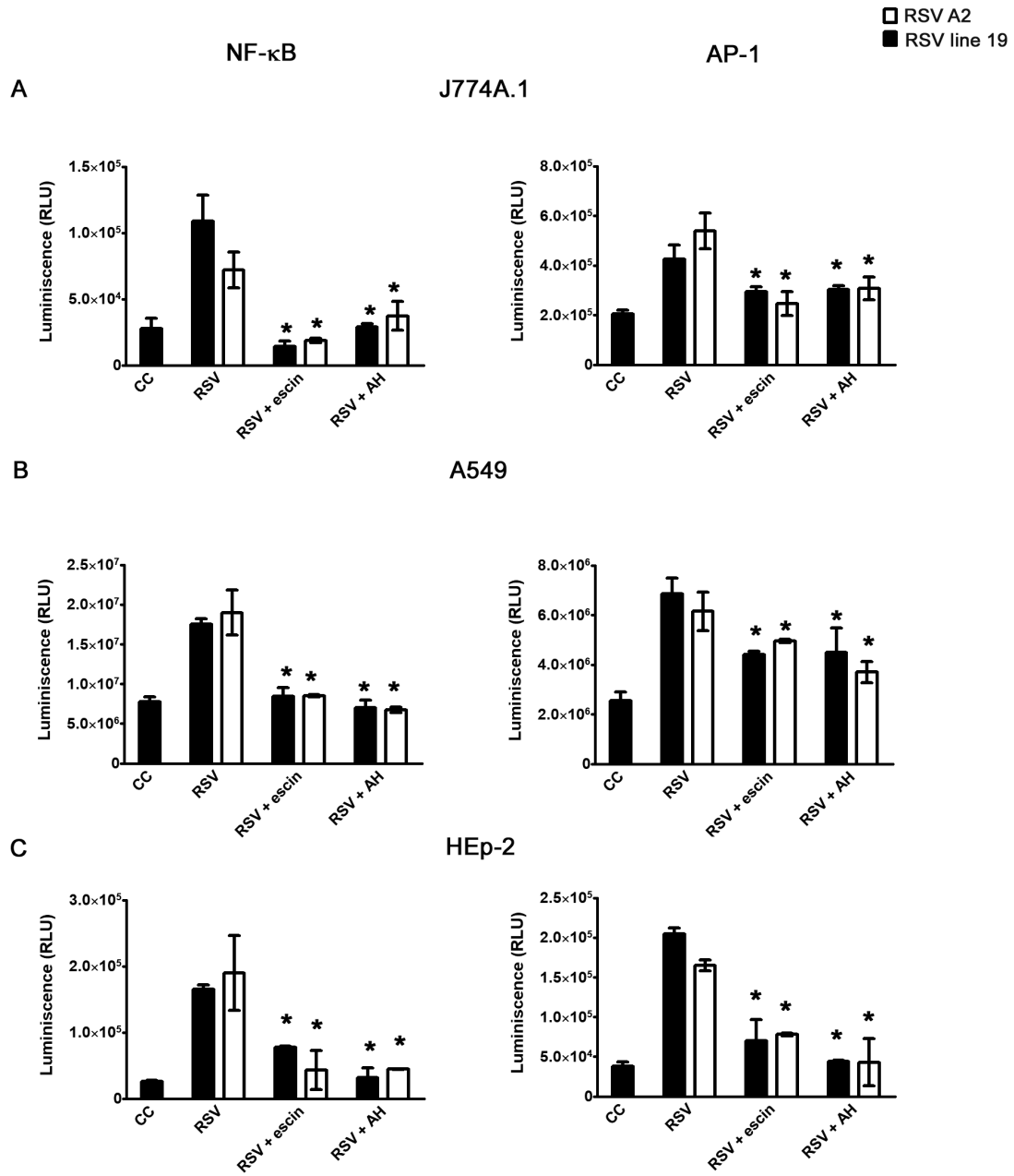


Figure 3

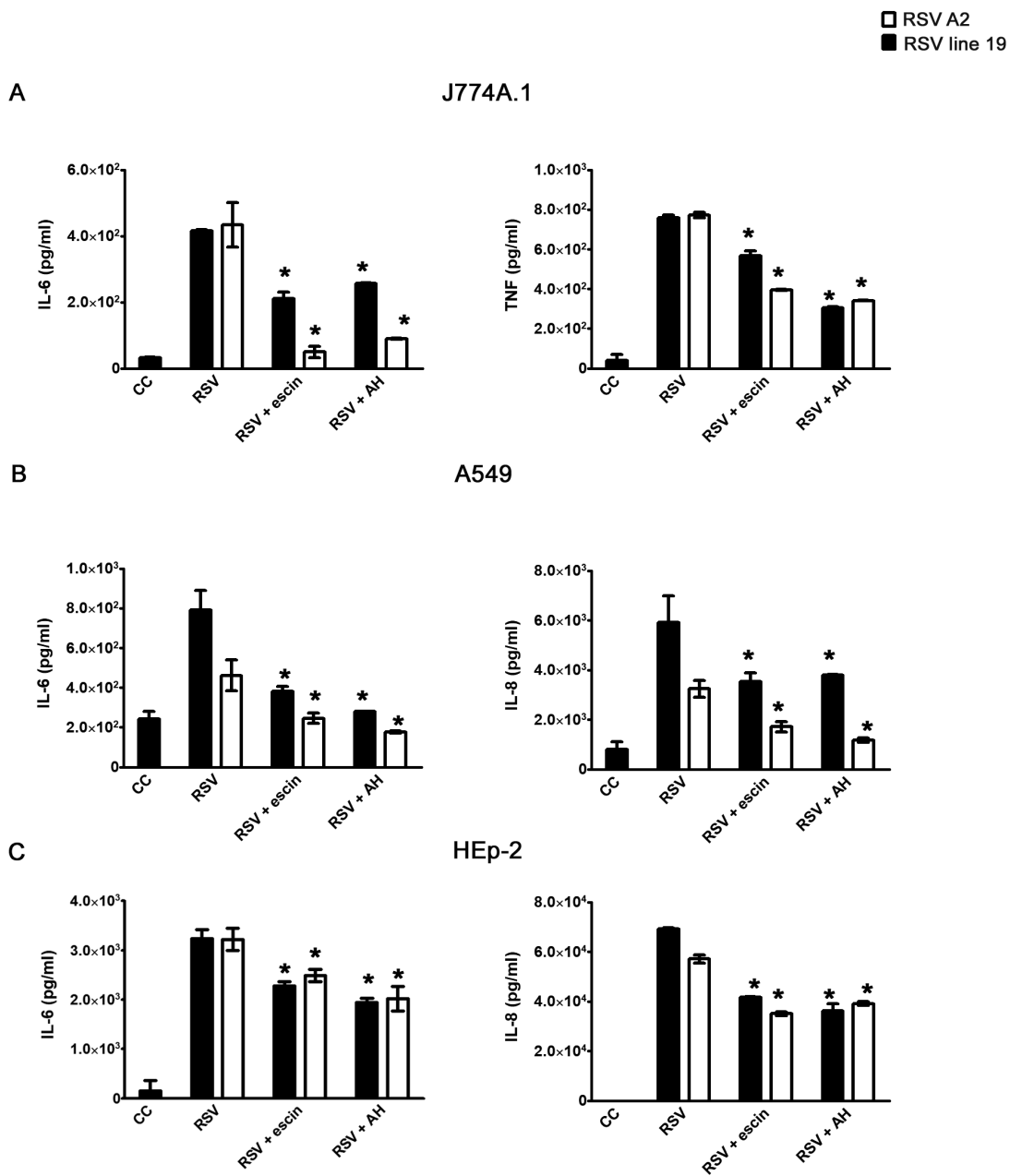
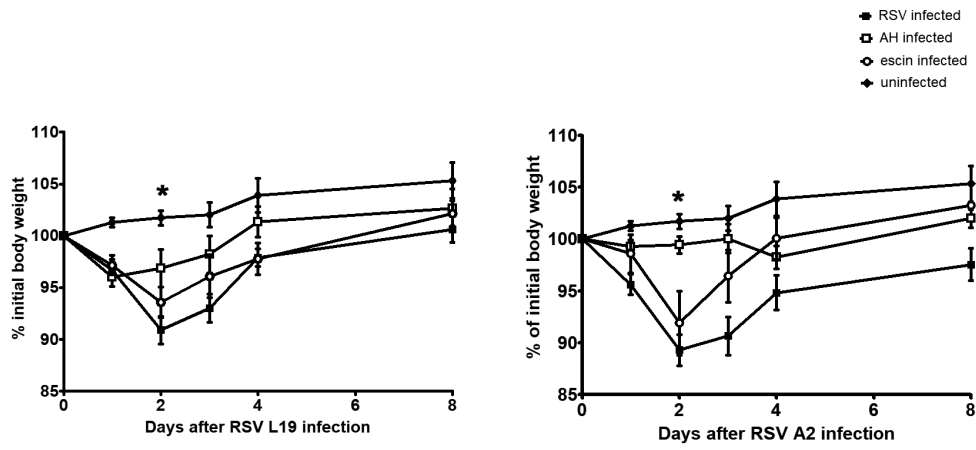
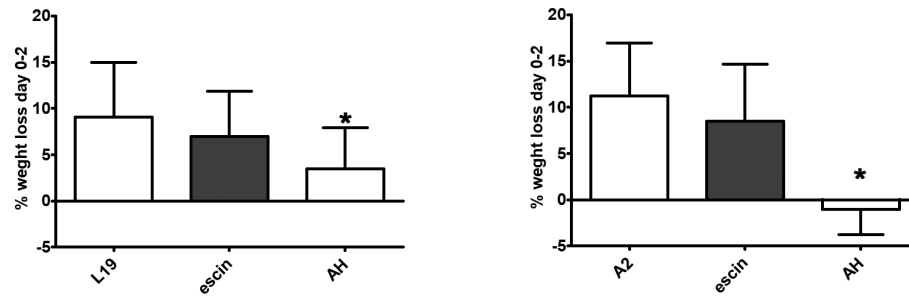


Figure 4

A



B



C

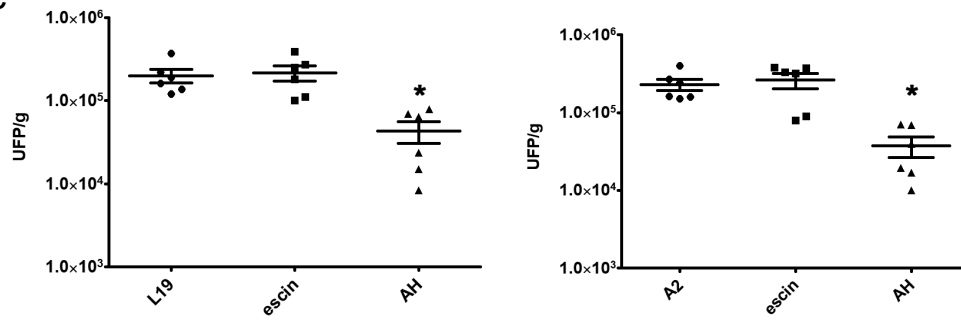


Figure 5

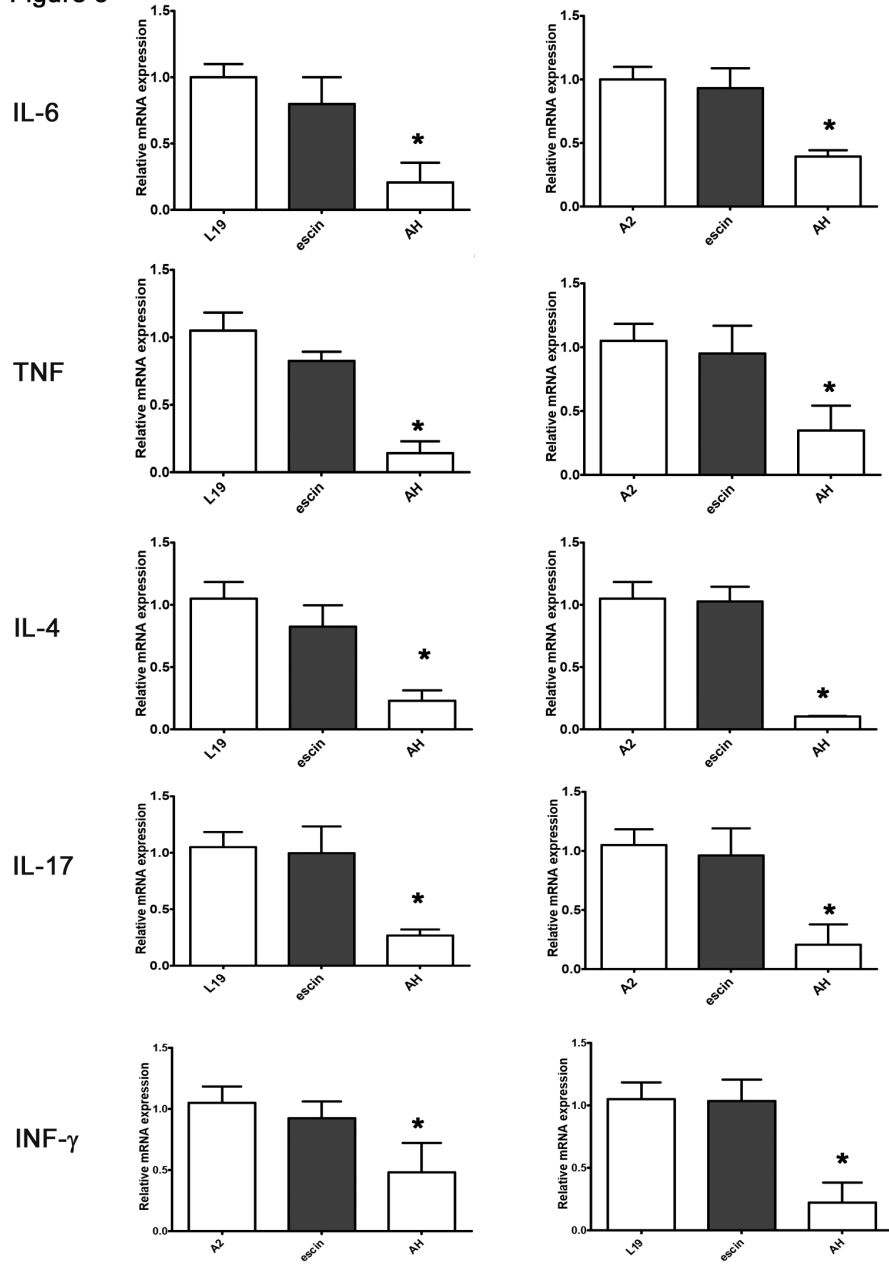
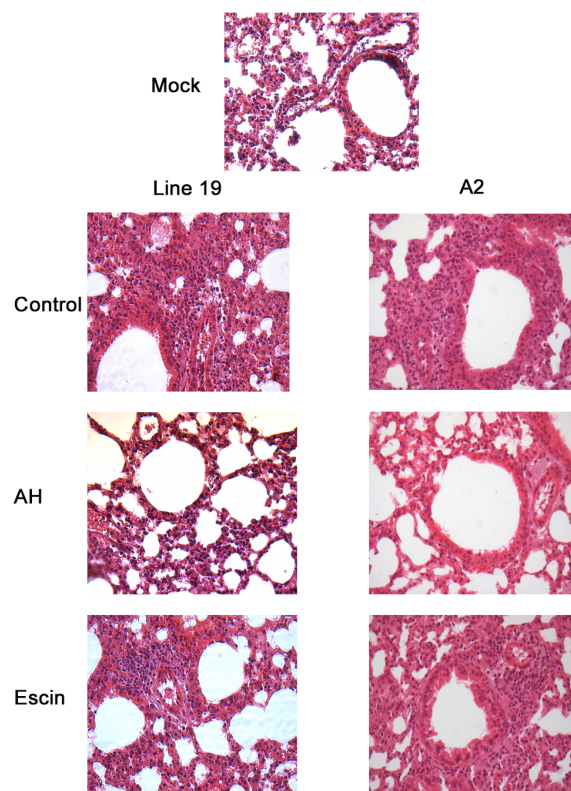


Figure 6



Highlights

Aesculus hippocastanum L. seed extract (AH) and β -escin show virucidal and antiviral activities against RSV *in vitro*

AH and β -escin show NF- κ B, AP-1 and cytokine modulating activities in RSV infected epithelial and macrophage cell lines

AH, but not β -escin, decreases weight loss and reduces lung titers and inflammation in a murine model of RSV infection.