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RESEARCH ARTICLE

# Virucidal activity of microalgae extracts harvested during phycoremediation of swine wastewater

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## Abstract

Phycoremediation of swine wastewater is a promising treatment since it efficiently removes nutrients and contaminants and, simultaneously, its biomass can be harvested and used to obtain a wide range of valuable compounds and metabolites. In this context, biomass microalgae were investigated for the phycoremediation of swine wastewater, and biomass extracts for its virucidal effect against enveloped and non-enveloped viruses. Microalgae were cultivated in a pilot scale bioreactor fed with swine wastewater as the growth substrate. Hexane, dichloromethane, and methanol were used to obtain the microalgae extracts. Extracts were tested for virucidal potential against HSV-1 and HAdV-5. Virucidal assays were conducted at temperatures that emulate environmental conditions (21 °C) and body temperature (37 °C). The maximum production of microalgae biomass reached a concentration of $318.5 \pm 23.6$ $mg_{DW}$ $L^{-1}$. The results showed that phycoremediation removed 100% of ammonia-N and phosphate-P, with rates ($k_1$) of $0.218 \pm 0.013$ and $0.501 \pm 0.038$ ($day^{-1}$), respectively. All microalgae extract reduced 100% of the infectious capacity of HSV-1. The microalgae extracts with dichloromethane and methanol showed inhibition activities at the lowest concentration (3.125 µg $mL^{-1}$). Virucidal assays against HAdV-5 using microalgae extract of hexane and methanol inhibited the infectious capacity of the virus by 70% at all concentrations tested at 37 °C. At a concentration of 12.5 µg $mL^{-1}$, the dichloromethane microalgae extract reduced 50–80% of the infectious capacity of HAdV-5, also at 37 °C. Overall, the results suggest that the microalgae can be an attractive source of feedstock biomass for the exploration of alternative virucidal compounds.

**Keywords** Enveloped virus · Non-enveloped virus · Infectivity inhibition · *Chlorella* biomass · Nitrogen removal · Phosphorus removal

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## Introduction

The nutrient-rich wastewater produced in the swine farm industry is conventionally treated by anaerobic digestion to reduce carbon loads followed by a tertiary treatment system, such as phycoremediation, to remove nitrogenous and phosphate compounds (Cheng et al. 2019). Phycoremediation has gained considerable attention, mainly for their efficiency in bioremediation and the potential for generating valuable raw material for various products of biotechnological importance, such as bioenergy production, nutrition, and pharmacological (Mohd Udaiyappan et al. 2017). Pharmacological compounds are of particular interest, and efforts have been focused on the potential of bioactive compounds for the control of microorganisms, since the worldwide scenario of diseases caused by microorganisms has been worsening (Falaise et al. 2016).

For the past 50 years, algae have been studied for their antiviral potential, with successful results in experiments conducted under controlled laboratory conditions and with a synthetic culture medium (Pagarete et al. 2021). The exact mechanisms of virus inhibition by microalgae extracts are still not completely understood (Joseph et al. 2020) but could be associated with the presence of polysaccharides (Hasui et al. 1995; Lee et al. 2006), proteins (Emad et al. 2010), fatty acids (Kamat et al. 1992), and terpene (Pereira et al. 2005; Cirne-Santos et al. 2020) compounds. However, it is well known that depending on the culture medium composition, the microalgae can change their metabolites and compounds production, changing the antimicrobial effect (Aremu et al. 2015).

In recent years, microalgae have been grown using swine wastewater as a base for the culture medium, a complex matrix that can influence cellular biochemical profile (Michelon et al. 2021). However, as far as we know, no study has been applied to evaluate the virucidal activity of microalgae extracts obtained from the biomass harvested from swine wastewater treatment systems.

Viral diseases have caused public health concern worldwide, since non-enveloped viruses are responsible for more than 500,000 deaths per year (WHO 2019); while enveloped viruses are responsible for the largest recorded pandemics, with the number of deaths increasing every day (Wigginton and Boehm 2020). Viral diseases are of particular concern because viruses present infections at low doses, which can vary from $10^1$ to $10^3$ particles (Gibson 2014), and long periods of survival in environmental matrices (Barardi et al. 2012).

In this sense, research has focused on finding low-cost alternatives or those associated with the simultaneous removal of other contaminants, for the inactivation of viruses. For that, some viruses have been used as a research model, such as the human *Herpes Virus Simplex* type 1 (HSV-1), which are enveloped and double-stranded DNA viruses (Silva et al. 2010), and *Human Adenovirus* type 5 (HAdV-5), which are non-enveloped and double-stranded DNA viruses (Rafie et al. 2021).

The aim of this study was to investigate the phycoremediation applied to the treatment of swine wastewater for obtaining biomass extracts, using different solvents, to assess the virucide potential against enveloped and non-enveloped viral models.

## Material and methods

### Consortium of microalgae and phycoremediation of swine wastewater

The microalgae consortium (predominantly *Chlorella* spp. — Chlorophyta) used in this study was characterized and identified previously in Michelon et al. (2015). The microalgae were grown on a pilot scale in an open 500 L bioreactor (121.2 cm Ø i.d.; 58.4-cm height), installed in a greenhouse with controlled temperature (25 °C) and exposed to natural sunlight (photosynthetic photon flux density of 336.5 ± 410.4 μmol $m^{-2}$ $s^{-1}$). The culture medium was prepared with 50 L of swine wastewater digestate effluent diluted in 300 L of tap water. The composition of the medium was (mg $L^{-1}$): total organic carbon (120 ± 3.4), alkalinity as $CaCO_3$ (200 ± 9.2), ammonia-N (141.6 ± 12.7) and phosphate-P (12.9 ± 2.7). pH was 7.9 ± 0.9.

The bioreactor was inoculated with 150 L of inoculum, with a dry weight (DW) concentration of 60 ± 5.1 $mg_{DW}$ $L^{-1}$ of biomass. The agitation was kept by means of mechanical pumps at 1200 L $h^{-1}$ (Sarlobetter®, S300, Brazil). After 11 days of inoculation, biomass was harvested by centrifugation at 3000 × g (EVODOS T10, Netherlands), immediately frozen (− 20 °C) and then lyophilized (Model 030-JJ LJI, Scientific, Brazil) for further assays.

### Wastewater chemical analysis

Total organic carbon was measured by thermal catalytic oxidation using a TC/TN analyzer (Multi C/N 2100, Analytik Jena, Germany). Temperature was set at 900 °C. Oxygen was used as carrier at flow rate of 160 mL $min^{-1}$. The samples were filtered using 0.45-μm membrane filters (Millipore, USA) acidified with phosphoric acid (40% w $w^{-1}$) (Sigma-Aldrich, EUA) and injected (250 μL) directly into an analyzer. Calibration curves were prepared by serial dilution of a stock solution of 1000 mg $L^{-1}$ biphthalate (Synth, Brazil).

Alkalinity (measured as $CaCO_3$ $L^{-1}$) was determined by automatic titration (Metrohm 848 Titrino Plus, Switzerland) using sulfuric acid (0.1 mol $L^{-1}$, Merck, Germany) as titrant. Ammonia-N concentration was determined by a series of colorimetric assays performed automatically by a flow injection analysis system (FIAlab 2500 system, USA) (APHA 2012). Briefly, samples (10 mL) were filtered using a 0.45-μm membrane filter (Millipore, USA) then dispensed in the autosampler. Ammonia-N was measured at a wavelength of 650 nm. Calibration curves were prepared by a serial dilution of ammonia-N stock solution (2–10 mg $L^{-1}$, Merck, Germany).

The concentration of phosphate-P was determined by the ascorbic acid colorimetric method (4500-P) (APHA 2012). The reagent solution was prepared using 50-mL sulfuric acid (5 N; Sigma-Aldrich, USA), 5-mL antimony potassium tartrate solution (Sigma-Aldrich, USA), 15-mL ammonium molybdate solution (Synth, China), and 30-mL ascorbic acid solution (Synth, China). Then, 0.8 mL of this solution was added to 5 mL of previously filtered samples (0.45-μm membrane filters, Millipore, USA).

After 10 min, the absorbance of each sample was measured on a UV–visible spectrophotometer (Varian, Cary® 50 UV–Vis, USA) at 880 nm. Standard curves were prepared by a serial dilution of a phosphate-P stock solution (0.05–0.2 mg $L^{-1}$) (Merck, Germany).

A satisfactory correlation ($r^2 = 0.97$) between gravimetric assay (APHA 2012) and optical density ($OD_{750}$) ($mg_{DW}$ $L^{-1} = 241.88 \times OD_{750}$ nm–36.522) was determined for the dry matter biomass concentration. The growth of microalgae over time was measured using a spectrophotometer (Varin, Cary® 50 UV–Vis, USA) at 750 nm. Light intensity and pH were measured using a Luximeter (DX-100, Japan) and pH meter (Hanna Instruments, HI8424, USA), respectively.

## Solvent extraction

A quantity of 20 g lyophilized biomass was successively extracted by a serial of exhaustive extraction with hexane (≥ 97.0%; Sigma-Aldrich, USA), dichloromethane (≥ 99.7%; Sigma-Aldrich, USA) and methanol (≥ 99.9%; J.T. Baker, USA) at a concentration ratio of 1:5 (g:mL). Extracts were dried using a rotatory evaporator (Fisaton 803, Brazil) and kept under a vacuum at 50 °C to eliminate any residual concentrations of the solvents. The extracts were then resuspended in dimethyl sulphoxide (DMSO) (≥ 99.7%; Sigma-Aldrich, USA) at 50 mg $mL^{-1}$.

## Application on viral models

### Viruses and cell line

The cell lines used were kidney epithelial derived from African green monkey (VERO cells) (ATCC: CCL81) and adenocarcinomic human alveolar basal epithelial (A549 cells) (ATCC: CCL-185), grown in a minimum essential medium (MEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), and maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$.

Viral stock of *Herpes Simplex Virus* type 1 (KOS strain; Faculty of Pharmacy, University of Rennes, France) was propagated on VERO cells, and the viral titers were determined by a standard plaque assay (Burleson et al. 1992) and stored at − 80 °C. The *Human Adenovirus* type 5 viral stock was propagated on A549 cells, titrated as described by Rigotto et al. (2011) and stored at − 80 °C.

### Cytotoxicity assay

VERO and A549 cells were seeded (2.5 $10^4$ $well^{-1}$ in 96-well plates) and, after 24 h, the confluent cells were exposed for 48 h to different concentrations, ranging from 0.488 to 500 µg $mL^{-1}$, of microalgae-extract samples. After incubation, cell viability was assessed by sulforhodamine B (Sigma-Aldrich, USA) assay, that measure total protein mass, which is related to cell viability (Vichai and Kirtikara 2006). The percentages of viable cells were plotted against each sample concentration, and the cytotoxic concentration $CC_{50}$ values (concentration that inhibited cell viability by 50% when compared to untreated controls) were determined based on concentration–response curves using Graphpad Prism 6.0 (GraphPad software, La Jolla, CA).

### Virucidal assay

The virucidal assay followed the procedures described by Silva et al. (2010), where mixtures of the samples at six different concentrations (3.125, 6.25, 12.5, 25.0, 50.0, and 100.0 µg $mL^{-1}$) and $4 \times 10^4$ PFU of HSV-1 or HAdV-5 in a serum-free minimum essential medium eagle (MEM; Gibco, USA) or serum-free Dulbecco's modified eagle medium (DMEM; Gibco, USA), respectively, were co-incubated during 15 min at room temperature (21 °C) or at 37 °C. These temperatures were chosen aiming to emulate the environmental average temperature (21 °C) and body temperature (37 °C).

The samples were then diluted to non-inhibitory concentrations (1:100) in MEM, and the residual infectivity was determined by plaque number reduction assay. For this, 400 µL of each dilution was adsorbed for 1 h at 37 °C on VERO cells or A549 cells. Cell cultures were then overlaid with MEM 2 × containing 1.5% carboxymethylcellulose (CMC; Sigma-Aldrich, USA) or DMEM 2 × supplemented with 4% fetal bovine serum (FBS; Gibco, USA), 2% penicillin and streptomycin (PS; Gibco, USA), 2% magnesium chloride (1 M; Sigma-Aldrich, Brazil), and 2% pyruvic acid (100 mM; Sigma-Aldrich, USA) containing 0.6% bacteriological agar (BD — Becton, Dickinson and Company Sparks, MD 21,152 USA) for 2 or 7 days at 37 °C. Cells were fixed and stained with naphthol blue-black (Sigma-Aldrich, USA), and viral plaques were counted by using a stereomicroscope.

# Results and discussion

## Phycoremediation of swine wastewater and biomass production

The results of the phycoremediation applied in the removal of ammonia-N and phosphate-P are shown in Fig. 1a. It is possible to observe a 100% removal with an exponential profile in 7 and 11 days for ammonia-N and phosphate-P, respectively. The removal of ammonia-N and phosphate-P by phycoremediation process adjusted to the pseudo-first-order kinetics (Fig. 1b). The calculated pseudo-first-order kinetic rates ($k_1$) were 0.218 ± 0.013 and 0.501 ± 0.038 ($day^{-1}$) for ammonia-N and phosphate-P, respectively.

Overall, these data corroborate what has been reported in the literature about phycoremediation to be efficient for the removal of ammonia-N and phosphate-P from wastewater (Aslan and Kapdan 2006; Prandini et al. 2015; Apandi et al. 2019).

The maximum concentration of microalgae biomass reached 318.5 ± 23.6 $mg_{DW}$ $L^{-1}$ after 11 days of phycoremediation (Fig. 1a). Similar results with microalgae grown from swine wastewater effluent have been reported previously (Abou-Shanab et al. 2013; Luo et al. 2016). The estimated specific growth rate of 0.248 ± 0.025 $day^{-1}$ (Fig. 1b) was within typical values reported for microalgae growth in swine wastewaters (Ji et al. 2013; Luo et al. 2016).

## Yield of biomass extracts

The yields obtained from the extracts, applying the hexane, dichloromethane, and methanol extractors, were 0.4, 4.8, and 5.6% of dry weight, respectively. Santoyo et al. (2009) reported similar yield values when applying hexane extractor in a pressurized liquid extraction. The obtaining of different bioactive compounds is closely related to the extractor used, mainly due to the polarity interaction between the compounds and solvent. The polarity of the solvents used in the present study increases as follows: hexane < dichloromethane < methanol. In this context, considering that hexane is an apolar solvent, it is possible to infer that it would extract lipophilic compounds from the biomass. Additionally, the low lipid content observed in microalgae grown with swine wastewater (Michelon et al. 2015) may explain the lower yield observed by hexane when compared to the other solvents. Methanol has polar characteristics and, therefore, interacts with hydrophilic compounds (Mäki-Arvela et al. 2014).

A theoretical scenario was created to simulate a route to evaluate the potential for producing microalgae extracts from swine wastewater. The layout of a cultivation system was considered according to Michelon et al. (2021), and the biomass production and extract yields are observed in the present study. The results showed a possible estimate of annual extract production of 0.17, 2.0, and 2.40 t $ha^{-1}$ $year^{-1}$ for hexane, dichloromethane, and methanol, respectively. This can be an attractive model of circular economy integrated with the current agribusiness scenario (Robles et al. 2020).

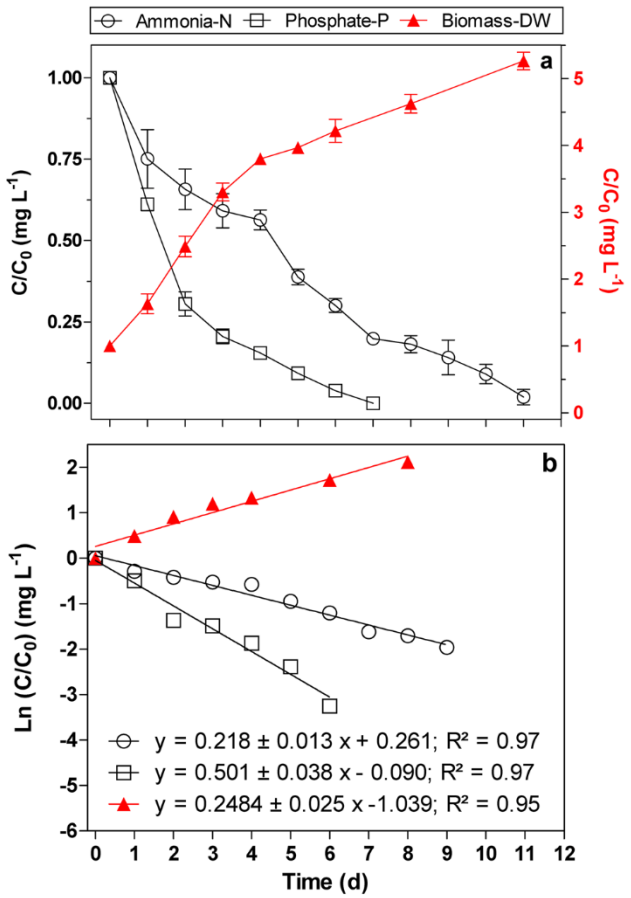


**Fig. 1** Nutrient removal and biomass concentration during swine wastewater phycoremediation (a); values of $k_1$ to kinetics of nutrient removal, and kinetics of biomass growth (b). Experimental conditions: $[\text{ammonia-N}]_0 = 41.5 \pm 1.2$ mg $L^{-1}$, $[\text{phosphate-P}]_0 = 12.9 \pm 1.5$ mg $L^{-1}$, $[\text{Biomass-DW}]_0 = 60 \pm 5.1$ $mg_{DW}$ $L^{-1}$, T = 25 °C and light intensity = 336.5 ± 410.4 µmol $m^{-2}$ $s^{-1}$

## Cytotoxicity evaluation

According to the results presented in Table 1, none of the samples showed cytotoxicity in VERO cells and only the hexane extract was not toxic to A549 cells.

## Virucidal activity

The extracts with dichloromethane and methanol reduced 100% of the infectious capacity of HSV-1 at the lowest concentration tested (3.125 µg $mL^{-1}$) when compared to the untreated control, at both temperatures tested (Fig. 2 a and b). The hexane extract was able to inactivate up to 100% of HSV-1, presenting a concentration–response profile regardless of temperature (21 or 37 °C).

The virucidal test against HAdV-5 at 21 °C showed that the extracts of microalgae in hexane and methanol, at 3.82 and 3.125 µg $mL^{-1}$, respectively, reduced by more than 50% of the infectious capacity of the virus. The microalgae extract in dichloromethane reached a maximum of 60% inhibition of viral infectivity in 100 µg $mL^{-1}$ at 21 °C (Fig. 3a). The virucidal test performed at 37 °C using hexane and methanol extracts inhibited above 60% the virus infectious capacity in all tested concentrations (Fig. 3b). In addition, the microalgae extracted in dichloromethane at 12.5 µg $mL^{-1}$ reduced between 50 and 80% of the infectious capacity of the HAdV-5 virus at 37 °C.

**Table 1** Cytotoxicity of microalgae extracts grown in swine wastewater

| Concentration tested (μg mL$^{-1}$) | VERO | | A549 | |
|---|---|---|---|---|
| | $CC_{50}$[a] | ($CI_{95\%}$)[b] | $CC_{50}$[a] | ($CI_{95\%}$)[b] |
| Hexane | 384.8 | 212.9 to 695.4 | 233.1 | 202.6 to 268.1 |
| Dichloromethane | 239.9 | 162.3 to 354.6 | 52.0 | 38.31 to 70.57 |
| Methanol | 219.3 | 136.5 to 352.2 | 30.5 | 23.42 to 39.66 |

[a]CC50: inhibitory concentration of 50% cell growth was calculated through a nonlinear fit-curve (log of sample concentration versus normalized response-variable slope)

[b]CI 95%: 95% confidence interval

Different solvents can be used to extract different compounds from microalgae with virucidal properties. Santoyo et al. (2010) investigated the use of different solvents (acetone, ethanol, and water) on *Chlorella vulgaris* compounds extraction against HSV-1. Ethanol and water extracts were efficient to inhibit the in vitro virus replication, demonstrating $IC_{50\%}$ values of 80.2 and 61 μg mL$^{-1}$, respectively. The antiherpetic properties were correlated with the presence of polysaccharides (Santoyo et al. 2010). Abdo et al. (2012) investigated water and methanol extracts of five freshwater microalgae [(Cyanobacteria: *Anabaena sphaerica*, *Chroococcus turgidus*) (*Pseudanabaena limnetica* formerly *Oscillatoria limnetica*), (*Arthrospira platensis* formerly *Spirulina platensis*) and Chlorophyta: *Cosmarium leave*)] on non-cytotoxic concentrations of 2 mg mL$^{-1}$, against *Human adenovirus* type 40 and verified that only extracts from *Arthrospira platensis* (*Spirulina platensis*) were efficient on the reduction of this virus (23.3% and 50% using water and methanol, respectively). Methanol extract of *Arthrospira platensis* (*Spirulina platensis*) at 6.8 μg mL$^{-1}$ showed antiherpetic activity (Chirasuwan et al. 2009).

Other studies tested microalgae extracts against HSV-1 and reported inhibitory activity related to attachment, adsorption, penetration, or replication (Ohta et al. 1998; Santoyo et al. 2010, 2012). The antiviral activity against non-enveloped viruses also have shown successful results, as reported by Afify et al. (2018), that tested *Tetradesmus obliquus* (*Scenedesmus obliquus*) (Chlorophyta) extracts against *Coxsackie* B3 virus, and the mechanism involved was the inhibition of the attachment, penetration, and adsorption of the viral particles.

In summary, the production of bioactive compounds integrated with other applications such as wastewater treatment, $CO_2$ biofixation, and bioenergy, highlights the circular economy concept strongly associated with phycoremediation (Chu and Phang 2019). The low cytotoxicity and high virucidal activity of some microalgae compounds reinforce their potential against viruses, playing an important role in the production of nutraceuticals, and in human and animal diseases.

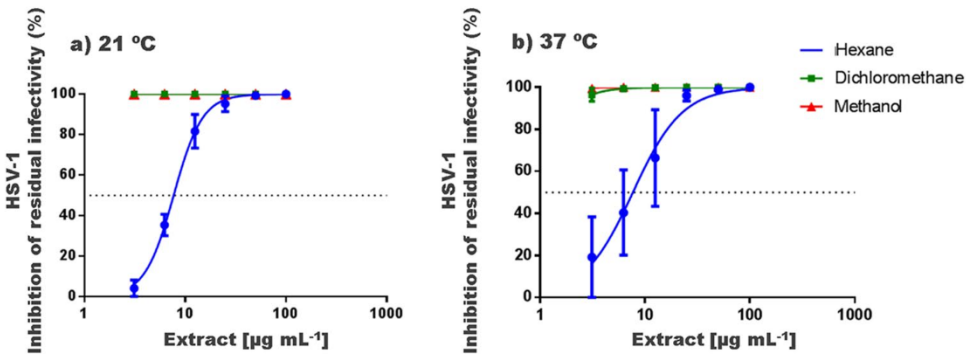


**Fig. 2** Inhibition of HSV-1 infectivity after exposition to microalgae biomass extracts at 21 °C (a) and 37 °C (b)

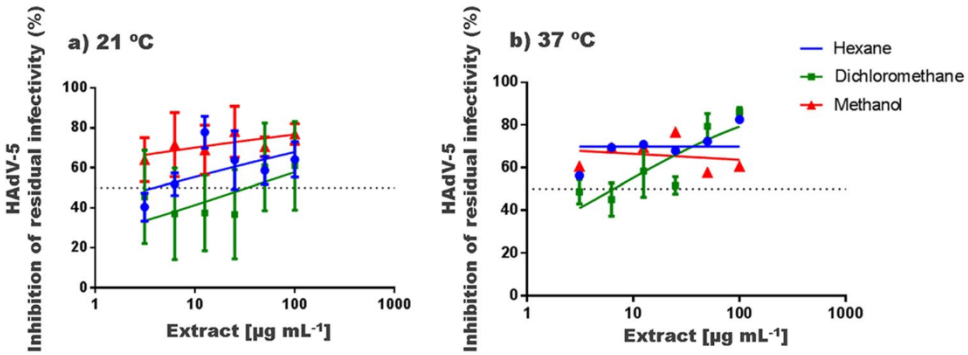


**Fig. 3** Inhibition of HAdV-5 infectivity after exposition to microalgae biomass extracts at 21 °C (a) and 37 °C (b)

## Conclusions

Phycoremediation was efficient in removing ammonia-N and phosphate-P from swine wastewater. The best yields of the extracts were obtained with methanol and dichloromethane solvents. The virucidal effects of microalgae extracts obtained with methanol and dichloromethane showed greater efficiency in controlling enveloped than non-enveloped viruses.

The main limitation for large-scale commercial production of microalgae is the cost associated with the culture medium. In this way, we reinforce in this study that the microalgae biomass can be successfully cultivated with swine wastewater, and then harvested for the exploration of pharmaceuticals products; in this sense, becoming an option for the valoration of waste. The search and development of new virucidal agents are very encouraging, as it is an expanding market with real possibilities for application, especially considering the current scenario of the spread of viral diseases, causing social and economic losses for the population and the industry.

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**Author contribution** William Michelon: Conceptualization, data curation, formal analysis, investigation, methodology, roles/writing — original draft, writing — review and editing. Isabella Dai Pra Zuchi: Conceptualization, data curation, formal analysis, investigation, methodology, roles/writing — original draft. Jacqueline Graff Reis: Conceptualization, data curation, formal analysis, investigation, methodology, roles/writing — original draft. Alexandre Matthiensen: Conceptualization, supervision, validation, writing — review and editing, project administration. Aline Viancelli: Conceptualization, methodology, writing — review &and editing. Ariadne Cristiane Cabral: Conceptualization, methodology, writing — review and editing. Izabella Thaís Silva: Formal analysis, investigation, methodology, writing — review and editing. Gislaine Fongaro: Formal analysis, investigation, methodology, writing—review & editing. Hugo Moreira Soares: Conceptualization, supervision, validation, writing — review and editing.

**Data availability** Not applicable.

## Declarations

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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