นิพนธ์ต้นฉบับ

การศึกษาฤทธิ์ต้านเชื้อไวรัสก่อโรคเริมของสาหร่ายสีเขียว *Chlorella* sp.

ยิ่งมณี ตระกูลพัว* และ ชมพูนุช สาตแฟง*

บทคัดย่อ

วัตถุประสงค์ : เพื่อศึกษาฤทธิ์ต้านเชื้อไวรัสก่อโรคเริมของสาหร่ายสีเขียว *Chlorella* sp.

วัสดุและวิธีการ : ทดสอบความเป็นพิษของน้ำกรองจากสาหร่ายสีเขียว *Chlorella* sp. กับ Vero cell จากนั้นนำน้ำกรองจากสาหร่ายในความเข้มข้นที่ไม่เป็นพิษต่อเซลล์เพาะเลี้ยงมาทดสอบผลต้านเชื้อไวรัสก่อโรคเริมชนิดที่ 1 และ 2 และทดสอบผลต่อการเพิ่มจำนวนของไวรัสทั้ง 2 ชนิด

ผลการทดลอง : พบว่าค่า CD_{50} ของน้ำกรองจากสาหร่ายสีเขียว *Chlorella* sp. ต่อ Vero cell เท่ากับ 338 µg/ml ผลการยับยั้งไวรัสสามารถตรวจสอบได้เมื่อทดสอบน้ำกรองจากสาหร่ายกับไวรัสขณะที่ไวรัสเกาะติดกับเซลล์ โดยเมื่อทดสอบกับน้ำกรองจากสาหร่ายที่ความเข้มข้นเท่ากับ 97.6 µg/ml ค่าการยับยั้งเชื้อ HSV-1 และ HSV-2 เท่ากับ 50.8 % และ 45.8 % ตามลำดับ อย่างไรก็ตามไม่สามารถตรวจพบผลการยับยั้งการติดเชื้อไวรัสเมื่อเติมน้ำกรอง จากสาหร่ายหลังจากไวรัสเกาะติดกับเซลล์ และไม่พบการยับยั้งการเพิ่มจำนวนของไวรัสเนื่องจากค่า log ของปริมาณไวรัส ที่ทดสอบไม่แตกต่างจากไวรัสควบคุม

สรุปผลการทดลอง : สาหร่ายสีเขียว *Chlorella* sp. สามารถยับยั้งการติดเชื้อไวรัสก่อโรคเริมได้โดยพบผลการยับยั้งเชื้อไวรัสขณะที่ไวรัสเกาะติดกับเซลล์ และพบการยับยั้งเชื้อไวรัสก่อโรคเริมชนิดที่ 1 มากกว่าชนิดที่ 2 **วารสารเทคนิคการแพทย์เชียงใหม่ 2548; 38: 179-184.**

คำหัส: การต้านเชื้อไวรัส, ไวรัสก่อโรคเริม, สาหร่ายสีเขียว, *Chlorella* sp.

* สาขาวิชาจุลชีววิทยา ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

Abstract : The study of anti-herpes simplex virus activity of green algae, *Chlorella* sp.

Tragoolpua, Y.* and Satafang, C*.

Objective: To study anti-herpes simplex virus (HSV) activity of green algae, *Chlorella* sp.

Materials and methods: Green algae, *Chlorella* sp. filtrate was tested for cytotoxicity on Vero cell. Then, the algal filtrate at nontoxic concentrations was further determined for inhibition of herpes simplex virus type 1 and type 2 infection, and also viral replication.

Results: CD_{50} of *Chlorella* sp. filtrate on Vero cell was 338 mg/ml. Antiviral activities were observed when the viruses were treated with algal filtrate at the same time of viral adsorption. Inhibition of HSV-1 and HSV-2 was 50.8 % and 45.8 % respectively after treatment with 97.6 mg/ml of *Chlorella* sp. filtrate. However, the inhibition of viral infection was not found when the algal filtrate was applied after viral adsorption, and inhibition of viral replication was not observed since the log of virus titer after treatment was not different from control virus.

Conclusion: Herpes simplex virus infection was inhibited by Green algae, *Chlorella* sp. Anti-HSV activities of the algae were observed at adsorption step and the activity against HSV-1 was higher than HSV-2. **Bull Chiang Mai Assoc Med Sci 2005; 38: 179-184.**

Key words: antivirus, herpes simplex virus, green algae, *Chlorella* sp.

* Microbiology Section, Department of Biology, Faculty of Science, Chiang Mai University

Introduction

Herpes simplex viruses are divided into 2 types. Both types can cause primary and recurrent infections. Treatment of herpes simplex virus infection has been performed using several antiviral agents. Acyclovir is nucleoside analogues, which is selectively incorporated into herpes simplex virus DNA and inhibited viral DNA polymerase and virus replication. It has proved effective against acute infection such as cold sores, eye infection and genital infection. However, it has no activity on latent infection and resistant mutants arise during long term treatment.[1]

Attempts to search for antiviral activity from algae were performed in several studies. Anti-herpes simplex virus activities have been reported from blue green algae[2,3], brown algae[4,5] and red algae.[6,7,8] Unicellular green algae, *Chlorella* sp. is a freshwater algae. It contains a very high amount of chlorophyll, which is essential for photosynthesis process. Several species of chlorella such as *Chlorella vulgaris* and *Chlorella pyrenoidosa* are commonly used in nutritional supplements. Moreover, chlorella has been shown to have activities on cancer treatment[9] and immune enhancement[10]. However, studies on anti-viral activities have not been performed. Therefore, anti-HSV activity of *Chlorella* sp. was investigated in this study.

Material and methods

Viruses, cell lines, green algae and antiviral agent

Herpes simplex virus type 1 and 2 (local isolates) were obtained from Assist. Prof. Dr. Wasna Sirirungsi, Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Chiang Mai University.

The viruses were propagated on Vero cell line, originating from African green monkey (*Cercopithecus aethiopes*) kidney, which was grown as a monolayer in Dulbeco's Modified Eagle Medium (D-MEM; GIBCO BRL) plus 10% heat inactivated fetal calf serum (Starrate), 10mM HEPES buffer (AMRESCO), 100 µg/ml Streptomycin, 100 units/ml Penicillin and 1 µg/ml Fungizone (Squibb Industria Farmaceutica S.A).

Green algae, *Chlorella* sp. was commercially available and was kindly provided by Assoc. Prof. Dr. Yuwadee Peerapornpisal, Department of Biology, Faculty of Sciences, Chiang Mai University. The tablet of dry powder of *Chlorella* sp. was suspended in D-MEM and *Chlorella* sp. filtrate was obtained after filtration through 0.45 µm membrane.

Antiviral agent, acyclovir (ACV, Virogon) was purchased as tablets and was suspended in distilled water before use.

Cytotoxicity test of *Chlorella* sp. filtrate

The algal filtrate was tested for cytotoxicity on Vero cells. The filtrate at concentration of 25 mg/ml was two-fold serial diluted and 100 µl of each dilution was added into quadruplicate wells on 96-well tissue culture plate. Then, $1x10^6$ cells/ml of Vero cell suspension was added into each well. After incubation at 37°C for 3-4 days, the cells were stained with 0.1% crystal violet in 1% ethanol for 5-10 minutes. The 50% cytotoxic dose (CD_{50}), which is the concentration of the algal filtrate that can induce cell detachment for 50% comparing to cell control was determined.

Plaque inhibition assay

Viruses (100 plaques/100 µl) were added into each well of 24-well tissue culture plate containing Vero cell monolayer. After serial two-fold dilution of *Chlorella* sp. filtrate, 250 µl of the filtrate at non-toxic concentrations was added into duplicate test wells at the same time of viral adsorption. The algal filtrate was also added after 1 hour viral adsorption at room temperature and viral inoculums were washed out whereas, acyclovir at concentration that inhibited viral infection for 100% was added into drug control wells. Then, mixture of 2% sodium carboxymethyl cellulose and 10% Dulbeco's Modified Eagle Media were prepared, and it was applied to each well as overlay medium. The plate was further incubated at 37°C for 3-4 days, and infected cells were stained with 0.1% crystal violet in 1% ethanol for 5-10 minutes. Plaques were visualized and counted comparing to control.

Inhibition of viral replication assay

$1x10^6$ PFU/ml of viruses was adsorbed into 25 cm^2 tissue culture flask containing Vero cell monolayer for 1 hour at room temperature. Then, infected cells were washed once with phosphate buffered saline and *Chlorella* sp. filtrate was added into duplicate flasks, whereas media was added into control flasks. At 6, 12, 24 and 30 hours after incubation at 37°C, infected cells were collected and frozen at -70°C. Viruses in supernatant were collected at -70°C after infected cells were frozen and thawed twice. Titer of viruses at different time was investigated by plaque titration assay.

Results

Antiviral activity of *Chlorella* sp. was determined in this study. Cytotoxicity of *Chlorella* sp. filtrate was determined on Vero cells and CD_{50} was shown at 338 µg/ml. Therefore, algal filtrate at non-toxic concentrations was tested against herpes simplex virus type 1 and type 2 comparing to 31.25 µg/ml of ACV.

After treatment of HSV with 48.8 and 97.6 µg/ml of *Chlorella* sp. filtrate at the same time of viral adsorption, inhibition of HSV-1 was 30.2 % and 50.8 % whereas inhibition of HSV-2 was 16.7 % and 45.8 % respectively comparing to 100% inhibition when using ACV. The inhibition of HSV-1 infection was decreased to 3.5 % when the virus was treated with 97.6 mg/ml of the algal filtrate after viral adsorption and viral inoculums were washed out whereas inhibition of HSV-2 infection was not observed (Table 1).

Table 1 Inhibition of HSV when tested with *Chlorella* sp. and antiviral drug, acyclovir

Treatment	Inhibition (%)[a]			
	HSV-1		HSV-2	
	during viral adsorption	after viral adsorption	during viral adsorption	after viral adsorption
Chlorella sp. (mg/ml)				
48.8	30.2	0	16.7	0
97.6	50.8	3.5	45.8	0
Acyclovir (µg/ml)	100	100	100	100
31.25				

[a] Results are averaged from 2 experiments

Inhibition of HSV replication assay was performed after treatment with a highest non-toxic concentration at 195.2 µg/ml *Chlorella* sp. filtrate. Virus titers after treatment were determined and results showed that log of HSV-1 titer when treatment with algal filtrate at 12 hours after viral infection was 1. Titer of control HSV-1 was higher than algal treated virus at 12 hours after viral infection since log of virus titer was 3.6. However, at 24 and 30 hours after infection, log of HSV-1 titers after treatment with algal filtrate was 4.3 and 5.3 comparing to 4.4 and 5.7 of control viruses (Table 2). Log of HSV-2 titers after treatment with algal filtrate at 12, 24 and 30 hours was 2, 4.5 and 5.5 comparing to 2.3, 4.5 and 5.5 of control viruses (Table 2). Therefore, titers of HSV-1 and HSV-2 after treatment with the algal filtrate at 24 and 30 hours were not different from control viruses.

Table 2 Log of HSV titer at 12, 24 and 30 hours after viral infection when treatment with *Chlorella* sp. comparing to control

Time after viral infection (hrs)	Log of virus titers			
	Control		Treatment with *Chlorella* sp.	
	HSV-1	HSV-2	HSV-1	HSV-2
12	3.6	2.3	1.0	2.0
24	4.4	4.5	4.3	4.5
30	5.7	5.5	5.3	5.5

Discussion

Study of *Chlorella* sp. was performed on several aspects. Immune enhancement of bacterial infected mice was observed as the bacteria were destroyed and infected mice still survived after treatment the mice with *Chlorella vulgaris.*[10,11] Extract of *Chlorella vulgaris* inhibited murine cytomegalovirus infection by stimulation of interferon production[12] and also exhibited antitumor effect in mice.[13]

Anti-HSV activities of *Chlorella* sp. filtrate on Vero cell were shown in this study. The inhibition of virus after treatment with algal filtrate at the same time of viral adsorption was higher than treatment after viral adsorption and the antiviral effect of the algal filtrate against HSV-1 was higher than HSV-2. Activities against virus replication were also determined at different time point when the viruses were treated after adsorption. It showed that log of HSV titers were not reduced after treatment with the algal filtrate. Therefore, the results indicated that the viruses were inhibited at adsorption step.

Other algae such as *Spirulina platensis*, which is blue green algae, has been shown to have anti-HSV-1 activity[2] and calcium spirulan was found as an active principle in *S. platensis.*[3] Fucoidan isolated from brown algae, *Leathesia difformis* showed activities against HSV and human cytomegalovirus.[4] Diterpenes from two brown algae, *Dictyota dichotoma* and *Dictyota linearis* showed antiviral activity after evaluation against HSV-1 and poliovirus[5]. Moreover, *Cryptopleura ramosa*, *Porphyridium* sp., *Gymnogongrus griffithsiae* and *Cryptonemia crenulata* red algae were found to inhibit HSV-1 and HSV-2 infection on Vero cells.[6,7,8] Sulfated galactan isolated from red algae were observed to contain anti-HSV activities by interfering with the interaction of HSV with heparan sulfate receptor molecules during viral adsorption[8]. Sulfate polysaccharide also showed activities against HIV and other enveloped viruses, and the presence of sulfate group in the molecule was essential for the antiviral properties. [14,15]

Although, anti-HSV of *Chlorella* sp. filtrate was observed at adsorption step in this study. Further study should be carried on to clarify the interfering mechanism involved the binding of virus to receptor molecule and isolation of the compound from *Chlorella* sp., which possess anti-HSV activity.

Acknowledgement

This work was supported by the Faculty of Sciences, Chiang Mai University, Thailand.

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