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***Ficus religiosa* L. bark extracts inhibit Human Rhinovirus and Respiratory Syncytial**

Virus infection *in vitro*

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Running title: Antiviral activity of *Ficus religiosa* L. bark extracts

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ABSTRACT
ETHNOPHARMACOLOGICAL RELEVANCE:

Ficus religiosa L. is one of the most relevant members of the family of *Moraceae*. It is the most sacred tree of South Asia, and it is used in traditional Ayurvedic and Unani medicine to cure respiratory disorders like cough, wheezing and asthma. Some studies were performed to investigate the anti-asthmatic potential of *F. religiosa* bark, leaves and fruit extracts but none of them tested their antiviral activity against viruses responsible for the exacerbation of wheezing and asthma.

AIM OF THE STUDY:

The present study was undertaken to investigate the antiviral activity of *F. religiosa* L. extracts against respiratory viruses such as human respiratory syncytial virus (RSV) and human rhinovirus (HRV).

MATERIALS AND METHODS:

The antiviral activity of *F. religiosa* L. was tested *in vitro* by plaque reduction and virus yield assays and the major mechanism of action was investigated by virus inactivation and time-of-addition assays.

RESULTS:

F. religiosa L. methanol bark extract was the most active against HRV with an EC_{50} of 5.52 µg/mL. This extract likely inhibited late steps of replicative cycle. Water bark extract was the most active against RSV with an EC_{50} between 2.23 and 4.37 µg/mL. Partial virus inactivation and interference with virus attachment were both found to contribute to the anti-RSV activity. Replication of both viruses was inhibited in viral yield reduction assays.

CONCLUSIONS:

The results of the present study demonstrate that *F. religiosa* L. is endowed with antiviral activity against RSV and HRV *in vitro*. Further work remains to be done to identify the active components and to assess the therapeutic potential *in vivo*.

1. INTRODUCTION

Respiratory infections represent one of the most important and widespread class of human diseases. Primary infections take place mostly during early childhood, with more than 50% of children experiencing lower respiratory tract (LRT) diseases. According to molecular diagnostics, most of the wheezing episodes occurring during the first five years of life are associated with viral infections (Jackson et al.*,* 2008), with respiratory syncytial virus (RSV), human rhinovirus (HRV), and human metapneumovirus (Jartti et al., 2004; Calvo et al., 2007; Garcia-Garcia et al., 2007; Fujitsuka et al., 2011) being the most common viruses identified during early-life wheezing illnesses. Data gathered so far demonstrate that both RSV and HRV represent an important risk factor associated with wheezing illness and subsequent increased risk of asthma development (Sigurs et al., 2010; Jackson et al., 2012) RSV is the major cause of LRT infections (LRTIs) in the first months of life. Epidemiological studies have clearly demonstrated that severe RSV bronchiolitis is frequently associated with subsequent persistent wheezing, childhood asthma or both (Stein et al., 1999; Gern and Busse, 2002; Henderson et al., 2005; Jackson et al.; 2008; Koponen et al., 2012). HRVs are the most commonly identified viruses involved in wheezing exacerbations in older infants and adolescents (Johnston et al., 1995; Busse and Gern, 1997; Rakes et al., 1999; Wennergren and Kristjansson, 2001; Heymann et al., 2004; Blanken et al., 2013).

Moreover HRVs cause upper respiratory tract infections at all ages and are the main cause of LRTIs leading to hospitalisation in infants and young children outside of the winter RSV bronchiolitis season (Montalbano and Lemanske, 2002; Kotaniemi-Syrjänen et al., 2003;;

Hayden, 2004; Korppi et al., 2004; Miller et al., 2007; Wiehler and Proud, 2007; Peltola et al., 2008; Lachowicz-Scroggins et al., 2010; Jartti and Korppi, 2011; Rollinger and Schmidtke,2011; Skevaki et al., 2012; Aya Takeyama et al., 2014).

At present, specific antiviral molecules are available only against RSV, namely Palivizumab, a RSV-specific monoclonal antibody used for immunoprophylaxis in high risk preterrm infants only due to its high cost (Wu et al., 2008) and ribavirin, a guanosine analogue which use is controversial because of its low efficacy and non negligible side effects (Sidwell and Barnard, 2006).

F. religiosa L. is one of the most relevant members of the family of *Moraceae*, native of Nepal, India, Bangladesh, Myanmar, Pakistan and Sri Lanka. It is the most sacred tree of South Asia, to both Hindus and Buddhists, holding great relevance in Indian culture and being referenced in several ancient Indian treatise and holy texts (Prasad et al., 2006). Therapeutic uses of *F. religiosa* L. in Hindu and South-Asian traditional systems of medicine like Ayurveda and Unani are well documented, including the use to treat symptoms of respiratory disorders like cough, wheezing and asthma (Singh et al., 2011). Although several studies attempted to validate its traditional medicinal uses focusing on the anti-asthmatic potential of *F. religiosa* L. bark, leaves and fruit extracts (Joshi and Joshi, 2000; Sharma et al., 2001; Singh et al., 2002; Mahishi et al., 2005; Kunwar and Bussmann, 2006) none assessed the antiviral activity against respiratory viruses.

The present study was undertaken to investigate *in vitro* the antiviral activity *F. religiosa* L. bark and leaves extracts against two respiratory viruses, namely RSV and HRV, which are associated with wheezing illness and asthma exacerbation. Here, we report on the

cytotoxicity, the antiviral potency, and the probable mechanisms of antiviral action of the extracts.

2. MATERIALS AND METHODS.

2.1. Plant material. The bark and leaves of *F. religiosa* L. were collected from medicinal plant garden of Birla Institute of Technology, Mesra, Ranchi and authenticated by Botanical Survey of India, Central National Herbarium, Botanical Garden, Howrah (Letter No. CNH/11/2014/Tech.II/ dated 26-03-2014). A specimen has been deposited at the herbarium (voucher no, C-130176: PA-8).

2.2. Preparation of extracts. The fresh bark and leaves of *F. religiosa* L. were completely dried at 40–45°C and pulverized in a knife grinder to fine powder (250 μm-particle diameters). The powdered bark (250 g) was then successively extracted by soxhlation using petroleum ether (60-80), chloroform, ethyl acetate and methanol. Water extract of bark was prepared by decoction. The powdered leaves were extracted by maceration using methanol and water at room temperature. The extracts were filtered and evaporated to dryness using a rotator evaporator under controlled temperature and reduced pressure. The obtained extracts were lyophilized, stored in desiccators (Singh et al., 2013; Silva et al., 2014). Before use for antiviral assays, extracts were resuspended in a final concentration of 25 mg/ml. Water extracts were resuspended in sterile water while other extracts were resuspended in sterile DMSO. Every experiment was performed with freshly resuspended extracts.

2.3.   Phytochemical investigations of *F. religiosa* L. extracts.

The plant extracts were submitted to preliminary phytochemical screening according to the methods previously described (Egwaikhide and Gimba, 2007; Manosroi et al., 2010). Briefly, flavonoids, tannins, saponins, alkaloids and steroids/triterpenoids, were detected by Shinoda test, FeCl$_3$, Frothing test, Dragendorff's reagent and Salkowski test respectively.

Table 1 Phytochemical analysis of *F. religiosa* L. extracts

Source	Extract	% Yield	Saponins	Carbohydrate	Alkaloids	Flavonoids	Tannins	Steroids
Leaves	Water	6.23	+	+	-	+	-	-
	Methanol	8.41	+	-	-	+	+	+
Bark	Ethylacetate	5.27	-	-	+	-	+	-
	Water	9.26	+	+	-	+	+	-
	Chloroform	8.14	-	-	+	-	+	+
	Methanol	10.24	+	-	-	+	+	+

2.4.   Cells. Human epithelial adenocarcinoma HeLa cells (ATCC® CCL-2™) were propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Gibco-BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany),

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at 37°C in an atmosphere of 5% of CO_2. Human epithelial cells Hep-2 (ATCC CCL-23) and A549 (ATCC CCL-185) were grown as monolayers in Eagle's minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% FBS and 1% antibiotic-antimycotic solution.

2.5.   Viruses. RSV strain A2 (ATCC VR-1540) was propagated in Hep-2 and titrated by the indirect immunoperoxidase staining procedure using an RSV monoclonal antibody (Ab35958; Abcam, Cambridge, United Kingdom) as described previously (Cagno et al., 2014).

Human rhinovirus 1A (ATCC® VR-1559) was propagated in HeLa cells, at 33°C, in a humidified 5% CO_2 incubator. When the full cytopathic effect (CPE) developed, cells and supernatants were harvested, pooled, frozen and thawed three times, clarified and aliquoted. Viruses were stored at −70°C. Rhinovirus titers were determined by the standard plaque method as described previously (Civra et al., 2014).

2.6.   Cell viability. Cell viability was measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Confluent cell cultures seeded in 96-well plates were incubated with different concentrations of extracts in triplicate under the same experimental conditions described for the antiviral assays, . Cell viability was determined by the CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Absorbances were measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. The effect on cell viability at different concentrations of the compound was expressed as a percentage, by comparing absorbances of treated cells with the ones of cells incubated with culture medium alone for water extract, or with culture medium supplemented with DMSO in equal volume for other extracts. The 50%

cytotoxic concentrations (CC_{50}) and 95% confidence intervals (CIs) were determined using Prism software (Graph-Pad Software, San Diego, CA).

2.7.   Rhinovirus inhibition assay. HeLa cells were first seeded (at 8×10^4 cells/well) in 24 well plates. 24 hours later the extracts were serially diluted in medium (from 300 to 0.4 μg/ml) and added to cell monolayers. After 2h of incubation (37°C, 5% CO_2), medium was removed and infection was performed with 200 μL/well with HRV 1A (multiplicity of infection (MOI) 0.0002 plaque forming units (PFU)/cell) and different concentrations of the extracts. The infected cells were incubated at 34°C for 1h, allowing the virus to attach and enter the cells. After incubation, cells were washed with medium, and overlaid with a 1:1 combination of 1.6% SeaPlaque Agarose and 2X DMEM containing the extract. Depending on the extract, control wells (100% of infection) were prepared by treating cells with equal volumes of DMSO or sterile water. The plates were incubated at 34°C for 3 days. After incubation, the plates were fixed with 7.5% formaldehyde (Fluka) and stained with crystal violet (Sigma, St. Louis, Mo.). The number of plaques formed was counted. To test the ability of extracts to limit the production of viral progeny resulting from multiple cycles of viral replication, HeLa cells were plated in 24-well trays, until they reached confluence. Each well was inoculated with 200 μL of a stock of HRV 1A (MOI 0.0002 PFU/cell) and incubated for 1h. After incubation, cells were washed with medium, and fresh medium was added (DMEM supplemented with 2% FBS) containing different concentrations of extracts. Infected cells and supernatants were harvested 5 days post-infection (when a complete CPE was visible), and titrated by using the plaque method as described above.

2.8.   RSV inhibition assay. A549 cells were first seeded (at 8×10^3 cells/well) in 96 well plate. The extracts were serially diluted in medium (from 300 to 0.4 μg/ml) and

incubated with cells for 2 h at 37°C, then mixtures of compound and virus (MOI 0.01 PFU/cell) were added to cells to allow the viral adsorption for 3h at 37°C; the monolayers were then washed and overlaid with 1.2% methylcellulose medium containing serial dilutions of extracts. Depending on the extract, control wells (100% of infection) were prepared by treating cells with equal volumes of DMSO or sterile water. Three days post-infection, cells were fixed with cold methanol and acetone for 1 min and subjected to RSV-specific immunostaining. Immunostained plaques were counted, and the percent inhibition of virus infectivity was determined by comparing the number of plaques in treated wells with the number in untreated control wells. For the virus yield reduction assay, A549 and Hep-2 cells were infected with RSV MOI 0.05 PFU/cell and following virus adsorption (3h at 37°C), the viral inoculum was removed and cultures were exposed to different concentrations of extracts and incubated until control cultures displayed extensive cytopathology. Supernatants and cells were harvested and cell-free virus infectivity titers were determined in duplicate by the plaque assay in A549 or Hep-2 cell monolayers. Percents of inhibition were determined by comparing the titer measured in the presence of the compounds to that measured in untreated wells.

2.9.   Virus inactivation assay. Approximately 10^5 PFU of rhinovirus 1A and of RSV and 33 µg/mL respectively of bark methanol or acqueous extract were added to medium and mixed in a total volume of 100 µL. The virus-compound mixtures were incubated for 0 or 2h at 37°C then diluted serially to the non-inhibitory concentration of test compound, and the residual viral infectivity determined by standard plaque method as described above.

2.10.   Time of addition assay. Serial dilutions of extracts were added on cells 2h before infection or during infection or post infection. After the incubation time described before, viral plaques were counted.

2.11. **Attachment assay**. Serial dilutions of extracts were mixed with RSV and added to cooled cells and incubated for 2h at 4°C to ensure viral attachment but not entry. After two gentle washes, cells were overlaid with 1.2% methylcellulose medium, shifted to 37°C for 24 or 72h and successively syncytia were counted.

2.12. **Entry assay**. For entry assay, RSV at a MOI of 0.01 PFU/cell was adsorbed for 2h at 4°C on pre chilled confluent A549 cells. Cells were then washed with cold MEM three times to remove unbound virus, treated with different concentrations of extract, and incubated for 3h at 37°C. Unpenetrated viruses were inactivated with acidic glycine for 2 min at room temperature, as previously described (Cagno et al., 2015). Cells were then washed with warm medium three times and treated as described above for plaque reduction assay.

2.13. **Data analysis.** All results are presented as the mean values from three independent experiments. The half maximal effective concentration (EC_{50}) values for inhibition curves were calculated by regression analysis using the program GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, U.S.A.) to fit a variable slope-sigmoidal dose–response curve. A selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} value. The statistical significance of differences between EC50s obtained in the time of addition assays was assessed by extra sum-of-squares F test GraphPad Prism 5.00 (GraphPad Software). Inhibition of viral yield in the presence and absence of extracts was compared by analysis of variance (ANOVA) followed by a Bonferroni test if P values showed significant differences, using GraphPad Prism 5.00 (GraphPad Software).

3.   RESULTS AND DISCUSSION

Table 1 reports the qualitative preliminary results about the presence/absence of some chemical classes of metabolites in the extracts. The antiviral activity of *F. religiosa* L. was initially investigated by a complete protection assay in which increasing concentrations of bark and leaves extracts were added to the cell culture before, during, and after the infection with HRV or RSV to generate dose-response curves. Ribavirin was tested in parallel as a reference compound for RSV while no drug is currently available for HRV. The EC_{50} values shown in Table 2a reveal a different spectrum of activity against the two viruses. Methanol bark extract (in which saponins, flavonoids, tannins and steroids were revealed), proved to be the most active against HRV (EC_{50}: 5.52 µg/mL) while water bark extract positively tested for saponins, carbohydrates, flavonoids and tannins was the most active against RSV (EC_{50}: 4.37 µg/mL). Results on RSV inhibition were confirmed also on Hep-2 cells (Table 2b). Of note, the most active extracts showed favorable SI values, demonstrating that the antiviral effect was not a consequence of cytotoxicity. Notably, the cell monolayers corresponding to the untreated controls (100% of infectivity) were treated with volumes of DMSO ranging from 1.2% (v/v) to 0.0048% (v/v) in cell media: these low volumes of DMSO are non cytotoxic and do not influence HRV or RSV replication (data not shown).

Table 2a Antiviral activity of *F. religiosa* L. extracts

Virus	Source	Extract	EC_{50}* ($\mu g/mL$) – 95% C.I. [#]	EC_{90}[§] ($\mu g/mL$) – 95% C.I.	CC_{50}[†] ($\mu g/mL$)	SI[‡]
HRV	Leaves	Water	n.a.	n.a.	>300	n.a.
		Methanol	n.a.	n.a.	>300	n.a.
	Bark	Ethyl acetate	61.33 (35.46-106.1)	n.a.	>300	>4.89
		Water	55.72 (35.72-86.92)	94.73 (52.04-172.4)	>300	>5.38
		Chloroform	22.26 (17.89-27.68)	52.25 (36.80-74.19)	216.7	9.73
		Methanol	5.52 (4.64-6.56)	12.16 (8.62-17.14)	66.49	12.1
RSV	Leaves	Water	10.61 (6.80-16.56)	59.39 (22.19-158.9)	>300	>28.3
		Methanol	n.a.	n.a.	>300	n.a.
	Bark	Ethyl acetate	35.48 (28.26-44.54)	124.7 (73.11-112.8)	293.1	8.25
		Water	4.37 (3.18-6.01)	18.34 (9.032-37.22)	>300	>69.0
		Chloroform	12.71 (8.51-18.96)	45.04 (17.48-116.1)	74.7	5.87
		Methanol	4.83 (4.62-	32.27 (15.44-	53.2	11.0

| | | | 8.50) | 67.45) | | |
| Reference compound | Ribavirin | | 5.01 (3.64-6.91) | 10.47 (4.99-21.96) | >300 | >59.9 |

*EC_{50} half maximal effective concentration

#C.I. confidence interval

§EC_{90} 90% effective concentration

†CC_{50} half maximal cytotoxic concentration

‡SI selectivity index

n.a. not assessable

Table 2b Anti-RSV activity on Hep-2 cells

Virus	Source	Extract	EC_{50} (µg/mL) – 95% C.I.	EC_{90} (µg/mL) – 95% C.I.	CC_{50} (µg/mL)	SI
RSV	Bark	Water	2.23 (1.15-3.28)	20.56 (28.52-49.6)	188.5	84.5
	Control Compound	Ribavirin	6.67 (4.89-6.09)	17.95 (9.95-32.4)	>300	>45.0

Based on the favourable SIs, methanol and water bark extracts were selected for further studies, in order to identify their major mechanism of action. To explore whether the extracts directly inactivate HRV or RSV particles, a virus inactivation assay was performed with a concentration of extracts that reduces almost completely virus infection (>EC_{90}). To this end, HRV and RSV aliquots (10^5 PFU) were incubated with 33 µg/mL of respectively methanol and water bark extract at 37°C for 0 or 2h. After incubation, the HRV and RSV samples were titrated respectively on HeLa or A549 cells using high dilutions at which extracts were no

longer active as an antiviral. As shown in Fig. 1, the water extract produced a modest yet significant loss of RSV titer (P<0.01) only when the incubations were carried out for 2h at 37°C. By contrast, no virucidal activity was observed for HRV at any assay condition.
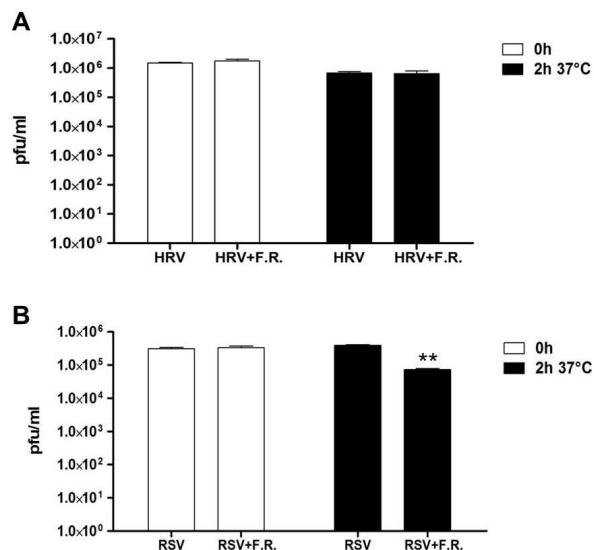

Figure 1. Evaluation of virus inactivation by *F. religiosa* L. methanol and water bark extracts respectively on infectious HRV (panel A) or RSV particles (panel B). On the *y* axis, the infectious titers are expressed as plaque-forming units per mL (PFU/mL). On the two graphs, error bars represent the SEM of 3 independent experiments. ** p < 0.01

These results indicate that a direct virus inactivation is not (HRV) or is only a minor (RSV) mechanism of action of the extracts, suggesting that they most probably target cell-surface or intracellular events involved in essential steps of the HRV and RSV replicative cycles. Therefore, we performed a series of time-of-addition assays, in which the extracts were added on cells only before, or during, or after infection to assess which step(s) of viral replication is inhibited by these extracts. As shown in Figure 2, the extracts showed different activity profiles against HRV and RSV. When the methanol extract was tested against HRV, it was possible to determine an EC_{50} for each of the three different protocols, although the extract

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showed a significantly (p<0.001) higher antiviral activity when added after the virus inoculum (EC_{50} = 6.40 µg/mL). By contrast, the water extract exerted a dose-dependent inhibitory activity against RSV only when added during infection with an EC_{50} value of 3.32 µg/mL.
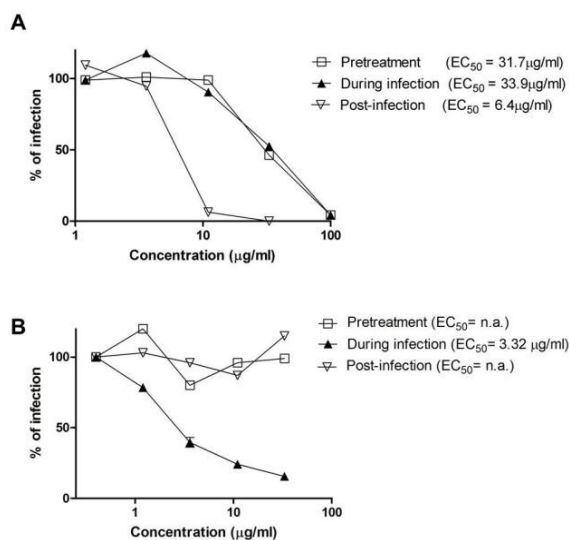

Figure 2 Time-of-addition assays. Cells were treated with methanol bark extracts for HRV experiments (panel A) or water bark extracts for RSV experiments (panel B) for 2h before infection (pre-treatment), for 1h during infection or by adding the extracts immediately after infection (post-infection). The virus titers in the treated samples are expressed as a percentage of the titer obtained in the untreated control. Where possible, the statistical significance of differences between EC50s was assessed by extra sum-of-squares F test.

Results from the time-of-addition assays suggest that the water extract may target early steps of the RSV replicative cycle (i.e. virus attachment or entry into cells). To test this hypothesis, we carried out an attachment assay, an experimental condition in which the virus is allowed to bind to the surface of the host cells, in the presence or absence of the water extract of *F. religiosa* L., but do not undergo cell entry. As shown in Fig. 3, the extract inhibited RSV

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infectivity with an EC_{50} (3.65 µg/mL) even lower than the one reported in Table 1. By contrast, when the antiviral activity was tested using an entry assay, in which the water extract was added immediately after virus attachment, only a weak inhibition was observed with an EC_{50} of 48.9 µg/mL.

Taken together, these results indicate that the antiviral activity of the water extract against RSV depends, at least in part, on its capacity to prevent the attachment of the virus particles to the cell surface.
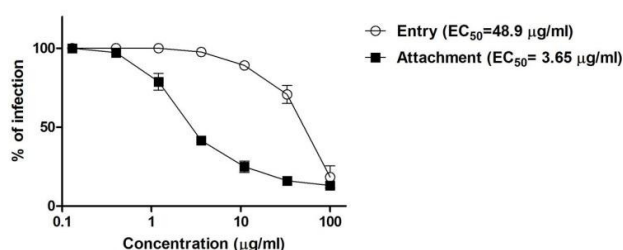

Figure 3. RSV entry and attachment assays. The water bark extract of *F. religiosa* L. was added to the cell culture during RSV-cell binding (attachment assay) or RSV-cell penetration (entry assay). The number of infected cells in the treated samples is expressed as a percentage of the untreated control. Each point represents mean and SEM for triplicates.

The antiviral activity of *F. religiosa* L. extracts was evaluated further by viral yield reduction assay – a more stringent test which allows multiple cycles of viral replication to occur before measuring the production of infectious viruses. As shown in Figure 4, methanol and water bark extracts treatment reduced the titer of viral progeny respectively of HRV and RSV in a dose-dependent manner, showing EC_{50} values ranging from 3.96 µg/mL to 15.2 µg/mL, respectively. These results indicate that besides preventing initial viral infection of HRV and RSV, *F. religiosa* L. extracts can also limit ongoing infection *in vitro*. This feature might be

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relevant for *in vivo* infections, characterized by the continuous release of virions by infected
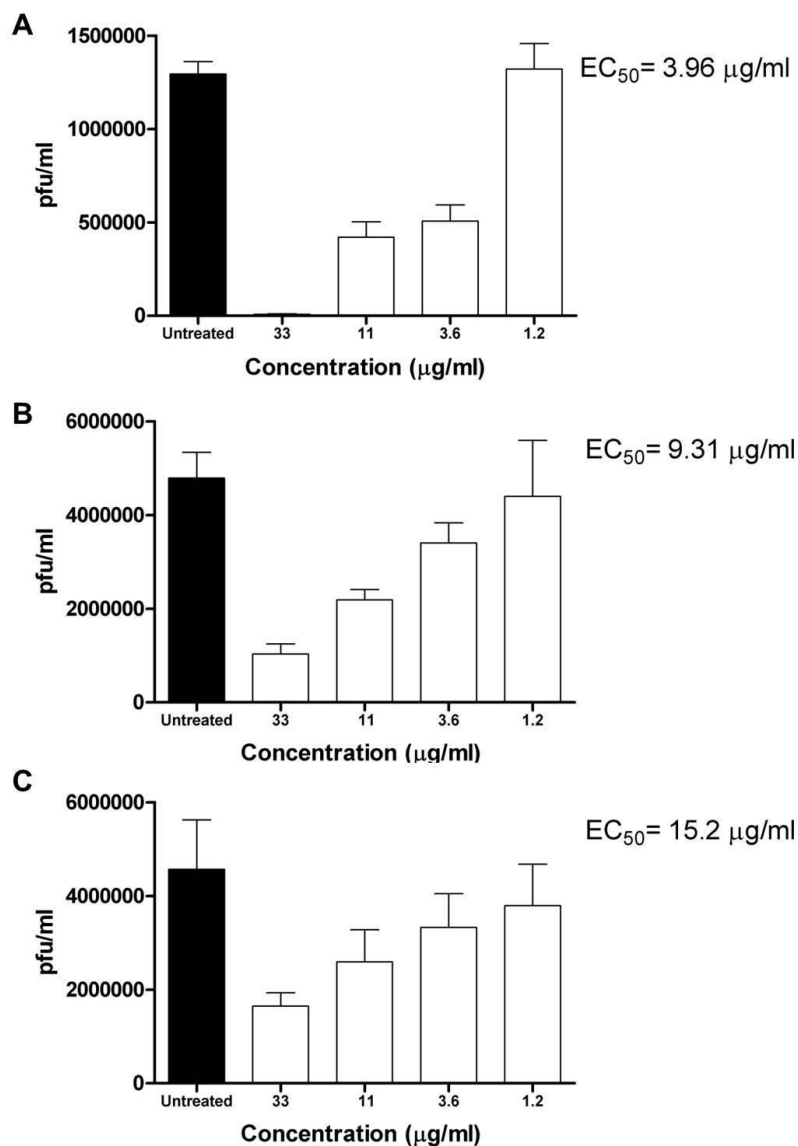
cells that promptly interact with neighboring cells.

Figure 4 . HRV and RSV yield reduction assay. The methanol (panel A) or water (panel B

A549 cells, and C Hep-2 cells) bark extract of *F. religiosa* L. were added to the cell culture

after infection. When the cytopathic effect involved the whole monolayer of untreated cells,

the supernatant was harvested and titrated. Plaques were counted and percent infection

calculated by comparing treated with untreated (control) wells in order to determine EC_{50}

values. Viral titers (expressed as PFU/mL) are shown as means plus SEM for three independent experiments . *, P<0.05.

Some conclusions can be drawn from the data presented so far. The most active extract against RSV is the water bark extract and the mechanism of action involves a modest virus inactivation activity and a marked inhibition of virus attachment. On the other hand, the methanolic bark extract was the most effective against HRV and proved active only when added to the cells after virus inoculum indicating that later steps of the virus replicative cycle are inhibited (e.g. virus uncoating, genome replication, virus assembly or exit). A more exhaustive study of the mechanism of action of both extracts will be obtained with a bioassay-guided fractionation procedure to identify the active compounds and verify their mode of action. A common feature between the two extracts is their inhibitory activity in virus yield reduction assays suggesting a possible therapeutic activity to treat ongoing infections *in vivo.*

Overall, the results of the present study demonstrate that *F. religiosa* L. constitutes a natural source of antiviral substances endowed with antiviral activity against two respiratory viruses which are associated with wheezing illness and asthma exacerbation. Thus, they are consistent with the use of *F. religiosa* L. in traditional medicine to treat respiratory disorders (Singh et al., 2011). Further work remains to be done to identify its antiviral constituents and to assess its therapeutic potential *in vivo*.

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FIGURE LEGENDS

Figure 1. Evaluation of virus inactivation by *F. religiosa* L. methanol and water bark extracts respectively on infectious HRV (panel A) or RSV particles (panel B). On the *y* axis, the infectious titers are expressed as plaque-forming units per mL (PFU/mL). On the two graphs, error bars represent the SEM of 3 independent experiments. ** $p < 0.01$

Figure 2 Time-of-addition assays. Cells were treated with methanol bark extracts for HRV experiments (panel A) or water bark extracts for RSV experiments (panel B) for 2h before infection (pre-treatment), for 1h during infection or by adding the extracts immediately after infection (post-infection). The virus titers in the treated samples are expressed as a percentage of the titer obtained in the untreated control. Where possible, the statistical significance of differences between EC50s was assessed by extra sum-of-squares F test.

Figure 3. RSV entry and attachment assays. The water bark extract of *F. religiosa* L. was added to the cell culture during RSV-cell binding (attachment assay) or RSV-cell penetration (entry assay). The number of infected cells in the treated samples is expressed as a percentage of the untreated control. Each point represents mean and SEM for triplicates.

Figure 4 . HRV and RSV yield reduction assay. The methanol (panel A) or water (panel B A549 cells, and C Hep-2 cells) bark extract of *F. religiosa* L. were added to the cell culture after infection. When the cytopathic effect involved the whole monolayer of untreated cells, the supernatant was harvested and titrated. Plaques were counted and percent infection calculated by comparing treated with untreated (control) wells in order to determine EC_{50} values. Viral titers (expressed as PFU/mL) are shown as means plus SEM for three independent experiments. *, P<0.05.