In Vitro Inhibition of SARS-CoV-2 Infection by Dry Algae Powders

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Abstract

Chlorella spp., Spirulina spp., and fucoidan dry powders, are commercialized as food supplements and are considered safe for human consumption. Their broad-spectrum antiviral properties have been studied, however, their effect against SARS-CoV-2 remains unknown. We investigated the potential antiviral activity of three algae powders: Chlorella vulgaris, Spirulina máxima and fucoidan purified from marine brown algae Sargassum spp. against SARS-CoV-2 infection in vitro. Vero cells were incubated with 70 µg/ml of each algae powder and either 50 or 100 TCID50/ml of SARS-CoV-2. Chlorella powder inhibited SARS-CoV-2 infection; viral RNA was signifcantly reduced in supernatants at 24, 48, 72, and 96 hrs post-infection, the highest difference in viral load (8000-fold) was observed after 96 hrs. Spirulina maxima powder partially inhibited SARS-CoV-2 infection since no cytopathic effect was observed in 87.5% of infected cultures and viral RNA decreased 48 hrs after infection, reaching a 250-fold difference at 72hrs. In conclusion, our preliminary in vitro assays suggest that C. vulgaris and S. máxima dry algae, may potentially be used to fight COVID-19.

1. Introduction

Coronavirus disease (COVID-19) is an infectious disease characterized by fever, sore throat, loss of smell, and in severe cases, breathing difficulty and chest pain [1]. In December 2019 a cluster of unknown-origin pneumonia cases were reported in the city of Wuhan, Hebei province [2]. The etiological agent was identified as a novel coronavirus soon after [3]. Since then, COVID-19 has spread worldwide and was declared a global pandemic on March 11, 2020 by the world health organization (WHO) [4]. At the time of writing, over 422 million cases and 5.8 million deaths have been reported worldwide [5]. COVID-19 is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a single stranded RNA virus belonging to the betacoronavirus genus [6]. The genome of RNA viruses mutates quickly due to the action of the error-prone viral RNA polymerase. Fast mutation rates promote rapid viral diversification [7]. Rapid mutation in SARS-CoV-2, has led to the continuous emergence of new variants, prompting WHO to characterize them into variants of interest (VOI) and variants of concern (VOC), according to the perceived risk to public health. As of November 2021, WHO has declared 5 VOC: alpha, beta, gama, delta and omicron [8]. Mass immunization programs have been established worldwide with considerable success, especially in developed nations. To date 66% of the extended European area and 59.7% of the US population have received full immunization [9, 10]. However, breakthrough infections in vaccinated individuals have been reported and correlated with both the time from infection and infection with novel variants, thus, a future increase in breakthrough infection rates is likely [10–15, 16]. The emerging variants and the increase of the breakthrough infections phenomenon has highlighted the need to develop novel antiviral compounds. Several vascular plant compounds, including alkaloids, flavonoids, polyphenols, and tannins, have been reported to inhibit either SARS-CoV-2 replication, or the activity of viral functional components [17]. Epigallocatechin-3-gallate (EGCG), a flavonoid from tea, has been found to inhibit SARS-CoV-2 infection in vitro, possibly by inhibiting the activity of the virus 3CL-protease, responsible for the viral polyprotein maturation [18–20]. Naringerin, another flavonoid present in grapes,

has been shown to inhibit SARS-CoV-2 infection in vitro [21]. Berbamine, an alkaloid involved in Ca2 + signaling, has also shown anti-SARS-CoV-2 activity in vitro [22].

In some research, micro and macro algae have been shown to produce antiviral agents. For instance, Hayashi et al. (1996) found in Spirulina platensis (Arthrospira platensis) an antiviral compound (calcium spirulan) that inhibits the entry of the enveloped viruses; herpes simplex (HIV-1), human cytomegalovirus (CMV), measles virus, mumps virus, and influenza A virus [23]. In 1998, Ayehunie et al. demonstrated that aqueous extract from S. platensis inhibited HIV-1 replication in human T-cell lines, peripheral blood mononuclear cells (PBMC), and Langerhans cells (LC) [24]. Later polysaccharide fractions of S. platensis were analyzed, showing strong antiviral activity against CMV, HSV-1, HSV-2, HSV-6, Pseudorabies virus (PRV), and human immunodeficiency virus type 1 (HIV-1) [25, 26]. Jang and Park found that a compound isolated from S. maxima, inhibited HIV-1 infection in the human T cell line MT4 [27]. A similar observation was found when testing a raw extract of Chlorella peruviana, which showed antiviral activity by inhibiting the replication of dengue virus serotype 2 (DENV-2), in vero-76 cells [28]. In another study C. vulgaris polysaccharides presented antiviral activity against replication of grass carp reovirus (GCRV), in vitro and in vivo [29]. In terms of macroalgae, the species of Sargassum henslowianum and S. naozhouense have been reported to inhibit HSV-1 in vitro, by cytopathic effect inhibition and reduction of plaque assay respectively [30, 31]. According to the above, a possible antiviral treatment against SARS-CoV-2 could be obtained from some micro and macro algae [32]. Raposo et al. summarized the bioactivity and applications of sulphated polysaccharides from various algae, including antiviral activities against a variety of viruses [33]. Recently, Song et al. reported that the sulphated polysaccharides fucoidan and carrageenan showed significant antiviral activities at concentrations of 3.90 ~ 500 μg/mL against SARS-CoV-2 [34]. Even though the capacity to produce antiviral compounds and the antiviral activity of whole extracts is well established, to the best of our knowledge, there are no reports of their effect against SARS-CoV-2 for any of the algae studied herein. In this context, we decided to investigate the effect of whole dried C. vulgaris and S. maxima, and purified fucoidan Alquimar® obtained from Sargassum spp. against SARS-CoV-2 infection in vitro.

2. Results

2.1 Samples cytotoxicity

To identify the maximum non-cytotoxic concentration, three different concentrations of each sample were tested (50, 70, and 100 μg/ml. The 50 and 70 μg/ml concentrations exhibited no detectable damage such as loss of confluence, cell rounding or vacuolization (Fig. 1b), when compared to the untreated control (Fig. 1a). Some modifications in the cell monolayer were observed at 100 μg/ml. Thus, the 70 μg/ml concentration was selected for further antiviral assays. MTT assays showed a cell viability of 78.1%, 76.0% and 67.5% for Fucoidan, Spirulina and Chlorella 72 hrs post treatment at a sample concentration of 70 μg/ml.

2.2 Antiviral assays

To determine the possible antiviral activity of the three different algae samples against SARS-CoV-2, two types of experiments were carried out (simultaneous and pretreatment), in presence of 50 and 100 TCID50/ml of SARS-CoV-2. In the assays in presence of C. vulgaris no CPE appearance was recorded for both types of experiments, in any of the replicates, using 50 or 100 TCID50/ml of SARS-CoV-2 (Fig. 1). In the case of S. maxima at the same concentration, no CPE was observed when challenged with 50 TCID50/ml of the virus for both, simultaneous and pretreatment assays; however, when the viral concentration was increased to 100 TCID50/ml, CPE appeared in 50% and 75% of the replicates, for pretreatment and simultaneous assays respectively. Assays in presence of fucoidan samples at 70 µg/ml concentration also presented protection against viral infection. In presence of 50 TCID50/ml, CPE appeared in 37.5% of the replicates for both pretreatment and simultaneous assays; however, when the viral concentration was increased to 100 TCID50/ml, CPE were observed in a 50% and 75% of the replicates, for pretreatment and simultaneous assays respectively (Table 1).

Table 1
Comparison in the percentage of infection (appearance of CPE) between 50 and 100 TCID50/ml.

Solvent		**50 $TCID_{50}$/ml***		**100 $TCID_{50}$/ml**	
DMEM	Sample	Pretreated	Simultaneous	Pretreated	Simultaneous
	Fucoidan	37.50%	37.50%	50%	75%
	Spirulina	0%	0%	50%	75%
	Chlorella	0%	0%	0%	0%
DMSO					
	Fucoidan	0%	0%	75%	87.5%
	Spirulina	0%	0%	12.5%	25%
	Chlorella	0%	0%	0%	0%

*In the assays with 50 TCID50/ml each treatment had 8 replicates while with 100 TCID50/ml the experiments had 4 replicates. The results are given in percentage of infection (CPE appearance in the replicates).

2.3 Algae bioavailability depending on type of solvent.

To assess if the solvent type could improve samples bioavailability, simultaneous and pretreatment assays were carried out using DMEM and DMEM plus 4% DMSO with 50 and 100 TCID50/ml of SARS-CoV-2 inoculations. Surprisingly, when C. vulgaris, S. maxima, and the fucoidan samples were challenged against 50 TCID50/ml, all samples showed 0% of infection since no CPE appeared in any of the replicates for both assays. Furthermore, when the viral concentration was increased to 100 TCID50/ml, S. maxima decreased the infection of the cell cultures from 50% using only DMEM, to 12.5% with DMSO in pretreatment and from 75–25% in the simultaneous assay. However, the Fucoidan did not show a

decrease in protection, but an increase with respect to the type of solvent. Infection increased from 50–75% in the pre-treatment assay and from 75–87.5% in the simultaneous assay (Table 1).

2.4 Protection against SARS-CoV-2 infection in vitro.

To further investigate the inhibitory effect of the different algae samples on SARS-CoV-2 infection, viral genome copies were quantified by RT-qPCR. Both C. vulgaris and S. maxima showed a significant difference in viral load when measured by RT-qPCR. The strongest antiviral effect was observed in the cells treated with C. vulgaris powder in both simultaneous and pretreatment schemes with the highest effect at 96 hrs post-infection (pi) with more than 1000-fold difference between treatment and control. Dry S. maxima powder also showed a powerful inhibitory effect, especially in the simultaneous scheme, however, it only showed partial protection in the pretreatment experiments with some of the replicates presenting a high viral load and others showing a clear decrease in viral load (Fig. 2).

2.5 C. vulgaris antiviral activity.

Chlorella strongly inhibited SARS-CoV-2 infection in vitro, in fact, a slight decrease in viral load was observed over time with the lowest concentration 103.2 for pretreatment and 103.1 for simultaneous assays were measured at 96 h pi. The biggest inhibition was measured at 96 h pi with both pretreatment and simultaneous experiments showing 1000-fold decrease in viral load, this difference was statistically significant with P-values of < 0.000 in the simultaneous assays and < 0.0 in the pretreatment assays (Fig. 2A and 2B).

2.6 S. maxima antiviral activity.

RT-qPCR of supernatants recovered from cells infected with 100 TCID50/ml and treated with S. maxima powder diluted in DMEM in simultaneous assays, showed a sharp reduction in genome copy numbers with respect to the control. The highest difference was observed at 72h, where an average of 104.5 genome copies were estimated for cells treated with the algae, in contrast to an average of 106.9 estimated for the positive control; this difference was statistically significant with a P-value of < 0.0 (Fig. 2B). High variability between replicates was observed due to infection of some of the cultures.

2.7 Fucoidan antiviral activity.

Supernatants of cells treated with fucoidan in the simultaneous scheme showed a decrease in viral RNA 24, 48, 72 and 96 hrs post- infection (Fig. 2B), the strongest decrease (> 10-fold) was measured at 72 hrs. Viral RNA also decreased in cell cultures in the pretreatment scheme at 48 and 72 hrs post-infection, however, these differences are not statistical significant.

3. Discussion

In recent years, the use of dry Chlorella and Spirulina as food supplements and nutraceuticals has increased substantially, both Chlorella and Spirulina are considered an excellent source of non-animal protein and are used widely as a supplement, especially in vegan diets [35]. These algae have not only

been proved safe for human consumption but are also easy to produce and available at a low price worldwide. The results presented herein suggest that they also have potential to be used as virus inhibitors against SARS-CoV-2.

C. vulgaris powder showed strong protection of the Vero cells cultures for all kinds of essay, solvent used, and viral concentration, since no CPE was observed in any replicates. Interestingly, the viral load measured in the supernatants of cell cultures showed sustained decrease form 103.7 and 103.9 at 24hrs post-infection to 103.2 and 103.1 viral genome copies for pretreatment and simultaneous experiments respectively. This decrease suggests that C. vulgaris inhibits SARS-CoV-2 replication since the estimated viral load consistently decreased over time. These results are in line with previous studies of animal viruses, such as grass carp reovirus and cyprinid herpesvirus 3 [29, 36] where chlorella was shown to inhibit viral replication.

S. maxima powder also showed protection of cell cultures, no CPE was detected when 70 µg/ml of dry algae powder, dissolved in DMEM medium or DMSO, were added to cultures challenged with 50 TCID50/ml of SARS-CoV-2. However, it only showed partial protection when the viral concentration was increased to 100 TCID50/ml. The viral load present in supernatants of cells challenged with 100 TCID50/ml showed a great variability among replicates, this is probably due to viral infection of some replicas since S. maxima only provided partial protection at this viral concentration. Also, we demonstrated that these algae were more bioavailable in DMSO solution. Similar results were presented by Hernandez-Corona [26], who inhibited 200 TCID50/ml of HSV-1 and HSV-2 using 69 and 300 µg/ml of S. maxima respectively.

The dry fucoidan powder showed a minor antiviral activity compared to C. vulgaris and S. maxima. However, is important to note that fucoidan did show antiviral activity against SARS-CoV-2. When fucoidan was dissolved in DMEM medium, infection was observed only in 37.5% cultures infected with 50 TCID50/ml. Infection rate increased to 62.5% when DMSO was used as solvent. This suggests that fucoidan bioavailability might be better in aqueous solutions such as DMEM especially considering that fucoidan is a water-soluble polysaccharide [37]. Our results are in line with those of Song et al. who previously reported fucoidan's inhibitory effect against SARS-CoV-2 [34]. Hidari found that algae derived polysaccharides inhibit DENV replication by preventing cell internalization. [38]. In accordance with this, Elizondo-Gonzalez found that fucoidan blocked early stages of Newcastle Disease virus infection [39]. It is possible that fucoidan might also be preventing SARS-CoV-2 infection by preventing viral internalization. It is also worth noting that fucoidan has been found to promote recovery of mitochondrial membrane potential in PBMCs from COVID-19 recovered patients, indicating that fucoidan may be a potential treatment to diminish long-term sequelae of the disease [40]. This is particularly promising considering that fucoidan is isolated from algae that produce environmental disruption and pollution on Mexican coasts.

Taken together, the data provided by this work shows that both dry C. vulgaris and S. maxima powders are potent inhibitors of SARS-CoV-2 in vitro. Fucoidan showed antiviral activity in some of our

experiments and should not be disregarded especially considering the recent evidence of mitochondrial membrane potential recovery. Further characterization is needed to identify the specific agents of each alga responsible for the antiviral activity and their action mechanism. Finally, the results of the present investigation suggest that these algae may have potential use in the treatment of SARS-CoV-2 infections; however, further research, preclinical and clinical studies are needed to support this assumption.

4. Materials And Methods

All experiments involving SARS-CoV-2 were carried out in the CIATEJ BSL-3 facility. The SARS-CoV-2 strain used in the experiments was a clinical isolate provided by the Hospital Civil de Guadalajara Fray Antonio Alcalde, identified by RT-PCR using the primers and probes described by WHO for the diagnostic detection of the E and RdRp genes of the SARS-CoV-2 virus.

Vero CCL-81 cells (American Type Culture Collection) were maintained with Dulbecco's Modified Eagle Medium (DMEM) containing L-glutamine (30 µg/mL, Sigma-Aldrich) and 10% of Fetal Bovine Serum (FBS) for growth medium (GM) or 2% of FBS for maintenance medium (MM) at 37°C in a humidified atmosphere with 5% CO2.

To evaluate a possible antiviral effect against SARS CoV-2, powder samples of C. vulgaris, S. maxima and a sample of fucoidan Alquimar ® (250.61 kDa) described by Díaz-Resendiz et al. (2022) [35], all provided by the company Creamos mas, were used in this study. Each algae was rehydrated with 1 ml of DMEM or 1 ml of dimethyl sulfoxide (DMSO) to prepare a final concentration of 10 mg/ml.

To identify the maximum non-cytotoxic concentration for the experiments, three different concentrations of each sample were tested in Vero cells and cytotoxicity was determined microscopically by observation of cell morphological changes under inverted microscope. To confirm results of the selected concentration, cytotoxicity was measured by the MTT assay to determine the capability of living cells to convert a soluble tetrazolium salt [3- (4,5-dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide (MTT) to insoluble formazan crystals. Vero cells were seeded in 96-well plates 24 hours before assay. Then the cells were treated with 100 µl of each sample at 70µg/ml concentration in MM, later the plate was incubated for 48 hours to determine the optimal effect of samples. Subsequently, the plate was carefully emptied, 10µl / well of MTT solution (0.5 mg / mL) were added and the plate was incubated for 2 hours. After incubation, 100 µl of isopropanol were added to each well and gently mixed until all crystals had dissolved. Once formazan was re-solubilized, absorbance was measured at 570 nm in a microplate reader to determine concentration. Control cells were incubated without samples. The percentage of cytotoxicity was calculated as (A-B)/A x 100, where A is the mean optical density of untreated wells and B is the optical density of wells with algae samples.

In order to assess the algae antiviral protection against SARS-CoV-2, and at the same time, assess whether the algae samples could confer some prophylaxis against the virus, two types of experiments were carried out using Vero cells. The first experiment was designated as "simultaneous" (70 µg/ml of sample, plus 50 or 100 TCID50/ml of the virus incubated 1 hr at 37°C), and the second experiment

(designed to assess prophylactic potential), was designated as “pretreatment” (Vero cells monolayers were pretreated with 70 µg/ml of sample x 48 hrs, then the medium was discarded and 70 µg/ml of fresh sample, plus 50 or 100 TCID50/ml of the virus were added, and incubated for 1 hr at 37° C). Both the “simultaneous” and “pretreatment” experiments were performed using the rehydrated samples (either in DMEM or 4% DMSO plus DMEM) at 10 mg/ml concentration. All experiments were performed in 96-well plates kept in an incubator at 37°C with 5% CO2 for 5 days and examined daily under the inverted microscope for evidence of viral cytopathic effect (CPE). Images were captured with a camera Optikam WiFi – 4083.

To assess viral load and cytopathic effect (CPE) inhibition between cell cultures treated with the algae samples, compared to untreated cultures (virus only), viral infection kinetics of simultaneous and pretreatment experiments were performed, using 100 TCID50/ml of the virus, plus 70 µg/ml of each sample. For this, cell culture supernatants were carefully harvested from each well of the 96-well microplates, at 24, 48, 72, and 96 hrs post-infection of each treatment, and kept in an ultra-freezer at -80°C for further use. For viral load quantification, the harvested supernatants from each treatment (by triplicate) per kinetic day, were thawed and RNA extraction was performed using the QIAamp Viral RNA kit (QiagenTM, Hilden, Germany), according to the manufacturer instructions. The extraction was carried out from 100 µl of supernatant and the RNA was resuspended in 60 µl of RNase-free water and stored at -80°C for later use.

Since greater differences in CPE appearance during the antiviral assays were recorded when 100 TCID50/ml were used for viral quantification, supernatants of the cultures from these experiments were collected to measure viral load. RT-qPCRs were performed using the primers and probes described by WHO for the diagnostic detection of the SARS-CoV-2 that amplify a 113 nt region of the virus E gene; (forward E_Sarbeco_F1 5’ ACAGGTACGTTAATAGTTAATAGCGT 3’, reverse E_Sarbeco_R2 5’ ATATTGCAGCAGTACGCACACA 3’ and prove E_Sarbeco_P1 5’ FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1 3’) approved for its use in Mexico by Instituto de Diagnóstico y Referencia Epidemiológicos Dr. Manuel Martínez Báez (InDRE). Super ScriptTM III PlatinumTM One-Step qRT-PCR System kit (Invitrogen) was used to perform the RT-PCRs in a CFX96 Real-Time System thermocycler instrument (Bio-Rad). To quantify the treatment viral loads, a standard curve with four triplicated dilutions was generated, using a plasmid containing SARS-CoV-2 genome fragments, recognized by the envelope gene probe, provided by IBT, UNAM. Finally, to estimate the viral load, the average Ct of each dilution was used to perform a simple linear regression.

The statistical significance of control and treatment groups were assessed using a one-way ANOVA and T-test, to determine if there was a significant difference. Data analysis was carried out using IBM SPSS statistics. A P value < 0.05 was considered statistically significant.

Declarations

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Data Availability Statement: The data supporting the findings of this study is available from the corresponding author upon reasonable request.

Conflicts of Interest: "The authors declare no conflict of interest."

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Institutional Review Board Statement: This study was approved by the Centro de investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C. (CIATEJ) Biosecurity Committee (First Ordinary session March 2021).

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Figures

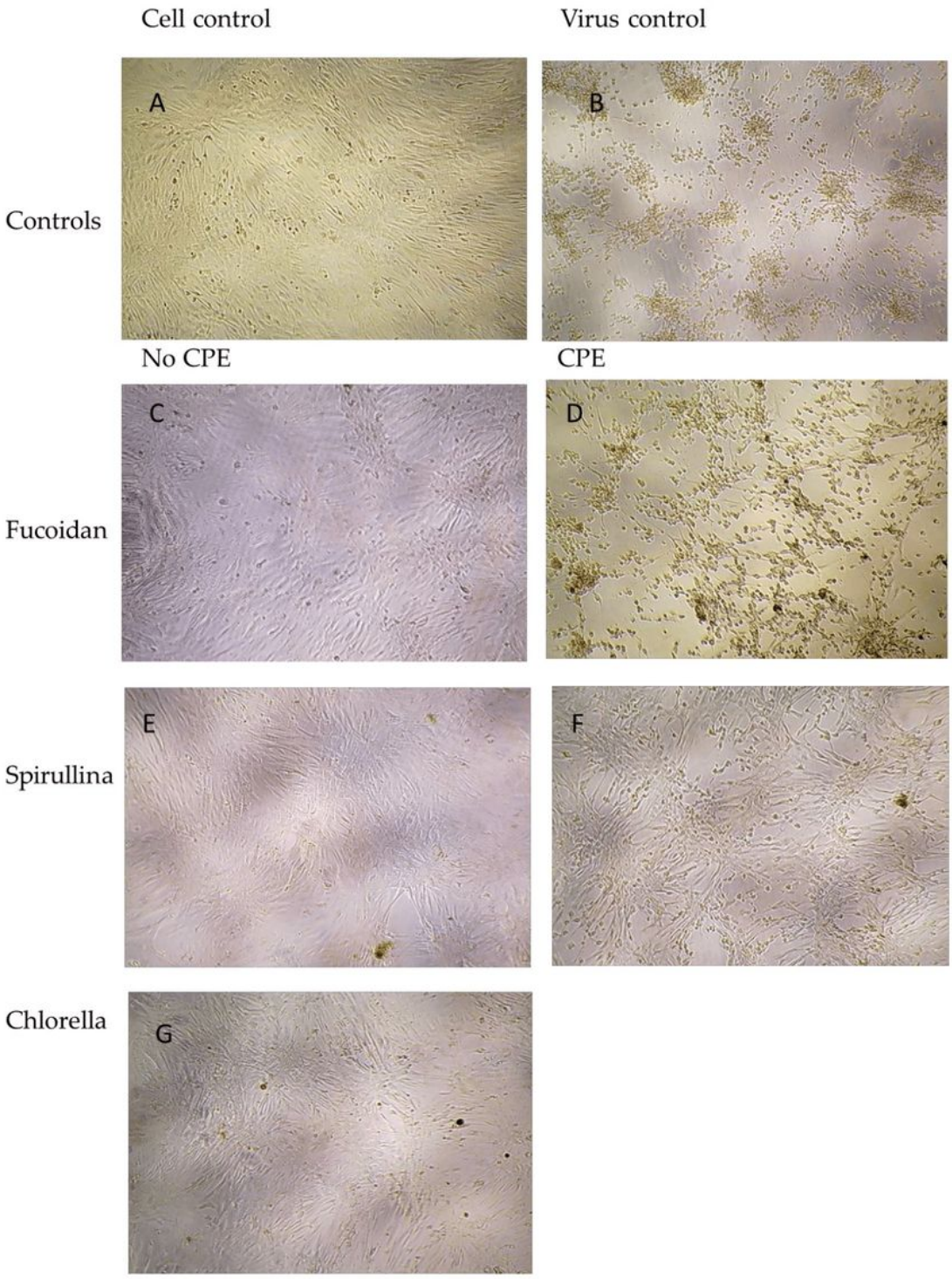

Figure 1

Inverted microscopic photographs representative of cultures at 96 hours post infection. Vero cells where infected with 100 TCID50/ml of SARS-CoV-2 and treated with 70 µg/ml of algae. A: Uninfected Vero cells; B: Vero cells infected with of SARS-CoV-2 presenting CPE consisting of cell detachment and culture degeneration; C: Vero cells treated with fucoidan. Some of the cell cultures show CPE. D: Cells treated with S. maxima, CPE is present in some of the cultures. G: Cells treated with C. vulgaris, no CPE was detected in any of the cultures.

Figure 2

Quantification of viral load after infection. Mean values and mean ± SEM are shown. A: C. vulgaris. B: S. máxima. C: fucoidan. Statistical significance is indicated by * P-value < 0.0, ** P-value < 0.00 and *** P-value of <000.