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## Inhibitory properties of crude microalgal extracts on the in vitro replication of cyprinid herpesvirus 3

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Microalgae are possible sources of antiviral substances, e.g. against cyprinid herpesvirus 3 (CyHV-3). Although this virus leads to high mortalities in aquacultures, there is no treatment available yet. Hence, ethanolic extracts produced with accelerated solvent extraction from six microalgal species (*Arthrospira platensis*, *Chlamydomonas reinhardtii*, *Chlorella kessleri*, *Haematococcus pluvialis*, *Nostoc punctiforme* and *Scenedesmus obliquus*) were examined in this study. An inhibition of the in vitro replication of CyHV-3 could be confirmed for all six species, with the greatest effect for the *C. reinhardtii* and *H. pluvialis* crude extracts. At still non-cytotoxic concentrations, viral DNA replication was reduced by over 3 orders of magnitude each compared to the untreated replication controls, while the virus titers were even below the limit of detection (reduction of 4 orders of magnitude). When pre-incubating both cells and virus with *C. reinhardtii* and *H. pluvialis* extracts before inoculation, the reduction of viral DNA was even stronger (> 4 orders of magnitude) and no infectious viral particles were detected. Thus, the results of this study indicate that microalgae and cyanobacteria are a promising source of natural bioactive substances against CyHV-3. However, further studies regarding the isolation and identification of the active components of the extracts are needed.

The breeding of common carp (*Cyprinus carpio*) as edible fish, with a worldwide commercial production of about 30 million tons, and koi as ornamental fish plays a key role in global aquaculture<sup>1</sup>. However, both—carp and koi—have been threatened worldwide by an animal disease caused by the cyprinid herpesvirus 3, CyHV-3, also called koi herpesvirus (KHV), belonging to the *Alloherpesviridae* family<sup>2</sup>. CyHV-3 is an enveloped DNA virus with a double-stranded DNA in an icosahedral capsid of a total diameter of 170 to 230 nm<sup>3–5</sup> that can cause the koi herpesvirus disease (KHVD) resulting in high mortality rates leading in turn to great financial damage<sup>6–8</sup>. Despite the wide spread of CyHV-3, no treatment against KHVD is currently available<sup>9</sup>.

Commercial antivirals are based on the inhibition of virus adsorption and entry, protein synthesis and DNA replication or damage of mature virions. The majority of antivirals that are currently in use—such as acyclovir applied against herpes simplex viruses (HSV)—inhibit viral DNA synthesis<sup>10</sup> by competing with natural guanosine triphosphates for chain incorporation and thus disturbing viral DNA replication<sup>11,12</sup>. Key problems of these presently available antivirals are the substantial number of undesirable side effects, dose-dependent cell toxicity and the risk of developing resistance. Moreover, treatment of viral infections in animals faces problems due to possible drug residues, which preclude their use as food, and the issue of possible different cytotoxic effects of conventional human drugs on animals<sup>10,13</sup>. Furthermore, additional factors such as antiviral application, its water solubility and stability must be considered, in particular when treating fish in aquacultures. While current activities focus on the search for alternative antiviral substances, natural sources of antiviral agents are of growing interest for direct use as well as for further drug development for aquaculture<sup>14,15</sup>.

Microalgae, with their enormous biodiversity, comprising an estimated number of species of up to several million as well as their ability to adapt to different habitats, are a potent source of bioactive compounds such as pigments, proteins, lipids and polysaccharides<sup>16–18</sup>. For example, the already industrially used algal pigments astaxanthin,  $\beta$ -carotene or phycobilins, are known for their range of bioactive effects, especially their antioxidative properties<sup>19</sup>. Microalgal (glyco)proteins have antiviral effects against human immunodeficiency virus (HIV), HSV and human cytomegalovirus (HCMV) by preventing/inhibiting cell-virus fusion<sup>20</sup>. Furthermore, microalgal sulfolipids, e.g. sulfoquinovosyl diacylglycerides (SQDG), are also regarded as potent antivirals<sup>20–22</sup>. Gustafson et al.

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Species	Taxonomy	Extraction method	Yield, % w/w
<i>Arthrospira platensis</i>	Cyanobacteria	Basic	5.6 ± 0.2
		Enhanced	32 ± 0.8
<i>Chlorella kessleri</i>	Chlorophyta	Basic	20 ± 0.8
		Enhanced	n.e
<i>Chlamydomonas reinhardtii</i>	Chlorophyta	Basic	18 ± 0.6
		Enhanced	29 ± 0.8
<i>Haematococcus pluvialis</i>	Chlorophyta	Basic	22 ± 0.3
		Enhanced	29 ± 1.8
<i>Nostoc punctiforme</i>	Cyanobacteria	Basic	8.5 ± 0.2
		Enhanced	11 ± 0.4
<i>Scenedesmus obliquus</i>	Chlorophyta	Basic	2.8 ± 0.5
		Enhanced	n.e

**Table 1.** Yield (% w/w, based on dry weight) of biomass extract obtained by accelerated solvent extraction using different temperatures and solvent compositions. Basic extraction: RT, 95% EtOH v/v. Enhanced extraction: 100 °C, 80:20 EtOH:H<sub>2</sub>O, v/v. n.e. Not examined.

showed already in 1989 that SQDG extracted and purified from cyanobacteria impair the replication of HIV<sup>23</sup>. In particular, sulphated polysaccharides of microalgal origin such as calcium spirulan and carrageenans show antiviral effects against HIV, influenza and especially against human herpesviruses such as HSV<sup>24–28</sup>. Moreover, there is evidence that among the defense mechanisms that algae developed evolutionarily antiviral substances against *Phycodnaviridae*. Members of this virus family infect a variety of microalgae and have a similar morphology and replication cycle as herpesviruses<sup>29,30</sup>. Thus, it is assumed that microalgal species contain defensive bioactives against viral infections that can also be effective against herpesviruses. Therefore, several studies have already reported on bioactives extracted from microalgal biomass that are effective against various human viruses and especially against herpesviruses<sup>31–34</sup>. Nevertheless, there is only little known about antiviral substances of microalgal origin effective against fish viruses and even less against CyHV-3 in particular<sup>15,35</sup>. While there is one study conducted by Reichert et al. focusing on the antiviral properties of extracellular polysaccharides from *Arthrospira platensis* against CyHV-3<sup>15</sup>, studies on extracted intracellular compounds originating from different species have not been conducted so far.

To examine microalgae for biologically active substances, the latter are usually extracted from the biomass to be tested for their activity. Depending on the properties of potential bioactives, different methods and solvents have to be selected in order to achieve the highest possible extraction efficiency. While for unpolar compounds (e.g. lipids) inorganic solvents such as for example hexane can be used, solvents for extraction of more polar molecules commonly consist of water and ethanol<sup>36,37</sup>. Accelerated solvent extraction (ASE) is often the method of choice when the target metabolites are at low concentrations and not of high stability. During ASE, increased pressures (3 to 200 bar), mostly elevated temperatures (up to 200 °C) and organic or aqueous solvents are used for the isolation of substances from solid samples<sup>38</sup>. The relatively low volume of required extraction solvent has the advantage of lower dilution of the extracted substances<sup>39</sup> and additionally, sensitive metabolites are protected from degradation processes by the oxygen-free and low-light environment. Moreover, in comparison to conventional extraction methods, ASE not only requires shorter extraction times but also enables significantly higher extraction yields<sup>40,41</sup>. ASE has become a widely used and reliable technique for the extraction of various valuable products from natural sources, including phenolic compounds, polysaccharides, fats and pigments<sup>42–44</sup>. Also, the extraction of antiviral agents from microalgae using this technique has previously been reported<sup>32</sup>.

In this study, ethanolic extracts from six microalgal species were prepared by ASE and, unlike to other approaches, the effect of these crude extracts on the in vitro replication of CyHV-3 was evaluated by analyzing the viral DNA as well as the actual virus titer. So far, there have been no such studies on the antiviral properties of microalgal crude extracts concerning CyHV-3. Therefore, this study contributes to the knowledge on microalgae as a source of the antiviral bioactives against this pathogen, which threatens one of the most important fish in aquaculture.

## Results and discussion

**Extract preparation with the basic protocol.** In this work, ASE was chosen for the extraction of potentially bioactive substances from microalgal biomass under defined conditions. Due to reports classifying EtOH as a safe and environmentally friendly solvent suitable for the extraction of natural value products from microalgae<sup>36,45</sup>, 95% EtOH was chosen. Considering a possible thermolability of target molecules, the extraction process was initially performed at room temperature (RT) (basic extraction). As summarized in Table 1, the obtained yields varied between 2.8 and 22% depending on the biomass. There was no clear correlation between extraction yield and taxonomic affiliation of the organisms, since both the best and the worst extraction were achieved with species from the division of chlorophyta.

**Effects of the basic extracts on virus replication and cellular vitality.** To obtain a first assessment of possible inhibitory effects of the basic crude extracts on viral replication, common carp brain (CCB) cells were

infected with CyHV-3 along with different extract concentrations and were incubated for 72 h at 25 °C without removing the viral supernatant after inoculation. In this set-up, the samples were only analyzed by characterizing the CyHV-3 DNA copy number, always in comparison to the replication control (without extract addition). With this fast-forward test, viral replication could be monitored, but not the quantity of infectious viral particles. Therefore, samples showing a possible inhibiting effect on viral DNA replication were additionally analyzed regarding their viral titer using the 50% tissue culture infection dose assay (TCID<sub>50</sub>). To exclude that a reduced viral replication was merely a result of damaged host cells, the cytotoxic influence of the extract on CCB cells was examined using the same extract concentrations.

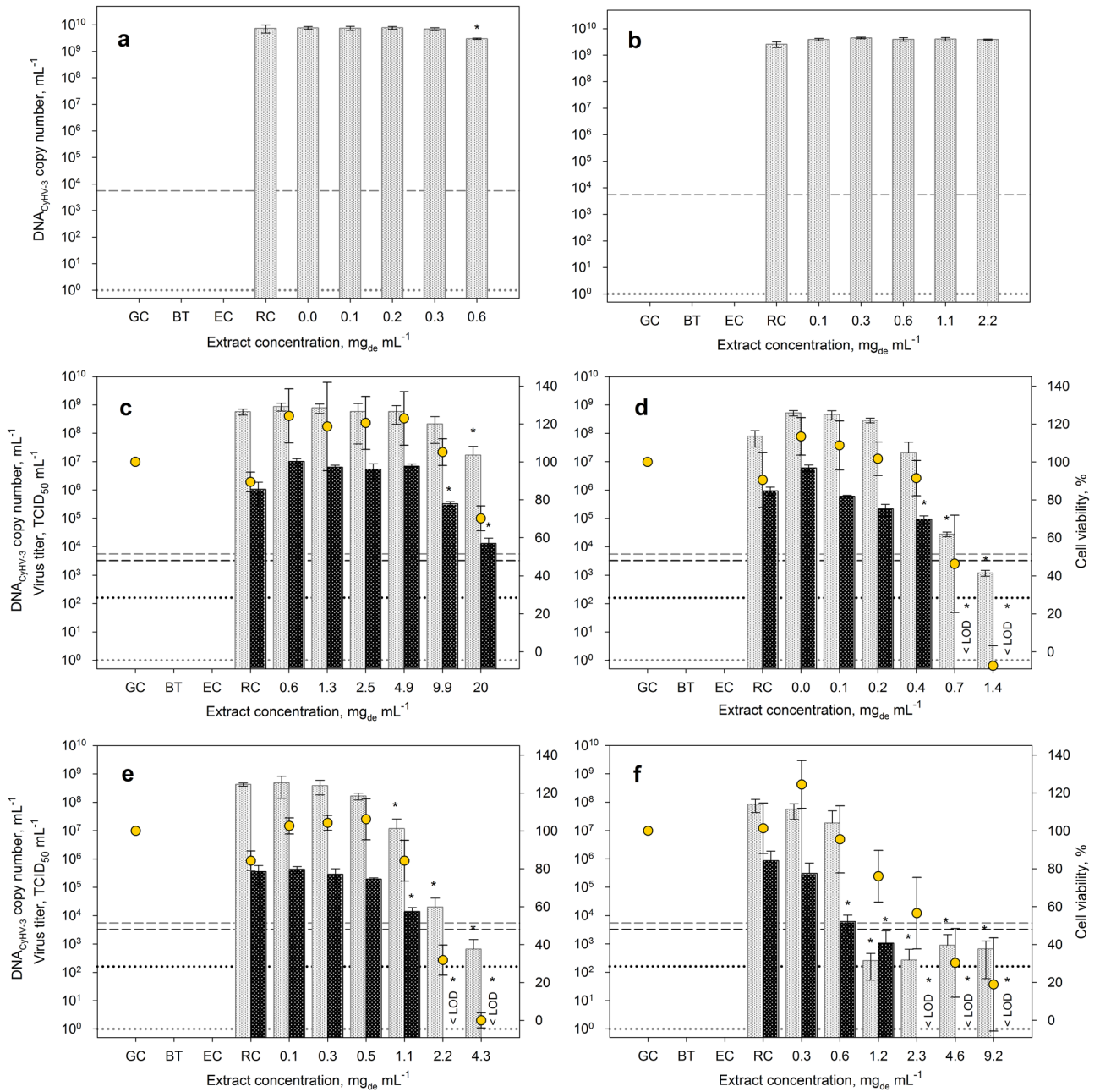
Since the yields of the basic extracts differed by up to a factor of 9, the maximum concentrations that could be used for the first effectiveness evaluation were very different, as shown in Fig. 1. Furthermore, in case of *H. pluvialis* and *A. platensis*, an additional dilution factor must be considered because of the maximal applicable solvent concentration and an increased solvent use due to poor solubility of these basic extracts. We therefore examined whether the extract concentrations were sufficient to determine half maximal inhibitory concentration (IC<sub>50</sub>), half maximal effective concentration (EC<sub>50</sub>) and selectivity index (SI), since these values allow for an evaluation of the strength of the antiviral effect in comparison to the cytotoxic effect. In general, the highest possible SI should be aimed for. Acyclovir, which is often used against herpes viruses, for example, can achieve an SI well over 100 for HSV-1<sup>46,47</sup>. The IC<sub>50</sub> and EC<sub>50</sub> values were determined on the basis of the corresponding dose–response curves (Fig. S1, Supporting Material) and are summarized in Table 2. Samples treated with the basic extracts of *A. platensis* and *H. pluvialis* showed no or only minor differences in viral DNA concentration compared to the replication control (Fig. 1a,b). The maximum concentrations of 0.6 mg<sub>de</sub> mL<sup>-1</sup> and 2.2 mg<sub>de</sub> mL<sup>-1</sup>, respectively, were not sufficient to affect the cell vitality or viral replication. Therefore, neither EC<sub>50</sub> nor IC<sub>50</sub> could be determined. In contrast, using *C. kessleri*, *N. punctiforme*, *S. obliquus* and *C. reinhardtii* extracts both cell viability and replication of CyHV-3 were affected in a dose-dependent manner (Fig. 1c–f, S1). Depending on the biomass used, these effects showed up differently. Due to the strongest cytotoxic effect, only a low SI could be determined for the extract of *S. obliquus* (Table 2). The calculated SI for *C. kessleri* was also comparatively low, since both inhibitory and cytotoxic effects occurred only at high concentrations resulting in high IC<sub>50</sub> and EC<sub>50</sub> values. The extracts of *C. reinhardtii* and *N. punctiforme* showed a comparable antiviral activity to the extract from *S. obliquus*, but at the same time an approximately threefold lower cytotoxicity, which resulted in higher SI values of 4.3 and 4.1, respectively (Table 2). A more detailed analysis of the antiviral activity at extract concentrations with only moderate effects on the cell viability (Table 3) also showed that the extracts from *C. reinhardtii* and *N. punctiforme* were the most promising ones, which led to a reduction of viral DNA by 0.7 and 1.6 orders of magnitude, respectively and virus titer below the limit of detection (Fig. 1e,f). However, in comparison to *N. punctiforme*, the viral titer was reduced stronger (2.2 orders of magnitude in comparison to 1.4) when using the extract of *C. reinhardtii*.

Due to the significant reductions of viral DNA copies, an inhibiting influence on the replication of CyHV-3 by the crude extracts of *C. kessleri*, *S. obliquus*, *N. punctiforme* and *C. reinhardtii* can be assumed, despite some cytotoxic effects of the latter (Table 2). These results are in good agreement with the already reported antiviral effects of *N. punctiforme* and *S. obliquus* extracts against enveloped DNA viruses<sup>21,48,49</sup>. In the mentioned studies polysaccharides (e.g. nostoflan), short-chain free fatty acids or glycolipids such as SQDG were identified as active compounds. Furthermore, it is believed that these compounds are also the reason for the antiviral effects of *C. kessleri* and *S. obliquus* extracts<sup>50–53</sup>. Although, the active substances and their mechanism of action have not yet been investigated in more detail in this work, our data shows that even the crude extracts, produced under basic extraction conditions and not yet purified, have an antiviral effect against CyHV-3.

**Extract preparation with an enhanced protocol.** As already mentioned, the extraction conditions play a major role in the extraction efficiency and the substances extracted, determined by the used biomass. Depending on the selected solvent and the extraction temperature, different substances can be dissolved from the biomass to different extent. We therefore started a second extraction run to test how modified extraction conditions might affect and improve the yield and the virus inhibiting effect of the extracts. In this second set-up, a focus was put on *C. reinhardtii* and *N. punctiforme* after the most promising effects were observed for the corresponding basic extracts. The extraction of *A. platensis* and *H. pluvialis* was also re-examined to test whether other extraction conditions would result in higher applicable concentrations and perhaps other bioactive effects, as these two microalgae has shown antiviral properties against various viruses before<sup>32,54</sup>. Especially *A. platensis* is known to be a source of extra- and intracellular polysaccharides that were identified in several studies as potent antiviral substances against e.g. HSV and also CyHV-3<sup>15,49,55</sup>. Thus, it was considered that the parameters chosen for the basic extraction might have not been suitable to extract these metabolites. Therefore, the water content of the extraction solvent was increased to 20% (v/v) to facilitate the extraction of more polar compounds. Additionally, the influence of an elevated extraction temperature (100 °C) for the isolation of anti-CyHV-3 bioactives was evaluated in this second experimental set-up called enhanced extraction.

For all four species, the extraction yield could be raised by factors between 1.3 and 5.7 by applying the second protocol as can be inferred from Table 1. These results suggest that indeed more metabolites were extracted from the applied biomass when using an increased water content in the solvent and a higher extraction temperature, which is in good agreement with previously published data<sup>56,57</sup>. Moreover, the improved extraction procedure enabled us to perform further studies on the antiviral properties of the selected species with higher concentrations of the extracts.

**Effects of the enhanced extracts on virus replication and cellular vitality.** Applying the enhanced extracts CyHV-3 infection experiments were performed again using the above-explained procedures, but now



**Figure 1.** Effect of various concentrations of ethanolic extracts of *A. platensis* (a), *H. pluvialis* (b), *C. kessleri* (c), *S. obliquus* (d), *N. punctiforme* (e) and *C. reinhardtii* (f) on viral replication of CyHV-3. Each bar of viral DNA copy numbers (shown in gray) and infectious viral titer (shown in black) represents the mean of three biological replicates ± SD. In addition, the vitality of CCB cells normalized to the growth control is shown. Initial viral DNA copy number and virus titer are shown by dashed lines, while the respective limit of quantification is represented by dotted lines. RC—represents replication controls with the CCB cells infected with the same virus load without extract addition; GC—corresponds to growth controls (mock infected without virus and extract), BT—relates to blind samples and EC—displays extract controls containing the highest applied extract concentration but no virus in cell culture medium. If the virus titers could no longer be detected using TCID<sub>50</sub>, this is marked with <LOD (limit of detection). \*p ≤ 0.05 (compared to replication control, RC).

with a deviation affecting the incubation post infection. It cannot be ruled out that virus particles, whose penetration has been initially prevented by the extract, subsequently infect cells anyway after prolonged exposure to the cells because of a possible weakening extract effect due to for example oxidizing impacts. In such a case, higher infectious titers would be measured than induced by a fresh extract. Therefore, based on previous studies<sup>32,55,58</sup> and since we assumed that 1 h is sufficient for the virus to enter the cells, further experiments were carried out without constant virus exposure. Thus, to remove unabsorbed viruses after the infection phase the infectious supernatant of all samples and also the supernatant of the replication controls was discarded after inoculation.



Species	EC <sub>50</sub> , mg <sub>de</sub> mL <sup>-1</sup>	IC <sub>50</sub> , mg <sub>de</sub> mL <sup>-1</sup>	SI, -
<i>A. platensis</i>	–	–	–
<i>C. reinhardtii</i>	1.7 ± 0.7	0.4 ± 0.1	4.3 ± 2.0
<i>C. kessleri</i>	24 ± 5.2	8.4 ± 6.0	2.9 ± 2.7
<i>H. pluvialis</i>	–	–	–
<i>N. punctiforme</i>	1.8 ± 0.1	0.4 ± 0.1	4.1 ± 0.5
<i>S. obliquus</i>	0.6 ± 0.1	0.4 ± 0.1	1.4 ± 0.7

**Table 2.** Cytotoxicity (EC<sub>50</sub>) and antiviral activity (IC<sub>50</sub>) of microalgal extracts obtained with the basic extraction protocol. EC<sub>50</sub> 50% effective concentration, IC<sub>50</sub> 50% inhibitory concentration, SI selectivity index. For associated dose–response curves see Fig. S1 (Supporting Material).

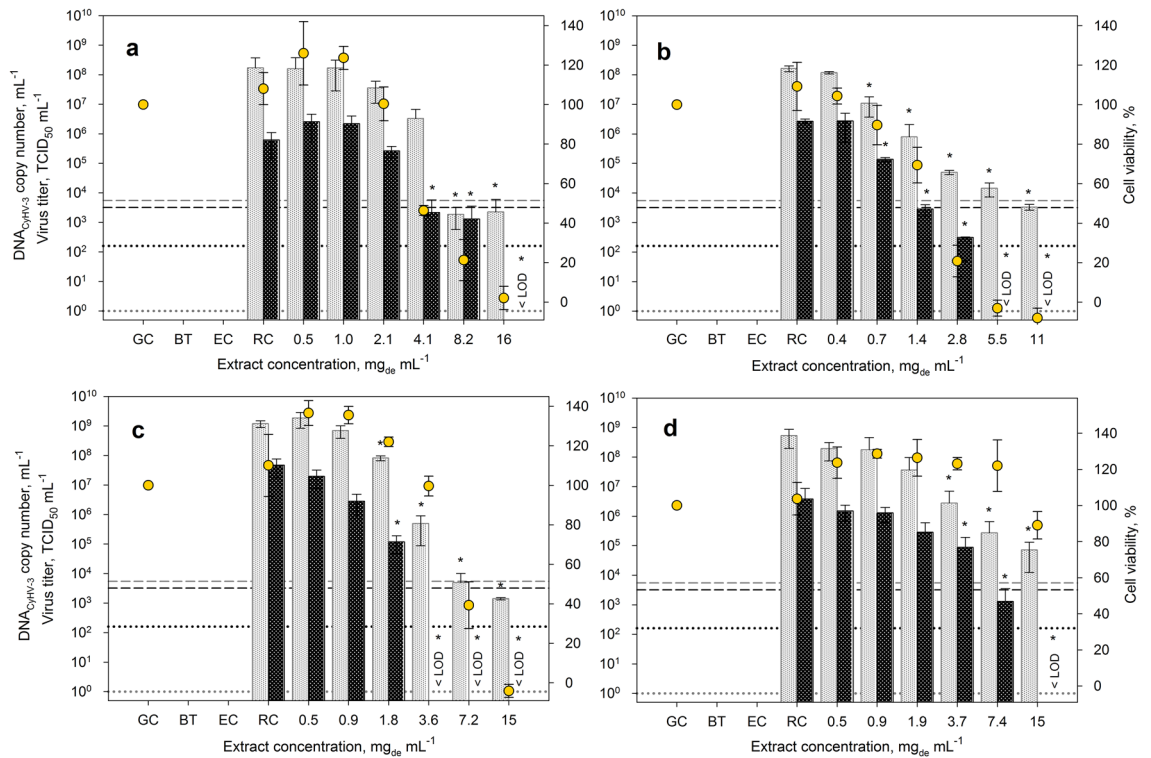
Species	Concentration, mg <sub>de</sub> mL <sup>-1</sup>	Cell viability, %	Reduction of DNA, orders of magnitude (%)	Reduction of virus titer, orders of magnitude (%)
<i>C. kessleri</i>	9.9	105	0.4 (60)	0.5 (68)
<i>N. punctiforme</i>	1.1	86	1.6 (98)	1.4 (96)
<i>S. obliquus</i>	0.4	90	0.6 (75)	1.0 (90)
<i>C. reinhardtii</i>	0.6	96	0.7 (80)	2.2 (>99)

**Table 3.** Effects of *C. kessleri*, *N. punctiforme*, *S. obliquus* and *C. reinhardtii* extracts obtained with the basic extraction protocol on CyHV-3 replication. Reduction of viral DNA copy number and virus titer was analyzed at concentrations at which cell viability was only slightly affected.

Subsequently, fresh cell culture medium with the appropriate extract concentrations was added to the cells. As a result, using the enhanced extracts, a dose-dependent inhibition of viral replication could now be observed for the extracts of all four species (Fig. 2). Although, no effects on the CyHV-3 replication were seen in the previous experiments for *A. platensis* and *H. pluvialis*, now inhibition was registered when using the enhanced extraction procedure. Applying the *A. platensis* extract at high concentrations, a reduction in CyHV-3 DNA copy numbers occurred up to approximately 5 orders of magnitude (Fig. 2a). Hence, to verify the data, the infectious viral particles were analyzed using TCID<sub>50</sub>. For higher extract concentrations viral titers lay even below the limit of detection of the assay, corresponding to a reduction of over 5 orders of magnitude. However, also cytotoxic effects of the *A. platensis* enhanced extract on the CCB cells were observed. Thus, the EC<sub>50</sub> and IC<sub>50</sub> values calculated from these results led to a relatively low SI (Table 4). As described above for *A. platensis*, also for *H. pluvialis* higher extract concentrations could be applied for the antiviral studies due to the improved extraction procedure. Consequently, inhibiting effects were also revealed for this alga. In this case, significant reductions of viral DNA of up to 4 orders of magnitude (99.99%) occurred, while the viral titer was no longer detectable (reduction of over 4 orders of magnitude) (Fig. 2d). It was noticeable that no significant signs of damage to the CCB cells were detectable via the applied cytotoxicity assay even at such elevated extract concentrations. Up to concentrations of 7.4 mg<sub>de</sub> mL<sup>-1</sup> the viability of CCB cells was actually exceeding the growth control and decreased to a minimum of yet 90% at the highest extract concentration used here. These high viabilities at elevated extract concentrations led to an increased EC<sub>50</sub> value, resulting in a comparably high SI of 60 (Table 4). Such normalized cell viabilities of above 100% were already found when examining the influence of cytotoxic compounds by the MTT assay, which measures the metabolic activity of the cells<sup>59,60</sup>. This phenomenon could be explained by the hypothesis of hormesis, constituting a positive biological response to low concentrations of potentially toxic substances<sup>61,62</sup>. Overall, using enhanced *A. platensis* and *H. pluvialis* extracts and the improved procedure, viral replication was significantly inhibited also without cytotoxic effects (Table 5). For both algae the dose-dependent inhibitory effect on CyHV-3 replication is in good agreement with the above-mentioned data indicating antiviral effects of *A. platensis* extracts and isolated compounds against herpesviruses such as HSV-1, HCMV and human herpesvirus 6 (HHV6), that were supposed to inhibit viral adsorption and entry into the target cells<sup>25,49</sup>. Also, for extracts of *H. pluvialis* an antiviral effect against HSV-1 was previously reported, Santoyo et al. identified polysaccharides and short-chain free fatty acids as possible antiviral substances<sup>32</sup>. It was again suggested that the mentioned bioactive substances can hinder the virus to penetrate the cell or even damage the lipid envelope of virions and therefore inactivate the virus<sup>53,63,64</sup>. The extracts obtained in this work may also contain such bioactives responsible for the antiviral effects of the crude extracts; however, this must be investigated further.

Moreover, the data presented for these two species showed in which manner the selection of the experimental conditions and analysis methods could affect the outcome of such screening studies. If only the preliminary experiments with *A. platensis* and *H. pluvialis* were considered, the inhibitory activity of their extracts on CyHV-3 would remain unrecognized, due to the lower tested extract concentrations and the lack of effects on the viral DNA. However, when varying the experimental conditions and extending the analysis to the viral titer, very promising results regarding an antiviral effect against the targeted virus could be registered.

Furthermore, the influence of the changed extraction conditions was examined for *N. punctiforme* and *C. reinhardtii*. As for the preliminary experiments, a dose-dependent reduction of replicated viral DNA could be detected for both enhanced microalgal extracts (Fig. 2b,c). Moreover, a steadily decreasing titer of infectious



**Figure 2.** Effect of ethanolic extracts of *A. platensis* (a), *N. punctiforme* (b), *C. reinhardtii* (c) and *H. pluvialis* (d) on viral replication of CyHV-3. CCB cells were infected with CyHV-3, treated with extract during and after inoculation and were incubated at 25 °C for 1 h. Infectious supernatant was replaced with extract (in medium) and cells were incubated at 25 °C for 72 h. Each bar of viral DNA copy numbers (shown in gray) and infectious viral titer (shown in black) represents the mean of three biological replicates ± SD. In addition, the vitality of CCB cells normalized to the growth control is shown. Initial viral DNA copy number and virus titer are shown by dashed lines, while the respective limit of quantification is represented by dotted lines. RC—represents replication controls with the CCB cells infected with the same virus load without extract addition; GC—corresponds to growth controls (mock infected without virus and extract), BT—relates to blind samples and EC—displays extract controls containing the highest applied extract concentration but no virus in cell culture medium. If the virus titers could no longer be detected using TCID<sub>50</sub>, this is marked with < LOD (limit of detection). \*p ≤ 0.05 (compared to replication control, RC).

Species	EC <sub>50</sub> , mg <sub>de</sub> mL <sup>-1</sup>	IC <sub>50</sub> , mg <sub>de</sub> mL <sup>-1</sup>	SI, -
<i>A. platensis</i>	3.5 ± 0.5	1.7 ± 0.1	2.1 ± 0.4
<i>C. reinhardtii</i>	5.8 ± 1.1	0.9 ± 0.1	6.4 ± 1.3
<i>H. pluvialis</i>	24 ± 5.8	0.4 ± 0.1	60 ± 30
<i>N. punctiforme</i>	1.9 ± 0.1	0.5 ± 0.1	4.3 ± 1.1

**Table 4.** Cytotoxicity (EC<sub>50</sub>) and antiviral activity (IC<sub>50</sub>) of microalgal extracts obtained with the enhanced extraction protocol. For associated dose–response curves see Fig. S2, Supporting Material. EC<sub>50</sub> 50% effective concentration, IC<sub>50</sub> 50% inhibitory concentration, SI selectivity index.

Species	Concentration, mg <sub>de</sub> mL <sup>-1</sup>	Cell viability, %	Reduction of DNA, orders of magnitude (%)	Reduction of virus titer, orders of magnitude (%)
<i>A. platensis</i>	2.1	100	0.7 (63)	0.4 (58)
<i>C. reinhardtii</i>	3.6	100	3.4 (>99.9)	> 4 (>99.99)
<i>H. pluvialis</i>	7.4	>100	3.3 (>99.9)	3.5 (>99.9)
<i>N. punctiforme</i>	0.7	90	1.2 (94)	1.3 (95)

**Table 5.** Effects of *A. platensis*, *C. reinhardtii*, *H. pluvialis*, and *N. punctiforme* extracts obtained with the enhanced protocol on CyHV-3 replication. Reduction of viral DNA copy number and virus titer was analyzed at concentrations at which cell viability was not affected.

viral particles was recognizable with increasing extract concentrations. Even at concentrations where replication of viral DNA was not yet affected, a reduced titer was found. Yet, it is noticeable that the  $IC_{50}$  values of the enhanced extracts could not be improved for both organisms.

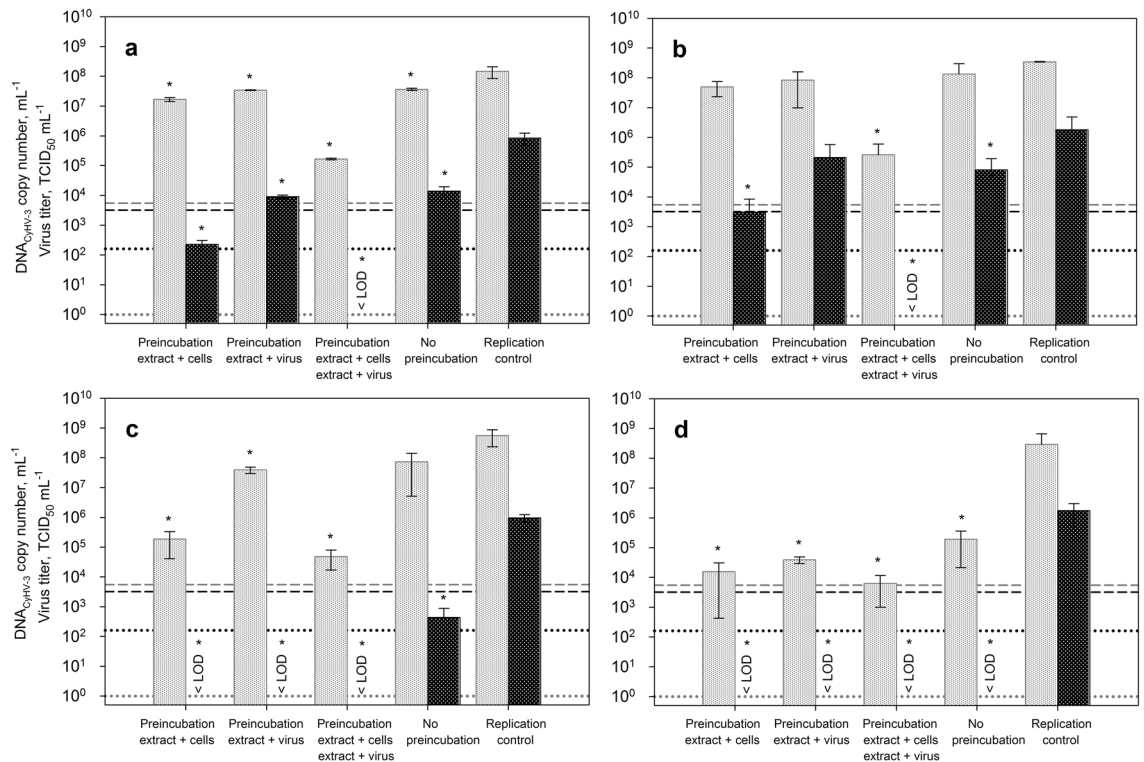
Quite the contrary, in the case of *C. reinhardtii* it was even slightly worse (Tables 2 and 4). Thus, the enhanced extraction led to higher concentrations and biomass yields, but to a lower inhibiting effect. This underpins the fact that with crude extracts an increased extraction efficiency does not necessarily correspond to a stronger effect. Nevertheless, whereas the SI values of the basic and enhanced extracts from *N. punctiforme* were comparable, the SI of the enhanced *C. reinhardtii* extract was improved by a factor of 1.5 since a reduced cytotoxic effect overcompensated the slight loss of antiviral activity. Table 5 shows that, among all considered microalgae in this study, the greatest inhibitory effect without damaging the CCB cells could be reached with the *C. reinhardtii* extract resulting in a reduction of viral DNA by 3.4 (>99.9%) and viral titer by over 4 (>99.99%) orders of magnitude. Although no analysis of the specific extract composition of *C. reinhardtii* was performed in this work, there is some data in the peer-reviewed literature on bioactive compounds contained in extracts of this species. In addition to carbohydrates such as sulphated polysaccharides, (glyco)proteins and (glyco)lipids were previously isolated from *C. reinhardtii* depending on the selected extraction conditions<sup>51,65–68</sup>. These bioactive substances could be able either to hinder virus-cell adsorption and membrane fusion or damage the lipid envelope of virions and therefore inactivate the virus, as observed for such compounds originating from other species. Though the first examinations of the total protein and lipid content of the selected extracts were performed in this work (Table S4 and S5, Supporting Material), no conclusions about active fractions could be drawn from these data. Thus, to identify the bioactive substances that are responsible for the antiviral effect of the crude extract of *C. reinhardtii*, further investigations including fractionation, antiviral activity and content analysis of the extracts are necessary.

Although various bioactive compounds and effects have been described in literature for extracts of *C. reinhardtii*, no inhibitory impact on the replication of viruses have been reported so far apart from the results obtained in this work. Again, this suggests that in studies regarding possible bioactive metabolites, both the choice of experimental conditions and the methods of analysis play an important role. Therefore, it is possible that *C. reinhardtii* was not yet considered as an interesting candidate as a source of antiviral compounds because, for example, other extraction methods or only one-sided analyses were used for evaluation and the corresponding data was not published. Hence, even if antiviral effects of extracts of certain species on selected viruses cannot be directly determined, these species should not be excluded from further investigations prematurely.

From the point of view of a future application and further studies, especially non-cytotoxic extract concentrations that are showing inhibitory effects on the viral replication are of great interest. With this in mind, the *C. reinhardtii* extract inhibited viral replication the most with unaffected cell viability (Table 5). On the other hand, the enhanced extract of *H. pluvialis* showed the lowest cytotoxicity and a high inhibitory influence on CyHV-3 which led to a relatively high SI of 60, which is significantly higher than the SI of 6.4 reached with *C. reinhardtii* (Table 4). However, keeping in mind, that crude extracts were used in this study, the latter might be optimized in further research for example by the identification, isolation and purification of the biologically active compounds. In general, biological extracts contain a variety of compounds, meaning that it is always unclear which biological effects are caused by which molecules. Thus, it has always to be analyzed whether a measured cytotoxic activity is a feature of the same molecule as the antiviral activity in order to avoid excluding active compounds from screenings only due to high cytotoxic effects of their crude extracts<sup>69</sup>. Therefore, the isolation of the active antiviral or inhibitory agent or rather substance class might help to eliminate substances which affect the host cells cytotoxically and therefore improve the SI accordingly.

Since the SI of antiviral extracts and substances depends not only on the species, extraction method and the degree of purification, but also on the virus and the host cells used, cross-study comparisons must be done with caution and are better suited for comparing orders of magnitudes than concrete values. In a similar study performed with hot water extracts from *A. platensis*, the replication of HSV-1 and HSV-2 was inhibited by 50% at a concentration of 0.3 mg mL<sup>-1</sup> showing  $IC_{50}$  values in the same order of magnitude and an SI of 26<sup>49,58</sup>. However, when using purified products such as sulphated polysaccharides and SQDG, significantly lower  $IC_{50}$  values were achieved where replication of HSV and HCMV was inhibited by 50% at concentrations in the range of µg mL<sup>-1</sup><sup>21,27</sup>. Moreover, exopolysaccharides extracted and purified from *A. platensis* resulted in an  $IC_{50}$  of less than 2.1 µg mL<sup>-1</sup> but also in a low SI of 3 for CyHV-3<sup>70</sup>. Using *H. pluvialis* extracts, 99 µg mL<sup>-1</sup> of the polysaccharide-rich fraction reduced virus infectivity by 50% resulting in an SI of 19<sup>32</sup>.

Summarizing, using the enhanced extraction protocol for the four selected species—*A. platensis*, *N. punctiforme*, *C. reinhardtii* and *H. pluvialis*—antiviral effects were observed after application of the extracts in the range of lower mg<sub>de</sub> mL<sup>-1</sup>, although so far only unpurified crude extracts have been applied. A substantial reduction in virus replication (based on both viral DNA and titer) could be proven for all of them even at still high cell viabilities. We could demonstrate that ASE enables the extraction of bioactive substances and also that the application of elevated temperature for the isolation process did not negatively affect the antiviral properties of the extracts tested here. On the contrary, elevated temperature and water content in the extraction solvent led to a higher extraction yield and enabled the identification of antiviral effects even for extracts of the species which, based on the preliminary experiments, seemed not to have such properties. Within the investigated species, the clearest antiviral effects were registered for the *C. reinhardtii* and *H. pluvialis* extracts with the highest SI values calculated in this study. To the best of our knowledge, this is the first report of antiviral properties of extracts of *C. reinhardtii*. Furthermore, our work shows that the quantification of the actual infectious virus titer in addition to the viral DNA copy number is of great importance in order to assess the antiviral properties of such extracts. While in some cases viral DNA could still be produced by the extract treated and CyHV-3 infected cells, no infectious viral particles could be detected via titration assay. Moreover, a titer reduction under extract



**Figure 3.** Effect of different pre-incubation modes with *A. platensis* (a), *N. punctiforme* (b), *C. reinhardtii* (c) and *H. pluvialis* (d) extract (concentrations: 2.1 mg<sub>de</sub> mL<sup>-1</sup>, 0.7 mg<sub>de</sub> mL<sup>-1</sup>, 1.8 mg<sub>de</sub> mL<sup>-1</sup> and 7.1 mg<sub>de</sub> mL<sup>-1</sup>) on viral replication of CyHV-3. Either cells, virus suspension or both were pre-incubated with the ethanolic extracts before inoculation with CyHV-3 at 25 °C for 1 h. Infectious supernatant was replaced with extract (in medium) and cells were incubated at 25 °C for 72 h. The viability of the cells was approximately 100% for each experiment, only for the incubation with *H. pluvialis* the cell viability was slightly affected and reduced to 90%. Each bar of viral DNA copy numbers (shown in gray) and infectious viral titer (shown in black) represents the mean of three biological replicates ± SD. In addition, the vitality of CCB cells normalized to the growth control is shown. Initial viral DNA copy number and virus titer are shown by dashed lines, while the respective limit of quantification is represented by dotted lines. If the virus titers could no longer be detected using TCID<sub>50</sub>, this is marked with < LOD (limit of detection). \*p ≤ 0.05 (compared to replication control, RC).

application was often more pronounced in comparison to the reduction of viral DNA. Thus, if only viral DNA was monitored the antiviral properties of some extracts might have remained unnoticed.

**Influence of pre-treatment on the antiviral activity of the enhanced extracts.** In order to obtain information about the virus replication phase affected by the inhibiting extracts, different pre-incubation set-ups were examined. Either CCB cells or CyHV-3 suspensions were pre-incubated for 1 h with the selected concentrations of extracts before inoculation. In addition, a combined mode was examined, where both, cells and virus suspension, were pre-incubated with the extracts. After this pre-incubation, the assays for antiviral activity were carried out as described above. Using this strategy, it should be possible to differentiate, whether the inhibiting agents influence either the cells or the viral particles or even both. For comparison, samples with no extract pre-incubation (corresponding to the previous set-up for the investigation of antiviral properties of the extracts) and the untreated replication controls were prepared along with the other samples.

The results of the pre-treatment experiment with the enhanced extracts from *A. platensis*, *N. punctiforme*, *C. reinhardtii* and *H. pluvialis* are illustrated in Fig. 3a–d. For all samples where one of the four extracts was applied, varying inhibitory effects on viral DNA and titer were observed. Depending on whether cells, the virus suspension, both or neither of them were pre-incubated with the corresponding extract, the reductions in viral DNA or titer occurred to different degrees. Thus, while the least effect on viral infection was always observed when no pre-incubation was applied, the pre-treatment of both cells and virus prior to the inoculation with respective extracts resulted in significantly lower viral DNA and titer for all four species. When pre-incubating the extracts of *A. platensis* and *N. punctiforme* with either cells or virus suspension, only a slight influence on the number of copies of viral DNA was registered. Simultaneously, the same use of these extracts caused a more pronounced reduction of infectious virus particles compared to viral DNA—as it has also been observed in previous experiments. The application of *C. reinhardtii* extract to either cells, virus or both led to a reduction of viral DNA and viral titer below the limit of detection. However, even stronger effects were observed for each scenario in this set-up when using the extract of *H. pluvialis*. All in all, a more pronounced interference with viral replication



could be recognized when the cells were in contact with the extract before inoculation. Consequently, it can be assumed that the extract affects components of the cells like impairing viral adsorption to the cells.

However, the data obtained especially for the combined pre-incubation set-up suggest that the antiviral effect is not solely based on the inhibition of virus binding to the host cells, as viral replication was much more impeded in the combined mode in comparison to just treating the cells. Most likely, also following replication steps such as virus assembly or release can be affected by the extracts used in this work. Furthermore, since infectious virus particles were also not detected after the virus stock was pre-treated with the extracts of *C. reinhardtii* and *H. pluvialis*, some virucidal properties of the latter can be assumed. Thus, the inhibitory effects on the replication seem to be a combination of the interference of bioactive substances with the attachment of virus particles to the target cells, preventing viral entry as well as a possible activation of some cell defense mechanisms. Therefore, the production of viral proteins and thus in a consequence the assembly of mature virions could possibly impeded. Since a drop in the titer below the initially added values was observed for most extracts, they can also have virucidal effects, at least for the virus investigated here. Nevertheless, based only on the presented here data a clear designation of mode of action and an appointment of the active compounds of these extracts is not yet possible. Altogether, the pre-incubation data confirmed the inhibitory effects of the crude extracts of *A. platensis*, *N. punctiforme*, *C. reinhardtii* and *H. pluvialis* and forms a basis for identifying the bioactive substances and their mechanisms of action in further studies.

In summary, we showed that ethanolic extracts of six different chlorophyta and cyanobacteria obtained by ASE inhibited the in vitro replication of CyHV-3, suggesting bioactives with antiviral properties against the selected virus present in these crude extracts. Although further research regarding the identification and purification of the antiviral substances and investigation of their modes of action is necessary, the work presented here confirms the immense potential of phototrophic microorganisms when searching for antiviral agents. In particular, further examination of the more closely studied microalgal species *A. platensis*, *H. pluvialis* and *C. reinhardtii*, which are already well characterized and used in food and pharmaceutical industry, is very promising regarding their future use for treatment against CyHV-3.

## Material and methods

**Chemicals.** Dulbecco's modified Eagle medium (DMEM, Invitrogen Thermo Fisher Scientific Waltham, USA) was used as cell culture medium for cell maintenance in this work. Fetal calf serum (FCS) was obtained from Biochrom AG (Berlin, Germany). Analytical grade salts, titrants, ethanol (EtOH,  $\geq 99.9\%$ ), DMSO and the buffer substance 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Pufferan,  $\geq 99.5\%$ ) were purchased from Carl Roth GmbH (Karlsruhe, Germany). PBS (pH 7.4) was prepared by mixing 137 mM NaCl with 2.7 mM KCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$  and 1.5 mM  $\text{KH}_2\text{PO}_4$ , all dissolved in ultrapure water ( $\text{H}_2\text{O}_w$ ) (Millipore, Darmstadt, Germany), autoclaved at 121 °C for 20 min and stored at room temperature (RT) in darkness until use. Antibiotic and antimycotic solution A5955 (with 10 000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL), Igepal (Nonident P40 Substitute) and Accutase were purchased from Sigma-Aldrich (St. Louis, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany).

**Cell cultures and virus stock.** Common carp brain cells (CCB, passage 83, provided by the Friedrich-Loeffler-Institut (FLI), Greifswald, Germany) were maintained in 25-cm<sup>2</sup> cell culture flasks (T-25, Sarstedt, Nümbrecht, Germany) in DMEM supplemented with 25 mM HEPES and 10% FCS at 25 °C<sup>71</sup>. CyHV-3 (KHV-TP 30)<sup>72</sup>, isolated by Dr. Peiyu Lee (Taiwan, 2005), was provided by the FLI (Greifswald, Germany). CyHV-3 stocks were prepared by inoculation (multiplicity of infection, MOI=0.2) of CCB cells seeded in DMEM with a density of 60 000 cells cm<sup>-2</sup> in a cell disc (1000 cm<sup>2</sup>, Greiner Bio-One GmbH, Kremsmünster, Germany). Infected cells were incubated at 25 °C for three days post infection (p.i.) and replication was stopped by freezing the whole cell culture at -80 °C. Finally, frozen cells and medium were thawed, resuspended, aliquoted, introduced to titer determination and frozen at -80 °C for further use.

**Algal biomass.** In this study, four different strains of chlorophyta (*Chlamydomonas reinhardtii*, *Chlorella kessleri*, *Haematococcus pluvialis*, *Scenedesmus obliquus*) and two cyanobacterial strains (*Arthrospira platensis*, *Nostoc punctiforme*) were used for extraction. The biomass was taken from an in-house microalgae biomass collection that had previously been produced in various research activities relating to microalgae (freeze-dried after harvesting and stored at -20 °C)<sup>73</sup>. For further information see Table S1 (Supporting Material).

**Preparation of ethanolic extracts.** Biomass extracts were prepared using an accelerated solvent extractor (ASE 350, DIONEX, Sunnyvale, USA). The extraction was conducted using EtOH:H<sub>2</sub>O mixtures with different ratios at two different temperatures. For the basic extraction 95% v/v EtOH and RT was chosen, whereas enhanced extracts were prepared using an increased water content (80:20 EtOH:H<sub>2</sub>O, v/v) and applying 100 °C. Accordingly, 500 mg of freeze-dried biomass was placed in extractions cells (V=22 mL) and sealed with cellulose filters (Thermo Fisher Scientific, Waltham, USA) on both sides. Next, the extraction cells were filled with solvent and based on literature static extraction was performed for 30 min at a pressure of 100 bar for both extraction procedures<sup>41,56</sup>. N<sub>2</sub> was used to purge the solvent from the cells into 60-mL vials that were closed with Teflon septa. To avoid contamination and carry-over of extracts the system was rinsed with solvent between extractions. Next, the extraction solvent was removed using a centrifugal evaporator (SPD131DDA, Thermo Fisher Scientific, Waltham, USA, RT, p=10<sup>-3</sup> bar). To minimize sample degradation due to oxidation, the dried extract residue was overlaid with N<sub>2</sub> and stored in the dark at -20 °C until use. Each biomass extraction was performed in triplicates together with one blank sample (an empty extraction cell). The extraction yield was

determined by dividing the exact dry weight of the extract residue by the initial dry biomass used for extraction. Afterwards, the dried extract residues were resuspended in non-toxic concentrations of DMSO (1 vol.-% at the lowest dilution level; continuously decreasing DMSO concentrations with increasing dilution) or EtOH ( $\leq 1$  vol.-%) and selected volumes of cell culture medium (DMEM). Samples were additionally placed in an ultrasonic bath for 3 min (SONOREX, Bandelin, Berlin, Germany). In order to remove undissolved particles and to ensure sterility, redissolved extracts were filtered through syringe filters (0.22  $\mu\text{m}$ , polyether sulfone, Sarstedt, Nümbrecht, Germany) in sterile 1.5-mL reaction vessels (Sarstedt, Nümbrecht, Germany) and kept at 8 °C in darkness until used. Extracts were analyzed regarding both the total protein and lipid content. For protein quantification the Bradford assay according to the manufacturer's specifications (Thermo Fisher Scientific, Waltham, USA) was carried out using a dilution series of bovine serum albumin (BSA) as standard. Lipids were quantified by first extracting the fatty acids and subsequently analyzing the dried residues gravimetrically as described by Beck et al.<sup>74</sup>.

**Evaluation of cytotoxic and antiviral effects of microalgal extracts.** The cytotoxic potential of the extracts was examined using the colorimetric viability MTT assay. Briefly, CCB cells were seeded into 96-well plates with a density of 40 000 cells  $\text{cm}^{-2}$  in 100  $\mu\text{L}$  DMEM supplemented with 25 mM HEPES, 0.5 M  $\text{NaHCO}_3$ , 10% FCS, 100 U  $\text{mL}^{-1}$  of penicillin G, 0.1 mg  $\text{mL}^{-1}$  of streptomycin sulphate and 0.25  $\mu\text{g mL}^{-1}$  of amphotericin B and incubated at 25 °C for 24 h. Next, the medium was removed from the wells and replaced with 100  $\mu\text{L}$  DMEM containing six consecutive dilutions (1:2) of the redissolved extracts ( $n=6$  for each concentration) and incubated for 72 h at 25 °C. Additionally, DMEM with 1% DMSO/EtOH was used as 100% viability controls ( $n=6$ ) and DMEM containing 20% DMSO as 0% viability controls ( $n=6$ ). After incubation, the medium was discarded and replaced with 100  $\mu\text{L}$  of MTT solution, which was prepared from MTT (0.5% w/v MTT in 0.9% w/v NaCl solution) and DMEM in the ratio of 2:1 (v/v). The plate was incubated again for 4 h at 25 °C, followed by centrifugation at 3 220 g for 10 min. After discarding the supernatant, 20  $\mu\text{L}$  of Igepal (0.4%) was added to lyse the cells and the plate was incubated for 5 min under shaking at 1000 rpm (Titramax 100, Heidolph, Schwabach, Germany). Next, formazan crystals were dissolved by adding 180  $\mu\text{L}$  of DMSO and shaking the plate again for 5 min at 1000 rpm. Finally, the absorbance of the samples and controls was measured at a wavelength of 570 nm using a plate reader (Multilabel Reader, Perkin Elmer, Waltham, USA). The average of the absorbance values of the 0% viability control was subtracted from all other values which were then normalized to the growth control (100% viability control). The determined cell viabilities were plotted against the corresponding extract concentrations and half-maximal effective concentrations ( $\text{EC}_{50}$ ) calculated using SigmaPlot and a Four Parameter Logistic Curve.

To evaluate the antiviral effects of the microalgal extracts CyHV-3 DNA was determined using a real-time qPCR (TaqMan) procedure as described by Gilad et al. with some modifications<sup>75</sup>. Firstly, viral DNA was extracted from infected CCB cells using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. In addition, Proteinase K was used for an improved release of the nucleic acids and the breakdown of proteins during cell lysis. The obtained DNA extract (100  $\mu\text{L}$ ) was stored at -20 °C until further investigation and subjected to qPCR analysis using 96-well plates (Bio-Rad, Hercules, USA), the Thermocycler CFX connect system (Bio-Rad, Hercules, USA), and the temperature profile recommended by the manufacturer (see Table S2, Supporting Material). iTaq Universal Probes Supermix (Bio-Rad, Hercules, USA) was used with CyHV-3 specific primers and probes (Sigma-Aldrich, St. Louis, USA). Further information is given in the Supporting Material (Table S3). Glucokinase expression was used as an internal control and analyzed using glucokinase-specific primers and probes (Sigma-Aldrich, St. Louis, USA). A logarithmically diluted CyHV-3 standard series was prepared for the quantification of viral DNA. The half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) of the extracts was determined based on the CyHV-3 DNA copy numbers, normalized to the values of the replication control and plotted against the extract concentrations by SigmaPlot (Four Parameter Logistic Curve).

Infectious CyHV-3 viral particles were estimated using the endpoint dilution assay (50% tissue culture infective dose assay,  $\text{TCID}_{50}$ ) as outlined by Reed & Muench and described previously by Mletzko et al. as well as Amtmann et al.<sup>76–78</sup>. The virus titer was registered as  $\text{TCID}_{50} \text{ mL}^{-1}$ .

To assess the influence of the extracts on the viral replication, the number of CyHV-3 DNA copies and the virus titers of the samples were compared with the values of the untreated virus replication control. To quantify this comparison, the logarithmic reduction factor R, which indicates the difference between two sample groups as orders of magnitude, was used. R was calculated according to Eq. (1).

$$R = \log_{10}(\text{replication control}) - \log_{10}(\text{extract treated sample}) \quad (1)$$

In order to assess whether the differences between replication control and samples are statistically significant, a one-way ANOVA (Excel) with a significance level of  $\alpha=0.05$  was carried out.

Finally, to evaluate the strength of the antiviral effect compared to the cytotoxic effect, the selectivity index (SI) was determined using  $\text{EC}_{50}$  and  $\text{IC}_{50}$  according to Eq. (2).

$$\text{SI} = \frac{\text{EC}_{50}}{\text{IC}_{50}} \quad (2)$$

**Investigation of antiviral/inhibitory and cytotoxic properties of ethanolic extracts of various microalgae against CyHV-3 and CCB cells.** Generally, the cytotoxic and inhibitory properties of the extracts were examined by incubation of CCB cells with or without inoculation with CyHV-3, after addition of consecutive (1:2) extract dilutions ( $n=6$ ) in 96-well plates. CCB cells were inoculated with a defined viral titer

to obtain a MOI of 0.01. For the investigation of antiviral properties of the microalgal extracts, CCB cells were seeded in 96-well plates (60 000 cells  $\text{cm}^{-2}$ ) and incubated at 25 °C for 24 h prior to the treatment. As in comparable studies extracts were tested in five to six different concentrations, obtained by consecutive dilutions (1:2) with DMEM as described above and mixed with virus stock to reach the appointed MOI<sup>32,34,58</sup>. The concentration of the extracts was determined by taking the dry extract weight, the solvent volume and the dilution level into account and expressed in mg of dried extract per mL ( $\text{mg}_{\text{de}} \text{mL}^{-1}$ ). Firstly, the basic extracts (extracted at RT with 95% v/v EtOH, n = 3) were added to the CCB cells together with the virus suspension. Samples were then incubated for 72 h at 25 °C. After incubation viral replication was stopped by freezing at –80 °C for at least 4 h and the samples were analyzed using qPCR (as described above) for CyHV-3 DNA copy numbers<sup>75</sup>. In further experiments, extracts produced at an elevated extraction temperature (100 °C) using EtOH:H<sub>2</sub>O mixture (80:20, v/v) were examined. These enhanced extracts were diluted as described above and mixed with the virus stock to reach the same MOI. Extract/virus stock solutions prepared in this manner were added to the seeded CCB cells which then were incubated for 1 h at 25 °C. Based on other studies and since the period of 1 h is normally sufficient for viruses to penetrate the cells, the inoculum was discarded after this time<sup>32,34</sup>. Cells were then overlaid with DMEM containing the same concentrations of the extracts and incubated for 72 h (25 °C) before viral replication was stopped by freezing as described above. Virucidal effects of biological substances are often evaluated based only on the plaque formation by infectious viruses<sup>32,34,58</sup>. However, to get a deeper knowledge about potential antiviral effects, in this study we considered both infectious viral titers and viral DNA. The samples were analyzed using the analytical procedures for CyHV-3 DNA copy numbers and viral titer as described above. Additionally, MTT assays with the same concentrations of the extracts were performed to evaluate their cytotoxic effects on CCB cells. Samples containing the same virus load without extract addition were used as replication controls. Growth controls just with DMEM (without virus and extract) and extract controls containing the highest applied extract concentration but no virus in DMEM were also prepared in parallel. For each extract (n = 3) samples were analyzed using both qPCR and TCID<sub>50</sub>, resulting in a total of three biological replicates for viral DNA and titer per species.

**Influence of pre-treatment on the antiviral effects of microalgal extracts.** To investigate the possible mode of antiviral action of the extracts, various set-ups of extract addition and inoculation of the cells were examined. The experiment was performed analogously to the procedure described above using CCB cells seeded with an identical density (60 000 cells  $\text{cm}^{-2}$ ) into 96-well plates and pre-incubated for 24 h at 25 °C. Inoculation with CyHV-3 (1 h, 25 °C) was carried out using four different virus/extract addition procedures: (1) Cells were pre-treated with a previously defined, non-toxic concentration of extract in DMEM (1 h, 25 °C) and inoculated with CyHV-3 stock solution containing the same concentration of the extract. (2) The virus stock was mixed with the same non-toxic concentration of extract (1 h, 25 °C) and then used to infect CCB cells. In a third set-up, (3) the combination of the aforementioned cell and virus pre-treatment was tested and finally, for comparison, (4) cells were infected with a CyHV-3/extract mixture without any pre-incubation (neither cells nor extract). Regardless of the procedure, the supernatant used for inoculation was discarded after 1 h and the cells were overlaid with the corresponding extract/cell culture solution and incubated for 72 h at 25 °C before stopping the viral replication. Resulting samples were analyzed via qPCR and TCID<sub>50</sub> for CyHV-3 DNA and titer.

### Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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## Author contributions

All authors have contributed substantially to the study conception and design. Material preparation, data collection and analysis were performed by Stefanie Fritzsche. The first draft of the manuscript was written by Stefanie Fritzsche and all authors commented on previous versions of the manuscript. All authors read and approved the submitted version and have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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## Competing interests

The authors declare no competing interests.

## Additional information

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