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Grupo Inmunovirología
[Faculty of Medicine]
[Immunovirology Group]

Final Report

Study:

"Quantifying *in vitro* antiviral activity of natural compounds against SARS-CoV-2"

Reporting Date: August 26, 2020

1. BACKGROUND

Pharmacare Laboratories company submitted a compound denominated **Sambucol** (see Photograph 1), to the Immunovirology Group Laboratory of Universidad de Antioquia (UdeA), in order to determine its antiviral potential against the SARS-CoV-2 virus isolated at Universidad de Antioquia.

2. METHODOLOGY

Materials and Reactives

The analysis of the antiviral activity of the submitted compound was conducted through the SARS-COV-2 virus isolation (at the Immunovirology Group of UdeA) on Vero E6 cell line. Vero E6 cells were maintained in a DMEM culture medium supplemented with 5% FBS (fetal bovine serum) at 5% CO2 and temperature of 37°C. The titer of SARS-CoV-2 virus isolated in the laboratory was determined using the plating technique and Tissue Culture Infectious Doses 50 in Vero E6 cells, according to a protocol previously described in literature (1) *. The titer obtained was 4.2 x 10^6 Plaque Forming Units - PFU/mL.

*1. Fan H.H., et al., Repurposing of clinically approved drugs for treatment of coronavirus disease 2019 in a 2019-novel coronavirus (2019-nCoV) related coronavirus model. Chin. Med. J. 2020;6. doi: 10.1097/CM9.0000000000000797.

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Cytotoxicity Test

Cytotoxicity (CTX) was determined by using the MTT assay which is based on the metabolic reduction of 3-(4,5- dimethylthiazole-2-yl)-2,5-diphenyltetrazol bromide made by the mitochondrial succinate dehydrogenase enzyme in a blue compound (formazan) enabling to establish the mitochondrial functionality of the treated cells. This method has been widely used to measure cellular proliferation, survival and antiviral activity; thus the amount of formazan is directly proportional to cell viability (2). Initially, the cells were seeded at a density of 1 x 10^5 cells per well in a plate of 96 wells in 200 µL of DMEM with 10% FBS cultured 24 hours at 5% CO_2 and temperature of 37°C. Then, cells were treated with various compound concentrations made in triplicate. Forty-eight hours after treatment, the supernatant was removed and an MTT solution was added to the culture (0.5 mg/mL). 130 µL/well of DMSO (dimethyl sulfoxide) were added after two hours of incubation. Plates were left in agitation for 15 minutes and finally measured in a spectrophotometer at 550nm. Untreated controls were also included with cell viability assumed to be 100%. Each experimental condition was evaluated in triplicate in two independent experiments (n = 6). For antiviral assays, compound concentrations which showed less than 20% cytotoxicity were measured.

2. Shen L., et al. High-throughput screening and identification of potent broad-spectrum inhibitors of coronaviruses. J. Virol. 2019;93(12).

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Antiviral Activity Assay

Vero E6 cells were seeded in 96-well plates at a density of 1 x 10^5 cells/well in 100 µL/well of DMEM with 2% FBS at 5% CO_2 and 37°C, 24 hours before being used in this experiment. On the day of the assay, non-cytotoxic compound dilutions were added to Vero E6 cells replicated in 3 wells during 1 hour. After this time, the compound was removed and the cells were infected with the SARS-CoV-2 virus at an MOI (multiplicity of infection) of 0.1 during 1 hour. After that period, the virus remnant that failed to enter the cells was removed and replaced by fresh culture, adding new compound dilutions. Cells were cultured during 48 hours, and at the end of this period, an MTT assay, previously described, was carried out to determine the antiviral activity.

The following controls were applied to this experiment: negative controls (cells + DMEM) and positive controls (cells + DMEM + virus).

The following formula was used to calculate the antiviral activity:

$$\% AVA = 100 \times [(A-B) / (C-B)]$$

Where A corresponds to the DO of infected cells treated with the compound, B to the DO of infected cells not treated with the compounds (positive control of infection) and C to the DO of non-infected cells (negative control of infection).

Additionally, the highest dilution in which antiviral activity was observed comparing compound-treated infected cells vs non treated infected cells, with compound cytotoxicity under 20%, was used to determine the titer of the virus in a plating assay.

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The plating assay is a technique considered as the gold standard to determine the viral titer; therefore, this is the technique of choice to efficiently determine the reduction in the viral titer and, therefore, the antiviral activity in this type of experiments.

Lastly, statistical analysis for all assays show the mean with the respective standard deviation in each dilution. Parametric or non-parametric statistical tests were conducted to identify differences between the conditions of each experiment. A value of $p < 0.05$ was considered statistically meaningful.

3. RESULTS

Regarding the cell viability assay with the **Sambucol** compound, Figure 1 shows that the undiluted condition and the 1:2, 1:4 and 1:8 dilutions were found to be cytotoxic with mean cell viability values of 24%, 34%, 21% and 47% respectively. Whereas dilutions equal to or greater than 1:16 showed an average cell viability higher than 91%, this and later dilutions were selected to carry out antiviral assays of the **Sambucol** compound (see Figure 1).

Regarding the antiviral activity assay measured by MTT using SARS-CoV-2 at an MOI of 0.1, as shown in Figure 2, the **Sambucol** compound, in a 1:32 dilution achieved 33% of average antiviral activity while dilutions of 1:64, 1:128 and 1:256 showed an average antiviral activity of 32%, 18%, and 6% respectively. Other dilutions showed little or no antiviral activity; in addition, a statistically meaningful difference was observed between these dilutions and the Chloroquine, the positive control of inhibition of

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viral replication used in this experiment, which showed an average inhibition of 73% (see Figure 2).

Lastly, the virus titer was determined by plating essay based on the supernatant of the 1:32 dilution of the **Sambucol** compound, obtained through the antiviral activity assay; such dilution was chosen according to criteria mentioned in the methodology section. The plating assay reflects that the viral titer in presence of treatment with the compound was on average 2.58×10^6 PFU/mL, while the virus titer in untreated controls was on average 4.38×10^6 PFU/mL which means an inhibition of 41% of infectious viral particles of SARS-CoV-2 by the **Sambucol** compound (see Figure 3).

4. CONCLUSION

It can be concluded that the **Sambucol** compound inhibits 41% of the infectious viral particles of SARS-CoV-2.

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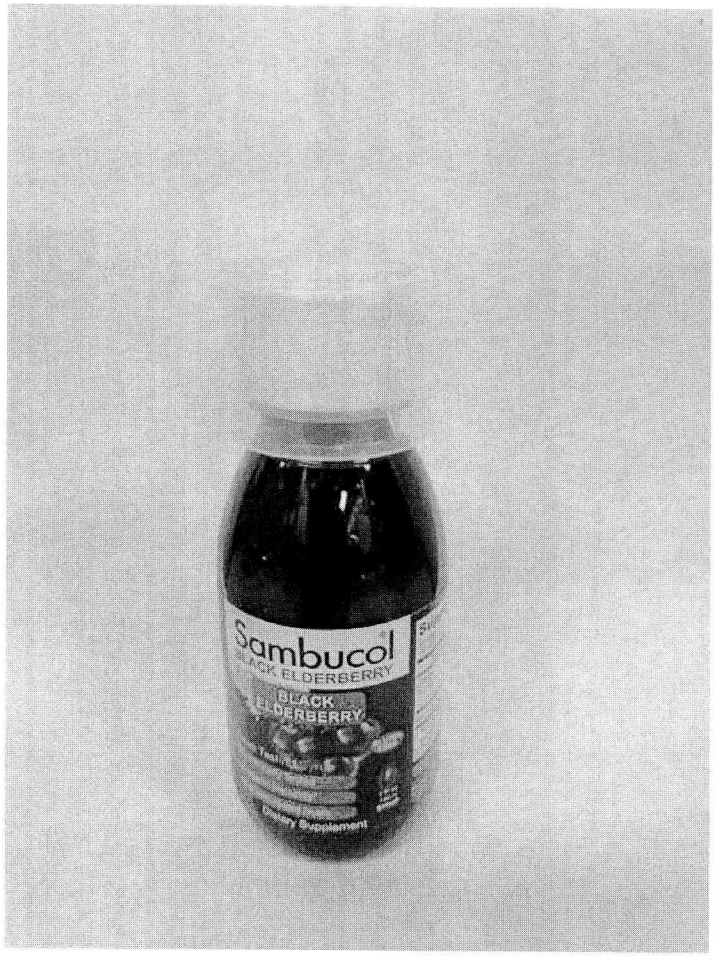

Photograph 1

Sambucol compound submitted to the laboratory of the Immunovirology Group of Universidad de Antioquia.

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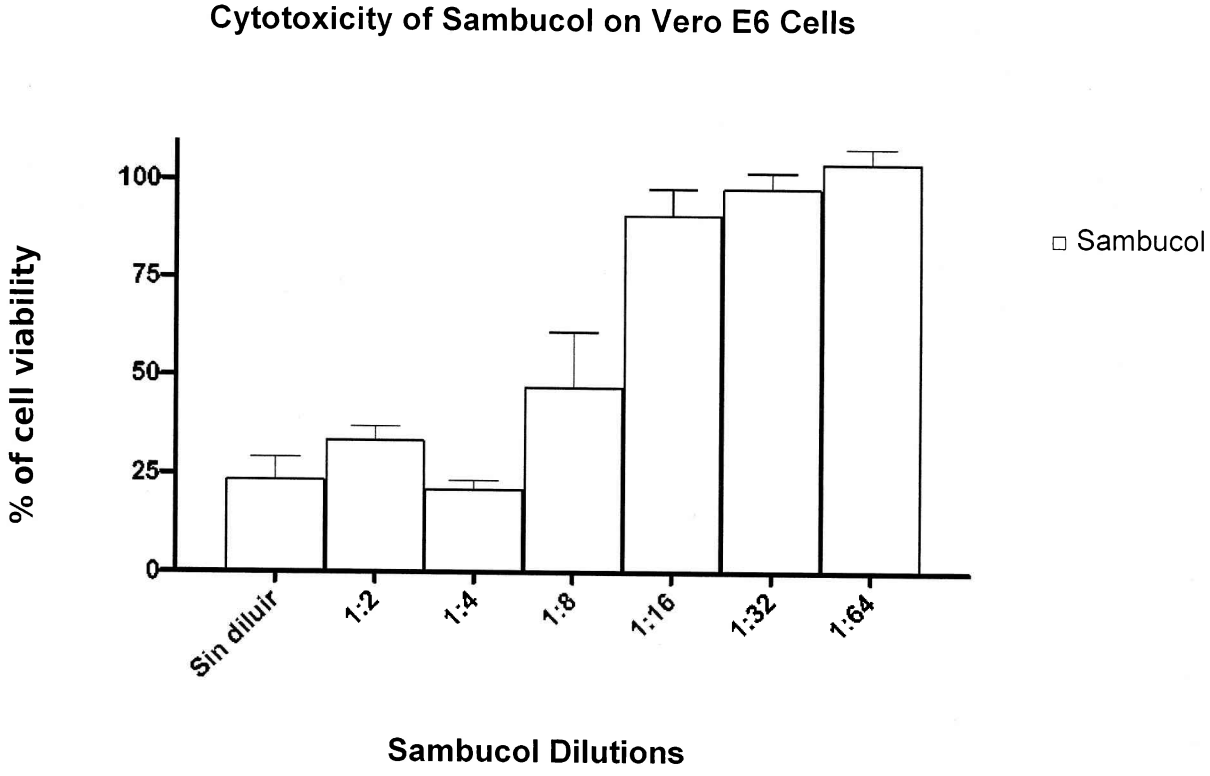

Figure 1.

Cell viability assay measured by MTT that shows the percentage of living cells after treatment with different concentrations of the compound and after 48 hours of cell culture. The graph shows the average of each measurement and the standard deviation. Two experiments were conducted with 4 replicas each.

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Antiviral Activity of Sambucol during the infection with the SARS-CoV-2 Virus

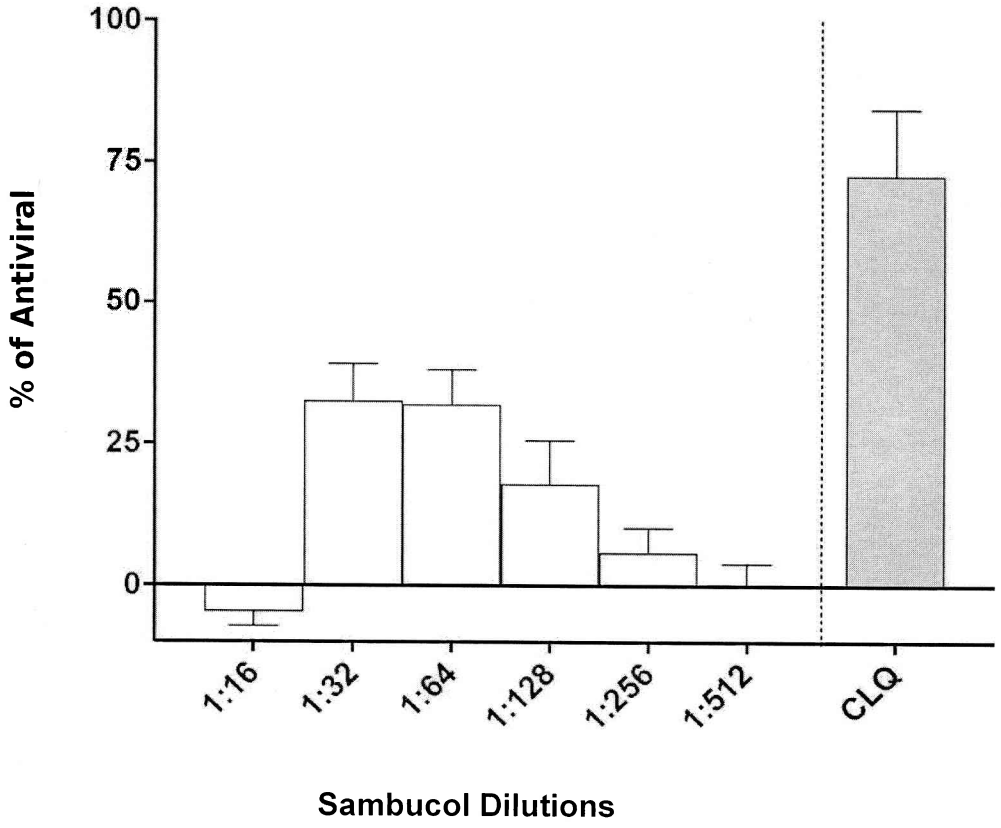

Figure 2

Percentage of antiviral activity calculated based on the cell viability assay. The graph shows the percentage of antiviral activity of **Sambucol** compound in various dilutions when using the virus SARS-CoV-2 at an MOI of 0.1. The average of each measurement and the standard deviation can be observed. Two experiments were conducted with 4 replicas each. CLQ: Chloroquine, positive control of inhibition of viral replication.

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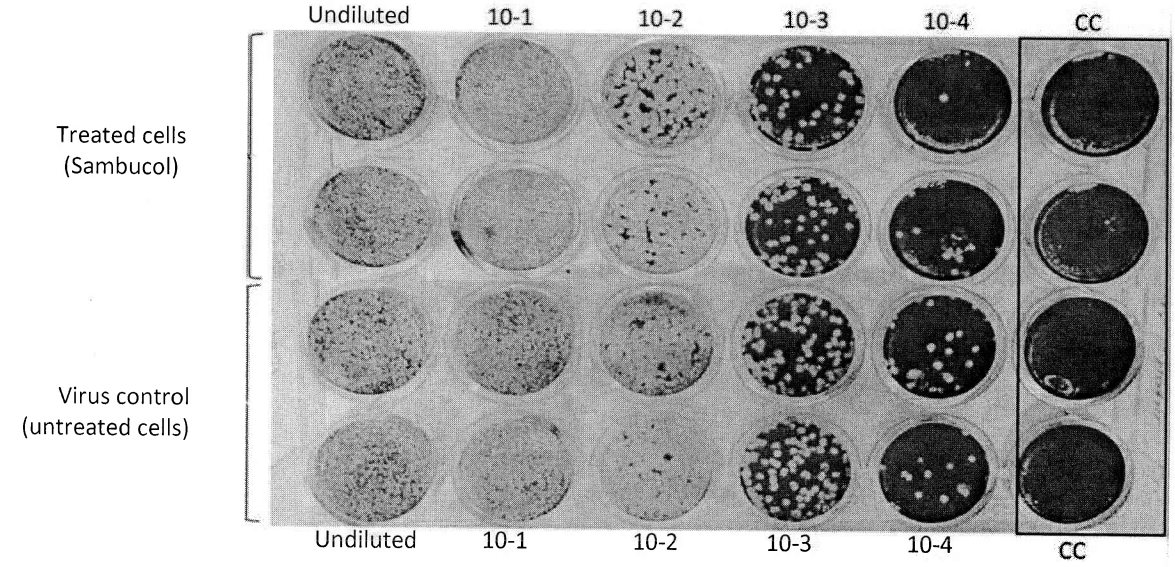

Figure 3

The plating assay shows plates formed by the SARS-CoV-2, obtained from the supernatant of the untreated control cells and from the **Sambucol** treated cells in the antiviral assay. Dilutions from 10^{-1} to 10^{-4} as well as the undiluted condition are observed. Result is stated as PFU/mL.

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* *

[Translator's comments in brackets]: [As a sworn translator, duly authorized by the Ministry of Justice of Colombia, according to Resolution No. 2035 of 1994, I certify that the foregoing is a true and accurate translation of the original document written in Spanish I have had before me. August 31, 2020 E-mail: claudia_guzman@live.com]

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