DOI:10.1021/jf100369h

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Pressurized Liquid Extraction as an Alternative Process To Obtain Antiviral Agents from the Edible Microalga *Chlorella vulgaris*

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The antiviral properties of pressurized liquid extracts (PLE) (acetone, ethanol, and water) obtained from the edible microalga *Chlorella vulgaris* were evaluated against herpes simplex virus type 1 (HSV-1). None of the extracts tested showed extracellular direct virucidal activity against the virus, although a pretreatment of Vero cells with 75 μg/mL of water and ethanol extracts before virus addition inhibited 70% of the virus infection. Moreover, water and ethanol extracts were able to significantly inhibit the in vitro virus replication, showing $IC_{50\%}$ values of 61.05 and 80.23 μg/mL respectively. To identify the type of compounds responsible for the antiviral activity found in the water extract, the polysaccharide fraction was isolated. This activity was found to correlate with polysaccharides, because the polysaccharide-rich fraction (46% concentrated) showed higher antiviral activity than the complete water extract. A concentration of 75 μg/mL of this fraction inhibited 90% virus infection when added as a pretreatment and showed an $IC_{50\%}$ value of 33.93 μg/mL for intracellular virus replication. GC-MS characterization of the ethanol extract showed that the antiviral activity of this extract could be partially related with the presence of phytol, although other compounds could be involved in this activity.

KEYWORDS: *Chlorella vulgaris*; edible microalga; pressurized liquid extraction; antiviral activity; herpes simplex virus type 1

INTRODUCTION

Seaweeds have been traditionally used as food in Asian countries and to a lesser extent also in Europe and America. Edible seaweeds are considered as a source of a wide spectrum of nutrients with potential health-beneficial properties. Moreover, these organisms have long been used for therapeutic purposes and, in the past decade, they have become the focus for extensive screening of novel compounds with interesting biological activities that may lead to therapeutically useful agents (*1*−*3*). The fact that algae and microalgae may produce antiviral agents is already well-known, because several studies reported novel marine chemicals with antiviral activity (*4*). Rodriguez et al. (*5*) isolated three galactan polysaccharide fractions from the alga *Callophyllis variegata*, which showed potent antiviral activity against herpes simplex (HSV) types 1 and 2 and dengue type 2, together with low cytotoxicity. Lee et al. (*6*) also described a sulfated polysaccharide naviculan from *Naviculata directa*, which inhibited HSV-1 and HSV-2 by interfering with early stages of viral replication. Iwashima et al. (*7*) discovered that three plastaquinones isolated from the marine alga *Sargassum micracanthum* inhibited cytomegalovirus and measles virus, and de Souza et al. (*8*) described two diterpenes isolated from the marine alga *Dictyota menstrualis*, which inhibited HIV-1 reverse transcriptase enzyme. Soares et al. (*9*) also reported the in vitro antiviral effect of meroditerpenes isolated from the seaweed *Stypopodium zonale* against HVI-1. However, despite these data, the antiviral activity of only a few alga and microalga species has been screened and the antiviral potential of most of them remains unknown.

Chlorella vulgaris is a green microalga that has been used as an alternative medicine in the Far East since ancient times, and it is known as a traditional food in the Orient, being considered as a potential source of a wide spectrum of nutrients (e.g., carotenoids, protein), and widely produced and marketed as a food supplement in Japan, China, the United States, and Europe (*10*). *C. vulgaris* biomass has already been used experimentally in feed and food products because it can provide a coloring effect and other functional characteristics such as antioxidant activity (*11*, *12*). Furthermore, *C. vulgaris* has been documented to exhibit an indirect antitumor effect (*13*) and a protective effect against bacteria (*14*).

Pressurized liquid extraction (PLE) is an emerging technique that presents important advantages over traditional extractions. Traditional solvent extraction techniques use large quantities of toxic organic solvents, are labor intense, need long extraction times, possess low selectivity and/or low extraction yields, and can expose the extracts to excessive heat, light, and oxygen. In contrast, PLE uses less solvent in a shorter period of time, is

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 Published on Web 07/09/2010

automated, and involves retaining the sample in an oxygen- and light-free environment (*15*, *16*). Whereas other environmentally friendly techniques, such as supercritical fluid extraction (SFE), are frequently used to obtain functional compounds from natural sources, PLE has not been widely applied as a routine tool in natural product extraction. However, recent studies have used PLE for the extraction of compounds from natural sources (*17*, *18*).

The present work studies the antiviral activity against herpes virus type 1 of PLE extracts obtained from the edible microalga *C. vulgaris*. The responsible compounds and their potential mechanism of action are also postulated.

MATERIALS AND METHODS

Samples and Chemicals. *C. vulgaris* (BNA 10-007, National Bank of Algae, Canary Islands, Spain) was grown in modified Bold's Basal Medium (*19*) enriched with $NaNO_3$ (0.75 g/L). Cells (green phase) were cultured photoautotrophycally during 2 weeks in 20 L carboys and were bubbled with air continuously (24 h) with a flow rate sufficient to maintain strong agitation, at 25 °C, in light/dark cycles (16:8) with white fluorescent lamps (80 μmol m^{-2} s^{-1}). The pH of the medium fluctuates from 6.5 to 7.5. Cells were collected by centrifugation, freeze-dried, and stored under inert atmosphere until extraction.

Phytol was purchased from Sigma Chemical Co. (Madrid, Spain). 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, MO).

Pressurized Liquid Extraction (PLE). Extractions of *C. vulgaris* were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA) equipped with a solvent controller. Three different solvents (i.e., acetone, ethanol, and water) were used to obtain extracts with different compositions. Extractions were performed at 150 °C, whereas the extraction time was 20 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 acetone 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.

The extraction procedure was as follows: (i) sample was loaded into cell, (ii) cell was filled with solvent to a pressure of 1500 psi (1 psi = 6894.76 Pa), (iii) heat-up time was applied, (iv) static extraction took place (i.e., at 20 min) 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 N_2 gas, and (vii) depressurization took place. Between extractions, a rinse of the complete system was made 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 (Büchi Labortechnik AG, Flawil, Switzerland) was used for the extracts obtained using organic solvents. For water extracts, a freeze-dryer (Labconco Corp., Kansas City, MO) 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) (Gibco, Spain) supplemented with 5% fetal bovine serum (FBS), 1% penicillin−streptomycin, 1% Hepes buffer 1 M, 1% nonessential amino acids, and 1% L-glutamine. Maintenance medium for Vero cells was as described above but with 2% FBS.

HSV-1 (KOS) was obtained from ATCC, prepared in aliquots, and stored at −80 °C until use. Virus titer was determined by plaque reduction assay in Vero cells and expressed as plaque-forming units (pfu) per milliliter.

Cytotoxicity Assay. The cytotoxic effect of the different extracts on Vero cells was tested using the MTT assay, according to a published method (*20*). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) is a yellow water-soluble tetrazolium dye that is reduced by live cells, but not dead cells, 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 48 h at 37 °C. Cells were then washed with PBS, and 0.5 mg/mL of MTT was added to each well and incubated for 4 h at 37 °C. Supernatants were discarded and formazan crystals dissolved in an extraction solution (10% sodium dodecyl sulfate in a mixture of dimethylformamide and water 1:1 v/v, adjusted to pH 4.7 with acetic acid) overnight (14 h) at 37 °C. 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 by 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 were mixed and incubated at 37 °C for 1 h with MEM containing different extract concentrations or MEM alone (control). Samples were then diluted and used to infect confluent Vero cells for 1 h at 37 °C. After incubation, the virus inocula was removed, and the cells were washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37 °C. The infected cells were fixed with acetone/methanol (50:50) at 4 °C and stained with a 1% solution of crystal violet, and the number of the plaques was 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] × 100.

Influence of Various Treatment Periods on the Anti-HSV-1 Activity of the Extracts. Vero cells and viruses were incubated with the extracts at different stages during the viral infection cycle to determine the mode of antiviral action. (1) In cell pretreatment, monolayers of Vero cells in 24-multiwell plates were pretreated with MEM containing different concentrations of the extracts for 3 h at 37 °C. Cells were then washed with PBS and infected with 120 pfu of HSV-1. After incubation for 1 h at 37 °C, the virus inocula was removed, and the cells were washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37 °C. The infected cells were fixed and stained, and the number of the plaques was counted. Control consisted of untreated cells infected with HSV-1. (2) During the adsorption period, cells were infected with 120 pfu of HSV-1 in the presence of different concentrations of the extracts for 1 h at 37 °C. Then, the virus inocula and the extract were removed and the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37 °C. The infected cells were fixed and stained, and the number of plaque was counted. Control consisted of cells infected without extract. (3) For intracelullar replication, cells were infected with 120 pfu of HSV-1. After incubation for 1 h at 37 °C, the virus inocula was removed, and the cells were 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 37 °C, the infected cells were fixed and stained, and the number of plaque was counted. The concentration of a substance required to reduce the 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 the method of Jeurink et al. (*21*). Briefly, the freeze-dried water extracts were mixed with sterile distilled water at 90 °C (1 g/100 mL) for 20 min and cooled to 4 °C. Polysaccharides were precipitated by adding 2 volumes of cold ethanol, vigorous stirring, and allowing polysaccharide precipitation overnight at 4 °C. The precipitated polysaccharides were collected by centrifugation (10000*g* for 20 min at 4 °C) and redissolved 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 h with three or four changes of the distilled water. After dialysis, the polysaccharides were lyophilized, and the sample weight was estimated. The lyophilized polysaccharides were stored at −20 °C until further use.

The polysaccharide extracts were analyzed for their total carbohydrate content with the modified phenol−sulfuric acid method described by Fox and Robyt (*22*). Test solutions (25 μL) or standards of known glucose concentrations with 25 μL of 5% (w/v) phenol were added to an Eppendorf tube. The tube was stirred in a vortex for 30 s and placed on crushed ice,

125 μL of concentrated H_2SO_4 was added, and the mixture was heated in a water bath at 80 °C for 30 min. After that, the absorbance was determined at 490 nm.

GC-MS Analysis of Carbohydrate Content. First, freeze-dried polysaccharides were hydrolyzed with 4 M TFA at 100 °C for 4 h, and the TFA solution was evaporated to dryness in a Rotavapor R-210 (Büchi Labortechnik AG, Flawil, Switzerland) at room temperature. β-Phenylglucoside was used as internal standard (I.S.) at a concentration of 0.2 mg/mL. Dry residues were treated with 500 μL of pyridine (containing 2.5 g of hydroxylamine hydrochloride/100 mL) and heated for 30 min at 70 °C. The cooled samples were then trimethylsilylated with 1000 μL of HMDS and 100 μL of TFA for 60 min at 100 °C. Thereafter, the solutions were ready for analysis. The amount of derivatized stock solutions injected into the GC-MS system was 2 μL. Glucose, fructose, arabinose, galactose, xylose, *myo*-inositol, 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, Palo Alto, CA). The system was controlled by means of Agilent MSD Chemstation software. To analyze the volatile fraction 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 250 °C in split mode (ratio 1:20). Helium was used as carrier gas (7 psi). The oven temperature was programmed as follows: from 60 °C as the initial temperature (maintained for 2 min) to 120 °C in 3 min at 20 °C/min, then from 120 to 155 °C in 5.83 min at 6 °C/min, maintaining this temperature for 10 min before reaching 250 °C at 13 °C/min in 7.30 min; this temperature was maintained for 12 min and then raised from 250 °C to a final temperature of 330 °C in 4 min at 20 °C/min. 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. The volatile fraction of the ethanol extract obtained at 150 °C was analyzed using the same apparatus and column as described before. The injector was heated to 250 °C in split mode (ratio 1:20). The oven temperature was programmed as follows: from 40 °C as the initial temperature (maintained for 2 min) to 150 °C in 24 min at 5 °C/min,and then from 150 °C to a final temperature of 300 at 15 °C/min. 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. To do this, a mixture of hydrocarbons (*n*-undecane to *n*-octacosane, Sigma-Aldrich Chemie, Steinheim, Germany) dissolved in *n*-hexane was employed.

RESULTS AND DISCUSSION

PLE is an environmentally friendly technique that presents important advantages over traditional solvent extraction techniques. In this work, PLE has been used to obtain different extracts from the edible microalga *C. vulgaris* to investigate potential antiviral agents. Acetone, ethanol, and water were selected as solvents with the aim to cover a wide range of dielectric constants and to evaluate the influence of the solvent polarity in the extraction of antiviral compounds from the microalga. Extraction temperature and time were fixed at 150 °C and 20 min, respectively, on the basis of previous results obtained with different seaweeds (*23*, *24*). The extraction yield (percent dry weight) obtained was maximum when the extractions were carried out with ethanol (17.6%) and minimum with acetone (9.9%), whereas extractions with water provided extraction yields around 13.8%.

Cytotoxicity of PLE Extracts from *C. vulgaris*. The acetone, ethanol, and water extracts obtained from *C. vulgaris* were initially evaluated for cytotoxicity on preformed monolayers of Vero cells by the MTT method. The CC_{50} data obtained (**Table 1**) indicated that all extracts showed a low toxicity, although water extracts were less cytotoxic than acetone and ethanol extracts.

Table 1. Antiviral Activities of Different Extracts Obtained from the Microalga *C. vulgaris*, Polysaccharide-Rich Fraction, and Phytol against Herpes Simplex Virus Type 1[a]

sample	CC_{50} (mg/mL)	IC_{50} (μg/mL)	SI
acetone	1.39 ± 0.09	99.50 ± 8.25	13.97
ethanol	1.32 ± 0.10	80.23 ± 6.76	16.45
water	1.61 ± 0.11	61.05 ± 4.31	26.37
polysaccharide-rich fraction	1.66 ± 0.14	33.93 ± 3.25	49.04
phytol	0.96 ± 0.12	42.35 ± 4.02	22.67

[a] Vero cells were infected with HSV-1 (120 PFU) and incubated at 37 °C for 1 h. The unabsorbed viruses were removed and the cells washed with PBS. The infected cells were further incubated in the presence of different concentrations of the supercritical extracts at 37 °C for 48 h. The cells were then fixed and stained, and the number of plaques was counted. 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}.

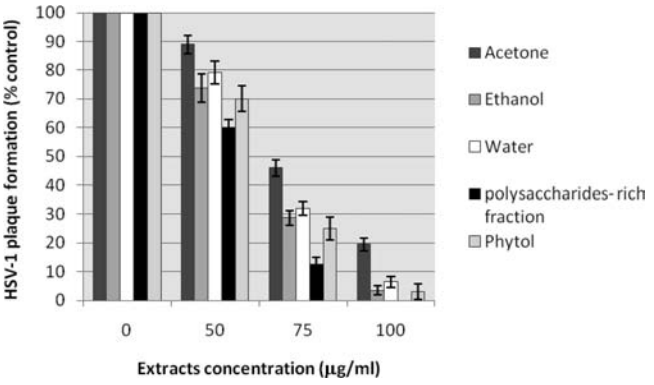

Figure 1. Effect of cell pretreatment with *C. vulgaris* extracts, polysaccharide-rich fraction, and phytol pure standard on HSV-1 infectivity. Vero cells were pretreated with the different substances for 3 h at 37 °C, then washed, infected with HSV-1 (120 pfu), and incubated at 37 °C for 1 h. The unabsorbed viruses were removed and the cells washed with PBS. The infected cells were incubated at 37 °C for 48 h. The cells were then fixed and stained, and the number of plaques was counted. Each bar is the mean of four determinations ± standard deviation.

Virucidal Activity of PLE Extracts from *C. vulgaris*. To investigate the direct inhibitory effect of the extracts against HSV-1, a virus suspension was treated at 37 °C for 1 h with different concentrations of the *C. vulgaris* extracts. Preincubation of HSV-1 with *C. vulgaris* 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 > 10 mg/mL for all of the extracts, indicating that PLE extracts almost lacked extracellular virucidal activity.

Influence of Various Treatment Periods on the Anti-HSV-1 Activity of the *C. vulgaris* Extracts. Vero cells were pretreated for 3 h with different concentrations of the microalga extracts. Afterward, extracts were removed and cells washed and infected with the HSV-1 virus. Results indicated that cells pretreated with 75 μg/mL of ethanol and water extracts inhibit virus infection by approximately 70% (**Figure 1**), whereas the same concentration of acetone extract reduced the virus infectivity by only 55%. These data suggested that all extracts interfered with the HSV-1 infection process at the initial infection steps, perhaps by blocking virus attachment or adsorption to Vero cells.

To investigate the influence of extracts on virus adsorption, cells were infected with HSV-1 in the presence of different

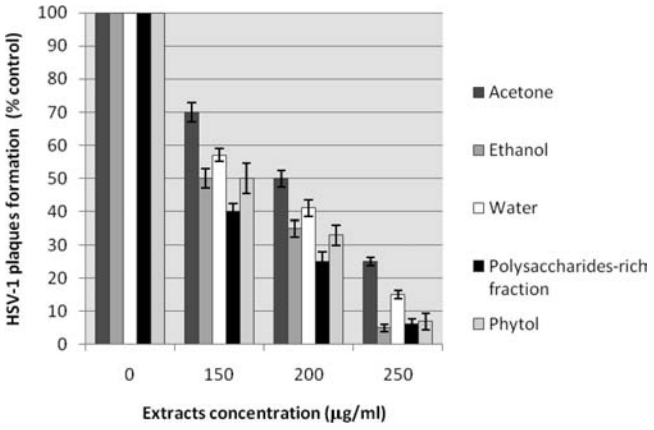

Figure 2. Effect of *C. vulgaris* extracts, polysaccharide-rich fraction, and phytol pure standard on HSV-1 adsorption period. Vero cells were infected with HSV-1 (120 pfu) in the presence of different concentrations of the compounds for 1 h at 37 °C. Then, the virus inocula and the different substances were removed and the cells washed with PBS and maintained for 48 h at 37 °C. Each bar is the mean of four determinations ± standard deviation.

concentrations of the extracts for 1 h at 37 °C. Then, the virus inocula and the extract were removed and the cells washed with PBS and maintained for 48 h at 37 °C. Addition of 150 and 200 μg/mL of water and ethanol extracts reduced virus infectivity by 45 and 60%, respectively (**Figure 2**). In that case, also, acetone extracts were less effective than the water and ethanol ones. Comparison of the data obtained during the adsorption stage with those found in the pretreatment step showed that when water and ethanol extracts were applied at 75 μg/mL as cell pretreatment, the infectivity was reduced 70%; meanwhile, the same concentration added during the adsorption period did not produce any reduction, indicating that extracts were more effective when applied as pretreatment.

The antiviral activity on the intracellular replication of the virus was evaluated by adding different concentrations of the *Chlorella* extracts to previously HSV-1 infected Vero cells and incubated for 48 h at 37 °C. All extracts showed a dose-dependent inhibition of virus replication. In this assay, water extract was more efficient against HSV-1 replication than ethanol and acetone extracts, showing the lowest IC_{50} values (**Table 1**). However, ethanol extract also showed an important inhibition of virus intracellular replication (IC_{50} = 80.23 μg/mL). By comparison of these data with those obtained during pretreatment period, for the same water and ethanol extract concentrations, that is, 75 μg/mL, it can be observed that this concentration reduced virus infectivity by 70% when added during the pretreatment period and by 68% for the water extract and by 46% for the ethanol extract when added after virus infection. Thus, the *C. vulgaris* water extract was effective as an inhibitor of the intracellular virus replication as well as an agent able to disrupt virus attachment to the cell; however, the ethanol extract was more effective in inhibiting intracellular virus replication.

To better define the anti-HSV-1 compounds presented in *Chorella* water extract and taking into account that antiviral activities of alga water extracts have been frequently related to diverse types of polysaccharides (*25*), the isolation of a polysaccharide-rich fraction from the water extract was carried out. Besides, the *Chlorella* ethanol extract also showed important antiviral effects, so this extract has been analyzed by GC-MS to identify potential antiviral components.

Antiviral Activity of Polysaccharide-Rich Fraction Isolated from *C. vulgaris* Water Extract. The percentage of carbohydrates found in the lyophilized polysaccharide-rich fraction obtained from the water extract after the extraction procedure was 46%. Besides, a GC-MS analysis of carbohydrate composition of this fraction showed the presence of glucose as its main component (**Table 2**), which is in agreement with the presence of β-1,3-glucan as the main polysaccharide described in *Chlorella* species cell wall (*26*, *27*). Moreover, according to galactose, mannose, and rhamnose levels found in this fraction, minor amounts of galactans, mannans, and rhamnans could be present in this fraction.

Table 2. Carbohydrate Composition of Polysaccharide-Rich Fraction Obtained from *Chlorella vulgaris* Water Extracts

carbohydrate	% dry basis	carbohydrate	% dry basis
glucose	50.54	mannose	12.03
galactose	15.29	xylose	3.8
fucose	1.12	rhamnose	11.30
arabinose	4.72	*myo*-inositol	1.19

To correlate the antiviral activity found in the water extract to the polysaccharide-rich fraction, the cytotoxicity and antiherpetic assays were measured under the same conditions as previously described for the water extract, except for virucidal effects. This activity was not measured in the polysaccharide fraction because the water extract presented a very small virucidal activity. The cytotoxicity assays of this polysaccharide-rich fraction (**Table 1**) indicated toxicity values similar to those of the water extract.

When Vero cells were pretreated with 75 μg/mL of the polysaccharide-rich fraction, virus infection was inhibited by approximately 90% (**Figure 1**). However, when the water extract was applied at this concentration, only 70% inhibition was achieved. Moreover, if 150 μg/mL of polysaccharide-rich fraction was applied during the virus adsorption period, the infectivity was reduced by 60% (**Figure 2**), providing a reduction of 45% when the *Clorella* water extract was used. Consequently, polysaccharides could be identified as the compounds responsible of the antiviral activity found in *C. vulgaris* water extract when added prior to the virus or simultaneously with the virus.

The antiviral activity of the polysaccharide-rich fraction on the intracellular replication of the virus was also evaluated, showing an IC_{50} value of 33.93 μg/mL (**Table 1**). This value indicated that the polysaccharide fraction presented almost a double antiviral action on the intracellular replication step as the water extract. It is worth mentioning that this enriched extract presented a very high selectivity index, painting it as an interesting source of antiviral compounds.

These results are in agreement with the mechanism by which alga and microalga 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, consequently leading to the blockade of viral entry. During in vitro assays, these compounds seemed to be effective only when added prior to the virus, simultaneously with the virus, or immediately after virus infection (*25*). Thus, several authors reported the antiviral activity of alga polysaccharides, mainly galactans from red seaweeds and fucans from brown seaweeds. Duarte et al. (*28*) indicated that sulfated 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. (*29*) also reported that the mode of action of sulfated fucans from *Cystoseira indica* could be mainly ascribed to an inhibitory effect on virus adsorption. With regard to green algae, Ghosh et al. (*30*) reported the in vitro antiherpetic activity of polysaccharide fractions from *Caulerpa racemosa* when these fractions were added immediately after virus infection. The antiviral activity

Table 3. Volatile Compounds Identified in *Chlorella vulgaris* PLE Extract (Ethanol, 150 °C) by GC-MS, Total Peak Area, Peak Area Contribution (Normalized Area Percent), and Retention Index (RI)

peak	retention time (min)	RI	compound	normalized area (%)	area (area counts)
1	25.52	1494.6	phenol, 2,4-bis(1,1-dimethylethyl)-	0.89	4729227
2	25.57	1667.06	phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl	1.11	5936951
3	27.88	1689.44	cyclododecane	1.37	7289982
4	27.95	1821.76	heptadecane	2.38	12710600
5	29.24	1852.91	neophytadiene	15.86	84551244
6	29.3	1871.8	2-hexadecene, 3,7,11,15-tetramethyl-, [*R*-(*R**,*R**-(*E*))]-	2.11	11261040
7	29.44	1925.83	2-hexadecen-1-ol, 3,7,11,15-tetramethyl, [*R*-(*R**,*R**-(*E*))]- (derivative)	2.62	13956311
8	29.59	1932.33	neophytadiene (derivative)	4.43	23599947
9	30.05	1949.72	9,12,15-octadecatrienoic acid, methyl ester	12.35	65818031
10	30.19	1955.57	hexadecanoic acid	15.46	82437296
11	31.25	1977.36	2-hexadecen-1-ol, 3,7,11,15-tetramethyl, [*R*-(*R**,*R**-(*E*))]- (phytol)	14.00	74645630
12	31.43	2101.92	9,12-octadecadienoic acid (*Z*,*Z*)	10.78	57471818
13	37.98	2123.14	vitamin E (α-tocopherol)	0.63	3360234
14	38.08	2130.52	cholest-5-en-3-ol, (3*β*)-	3.35	17862094
15	38.58	2148.96	ergosta-5,22-dien-3-ol, (3*β*,22*E*)-	4.65	24800236
16	39.05	2156.2	ergosta-5,8,22-trien-3-ol, (3*β*,22*E*)-	8.00	42638138
				100.00	533068779

of polysaccharides isolated from cyanobacteria were also studied, and Rechter et al. (*31*) 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 primarily targeted to virus entry.

GC-MS Characterization of PLE Ethanol Extract from *C. vulgaris*. In an attempt to identify the compounds responsible of the antiviral activity found in the ethanol extract from *C. vulgaris*, a characterization by GC-MS of this extract was performed. Results obtained are shown in **Table 3**, where a tentative identification has been performed on the basis of the comparison of mass spectra and retention index (RI). As can be observed, 16 compounds were identified. Some of them were detected in large amounts, such as neophytadiene, phytol, hexadecanoic acid, and the methyl ester of 9,12,15-octadecatrienoic acid. With regard to potential compounds responsible for the antiviral activity, the presence of neothytadiene and phytol can be pointed to. Neophytadiene, a hydrocarbon belonging to the phytan family, has been detected in essential oil from tobacco leaves (*32*) as well as in certain microalgae (*18*, *33*) and has demonstrated some antimicrobial activity (*34*). Moreover, phytol has also been described in microalgae (*18*) and in different plant essential oils with proven antimicrobial activity (*35*). Consequently, the determination of antiviral activity, in the same conditions as previously, of these two compounds was desired. However, only the antiviral activity of phytol was evaluated, due to the lack of a commercial pure standard for neophytadiene. The data obtained indicated that phytol presented a cytotoxicity value higher than that of the *Chlorella* ethanol extract (Table**1**). When Vero cells were pretreated with 75 μg/mL phytol, virus infection was inhibited by approximately 65−70% (**Figure 1**), a value similar to that found for the ethanol extract. Moreover, during the virus adsorption period, 150 μg/mL phytol reduced the infectivity by 50% (**Figure 2**); similar data were obtained when the *Chlorella* ethanol extract was used. As a conclusion, it could be pointed out that phytol has a clear contribution to the total antiviral activity of ethanol extracts when added prior to the virus or simultaneously with the virus, although probably the whole activity could not be ascribed to only this compound.

The activity of phytol on the intracellular replication of the virus was also evaluated, showing an IC_{50} value of 42.35 μg/mL (**Table 1**). This value indicated that phytol presented almost double the antiviral action on the intracellular replication step as the ethanol extract; thus, this compound could be responsible for the activity at this step found in the *C. vulgaris* ethanol extract.

In conclusion, PLE extracts from the edible microalga *C. vulgaris* presented important antiviral activities against herpes simplex type 1, over all water and ethanol extracts. These extracts mainly inhibit HSV-1 intracellular replication, although they were also able to disrupt the virus attachment step. Polysaccharides presented in the water extract could be pointed out as the compounds responsible for the antiviral activity found in this extract. The activity of the ethanol extract seemed to be partially related with the presence of phytol, although other compounds could be involved in this activity.

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Received for review February 1, 2010. Revised manuscript received June 22, 2010. Accepted June 29, 2010. This work has been financed by the Spanish Ministry of Education and Science (AGL2005-06726-C04), the program CONSOLIDER-INGENIO 2010 (CDS2007-00063), and the regional program ALIBIRD-CM S-0505/AGR-0153 from the Comunidad de Madrid, Spain. M.P. thanks CSIC for her I3P fellowship.