Antiviral compounds obtained from microalgae commonly used as carotenoids sources

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ABSTRACT

Pressurized liquid extraction (PLE), an environmentally friendly technique, has been used to obtain antiviral compounds from microalgae commonly used as carotenoids sources: *Haematococcus pluvialis* and *Dunaliella salina.* The antiviral properties of PLE extracts (hexane, ethanol and water) were evaluated against herpes simplex virus type 1 (HSV-1) at different stages during viral infection. Pre-treatment of Vero cells with 75 μg mL^{-1} of *H. pluvialis* ethanol extract inhibited virus infection by approx. 85%, whereas the same concentration of water and hexane extracts reduced the virus infectivity 75% and 50% respectively. *D. salina* extracts were less effective than *H. pluvialis* extracts and presented a different behaviour, since water and ethanol extracts produced a similar virus inhibition (65%). Moreover, *H. pluvialis* ethanol extract was also the most effective against HSV-1 intracellular replication.

The antiviral activity of water PLE extracts was found to correlate with polysaccharides, since the polysaccharide-rich fraction isolated from these extracts showed higher antiviral activity than the original water extracts. A GC-MS characterization of the *H. pluvialis* ethanol extract showed the antiviral activity of this extract could be partially related with the presence of short chain fatty acids, although other compounds could be involved in this activity; meanwhile, in the case of *D. salina* ethanol extract other compounds seemed to be implied, such as: β-ionone, neophytadiene, phytol, palmitic acid and α-linolenic acid.

Results demonstrated the use of PLE allows obtaining antiviral compounds from microalgae used as carotenoids sources, which gives both microalgae biomass an added value.

INTRODUCTION

Microalgae have been used as food in ancient civilizations in Asia, Africa and South America for centuries. However, microalgae biotechnology only really began to develop in the middle of last century. Since then, these microorganisms have become the focus for extensive screening of novel compounds with interesting biological activities which may lead to therapeutically useful agents (Mendes et al., 2003; Mayer and Hamann, 2005; Spolaore et al, 2006). At present, microalgae offer great possibilities for the isolation of natural substances of significant commercial interest in industries such as pharmaceuticals, alimentary or cosmetic products. This fact makes microalgae raw materials with a great deal of added value.

The fact that algae may produce antiviral agents is already well-known (Damonte et al., 2004; Mayer et al, 2009). However, only few reports have shown the existence of compounds inhibiting viral infection from marine microalgae. Calcium spirulan, a sulfated polysaccharide isolated from *Spirulina platensis* has been indicated to possess antiviral activity (Hayashi et al., 1996; Lee et al., 2001). Rechter et al. (2006) also described spirulan-like molecules with pronounced antiviral activity in the absence of cytotoxic effects. Moreover, Fabregas et al. (1999) screened the in vitro inhibition of viral replication of extracts obtained from several marine microalgae, indicating that the aqueous extracts from *Porphyridium cruentum, Chorella autotrophica and Ellipsoidon sp.* produced a significant inhibition of the in vitro replication of haemorrhagic septicemia virus and African swine fever virus. The antiviral activity of the red microalga *Porphyridium sp.* was also confirmed by Mahmoud et al. (2002) against herpes simplex viruses.

Dunaliella salina is a unicellular green alga belonging to family Chlorophyceae that accumulates carotenoids in response to stress conditions and under ideal conditions can yield 400 mg β-carotene m^{-2} of cultivation area (Dufossé et al., 2005). *Haematococcus pluvialis* is also a green microalga known for its ability to accumulate astaxanthin, a ketocarotenoid, up to 0.2-2% (on dry weight basis) (Sommer et al., 1991). *H. pluvialis* changes from a motile, flagellated cell to a non-motile, thick-walled aplanospore during the growth cicle; the astaxanthin is contained in the aplanospore (Borowitzka et al., 1991). At present, these two microalgae are cultivated commercially to produce β-carotene and asthaxanthin, since these natural carotenoids are potent antioxidants and strong coloring agents (Dufossé et al., 2005). However, *D. salina* and *H. pluvialis* biomass has been poorly screened for the presence of other active compounds. Thus, several studies reported the presence of antimicrobial compounds (Herrero et al., 2006, Santoyo et al., 2009) and extracellular polymeric substances (Mishra and Jha, 2009) in these microalgae, but no studies have evaluated their potential to produce antiviral agents.

Pressurized liquid extraction (PLE) is an emerging technique that has important advantages over traditional extraction ones. Traditional solvent extraction techniques use a large quantities of toxic organic solvents, are labor intensive, need long extraction times, posses low selectivity, and/or low extraction yields and can expose the extracts to excessive heat, light and oxygen. In comparison, PLE uses less solvent in a shorter period of time, is automated and involves retaining the sample in an oxygen and light-free environment (King, 2000). Whereas other environmentally-friendly techniques, such as supercritical fluid extraction (SFE), are frequently used to obtain functional compound from natural sources, PLE has not been widely applied as a routine tool in natural product extraction. However, recent studies have proposed PLE as an efficient

extraction technique for bioactive compounds from algae and microalgae (Denery et al., 2004, Santoyo et al, 2006; Rodriguez-Meizoso et al., 2008), including antiviral compounds (Santoyo et al., 2010a,b).

The goal of the present work was to study the ability of PLE to obtain antiviral compounds against Herpes simplex virus type 1 (HSV-1) from microalgae used as carotenoids sources: *D. salina* and *H. pluvialis.* Simultaneously the antiviral activity of the extracts at different stages during viral infection cycle was also determined and attempts were made to effectively correlate this activity with the chemical profile of the PLE extracts.

MATERIALS AND METHODS

Haematococcus pluvialis (BNA 10/024, National Bank of Algae, Canary Islands, Spain), were grown in modified Bold´s Basal Medium (Nichols & Bold, 1964) enriched with $NaNO_3$ (0.75 g L^{-1}). Cells (green phase) were cultured in 20 L carboys bubbled with air, at 25°C, in light:dark cycles (16:8) with white fluorescent lamps providing 80 µmol m^{-2} s^{-1}. To induce astaxanthin biosynthesis (red phase) exponentially grown cultures were transferred to nitrogen deprived medium and continuously at an irradiance of 200 µmol photons m^{-2} s^{-1} during 6 days. Cells were collected by centrifugation, freeze dried and stored at inert atmosphere until extraction.

Dunaliella salina sample consisted on freeze dried microalgae supplied by NBT Ltd. (Jerusalem, Israel) stored under dry and dark conditions until use.

Glucose, fructose, arabinose, galactose, xylose, myo-inositol, rhamnose, fucose, mannose, tagatose, glucuronic acid, gluconic acid, galactonic acid, sorbitol, manitol,

xylitol, arabitol, *β*-phenyl-glucoside, pyridine, trifluoroacetic acid (TFA) and hexamethyldisilazane (HMDS) were acquired from Sigma-Aldrich (St. Louis, USA).

Pressurized liquid extraction (PLE)

The samples were pretreated by freezing and mashing the microalgae with liquid nitrogen in a ceramic mortar. The process was repeated three times in order to increase extraction.

Extractions of microalgae were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. Three different solvents (i.e., hexane, ethanol, and water) were used to obtain extracts with different compositions. When employing *H. pluvialis* biomass, extractions were performed at 100ºC for 20 min, whereas for *D. salina* extractions were carried out at 160ºC for 15 min. All extractions were done using 11 mL extraction cells, containing 1.5 g of sample in ethanol extracts, 1 g in water extracts and 0.8 g in hexane extracts. When water was used for the extraction, the extraction cell was filled with sand between the sample (6.0 and 2.0 g of sand at the bottom and top, respectively) to prevent the clogging of the system.

Extraction procedure was as follows: (i) sample was loaded into cell, (ii) cell was filled with solvent up to a pressure of 1500 psi (1 psi. = 6894.76 Pa), (iii) heat-up time was applied, (iv) static extraction took place in which all system valves were closed, (v) cell was rinsed (with 60 % cell volume using extraction solvent), (vi) solvent was purged from cell with N2 gas and (vii) depressurization took place. Between extractions, a rinse of the complete system was made in order to overcome any carry-over. The extracts obtained were protected from light and stored under refrigeration until dried.

For solvent evaporation, a Rotavapor R-210 (from Büchi Labortechnik AG, Flawil, Switzerland) was used for the extracts obtained using organic solvents. For water extracts, a freeze-dryer (Labconco Corporation, Missouri, USA) was employed.

Antiviral assays

Cells and viruses

Vero cells (African green monkey kidney cell line) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. They were used as host for HSV-1. The cells were grown using Eagle's Minimum Essential Medium (MEM) supplemented with 5% foetal bovine serum (FBS), 1% penicillin-streptomycin, 1% hepes buffer 1M, 1% non essential amino acids and 1% L-glutamine. Maintenance medium for Vero cells was as described above but with 2% FBS.

Herpes virus simplex type 1 (HSV-1) (KOS) was obtained from the Americam Type Culture Collection (ATCC), Rockville, MD, prepared in aliquots and stored at –80ºC until use. Virus titer was determined by plaque assay in Vero cells and expressed as plaque forming units (pfu) per ml.

Cytotoxicity assay

The cytotoxic effect of the different extracts on Vero cells was tested using MTT assay, according to a published method (Mosmann, 1983). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, Spain) is a yellow water soluble tetrazolium dye that is reduced by live cells, but not dead to a purple formazan product that is insoluble in aqueous solutions. Monolayers of Vero cells in 24-multiwell plates were incubated with MEM containing different concentrations of the extracts for 48h at 37ºC. Cells were then washed with PBS and 0.5 mg mL^{-1} of MTT was added to each

well and incubated 4h at 37ºC. Supernatants were discarded and formazan crystals dissolved in an extraction solution (10% sodium dodecyl sulphate in a mixture of dimethyl formamide and water 1:1 v/v, adjusted to pH 4.7 with acetic acid) overnight at 37ºC. Formazan quantification was performed by measuring the optical density at 570 nm using a multiscanner autoreader (Sunrise, Tecan, Germany) with the extraction solution as a blank. The data were plotted as dose-response curves, from which the concentration required to reduce 50% the number of viable Vero cells (CC_{50}) after 48 h of incubation with the different extracts was obtained.

Evaluation of virucidal activity

Virus samples containing 10^5 pfu mL^{-1} were mixed and incubated at 37ºC for 1h with MEM containing different extracts concentrations or MEM alone (control). Samples were then diluted and used to infect confluent Vero cells for 1h at 37ºC. After incubation, the virus inocula was removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37ºC. The infected cells were fixed acetone:methanol (50:50) at 4ºC, stained with a 1% solution of crystal violet and the number of the plaques counted. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control) – (mean number of plaques in test)] / (mean number of plaques in control) x 100

Influence of various treatment periods on the anti-HSV-1activity of the extracts

Vero cells and viruses were incubated with the extracts at different stages during the viral infection cycle in order to determine the mode of antiviral action. (1) Cells pretreatment: monolayers of Vero cells in 24-multiwell plates were pretreated with MEM containing different concentrations of the extracts for 3h at 37ºC. Cells were then washed with PBS and infected with 120 pfu of HSV-1. After incubation for 1h at 37ºC, the virus inocula was removed, the cells washed with PBS and then overlaid with

maintenance medium (with 0.5% agarose) for 48 h at 37ºC. The infected cells were fixed, stained and the number of the plaques counted. Control consisted of untreated cells infected with HSV-1. (2) Adsorption period: cells were infected with 120 pfu of HSV-1 in presence of different concentrations of the extracts for 1h at 37ºC. Then, the virus inocula and the extract were removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37ºC. The infected cells were fixed, stained and the number of the plaques counted. Control consisted of cells infected without extract. (3) Intracelullar replication: cells were infected with 120 pfu of HSV-1. After incubation for 1h at 37ºC, the virus inocula was removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) containing different concentrations of the extracts or only medium (control). After incubation for 48 h at 37ºC, the infected cells were fixed, stained and the number of the plaques counted. The concentration of a substance required to reduce plaque number in Vero cells by 50% (IC_{50}) as compared to control, was calculated from the dose-response curves generated from the data.

Isolation of polysaccharides and determination of total carbohydrate content

Polysaccharides were isolated according to Jeurink et al. (2008). Briefly, the freeze-dried water extracts were mixed with sterile distilled water at 90ºC (1g 100 mL^{-1}) for 20 min and cooled down to 4ºC. Polysaccharides were precipitated by adding two volumes of cold ethanol, vigorous stirring, and allowing polysaccharide precipitation overnight at 4ºC. The precipitated polysaccharides were collected by centrifugation (10,000*g* for 20 min at 4ºC), re-dissolved in distilled water and the whole precipitation procedure was repeated once. The precipitated polysaccharides were dialyzed with Spectra/Por 3 molecular-porous membrane tubing (MWCO 3500; Spectrum Medical Industries Inc.)

against distilled water to remove small compounds during at least 24 hours with three or four changes of the distilled water. After dialysis, the polysaccharides were lyophilised and the sample weight estimated. The lyophilised polysaccharides were stored at –20ºC until further use.

The polysaccharides extracts were analysed for their total carbohydrate content with the modified phenol-sulphuric acid method described by Fox and Robyt (1991). Test solutions (25 μL) or standards of know glucose concentrations with 25 μL of 5% (w/v) phenol were added to an eppendorff. The tube was stirred in a vortex for 30s, placed on crushed ice, added 125 μL of concentrated H_2SO_4 and heated in a water bath at 80ºC for 30 min. After that, the absorbance was determined at 490 nm.

GC-MS analysis of carbohydrate content

Firstly, freeze-dried polysaccharides were hydrolyzed with 4 M trifluoroacetic acid (TFA) at 100°C for 4 h and the TFA solution evaporated to dryness in a Rotavapor R-210 (Büchi Labortechnik AG, Switzerland) at room temperature. *β*-phenyl-glucoside was used as internal standard (I.S.) at a concentration of 0.2 mg mL^{-1}. Dry residues were treated with 500 μL pyridine (containing 2.5 g hydroxylamine hydrochloride/100 mL) and heated for 30 min at 70ºC. The cooled samples were then trimethylsilylated with 1000 μL HMDS and 100 μL TFA for 60 min at 100ºC. Thereafter the solutions were ready for the analysis. The amount of derivatized stock solutions injected into the GC-MS system was 2 μL. Glucose, fructose, arabinose, galactose, xylose, myoinositol, rhamnose, fucose mannose, tagatose, glucuronic acid, gluconic acid, galactonic acid, sorbitol, manitol, xylitol and arabitol were used as reference standards.

GC-MS analyses were performed in an Agilent-6890N GC system with a programmed split/splitless injector coupled to an Agilent-5973N quadrupole mass spectrometer (Agilent, USA). The system was controlled by means of Agilent MSD Chemstation software. To analyze the derivatized solutions a 30 m long, 0.25-mm internal diameter fused silica capillary column coated with a 0.25-μm layer of SE-54 (HP-5MS, Agilent) was used. The injector was heated to 250ºC in split mode (ratio 1:20). Helium was used as carrier gas (7 psi). The oven temperature was programmed as follows: from 60ºC as the initial temperature (maintained for 2 min) to 120ºC in 3 min at 20 ºC min^{-1}, then from 120ºC to 155ºC in 5.83 min at 6ºC min^{-1}, maintaining this temperature 10 min before reaching 250ºC at 13ºC min^{-1} in 7.30 min, this temperature was maintained for 12 min and then from 250ºC to a final temperature of 330ºC in 4 min at 20ºC min^{-1}. The final temperature was held for 10 min. Sugars were identified by MS in SCAN mode, using a mass interval ranging from 35 to 450. Their spectra were compared with those in a MS library (Wiley Registry of Mass Spectral Data), and with standards.

GC analysis of volatile fraction

Volatile fraction of the hexane and ethanol extracts obtained was analyzed using the same apparatus and column as described before. The injector was heated to 250ºC in split mode (ratio 1:20). The oven temperature was programmed as follows: from 40ºC as the initial temperature (maintained for 2 min) to 150ºC in 24 min at 5 ºC min^{-1}, and then from 150ºC to a final temperature of 300ºC at 15 ºC min^{-1}. Volatiles were tentatively identified by MS in SCAN mode, using a mass interval ranging from 35 to 450. Their spectra were compared with those in an MS library (Wiley Registry of Mass Spectral Data), with data found in the literature and with standards when available.

Additionally, to identify compounds more precisely, their linear retention indices (RIs) were calculated.

RESULTS

Usually, the first step of the screening includes the selection of the different solvents (covering a wide range of dielectric constants) and the optimization of extraction conditions (extraction temperature and time). Hexane, ethanol and water were selected as solvents to evaluate the influence of their polarity in the extraction of antiviral compounds from the microalgae. Extractions temperature and time were fixed at 100ºC and 20 min for *H. pluvialis* and at 160ºC and 15 min for *D. salina,* based in previous results obtained with these microalgae in our laboratatory (Herrero et al, 2006, Santoyo et al., 2009). The extraction yield (% of dry weight) obtained from *H. pluvialis* biomass (table 1) was maximum when the extractions were carried out with water and minimum with hexane. However, when *D. salina* biomass was used for the extractions, the highest yield was obtained with ethanol, followed by hexane and finally water. These results suggest that medium-highly polarity substances are more abundant in *H. pluvialis* whereas *D. salina* contained mainly medium-low polarity compounds.

Prior to evaluating the antiviral activity of the different extracts, their cytotoxicity on preformed monolayers of Vero cells was studied, using the MTT method. The CC_{50} (Table 2) indicated that all extracts showed a low toxicity, although hexane extracts were more cytotoxic than ethanol and water ones for both microalgae.

Virucidal activity of PLE extracts from *H. pluvialis* and *D. salina*

In order to analyze the possibility that PLE extracts may act directly on the virus particle leading to infectivity inactivation, a virucidal assay against HSV-1 was carried out. A virus suspension was treated at 37º C for 1h with different concentrations of the microalgae extracts. Pre-incubation of HSV-1 with both microalgae PLE extracts resulted in dose-dependent reduction of remaining virus infectivity when compared with the untreated control. However, the concentrations with 50% virucidal activities against the virus were higher than 10 mg mL^{-1} for all extracts, indicating that PLE extracts almost lacked extracellular virucidal activity.

Influence of various treatment periods on the anti-HSV-1 activity of the *H. pluvialis* and *D. salina* extracts.

Further experiments were performed to determine if *H. pluvialis* and *D. salina* extracts presented antiviral activity against HSV-1. Extracts were added to Vero cells before virus infection, simultaneously with virus and after virus infection. When cells were pre-treated for 3 h with different concentrations of extracts, before virus infection, the results (Figure 1) indicated an inhibition in virus infectivity. Thus, 75 μg mL^{-1} of *H. pluvialis* ethanol extract inhibited virus infection by approx. 85%, whereas the same concentration of water and hexane extracts reduced the virus infectivity 75% and 50%, respectively. *D. salina* extracts were less effective than *H. pluvialis* ones and showed a different behaviour, since water and ethanol extracts produced a similar virus inhibition (65%).

Additional assays were performed with extracts added simultaneously with virus to assess their effects. Under these conditions, the efficacy of the extracts decreased (Figure 2) since 150 μg mL^{-1} of *H. pluvialis* ethanol extract reduced virus infectivity by

75%. Water and hexane extracts were also less effective than when added before the virus. When 150μg mL^{-1} of *D. salina* ethanol and water extracts were applied, the virus infectivity was only reduced by 20%.

The antiviral activity on the intracellular replication of the virus was evaluated adding different concentrations of the extracts to previously HSV-1 infected Vero cells and incubated for 48 h at 37ºC. All extracts showed a dose-dependent inhibition of virus replication. In this assay, *H. pluvialis* ethanol extract was also more efficient against HSV-1 replication than water and hexane extracts, showing the lowest IC_{50} values (Table 2). However, when *D. salina* extracts were applied, the highest inhibition of virus intracellular replication was obtained with water extracts.

Taking into account that antiviral activities of several microalgae water extracts have been frequently related to sulphated polysaccharides (Lee et al, 2001; Rechter et al. 2006), a polysaccharide-rich fraction from the water extract was extracted. Further more, since microalgae ethanol and hexane extracts also showed important antiviral effects, these extracts were analysed by GC-MS in order to identify potential antiviral components.

Antiviral activity of polysaccharide-rich fraction isolated from *H. pluvialis* and *D. salina* water extracts

The percentage of carbohydrates found in lyophilised polysaccharide-rich fraction obtained from the water extracts was 36.76% for *H. pluvialis* and 42.95% for *D. salina*. A GC-MS analysis of carbohydrate composition of fractions from the two microalgae showed a quite different composition between them (Table 3). Polysaccharide-rich fraction from *H. pluvialis* showed the presence of mannose as its main component,

together with high amounts of glucose and galactose, whereas *D. salina* fraction contained a 94.34% of glucose.

In order to correlate the antiviral activity found in the water extracts to the polysaccharide-rich fractions, the cytotoxicity and antiherpetic assays were measured under the same conditions as previously described for the water extracts, except for virucidal effects. This activity was not measured in the polysaccharide fractions since water extracts had a very small virucidal activity. The cytotoxicity assays of these polysaccharide-rich fractions (Table 2) indicated lower toxicity than the water extracts.

When Vero cells were pre-treated with microalgae polysaccharide-rich fractions, the inhibition of the virus infection increased by 10-15% (Figure 1), compared to the water extracts. Moreover, if polysaccharide-rich fractions were applied during virus adsorption period, the virus infectivity was also reduced (Figure 2), respect to values shown when microalgae water extracts were used. This suggests that polysaccharides are the compounds responsible of the antiviral activity found in the water extracts when added either before the virus or simultaneously with the virus.

The antiviral activity of the polysaccharide-rich fractions on the intracellular replication of the virus was also evaluated, showing IC_{50} values lower than those obtained when water extracts were applied, for both microalgae (Table 2). The polysaccharide fractions increased the antiviral action of the water extracts on the intracellular replication step by 25-37%.

GC-MS characterization of ethanol and hexane extracts from *H. pluvialis* and *D. salina*.

In an attempt to identify the compounds responsible of the antiviral activity found in ethanol and hexane extracts, these extracts were characterized by GC-MS. The results

are shown in Tables 4 and 5, where a tentative identification has been carried out based on the comparison of mass spectra and retention index (RI).

Several compounds were identified in the *H. pluvialis* samples, mainly fatty acids, alkanes, phenols and compounds such as neophytadiene (table 4). By comparing the ethanol and hexane extracts, the main difference is due to the presence of short chain fatty acids such as propanoic/lactic and butanoic acids in the ethanol extract. In total, the short chain fatty acids account for more than 52% of the total content of the volatile composition of the sample.

Table 5 shows the volatile compounds detected in the *D. salina* samples. As can be observed, 16 compounds could be identified, mainly naptalene, β-ionone, neophytadiene, hexacecanoic acid and 9,12,15-octadecatrienoic acid methyl ester. By comparing the relative amount of each compound (in terms of normalized areas %), *D. salina* hexane and ethanol extracts seem to have a similar composition.

Disccusion

This work studied the ability of PLE to obtain antiviral compounds against HSV-1 from microalgae traditionally used as carotenoids sources: *D. salina* and *H. pluvialis.* Pressurized liquid extraction is an emerging extraction technique proposed to obtain bioactive compounds from different algae, including antiviral compounds (Santoyo et al. 2010a, b).

Dunaliella and *Haematococcus* PLE extracts (hexane, ethanol, and water) were added at different stages during viral infection cycle to determine its mode of action. Extracts were added to Vero cells prior to viral infection, simultaneous with the virus and after viral infection, and also virucidal assays were carried out. The results indicated that all

the extracts tested lacked extracellular virucidal activity, although when cells where pre-treated for 3 h with those extracts, prior to virus infection, 75 μg mL^{-1} of the extracts produced an important reduction in virus infectivity. Thus *H. pluvialis* ethanol extract stood out by inhibiting virus infection by approx. 85%, whereas *D. salina* water extract reduced virus infectivity by 70%. Assays performed with the extracts added to cells simultaneously with the virus also demonstrated an important reduction of viral infection, although under these conditions, the efficacy of the extracts decreased. Taking *H. pluvialis* ethanol extract as reference, 150 μg mL^{-1} of the extract were necessary to inhibit virus infection by 75%. Finally, PLE extracts also provided an important inhibition of virus intracellular replication, outstanding also in this case, *H. pluvialis* ethanol extract. All these data suggest that the inhibitory action of the extracts against HSV-1 replication is unlikely to be mediated by its virucidal activity or some non-specific interference on viral particles. The mechanism by which *H. pluvialis* and *D. salina* extracts inhibit viral replication could be explained by the inhibition of viral attachment to the host cells, virus–cell fusion, and/ or virus penetration.

Taking into account that antiviral activities of algae water extracts have been frequently related to diverse types of polysaccharides (Damonte et al, 2004), the isolation of a polysaccharide-rich fraction from the water extracts was carried out. Besides microalgae ethanol and hexane extracts also showed important antiviral effects, so these extracts have been analyzed by GC–MS to identify the potential antiviral components.

Though *Dunaliella salina* is often described as naked or wall-less, electron micrographs revealed the existence of a fibrous extracelular matrix on the cell surface, mainly consists of polysaccharides (Nakayama et al, 1996). It is thought that the production of these exopolysaccharides is an additional mechanisms to keep the cells safe from salinity (Abbasi and Amiri, 2008; Mishra and Jha, 2009). There were few studies focus

on these exopolysaccharides composition. Dai et al (2010) reported that glucan, sulfated proteoglycan and sulfated heteropolysaccharide mainly containing glucose were the main components of these exopolysaccharides. Zheng et al (1997) and Dai et al (2010) studies showed that monosaccharide composition of the exopolysaccharides from *D. salina* was mainly constituted by a large quantity of glucose followed by minor amounts of galactose, xylose, mannose and rhamnose. Polysaccharide-rich fraction showed similar monosaccharides composition. Therefore, it can be inferred glucan, and minor amounts of heteropolysaccharides were its main constituents.

Opposite to *D. salina* cells, *H. pluvialis* mature red cysts have been described to possess a thick cell wall. Several studies have shown algaenan, a complex cell wall polymer that is resistant to several chemical and enzimatic treatments, together with diverses polysaccharides are the main components of *H. pluvialis* cell wall (Montsant et al, 2001; Hagen et al, 2002; Damiani et al, 2006). Regarding to polysaccharide fraction, Hagen et al (2002) reported mannans as the main polysaccharide of the multilayered cell wall aplanospores, where minor amounts of cellulose from the original cristalline layer remain in the cysts. Similar results were obtained by Damiani et al (2006). Therefore, in this study polysaccharide-rich fraction obtained from *H. pluvialis* seems to be composed mainly by mannans together with other heteropolysaccharides.

The antiviral assays performed with this fraction indicated that polysaccharides could be the compounds responsible of the antiviral activity found in the water extracts. These results are in agreement with the mechanism by which microalgae polysaccharides are reported to inhibit virus replication. In that sense, the inhibitory effect of these compounds appeared to be based mainly on their ability to interfere with the initial attachment of the virus to the target cell, and consequently leading to the blockade of viral entry. During in vitro assays, these compounds seemed to be effective only when

added simultaneously with the virus or immediately after virus infection (Damonte at al., 2004). Thus, several authors reported the antiviral activity of alga polysaccharides, mainly galactans from red seaweeds and fucans from brown seaweeds. Duarte et al. (2001) showed that sulphated galactans from the marine alga *Bostrychia montagnei* inhibited HSV-1 replication when these compounds were added during the virus adsorption period and Mandal et al. (2007) also reported that the mode of action of sulphated fucans from *Cystoseira indica* could be mainly ascribed to an inhibitory effect on virus adsorption. Regarding green algae, Ghosh et al. (2004) reported the in vitro anti-herpetic activity of polysaccharide fractions from *Caulerpa racemosa* when cells were infected in presence of these fractions. The antiviral activity of polysaccharides isolated from cyanobacteria also have been studied and Rechter et al. (2006) indicated that antiviral effects of polysaccharides from *Artrospira platensis* against herpes viruses were more pronounced when the cells were preincubated with these compounds prior to the addition of the virus, indicating that antiviral action may be primary targeted to virus entry.

In an attempt to identify the compounds responsible of the antiviral activity found in ethanol and hexane extracts from *H. pluvialis,* a characterization by GC-MS of these extract was performed. The main difference between these extracts was the high amount of short chain fatty acids such as propanoic/lactic and butanoic acids found in ethanol extract. Also ethanol extract presented an important quantity of hexadecatrienoic acid which was not detected in hexane extract. However both extracts had a high percentage of hexacecanoid acid (palmitic acid) and 9,12,15-octadecatrienoic acid (α-linolenic acid). These results are in agreement with the highest antiviral activity showed by the ethanol extract since lactate is a recognized antimicrobial agent (Cherrington et al., 1991; Mbandi and Shelef, 2002). Besides, hexadecatrienoic acid has also been reported

to present a moderate antiviral activity (Guang-Zhou et al, 2004). The antiviral activity of hexane extract could be explained by the presence of the hexadecanoid acid (palmitic acid) that have been proposed to possess antibacterial and antiviral activities in some cases (Yff et al. 2002; Orhan et al. 2009).

The GC-MS analysis of *Dunaliella* hexane and ethanol extracts allowed identification of several compounds with antimicrobial and antiviral effects, such as: β-ionone, neophytadiene, phytol, hexadecanoic acid and 9, 12, 15-octadecatrienoic acid (Aranzaldi et al., 1999; Yff et al., 2002; Orhan et al., 2009; Santoyo et al., 2010a). Since most of these compounds are present in a higher amount in the ethanol extract than in the hexane extract, these data could explain why ethanol extract showed a higher antiviral activity.

In conclusion, all PLE extracts from *H. pluvialis* and *D. salina* presented important antiviral activities against herpes simplex type 1, overall *H. pluvialis* ethanol extract. These extracts mainly disrupt the step of attachment of the virus, although they were also able to inhibit HSV-1 intracellular replication. Polysaccharides in the water extracts of both microalgae can be suggested as the compounds responsible for the antiviral activity found in these extracts. The activity of the *H. pluvialis* ethanol extract could be partially related with the presence of short chain fatty acids, although other compounds could be involved in this activity; whereas, in the case of *D. salina* ethanol extract other compounds seemed to be involved, such as: β-ionone, neophytadiene, phytol, palmitic acid and α-linolenic acid.

It is worth mentioning that the use of PLE allows antiviral compounds to be obtained from microalgae used as carotenoids sources, which gives both microalgae biomass an added value.

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Table 1. PLE conditions (solvent, temperature and extraction time) of *Haematococcus pluvialis* and *Dunaliella salina* extractions and yield produced (% dry weight).

Sample	Solvent	Extraction temperature (ºC)	Extraction time (min)	Yield (%)
Haematococcus pluvialis	Hexane	100	20	5.88
	Ethanol	100	20	14.26
	Water	100	20	21.19
Dunaliella salina	Hexane	160	15	17.7
	Ethanol	160	15	31.4
	Water	160	15	9.2

Table 2. Antiviral activities of different extracts obtained from the microalgae *Haematococcus pluvialis* and *Dunaliella salina* against herpes simplex virus type 1.

Sample	CC_{50} (μg mL^{-1})	IC_{50} (μg mL^{-1})	SI
Haematococcus Hexane	677.24 ± 14.27	189.58 ± 3.18	3.57
Haematococcus Ethanol	736.24 ± 10.45	99.59 ± 2.36	7.39
Haematococcus Water	1608.82 ± 16.32	133.98 ± 5.53	12.01
*Haematococcus P*olysaccharide-rich fraction	1866.78 ± 23.14	98.61 ± 3.78	18.93
Dunaliella Hexane	486.56 ± 9.02	168.81 ± 5.25	2.88
Dunaliella Ethanol	622.94 ± 12.42	152.73 ± 4.56	4.07
Dunaliella Water	1578.95 ± 21.30	137.53 ± 7.69	11.48
Dunaliella Polysaccharide-rich fraction	1711.45 ± 29.31	85.34 ± 5.89	20.05

CC_{50} (cytotoxic concentration 50%): concentration required to reduce 50% the number of viable Vero cells after 48 h of incubation with the compounds. IC_{50} (inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Each value is the mean of four determinations ± standard deviation. SI (selectivity index): ratio CC_{50}/IC_{50}.

Table 3. Carbohydrate composition of polysaccharide-rich fractions obtained from *Haematococcus pluvialis* and *Dunaliella salina* water extracts.

Carbohydrate	*Haematococcus pluvialis* (%)	*Dunaliella salina* (%)
Glucose	19.70	94.34
Galactose	17.49	1.9
Fucose	3.18	--
Arabinose	4.53	--
Mannose	52.22	1.02
Xylose	1.87	1.07
Rhamnose	0.85	0.88
Myo-inositol	0.15	--
Glucuronic acid	--	0.77

Table 4. GC-MS identification, peak area contribution (normalized area %) and retention indices (RI) of compounds found in ethanol and hexane PLE extracts of *Haematococcus pluvialis* (100°C). Rt: retention time; NI: not identified.

Rt	Identification	RI	Ethanol Normalized Area (%)	Hexane Normalized Area (%)
4.9	Octane		-	43.2
5.5	Butanoic acid	815	11.8	-
6.1	Cyclobutanone,2,3,3,4- tetramethyl	837	-	2.7
7.9	Propanoic acid,2-hydroxy, methyl ester (methyl lactate)	903	40.3	-
11.7	L-Limonene	1025	-	3.0
13.4	NI	1080	-	1.7
19.8	NI	1302	-	2.5
20.0	NI	1311	-	3.7
20.3	NI	1319	-	2.5
24.9	Phenol 2,4-bis (1,1-dimethylethyl)	1511	-	2.5
25.0	Phenol 2,6-bis (1,1-dimethylethyl)	1513	2.9	3.4
26.8	Methanone, diphenyl	1637	2.5	-
27.4	Cyclododecane	1701	2.2	-
27.6	Octadecane	1719	-	1.3
28.8	Neophytadiene	1844	1.7	2.4
29.3	Nonadecane	1905	0.9	2.4
29.8	Hexadecanoic acid	1965	13.5	4.0
30.0	Hexadecanoic acid, ethyl ester	1995	5.5	12.8
31.0	Hexadecatrienoic acid, methyl ester	2123	7.3	-
31.1	Ethyl linoleate	2168	4.5	4.7
31.2	9-Octadecenoic acid(Z)-ethyl ester	2171	2.9	3.0
31.3	9,12,15-Octadecatrienoic acid, ethyl ester	2174	3.8	4.6
TOTAL SUM			2127319	318255

Table 5. GC-MS identification, total peak area, peak area contribution (normalized area %) and retention indices (RI) of compounds found in the PLE extracts (hexane and ethanol at 160 ºC) from *Dunaliella salina.* Rt.: retention time; NI: not identified.

Rt. (min)	Compound	RI	Hexane Normalized Area (%)	Ethanol Normalized Area (%)
4.4	Benzene, methyl		2.15	1.06
6.9	Benzene, 1,4,-dimethyl	866.4	1.94	0.81
14.2	2,6-dimethyl-cyclohexanol	1107	0.79	1.11
17.5	β-cyclocitral	1220	0.93	1.05
18.6	NI	1259	1.83	1.64
19.2	3,3-dimethyl-2,7-octanedione	1281	2.26	2.14
21.3	Naptalene,1,2,3,4-tetrahydro-1,1,6-trimethyl	1359	10.78	6.97
22.5	Naptalene, 2,7-dimethyl	1405	2.94	2.31
23.1	α-ionone	1430	2.18	3.10
24.5	β-ionone	1488	8.88	10.17
25.4	2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl	1539	5.45	7.20
27.3	1-heptadecene	1688	4.30	6.30
28.8	neophytadiene	1841	6.47	8.83
29.8	Hexadecanoid acid	1969	12.95	13.59
30.8	2-hexadecen-1-ol,3,7,11,15-tetramethyl	2115	3.64	3.90
31	9,12,15-octadecatrienoic acid methyl ester	2145	16.12	14.79
31.1	NI	2160	9.71	7.96
37.2	Vitamin E	3476	6.71	7.06
TOTAL SUM			35775710	63653028

FIGURE LEGENDS

Figure 1. Effect of pre-treatment with *H. pluvialis* and *D.salina* extracts and polysaccharide-rich fractions on HSV-1 infectivity. Each bar is the mean of four determinations ± standard deviation. From left to right: *Haematococcus* hexane, *Haematococcus* ethanol, *Haematococcus* water, *Haematococcus* polysaccharides-rich fraction, *Dunaliella* hexane, *Dunaliella* ethanol, *Dunaliella* water, *Dunaliella* polysaccharides-rich fraction.

Figure 2. Effect of *H. pluvialis* and *D.salina* extracts and polysaccharide-rich fractions on HSV-1 absorption period. Each bar is the mean of four determinations ± standard deviation. From left to right: *Haematococcus* hexane, *Haematococcus* ethanol, *Haematococcus* water, *Haematococcus* polysaccharides-rich fraction, *Dunaliella* hexane, *Dunaliella* ethanol, *Dunaliella* water, *Dunaliella* polysaccharides-rich fraction.

Figure 1

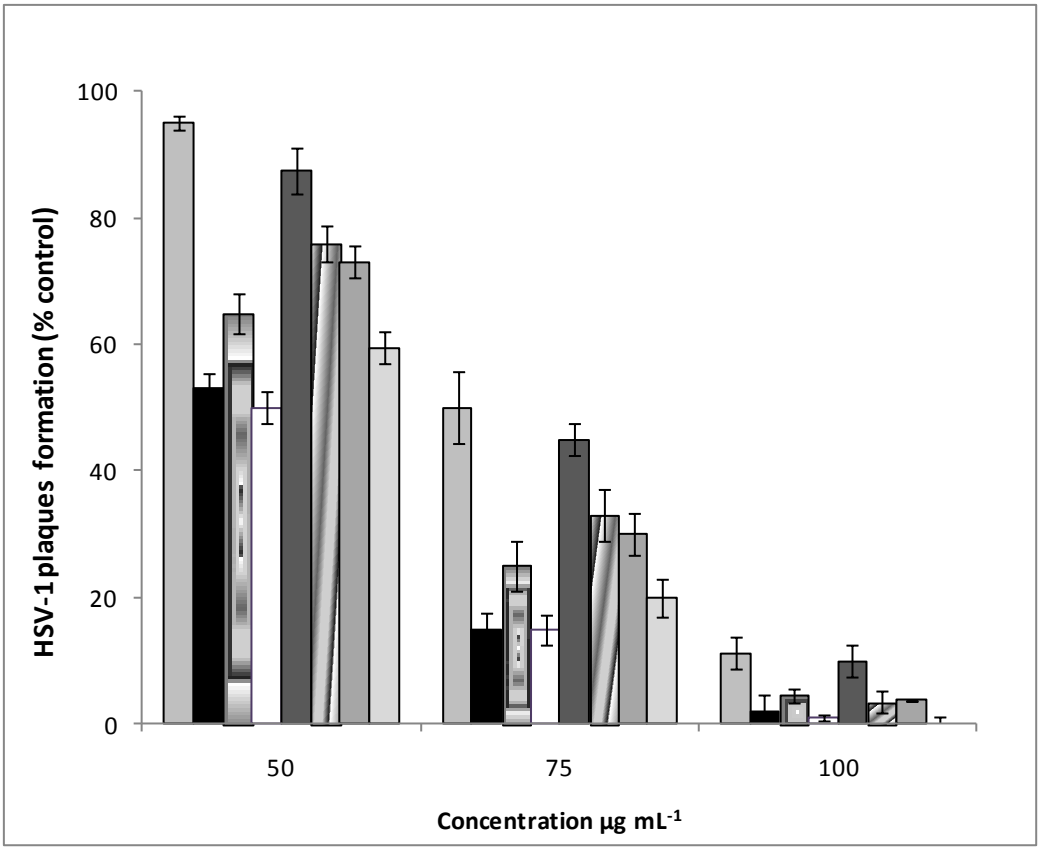
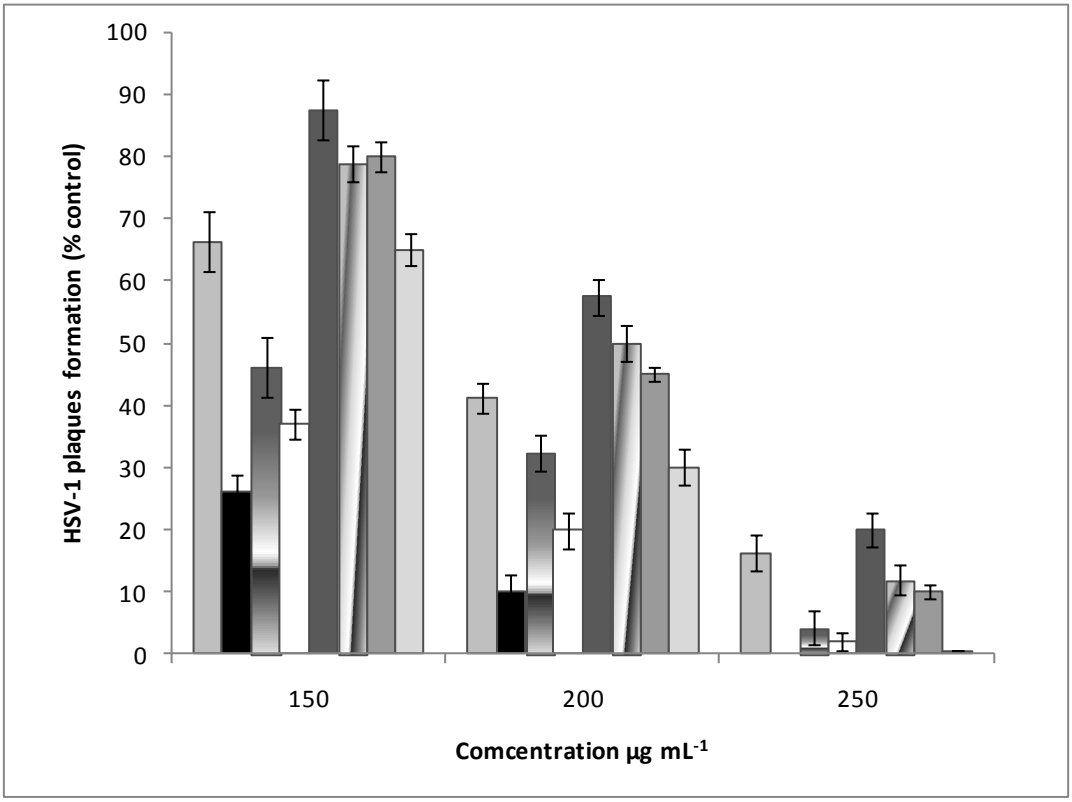

100
80
60
40
20
0
HSV-1 plaques formation (% control)
50
75
100
Concentration µg mL-1

Figure 2


100
90
80
70
60
50
40
30
20
10
0
HSV-1 plaques formation (% control)
150
200
250
Comcentration µg mL-1