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# Secondary Metabolites in Functionalized Titanium Dioxide ($TiO_2$) Nanoparticles: A Novel and Safe Virucide Against Sars-Cov-2

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**Keywords:** Secondary metabolites, Titanium dioxide, $TiO_2$, Functionalized nanoparticles, SARS-CoV-2, Antiviral, Citrus extracts, antiseptics.

**Abstract.** Nanotechnology and nanomedicine have been shown to provide a novel and safe platform to combat a variety of viruses like SARS-CoV-2. Secondary metabolites implanted into a carrier of functionalized titanium dioxide ($TiO_2$) nanoparticles (SMNP) were tested for efficacy versus SARS-CoV-2 infectivity and cytotoxicity on healthy cells. Viral load; from a clinical point of view, it is not as important as the number of infective viral particles, which relates to the viral particles capable of causing the disease. To measure viral infectivity SARS-CoV-2 was placed into cell cultures and evaluating the destructive effect on cultured cells. In this system, SMNP demonstrated a significant reduction of viral infectivity *in vitro*. Lytic plaques of viral infectivity were observed at a dilution of $4x10^{-8}$ in VERO E6 cells, while SARS-CoV-2 pre-incubated with the SMNP compound, tissue damage was observed only up to the $3x10^{-5}$ dilution. SMNP reduced the number of infective viral particles by 3 orders of magnitude. Surprising minimal toxicity to healthy cells was observed when compared to other commercially available antiseptics (glutaraldehyde, chlorine, chlorhexidine, ethanol, and Lysol™), cell viability decreased only by 5.5%. SMNP is a safe and effective antiviral against SARS-CoV-2, and further studies are warranted to explore this compound further.

## Introduction

COVID-19 currently represents one of the leading causes of morbidity and mortality worldwide, which needs to be addressed immediately, for it will continue to cause not only personal sorrow but substantial economic loss for the foreseeable future [1, 2]. COVID-19 has challenged healthcare systems around the globe, which will only be further burdened by the demand for attention for all other neglected diseases. Authorities are struggling to minimize mortality and overcome the economic downturn [3, 4].

According to the available data, SARS-CoV-2 virus can be transmitted through direct contact with an infected person and indirectly through contact with surfaces in their immediate surroundings or with objects used by an infected person [5]. Indirect transmission of SARS-CoV-2 is the route of infection that does not involve direct contact from person to person [6]. There are currently no precise data on the proportion of infections occurring by this route, but the potential for spreading through

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indirect contact is considered to be so high that more effective and safe measures are needed. To date, most control programs aim to reduce the spread of the virus through social distancing, blocking, tracing, and quarantine. Decreasing the rate of non-contact transmission is critical to reducing the number of cases of infection below the hypothetical healthcare capacity [7]. Cities like Wuhan, China, have managed to keep the number of infections low since April through a permanent sanitation campaign in all public places, among other actions [8]. The problem with this is that disinfectants used for disinfection purposes also have risks associated with them and range from skin irritation to long-term effects, while their use will increase [9, 10]. This is why it is necessary to develop effective but low or zero toxicity sanitizers and disinfectants.

On the other hand, nanotechnology has demonstrated potential in addressing serious healthcare challenges [11], and nanomaterials have been used as delivery platforms or even as antivirals themselves [12, 13, 14]. Biotechnology is also effective; citrus-derived compounds are known to stimulate immunity, having shown clear clinical benefit in fighting viral infections [15, 16]. Natural secondary metabolites have shown antiviral activity. Their inhibitory or stimulatory effect on key metabolic enzymes influence signaling pathways, cellular function, and gene expression [17].

Our research team has developed an organic nano-compound in the laboratory. Assorted citrus-derived secondary metabolites were implanted into a carrier of functionalized titanium dioxide ($TiO_2$) nanoparticles measuring less than 5 nm [18, 19, 20]. In the face of the pandemic, it seemed pertinent to study the effect of this unique functionalized nanoparticle (SMNP). Therefore, the aim of this work was to test the *in vitro* efficacy against SARS-CoV-2 infectivity and its toxicity on healthy cells.

## Experimental

The current study was divided into two sections: efficacy (the ability of the nano-compound to decrease SARS-CoV-2 infectivity *in vitro*); and cytotoxicity (how healthy cells tolerate the nano-compound as compared to other commercially available antiseptics and disinfectants) (Table 1).

Table 1. Commercial antiseptics and disinfectants used in this work.

| PRODUCT | USAGE | COMPOSITION |
|---|---|---|
| Lysol® | Trademark for a cleaning product | Organic Alcohol, Organic Amine, Fragrance, Cationic Surfactant, Inorganic Base, Antioxidant. |
| Ethanol | Gels and alcohols | Made from ethyl alcohol |
| Chlorine | Sodium Hypochloride | Solution at 4% |
| Glutaraldehyde | Used in sterilizers and mouthwashes | Normally used up to 4% |
| Chlorhexidine | Used in antiseptics and surgical soaps | Normally used at 1% |

**Nanoparticle functionalization and attachment with secondary metabolites.** Initially, an organic nano-compound was developed supported in an inorganic metal oxide carrier, which was then functionalized through an impregnation process and dispersed with citrus-derived extracts containing different secondary metabolites. This was done with temperature control to stabilize the interactions within the network of the support, creating a nanoparticle of 2-5 nm in size. The process included the addition of hydroxyl, phosphate, sulfate, chloride, amino, methyl, and folate terminals to 1.4% each in order to enhance delivery to action sites and improve effectiveness. The manufacturing process is described in the following patents: USA Patent 10,342,840 B2 [18]; IMPI Patent 339086 [19]; Japanese Patent JP 6625051 B2 [20].

Functionalization of the nanoparticle allows for control of several parameters, including support acidity, BET (Brunauer, Emmett and Teller) area, pore size distribution, particle size, functionalization extent, and dispersion of adsorbed extracts onto the support base, which are

important in order to increase its action. In order to perform the inclusion of the organic extracts, the titanium dioxide particles must have a surface area greater than or equal to 50 $m^2/g$. These matrix-supported extracts must be fully dispersed within the carrier in order to obtain a high cracking efficiency.

**Efficacy.** In the clinical setting, viral load is not as important to assess as viral infectivity, which relates to the viral particles capable of causing the disease. Lytic plate test was used as a measure of viral infectivity. This test involves placing SARS-CoV-2 into cell cultures and evaluating the destructive effect on cultured cells.

The SARS-CoV-2 virus was isolated from biological samples of nasopharyngeal lavage from a patient diagnosed as COVID-19 and confirmed positive for SARS-CoV-2 mediated qRT-PCR assay.

The SARS-COV-2 virus was titrated by lithic plate assay as previously reported by Cabello and col. (2009) [21]. VERO E6 (ATCC® CRL-1586™) monolayers were inoculated with 100 µl of serial log dilutions of SARS-CoV2 in serum-free medium ($10^0$ to $10^{-9}$) in duplicate and incubated for 2 hours at 37°C with 5% $CO_2$. The viral inoculum was removed with washing, and the cells were overlayed with 1.5 ml of incomplete medium, plus 2.5% methylcellulose. Cultures were incubated until the CPE was above 90% (6–8 days) and then stained with 1% crystal violet. Control wells had the VERO E6 cell culture with culture media, while the study sample had the same VERO E6 cells; however, the infective SARS-CoV-2 viral particles had been pre-incubated with the compound (SMNP). Viral-related cell damage was evaluated by assessing lytic plates on microscopy.

Infective viral particles per volume unit were determined. [22] For this, cells were incubated for four days at 37.5 ºC in high humidity with 5% $CO_2$ atmospheric concentration. Then cell cultures were stained with 1% crystal violet and reviewed every 24 hours until tissue damage was seen.

Dilution required for clearance of lytic plates, thus reflecting absence of viral infectivity, was compared between groups.

**Cytotoxicity. *L929 Agar Overlay Test.*** NCTC clone 929 [L cell, L-929, derivative of Strain L] ATCC® CCL-1™. L929 cells were seeded at approximately 35,000 to 640,000 cells per 60 mm dish in 5 ml of DMEM10 at 1-5 days before the assay. The dishes were incubated at 37 ± 1 °C in a humidified atmosphere containing 5 ± 1% $CO_2$. Prior to the agar overlay step, the monolayer was inspected for subconfluence and appropriate cell morphology. Dishes which were over-confluent (with primarily rounded cells) or which show abnormal cell morphology were discarded. Sterile, melted 2.5% agar was mixed 1-part agar to 1.5 parts DMEM10 and allowed to cool between 45 °C and 55 °C to yield DMEM6A with 1% agar. The medium was removed from each dish, and 5 ml of the 1% agar/medium solution (DMEM6A) was carefully overlaid on the cell monolayer. Once the agar had solidified 5 ml of 0.01% neutral red solution in PBS was pipette into the dish to stain the cells. The dishes were returned to the $CO_2$ incubator for 30 ± 5 minutes at 37 ± 1 °C, and then the remaining neutral red solution was removed. Samples were applied via a filter disk (approximately 1 $cm^2$ area). The disk was saturated with no more than 50 µl of the sample before application to the agar. The disk was applied to the agar immediately. The dishes were then incubated at 37 ± 1 °C and 5 ± 1% $CO_2$ for approximately 24 hours.

***Cytotoxicity assay (MDCK).*** Trials were performed using Canine Kidney MDCK (NBL-2) (ATCC® CCL-34™) cell cultures. MDCK cell cultures were exposed over 10 minutes to different common-use antiseptic and disinfectant products. Cell viability was tested using trypan blue, a dye that needs to penetrate the cell membrane in order to stain the cytoplasm so that only dead cells with ruptured membranes will stain blue under the microscope.

Cell cultures were exposed to different antiseptics and disinfectants with steady concentrations at different volumes and counted on a cytometer. Registries were averaged and plotted for descriptive purposes.

***In vitro* XTT cell viability assay.** The XTT assay was performed to determine the viability of VERO.E6 cells after treatment with SARS-CoV-2, pre-incubated with SMNP at different dilutions ($10^{-1}$- $10^{-11}$). The cells were seeded in a 96-well plate at a density of $3.2x10^4$ cells per well in 100 µL of culture medium and cultured in a humidified atmosphere with 5% $CO_2$ atmosphere at 37 °C. After incubation for 48 h at 37 °C, 50 µl of XTT solution was added to each well according to the kit manufacturer's instructions. The absorbance was read at 490 nm. Cell viability was determined using the formula shown in the manufacturer's protocol.

## Results and Discussion

The control of the spread of SARS-CoV-2 by contamination of surfaces should be considered a mandatory step [23]. In high-risk locations such as hospital facilities, this situation becomes more important [24, 25]. With this goal and given the growing need to have an effective antiseptic compound but without possible toxic effects, nanotechnology could give us the answer [26]. The synthesis and use of materials with dimensions on a molecular scale (diameter ≤100 nm) has become increasingly utilized for different applications and is of great interest as an approach to killing microorganisms and viruses. These particles exhibit characteristic effects owing to their high surface-area–to-volume ratio with unique chemical and physical properties. On this basis, secondary metabolites were attached to a carrier of functionalized titanium dioxide ($TiO_2$) nanoparticles of less than 5 nm, which we call SMNP.

**Efficacy.** The primary goal of this study was to determine if SMNP could inhibit SARS-CoV-2 infectivity *in vitro*. To determine the effect of the SMNP on viral infectivity, damaged cell areas were assessed as number of lytic plates in VERO E6 cells exposed to SARS-CoV-2. A basic sample (SARS-CoV-2 only) for the control group, and SARS-CoV-2 were pre-incubated with and without the compound SMNP before adding to the culture. In Figure 1 cell damage by COVID-19 infection is seen as rounded cells (Red arrow) followed by the formation of syncytium and hollow spaces (cell lysis) (Yellow arrows).

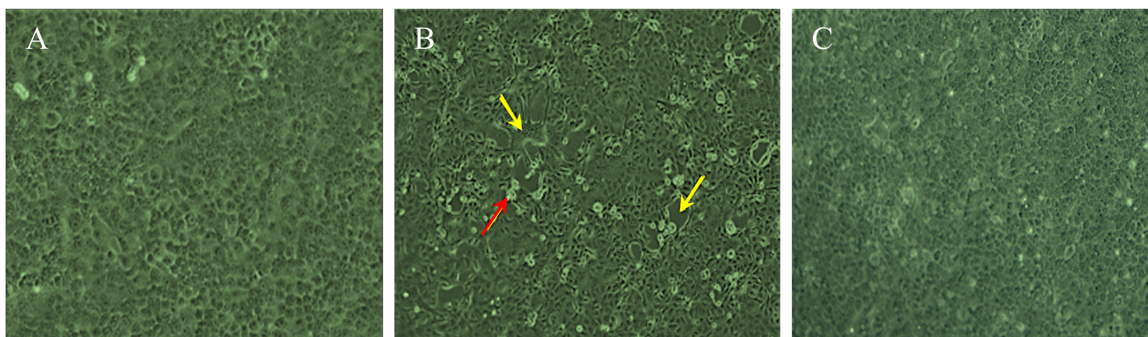


Figure 1. Microscopic view [40x]: A) VERO E6 cells. B) VERO E6 cells infected with SARS-CoV-2. C) VERO E6 cells infected with SARS-CoV2 pre-incubated with SMNP.

Results showed lytic plates from viral infectivity at a dilution of $4x10^{-8}$ in plates containing SARS-CoV-2, whilst in VERO E6 cells pre-incubated with the compound (SMNP), tissue damage was observed at a dilution of $3x10^{-5}$, reflecting 750-fold faster tissue damage than in the pre-incubated plates with SMNP. Infectious viral particles are seen as white spots (lytic plaques) on the blue-stained plate with violet crystal. In both cultures, plates with infected cells, and thus visible tissue damage, were diluted until no virus-related damage was seen. One of the wells on each culture box was left uninfected as a control. Inactivated virus was also used as another negative control (Figure 2).

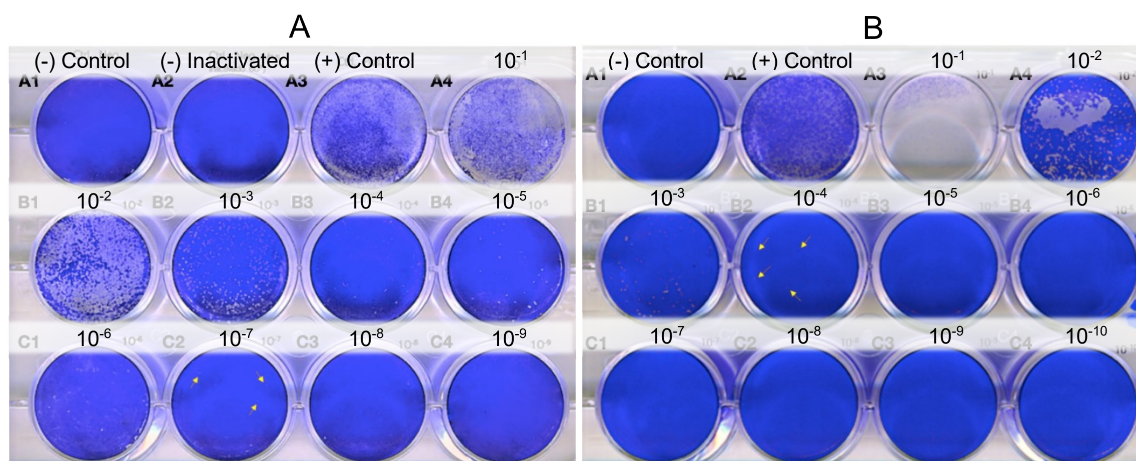


Figure 2. A) VERO E6 cells infected with SARS-CoV-2 without exposure to compound SMNP. B) VERO E6 cells infected with SARS-CoV-2 and pre-incubated for 5 minutes with compound SMNP.

In order to measure the protective effect of SMNP on viability of VERO cells infected with SARS-CoV-2. Cell viability was measured by absorbance values obtained by using serial dilutions of SMNP or culture medium (control) on VERO E6 cells. This was done in the presence and absence of SMNP using *in vitro* XTT cell viability assay. Results are shown in Figure 3.

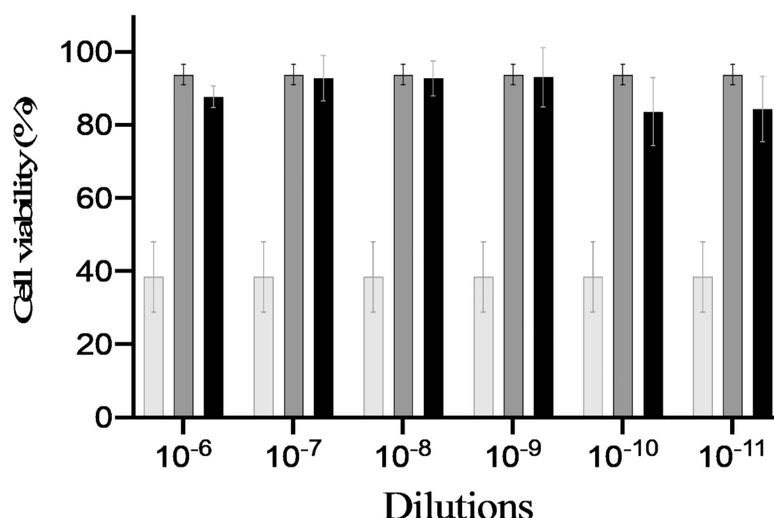


Figure 3. VERO E6 cells infected with SARS-CoV-2 (■ viral control). Culture medium added to VERO E6 cells (■ cellular control). VERO E6 cells infected with SARS-CoV-2 pre-incubated with SMNP for 5 minutes (■).

On the other hand, among the most used sanitizers and disinfectant, chlorine and chlorine compounds, glutaraldehyde, and chlorhexidine can be mentioned, among others. And while it is true that these compounds show adequate effectiveness when used against viruses, we cannot forget that they have toxicological effects, together with their risk of being allergenic and polluting.

**Cytotoxicity.** L929 Agar Overlay Assay was used to evaluate the cytotoxic potential of SMNP. This study was designed to comply with the testing methods described in the ISO 10993-5 standard, 2009, and USP 23, Biological Reactive Tests *in vitro*. Results are shown in Table 2.

Table 2. Description of Reactivity Zone. Grade 0: No detectable zone; grade 1: Some malformed or degenerated cells; grade 2: Zone limited to area under specimen; grade 3: Zone extending specimen size up to 1.0 [cm]; grade 4: Zone extending farther than 1.0 [cm].

| Sample ID | Grade | Reactivity (Means across of three replicates) | |
|---|---|---|---|
| Negative control | 0 | None | No detectable zone around or under specimen |
| Positive control | 4 | Severe | Zone extending further than 1.0 cm beyond specimen |
| SMNP | 0 | None | No detectable zone around or under specimen |

Each antiseptic and disinfectant were tested separately, exposing MDCK cell cultures to different concentrations, up to the commercial concentration of each product for 10 minutes, with a control well without the product.

As Figure 4 shows, glutaraldehyde was the least toxic; however, even this sanitizer caused 50% of cell death when used at a concentration of 4%.

A similar cytotoxic effect was obtained with chlorine on MDCK cells, as is shown in Figure 4. The highest chlorine concentration tested was 4%. After exposure for 10 minutes at this concentration, 59% of cells died. Cytotoxicity was evident even at concentrations as low as 0.25%, inducing a 23% rate of cell death.

In our study, chlorhexidine 1% produces the highest rate of cellular toxicity, with a 79% rate of cell death at this concentration. It is worth noticing that concentrations as low as 0.062% caused 32% of the cell population to die.

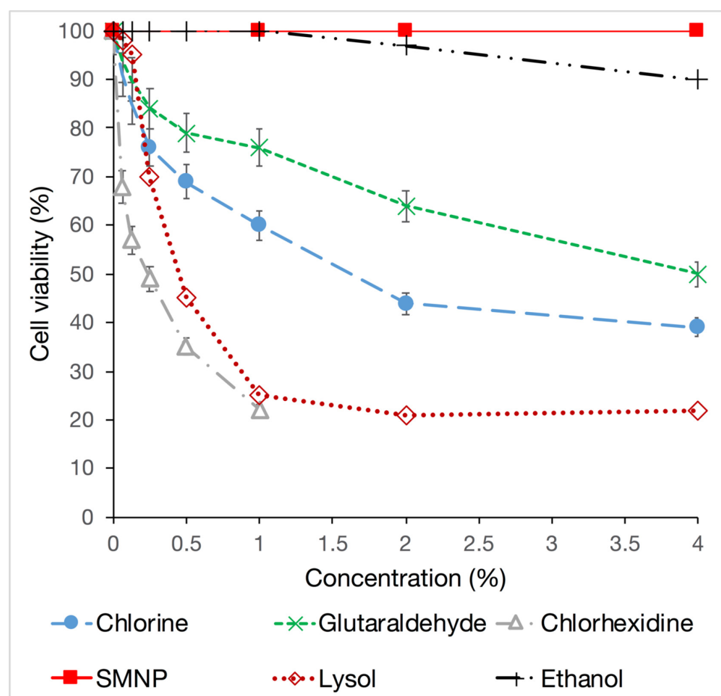


Figure 4. Cytotoxic effect of glutaraldehyde, chlorine, chlorhexidine, ethanol, and Lysol™ and SMNP after exposure for 10 minutes on MDCK (NBL-2) (ATCC® CCL-34™) cell culture.

For the antiseptics and disinfectants (Ethanol and Lysol™) a significant cytotoxic effect was observed using the same low concentrations of the other products (0.5, 1, 2, and 4 %). However, it was not possible to correlate product concentration exposure to cell damage, as they were found to completely destroy the cell culture at a commercial concentration.

In contrast, when exposing the cell cultures to SMNP, surprising results were obtained. Even at concentrations as high as 100% (25 times more than the one used for the rest of the antiseptics and disinfectants), cell viability decreased only by 5.5%.

**Statistical analysis.** All experimental measurements are reported as means across at least three replicates. Categorical variables as counts and percentages. The results were analyzed by Tukey test (Fig. 5A), boxplot (Fig. 5B), and ANOVA.

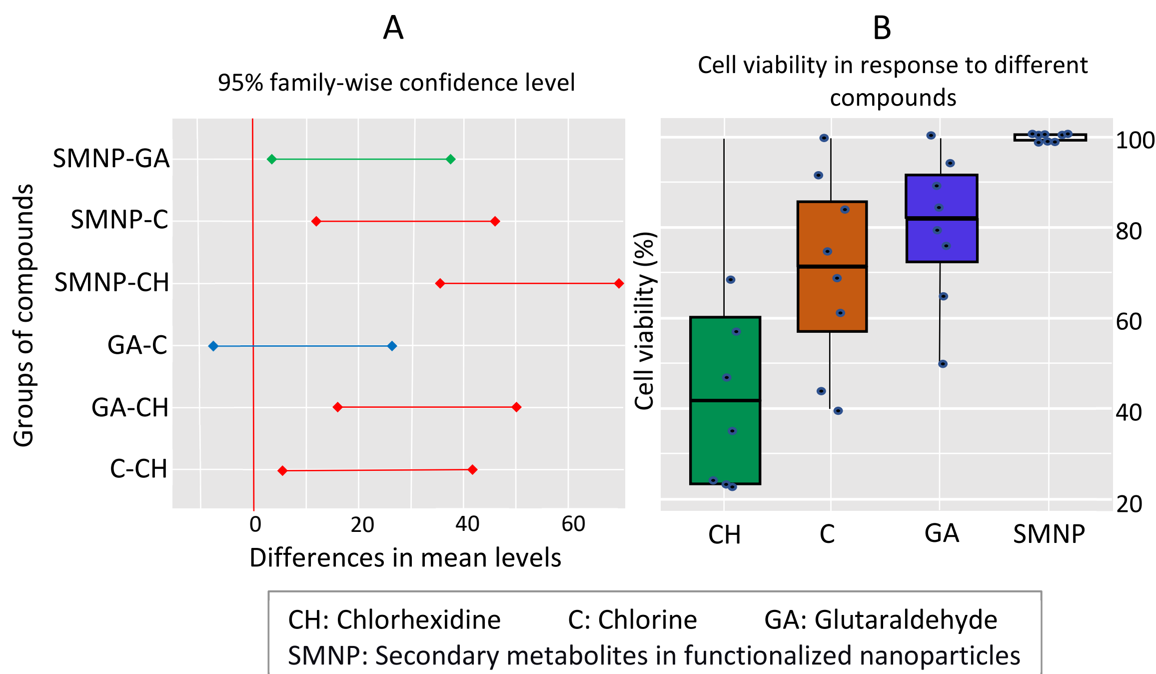


Figure 5. A) Comparison of confidence intervals was made at 95%. B) Box and whisker plot, where a summary of the data is shown in five descriptive measures, their morphology, and symmetry.

The p-values obtained are significant (6.45 $e^{-05}$ and 0.000468) therefore, one or more of the compounds generates a statistically significant difference in cell viability. A 95% confidence interval comparison was made. In peer comparisons, the difference in the mean between the compounds between Glutaraldehyde and Chlorine is not significant, while the rest of the comparisons were statistically significant (Fig. 5A). Those intervals containing zero indicate that there is no difference.

These results reveal that a unique nano-compound (SMNP) significantly inhibited SARS-CoV-2 infectivity *in vitro*. In cell cultures inoculated with SARS-CoV-2 pre-incubated with SMNP, viral infectivity cleared at a dilution of $4x10^{-5}$; while in those cell cultures inoculated with the virus NOT exposed to the compound, infectivity was lost until the cell culture was diluted to $3x10^{-8}$, representing a 750-fold (three logarithms) decline in viral infectivity.

Sundararaj et al. (2020) [27] evaluated silver nanoparticles (AgNP) and found that 10 nm nanoparticles were effective in extracellular inhibiting SARS CoV-2 at concentrations between 1 and 10 ppm; however, they observed a cytotoxic effect at concentrations of 20 ppm or more. These results indicate that AgNPs are very powerful against SARS-CoV-2, but, as the authors report, should be used with caution because of their cytotoxic effects and also because of their potential to harm environmental ecosystems when inadequately removed.

SARS-CoV-2 belongs to the family Coronaviridae and, like the rest of the human coronaviruses (HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1) has a lipid envelope [28], which makes them easily susceptible to various sanitizers and disinfectant. In other words, destroying coronaviruses outside the body is relatively easy. The real challenge is to do it without having cytotoxic effects.

With this concept in mind, SMNP also proved to be far less toxic on healthy cultured cells when compared to other commonly used antiseptics and disinfectants *in vitro*. Chlorine and chlorine compounds show good effectiveness against viruses but are known to cause dermatological effects such as redness, edema, burning, pain, blisters, and even tissue necrosis. Glutaraldehyde, through reticulation with protein, can produce yellow-brown discoloration in the skin. With chlorhexidine, despite its relatively non-toxic profile, there have been incidences of anaphylactic reactions [10]. This is consistent with our results, where the cellular mortality rate for the antiseptics and disinfectants

analyzed was from 40% to 80%. While for SMNP, it was less than 6%, which shows that the compound is safe *in vitro*. Being less toxic to the clinical user could be very beneficial since, as already mentioned, allergic and topical reactions represent a fairly common side effect of excessive use of disinfectants.

Chlorhexidine 1% is extensively used daily in operating rooms across the globe. It is the most commonly used skin preparation before the incision, and most surgeons scrub with it. It was also recently used as a hand sanitizer gel during the latest H1N1 A influenza pandemic [29].

The appearance of side effects such as allergic reactions will be inevitable with the growing need for sanitizers and disinfectants. Therefore, if these *in vitro* experiments achieve translational significance, the potential positive impact on health and economy could be extensive. Further studies are needed in order to establish the clinical effectiveness and safety of SMNP *in vivo*.

## Conclusion

In summary, these functionalized nanoparticles (SMNP) inhibit SARS-CoV-2 infectivity *in vitro* with minimal cytotoxicity. SARS-CoV-2 viral replication was effectively inhibited *in vitro* when pre-incubating infective viral particles with SMNP. When compared to other commercially available antiseptic and disinfectant products, SMNP demonstrates minimal cytotoxicity.

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