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|  | PROGRESS REPORT Virology & Molecular Biology Services | Protocol ID: MB-2029 |
| | | Report Amend #: N/A |

REPORT TITLE ***In vitro* evaluation of antiviral activity of red algae against herpes simplex viruses**

STUDY PROTOCOL ID **MB-2029**

DEVELOPMENT PHASE **Pre-Clinical Research and Development**

NAME OF TEST ARTICLE ***Dumontiaceae 1***

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1. Executive Summary

The antiviral effect of marine algae (*Dumontiaceae 1*) against herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) was evaluated in cultured cells using plaque reduction assay. The crude extracts containing polysaccharide were prepared by ethanol precipitation and subsequently used in pre-treatment of the cells prior to HSV infection and treatment of HSV infected cells. The results of pretreatment of cells showed that the algae extracts were highly active against both HSV-1 and HSV-2 infections with the 90% inhibitory concentrations (IC₉₀) approximately 0.005% for HSV-1 and 0.013% for HSV-2.

The antiviral activities of *Dumontiaceae 1* extracts were also examined by treatment of cells that were already infected with either HSV-1 or HSV-2. Inhibition of HSV replication was observed with IC₉₀s approximately 0.095% for HSV-1 and 0.19% for HSV-2. Comparison of the plaque sizes in *Dumontiaceae 1* -treated and untreated cells suggests that the *Dumontiaceae 1* extracts may inhibit the cell-to-cell transmission of HSV.

In conclusion, our data indicate that the *Dumontiaceae 1* extracts are highly active on preventing HSV transmission and also active on cells that are already infected with either HSV-1 or HSV-2.

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2. Materials and Methods

1. Testing compound

The marine algae (*Dumontiaceae 1*) was received from Blue Moon Marine.

2. Virus and cells

HSV reference strains F (HSV-1) and G (HSV-2) (ATCC No. VR-733 and VR-734, respectively) were used in the experiments. A HSV susceptible cell line, Vero cells (African green monkey kidney cells, ATCC No. CCL-81), was used in the virus plaque reduction assays. The culture medium for Vero cells was 5%MEM (Minimum Essential Medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin).

3. Preparation of extracts

The algae extracts were prepared essentially as described by Ehresmann *et al.* (J. Phycol. 13: 37-40, 1977) with minor modification. One gram of *Dumontiaceae 1* was washed in 25 ml of sterile deionized water three times and collected by centrifugation, then resuspended in 10 ml of 95% ethanol. The mixture was return to boiling for 2 min and sonicated for 3 min x 5. 10 ml of 95% ethanol was added and brought to 65°C for 5 min. The mixture was then cooled to room temperature and filtered through Whatman #1 paper. The cellular residue was air-dried overnight and added to 20 ml sterile water which had been heated to 80°C with constant stirring at this temperature for 2 h. After centrifugation of the viscous extract for 5 min, two volumes of 95% ethanol were added to the supernatant. The resulting precipitates were air-dried, weighed and dissolved in PBS buffer.

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4. Virus plaque reduction assay

Confluent Vero cells were washed with PBS and subsequently infected with either HSV-1 or HSV-2 (200 pfu/well) for 1 h at 37°C. After viral inoculum was removed, infected cells were washed with PBS and overlaid with 0.5% methylcellulose in culture medium (equal volume of 1% methylcellulose mixed with 2 x culture medium) containing *Dumontiaceae 1* extracts at concentrations of 0, 0.005%, 0.01%, 0.05%, 0.1%, and 0.5%. The cells were incubated at 37°C for 48 h. When plaque size was adequate, the cells were fixed with 10% formalin for 10 min, and stained with 0.5% crystal violet for 10 min. The dye was removed by washing with tap water and left to dry in a fume hood. The plaques were then counted.

5. Pre-treatment of cells with the algae extracts

Confluent Vero cells were incubated with the *Dumontiaceae 1* extracts at concentrations of 0, 0.005%, 0.01%, 0.05%, 0.1%, and 0.5% at 37°C for 1 h. 200 pfu virus was then added to the cells and incubated at 37°C for 1h. After viral inoculum was removed, the cells were washed with PBS and overlaid with methylcellulose in culture medium. After 2-3 days incubation, the cells were fixed, and stained.

6. Data analysis

All data were generated from duplicate or triplicate wells in two independent experiments. The effect of a test compound at varying concentrations is expressed as % of control (the mean plaque counts in drug-treated wells/the mean plaque counts in control wells). The 50% and 90% inhibitory concentrations (IC₅₀ and IC₉₀: inhibitory concentration giving 50% and 90% plaque reduction, respectively) were calculated using computer program CalcuSyn (Biosoft, St Louis). All figures were generated using Microsoft Excel.

3. Results

3.1. Antiviral effect of Dumontiaceae 1 on HSV determined by pre-treatment of cells

To examine the efficacy of *Dumontiaceae 1* extracts on blocking HSV entry into cells and thus have a role on preventing transmission of HSV, pre-treatment of cells with the *Dumontiaceae 1* extracts was performed. The susceptible cells were incubated with different concentrations of the algae extracts at 37°C for 1 h, followed by immediate infection with either HSV type 1 or 2. After viral inoculum was removed, the cells were washed with PBS and overlaid with methylcellulose containing no drug for plaque assay.

The results of pre-treatment of cells with the *Dumontiaceae 1* extracts were shown in Table 1 and Fig. 1. Strong inhibitory effects on both HSV-1 and HSV-2 were obtained in this treatment. The IC₅₀s were approximately 0.0023% for HSV-1 and 0.005% for HSV-2, while the IC₉₀s were approximately 0.005% for HSV-1 and 0.013% for HSV-2. This indicates that the *Dumontiaceae 1* extracts can block HSV entry into the cells.

Table 1. The antiviral effect of *Dumontiaceae 1* extracts on HSV determined by pre-treatment of cells (% of control)

| Concentrations (%) | 0 | 0.005 | 0.01 | 0.05 | 0.1 | 0.5 |
|--------------------|-----|-------|------|------|-----|-----|
| HSV-1 | 100 | 9.2 | 3.5 | 5.0 | 1.4 | 0 |
| HSV-2 | 100 | 36.8 | 38.2 | 10.3 | 3.7 | 0.7 |

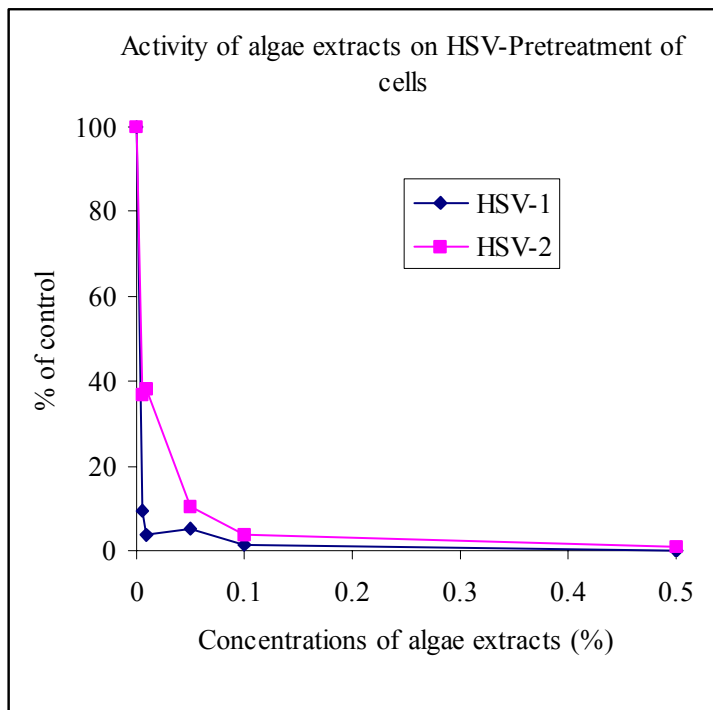


Fig. 1. The antiviral effect of Dumontiaceae 1 extracts on HSV determined by pre-treatment of cells and plaque reduction assay. The effect of the compound at varying concentrations is expressed as % of control (the mean plaque counts in drug-treated wells/the mean plaque counts in control wells).

3.2. The antiviral effect of Dumontiaceae 1 on HSV infected cells

To examine the efficacy of Dumontiaceae 1 extracts on HSV infected cells and thus have a role in treatment of HSV infections, the susceptible cells were infected with either HSV-1 or -2, then incubated with different concentrations of the Dumontiaceae 1 extracts. The antiviral effect was then determined by plaque reduction assay. The results were summarized in Table 2 and Fig. 2, which showed that the *Dumontiaceae 1* extracts were highly active on HSV-1 or HSV-2 infected cells. The IC₅₀s in this treatment were approximately 0.026% for HSV-1 and 0.017% for HSV-2, while the IC₉₀s were approximately 0.095% for HSV-1 and 0.19% for HSV-2. We have noticed that the size difference of plaques between Dumontiaceae 1 extracts-treated and untreated cells. The plaque sizes in Dumontiaceae 1-treated cells at the

concentrations higher than 0.05% were smaller than that of untreated cells. This may indicate that the Dumontiaceae 1 extracts inhibited the cell-to-cell spread of the HSV.

Table 2. The antiviral effect of Dumontiaceae 1 on HSV infected cells (% of control)

| Concentrations (%) | 0 | 0.005 | 0.01 | 0.05 | 0.1 | 0.5 |
|--------------------|-----|-------|------|------|------|-----|
| HSV-1 | 100 | 97.8 | 81.5 | 35.7 | 6.6 | 0 |
| HSV-2 | 100 | 75.0 | 54.3 | 27.4 | 28.0 | 3.0 |

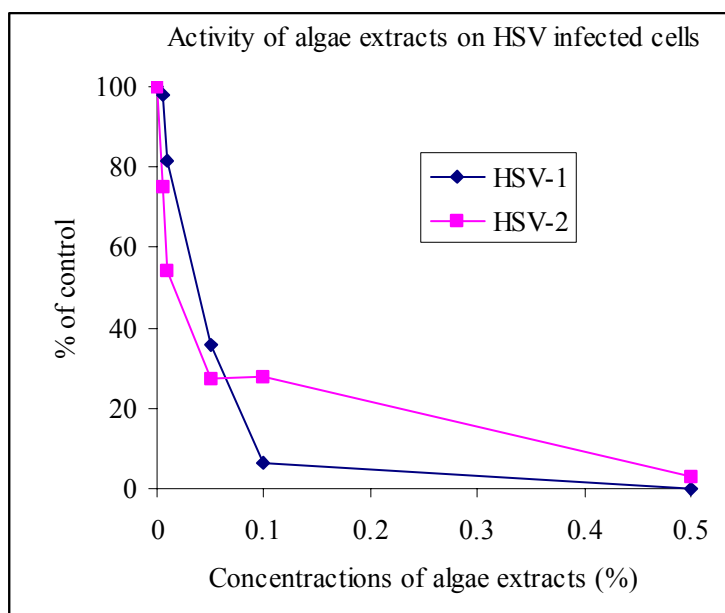


Fig. 2. The antiviral effect of Dumontiaceae 1 on HSV infected cells determined by plaque reduction assay. The effect of the compound at varying concentrations is expressed as % of control (the mean plaque counts in drug-treated wells/the mean plaque counts in control wells).

4. Conclusions

In conclusion, the Dumontiaceae 1 extracts showed direct inhibitory activities on both HSV-1 and HSV-2. Strong inhibitory effects were observed on HSV reference strains when the cells were pre-incubated with the Dumontiaceae 1 extracts prior to virus infection. This suggests that the Dumontiaceae 1 extracts can block HSV entry and thus prevent the transmission of HSV. Antiviral activities were also observed on cells that were already infected with HSV reference strains. This indicates that the Dumontiaceae 1 extracts also have therapeutic use for treatment of HSV infections.

Table 3. Summary of IC₅₀ and IC₉₀ of Dumontiaceae 1 extracts

| Drug treatment | Viruses | IC ₅₀ | IC ₉₀ |
|-----------------------|---------|------------------|------------------|
| Pretreatment of cells | HSV-1 | 0.0023% | 0.005% |
| | HSV-2 | 0.005% | 0.013% |
| On infected cells | HSV-1 | 0.026% | 0.095% |
| | HSV-2 | 0.017% | 0.19% |