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การศึกษาฤทธิ์การต้านเชื้อ Cyprinid herpesvirus 3 ของสารสกัดพญายอ (*Clinacanthus nutans*) ในเซลล์เพาะเลี้ยง

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บทคัดย่อ: เคเอชวีเป็นเชื้อไวรัสที่ก่อให้เกิดโรคที่รุนแรงและมีอัตราการตายสูงในปลาไนและปลาคาร์พทั่วโลก ก่อให้เกิดความสูญเสียทั้งในปลาสวยงามและปลาบริโภคทั้งในที่เพาะเลี้ยงและในธรรมชาติ พญายอเป็นพืชสมุนไพรไทยที่มีรายงานถึงประสิทธิภาพในการต้านเชื้อไวรัสเฮอร์ปีส์ซิมเพล็กซ์ในมนุษย์ในห้องทดลองและทางคลินิก การวิจัยครั้งนี้ทำการศึกษาผลของพญายอที่ทำการสกัดด้วยเอทานอลและส่วนของสารสกัดที่แยกโดยใช้คุณสมบัติการละลายของตัวทำละลาย 2 ชนิดในการต้านเชื้อไวรัสเคเอชวีโดยทำการศึกษาประสิทธิภาพในการต้านเชื้อไวรัสทั้งก่อนและหลังจากที่มีการติดเชื้อเข้าสู่เซลล์เพาะเลี้ยงเคเอฟเซลล์ การทดสอบความเป็นพิษของสารสกัดหยาบจากเอทานอลพบว่าความเข้มข้นที่ทำให้เซลล์ตายไป 50% ของเซลล์ทั้งหมดคือ 1,701.57 ไมโครกรัม/มิลลิลิตร สารสกัดส่วนไดคลอโรมีเทนคือ 522.47 ไมโครกรัม/มิลลิลิตร และสารสกัดส่วนบิวทานอลคือ 1,797.98 ไมโครกรัม/มิลลิลิตร สารสกัดหยาบจาก เอทานอลสามารถยับยั้งเชื้อไวรัสได้ที่ความเข้มข้น 250, 500 และ 1,000 ไมโครกรัม/มิลลิลิตร ทั้งก่อนที่มีการติดเชื้อเข้าสู่เซลล์ 1, 2, 3 และ 4 ชั่วโมง และหลังจากเชื้อติดเข้าสู่เซลล์แล้ว ในขณะที่สารสกัดส่วนไดคลอโรมีเทนและบิทานอลความเข้มข้นสูงสุดที่ไม่เป็นพิษต่อเซลล์ ไม่มีประสิทธิภาพในการยับยั้งเชื้อไวรัสเคเอชวีในเซลล์ทั้งก่อนและหลังการติดเชื้อ จากผลการศึกษาในครั้งนี้แสดงให้เห็นว่าสารสกัดจากพญายอมีประสิทธิภาพในการต้านเชื้อไวรัสเคเอชวีในปลาคาร์พ โดยสามารถที่จะนำไปประยุกต์ใช้และพัฒนาต่อไปเพื่อการรักษาโรคไวรัสเคเอชวีในอนาคตได้

คำสำคัญ: พญายอเคเอชวี ต้านเชื้อไวรัส ความเป็นพิษต่อเซลล์ เซลล์เพาะเลี้ยง

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Clinacanthus nutans (Burm. f.) Lindau Extract against Cyprinid Herpesvirus 3 in Koi Fin Cell Line

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Abstract: Cyprinid herpesvirus 3 (CyHV-3) or Koi herpesvirus (KHV) is a virulence viral infection in common carp and koi. The disease has caused global epizootic and economic loss in fish aquaculture and in the wild. *Clinacanthus nutans* (Burm. f.), belonging to the family Acantaceae, is a well-known medicinal plant used in Thai traditional medicine. Virucidal effects of the plant extract against human herpes simplex virus have been reported. In this study, *C. nutans* crude extract, dichloromethane fraction and n-butanol fraction were tested for antiviral activities against CyHV-3 in koi fin cell line. The extracts were tested for cytotoxicity, pre and post infection antiviral activities. The 50% cytotoxic concentration of crude extract was at 1,701.57 µg/ml, dichloromethane fraction at 522.47 µg/ml and n-butanol fraction at 1,797.98 µg/ml. Effective concentrations of the crude extract pre and post-infection were at 250, 500 and 1,000 µg/ml. The dichloromethane fraction and n-butanol fraction did not have antiviral activity. The results showed that crude extract expressed antiviral activity against CyHV-3 which can be applied as therapeutic agents in common carp and koi in the future.

Keywords: *Clinacanthus nutans* (Burm. f.) Lindau, Cyprinid Herpesvirus 3, Antiviral activity, Cytotoxicity, Cell line

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Introduction

Cyprinid Herpesvirus 3 (CyHV-3) or Koi Herpesvirus (KHV) has been reported in common carp (*Cyprinid Carpio* L.) and koi (*Cyprinid Carpio* koi) from many countries worldwide including Thailand. The first report of this disease was isolated from mass mortality of common carp in Israel in 1998

(Hedrick *et al*., 2000). It is highly virulence and contagious viral disease which causes high mortality. Many criteria for viral prevention were reported. For example, produce fish strains and crossbreeds for more resistance to virus (Ronen *et al*., 2005). Immunization is another option to prevent those fish from infection. Unfortunately, the use of inactivated virus vaccine (Yasumoto *et al*., 2006) or viral proteins has been proven unsuccessful. Two methods of fish immunization were developed and used only in Israel by immunizing fish with pathogenic virus (natural immunization) (Ronen *et al*., 2003) or with live attenuated virus vaccine (Perelberg *et al*., 2005). At present, there is no report of any drug or chemical that can effectively treat the virus in fish. *Clinacanthus nutans* (Burm. f.) Lindau is one of Thai herbal plants with antiviral activities. Extracts from leaves showed antiviral activity against Herpes Simplex Virus (HSV) and Varicella Zoster Virus (VZV) in human both in experiments and clinical trials (Jayavasu *et al*., 1992[a]; Jayavasu *et al*., 1992[b]; Thawaranantha *et al*., 1992; Sangkitporn *et al*., 1995). The herb extract was also reported to be effective against Yellow-head Rhabdovirus in Black tiger shrimp by feeding for 7 days (Direkbusarakorn *et al*., 1998). Moreover, it was reported to have *in vitro* antiviral activity against Infectious Hematopoietic Necrosis Virus (IHNV) and Oncorhynchus Masou Virus (OMV) which is herpesvirus disease in salmonid fish (Direkbusarakorn *et al*., 1996). This study was focused on the antiviral activity of *Clinacanthus nutans* Burm.f.) Lindau ethanol extracts against CyHV-3 in cell culture (*in vitro*). The results of this study would be useful for alternative treatment and prevention of Cyprinid Herpesvirus 3 disease in koi and common carp.

Materials and Methods

Virus isolation and cell cultures

Koi Fin Cell line (KFC) was obtained from Dr. Peiyu Alison Lee, The Central Taiwan University of Science and Technology. Cell was propagated in Dulbeco's Modified Eagle Medium (DMEM) (Gibco®) and 25% Leibovitz medium (L-15) (Gibco®) supplemented with 10% fetal calf serum (Gibco®) and antibiotics (Gibco®) at 22°C. Cells were cultured for 3 passages before using in the experiments. Cyprinid Herpesvirus 3 (CyHV-3) was isolated from infected koi that was confirmed by nested PCR (IQ2000TM KHV Detection System, Farming IntelliGeneTech. Corp®) at the Veterinary Medical Aquatic Animal Research Center, Faculty of Veterinary Science, Chulalongkorn University. The viral titer was determined by viral assays and was expressed as 50% Tissue Culture Infected Dose ($TCID_{50}$).

Preparation of herb and extraction

Fresh aerial parts of *Clinacanthus nutans* (Burm. f.) Lindau was collected from medicinal herb plantation in Chiang Rai province during October to December. The plants (weight 9.8 kg) were washed thoroughly and dried before blended. The sample was extracted with 95% ethanol at room temperature for 72 hours. The extracted solutions were filtered through filter sheet and the filtrates were concentrated on a rotary evaporator to give a dark green thick oil which was crude extract (3,764 g). The crude extract then was partitioned between water and dichloromethane (CH_2Cl_2) and the water layer then was extracted with n-butanol (C_4H_9OH). Removal of the solvent of each fraction gave the CH_2Cl_2 fraction as the dark green thick oil (192.6 g), the n-butanol fraction as brown wax (178.63 g). The crude extract, CH_2Cl_2 fraction and n-butanol fraction were dissolved in DMSO before tested to compare their antiviral activities against CyHV-3 in this study.

Cytotoxicity assay of C. nutans extracts

The cytotoxicity assays were performed in order to find the concentrations of crude extract, CH_2Cl_2 fraction and n-butanol fraction that were non-toxic to KFC which were used in antiviral activity tests. Briefly, KFC was seeded in 24-well plate at concentration 2.4 x 10^5 cells/ml and incubate at 24ºC for 24 hours. The crude extract, CH_2Cl_2 fraction and n-butanol fraction were prepared into 2-fold serial dilution for 5 concentrations before adding to cell 500 µl per well for 6 wells. Control groups were added with 500µl of L-15-2% medium and DMSO at 0.01%, 0.1% and 1% then incubated at 24ºC for 72 hours. The cell viability was counted by dying cell with 1% trypan blue and calculated for % cell viability. The cell viability was then calculated to compare % cell viability between experimental groups and control group using Chi square with IBM SPSS statistic version 22 (New York, USA). The results were considered statistically significant if the *P*-value less than 0.05. The concentrations that reduce the number of viable cells by 50% were evaluated as 50% Cytotoxicity Concentration (CC_{50}) by using doses-response curve between doses and percentages of survival cells and calculated by probit analysis with IBM SPSS statistic version 22 (New York, USA).

Pre-infection antiviral activity test

To compare the efficacy between crude extract, CH_2Cl_2 fraction and n-butanol fraction of *C. nutans* against CyHV-3 before infected to cell line, tests were performed as briefly: cells were seeded in 96-well plate and incubated at 24ºC for 24 hours. The extracts were prepared at the highest

concentrations that were non-toxic to cells. Then make 2-fold serial dilution with L-15-2% for 5 concentrations. The control group was added with L-15 2% without extract. The virus (MOI 0.1) was mixed with each extract at different concentrations. The mixtures were incubated at 24ºC for 1, 2, 3 and 4 hours before infected to cells. All plates were incubated at 24ºC for 14 days and cytopathic effect (CPE) were observed and recorded daily for 14-21 days. The end point dilutions ($TCID_{50}$) were calculated and the tests were done in triplications.

Post-infection antiviral activity test

To compare the efficacy between crude extract, CH_2Cl_2 fraction and n-butanol fraction extract of *C. nutans* against CyHV-3 after infected to cell line. The virus was prepared as 10-fold serial dilution for 5 concentrations with L-15-2% then added 50 µl of the virus per well in 96-well plate. Infected cell was incubated at 24ºC for 1 hour then removed the inoculums. The 2-fold serial dilutions of extracts (five concentrations) were added after removing the inoculums. The extracts were added 100 µl to each well for 8 wells per concentrations then incubated at 24ºC. The control group was added with L-15 2%. CPE was observed and recorded daily for 14-21 days. The end point dilutions ($TCID_{50}$) were calculated and the tests were done in triplications.

Statistical Analysis

The end point dilution of virus in the antiviral activity experiments were calculated by the method of Reed and Muench (1938). The concentration that reduced virus titer for more than 1 log10 when compared to the control group was the effective concentration that inhibits 90% viral replications (Haetrakul, 2009).

Results

*Cytotoxicity assay of C. nutans extracts*Crude extract of *C. nutans* at 2,000, 1,000, 500, 100 and 50 µg/ml, CH_2Cl_2 fraction at 1,000, 500, 100, 50 and 10 µg/ml, n-butanol fraction at 2,000, 1,500, 1,000, 500 and 100 µg/ml were tested for toxicity in cell line. The toxic concentration of extracts to KFC was determined by comparing % cell viability between experimental groups and control group. Crude extract at 1,000 µg/ml, CH_2Cl_2 fraction at 100 µg/ml and n-butanol fraction at 1,000 µg/ml were significantly different from the control group ($P \leq 0.05$), respectively. All concentrations of DMSO were not cytotoxic. The CC_{50} of crude extract was 1,701.57 µg/ml, dichloromethane fraction was 522.47 µg/ml and n-butanol was 1,797.98 µg/ml.

Pre-infection antiviral activity

The direct antiviral activity of *C. nutans* against CyHV-3 after mixing each fraction of herb extracts with the virus at room temperature for 1, 2, 3 and 4 hours are shown in figure 1-4. The viral titers of CyHV-3 in the control group were $1.75x10^4$, $1.39x10^4$, $1.15x10^4$ and $1.35x10^4$ $TCID_{50}$/ml at 1, 2, 3 and 4 hours before infected to the cell, respectively. In all incubation time, the minimal concentration of the crude extract that could reduce CyHV-3 at least 10 times (>1 log10) was 250 µg/ml.

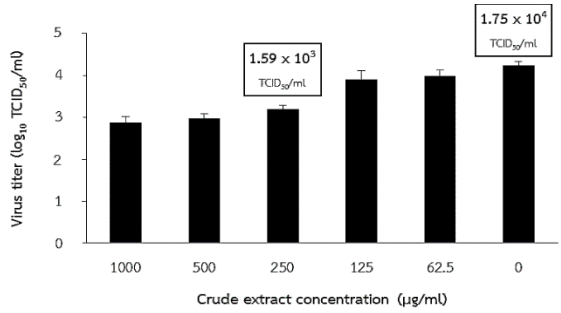

Figure 1 Pre-infection antiviral assay of crude extract and CyHV-3 at 1 hour, effective concentration started from 250 µg/ml

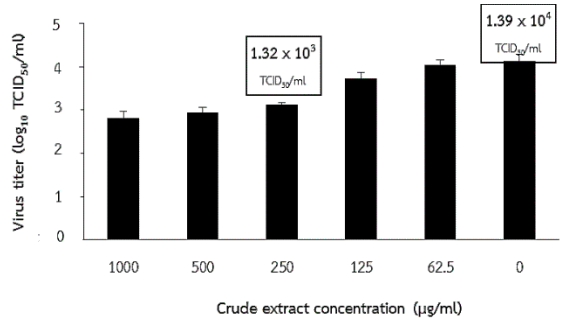

Figure 2 Pre-infection antiviral assay of crude extract and CyHV-3 at 2 hours, effective concentration started from 250 µg/ml

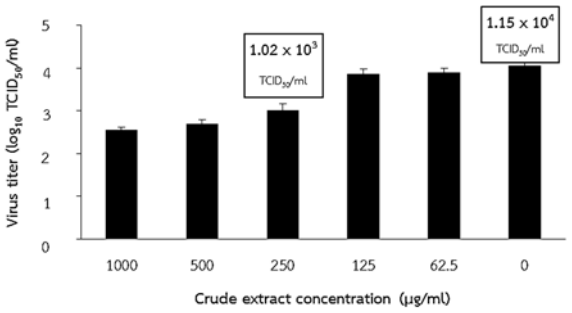

Figure 3 Pre-infection antiviral assay of crude extract and CyHV-3 at 3 hours, effective concentration started from 250 µg/ml

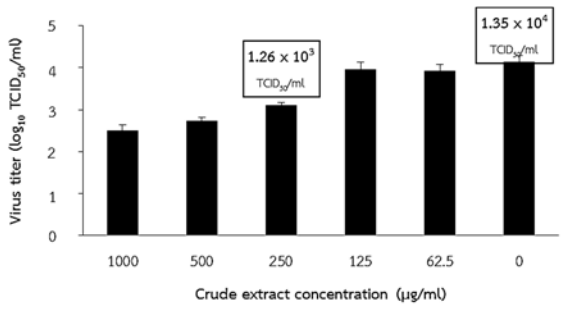

Figure 4 Pre-infection antiviral assay of crude extract and CyHV-3 at 4 hours, effective concentration started from 250 µg/ml

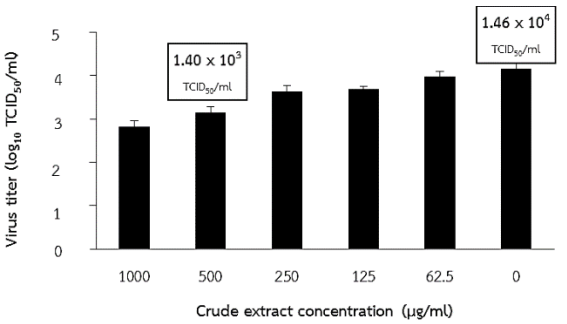

Figure 5 Post-infection antiviral assay of crude extract, effective concentration started from 500 µg/ml

Post-infection antiviral activity

CyHv-3 was used as a control and viral titer was $1.46x10^4$ $TCID_{50}$/ml. The results show that since 500 µg/ml of crude extract concentration, could reduce CyHV-3 >1 log10 (Fig.5). However, CH_2Cl_2 fraction and n-

butanol fractions at non-toxic concentrations could not reduce virus replication post-infection.

Discussion

Ethanol crude extract from *C. nutans* could reduce CyHV-3 pre and post infection in KFC. However, dichloromethane and n-butanol fractions could not reduce CyHV-3 atnon-toxic concentrations. Phytochemical analysis of *C. nutans* was reported to contain alkaloids, flavonoid (Satayavivad *et al*., 1996), chlorophyll (Sakdarat *et al.,* 2009) and cerebrosides (Tuntiwachwuttikul *et al*., 2004). Ethanol extracts of *C. nutans* were reported to have antiviral activities against Herpes simplex virus (HSV) before infection in the human cell line (Jayavasu *et al*., 1992[b]; Tuntiwachwuttikul *et al*., 2004; Vachirayonstien *et al*., 2010). The report of Vachirayonstien *et al*. (2010) also indicated that treating HSV with either less or higher purified extracts before infection gave great reductions of viral infectivity in HEp-2 cells. Chloroform extract of *C. nutans* was isolated to give chlorophyll a and chlorophyll b related compounds which had anti HSV-1 activity before infection (Sakdarat *et al.,* 2009). Dichloromethane fraction was ineffective which was contradicted to previous report on the effectiveness against HSV-1 pre-infection in the cell line (Kunsorn *et al*., 2013). N-butanol soluble fractions were reported to have 6 compounds of C-glycosyl flavones (Tuntiwachwuttikul *et al*., 2004) and 5 sulfur-containing glucosides (Teshima *et al*., 1998). The C-glycosyl flavones are flavonoids which contain anti-inflammatory activity (Satayavivad *et al*., 1996). The recent study showed an antiviral activity of monogalactosyl diglyceride and digalactosyl diglyceride from *C .nutans* extract against HSV-1 and HSV-2 post-infection (Pongmuangmul *et al*., 2016). The same pattern of antiviral activity resulted from synthesis monoglycosyl diglycerides showed high inhibitory activity against HSV in human cell line (Janwitayanuchit *et al*., 2003). The antiviral activities of *C. nutans* crude extract with ethanol gave significant results in reducing viral number after entering into the cells. The possibility of inhibition may occur during viral replication after infection. Some monoglycerides showed antiviral activity against virus by a destruction of viral envelopes (Thormar *et al*., 1994). The previous study of *C. nutans* alcohol extract showed deformity of CyHV-3 virion in KFC after the treatment under Transmission Electron Microscope (TEM) (Haetrakul, 2009). Pre-infection antiviral activities at 1, 2, 3 and 4 hours showed an efficiency of crude extract against CyHV-3 directly before entering into the cell. The concentration that significant reduced viral titer when comparing to control started from 250 µg/ml. Some researchers reported that CyHV-3 can surveil in water at

least 4 hours without hosts (Perelberg *et al*., 2003).So this crude extract can be applied for water treatment in common carp or koi ponds. The direct effect of *C. nutans* extract also reported to inhibit Infectious Hematopoietic Necrosis Virus (IHNV) and Oncorhynchus Masou Virus (OMV) in fish which showed 100% plaque reduction rate in cell line before infection (Direkbusarakorn *et al*., 1996).

Conclusion

Crude extract of *C. nutans* could inhibit cyprinid herpesvirus-3 pre and post infection in koi fin cell line. The present study showed direct detrimental effects of the extract to the virus and possibly interrupted viral replication process post infection. The use of crude extract from *C. nutans* against cyprinid herpesvirus 3 would be an alternative treatment to prevent and decrease mortality in infected fish. In vivo study should be performed to evaluate the toxicity of extract in fish and check for antiviral activities. This will be beneficial to common carp and koi aquaculture to prevent the losses of valuable fish and minimizing the economy loss.

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