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Re: Manuscript submission

Dear Editor,

We are submitting for publication in *Biochemical and Biophysical Research Communications*, our research manuscript entitled **"***Antiviral effect of an essential-oil extract derived from three traditional Cretan aromatic plants against viruses causing infections of the upper respiratory system***"** by M. Tseliou *et al*.

In a previous study, we have shown that an essential-oil extract based on three Cretan aromatic plants reduces the duration and severity of symptoms of patients with upper respiratory tract viral infections. In the current study, we tested whether this Cretan Aromatic Plants extract (CAPex) exhibits a direct antiviral activity against a wide range of respiratory viruses *in vitro*. Our results revealed a remarkable antiviral activity of CAPex against influenza A/H1N1 virus strains, influenza B and human rhinovirus 14 (HRV14). A series of experiments showed that both influenza AH1N1 and HRV14 replication and progeny virus were significantly decreased after the treatment with CAPex. Furthermore we are able to demonstrate that CAPex exerts its antiviral activity after A/H1N1 or HRV14 entry whereas it confers a preventive reactivity against RSV. Finally, CAPex resulted in a defective trafficking of influenza A Nucleoprotein, suggesting NP as a valid target of this extract. We conclude that the Cretan aromatic plants extract possesses antiviral activity and has the potential to be used as an herbal agent against influenza viruses and rhinovirus.

The presented work has not been published previously and it is not under consideration for publication elsewhere. This manuscript has been seen, reviewed and approved for publication by all contributing authors.

We are looking forward to hearing from you.

Sincerely yours,

George Sourvinos, PhD
Professor

Highlights

- The Cretan Aromatic Plants (CAPex) extract possesses impressive antiviral properties.
- CAPex inhibits Influenza A/H1N1, influenza B and human rhinovirus 14 (HRV14) infections.
- Both A/H1N1 and HRV replication and progeny virus are significantly decreased by CAPex.
- CAPex severely affects trafficking of FluA Nucleoprotein, suggesting it as a valid target of the herbal extract.
- CAPex has the potential to be used as a herbal agent against influenza and human rhinovirus.

Antiviral effect of an essential-oil extract derived from three traditional Cretan aromatic plants against viruses causing infections of the upper respiratory system

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Key words: Cretan aromatic plants, plant extract, influenza A, H1N1, rhinovirus 14, antiviral activity

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Abstract

Since an essential-oil extract based on three Cretan aromatic plants has been shown to reduce the duration and severity of symptoms of patients with upper respiratory tract viral infections, we tested whether this Cretan Aromatic Plants extract (CAPex) exhibits a direct antiviral activity against a wide range of respiratory viruses *in vitro*. Our study revealed a remarkable antiviral activity of CAPex against influenza A/H1N1 virus strains, influenza B and human rhinovirus 14 (HRV14) whereas no viral inhibition was found for influenza A/H3N2, Respiratory Syncytial Virus (RSV) and Adenovirus 5. Both influenza AH1N1 and HRV14 replication and progeny virus were significantly decreased after the treatment with CAPex. Pre-treatment with the plant extract demonstrated that CAPex exerts its antiviral activity after A/H1N1 or HRV14 entry in host cells whereas it confers a preventive reactivity against RSV. Furthermore, CAPex resulted in a defective trafficking of influenza A Nucleoprotein, suggesting NP as a valid target of this extract. We conclude that the Cretan aromatic plants extract possesses antiviral activity and has the potential to be used as an herbal agent against influenza viruses and rhinovirus.

1. Introduction

Influenza, commonly referred also as the ‘’flu’’ and common cold are among the most common upper respiratory tract viral infections, in humans, as well as in other mammals, with symptoms ranging from mild discomfort to pneumonia or even death. Influenza viruses, of type A, B and C [1], are characterized by an extremely high surface proteins (hemagglutinin and neuraminidase) mutation rate and species distribution [2] are remarkably epidemic and pandemic factors, resulting in about three million cases of severe illness and around 300,000 deaths per year, thence representing a severe health threat and a substantial economic burden both on health care systems and societies. [3]. Although influenza is the most common cause of serious viral respiratory infection, other respiratory viruses are also fatal, including respiratory syncytial virus (RSV), human rhinoviruses (HRVs) and human adenoviruses (HAdVs). RSV [4], most related with children’s respiratory infection, may also cause severe adult infection and pneumonia. HRVs represents the primary etiologic agent of the common cold, leading to significant morbidity [5], while HAdVs cause pneumonia, conjunctivitis and gastroenteritis [6].

Antiviral drugs represent the first line of treatment of respiratory viral diseases [7]. They include amantadine, rimantadine [8] and neuraminidase inhibitors [9; 10]. However, there are severe side effects associated with their administration, as well as increased toxicity [11; 12]. Subsequently, it is crucial to identify and validate novel efficient antiviral agents. Different plant extracts have been assayed as potential antiviral agents, in relation to an extensive empirical knowledge of their medical benefits [13; 14] *in vitro* [15] and *in vivo*, dealing with influenza [16; 17; 18; 19], RSV and HRV [18; 19; 20; 21].

In a previous cross-over clinical study, we have reported that an extract derived from three Cretan aromatic plants is effective in reducing the duration and severity of

symptoms of patients with upper respiratory tract viral infections, while, in the same time it decreases systemic inflammation, assayed by C-reactive protein (CRP) levels [22]. Our data were further confirmed by a post-market prospective study [23]. In the current study, the same mixture of Cretan Aromatic Plants extract (CAPex) was used in order to screen the antiviral potential in a wide range of respiratory viruses infecting the upper respiratory tract in a cell culture-based model.

2. Materials and Methods

2.1 Cretan Aromatic Plants essential-oil extract (CAPex)

CAPex (a mixture essential-oil extract of thyme or Spanish oregano (*Coridothymus capitatus* (L) Rchb. F. synonym of *Thymbra capitata* (L) Cav.), dictamnus or Cretan dittany (*Origanum dictamnus* L) and sage (*Salvia fruticosa* Mill., *Salvia pomifera* L.), isolated through steam distillation) was prepared at used in concentrations previously described [22]. Ribavirin (1-beta-d-ribofuranosyl-1,2,4-triazole-carboximaide, Sigma-Aldrich, Germany) and Amantadine (1-Adamantamine hydrochloride, 99+%, Acros Organics, USA) were included in the antiviral assays at a concentration of 25μg/ml, as positive controls [24; 25].

2.2 Cell culture, Virus infections and Cytotoxicity assays

MDCK (ATCC® CCL34™) cells and HeLa cells were grown according to standard conditions [26]. Human influenza A virus strains, influenza A FM/1/47 (H1N1) virus, influenza A PR/8/34 (H1N1) virus, influenza A Aichi/2/68/H3N2 (H3N2), influenza B Florida/4/2006 (Influenza B Florida), influenza B Brigit (Influenza B Russia), respiratory syncytial virus strain Long (RSV) and adenovirus C subtype 5 (Adeno 5) were obtained

from ATCC (Manassas VA, USA). Human rhinovirus B subtype 14 (HRV14) was kindly provided from the Universitätsklinikum Jena, Germany. Influenza strains, RSV and Adeno 5 were propagated in MDCK cells with serum free MEM containing 1µg/ml trypsin TPCK-treated (Sciex), 2mM of L-glutamine, 100 U/ml of penicillin and 0.1 mg/ml of streptomycin. HRV 14 was studied in HeLa cells with serum free DMEM containing 1µg/ml TPCK-treated trypsin, 100 U/ml of penicillin and 0.1 mg/ml of streptomycin. Evaluation of the virus titers [26; 27] as well as viral growth curves were also carried out according to standard protocols [28].

Analysis for *in vitro* cytotoxicity of CAPex was performed in MDCK and HeLa cells growing either with the daily dose of CAPex or with different dilutions (1:10, 1:100 and 1:1000 of the clinically administered concentration (15 ml extract/L, 1 ml/day) in DMSO, using the tetrazolium MTT assay in triplicates [26]. Additionally, the cytotoxicity of the tested substance onto the respective cells was monitored by microscopic examination.

2.3 Antiviral Assays

The antiviral activity of the CAPex in Influenza A and B strains, RSV, Adenovirus 5 and HRV14 was assayed in plaque-reduction assay (expressed in plaque forming units (PFU)) and in cytopathogenic effect (CPE), with ribavirin and amantadine as positive controls. After 3-4 days, cells were fixed with paraformaldehyde and cell monolayers were stained with crystal violet.

2.4 Time-of-Addition Assay

Cells were pre-treated with CAPex for 2 h and 24 h and then infected with corresponding viruses for 2 h. After viral adsorption, the cells were washed with PBS and cultured for 72h in the presence or absence of CAPex, then fixed with paraformaldehyde, stained with crystal violet and counted.

2.5 Immunofluorescence Analysis

$1x10^5$ MDCK cells were plated on glass coverslips in 24-well plates. After 24h, they were infected with influenza A FM/1/47/H1N1 for 1 h at 37^{o}C. MEM with or without CAPex was added and after 12 h, cells were fixed with 4% PFA and permeabilized with 0.1% TritonX-100. Cells were stained with a mouse monoclonal anti-NP (Santa Cruz), followed by a secondary FITC-labeled goat anti-mouse antibody (Santa Cruz). The nuclei were stained with DAPI. Fluorescent images were acquired with an epifluorescent Leica DMIRE2 microscope equipped with a Leica DFC300FX digital camera.

2.6 Real-time quantitative reverse transcription PCR (qRT-PCR)

Cells ($2X10^5$) were plated in 12-well plates and after 24h infected with influenza A FM/1/47/H1N1 or HRV14 for 2 h at 37^{o}C. Subsequently, cells were incubated in the absence (control) or the presence of CAPex or ribavirin for 12 h. Total RNA was extracted with NucleoSpin RNA kit (Macherey-Nagel). The in vitro quantification of influenza A and HRV 14 genome was performed using the Techne qPCR human influenza A (M1) and Techne qPCR for HRV14 genomes kit, respectively (Tecnhe^{TM}, Sweden), following the manufacturer's instructions, in a Stratagene Mx300P qPCR System. Relative expression levels of target genes were calculated from ct values.

2.7 Data Analysis

The cytotoxic concentrations of compounds were estimated from concentration effect curves after linear regression analysis. The quantification of viral inhibition by the compounds in relation to each tested virus represent mean ± SD values of three replicates, derived from three or four independent experiments. The measurements were compared by analysis of logged data (GraphPad Prism, V6) and significant differences were determined using a one-way ANOVA or Student's t-test ($p< 0.05$) as appropriate.

3. Results

3.1 In vitro cytotoxicity of CAPex

The potential *in vitro* cytotoxicity and metabolic effects of CAPex in MDCK and HeLa cells were assayed over 3 descending dilutions (1:10 to 1:1000) for 1 or 2 days, respectively. IC_{50} was <1:10 in MDCK cell (Fig. 1A) and <1:1 in HeLa cells (Fig 1B). No significant differences were observed between 24 and 48h (Fig. 2). Therefore, the dilutions of CAPex 1:100 for MDCK and 1:10 for HeLa cells were chosen to precede with the antiviral activity assays.

3.2 Determination of CAPex antiviral activity

The potential antiviral effect of CAPex against Influenza A and B strains, HRV14, RSV and Adeno 5 were assayed both in PFU and in cytopathogenic effect (CPE). Treatment, following infection, with the CAPex had a remarkable inhibitory effect on PFU of influenza A H1N1 (Figure 2 A&B). The decrease in the FluA/H1N1 infectivity was observed for both viral strains, being more profound in strain A/FM/1/47. Interestingly, the inhibitory effect of the CAPex was comparable to that of ribavirin and amantadine. On the contrary, no antiviral activity was observed in influenza A/H3N2

tested. Furthermore, treatment of A/H3N2-infected cells with increasing concentrations of CAPex did not induce any inhibitory effect, excluding a dose-dependent action on this strain (data not shown).

The antiviral activity of the CAPex was further examined in two strains of influenza B, B/Florida/4/2006 and B/Russia/69. A significant decrease in PFU was observed in the B/Florida/4/2006 strain (Figure 2C). CAPex was less effective in PFU assay of the B/Russia/69 strain (not shown). Additionally, CAPex induced a ~65% decrease of PFU of human rhinovirus 14 (HRV14) infected HeLa cells. This antiviral effect of the CAPex was even more evident compared to ribavirin (Figure 2D). On the contrary, CAPex was ineffective in RSV or HAV5 (data not shown).

3.3 Inhibitory activity of CAPex in a Time-of-Addition Assay

To clarify the mechanism of CAPex against influenza A/FM/1/47 and HRV14, we performed a time-of-addition assay, investigating at which stage CAPex inhibits viral function(s), within a single life cycle (Figure 2E). Pre-treatment of cells with CAPex either for long or short periods of time prior to viral infection, significantly inhibited influenza A/H1N1 replication (Figure 2F) and HRV14 (Figure 2G). Surprisingly, despite the lack of any antiviral activity after virus adsorption, CAPex demonstrated a remarkably preventive activity against RSV (-24 to -2 and -24 to 72 hours post infection) (Figure 2H). Short pre-treatment with CAPex did not inhibit the RSV infection (data not shown).

3.4 CAPex inhibits the replication of Influenza A H1N1 and Rhinovirus 14 and reduces the progeny virus

To further confirm the antiviral activity of CAPex against Influenza A H1N1 and HRV14, the replication of both viruses was tested in the absence or presence of CAPex. Quantification of viral genomes showed a significant decrease of copy number for both viruses in CAPex-treated, as compared to untreated cells (Figure 3 A&B). The inhibition of both viruses replication rate at the CAPex-treated cells was reflected in the significantly reduced progeny virus, assayed in terms of viral titers (Figure 3 C&D) and number of infected, compared to the non-treated cells (Figure 3 E&F). Combining the above results, we conclude that CAPex exhibits a preferential antiviral activity against Influenza A H1N1 and HRV14, affecting viral replication activity and suppressing viral growth.

3.5 Cretan aromatic plants extract cause defect of Influenza A H1N1 Nucleprotein trafficking

Influenza A nucleoprotein (NP) plays a crucial role in the virus life cycle forming, along with other viral proteins, a ribonucleoprotein complex (vRNP) which shuttles between the cytoplasm and the nucleus of the infected cells [reviewed in 29]. We examined the effect of the Cretan aromatic plant extracts on the NP nuclear trafficking by fluorescence microscopy. At 3h post-infection, NP was localized in the cytoplasmic sub-plasma membrane region, in both CAPex-treated and untreated cells (Figure 4). The subsequent exclusive localization of NP to the nucleus of the untreated cells at 6h post-infection was not paralleled in the CAPex-treated cells which presented reduced distribution of NP in the nucleus. At later times, (12h post-infection), NP localization changed to cytoplasmic facilitating the export of vRNPs in the control cells, in contrast to the constantly defective trafficking of NP to the nucleus which remarkably accumulated at the nuclear periphery. These observations suggest that the extracts from the Cretan aromatic plants

inhibit viral influenza A infection disturbing the programmed trafficking of the nucleoprotein to the nucleus.

4. Discussion

Recently, there has been a remarkable progress in the field of development new efficacious and safe antiviral therapies based on aromatic plants, avoiding drug resistance, cytotoxicity and other side effects [reviewed in 30]. In our study, we tested a mixture essential-oil extract of three Cretan aromatic plants (CAPex), previously reported in being efficient in decreasing the severity and the duration of upper respiratory tract viral infections [22; 23]. Cell viability assays showed low levels of cytotoxicity and a high EC_{50} value for the plant extract tested, which is important for its further investigation as an antiviral agent. Moreover, the applied CAPex concentrations are compatible to the peak plasma concentration of plant phenolics after *per os* administration (~1:100 of the administered dose) [31].

Herein, we demonstrate an *in vitro* antiviral activity of CAPex against various viruses responsible for infections of the upper respiratory tract. Foremost, we observed a remarkable inhibitory effect in influenza A/H1N1 strains and interestingly, comparable to the gold-standard antiviral drugs ribavirin and amantadine. On the contrary, no reactivity was evident against influenza A/H3N2 strain, even when cells were treated with increased concentrations of CAPex (data not shown). This was an unexpected result because both influenza A/H1N1 and influenza A/H3N2 virus strains are members of the same family, Orthomyxoviridae and there are studies showing similar activity of antiviral extracts against these two virus strains, such as the one with *Scutellaria baicalensis Georgi*, a Chinese herbal extract which inhibits A/H1N1 and A/H3N2 infection in cell culture [32].

Furthermore, CAPex exhibited a significant inhibitory activity against Influenza B virus, particularly on the B/Florida/4/2006 strain. The efficiency of antiviral inhibition observed for another influenza B virus strain varied, even when cells treated with higher doses of CAPex, suggesting a differential activity of the herbal extract also among influenza B virus strains. However, our cumulative results established a comprehensive suppressing mode of action for CAPex targeting Influenza B, similarly to previous studies which outline anti-influenza herbal compounds against different influenza B virus strains [33].

Additionally, CAPex exhibited a strong antiviral effect against the human rhinovirus 14. Both HRV-14 replication and progeny virus production were severely impaired after treatment of cells with CAPex. This laboratory finding was a great surprise, considering that such a correlation between CAPex and human rhinovirus was not found at the previous clinical study testing the same extract, possibly due to the very strict introduction criteria set for the participants [22]. On the contrary, treatment with CAPex had no effect against Adeno5, another non-enveloped virus. The difference between these two non-enveloped viruses was also shown for an aqueous extract derived from *Aloe arborescens* Mill [26]. The lack of viral sensitivity for CAPex was also indicated for RSV, member of the Paramyxoviridae, enveloped virus which is closely related to the Orthomyxoviridae. This was not expected because most of the antiviral herbal extracts studied up to now displayed similar activity against these two virus families [34].

The powerful antiviral properties of the aromatic plant extract prompted us to further investigate the mechanism of CAPex against influenza A/H1N1 and human rhinovirus 14. Our time-of-addition assay suggested that pre-treatment of cells for short or long periods of time with CAPex prior to the viral infection did not affect replication of both

influenza A/H1N1 and HRV14, indicating that this Cretan herbal extract exerts its antiviral activity after the virus entry in host cells. Unexpectedly, long pre-treatment with CAPex demonstrated remarkably antiviral activity against respiratory syncytial virus (RSV) compared to the absence of extract antiviral reaction after short pre-treatment or after the virus entry into the cells. This observation might implicate a different mode of CAPex action for RSV but further study of infection of this respiratory virus is required for final conclusions.

Influenza A Nucleoprotein (NP) has a fundamental role for the establishment of the successful viral infection, being the major component of the viral nucleoprotein complex and regulating vRNP shuttling between the cytoplasm and the nucleus. The proper trafficking of vRNP was inhibited by the Cretan aromatic plants extract, as observed by microscopy analysis, suggesting that CAPex may interfere with RNP export from the nucleus at an early stage and persists throughout the rest of the infection. Similar defects at the NP trafficking has been also described, predominantly by chemical synthesized compounds [35; 36]. Given the conserved sequence of NP among all influenza A viruses and the absence of any cellular equivalent protein, Nucleoprotein looks as an ideal target for the development of anti-influenza drugs. To that end, the extract from the Cretan aromatic plants could be involved in antiviral strategies as a very powerful, natural agent.

Taken together these results, we report antiviral activity of CAPex against a panel of respiratory viruses which is clearly and selectively against enveloped RNA viruses belonging to the Orthomyxoviridae and against a non-enveloped RNA virus belonging to the Picornaviridae. To that end our *in vitro* data confirm the reported *in vivo* significant orthomixovirus and picornavirus-specific responsiveness for CAPex.

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Figure Legends

Figure 1
***In vitro* cytotoxicity of CAPex.**

(A) MDCK and **(B)** HeLa cells were grown with serial dilutions of CAPex for 1 day and 2 days, respectively. The cytotoxicity of CAPex was measured by MTT assay. The relative cytotoxicity of test component dilutions was normalized by the control (medium with 0.003% DMSO), representing 100% cellular viability. Data are shown as means of four experiments in triplicate.

Figure 2

Antiviral activity of CAPex against respiratory viruses by plaque reduction assays.

Cells were infected with the indicated viruses at MOI 0.05 PFU/cell. Data (means ± SD) were derived from three replicates of four independent experiments. * p=0.01 to 0.1, ** p=0.001 to 0.01, *** p=0.0001 to 0.001, as compared to control (non-treated) cells.

Figure 3

CAPex inhibits viral replication and production of progeny virus.

(A) Quantification by qRT-PCR of FluA M1 genome copies in MDCK cells infected with FluA/H1N1 at MOI 0.5 PFU/cell, or **(B)** of HRV14 genome copies in HeLa cells infected with HRV14, for 12 h, in the absence or presence of CAPex **(C)** Growth curves of H1N1 in MDCK cells, infected with H1N1 at MOI 0.01 PFU/cell, in the absence or presence of CAPex. **(D)** Growth curves of HRV14 in HeLa cells, infected

with HRV14 at MOI 0.01 PFU/cell, in the absence or presence of CAPex. In C and D, viral titers were determined by plaque assay at the indicated time points. **(E)** MDCK cells were infected with FluA/H1N1 at MOI 10 PFU/cell for 6 h in the absence or presence of CAPex, stained with a mouse anti-influenza A NP antibody and FITC-conjugated secondary antibody (green). **(F)** The number of infected cells was counted from microscopy images in cells of the experiment described in **(E)**. The asterisks indicate statistical significance. ** p=0.001 to 0.01 as compared to untreated (control) cells. Data shown are means ±SD of at least three experiments in triplicate.

Figure 4

CAPex interrupts the Influenza A Nucleoprotein trafficking to the nucleus.

MDCK cells were infected with FluA/H1N1 (MOI 10) in the absence or presence of CAPex. At the indicated time points, cells were fixed and subsequently stained using influenza A anti-NP primary antibody followed by FITC secondary antibody (green). DAPI was used to visualize the nucleus area (blue). Bar: 20μm.

Figure 1

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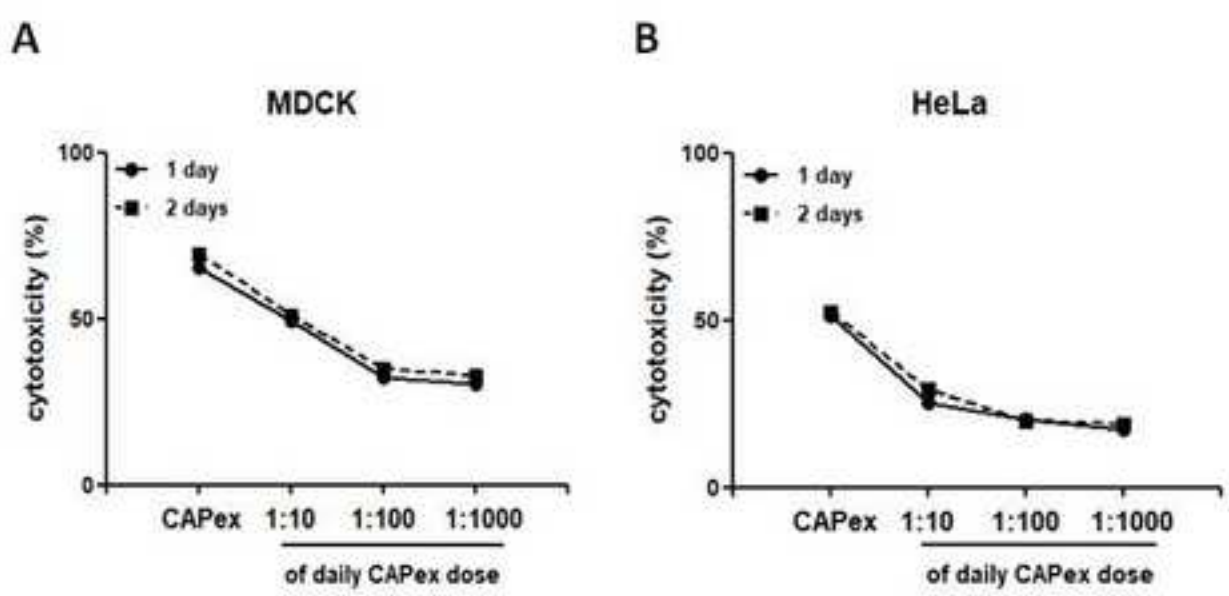

Figure 2

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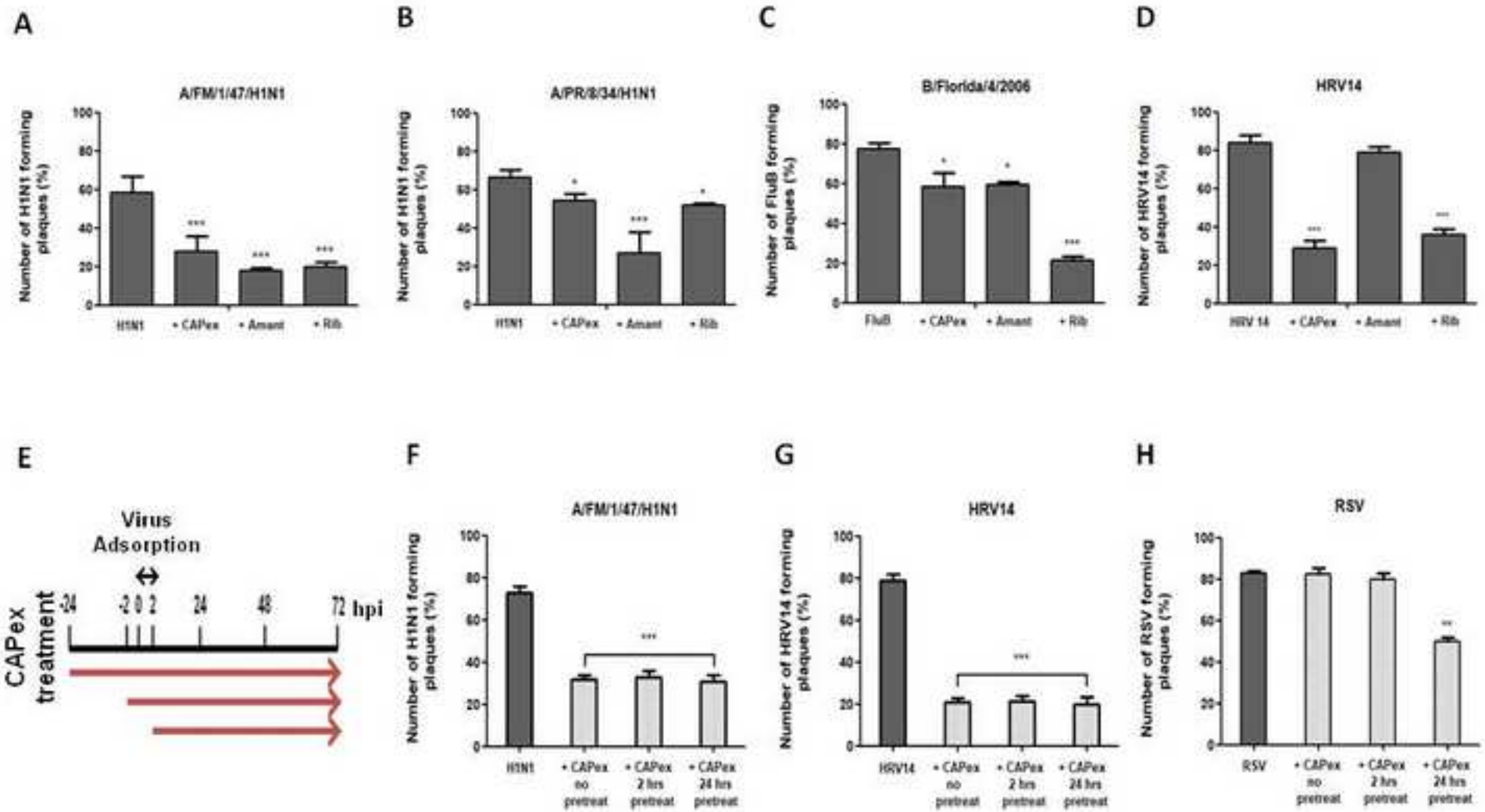

Figure 3

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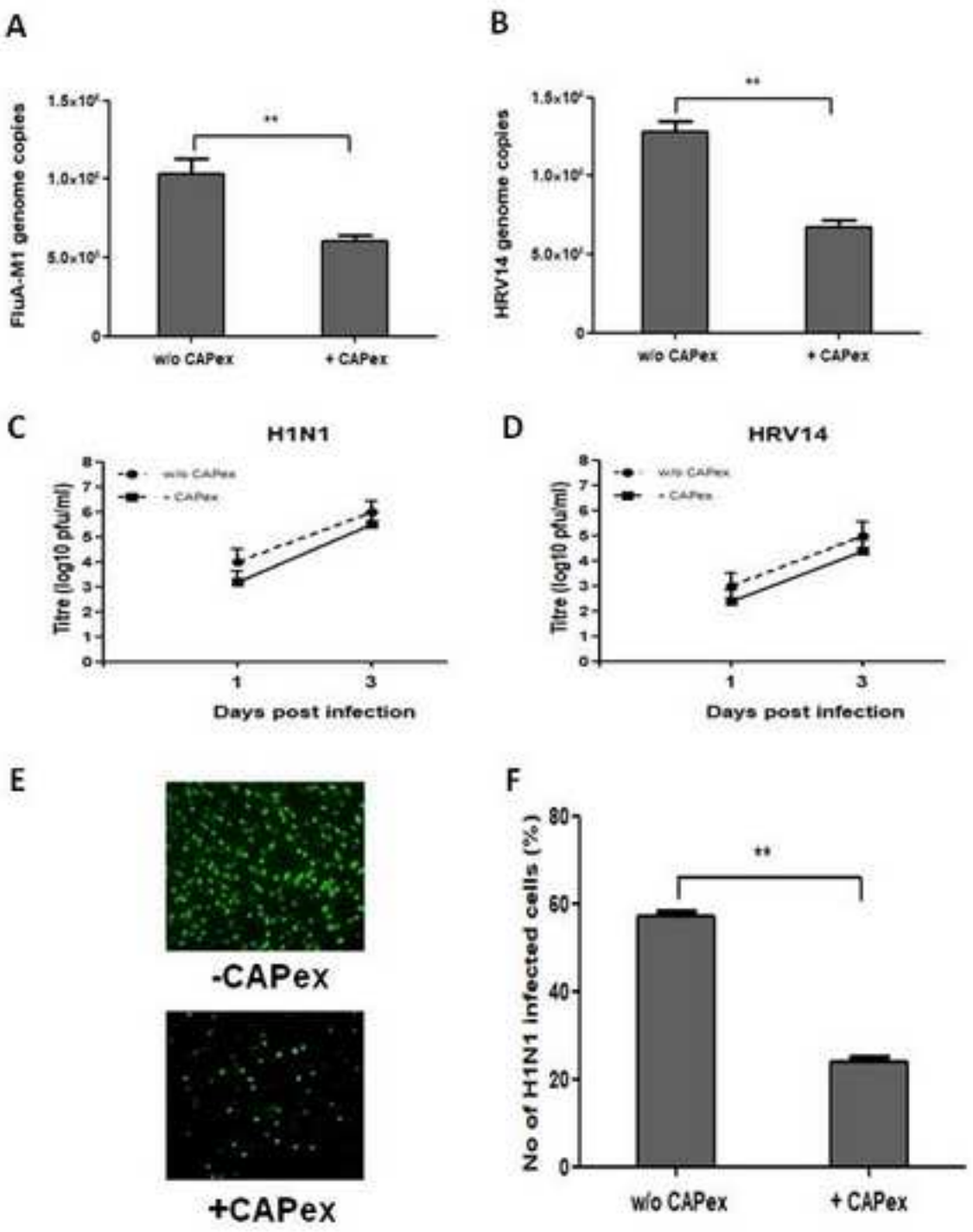

Figure 4

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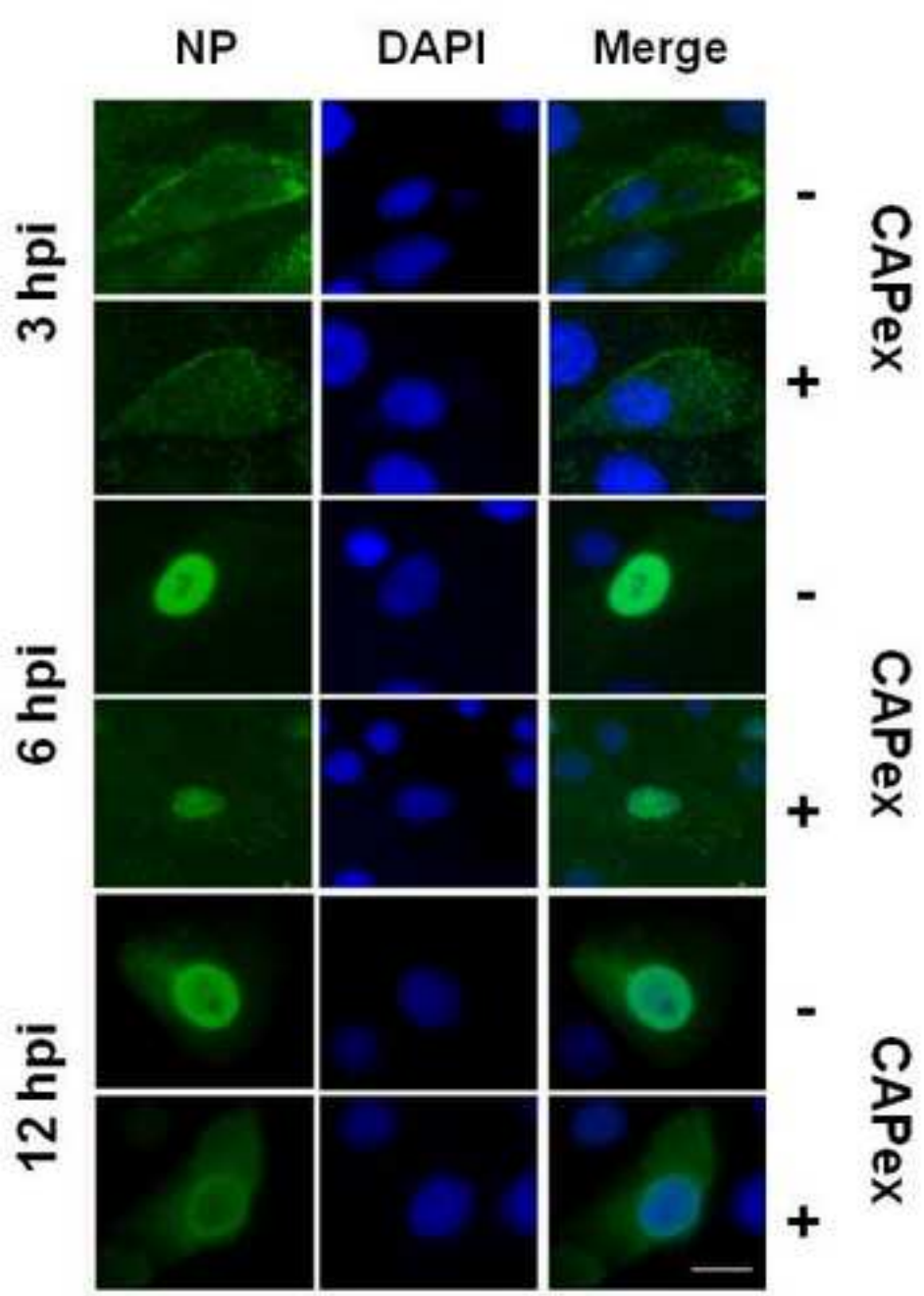

Conflict of Interest

SAP, CL and EC are inventors in patents CN102762218, EP2482831 and WO2011045557, with priority numbers WO2010GB01836 20100929 and GB20090017086 20090929, related to the antiviral activity of the CAPex.