# Neutralizing Activity of Usnic Acid and β-Cyclodextrins Complex against SARS-CoV-2 Spike Pseudovirus

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The rapid spread of SARS-CoV-2 and its infection severity require an urgent development of antiviral agents. In this respect, Usnic acid (UA), a natural dibenzofuran derivative, exerts antiviral activity against several viruses, though presenting very low solubility and high cytotoxicity. Here, UA was complexed with β-cyclodextrins (β-CDs), a pharmaceutical excipient used to improve drug solubility.

The cytotoxic activity, tested on Vero E6 cells, revealed no effect for β-CDs alone whereas significant cytotoxicity for the UA/β-CDs complex was recorded at concentrations ≥ 0.05%. The neutralizing activity towards the fusion of SARS-CoV-2 Spike Pseudovirus showed no effects for β-CDs alone whereas the UA/β-CDs complex, when pre-incubated with the viral particles, efficiently inhibited the Pseudoviral fusion of about 90 and 82% at non-cytotoxic concentrations of 0.03 and 0.01%, respectively.

In conclusion, although further evidences are needed to clarify the exact inhibition mechanism, UA/β-CDs complex could be useful in SARS-CoV-2 infection.

Keywords: Usnic acid; Usnic acid and β-cyclodextrins complex; SARS-CoV-2; COVID-19; antiviral activity

**SUPPLEMENTARY MATERIAL**

## 3. Experimental

### *3.1. Characterization of Usnic acid and β-cyclodextrins*

The UA (2,6-Diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan -1,3(2H,9bH)-dione) was produced by Zhejiang Yixin Pharmaceutical Co., Ltd. (China) and supplied by Vivatis Pharma Italia s.r.l. The characteristics of (+)-UA, according to certificate of analysis, are reported in Table S1. The β-CDs, produced and supplied by Vivatis Pharma Italia s.r.l. (Code: 0598; Batch N.: 181204), appear as a white crystalline powder, odorless, slightly sweet, almost insoluble in methanol, ethanol, propanol or ether and soluble in water. The β-CDs derived from enzymatic conversion from starch and possess a specific optical rotation ranging from +160° to +164°. Their purity corresponded to 98% (HPLC method) with traces of heavy metals ≤ 1.0 ppm.

### *3.2. Preparation of the Usnic acid and β-cyclodextrins complex*

The UA/β-CDs complex (1:1 *w/w*) were kindly supplied by Vivatis Pharma Italia s.r.l. In particular, dry extract of UA is selectively complexed with β-CDs to obtain an inclusion compound. The inclusion compound was obtained by a co-precipitation of (+)-UA and β-CDs, as reported by Nikolić et al. (2013). In brief, β-CDs were initially dissolved in water and, subsequently, the dry extract of UA was added to the solution under stirring. In the presence of appropriate concentration of β-CDs in water, precipitation of the inclusion compound began as the complexation reaction of the UA by β-CDs progressively proceed. The inclusion compound was collected after evaporation of the solution in a vacuum evaporator and, then, dried in a desiccator.

### *3.3. Solubilization of the β-cyclodextrins alone and the Usnic acid and β-cyclodextrins complex*

Solubility assays of the β-CDs alone and of the UA/β-CDs complex (1:1 *w/w*) were performed by dissolving the compounds at different concentrations (ranging from 0.5 to 0.0005% for β-

CDs alone and 1 to 0.001% for the complex) in both sterile distilled water and cell culture medium D-MEM (Corning, Italy). The solubility of UA alone cannot be performed due to its poor solubility (Table S1).

For an excellent dispersion of the β-CDs alone and of the UA/β-CDs complex in D-MEM, an energetic mixing was required if the solution forms a visible precipitate. To further improve solubility, the different solutions have been immerged in water at 50°C for 20 min.

The β-CDs alone in D-MEM at the concentrations of 0.5, 0.25, 0.05, 0.025, 0.015, 0.005, 0.0025 and 0.0005% were completely dissolved. On the contrary, the UA/β-CDs complex solubilized in D-MEM at the concentrations of 1%, 0.5% and 0.1% showed a quantity of precipitate which is proportional to the concentration of the compound (Table S2). Therefore, for these latter solutions, the supernatants were employed for the cytotoxicity tests. For solutions of the UA/β-CDs complex solubilized in D-MEM at concentrations ≤ 0.05%, no precipitate was observed, hence, an aliquot of each solution was used for the cytotoxicity test.

Since identical solubility was observed for both β-CDs alone and the UA/β-CDs complex in distilled water and D-MEM, in vitro experiments were carried out by solubilizing the substances in D-MEM to avoid putative cell monolayer damage and maintain the same cell viability exerted in the absence of the compounds. The distilled water is known to compromise cell viability.

### *3.4. Vero E6 cells and SARS-CoV-2 Spike Pseudovirus*

The African green monkey kidney–derived Vero E6 was purchased from American Type Culture Collection (ATCC, USA). Vero E6 cells were cultured in D-MEM supplemented with 1% of L-glutamine, 10% of fetal bovine serum (FBS) (Euroclone, Italy) and 1% of penicillin/streptomycin (Merck, Italy) and incubated at 37°C in humidified incubators with 5% $CO_2$. The cell line was propagated in 75 $cm^2$ cell culture flask (Corning, Italy) with 10 mL of complete D-MEM in order to obtain an adequate number of cells to inoculate 96-well plates.

Pseudovirus, an HIV-based luciferase lentivirus pseudotyped with SARS-CoV-2 full length S

glycoprotein of Wuhan strain, was purchased from Creative Biogene (USA) (SARS-CoV-2 S Pseudotyped Luciferase Lentivirus, cat. CoV-002).

The Pseudovirus presents SARS-CoV-2 S glycoprotein as the only surface protein that mediates viral fusion with host cells. The stock of Pseudovirus, harboring the SARS-CoV-2 S protein, contains $10^7$ transduction units (TU)/mL, where the TU is the unit of measurement used to define the quantity of Pseudovirus. Of note, the Pseudovirus also contains the reporter gene for the firefly luciferase enzyme which allows to quantify the luminescence emitted by the intracellular viral particles.

### *3.5. Cytotoxicity assays*

To establish the maximal non-cytotoxic dose of the β-CDs alone and of the UA/β-CDs complex, the previous described serial dilutions of compounds in D-MEM were incubated at 37°C with semi-confluent Vero E6 cells grown in 96-well tissue culture plates (Flow Laboratories) for 8 and 24h.

The MTT assay (Sigma Aldrich, Italy), colorimetric test based on the reduction of a yellow tetrazolium salt to formazan by metabolically active cells, was used to verify the possible cytotoxic activity of the compounds at different concentrations. More intensive violet color is related to a greater number of viable cells.

Briefly, for the cytotoxicity experiments, the cells previously detached from the 75 $cm^2$ flasks were seeded in 96-well plates at a final concentration of $1x10^4$ cells/well and incubated for 24h at 37°C in a humidified incubator with 5% of $CO_2$. After 24h of incubation, the culture medium was removed and 100 µl of the β-CDs alone and UA and β-CDs complex, previously dissolved in D-MEM, were added at different concentrations. The multiwells were incubated for 8 and 24h at 37°C in a humidified incubator with 5% of $CO_2$.

After 8 and 24h of incubation, the culture medium with the different concentrations of the β-CDs alone and UA and β-CDs complex was removed from the multiwells, and the cell monolayers were washed with 100 µl/well of phosphate buffered saline (PBS). Then, 100

µl/well of a 0.5 mg/mL solution of MTT dissolved in PBS were added and the multiwells were incubated in the dark for 3h at 37°C in a humidified incubator with a 5% of $CO_2$.

After 3h, the MTT solution was removed, the multiwells were washed with PBS and 100 µl/well of dimethyl sulfoxide (DMSO) were added. The multiwells were maintained at room temperature in the dark for further 15 min. Subsequently, the resulting solution was evaluated by spectrophotometric absorbance at the wavelength of 570 nm with correction at 690nm in order to define cell viability. For cytotoxic assay, a cell viability less than 70% classifies a substance or a complex as cytotoxic (Cannella et al. 2019).

### *3.6. Pseudovirus neutralization assay*

For neutralization assays, Vero E6 cells were seeded in 96-well tissue culture plates ($1\times10^4$ cells/well) for 24h at 37°C in a humidified incubator with 5% $CO_2$.

Cell confluence conditions were set following instructions provided by Pseudovirus manufacturer. Preliminarily, Pseudoviral fusion in Vero E6 cells, an epithelial cell line largely used in SARS-CoV-2 studies, was tested at a MOI of 100, 10, 1 and 0.1, corresponding to $10^6$, $10^5$, $10^4$ and $10^3$ TU of Pseudovirus, respectively. As reported in Figure S3, the optimal concentration of Pseudovirus luminescence is obtained with MOI 10, while MOI 100, 1 and 0.1 did not fall in the sensitivity limit of the luminescence method. Therefore, MOI 100, 1 and 0.1 were excluded.

To evaluate the inhibition of Pseudovirus fusion to host membrane, different concentrations of the β-CDs alone and of the UA/β-CDs complex were tested on Vero E6 cells. For studies on the interaction of the β-CDs alone and of the UA/β-CDs complex with pseudoviral particles and/or host cells, the neutralization assay was carried out at a MOI of 10, in the presence or absence of the β-CDs alone or of the UA/β-CDs complex, according to the following experimental plan: i) to evaluate the entry efficiency of the pseudoviral particles, cells were treated with the Pseudovirus for 8h at 37°C; ii) to evaluate whether the β-CDs alone or the UA/β-CDs complex interferes with the viral fusion by binding viral surface components, the

Pseudovirus was preincubated with the β-CDs alone or the UA/β-CDs complex for 1h at 37°C and then the cells were infected with these suspensions for 8h at 37°C; iii) to evaluate whether the β-CDs alone or the UA/β-CDs complex interferes with viral attachment to host cells, cells were preincubated with the β-CDs alone or the UA/β-CDs complex for 1h at 37°C. The cells were then washed with PBS and infected with the Pseudovirus for 8h at 37°C; iv) to assess whether β-CDs alone or the UA/β-CDs complex interferes with both viral and host cell components, β-CDs alone or the UA/β-CDs complex was added together with the Pseudovirus to cell monolayer for 8h at 37°C.

The MOI indicates how many viral particles are inoculated and, therefore, how many viral particles infect a known number of cells per well.

At the end of the incubation, cells were washed twice with PBS, covered with D-MEM supplemented with 2% of FBS and incubated for 48h at 37°C in a humidified incubator with 5% $CO_2$. After 48h, cells were washed, lysed with cell culture lysis reagent (Promega, Italy) and the transduction efficiency was determined by luminescence analysis using firefly luciferase assay kit (Promega, Italy). The relative luminescence unit (RLU) in each well was detected using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, USA).

### *3.7. Statistical analysis*

For neutralization experiments, statistically significant differences were assessed by one-way ANOVA, with post-hoc Tukey test. All statistical analyses were run using Prism v9 software (GraphPad Software, USA).

All the results were expressed as the mean values ± standard deviation (SD) of three independent experiments. In each case, a $p$-value $\leq 0.05$ was considered statistically significant.

## Reference

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Nikolić V, Stanković M, Nikolić L, Nikolić G, Ilic´-Stojanović S, Popsavin M, Zlatković S, Kundaković T. 2013. Inclusion complexes with cyclodextrin and usnic acid. J Incl Phenom Macrocycl Chem. 76:173–182. doi: 10.1007/s10847-012-0187-8.

| CHARACTERISTICS | (+)-UA |
|---|---|
| Description | Yellow crystalline powder |
| Solubility | Soluble in chloroform and Ethyl acetate, slightly soluble in ethanol, and not/slow soluble in water |
| Origin | *Usnea Longissima* Ach. |
| Product code | 1405 |
| Batch number | 1405-1911-A001 |
| Purity (HPLC assay) | 98.7% |
| Granule size | 10-20 microns |
| Particle distribution | 0.2-15 μm |
| Loss on drying | 0.1% |
| Specific rotation | +502° |
| Melting point | 195-196°C |
| Heavy metals | ≤ 10 mg/kg |
| Arsenic | ≤ 1 mg/kg |
| Residual solvent (ethyl acetate) | 64 mg/kg |
| Total plate count | ≤ 1000 cfu/g |
| Yeast and mold | ≤ 100 cfu/g |
| *E. coli* | Negative |
| *Salmonella* spp. | Negative |

**Table S1.** Characteristics of (+)-Usnic acid (UA).

| Concentrations of β-CDs alone in D-MEM | Concentrations of the UA/β-CDs complex (1:1 *w/w*) in D-MEM |
|---|---|
| 0.5% (5 mg/mL) | 1% (10 mg/mL)* |
| 0.25% (2.5 mg/mL) | 0.5% (5 mg/mL)* |
| 0.05% (500 μg/mL) | 0.1% (1 mg/mL)* |
| 0.025% (250 μg/mL) | 0.05% (500 μg/mL) |
| 0.015% (150 μg/mL) | 0.03% (300 μg/mL) |
| 0.005% (50 μg/mL) | 0.01% (100 μg/mL) |
| 0.0025% (25 μg/mL) | 0.005% (50 μg/mL) |
| 0.0005% (5 μg/mL) | 0.001% (10 μg/mL) |

*Concentrations of the UA/β-CDs complex presenting precipitate at room temperature.

**Table S2.** Solubility test of different concentrations of both the β-cyclodextrins (β-CDs) alone and the Usnic acid (UA) and β-CDs complex (1:1 *w/w*) dissolved in Dulbecco's Modified Eagle Medium (D-MEM) at room temperature (the concentration values are expressed as percentage and as mg/mL ratio).

| Conditions | % Vero E6 viability | |
|---|---|---|
| | **8h** | **24h** |
| Control | 100 | 100 |
| UA/β-CDs complex at 1% | 15 | 7 |
| UA/β-CDs complex at 0.5% | 52 | 35 |
| UA/β-CDs complex at 0.1% | 54 | 41 |
| UA/β-CDs complex at 0.05% | 71 | 65 |
| UA/β-CDs complex at 0.03% | 83 | 85 |
| UA/β-CDs complex at 0.01% | 90 | 97 |
| UA/β-CDs complex at 0.005% | 90 | 98 |
| UA/β-CDs complex at 0.001% | 95 | 98 |

**Table S3.** Percentage of Vero E6 cell viability after 8 and 24 h of incubation with different concentrations of the UA/β-CDs complex.

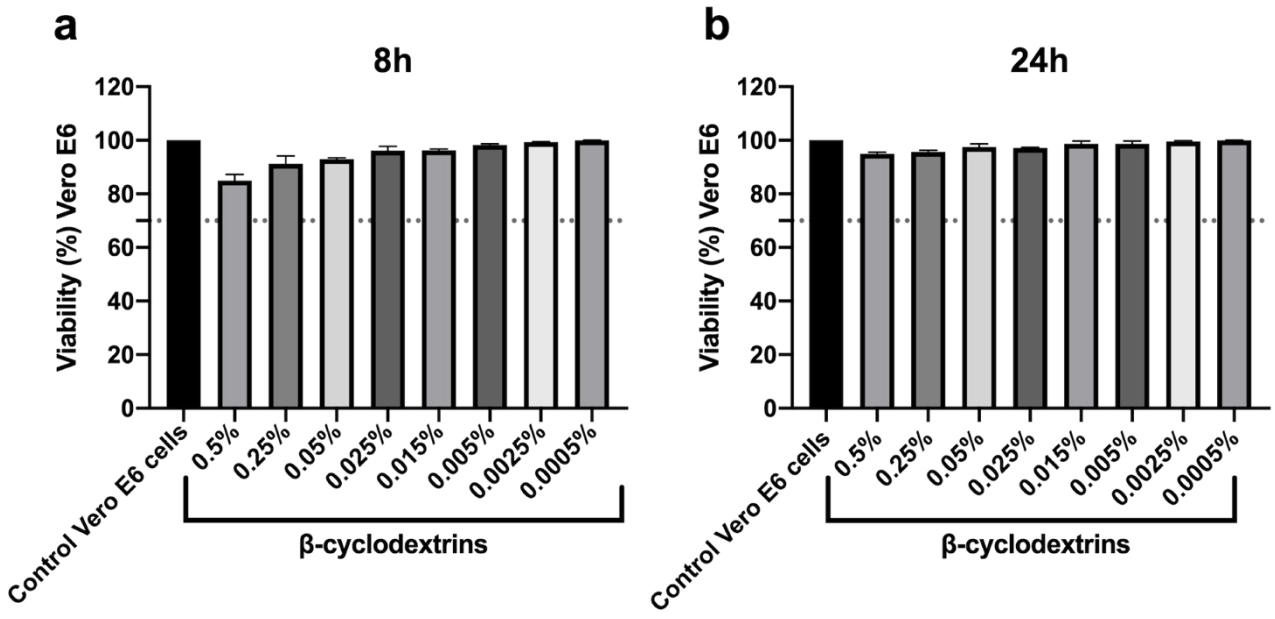


**Figure S1.** Percentage of Vero E6 cells viability after 8 (**a**) and 24h (**b**) of incubation with different concentrations of β-cyclodextrins alone. The dotted lines indicate the threshold value (70%) to define a substance as cytotoxic (Cannella et al. 2019).

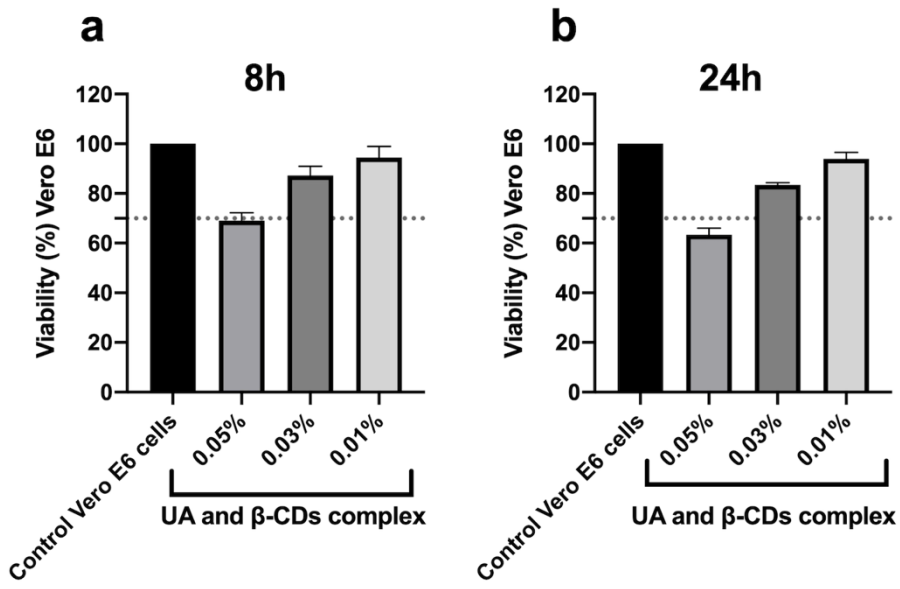


**Figure S2.** Percentage of Vero E6 cells viability after 8 (**a**) and 24 h (**b**) of incubation with different concentrations of the UA/β-CDs complex. The dotted lines indicate the threshold value (70%) to define a substance as cytotoxic (Cannella et al. 2019).

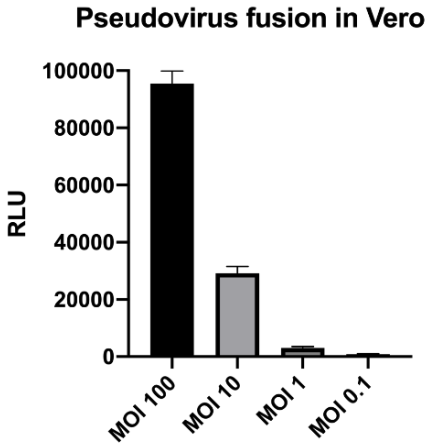


**Figure S3.** Luminescence of Pseudovirus observed in Vero E6 cells infected at multiplicity of infection (MOI) of 100, 10, 1, 0.1. RLU = Relative Luminescence Units.

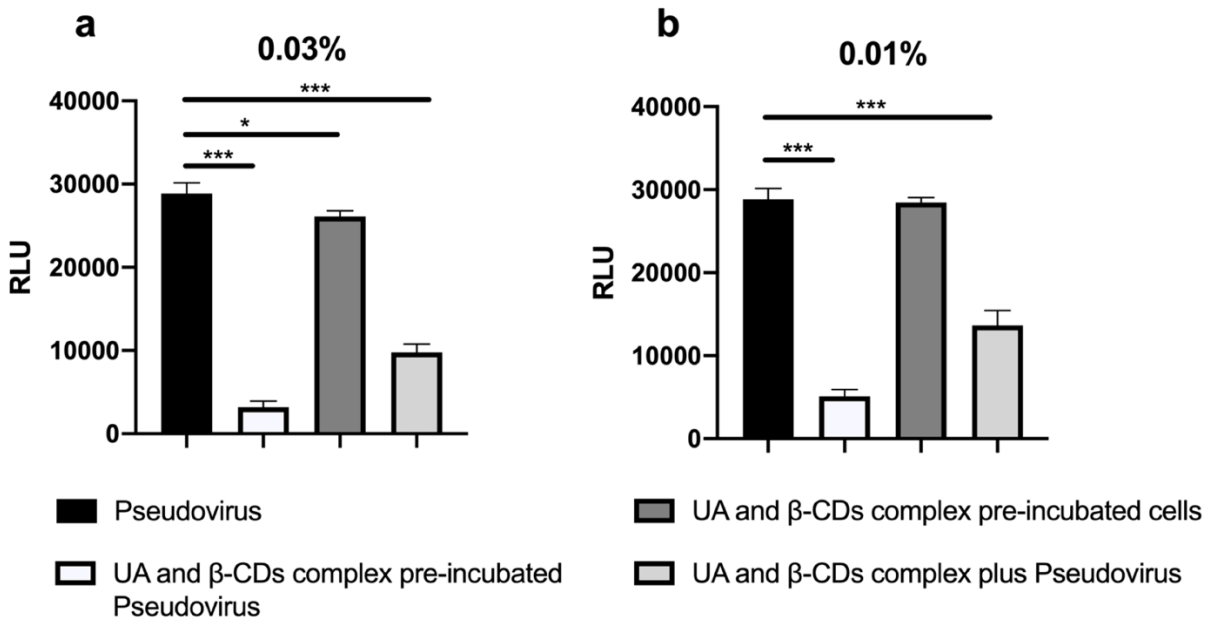


**Figure S4.** Luminescence of Pseudovirus observed in Vero E6 cells infected at MOI of 10 in the presence or absence of 0.03% (**a**) or 0.01% (**b**) of UA/β-CDs complex. See text for details. *: $p < 0.05$; ***: $p < 0.001$ (one-way ANOVA with post-hoc Tukey test). RLU = Relative Luminescence Units.