Trabajo científico

Actividad anti-dengue in vitro del alga marina *Hypnea cervicornis*

In vitro anti-dengue activity from marine alga *Hypnea cervicornis*

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Resumen

Una fracción del extracto acuoso del alga marina Hypnea cervicornis se sometió a evaluación contra el virus del dengue, DENV-2. La fracción obtenida por precipitación con sulfato de amonio mostró en un inmunoensayo reducción del 43 % de la proteína viral no estructural NS1 en el sobrenadante de células Huh-7 infectadas con el virus. Esa observación sugiere una inhibición del proceso de infección. La actividad se confirmó con experimentos de q-PCR observándose una reducción significativa del número de copias del genoma viral a nivel intracelular (1.17 log) con respecto al control positivo de infección. Adicionalmente, la fracción evaluada mostró altos títulos de hemaglutinación y un patrón de afinidad que sugiere que la actividad antiviral observada podría ser debida a lectinas.

Abstract

A fraction of the aqueous extract of seaweed Hypnea cervicornis was evaluated against dengue virus, DENV-2. The fraction obtained by ammonium sulphate precipitation showed in an immunoassay 43 % reduction of the NS1 non-structural viral protein in the supernatant of Huh-7 cells infected with the virus. This observation suggests an inhibition of the infection process. The activity was confirmed by q-PCR experiments with a significant reduction in the number of copies of the viral genome at the intracellular level (1.17 log) relative to the positive control of infection. Additionally, the fraction evaluated showed high hemagglutination titers and an affinity pattern suggesting that the observed antiviral activity could be due to lectins.

Palabras clave: fiebre del dengue, glicoproteínas, alga roja, Golfo de California, lectinas antivirales.

Key words: Dengue fever, glycoproteins, red alga, Gulf of California, antiviral lectins

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Introduction

Dengue is a viral disease that is transmitted through the bite of the *Aedes aegypti* and *Ae. albopictus* mosquitoes.[1] This disease is the most important of the re-emerging infectious diseases worldwide, particularly in the tropics and urban centers where rainfall, temperature, and rapid urban sprawl have resulted in the rapid spread of the disease.[2] Approximately 390 million people are infected annually, of which 96 million are severe dengue cases and 25,000 cases are fatal, primarily in infants.[3] Dengue fever and the most severe of manifestations of dengue: dengue haemorrhagic fever and dengue shock syndrome is caused for any of four-dengue virus serotypes (DENV1–4).[2] The dengue virus is a member of the genus Flavivirus (family *Flaviviridae*); the RNA is a single-stranded 10.7-kb virus, with positive polarity, encoding three structural proteins (envelope glycoprotein E, the protein associated with membrane M, and the capsid protein C) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).[4] Alternative strategies other than vaccines should be considered, since there is no specific treatment for dengue, and the developed vaccines are partially efficient.[5] It has been documented that marine organisms are invaluable source of bioactive compounds. At present, marine bioactive metabolites have been tested against herpes simplex virus, cytomegalovirus, measles virus, human immunodeficiency virus and dengue virus. So far, carrageenans, quinones, diterpenes and lectins have been active.[6,7] Algae from *Hypnea* genus (Gigartinales, Rhodophyta) are widespread tropical distributed. *Hypnea* species are considered as an interesting resource for the industry of phycocolloids because synthetize κ- and/or ι- carrageenan. In addition, those algae have been reported as a source of lectins and steroids. In particular, *Hypnea cervicornis* has been reported as source of anti-inflammatory and antinociceptive lectins.[8] Considering the above, the aim of the present study was to evaluate the anti-dengue activity, cytotoxicity and haemagglutination of a salted out fraction from the *H. cervicornis* aqueous extract.

Materials and Methods

Materials

Fresh samples of the red alga *H. cervicornis*[9,10] were collected in the Bahía de La Paz, Baja California Sur, Mexico (June, 2014). The algae were washed with fresh water, cleaned of epiphytes, and stored at -40 °C until processing. The samples were lyophilized and ground in a mill to facilitate extraction. Blood for haemagglutination assays was obtained from the Californian rabbit. D-(+)-mannose, D-(+)-glucose, D-(+)-galactose, D-(+)-arabinose, L-(+)-fucose, N-acetyl-D-glucosamine, human thyroglobulin, ovomucoid, mannans from *Saccharomyces cerevisiae* and concanavalin A were purchased from Sigma-Aldrich.

Extraction and fractionation of *H. cervicornis*

Five grams of freeze-dried *H. cervicornis* were homogenized in phosphate buffer (1:1 ratio; Ultra-Turrax, IKA Works). The homogenate was diluted with buffer to reach 5 volumes and left with constant agitation for 18 h at 4 °C. Particulate matter was removed through filtration followed by centrifugation at 15,000 × *g* for 20 min at 4 °C. the supernatant was added with ammonium sulphateto attain a concentration of 20 % (w/v) and maintained under gentle agitation for 18 h at 4 °C, and posterior centrifugation. The supernatant was taken and a second precipitation was done with ammonium sulphate to reach 80 % concentration. After 18 h, the precipitated was recovered by centrifugation and the obtained pellet (F20/80) was suspended in phosphate buffer and extensively dialyzed. The protein content in fraction F20/80 was quantified using the Bradford method.[11]

Cell lines and viral strains

The DENV-2-susceptible human liver cell line Huh-7 was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 5 % foetal bovine serum, 2 mM glutamine, and a mix of antibiotics (100 U penicillin and 100 µg/mL streptomycin) and incubated at 37 °C in an atmosphere of 5 % CO_2. The viral strain DENV-2 New Guinea was tested.

Cytotoxic assay

Huh-7 cells (5 × 10^3 cells/well) were seeded onto a 96-well microplate CytoTox 96® kit (Promega) and incubated at 37 °C overnight in a humidified incubator with 5 % CO_2. The medium was aspirated, and the cells were treated with several concentrations ranging between 0-100 µg/mL of sample. Concanavalin A at 50 µg/mL was used as reference based on studies using BHK cells.[12] After 24 h incubation, the activity of lactate dehydrogenase was measured in 50 µL of medium after the addition of enzyme substrate and reading the absorbance at 490 nm. The survival rate was calculated and the half cytotoxic concentration (CC_{50}) of fraction F20/80 was calculated. The determination was carried out in triplicate.

Antiviral assay

Huh-7 cells were seeded at a density of 5 × 10^3 cells/well. The cells were incubated for 24 h at 37 °C with 5 % CO_2 and subsequently infected with DENV-2 at a multiplicity of infection (MOI) of 10 in presence of 4.0 and 8.0 µg/mL of fraction F20/80 for 1 h. As proposed by Hung and co-workers,[12] some wells were treated with 50 µg/mL of concanavalin A as inhibition control. Infected and non-

infected mouse brain extract was used as positive and negative controls, respectively. Subsequently, the cells were washed three times with phosphates buffer-foetal bovine serum. The infection proceeded for 24 h. Subsequently, the supernatant was recovered to assess infection, using the dengue NS1 antigen and the cells were harvested to measure the copy number of viral particles inside the cell with real-time PCR (qPCR). The experiment was performed twice, in triplicate.

NS1 antigen detection in the supernatant of infected cells
The NS1 Ag Platelia assay was used to measure the secretion of the viral antigen NS1 in the supernatants from the antiviral experiments. The absorbance was read at 450/620 nm using a multiplate reader (Multiscan FC, Thermo Scientific). The absorbance readings were compared to the infection controls to calculate the percentage of the NS1 protein.

RNA copy viral detection
RNA isolation. Total RNA from Huh-7 cells was isolated using Trizol reagent (Life Technologies). Briefly, Huh-7 cells were homogenized in Trizol reagent and vigorously mixed with chloroform, followed by centrifugation to collect the aqueous phase. The RNA was precipitated after adding cold isopropanol. To increase the quality of the RNA, an additional precipitation was performed with 0.8 M LiCl. The RNA pellet was washed with ethanol and suspended in RNase-free water. The RNA was quantified using a nanophotometer, and the RNA quality was monitored using 2 % agarose gel electrophoresis and visualized on a UV transilluminator (BioDoc-It Imaging System).

cDNA synthesis. Total RNA was treated with DNase I (Invitrogen) for 1 h to remove genomic DNA. First-strand cDNA was synthesized through reverse transcription using a kit (ImProm-II Reverse Transcription System kit, Promega) and random oligonucleotides. The reaction mixture was added to 4 µL (20-40 ng/µL) RNA samples. Amplification was performed using a thermocycler (C1000, Bio-Rad Laboratories) following the manufacturer instructions. cDNA samples were prepared and stored at -20 °C.

Preparation of the DENV-2 standard for quantification. One fragment of DENV-2 protein C was amplified using the designed primers and cloned into the pGEM-T Easy plasmid (Promega), followed by transformation into competent *E. coli* cells. After 24 h, the positive clones were examined. The presence of the fragment was verified through PCR and agarose gel electrophoresis. The plasmid was extracted, purified and the concentration was measured. The plasmid DNA was diluted at a concentration range of 5.0×10^{-2} to 5.0×10^{-9} pg/µL, followed by the amplification of the protein C fragment and quantification through qPCR, dilutions were measured in triplicate in order to build the standard curve.

Absolute quantification of specific cDNA. The primers for the viral genome were designed based on a gene segment encoding the DENV-2 protein C (GenBank accession number AF038403). The forward primer: 5′-CAATATGCTGAAACGCGAGA-3′ and reverse primer 5′-TGCTGTTGGTGGGATTGTTA-3’ were used. The amplification was performed on a real-time thermocycler (CFX96TM, Bio-Rad Lab). The reactions were performed in a 96-well plate using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Lab) following the manufacturer instructions. The amplification protocol for all qPCR was 90 °C for 3 min, 39 cycles at 95 °C for 10 s, and 60 °C for 1 min. A dissociation curve was obtained by increasing the temperature 0.1 °C/s from 65 to 95 °C. Positive and negative controls were included, as well as reference sample having concanavalin A that protects the cell from infection. All cDNA and control samples were examined in triplicate. The number of viral copies of DENV-2 was estimated according to Whelan and co-workers.[13]

Haemagglutination assay
The presence of lectins in fraction F20/80 was determined through haemagglutination using a microtiter method, with a 96-well microtiter U-plate and papain-treated erythrocytes from rabbit blood as published by Hung and co-workers.[14] Haemagglutination was macroscopically observed. The haemagglutination activity was expressed as a titre, corresponding to the highest two-fold dilution capable to produce visible agglutination. Concanavalin A was used as a positive control. The assays were performed in triplicate.

Haemagglutination inhibition
Using the aforementioned sugars and glycoproteins, haemagglutination-inhibition assays were performed. The sugars were prepared at 100 mM and the glycoprotein was prepared at 500 µg/mL in 0.15 M NaCl. First, 25 µL of a two-fold serial dilution of sugar or glycoprotein were added to each well. An equal volume of the fraction F20/80 with a haemagglutination titre of four was added. After 1 h incubation at room temperature, 25 µL of erythrocytes suspension were added, mixed and incubated at room temperature for an additional 2 h. The assays were performed in triplicate. Erythrocyte button formation was considered as a positive result of agglutination inhibition. The lowest concentration (in mM) of a sugar or (in µg/mL) glycoprotein that completely inhibited haemagglutination (4 titres) was recorded as the inhibitory potency.

Statistical analysis

The data assuming normality and homogeneity of variance were subjected to one- and two-way ANOVAs. The data that did not assume normality or homogeneity of variance were subjected to Bonferroni's correction for multiple comparisons. Statistical significance was set at $P < 0.05$. All of the statistical and graphical analyses were performed using Prism GraphPad v. 6.01 software.

Results and discussion

The identification of new compounds from marine organisms with anti-dengue activity represents a feasible strategy for the developing of new drugs. In the present study, the antiviral activity of an ammonium sulphate precipitated fraction (F20/80) of the red algae *H. cervicornis* is documented. As a first step, the cytotoxic activity was evaluated. Fraction F20/80 had a CC_{50} = 24.01 µg/mL of soluble protein on Huh-7 cells. This is the first cytotoxicity report for this alga. The cytotoxicity value encountered allowed for the evaluation of antiviral activity. The second step was to determine if the fraction F20/80 confers complete or partial protection against DENV-2 in Huh-7 cells at two molecular levels: one was by the quantifying the amount of non-structural secreted protein (NS1) present in supernatant, and the other was by quantifying the amount of intracellular viral genome in the cells using qPCR. Hexameric lipoprotein NS1 is a widely used antigen in clinics to diagnose dengue disease.[15] The tested concentrations of the F20/80 fraction had a similar reduction of 42 % and 43 % of the NS1 antigen, respectively (Fig. 1A). The total NS1 antigen of cells treated with F20/80 was half those of positive control suggesting that F20/80 exerted a protective effect to infection. Although, the infection was not fully inhibited, the NS1 protein levels decreased significantly. The quantified viral copies of DENV in the protected cells were significantly reduced, 1.17 log compared to the positive control using the lowest concentration of the fraction (4.0 µg/mL) and by 1.01 log using the highest concentration (8.0 µg/mL; Fig. 1B). These results suggest the blocking in some way of the viral entry mechanism during the hour of contact with the fraction. Interestingly, the effects were not significantly different between the two concentrations treatments ($p < 0.0001$). Thus, the lowest concentration might be enough to halt one mechanism of entry though agonist inhibition. However, could exist different mechanisms of infection on Huh-7 cell line. Indeed, is accepted that the lectin concanavalin A inhibit the viral entry through two ways: by attaching the viral glycoprotein E or binding the HS receptor in BHK cells.[12] In our study, the lectin concanavalin A (50 µg/mL) shown a 48 % reduction of secreted NS1 antigen, and a reduction of 1.33 log in the copy number compared to the infection control. Notably, this molecule cannot completely block the infection in Huh-7 cell line, suggesting the existence of another entry mechanism in these cells. The effects on reduction of the infection with the treatments at both levels, protein and mRNA showed the same trend; nevertheless, the reduction of viral copy number was more dramatic than the reduction of the amount of NS1 antigen. Unlike qPCR, NS1 Ag Platelia is a semi-quantitative assay, that probable reflecting the fact that the NS1 protein is secreted from infected cells as a hexameric lipoprotein form, and the amount of antigen bound to each monomer of NS1 is one of the parameters measured in this analysis.[16] In both analyses, an antiviral effect was detected, independent of the concentration F20/80, which likely reflects the presence of several constituents in the fraction. Due the polar nature of the aqueous extract from *H. cervicornis*, it is possible that it contains polysaccharides and proteins.[17, 18] *Hypnea* genus has been previously reported as a source of carrageenan;[8] however, the high molecular weight and anionic nature of carrageenans, the low oral bioavailability, the anticoagulant properties, the toxic side effects and the incapability of the cellular penetration of target tissues[19] represent a great disadvantage for the developing new drugs.

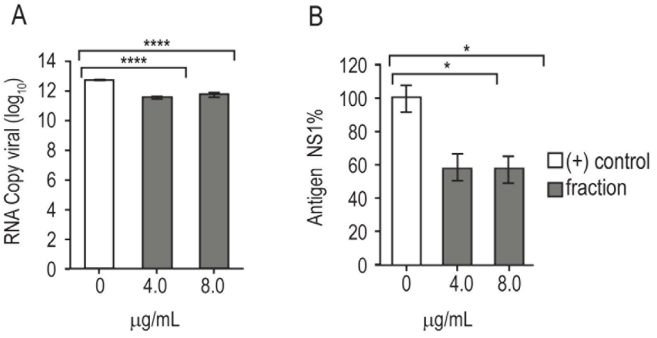

Figure 1. Effect of the fraction F20/80 of *Hypnea cervicornis* in the infection of DENV-2 on the human cell line Huh-7. The white bars represent the viral infection positive control, and the black bars represent the algae protein fraction treatments. The virus and the fraction were in contact for 1 h. The potential protective activity was measured 24 h after incubation. A) The percent of NS1 antigen secreted in the medium of infected cells. B) Copy number of intercellular viral particles of DENV-2. The absolute quantification was performed using qPCR. Statistical significance was determined using a one-way ANOVA, followed by Bonferroni's test (* = $P < 0.05$, *** = $P < 0.001$, **** = $P < 0.0001$).

Unlike others aqueous extracts, the ammonium sulphate precipitate gave a fraction with high content of proteins. The addition of higher amount of ammonium sulphate

reduce protein solubility, leading to protein precipitation and increasing concentration.[20] Seaweeds lectin-type proteins, have reported with potent antiviral activity against enveloped viruses, like the agglutinin KAA-2 from *Kappaphycus alvarezii*, agglutinin ESA-2 from *Eucheuma serra* and GRFT from *Griffithsia* sp.[21] however, none of them were tested against DENV.

The lectin are proteins or glycoproteins known for their ability to bind specifically and reversibly to glycans without altering the covalent structure; the specific interaction of algal lectins to high mannose type N-glycans embedded on cell surface of viral envelope or target cell, suppresses virus infection.[22] This suggests that the lectins present in the precipitate fraction could be responsible for the anti-viral effect. The high haemagglutination activity on papain-treated erythrocytes (titre of 2,048) also suggests the presence of lectins in the protein content of the algae fraction. This presence was confirmed by the haemagglutination inhibition, because the binding of lectins to erythrocyte membranes can only be interrupted through competing sugars.[23] Haemagglutination of fraction F20/80 was inhibited through the sugars mannose (25 mM) and N-acetyl-D-glucosamine (100 mM), the human glycoprotein thyroglobulin (31.25 μg/mL), and *S. cerevisiae* mannans (62.5 μg/mL). The mannan of *S. cerevisiae* and human thyroglobulin glycoproteins possess high mannose type N-glycans; in DENV, the glycosylation of the envelope protein E occurs with high-mannose and pauci-mannose types.[12] Therefore, it is probable that high-mannose binding lectins of the algal fraction bind the E glycoprotein and create steric hindrance with HS, acting as an inhibitor of viral entry.[24] In contrast with our results, the activity of a lectin isolated from *H. cervicornis* from Brazil, HCA, was inhibited through galactoside-binding activities, suggesting that the protein fractions of *H. cervicornis* from Bahía de La Paz contained a different lectin with antiviral activities, reinforcing the proposal of Nagano and co-workers on the existence of a family of lectins closely related whose expression varies geographically.[25]

Marine life can experience much greater differences in temperature, light, and pressure; there are even sulphur- or arsenic-based organisms. The greater variety of marine organisms provide more opportunities for biotechnological development of antiviral drugs. As the aqueous fraction tested in this study, the next step for this research will be the purification and characterization of the potential lectins with anti-DENV activity, as well to understand the mechanism of the inhibition of viral entry using cell lines bearing only one receptor.

Conclusion

The original contributions of this paper are: 1) the extract from the red alga *H. cervicornis* reducing dengue infection as measured at both the protein level (NS1) and mRNA, by reducing the presence of number of viral copies; 2) the affinity and high titre fractions of haemagglutination strongly suggest the involvement of lectins implicated in the anti-dengue activity; and 3) the dissimilarity in the profile of inhibition of haemagglutination suggests a different lectin than the one previously reported. These results suggest that purifying the extract of *H. cervicornis* for lectins should be the next step considered in order to determine if a lectin(s) is responsible for the anti-dengue response observed. To the best of our knowledge, this is the first report of anti-dengue response with an extract of the red alga *H. cervicornis*. The components present in the *H. cervicornis* aqueous fraction contain promising candidates for the development of antiviral drugs.

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