Anti-herpes simplex virus effect of algal polysaccharide extract from *Ulva reticulata*

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Abstract

Herpes simplex virus (HSV) is the causative agent of a fever blister, genital herpes and neonatal herpes. Treatment of HSV infection was performed by acyclovir. Acyclovir remains to have a side effect in the case of long-term usage, and the virus may develop drug resistance. Therefore, these problems are interesting to study the natural substance for the treatment of HSV infection and drug-resistant HSV infection. In the present, edible algae are supplied as a healthy food because algae contain high nutrition and have many active compounds that are beneficial to health. Therefore, the algal extract can be used as an alternative agent for the treatment of HSV infection. The purpose of this research was to investigate the inhibitory effect of the algal polysaccharide extracts from *Ulva reticulata* against herpes simplex virus type 1 and type 2 infection in Vero cell. The structure of polysaccharide was analyzed by FT-IR technique. The algal polysaccharide extract from *U. reticulata* represents S=O and C–O–S of sulfate group. Besides, the toxicity of algal polysaccharide extract on Vero cell was evaluated by MTT assay. The algal polysaccharide extract from *U. reticulata* showed low toxicity on the cells with 50% cytotoxic concentration was greater than 5000 µg/mL. Inhibition of HSV infection was determined on Vero cell using plaque reduction assay. The results indicated that the algal polysaccharide extracts from *U. reticulata* showed antiviral activity against HSV-1 upon treatment before, meanwhile and after viral adsorption with 50% effective concentration of 2,525.90, 58.32 and 263.95 µg/mL, respectively. Also, the algal polysaccharide extracts from *U. reticulata* showed antiviral activity against HSV-2 upon treatment before, meanwhile and after viral adsorption with EC_{50} of 163.26, 9.70 and 527.28 µg/mL, respectively. Besides, HSV-1 and HSV-2 viral particles were inactivated by the polysaccharide extract from *U. reticulata*. In summary, the polysaccharide extract from *U. reticulata* could protect Vero cell from HSV infection, inhibit HSV infection and inactivate HSV particles. Therefore, it will be useful to apply *U. reticulata* polysaccharide extract as a potential anti-HSV agent.

Keywords: *Algal polysaccharide, Green algae, Herpes simplex virus, Ulva reticulata*

1. Introduction

Herpes simplex virus is categorized as the group I double-stranded DNA virus, and they are a member of the *Herpesviridae* family (Chayavichitsilp et al., 2009). Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are pervasive human pathogens that cause localized skin infection. HSV-1 infection can cause herpes labialis, whereas HSV-2 infection can cause herpes genitalist (Crimi et al., 2019). HSV-1 is transmitted primarily through direct contact whereas HSV-2 infection is from sexual contact including exposure to mucous membrane that have lesions or infection from mucosal secretion. Moreover, HSV-2 can cause horizontal and vertical transmissions meanwhile asymptomatic shedding (Koelle & Corey, 2008).

Nucleoside analogues such as acyclovir, valacyclovir, penciclovir and famciclovir are standard therapies for the management of HSV infections (Emmert, 2000). Moreover, the epidemiology of HSV infection and drug-resistant HSV are increased. Drug resistant HSV is related to viral thymidine kinase and viral DNA polymerase genes mutation. The mutant viruses do not respond to commercial drug treatment (Piret & Boivin, 2011). In the present, it is interesting to use natural substances for the remedy of herpes simplex virus infection.

Several natural and chemical synthetic sulfated anion-polymers inhibit the multiplication of various mammalian pathogenic viruses. The antiviral activity of sulfated anion-polymers including the sulfated algal polysaccharides was described that negatively charged molecules of sulfated anion-polymers interfered the virus infection by interacting with the positive charges on the viral particles or on the host cell surface. Therefore, these interactions prevented the penetration of the virus into the host cells (Huleihel et al., 2001).

Marine algae are the main source of sulfated polysaccharide. The sulfated polysaccharide structure varies according to the algal species. The mostly sulfated polysaccharide found in marine algae is fucoidan and

laminarans of brown algae (Phaeophyceae), carrageenan of red algae (Rhodophyceae) and ulvan of green algae (Chlorophyceae) (Wijesekara, Pangestuti, & Kim, 2011). Moreover, the sulfated polysaccharide demonstrates several physicochemical characteristics and various biological attribute of potential interest for food, nutritional supplements, agricultural, cosmeceutical and pharmaceutical applications. Furthermore, the sulfated polysaccharide was represented special applications such as anticoagulant, antivirus, antioxidation, anticancer and immunomodulatory activities (Jung et al., 2002; Damonte, Matulewicz, & Cerezo, 2004; Leiro et al., 2007).

2. Objectives

The purpose of this research was to study the chemical composition and the chemical characteristics of the polysaccharide extract from *U. reticulata* and to investigate the inhibitory effect of the algal polysaccharide extract against herpes simplex virus infection.

3. Materials and Methods

3.1 Algal polysaccharide extraction

Specimens of *Ulva reticulata* was collected from Rusamilae Sub-District, Mueang Pattani District, Pattani Province, Thailand. The algal polysaccharide was obtained using a high-temperature water extraction method. Dried *U. reticulata* was boiled with distilled water in water bath at 98 °C for 1 hour. After cooling, the solution was filtrated and concentrated by rotary evaporator. The extract was precipitated by 95% Ethanol at 4 °C for overnight. After that, the precipitate was centrifugated and lyophilized to obtain algal polysaccharide from the extract (Paradossi et al., 1999).

3.2 Analysis of algal polysaccharide biochemical composition

The algal polysaccharide extract was analyzed for total carbohydrate content, protein content, sulfate content and other functional groups of algal polysaccharide structure. The total carbohydrate content was measured by a phenol-sulfuric acid assay using D-glucose solution as a standard (Dubois et al., 1956). The protein content was determined by the Lowry assay using bovine serum albumin solution as a standard (Lowry et al., 1951). The sulfate content was estimated after acid hydrolysis of the polysaccharides, according to the sulfate turbidity method using potassium sulfate solution as a standard (Dodgson & Price, 1962). The structural characteristics and the functional groups of the algal polysaccharide was analyzed by Fourier-transform infrared (FT-IR) spectroscopy using a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, USA) with transmittance mode. (Fernando et al., 2017).

3.3 Cell line and viruses

Vero cell from African green monkey kidney cell was cultured in a growth medium, Dulbecco's modified Eagle medium; DMEM (Gibco, UK) supplemented with 10% of inactivated fetal bovine serum, FBS (Gibco, UK), 100 ug/mL of streptomycin and 100 U/mL of penicillin (Gibco, UK). The cell was grown until confluence in humidified 5% CO_2 atmosphere at 37 °C. The DMEM containing 2% FBS is maintenance medium, and it is used for propagation of herpes simplex virus. The herpes simplex virus with standard strains was used in this study. The herpes simplex virus type 1 strain F (HSV-1F) and herpes simplex virus type 2 strain G (HSV-2G) were propagated on Vero cells. The titers of standard viruses were quantified by plaque titration assay.

3.4 Cytotoxicity Assay

Cytotoxicity test defines as a concentration of algal polysaccharide extract that can be used in subsequent antiviral studies. Vero cell was exposed to different concentrations of the polysaccharide extract in DMEM. The treated plate was incubated at 37°C in a 5% CO_2 incubator for 72 hours. Cell viability test was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, the 5 mg/mL of MTT reagent (Bio Basic Inc., Canada) was added to each well and incubated at 37°C for 4 hours. After that, the MTT-formazan product was dissolved by dimethyl sulfoxide, and the color of the formazan solution was detected using an ELISA microplate reader. The optical density of the formazan solution was measured at 540 nm with a reference wavelength of 630 nm. The percentage of cell viability was calculated

from the ratio between the absorbance value of treated cells and the absorbance value of untreated cells. Moreover, the 50% cytotoxic concentration (CC_{50}) represented the concentration that the extract reduced the cell viability by 50% (Mosmann, 1983).

3.5 Antiviral assay

Antiviral activities were evaluated by plaque reduction assay with three mechanisms, including antiviral activity upon treatment early, meanwhile and after viral adsorption.

The antiviral activity upon treatment before viral adsorption was studied. Vero cells monolayer grown in 24-well plate was treated with the non-toxic concentration of algal polysaccharide extract and incubated at 25°C for 1 hour. After incubation, the treated cells were washed once with phosphate-buffered saline solution. Then, the Vero cells were infected with HSV at the titers of 1×10^2 PFU/mL.

The antiviral activity upon treatment; meanwhile, viral adsorption was also investigated. The non-toxic concentrations of algal polysaccharide extract and HSV at the titers of 1×10^2 PFU/mL were inoculated onto Vero cells monolayer that was grown in 24-well plate. The mixtures were incubated at 25°C for 1 hour. After incubation, the infected cells were washed once with phosphate-buffered saline solution.

The antiviral activity upon treatment after viral adsorption was performed. Vero cells monolayer grown in 24-well plate was infected with HSV at the titers of 1×10^2 PFU/mL for 1 hour. The residual inoculum was eliminated, and the infected cells were washed once with phosphate-buffered saline solution. Then, the infected cells were treated with the non-toxic concentrations of algal polysaccharide extract comparing to antiviral agent, acyclovir (Sigma-Aldrich, USA).

After viral infection and treatment in all antiviral assays, the growth medium contained 0.5% carboxymethyl cellulose was added to the wells for viral plaque forming. The infected cells were further incubated at 37 °C in a 5% CO_2 incubator for 72 hours. After incubation, viral plaques were stained by 0.1% crystal violet in 0.5 % ethanol. Percentage of viral inhibition was calculated from the ratio between the amount of virus that inactivated by the algal polysaccharide extract and the amount of virus control. Moreover, the 50% effective concentration (EC_{50}) that demonstrated the concentration that the algal polysaccharide extract incur the viral inhibition by 50% was calculated. The selective index (SI) was calculated from the ratio between the CC_{50} and the EC_{50} (Deethae et al., 2018).

3.6 Virucidal assay

The virucidal assay elucidates as inactivation of virus particles by the algal polysaccharide extract. A suspension of HSV at the titers of 1×10^4 were mixed with an equivalent volume of the non-toxic concentration of algal polysaccharide extract and incubated at room temperature for 1, 2, 3, and 4 hours. After that, the virus titers were quantified by plaque titration assay.

3.7 Statistical analysis

The statistical analyses were performed using one-way ANOVA analysis of variance by IBM SPSS Statistics 20 software (IBM Corp., USA). The significances among all groups were determined by Tukey's honestly significant difference (HSD) post hoc test. P-values of less than 0.05 ($p < 0.05$) were considered as significant. The EC_{50} values were estimated using probit analysis by PriProbitNM 1.63 software (Kyoto University, Japan).

4. Results and Discussion

Yield and chemical content of the polysaccharide extract from *U. reticulata* in the present study were shown in Table 1. The yield after extraction of crude algal polysaccharide extract was 17.48%, which was higher than *U. pertusa* (14.2%), *U. intestinalis* (12.0%), and *U. clathrate* (7.7%) (Hernández-Garibay, Zertuche-González, & Pacheco-Ruíz, 2011). The analysis of the chemical content of algal polysaccharide extract showed that carbohydrate, protein and sulfate were 50.60, 15.25 and 3.25%, respectively. The total carbohydrate content of the polysaccharide extract from *U. reticulata* was related to carbohydrate content in *U. arasakii* (54.9%), *U. linza* (51.0%), and *U. rigida* (48.3%). Also, the protein content of the polysaccharide

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extract from *U. reticulata* related to protein content in *U. rigida* (10.0%), *U. compressa* (12.0%), and *U. clathrata* (8.2%). In contrast, the polysaccharide extract from *U. reticulata* demonstrated that sulfate content was lower than that found in *U. compressa* (12.9), *U. scandinavica* (13.1), and *U. gigantea* (11.9%) (Lai, Li, & Li, 1994; Lahaye et al., 1999; Zhang et al., 2013; Gadenne et al., 2015). The yield of crude algal polysaccharide extract and chemical content including carbohydrate, protein and sulfate were different in each algal extract and depended on species, nutritional and cultural environment.

Table 1 Yield and chemical content of algal polysaccharide extract from *U. reticulata*

	Yield	Total carbohydrate	Protein	Sulfate
Chemical content (% w/w)	17.48	50.60	15.25	3.25

The FT-IR spectrum is presented in Figure 1. The result was comparable with the previous study for algal polysaccharide extract from *U. clathrate*, *U. intestinalis*, *U. pertusa* and *U. rigida* (Pengzhan et al., 2003; Tako et al., 2015; Tabarsa et al., 2018). A characteristic band at 3279.8 cm^{-1} corresponded to the vibrations of -OH stretching groups and a band at 2930.4 cm^{-1} corresponded to the vibration of -CH stretching groups. The strong transmission at 1607.3 cm^{-1} indicated the vibration of C=O stretching that related to uronic acid. The signals at 1035.6 cm^{-1} attributed to the vibration of the C-O-C bridge of the glucosides and sugar ring. Specially, bands at 1229.1 cm^{-1} and 829.1 cm^{-1} attributed to vibration of S-O stretching of sulfated groups and vibration of C-O-S stretching of sulfate ester substitutions, respectively. These results correlated with the presence of sulfate content (3.25%) in the polysaccharide structure.

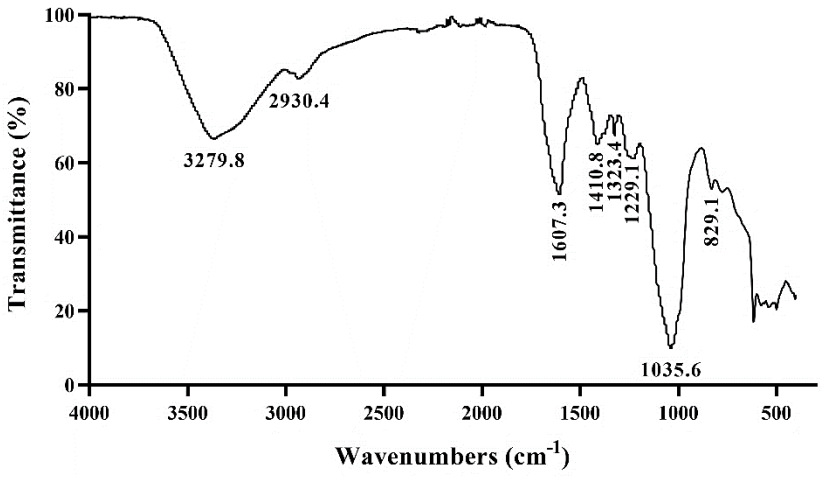

Figure 1 Infrared transmittance spectrum of an algal polysaccharide extract from *U. reticulata*

The algal polysaccharide extract from *U. reticulata* was evaluated *in vitro* for cytotoxic activity on Vero cells using concentrations of 156.25, 312.5, 625, 1250, 2500 and 5000 µg/mL and compared to the cell control (CC) and the vehicle control (VC). The absorbance of cell control was considered as a cell viability of 100%. The results in Figure 2 showed that the algal polysaccharide extract showed low toxicity on Vero cells. The 50% cytotoxic concentration (CC_{50}) value was greater than 5,000 µg/mL. This result was accordant with the previous studies that evaluated the cytotoxicity of other algal polysaccharide extract from *Ulva* sp. in cell culture such as *U. armoricana*, *U. intestinalis* and *U. pertusa* (Matloub et al., 2015; Hardouin et al., 2016; Song et al., 2016). Moreover, the algal polysaccharide extract represented lower toxicity than antiviral drugs that were approved by the FDA (Aguilar-Briseño et al., 2015).

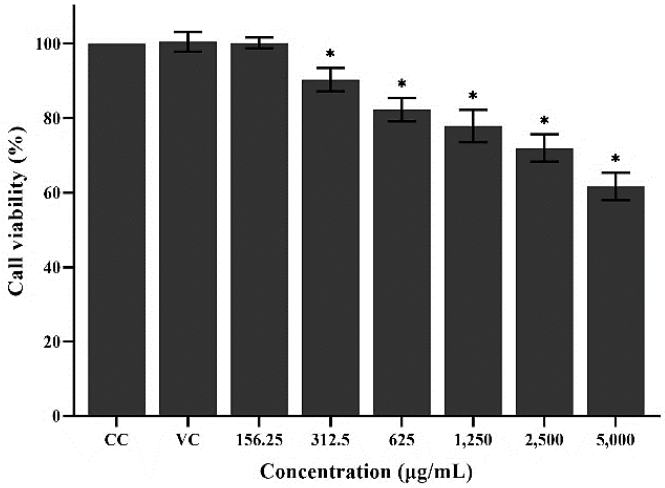

Figure 2 Cytotoxic effects of an algal polysaccharide extract from *U. reticulata* on Vero cells. Bar graph and error bars are based on mean ± SD of three experiments. The related * on the graph shows the significant differences values from cell control ($p < 0.05$).

The antiviral activity of the algal polysaccharide extract from *U. reticulata* against HSV was determined by plaque reduction assay. Vero cells and infected cell were treated with different concentrations of the algal polysaccharide extract. Besides, the antiviral efficiency of the algal polysaccharide extract against HSV was demonstrated. The algal polysaccharide extract at the concentration of 5,000 µg/mL showed potential inhibitory effects against HSV-1 and HSV-2 when the virus was treated before viral adsorption to Vero cells, with inhibition percentages of 66.96 ± 2.26% and 100%, respectively (Figure 3). Furthermore, the algal polysaccharide extract showed efficient inhibitory effects against both of HSV-1 and HSV-2 when the virus was treated while viral adsorption to Vero cells, with a percentage of inhibition by 100% at 5,000 µg/mL (Figure 4). Additionally, the algal polysaccharide extract at the concentration of 5,000 µg/mL also showed high potential inhibitory effects against both of HSV-1 and HSV-2 when the virus was treated after viral adsorption to Vero cells, with a percentage of inhibition by 100% (Figure 5).

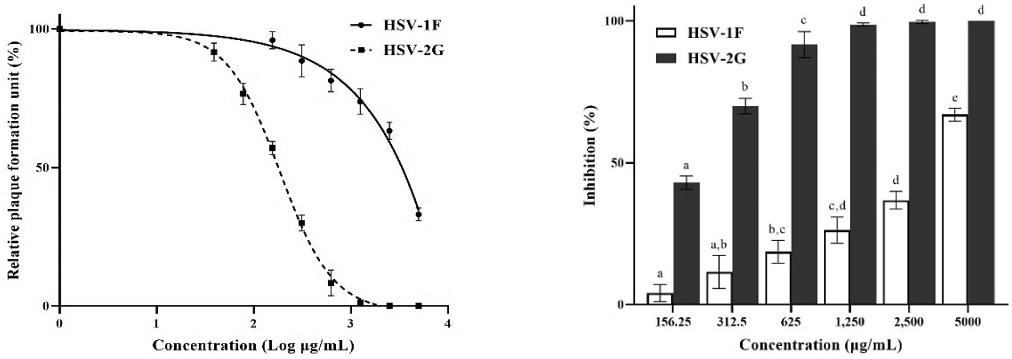

Figure 3 The plaque reduction (left) and inhibition (right) of HSV infection by algal polysaccharide extract from *U. reticulata* upon treatment before viral adsorption on Vero cell. Graphs and error bars are based on the mean ± SD of three experiments. The related alphabets on the graph show the significant differences values from each test group ($p < 0.05$).

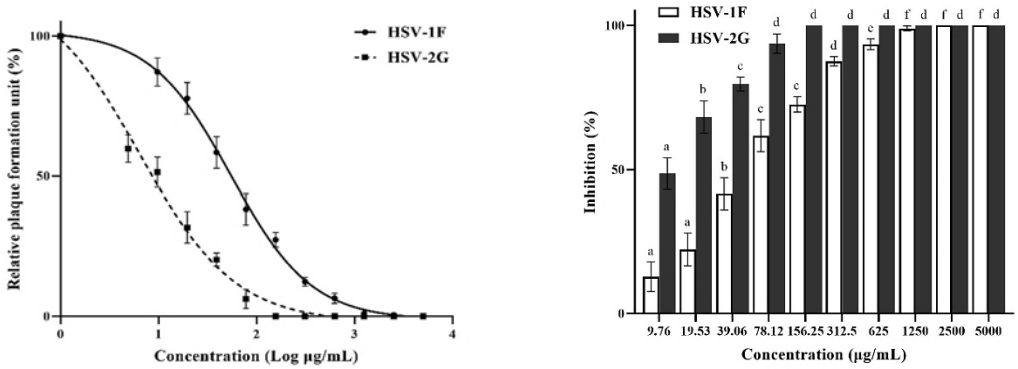

Figure 4 The plaque reduction (left) and inhibition (right) of HSV infection by algal polysaccharide extract from *U. reticulata* upon treatment meanwhile viral adsorption on Vero cell. Graphs and error bars are based on the mean ± SD of three experiments. The related alphabets on the graph show the significant differences values from each test group ($p < 0.05$).

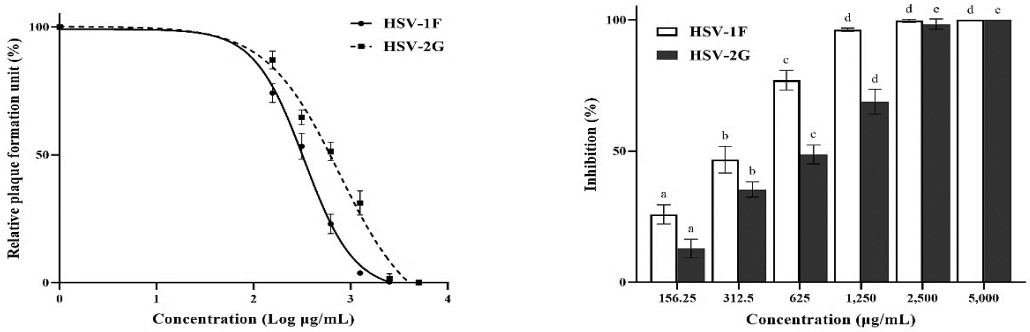

Figure 5 The plaque reduction (left) and inhibition (right) of HSV activity by algal polysaccharide extract from *U. reticulata* upon treatment after viral adsorption on Vero cell. Graphs and error bars are based on the mean ± SD of three experiments. The related alphabets on the graph show the significant differences values from each test group ($p < 0.05$).

The antiviral activity of the algal polysaccharide extract from *U. reticulata* against HSV upon treatment before viral adsorption on Vero cell corresponded to the algal polysaccharide extract that inhibited HSV infection by protection of Vero cell form viral infection. Similarly, the antiviral activity of the algal polysaccharide extract from *U. reticulata* against HSV upon treatment meanwhile viral adsorption on Vero cell related to the algal polysaccharide extract that blocked HSV infection by interfering the viral infection step to Vero cell. In contrast, the antiviral activity of the algal polysaccharide extract from *U. reticulata* against HSV upon treatment after viral adsorption on Vero cell associated with the algal polysaccharide extract that disturbed the viral multiplication steps after viral adsorption such as viral penetration, viral replication, viral gene expression, viral protein synthesis and viral assembly.

The 50% effective concentration (EC_{50}) values of the acyclovir against HSV-1 and HSV-2 when treatment after viral adsorption on Vero cell were 1.40 and 4.69, respectively. In these antiviral studies, The EC_{50} values of algal polysaccharide extract from *U. reticulata* against HSV-1 when treatment before, meanwhile and after viral adsorption on Vero cell were 2,525.90, 58.32 and 263.95 µg/mL, respectively. Then, the selective indices (SI) were greater than 1.98, 85.73 and 18.94, respectively. In addition, the EC_{50} values of the algal polysaccharide extract from *U. reticulata* against HSV-2 when treatment before, meanwhile and after viral adsorption on Vero cell were 163.26, 9.70 and 527.28 µg/mL, so the SI were greater than 30.63, 515.46 and 9.48, respectively (Table 2). Similar results were obtained previously when treatment

green algal polysaccharide, sulfated polysaccharide and ulvan against enveloped viruses (Lee et al., 2004; Lopes et al., 2017).

Table 2 Inhibition of HSV by algal polysaccharide extract from *U. reticulata* upon treatment early, meanwhile and after viral adsorption on Vero cell.

Viruses	Early viral adsorption		Meanwhile viral adsorption		After viral adsorption	
	EC_{50} (µg/mL)	SI	EC_{50} (µg/mL)	SI	EC_{50} (µg/mL)	SI
HSV-1	2,525.90	> 1.98	58.32	> 85.73	263.95	> 18.94
HSV-2	163.26	> 30.63	9.70	> 515.46	527.28	> 9.48

The inactivation of HSV-1 and HSV-2 viral particles by the algal polysaccharide extract from *U. reticulata* was quantified using plaque titration assay from treatment at 1, 2, 3 and 4 hours compared with the virus control. The inactivation effect on HSV viral particles was distinguished to be the increase when the incubation period of the algal polysaccharide extract and the virus were escalated (Figure 6). The result assessed that the algal polysaccharide extract at the concentration of 5,000 µg/mL reduced the plaque number of HSV-1 after treatment by 80.17 ± 5.01% at 3 hours after treatment and 85.37 ± 6.61% at 4 hours after treatment. However, the inactivation of HSV-1 at 1 hour and 2 hours after treatment was not a significant reduction when comparing to virus control. On the contrary, the algal polysaccharide extract showed high potential to inhibit HSV-2 viral particles greater than 50% from the first hour. A reduction in the plaque number of HSV-2 presented after treatment at 1 hour by 56.64 ± 8.03%. Furthermore, a reduction in the plaque number at 2, 3 and 4 hours after the treatment also showed high efficiency with 88.83 ± 3.03, 97.29 ± 0.51 and 99.59 ± 0.27%, respectively (Figure 7).

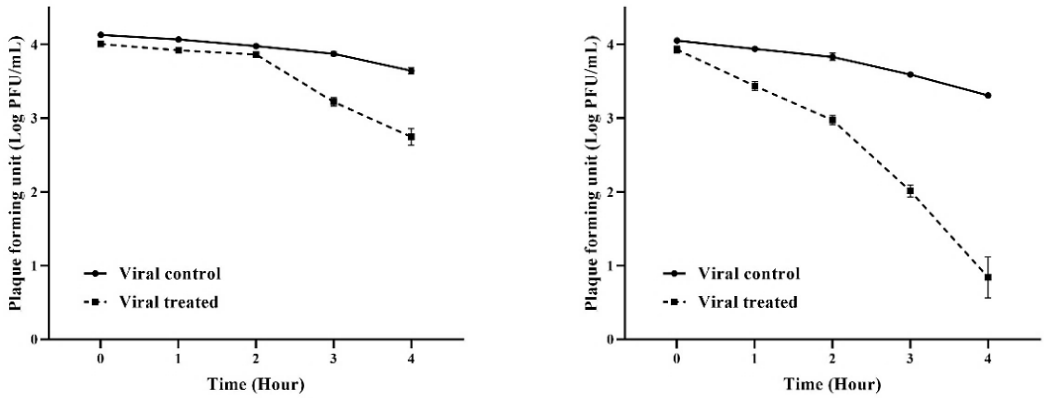

Figure 6 The virucidal activity of the algal polysaccharide extract from *U. reticulata* against HSV-1F (left) and HSV-2G (right) particles in comparison with the virus control.

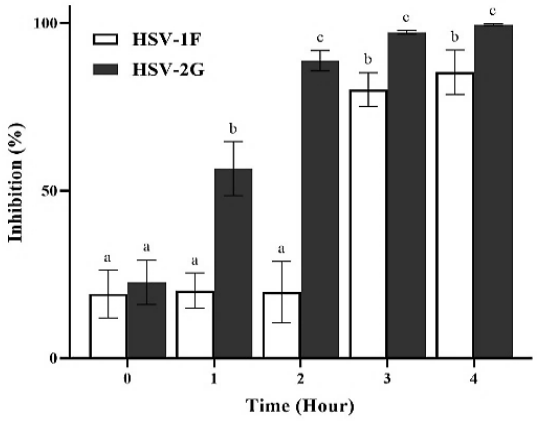

Figure 7 The inactivation of HSV particles upon treatment with the algal polysaccharide extracts from *U. reticulata* for 0, 1, 2, 3 and 4 hours. Bar graph and error bars are based on mean ± SD of three experiments. The related alphabets on the graph show the significant differences values from each test group ($p < 0.05$).

The results of this study indicated that algal polysaccharide extract from *U. reticulata* was sulfate contained polysaccharide. The structural characteristics demonstrated the functional group of the sulfate group and C=O group of uronic acid were similar to the ulvan analysis on the previous study. In accordance with the previous study, ulvan is the main component of the cell wall polysaccharide of *Ulva* sp. (Lahaye & Robic, 2007). Moreover, the chemical structure of ulvan correlated with its antiviral activity against enveloped viruses (Cunha & Grenha, 2016). Additionally, ulvan represented potency of antiviral activity against dengue virus, Japanese encephalitis virus, yellow fever virus, influenza virus, avian influenza virus and measles virus. Furthermore, ulvan demonstrated various biological activities, including anticoagulant, antithrombotic, antioxidant, antiviral, anticancer, antihyperlipidemic and immunomodulation activities (Kidgell et al., 2019). Therefore, it would be very useful to search and use natural compounds from *U. reticulata* algal extract that acted along with synthetic antiviral drugs on different targets of the HSV infection cycle.

5. Conclusion

Characterization of *U. reticulata* by chemical characteristics analysis demonstrated that the structure of the algal polysaccharide extract is a sulfated polysaccharide, which was similar to ulvan from *Ulva* sp. The algal polysaccharide extract showed low toxicity on Vero cell and high potential of anti-herpetic activity against both HSV-1 and HSV-2 infection upon treatment before, meanwhile, and after viral adsorption into Vero cells. Moreover, the algal polysaccharide extract demonstrated direct inactivating effect on HSV viral particles in the virucidal assay.

6. Acknowledgements

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