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Short communication

Antiviral activity of *Spirulina maxima* against herpes simplex virus type 2

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Abstract

Spirulina has been used in a variety of practical applications in biotechnology and medical sciences. This paper presents the antiviral activity found in a hot water extract (HWE) of a commercial preparation of *Spirulina maxima*, studied by a microplate inhibition assay, using several viruses. The HWE inhibited the infection for: herpes simplex virus type 2 (HSV-2), pseudorabies virus (PRV), human cytomegalovirus (HCMV), and HSV-1, and the 50% effective inhibition doses (ED_{50}) were 0.069, 0.103, 0.142, and 0.333 mg/ml for each virus, respectively. For adenovirus the inhibition was less than 20%, and no inhibition was found for measles virus, subacute sclerosing panencephalitis virus (SSPE), vesicular stomatitis virus (VSV), poliovirus 1 and rotavirus SA-11, at concentrations of 2 mg/ml of the HWE. The highest antiviral activity was for HSV-2, with a selectivity index of 128. The antiviral activity was not due to a virucidal effect. Herpesvirus infection was inhibited at the initial events (adsorption and penetration) of the viral cycle. To initiate the isolation and identification of the compound that exhibits the antiviral activity of *S. maxima*, some extracts made by using several solvents with different polarity were evaluated by microplate inhibition assay using HSV-2. The highest antiviral activity was detected in the methanol–water 3:1, which suggests that the antiviral activity is probably due to highly polar compounds.

Keywords: *Spirulina*; Inhibition; HSV-2; Hot water extract

Spirulina (*Arthrospira*) *platensis* and *Spirulina maxima* have a long history of use as a food for humans, traditionally for some Mexican (Aztecs) and African (Lake Chad area) peoples. (Ciferri and Tiboni, 1985). Spirulina is a planktonic blue–green alga found in some warm water alkaline volcanic lakes, and has been considered as a supplement in human and animal food (Dillon et

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al., 1995). Actually, it is being widely studied for its possible anticancer, antibacterial, and antiparasitic properties, and for several medical conditions such as allergies, ulcers, anemia, heavy-metal poisoning, and radiation poisoning (Al-Batshan et al., 2001; Dasgupta et al., 2001; Zhang et al., 2001; Rodriguez-Hernandez et al., 2001; Pinero Estrada et al., 2001; Hirahashi et al., 2002). *S. platensis* has been also studied for its antiviral properties (Hayashi et al., 1993; Ayehunie et al., 1998), which seem to be related to its sulfated polysaccharide named calcium spirulan (Hayashi et al., 1996a,b).

S. maxima grows naturally in México, in the Texcoco Lake, and several commercial preparations have been widely studied and evaluated for their possible toxic effects. Until now, *S. maxima* has not shown any side effects in several experimental animals (Chamorro et al., 1988a,b, 1989; Chamorro and Salazar, 1990; Salazar et al., 1996; Chamorro, 1997; Salazar et al., 1998). This paper presents the results of the antiviral activity found in a hot water extract (HWE) of a commercial preparation of *S. maxima*.

The HWE from *S. maxima* was prepared according to the procedure described by Hayashi et al. (1993), 100 g from a commercial stock of *S. maxima* produced by Sosa Texcoco S.A. de C.V., México, were extracted three times with 500 ml of boiling deionized water, and leaving the extract in the water for 1 h. Insoluble material was removed by centrifugation at $800 \times g$ for 10 min. The supernatant was filtered through a 0.22 μm pore filter, and then lyophilized. The powder was rehydrated with deionized water to prepare a 10% stock solution (HWE), which was evaluated for cell toxicity and antiviral activity.

Cytotoxicity was determined by the method described by Sudo et al. (1994), for that, a serial 2-fold dilution of HWE of *S. maxima* was prepared in a 96-well microtiter, using cell Medium (according to the cells used). A sample of 100 μl of each dilution was mixed with 100 μl of a cellular suspension (2×10^4 cells); eight wells were used for each extract dilution. Cells were incubated for 24 h and the percent of cell viability was determined by counting the cells with trypan blue, or the colorimetric MTT (tetrazolium salt) assay, in comparison with cells without extract. The 50% inhibitory dose (ID_{50}) was calculated by the Spearman–Kärber method (Mahy and Kangro, 1996). The HWE of *S. maxima* showed no cell growth inhibition at concentrations below 2 mg/ml, and the most sensitive cells to *S. maxima* toxic effect were the MRC-5 cells, ID_{50} 2.2 mg/ml (Table 1).

Therefore, all the antiviral experiments were carried out at concentrations lower than 2 mg/ml of the HWE, and in each one, a control for cell toxicity was included by using uninfected cells treated with the HWE in the same way as the virus infected-HWE treated cells.

The screening for antiviral activity of *S. maxima* using several viruses was done by inhibition of the viral cytopathic effect (CPE), according to the method described by Mahy and Kangro (1996). For that, a 2-fold dilutions of the HWE were made in a 96-well microtiter plate; 50 μl of each dilution were mixed with 50 μl of the viral suspension containing 200 $TCID_{50}$, and then 100 μl of cells (2×10^4 cells) were added. Eight wells were used for each extract dilution. Cells were incubated until the viral control showed CPE in all the inoculated wells. The antiviral activity detected by CPE inhibition, was calculated by the Spearman–Kärber method and expressed as 50% effective dose (ED_{50}), which was the lowest concentration of the HWE that reduced the viral infection of the control to a 50%. The in vitro selectivity index of the extract was determined by comparison of the inhibitory dose versus the effective dose (ID_{50}/ED_{50}). The tested viruses were herpes simplex virus type 2 (HSV-2 G strain), herpes simplex virus type 1 (HSV-1 MacIntyre strain), pseudorabies virus (PRV Shope strain), human cytomegalovirus (HCMV), adenovirus type 3, poliovirus 1 (Sabin 1 vaccine strain), rotavirus SA-11, measles viruses (Edmonston–Zagreb vaccine strain, and subacute sclerosing panencephalitis, SSPE Halle strain), and vesicular stomatitis virus (VSV Indiana strain). The HWE inhibited the infection of HSV-2, PRV, HCMV, and HSV-1, and the highest antiviral activity was against HSV-2, with a selectivity index of 128. There was no antiviral effect for the two measles viruses, VSV, SA-11, and poliovirus type 1 (Table 1).

Table 1
Antiviral effect of the HWE of *S. maxima* on several viruses

Virus	Cells	Cytotoxicity[a] ID_{50} (mg/ml)	Antiviral activity[b] ED_{50} (mg/ml)	Selectivity index (ID_{50}/ED_{50})
HSV-2	Vero	8.9±0.16	0.069±0.015	128
PRV	MDBK	7.9±0.12	0.103±0.002	76
HCMV	MRC-5	2.2±0.16	0.142±0.001	15
HSV-1	Vero	8.9±0.16	0.333±0.001	26
Adenovirus 3	Hep2[c]	5.2±0.1	< 20%[c]	–
Poliovirus 1	Vero	8.9±0.16	NA[d]	–
SA-11	MA-104	8.6±0.12	NA	–
Measles vaccine virus	Vero	8.9±0.16	NA	–
SSPE	Vero	8.9±0.16	NA	–
VSV	Vero	8.9±0.16	NA	–

Each value is the mean±S.D. of three independent experiments.
[a] Cytotoxicity of the HWE of *S. maxima* was determined by counting the cells with trypan blue, or MTT assay, 24 h after being in contact with the HWE, in comparison with cells without the extract. It is expressed as ID_{50} (minimum concentration required to inhibit control cell growth by 50%).
[b] Antiviral activity was determined by inhibition of viral cytopathic effect (CPE), and expressed as ED_{50} (minimum concentration required to reduce control virus infection by 50%).
[c] Less than 20% of inhibition with 2 mg/ml of *S. maxima*.
[d] NA, no antiviral activity.

To determine whether the antiviral activity of *S. maxima* was due to a virucidal activity, the HWE was diluted in 199 Medium to obtain final concentrations from 0.7 to 12 mg/ml, and 100 µl of each concentration were mixed with 100 µl of HSV-2 or HSV-1 containing 1000 $TCID_{50}$. Samples were incubated for 2 h at 37 °C, and the remaining infectious virus was determined by $TCID_{50}$ (Mahy and Kangro, 1996). The HWE did not modify the viral rate of infection, after being for 2 h directly in contact with either HSV-2 or HSV-1, even at concentrations of 12 mg/ml (Data not shown), suggesting that herpesvirus inhibition was not a result of a direct virus inactivation.

Due to the fact that the HWE of *S. maxima* did not show a direct virucidal effect on HSV-2, or HSV-1, we investigated which events of the viral infectious cycle were affected. For that, Vero cells grown in a 96-well microtiter plate were infected with 1000 $TCID_{50}$ of HSV-2. At different times after infection: 0, 30, 60 and 90 min, and then every hour up to 8 h post-infection, cells were washed twice with 199 Medium to remove the unbound virus. Then, different concentrations of the HWE of *S. maxima* were added. Cells were incubated for 4 days and scored for CPE, in comparison with infected untreated cells (Ramos-Kuri et al., 1996). We found that the extract competed with the virus, and inhibited the viral infection in a dose-dependent event, when it was administered on the first 2 h after viral infection (Fig. 1). These results suggested that *S. maxima* could interfere with the initial events of the viral infectious cycle, adsorption and penetration,

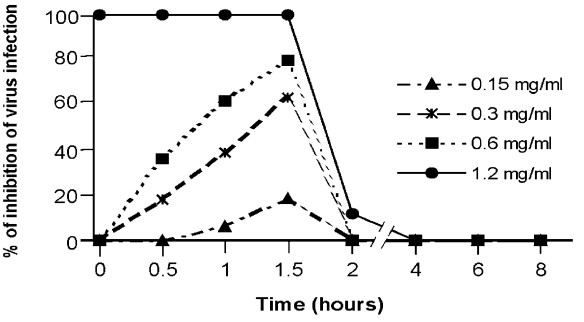

Fig. 1. Effect of *S. maxima* on HSV-2 infection when added to the cell medium at different times after viral infection. Vero cells were infected with 1000 $TCID_{50}$ of HSV-2. At different times post infection, unbound virus was removed, and then different concentrations of the HWE of *S. maxima* were added to the cells. Cells were incubated and scored for CPE in comparison with virus infected HWE untreated-cells.

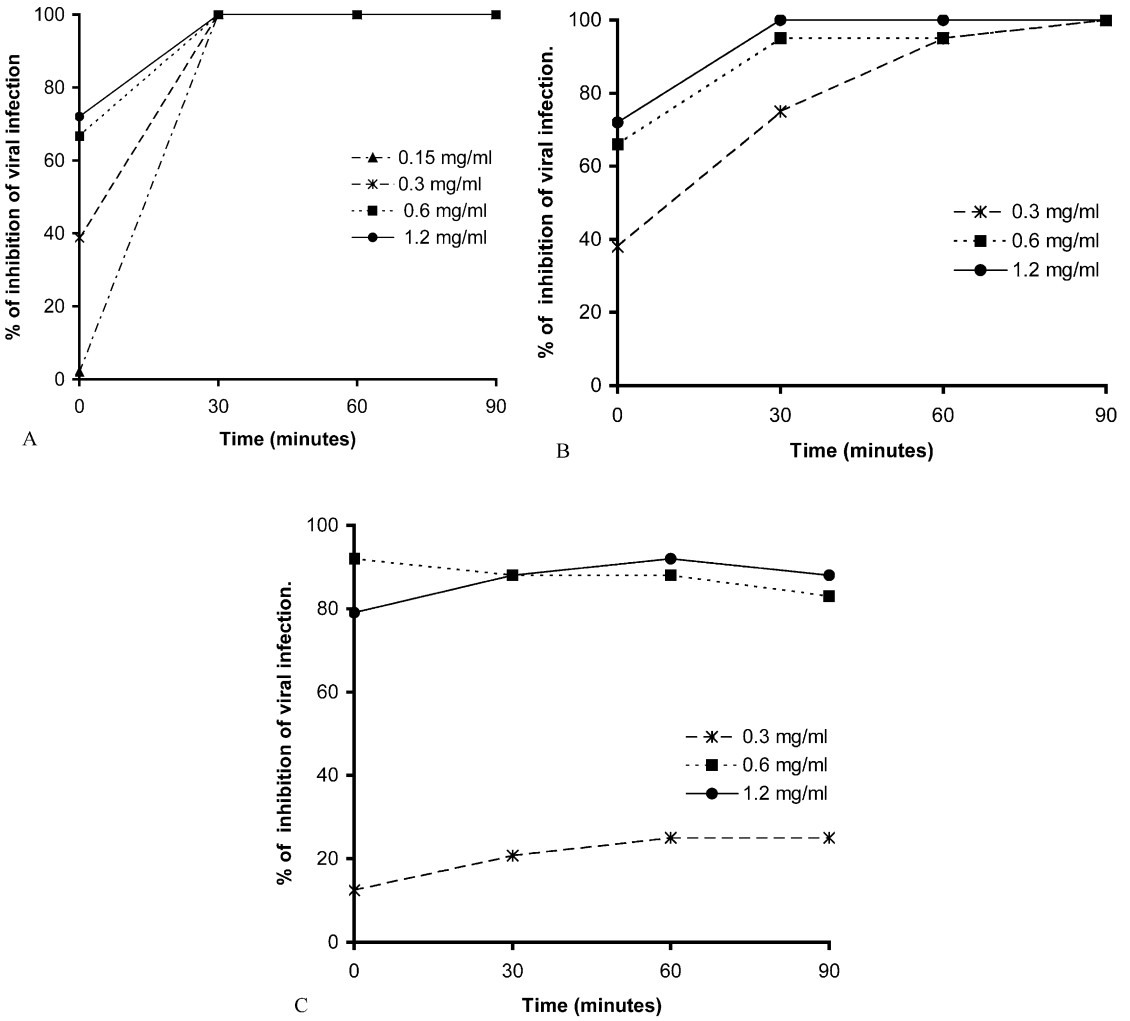

Fig. 2. Effect of *S. maxima* on the adsorption of several herpes viruses to Vero cells. (A) HSV-2; (B) HSV-1; and (C) PRV. Vero cells were exposed to different concentrations of the HWE of *S. maxima*. At different times post exposure it was washed off, and then cells were infected with 1000 $TCID_{50}$ of the indicated virus. The 0 time point indicates application of the HWE followed by virus infection immediately after removing the HWE.

events that usually take place on the first 2 h after virus infection of the cells.

To investigate whether the extract was blocking the first event of the virus infectious cycle, the adsorption of HSV-2 to the cells. Vero cells grown in a 96-well microtiter plate were inoculated with 100 µl of different concentrations of the HWE, and incubated. At different times post inoculation (0, 30, 60, 90 min), cells were washed twice with 199 Medium, to remove the unbound extract, and immediately, they were infected with 1000 $TCID_{50}$ of HSV-2. Cells were incubated for 4 days, and scored for CPE in comparison with untreated cells infected with HSV-2 (Ramos-Kuri et al., 1996). It was found that the HWE could block the HSV-2 infection in dose dependent kinetics (Fig. 2A). Similar results were found with the other herpes viruses (HSV-1, and PRV), (Fig. 2B and C).

These results suggested that *S. maxima* inhibited from the beginning the herpesvirus infection by

Table 2
Antiviral activity against HSV-2 of several extracts of *S. maxima* obtained with different solvents

Solvent	Cytotoxicity[a] ID_{50} (mg/ml)	Antiviral activity[b] ED_{50} (mg/ml)	Selectivity index (ID_{50}/ED_{50})
Hexane	2.7	No activity	–
Chloroform	1.95	0.14	13.9
Methanol	6.9	0.13	53
Methanol:water 3:1	37	0.1	370
Hot water	37	0.3	123

Each value is the mean of two experiments. *S. maxima* extracts were obtained by five times extraction with each solvent. After solvent evaporation, the residual material was rehydrated and tested for antiviral activity against HSV-2 by inhibition of CPE, and cell toxicity by trypan blue exclusion or MTT assay.

[a] Cytotoxicity is expressed as ID_{50} (Minimum concentration required to inhibit control cell growth by 50%).

[b] Antiviral activity is expressed as ED_{50} (Minimum concentration required to reduce control virus infection by 50%).

blocking virus adsorption and penetration to Vero cells, without a direct virucidal effect. This finding is similar to the one reported by Hayashi et al. (1993) for *S. platensis*. However, it is possible that the antiviral activity could be different, *S. platensis* blocked the penetration of HSV-1, without affecting the virus adsorption event to HeLa cells (Hayashi et al., 1993), but the *S. maxima* extract competed with the adsorption and penetration of HSV-2 to Vero cells. Furthermore, a sulfated polysaccharide named calcium spirulan has been identified as the antiviral principle of *S. platensis*, which has shown to have a high selectivity index for enveloped viruses such as HSV-1, HCMV, measles virus, mumps virus, influenza A virus, and HIV-1 (Hayashi et al., 1996a,b). However, when we tested the HWE of *S. maxima* against two measles viruses, Edmonston–Zagreb vaccine strain and SSPE, we could not detect any virus inhibition. Same result was obtained with VSV, which is another enveloped virus. Also, it is known that different naturally or chemically sulfated polysaccharides have shown antiviral properties against HSV and other viruses, and most of them seem to interfere with the initial events of the virus infectious cycle. For example, chemically oversulphated galactosaminoglycan sulphates have shown inhibition for some enveloped viruses such as HIV-1, HSV-1 and HCMV (Di Caro et al., 1999). An anionic polysaccharide obtained from *Prunella vulgaris*, a Chinese herb, has shown antiviral properties against HSV-1, HSV-2, but was inactive against HCMV, human influenza virus A and B, poliovirus type 1 or VSV (Xu et al., 1999). A rhamnan sulfate, a natural sulfated polysaccharide isolated from *Monostroma latissimun* has shown a potent antiviral effect on HSV-1, HCMV, and HIV-1 (Lee et al., 1999). Sulfated galactans from the marine alga *Bostrychia montagnei* inhibited HSV replication (Duarte et al., 2001); same result was obtained with a fucan sulfate from an edible brown alga *Sargassum horneri* (Preeprame et al., 2001).

We consider that it is important to isolate and characterize the antiviral activity of *S. maxima*, therefore, we initiated a preliminary attempt to isolate the compounds with antiviral activity, for that, different extracts were prepared at room temperature, by using 126 g of the *S. maxima* commercial stock, which was extracted five times with 300 ml of hexane. Other *Spirulina* samples were extracted with chloroform, methanol, methanol–water 3:1, and boiling water. All residual material after solvent evaporation at 40 °C, 333 mbars, was tested against HSV-2 by inhibition of CPE, as was described above. Infected-treated cells were scored for CPE, and mock infected for cytotoxicity. We found that the antiviral activity was extracted with high polarity solvents such as methanol–water 3:1, and hot water (Table 2), indicating that the antiviral activity could be due to highly polar compounds. Identification of compound with antiviral activity of *S. maxima* will be very helpful to understand the mechanism by which the HSV-2 infectious cycle is blocked at adsorption and penetration stages. Entry of HSV

to cells is a very complex event, which involve five viral glycoproteins (gC, gB, gD, gH, and gL) participating in three different stages, with several different molecules of the cell surface (Roizman and Knipe, 2001). Purification of the *S. maxima* extract, and evaluation of its antiviral activity in a mouse model for herpesvirus infection are on the way.

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