Antiviral and Virucidal activities of sulphated polysaccharides against Japanese Encephalitis Virus.

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1 **Abstract**

2 **Background:** Japanese encephalitis virus (JEV), a member of the family Flaviviridae, causes
3 severe neurological disorders in humans. JEV infections represent one of the most widely
4 spread mosquito-borne diseases, and therefore, it has been considered as an endemic disease.
5 An effective antiviral drug is still unavailable to treat JEV, and current drugs only provide
6 supportive treatment to alleviate the symptoms and stabilize patients' conditions. This study
7 was designed to evaluate the antiviral activity of the sulphated polysaccharides
8 "Carrageenan," a linear sulphated polysaccharide that is extracted from red edible seaweeds
9 against JEV replication in vitro. **Methods and Results:** Viral inactivation, attachment, and
10 post-infection assays were used to determine the mode of inhibition of Carrageenan. Virus
11 titers after each application were evaluated by plaque formation assay. MTT assay was used
12 to determine the 50% cytotoxic concentration (CC50), and ELISA-like cell-based assay and
13 immunostaining and immunostaining techniques were used to evaluate the 50% effective
14 concentration (EC50). This study showed that Carrageenan inhibited JEV at an EC50 of 15
15 µg/mL in a dose-dependent manner with CC50 more than 200 µg/mL in healthy human liver
16 cells (WRL68). The mode of inhibition assay showed that the antiviral effects of Carrageenan
17 are mainly due to their ability to inhibit the early stages of virus infection such as the viral
18 attachment and the cellular entry stages. **Conclusion:** Our investigation showed that
19 Carrageenan could be considered as a potent antiviral agent to JEV infection. Further
20 experimental and clinical studies are needed to investigate the potential applications of
21 Carrageenan for clinical intervention against JEV infection.

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23 **Keywords:** Japanese encephalitis virus, Antiviral activity, sulphated polysaccharides,
24 Carrageenan.

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1 Introduction

2 Flaviviruses infection has been disseminated across many countries in tropical countries [1].

3 Japanese Encephalitis virus (JEV) infections represent one of the most widely spread

4 mosquito-borne diseases, and therefore, it has been considered as an endemic disease,

5 predominantly occurs in South-East Asia and the Western Pacific regions [2, 3]. The

6 infection is mainly caused by the transmission of JEV to humans through mosquitoes from

7 the *Culex* genus, especially *Culex tritaeniorhynchus*. The symptom of JEV infection ranges

8 from mild to severe manifestation. Most infected individuals only experience mild symptoms

9 such as fever and headache. However, some (~ 1%) might develop severe clinical symptoms

10 such as high fever, spastic paralysis, and coma [1, 4]. There are approximately 44,000 new

11 cases of JEV with clinical symptoms per year with a 30% mortality rate, while 30-50 % of

12 survivors will suffer permanent neurological impairment [4, 5]. Although vaccines for JEV

13 are available, long term protective effects is still not known, and treatment usually requires at

14 least one booster dose after 12 months of vaccination. This requirement is highly

15 inconvenient and usually leads to low public compliance [6]. An effective antiviral drug is

16 still unavailable to treat JEV, and current drugs only provide supportive treatment to alleviate

17 the symptoms and stabilize patients' conditions [3]. As such, the development of active

18 antiviral agents for JEV is necessary to address the unmet medical needs.

19 Natural compounds show considerable antiviral activities through direct binding with viral

20 proteins or cell membrane receptors [7, 8]. Carrageenan is sulphated high-molecular-weight

21 polysaccharides extracted from red edible seaweeds and widely used in the food industry.

22 Chemically, Carrageenan is made up of repeating galactose units joined by alternating α -1,3

23 and β -1,4 glycosidic linkages. Sulphated polysaccharide was found to be very potent and

24 selective inhibitors of Human Immunodeficiency Virus-1 (HIV-1) properties [9, 10]. These

25 effects are believed to be the result of direct binding to HIV glycoprotein 120 (gp120) on the

1 surface of the viral particle that, in turn, inhibit the entry of HIV-1 into human cells [9]. In
2 this study, we report the antiviral properties and cytotoxicity of Carrageenan towards JEV on
3 WRL68 cells. The mechanisms of the inhibitory effects of Carrageenan were also
4 investigated in order to get a better understanding of Carrageenan as a potential antiviral
5 agent for combating JEV infections.

6 **Materials and Methods**

7 **Virus and cells**

8 The stocks of JEV were prepared in C6/36 cells, and viral titers were determined *via* plaque
9 assay using Vero cells (ATCC; Rockville, MD, USA). The cells were cultured in complete
10 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum
11 (FBS) as a growth medium or 2% FBS as maintenance medium. Iota Carrageenan and
12 heparin were purchased from Sigma (Sigma Aldrich) and dissolved in 5% DMSO. The final
13 concentration of DMSO was lower than 1% in all cell culture-based assays.

14 **Cytotoxicity assay**

15 Healthy liver cells (WRL68, 1×10^4 cells per well of a 96-well plate) were treated with
16 increasing concentrations (12.5, 25, 50, 100, and 200 $\mu\text{g/ml}$) of Carrageenan or heparin as a
17 positive control for 72 h. The cytotoxic effect of the test compound was determined based on
18 MTT (3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide) assay, as previously
19 described [11].

20 **Virus quantification by plaque formation assay**

21 A 10-fold serial dilution of culture supernatant of JEV infected cells was added to fresh Vero
22 cells grown in 6-well plates (1.2×10^6 cells) and incubated for 1 h at 37°C. The cells were
23 overlaid with DMEM containing 1.1% methylcellulose. Viral plaques were stained with
24 crystal violet dye after a 5-day incubation period. Virus titers were calculated according to
25 the following formula: Titer (p.f.u./ml) = number of plaques $\times$ volume of the diluted virus

1 added to the well $\times$ dilution factor of the virus used to infect the well in which the plaques
2 were enumerated [12].

3 **Evaluation of antiviral activities of Carrageenan on WRL68 cell line**

4 The cells were seeded into 24-well plates (1×10^5 cells per well) for 24 h at 37°C and 5%
5 CO₂. JEV culture supernatant was separately added to the cells at an M.O.I. of 1, followed by
6 incubation for two h with gentle shaking every 15 min to achieve optimal virus-to-cell
7 contact. The cells were washed twice with phosphate-buffered saline (PBS) after removing
8 the virus culture supernatant. Subsequently, the new complete DMEM containing 25 µg/ml
9 of Carrageenan or heparin was added to the cultures and further incubated for 72 h. Viral
10 titers were evaluated using plaque formation assays.

11 **Viral inactivation assay**

12 Virus inactivation assay was performed to assess the ability of Carrageenan to inactivate JEV,
13 and prevent subsequent infections directly. WRL68 cells were seeded in 24-well plates ($1 \times$
14 10^5 cells/well) for 24 h. The test compound (25 µg/ml) was mixed with the virus culture
15 supernatant (M.O.I of 2) and incubated at 37°C for 1 h and then inoculated onto the WRL68
16 cells with gentle shaking every 15 min for 2 h. The virus and test compound mixture were
17 then removed, and the cells were washed three times with PBS to remove any residual virus.
18 Then, fresh complete DMEM was added, and the cultures were further incubated for 72 h at
19 37°C supplemented with 5% CO₂. The viral titers of DENV2 and JEV suspension were then
20 determined via plaque formation assays.

21 **Viral attachment assay**

22 This assay was performed to show the ability of Carrageenan to inhibit DENV2 and JEV
23 entry into host cells. WRL68 cells were grown in 24-well plates (1×10^5 cells/well) for 24 h.
24 Cell culture media were removed and the cells were then washed three times with PBS. New
25 media containing virus and the test compound (25 µg/ml) mixture were separately added and

1 the cells were incubated for one h at 4°C. Subsequently, the media was removed and the cells
2 were washed extensively with cold PBS to remove the unabsorbed virus. The cells were
3 overlaid with DMEM containing 1.1% methylcellulose and incubated for five days. Viral
4 titers were quantified by counting infectious centers.

5 **Post-infection treatment**

6 The cells were seeded in 24-well plates (1×10^5 cells/well) for 24 h. Cell culture media were
7 removed and the cells were then washed three times with PBS. New media containing virus
8 (M.O.I of 2) was added and incubated at 37°C with gentle shaking every 15 min for two h.
9 The virus was then removed by washing the cells twice with PBS followed by the addition of
10 new complete DMEM containing (25 µg/ml) of the test compound. The cells were then
11 incubated for 72 h. The viral titers of JEV were then determined by plaque formation assays.

12 **Immunofluorescence assay**

13 Vero cells grown on coverslips were infected with JEV (M.O.I. of 2) in the presence or
14 absence of Carrageenan (10, 20 and 30 µg/ml). At 72 h post-infection, cell monolayers were
15 washed with cold PBS and fixed in methanol for 15 min at -20 °C for cytoplasmic
16 immunofluorescence. Indirect staining was carried out by using anti-JEV mouse antibodies
17 and fluorescein (FITC)-labeled goat anti-mouse IgG [13].

18 **Statistical analysis**

19 All assays were performed in triplicates, and the statistical analyses were performed using
20 GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). P values <0.05 were
21 considered significant. The error bars are expressed as $\pm$ SD.

22 **Results**

23 **Determination of potential cytotoxicity of Carrageenan**

24 The potential cytotoxicity effect of Carrageenan was evaluated using WRL68 cells and
25 heparin was employed as a positive control. The 50% cytotoxic concentration (CC50) was

1 higher than 200 $\mu\text{g/ml}$ for both Carrageenan and heparin The maximal non-toxic dose
 2 (MNTD) value was approximately 25 $\mu\text{g/ml}$ and showed at least 95% cell viability.
 3 Following this, all subsequent in vitro cell culture experiments were carried out using doses
 4 of less than 25 $\mu\text{g/ml}$ of the potential compounds (Fig. 1A and 1B).
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8 **Figure 1: Cell viability of WRL68 cells after treatment with carrageenan and the**
 9 **positive control, heparin and test the antiviral activity by plaque assay. (A) The CC_{50}**
 10 **value concentration was higher than 200 $\mu\text{g/ml}$ for carrageenan (B) The CC_{50} value**
 11 **concentration was higher than 200 $\mu\text{g/ml}$ for heparin. The MNTD value was approximately at**

25 µg/ml for both compounds that showed at least 95% cell viability. (C) Determination of viral inhibition by the test compounds using plaque formation assay. The cells were pre-treated separately with 25 µg/ml of each test compounds and infected with JEV at MOI of 1. after 72 hrs, a 10-fold serial dilution of culture supernatant of JEV- infected cells was added to fresh Vero cells grown in 6-well plates (1.2×10^6 cells). Viral plaques were stained with crystal violet dye after 5 days of incubation. MNTD=Maximal non-toxic dose.

Carrageenan inhibited JEV entry into host cells

Liver cell lines (WRL68) showed normal morphology before infection with JEV virus. After JEV-infection, the cells showed various cytopathic effects (CPE). Treatment with carrageenan or heparin showed approximately normal monolayer sheets with lessen CPE (Fig. 2). Then, the antiviral activity of carrageenan against JEV was investigated by plaque formation assay. Our results showed that carrageenan acts by inhibiting viral entry into WRL68 cells, and the inhibitory strength of carrageenan towards JEV was at a similar level as heparin. The results obtained from the direct virus inactivation assay (Fig. 3A) demonstrated that carrageenan had a significant inhibitory effect against JEV (6.7 ± 2.2 p.f.u./ml) compared to untreated cells (19.6 ± 1.8 p.f.u./ml) while no significant difference was observed for heparin as a positive control (7.2 ± 2.6 p.f.u./ml) compared to carrageenan (t-test, $p < 0.001$). Similarly, carrageenan also showed significant inhibitory effects at the viral attachment stage with 4.7 ± 3.1 p.f.u./ml inhibition for JEV infection compared to untreated cells (18.2 ± 2.4 p.f.u./ml) and no significant difference compared to heparin (3.8 ± 2.9 p.f.u./ml) as a positive control (t-test, $P < 0.01$) as presented in Figure 3B. We then incubated the WRL68 cells with JEV at 37°C for two h and subsequently treated the cells with the test compounds to determine their inhibitory effects against viral replication post-infection. The post-infection treatment showed that carrageenan reduced viral titer to 13.4 ± 2.2 p.f.u./ml compared to untreated cells (21.7 ± 2.6 p.f.u./ml). For post-infection assay heparin had showed similar inhibition towards JEV (13.2 ± 3.8 p.f.u./ml) infection (t-test, $P < 0.05$) (Fig. 3C). Moreover, treatment with carrageenan also showed significant protective effects which

- 1 retained the viability of infected cells to approximately 80% (t-test, $p < 0.01$) compared to
- 2 untreated cells that showed cell viability of 40% (Fig. 3D)

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Figure 2. Morphological changes of JEV-infected cells treated with Carrageenan or heparin. Uninfected cells showed normal cell morphology of normal liver cell lines WRL68. The untreated cells morphology post-infection showed various cytopathic effects (CPE). Treatment with carrageenan or heparin showed approximately normal monolayer sheet with lessen CPE. Cell morphology was examined using an inverted microscope at 200× magnification.

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Figure 3: Mode of inhibition of carrageenan against JEV replication. (A) Viral inactivation assay showed significant viral inhibition when viral particles were treated with carrageenan compared to untreated cells. Carrageenan showed similar inactivation potential compared to the positive control heparin. (B) Viral attachment assay showed that carrageenan significantly inhibited virus attachment to target cells that was similar to heparin.

(C) Post-infection treatment showed that the inhibitory effect of carrageenan against JEV was significant but at lower extent compared to the antiviral activity against viral entry (t-test $p < 0.01$). Moreover, treatment with carrageenan also showed significant protective effects which retained the viability of infected cells to more than 75% (t-test, $p < 0.01$).

Carrageenan inhibited JEV in a dose-dependent manner

Our investigations showed Carrageenan increased inhibition potentials against JEV; we next characterized the antiviral activity of Carrageenan against JEV using an ELISA-like cell-based assay (Fig. 4). Our results demonstrated that Carrageenan inhibited JEV replication in WRL68 cells in a dose-dependent manner. The 50% effective concentration (EC_{50}) for Carrageenan was approximately 15 $\mu\text{g/ml}$, which is similar to the positive control heparin (Fig. 4). The immunostaining experiment showed that the concentration level of virus particles was considerably decreased after applying increased concentrations from 10 $\mu\text{g/ml}$ to 30 $\mu\text{g/ml}$ of Carrageenan (Fig. 5).

Figure 4: Viral inhibition of JEV in WRL68 cells by carrageenan. Carrageenan inhibited JEV replication in WRL68 cells in a dose-dependent manner. The EC_{50} value for carrageenan was approximately 15 $\mu\text{g/ml}$ which is approximately similar to the positive control heparin.

Figure 5. The effect of carrageenan in reducing viral particles. The cells were grown on cover slips in 6-well plates and infected with JEV. The cells were then treated with increasing concentrations of carrageenan (A, 0 µg/ml; B, 10 µg/ml; C 20 µg/ml; D, 30 µg/ml) for 72 h. The fixed cells were treated with anti-JEV mouse antibodies and then treated with fluorescein (FITC)-labeled goat anti-mouse IgG. The results showed that the inhibitory effects were dependent on increasing concentrations of carrageenan.

Discussion

In response to the urgent need for effective anti-JEV drugs, this study was proposed to identify and evaluate new inhibitors for the treatment of JEV infections. Best of our knowledge, the potential of the use of Carrageenan to inhibit JEV has not been previously investigated. Our studies have indeed shown that Carrageenan demonstrated minimal cytotoxicity while exhibiting significant inhibitory effects towards JEV ($EC_{50} = 15 \mu\text{g/ml}$) infections in the normal human liver cell (WRL68), suggesting the potential of Carrageenan

1 as an antiviral agent to combat JEV infections. In all experiments, Heparin was used as a
2 positive control.

3 In this study, three assays were performed to elucidate the antiviral mechanisms of
4 Carrageenan. Our results indicate that Carrageenan inhibits JEV mainly through its direct
5 virucidal effects and interference within the early stages of viral infection, such as viral
6 attachment and cellular entry. We postulated that the direct antiviral activity of Carrageenan
7 might be due to their ability to interact with the virus envelop glycoprotein, particularly the
8 flavivirus E protein, which is responsible for JEV adsorption through the host membrane [18,
9 19]. Besides, Carrageenan also showed a significant inhibitory effect in the viral attachment
10 assay. In contrast to the direct virucidal activity, the prevention of viral attachment might be
11 attributed to the ability of Carrageenan to compete with both viruses for the cellular receptors
12 (i.e. heparin sulphate) required for cellular entry and subsequent infections [18, 20].

13 On the other hand, in the post-infection assay, Carrageenan showed lower but significant
14 antiviral activities towards JEV infection. It has been found that heparan sulphate (HS) is
15 involved in the first interaction with E glycoprotein to initiate a flavivirus multiplication
16 cycle in different types of vertebrate cells [21]. HS is a member of highly sulphated
17 glycosaminoglycans (GAGs), which is very abundant on the surface of most mammalian
18 cells and serves as a receptor for many microbial agents, including bacteria, parasites, and
19 viruses [22]. For DENV, the interaction with HS is unusual, owing to its specificity for a
20 highly sulphated form of HS [23]. Therefore, the polysaccharide,
21 curdlan sulphate showed a potential antiviral activity through interfering with the cellular
22 entry of DENV [24].

23 Our studies also showed that Carrageenan exhibited differing levels of antiviral strength
24 towards JEV, in each of the assays that have been carried out. Although the underlying
25 reasons are still unclear, we suspect it may be due to the variability in the viral glycoproteins

1 and cellular receptors, which are crucial for each virus to establish infection. On the other
2 hand, Heparin has shown similar inhibitory activity in the post-infection assay. Although the
3 potential use of Carrageenan to inhibit JEV, there are still some limitations that have not been
4 addressed in this study. Researches on polysaccharides as antivirals has shown that the
5 antiviral properties of polysaccharides are highly dependent on the structural complexity (i.e.
6 number and position of the branches, branch length and chemical modifications of the
7 polysaccharides) [25, 26]. However, it is difficult for researchers to accurately characterize
8 and purify a particular type of polysaccharide from the extracts of natural sources, which also
9 remains as an inherent limitation in this study. To this end, further research is still needed to
10 elucidate the exact mechanism of inhibition by Carrageenan and its association with the JEV
11 virus replication cycle. Downstream studies with suitable animal models are perhaps required
12 to evaluate the reproducibility of the inhibitory effects of Carrageenan *in vivo*.
13 In conclusion, our study has successfully demonstrated the inhibition of JEV replication by
14 Carrageenan with minimal cytotoxicity effects. Carrageenan could potentially be a promising
15 broad-spectrum antiviral agent targeting the flavivirus family, and thus warrants further
16 investigation.

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