ประสิทธิภาพของพญายอต่อเชื้อ Cyprinid Herpesvirus 3 ในเซลล์เพาะเลี้ยง koi fin cell และ ในปลาคาร์พ

นางสาวฐนิดา เหตระกูล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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THE EFFICACY OF *CLINACANTHUS NUTANS* (BURM.F.) LINDAU AGAINST CYPRINID HERPES VIRUS 3 IN KOI FIN CELL LINE AND FANCY CARP (*CYPRINUS CARPIO* KOI)

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ฐนิดา เหตระกูล : ประสิทธิภาพของพญายอต่อเชื้อ Cyprinid Herpesvirus 3 ในเซลล์เพาะเลี้ยง koi fin cell และ ในปลาคาร์พ (THE EFFICACY OF *CLINACANTHUS NUTANS* (BURM.F.) LINDAU AGAINST CYPRINID HERPESVIRUS 3 IN KOI FIN CELL LINE AND FANCY CARP (*CYPRINUS CARPIO* KOI)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพญ. ดร.นันทริกา ชันซื่อ, หน้า.

เคเอชวีเป็นเชื้อไวรัสที่ก่อให้เกิดโรคที่รุนแรงและมีอัตราการตายสูงในปลาไนและปลาคาร์พทั่วโลก ก่อให้เกิดความสูญเสียทั้งในปลาสวยงามและปลาบริโภคทั้งในที่เพาะเลี้ยงและในธรรมชาติ พญายอเป็นพืชสมุนไพรไทยที่มีรายงานถึงประสิทธิภาพในการต้านเชื้อไวรัสเฮอร์ปีส์ซิมเพล็กซ์ในมนุษย์ การวิจัยครั้งนี้ทำการศึกษาผลของพญายอที่ทำการสกัดด้วยเอทานอลและส่วนของสารสกัดที่แยกโดยใช้ไดคลอโรมีเทนและบิวทานอลในการต้านเชื้อไวรัสเคเอชวีโดยทำการศึกษาประสิทธิภาพในการต้านเชื้อไวรัสทั้งก่อนและหลังจากที่มีการติดเชื้อในเซลล์เพาะเลี้ยงเคเอฟเซลล์ การทดสอบความเป็นพิษของสารสกัดหยาบจากเอทานอล พบว่าความเข้มข้นที่ทำให้เซลล์ตายไป 50% ของเซลล์ทั้งหมดคือ 1,701.57 ไมโครกรัม/มิลลิลิตร สารสกัดส่วนไดคลอโรมีเทนคือ 522.47 ไมโครกรัม/มิลลิลิตร และสารสกัดส่วนบิวทานอลคือ 1,797.98 ไมโครกรัม/มิลลิลิตร สารสกัดหยาบจากเอทานอลสามารถยับยั้งเชื้อไวรัสได้ที่ความเข้มข้น 250, 500 และ 1,000 ไมโครกรัม/มิลลิลิตร ทั้งก่อนที่มีการติดเชื้อเข้าสู่เซลล์ 1, 2, 3 และ 4 ชั่วโมง และหลังจากเชื้อติดเข้าสู่เซลล์แล้ว ในขณะที่สารสกัดส่วนไดคลอโรมีเทนและบิวทานอล ไม่มีประสิทธิภาพในการยับยั้งเชื้อไวรัสเคเอชวีในเซลล์ทั้งก่อนและหลังการติดเชื้อ นำสารสกัดหยาบจากเอทานอลมาทดสอบฤทธิ์ในการต้านเชื้อไวรัสในปลาคาร์พ พบว่าสามารถยับยั้งเชื้อได้ทั้งก่อนและหลังจากที่มีการติดเชื้อ โดยความเป็นพิษของสารสกัดในปลาคาร์พมีค่ามากกว่า 5,000 ไมโครกรัม/มิลลิลิตร ในขณะที่ความเข้มข้นที่ได้ผลในการต้านเชื้อก่อนการติดเข้าสู่ร่างกายมีค่า 991.59 ไมโครกรัม/มิลลิลิตร 782.72 ไมโครกรัม/มิลลิลิตร 749.05 ไมโครกรัม/มิลลิลิตร และ 707.80 ไมโครกรัม/มิลลิลิตร เมื่อทำการบ่มสารสกัดกับไวรัสที่ 1, 2, 3 และ 4 ชั่วโมงก่อนการทำให้ปลาติดเชื้อ ความเข้มข้นที่ได้ผลในการต้านเชื้อหลังการติดเข้าสู่ร่างกายโดยทำการใส่สารสกัดหลังจากติดเชื้อไปแล้วที่ 0 และ 24 ชั่วโมง มีค่า 2,050.88 ไมโครกรัม/มิลลิลิตร และ 2,337.74 ไมโครกรัม/มิลลิลิตรตามลำดับ จากผลการศึกษาในครั้งนี้แสดงให้เห็นว่าสารสกัดจากพญายอมีประสิทธิภาพในการต้านเชื้อไวรัสในปลาคาร์พ โดยสามารถนำไปประยุกต์ใช้เพื่อการป้องกันและรักษาโรคไวรัสเคเอชวีในอนาคตได้

ภาควิชา อายุรศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา อายุรศาสตร์สัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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THANIDA HAETRAKUL: THE EFFICACY OF *CLINACANTHUS NUTANS* (BURM.F.) LINDAU AGAINST CYPRINID HERPESVIRUS 3 IN KOI FIN CELL LINE AND FANCY CARP (*CYPRINUS CARPIO* KOI). ADVISOR: ASSOC. PROF. NANTARIKA CHANSUE, Ph.D., pp.

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.) Lindau 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 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 *C. nutans* crude extract was chosen to perform antiviral activity test in koi carp. The results showed effective antiviral activity against CyHV-3 pre and post infection. The 50% lethal concentration of extract was higher than 5,000 µg/ml. The 50% effective doses (ED_{50}) were 991.59 µg/ml, 782.72 µg/ml, 749.05 µg/ml and 707.80 µg/ml at 1, 2, 3 and 4 hours pre-infection, respectively. The ED_{50} from post infection test were 2,050.88 µg/ml and 2,337.74 µg/ml at 0 and 24 hours, respectively. The results showed that crude extract expressed antiviral activity against CyHV-3 can be applied as therapeutic agent in common carp and koi aquacultures.

Department:	Veterinary Medicine	Student's Signature	.....
Field of Study:	Veterinary Medicine	Advisor's Signature	.....
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CHAPTER I
INTRODUCTION

1.1 Importance and rational

A herpesvirus infection that only affects koi or fancy carp (*Cyprinus carpio* koi) and common carp (*Cyprinus carpio* L.) was first reported in Israel (Hedrick et al., 2000). The virus was identified as Cyprinid Herpesvirus 3 (CyHV-3), was found to be highly virulent and rapidly spreading, and was soon found in all the countries around the world. The disease caused severe financial loss in common carp and koi culture industries. In Asia, the evidence of disease outbreak was reported in Indonesia in 2002 and Japan in 2003 (Sano, 2004). In Thailand, the first reported of the disease was found from koi exported to Germany in 2004 (Poonpanich and Prapreut, 2013). Thailand started the koi herpesvirus disease (KHVD) surveillance program since August 2002 under the Thailand National Strategy for Control of Aquatic Animal Diseases (Kanchanakan, 2005). CyHV-3 surveillance in 2004 revealed that the viral DNA was detected in koi carp from many places around Bangkok and perimeter (Chansue and Tangtrongpiros, 2005). The patient records at the Veterinary Medical Aquatic animal Research Center, Faculty of Veterinary Science, Chulalongkorn University was quite revealing. Using nested PCR, it was found in 2005 that almost 75% of sick koi were positive for CyHV-3 infection. The incidence was higher in winter.

The Department of Fisheries has been established a strategy plan to control transboundary movement of CyHV-3 diseases using quarantine measures. Fish quarantine inspector conducts inspection services at the port of entry. All live aquatic animal shipments are inspected at the port, then they will be sent to quarantine for at least 21 days at the quarantine facility that passed the standard biosecurity from The Department of Fisheries (Poonpanich and Prapreut, 2013). During the quarantine period, fish health inspector will visit and conduct health inspection of the imported animals by using single round PCR (Kanchanakan, 2005). Fish that is tested positive to the virus will be eradicated. Fish will be buried around

farm area or burnt at the contagious carcass management facility certified by the Department of Livestock Development (Poonpanich and Prapreut, 2013).

Most CyHV-3 infected fish was diagnosed with signs of secondary infections. Significant necrotic lesions appeared specifically on the gills. The mortality ranged from 80 to 100% in naïve pond when the water temperature ranged between 18 – 24ºC, which is permissive temperature for the virus. Experiments have been conducted attempting to prevent CyHV-3 infection using natural immunization (Ronen et al., 2003) and vaccination (Perelberg et al., 2005). Up until present, there is no effective drug or chemical reported to provide anti-CyHV-3 activities in fish. In recent years herbal medicine has become an alternative treatment for plethora of infections in humans. Herbal therapeutics is widely considered to be less toxic with limited side effects and inexpensive compared to synthetic drugs.

Clinacanthus nutans (Burm.f.) Lindau is one of the herbal medicinal plants of Thailand generally known for its antiviral properties. Its use can be traced to the distant past. Extracts from aerial parts and leaves show antiviral activity against Herpes Simplex Virus (HSV) and Varicella Zoster Virus (VZV) in human both in experiments and clinical trials (Jayavasu et al., 1992; Thawaranantha et al., 1992; Sangkitporn et al., 1995; Yoosook et al., 1999; Tuntiwachwuttikul et al., 2004; Jayavasu et al., 2013). The herb extract was also reported to be effective against Yellow-head Rhabdovirus in Black tiger shrimp (Direkbusarakom et al., 1998) and two viruses in fish (Direkbusarakom et al., 1996).

The present research was designed in the background that the use of alternative medicine is seemingly becoming popular as opposed to purified drugs. In case of purified drugs the major concerns are the development of resistance and the presence of residual contents both of which are potentially detrimental to the health of human consumers. The present study was conducted to evaluate the antiviral activity of herb extracts against CyHV-3 both in cell culture (*in vitro*) and koi (*in vivo*). One can hope that the outcome be a scientific evaluation of alternative treatment and prevention of Cyprinid Herpesvirus 3 disease in koi and common carp clinically and can be a model study for other herbal medicines or other viral infections in aquatic animals.

1.2 Objectives of study

1. To extract *C. nutans* by using dissolve properties in different diluents in cell culture.
2. To compare the anti-Cyprinid herpesvirus 3 (CyHV-3) activities of crude extract, dichloromethane fraction and n-butanol fraction in Koi fin cell line.
3. To study the antiviral activity effect of *C. nutans* extract against CyHV-3 infection in koi.
4. To find the effective concentrations of *C. nutans* extract against CyHV-3 infection in koi.

1.3 Hypotheses

1. The isolated fractions of *C. nutans* have higher antiviral activity effects against CyHV-3 than the crude extract in cell line.
2. The effective concentration of *C. nutans* extract is non-toxic to cell line and has antiviral activities in Koi fin cell line.
3. The extract can be used for prevention and treatment of CyHV-3 infected koi.
4. The effective concentration of extract against CyHV-3 infected koi is non-toxic to koi, with high therapeutic index.

1.4 Advantages of study

The new alternative treatment and prevention of Cyprinid Herpesvirus 3 infection with *C. nutans* extract was discovered. The primary dosage and safety for using in koi from this experiment could be applied to use for treatment in clinic. Apart from direct application with the fish, it could be used in treating the virus in water or environment which would reduce the transmission of virus. This could be used as basic information for other measures of treatment or prevention in CyHV-3 infected koi and common carp. The results also could be applied to study anti-herpesviral activity of *C. nutans* in other animal herpesviral infection.

CHAPTER II
LITERATURE REVIEW

2.1 Cyprinid Herpesvirus 3

2.1.1 Introduction

Cyprinid Herpesvirus 3 (CyHV-3) or Koi Herpesvirus (KHV) is highly virulence and contagious viral disease. It causes high mortality only in common carp (*Cyprinid Carpio* L.) and koi or fancy carp (*Cyprinid Carpio* koi). The first report of the disease was isolated from mass mortality of common carp and koi in Israel in 1998 (Hedrick et al., 2000). Unidentified mortality of common carp and koi were reported from many farms started from the northwest coast to the north central region. The disease also was reported later in the USA in the same year. All sizes of koi from retail facility and private owners in the mid region of the USA were found death after 2 weeks of returning from a koi show (Hedrick et al., 2000). After the first report, the disease was spread around the world due to the global fish trade and ornamental koi shows (Ilouze et al., 2006). CyHV-3 has been included on the list of fish diseases notifiable to the World Organization of Animal Health (OIE) at the 74th General Session in Paris (OIE, 2006). The disease has been detected among many countries in Europe, USA and Asia including Thailand (Chansue and Tangtrongpiros, 2005; Pikulkaew et al., 2009). The first report in Thailand was found from koi exported from Thailand to Germany in 2004. Since the first detection, all koi farms were investigated for CyHV-3 infection. Koi that was tested positive to the disease had to be destroyed under regulation of the Department of Fisheries. Koi imported from Japan was temporary inhibited for almost one year and all imported fish must be quarantined and screened for the disease since then (Appendix A). The disease caused severe financial and economic losses in common carp and koi culture industries around the world. This also impacted ornamental koi farms and fish owners in Thailand. Ornamental koi was recorded in the top three of fish export market in Thailand by the Inland Aquatic Animal Health Research Institute.

2.1.2 Classification and taxonomy

CyHV-3 is a double stranded DNA virus classified to be a member of the order *Herpesvirales* in a family *Alloherpesviridae* (Waltzek et al., 2005). The known Alloherpesviruses typically exhibit host specificity and at least 11 known fish *Alloherpesviridae* cause significant economic losses to aquaculture (Hanson et al., 2016). Virus structure classified by electron microscope showed a symmetry icosahedral capsid size 100 – 110 nm (Hedrick et al., 2005). The genome of this virus is a 295-kb encoded 156 protein coding open reading frames (ORFs), including 8 ORFs encoded by the repeat regions (Aoki et al., 2007). A total of 40 structural proteins consist of 3 capsid, 13 envelope, 2 tegument, and 22 unclassified proteins, were described by using liquid chromatography tandem mass spectrometry (Michel et al., 2010). Only ORF81 which encodes a type 3 membrane protein expressed on the CyHV-3 envelope have been studied (Aoki et al., 2007). Three strains of CyHV-3 were sequenced from Israel (CyHV-3 I), Japan (CyHV-3 J) and United states (CyHV-3 U). These strains exhibit high sequence identity even they are from different geographic origins (Aoki et al., 2007).

2.1.3 Characterization

CyHV-3 affects carp of all ages but more susceptible to younger fish (Perelberg et al., 2003). The mortality rate of infected fish is 80-100% and occurs within 6 to 22 days post infection (dpi), highest on day 8 to 12 (Hedrick et al., 2000; Perelberg et al., 2003). Temperature plays an important role on the virulence of this virus. The optimum temperature that induces the disease is between 18 to 26°C, but no fish mortality was observed at 13°C (Gilad et al., 2003). CyHV-3 DNA could be detected by PCR earlier in fish that was infected with virus by immersion at 25°C than at 18°C. Viral DNA was detected on day 3 from skin and gill samples, while at 18°C could be detected on day 6 post infection (Matras et al., 2012).

2.1.4 Pathogenesis

Many scientists postulated that the virus enters fish via gill (Gilad et al., 2004; Pikarsky et al., 2004; Dishon et al., 2005; Ilouze et al., 2006; Miyazaki et al., 2008).

Because infected fish has prominent lesion on gill, the virus was called carp interstitial nephritis and gill necrosis virus (Hedrick et al., 2000; Perelberg et al., 2003; Ronen et al., 2003; Pikarsky et al., 2004; Miyazaki et al., 2008). However, later report showed that the skin mediated the entry of CyHV-3 into fish by using bioluminescent imaging technique. The image showed the infection restricted to the posterior part of the fish especially the skin covering the fin and body mediated entry of virus into carp (Costes et al., 2009). Fish mucus was an important barrier to prevent virus infection. Virus entry was increased on the area that mucus was removed with or without wound (Raj et al., 2011). The periodontal pharyngeal mucosa was also confirmed to be the major portal of viral entry. The CyHV-3 was mixed with feed before feeding and observed by bioluminescence imaging, which was found positive after 1 – 2 days post infection (Fournier et al., 2012). After initial replication, CyHV-3 is suspected to spread rapidly by detecting the viral DNA in fish tissue. The amount of viral DNA from kidney and blood cells of infected fish was detected as early as 1 day post infection by using semiquantitative PCR (Pikarsky et al., 2004). Viral DNA could be detected from skin and gills sample on 3 dpi, later from liver, spleen and kidney on day 5 and brain on 6 dpi using modified conventional PCR method (Matras et al., 2012). Mucus sample from external swab also represent a good sample for detecting viral DNA in early infection stage (< 5 dpi) (Monaghan et al., 2015b). Virus replication in gut at the later stages of infection represents sources of viral excretion into the environment (Dishon et al., 2005). Fish mortality was considered due to loss of the osmoregulatory functions of the gills, kidneys, and gut (Gilad et al., 2004). The unique characteristic of Herpesviruses is Latency. Peripheral white blood cells and various tissues were reported to detect viral DNA during latency period (Eide et al., 2011). Temperature shifts could induce latency and reactivation. This was confirmed when no viral replication occurs at non permissive temperature in cell culture (Gilad et al., 2003; Imajoh et al., 2015). CyHV-3 DNA has been detected by real-time PCR at 65 dpi in clinically healthy fish that survived from viral infection (Gilad et al., 2004). Fish that survived from viral infection could be reactivated by temperature stress (Eide et al., 2011) or netting stress (Bergmann and Kempter, 2011). The virus persisted in a wild population of common carp after the outbreak for at least 2 years (Uchii et al., 2009).

Seasonal changes could also reactivated CyHV-3 in wild host population from persistent or latent infection (Uchii et al., 2014).

2.1.5 Clinical signs and pathological findings

The infected fish would show clinical signs around 2 to 3 days post-infection (dpi). Fish exhibits lethargy, appetite loss, sunken eyes, moderately disoriented and abnormal swimming, grasping. Skin shows hyperemia, hyper secretion of mucus, herpetic lesions or white patches on the skin and signs of secondary bacterial infection (Hedrick et al., 2000; Gilad et al., 2002). Gill frequently becomes necrotic, swollen and inflamed. Fish was lethargic then suddenly hyperactive and started to loss equilibrium before death. Some dead fish had hemorrhage at the base of fin (Hedrick et al., 2000). Histopathology showed intranuclear inclusion bodies in gill tissues and other organs such as kidney, spleen, liver, heart, intestine and brain (Pikarsky et al., 2004; Miyazaki et al., 2008; Pikulkaew et al., 2009). Pathological changes showed on the gill as early as 2 dpi. The gill was main target of histopathological changes. Gill lamellae were swollen or vacuolated, and exhibited nuclear degeneration. Gill lamellae fusion and clubbing of gill filaments were observed. Intranuclear inclusion body was observed as basophilic material within the nucleus of gill epitheliums (Miyazaki et al., 2008). In fish showed neurological signs had lesions in brain. Brain sections showed focal meningeal and parameningeal inflammation (Pikarsky et al., 2004). Perivasculitis with infiltration of small round cells was seen around some vessels (Miyazaki et al., 2008).

2.1.6 Diagnosis and detection

There are many methods for CyHV-3 infection diagnosis apart from clinical signs and histopathology. Viral isolation was the first method to be developed. Many types of fish cell lines were used for viral propagation. Hedrick et al. (2000) developed koi fin (KF-1) cell line for using in their laboratory. Koi fin cell (KFC), carp fin cell (CFC) and common carp brain (CCB) cell line are commonly used for CyHV-3 isolation. They were developed from gills, fin and brain of koi and common carp (Neukirch et al., 1999). New cell line named as CCF-K104 was developed from caudal

fin of common carp. It was used in temperature-shift experiment for studying CyHV-3 latency in cell line (Imajoh et al., 2015). After infected into cell line, the virus induced cell fusion and cytoplasmic vacuolation in cell culture. Cytopathic effect (CPE) or cell lysis will be obvious at 7 – 10 days post inoculation and completed after 14 days post infection. It caused cytopathic effect in Koi Fin Cell line (KFC) at 20 to 25 ºC while no growth at the temperature below 4ºC or higher than 30ºC (Gilad et al., 2003). Electron microscope was used to classify the virus (Hedrick et al., 2000; Miwa et al., 2007). Many techniques for detecting viral genes have been developed including conventional polymerase chain reaction (PCR) (Gilad et al., 2002; Gray et al., 2002; Bercovier et al., 2005), nested PCR (Bergmann et al., 2006; El-Matbouli et al., 2007), loop-mediated isothermal amplification (LAMP) (Gunimaladevi et al., 2004), TaqMan PCR and real time PCR (Gilad et al., 2004). Real-time PCR, nested PCR and semi-nested PCR are proven to be sensitive methods for viral DNA detection at early stage of infection (Bergmann et al., 2010b). Seven PCRs were tested to compare their sensitivity to detect viral DNA in the early stage of infection. The result showed single-round TK gene PCR, Nested TK gene PCR, single-round glycoprotein PCR, semi-nested PCR and real time PCR gave 80-100% positive from mucus swab within 1-4 dpi. Sample of mucus swab from fish survival 70 days after experiment could be able to detect by nested TK gene PCR, single-round PCR and real-time PCR (Monaghan et al., 2015a). However, single-round PCR detecting TK gene (Bercovier et al., 2005) is recommended by the world organization of animal health (OIE) to use as the gold standard for detecting CyHV-3 DNA. ELISAs have been developed to detect virus antigens in fish droppings samples (Dishon et al., 2005) and anti-CyHV-3 antibodies in blood of infected fish (Adkison et al., 2005; Abdullah et al., 2012). Immunohistochemistry has also been developed to be used for detecting CyHV-3 by producing a monoclonal antibody against ORF68 (Aoki et al., 2011). In the past few years, many scientists tried to develop diagnostic method with less time-consuming and no specialized equipment was required. The lateral flow device was developed for CyHV-3 diagnosis using non-invasive approach. This device was an immunochromatographic test for CyHV-3 antigen detection. It gave high specificity but require more copies of virus to be able to detect when compare to PCR

(Vrancken et al., 2013). Gold nanoparticles assay was developed for CyHV-3 DNA detection. The result showed that this method was rapid, precise and specific to detect virus in diseased fish. The test could detect viral DNA in very low number of copies which the sensitivity was 10 times lower than real-time PCR (Saleh and El-Matbouli, 2015).

2.1.7 Transmission

Fish survived from CyHV-3 infection had viral DNA in gill, heart, liver, eye, leukocytes and gastrointestinal epithelium which could excrete virus after stress was stimulated (Bergmann and Kempter, 2011; Eide et al., 2011). Although CyHV-3 caused high mortality only in common carp and koi, other cyprinids were reported to be able to detect viral DNA from their tissues (Bergmann et al., 2010b). Silver carp (*Hypophthalmichthys molitrix*), crucian carp (*Carassius carassius*), goldfish (*Carassius auratus auratus*), grass carp (*Ctenopharyngodon idella*) and golden ide (*Leuciscus idus*) did not show clinical signs after experimental challenge by cohabitation with CyHV-3 diseased koi (Ronen et al., 2003). Goldfish showed slightly swollen abdomen for 10 – 15 dpi with no mortality when infected with CyHV-3 by immersion or intraperitoneal injection. In contrast, koi with the same methods of infection showed clinical signs of the disease and had mortality up to 50% (Bergmann et al., 2010a). These are evidences of possible transmission by other fish species that can be carrier for CyHV-3. Virus is transmitted horizontally via the excrement of disease fish. The viral DNA was detected in fish droppings by using PCR and virus was confirmed to be active by cell culture and naïve fish inoculations (Dishon et al., 2005). Natural ecosystem is also suspected to be associated with disease transmission. Virus harvested from tissue cultures was proven to remain infective in water for at least 4 hours but not after 21 hours (Perelberg et al., 2003). The virus concentration method has been developed for detecting CyHV-3 DNA from water sample in natural lake that outbreak was occurred. The viral DNA was detected in high levels by using this method (Haramoto et al., 2009). The viral DNA was reported to be detected by quantitative real time PCR from planktons in the lake where the spread of the disease was reported (Minamoto et al., 2011). Soil and pond sediment were

suspected to be a reservoir for CyHV-3. Because the amount of viral DNA in natural sediment was detected a lot higher than in the water from the same location by using real time PCR (Honjo et al., 2012).

2.1.8 Prophylaxis and control

At present, there is no report of any drug or chemical that can effectively treat the virus in fish. Many criteria for viral prevention were reported. For example, to produce fish strains and crossbreeds for more resistance to the viral infection but it may not be practical for selecting resistance in ornamental koi (Israel, 2005). Immunization is another option to prevent those fish from infection. Unfortunately, the result by using inactivated virus liposome vaccine has been proven unsuccessful for protecting fish from disease (Yasumoto et al., 2006). Two methods of fish immunization were developed and used only in Israel are immunizing fish with pathogenic virus (natural immunization) or with a live attenuated virus vaccine (Perelberg et al., 2005). Natural immunization was developed base on the permissive temperature of virus during outbreak of the disease. Carp and koi fingerlings were exposed to virus by cohabitation with sick fish at 22 – 24°C for 2 - 5 days. Fish were then moved to non-permissive temperature at 30°C for 30 days. The survival rate of immunized fish was 60% (Ronen et al., 2003). The attenuated virus was isolated by serial transfer of viral isolate in cell line then was cloned in tissue culture. Cloned viruses were UV irradiated and were re-clone in order to insert mutations into the viral genome. The selected attenuated virus gave efficiently protection in immunized fish against challenge infection (Ronen et al., 2003; Perelberg et al., 2005). Recombinant CyHV3 vaccine was developed using BAC technology by using prokaryotic mutagenesis (Boutier et al., 2015). The result of using recombinant vaccine is still in the laboratory level which needs more study in field experiment. Many disinfectants, ultraviolet and heat were tested for their antiviral activity against CyHV-3. Iodophor, benzalkonium chloride, ethyl alcohol and sodium hypochlorite showed their efficacy to kill the virus at suitable dosage and time (Kasai et al., 2005). However, these agents are suitable to use only without fish or for eliminating the virus from fish utensils.

2.2 *Clinacanthus nutans* (Burm.f) Lindau

2.2.1 Introduction

Clinacanthus nutans (Burm.f) Lindau (*C. nutans*) is a Thai herbal plant in a family Acanthaceae. The local names in Thai are Phaya Yo, Phaya Plong Thong or Female Saletpangporn. In human, *C. nutans* has been used as a traditional medicine for treatment of skin lesions from Herpes simplex virus (HSV), Varicella-zoster virus (VZV) and snake or insect bite. Taxonomically this plant can be classified by kingdom: Plantae; phylum: Magnoliophyta; class: Magnoliopsida; subclass: Asteridae; order: Lamiales; family: Acanthaceae; genus: Clinacanthus Lindau; species: *C. nutans* (Burm. f.) Lindau. *C. nutans* leaves are simple, opposite, narrowly elliptic-oblong or lanceolate (2.5–13.0 cm long × 0.5–1.5 cm wide). The plant can grow up to 1 m tall with pubescent branches and cylindrical, striate, stems. The petiole is around 0.3–2.0 cm, sulcate pubescent. The leaves are apex acute or acuminate and exculpate; dentate or subentries margins. The leaf base are obtuse rounded or truncate and often oblique (Alam et al., 2016).

2.2.2 Phytochemistry

Phytochemical properties on various parts of *C. nutans* have been studied. C-glycosyl flavones; vitexin, isovitexin, shaftoside, orientin, isomollupentin 7-O-β-glucopyranoside and isoorientin were isolated from the methanolic extract of the stems and leaves of *C. nutans* (Teshima et al., 1997; Chelyn et al., 2014). The n-BuOH-soluble portion of a methanol extract gave five sulfur-containing glucosides from stem and leaves (Teshima et al., 1997). Chloroform extract from leaves was purified to give chlorophyll a and chlorophyll b related compound. They were 13^2-hydroxy-(13^2-S)-phaeophytin b, purpurin 18 phytylester and phaeophorbide a, 13^2-hydroxy-(13^2-R)-phaeophytin b, 13^2-hydroxy-(13^2-S)-phaeophytin a and 13^2-hydroxy-(13^2-R)-phaeophytin a (Sakdarat et al., 2009). Ages of plant and durations of storage were studied to be influenced in the phytochemical content of *C. nutans*. Total flavonoids were higher in younger plants than in older plants. Many phytochemical

compounds were decrease gradually due to longer period of plant storage (Raya et al., 2015).

2.2.3 Biological activity

In many literatures, a variety of biological activities and effects from leaves extract of *Clinacanthus nutans* have been described (Table 1).

Table 1 The biological activities of *C. nutans* extract that have been reported.

Bioactive constituents	**Activity**	**References**
stigmasteryl-3-O-β-D-glucopyranoside	Growth promoting activity	Boonyaratavej and Petsom (1991); Panyakom (2006)
13^2-hydroxy-(13^2-*S*)-chlorophyll b	Antioxidant activity	Panyakom (2006); Watanabe et al. (1993)
13^2-hydroxy-(13^2-*R*)-chlorophyll b	Antioxidant activity	Watanabe et al. (1993); Panyakom (2006)
Flavonoid	Anti-inflammatory activity	Satayavivad et al. (1996)
Flavone *C*-glycosides (shaftoside, orientin, isovitexin and vitexin)	Anti-inflammatory activity	Chelyn et al. (2014)
1-O-palmitoyl-2-Olinolenoyl-3-O-[α-D-galactopyranosyl-(1" 6')-O-β-D-galacctopyranosyl]-glycerol	Antiviral activity	Satakhun (2001)
1,2-Odilinolenoyl-3-O-β-D-galactopyranosyl-glycerol	Antiviral activity	Satakhun (2001)

Bioactive constituents	Activity	References
1,2-O-dilinolenoyl-3-O-b-d glucopyranosyl-sn-glycerol	Antiviral activity	Janwitayanuchit et al. (2003)
Cerebroside	Antiviral activity	Tuntiwachwuttikul et al. (2004)
Monoacyl monogalactosylglycerol	Antiviral activity	Tuntiwachwuttikul et al. (2004)
13^2-hydroxy-(13^2-*R*)-phaeophytin b	Antiviral activity	Sakdarat et al. (2009)
13^2-hydroxy-(13^2-*S*)-phaeophytin a	Antiviral activity	Sakdarat et al. (2009)
13^2-hydroxy-(13^2-*R*)-phaeophytin a	Antiviral activity	Sakdarat et al. (2009)
Monogalactosyl diglyceride	Antiviral activity	Pongmuangmul et al. (2016)
Digalactosyl diglyceride	Antiviral activity	Pongmuangmul et al. (2016)

2.2.4 Antiviral activity

In vitro studies showed that the extracts from *C. nutans* provide inhibition activity against Herpes Simplex Virus (HSV). Leaves extraction of *C. nutans* showed antiviral activities against Herpes Simplex Virus type 1 (HSV-1) (Tuntiwachwuttikul et al., 2004) and Varicella-Zoster Virus (VZV) (Thawaranantha et al., 1992). The ethanol leaves extracts were also inhibited Herpes Simplex Virus type 2 (HSV-2) or genital herpesvirus before infected to cell cultures but no intracellular inhibitory effects (Jayavasu et al., 1992; Yoosook et al., 1999). Pretreatment the cells with the crude extracts did not prevent HSV-2 infection (Jayavasu et al., 1992). Crude extract with methanol, dichloromethane and n-hexane was shown to inhibit HSV-1 and HSV-2 in cell culture (Kunsorn et al., 2013). Clinical use of *C. nutans* cream compared with Acyclovir in patients with HSV-2 and VZV showed the same effect. But *C. nutans* cream did not cause burning sensation to patient's skins which is side effect of Acyclovir (Jayavasu et al., 1992; Sangkitporn et al., 1995). Virucidal effect was studied against Newcastle Disease virus which is very important viral disease in avian. The

result showed direct antiviral activity of ethanol extract from *C. nutans* leaves before infection to cell line (Punyadarsaniya et al., 2015). Ethyl acetate soluble fraction from ethanol extract was isolated from *C. nutans* leaves. It gave 13 fractions which fraction 9 had anti-HSV-1 activity. Fraction number 9 was purified to be a mixture of nine cerebrosides and a monoacylmonogalactosylglycerol (Tuntiwachwuttikul et al., 2004). Vachirayonstien et al. (2010) studied the extracellular inhibitory effect of alcohol extract from *C. nutans* leaves against HSV-2 in cell culture. The viral DNA and protein quantities in the cells were investigated by western blot analysis. The result showed that the more purified the extract, the higher viral inactivation potency and also in a dose dependent manner. *C. nutans* extracts inhibited the virus before entering cells but had very low or no intracellular antiviral activity. However, there have been reports that the galactosyl diglycerides isolated from *C. nutans* and synthetic monoglycosyl diglycerides had an anti-HSV activities post-infection (Satakhun, 2001; Tuntiwachwuttikul et al., 2004). Three chlorophyll related compounds were isolated from chloroform extract showed antiviral activity against HSV-1. All compound showed percent inhibition as high as acyclovir in pre infection test in cell line. Post infection anti-HSV-1 showed low than 50% inhibition while acyclovir gave 100% inhibition effect (Sakdarat et al., 2009). Phenolic compound from water extract of *C. nutans* leaves was tested for cytotoxicity in brine shrimp. The researcher suggested the aqueous extract of *C. nutans* can be alternatively use because of less toxicity (Kosai et al., 2016).

2.2.5 Anti-inflammatory activity

The butanol extract from *C. nutans* leaves was reported to reduce inflammation in rats and active ingredient was identified as flavonoid. The 80% ethanol extract of aerial part showed a significant inhibition superoxide anion generation and the elastase release by activated neutrophils (Tu et al., 2014). Topical cream or lotion from *C. nutans* was sufficient for the relief of skin inflammation and insect bites (Satayavivad et al., 1996). Methanol extract of the leaves were tested by topical application. Rats were induced skin edema by using ethyl phenylpropiolate. The extract showed the most potent inhibition at 15 min with all dosages. The

underlying prevention of the release histamine and serotonin considered as initial inflammatory mediators of this pathway (Wanikiat et al., 2008). Oral administration of methanolic *C. nutans* extracts were given 1 hour before the rats were induced edema on the plantar side of the right hind paw by intradermal injection of carrageenan. The results showed significant reduction of foot volume of rats fed with plant extract compared with control group (Wanikiat et al., 2008).

2.2.6 Antioxidant activity

The anti-oxidant activity of *C. nutans* was reported from chloroform extract, methanol extract, water extract, ethanol extract and petroleum ether. The nitric oxide scavenging activity observed only in water extract and a relatively mild hydrogen peroxide scavenging activities recorded for chloroform, methanol and water extract (Yong et al., 2013). Petroleum ether extract of whole plant strongly scavenged 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) while methanolic extract of stem produced 70.0% radical scavenging activity. The crude ethanol extract presented highest scavenging activity of 67.65 ± 6.59% (Arullappan et al., 2014). The flavonoids, alkaloids and flavones may be affected with the higher radical scavenging activity (Teshima et al., 1997).

Many plants extracts were studied in order to replace the use of drugs and chemicals in aquatic animal aquaculture. Considering the side effects of drug treatment on human health and environmental residue, trending of natural products research in aquaculture had been increased since 2003 (Reverter et al., 2014). The activities of plant extracts in aquaculture were reported such as immunostimulants, appetite stimulators, growth promoters, antibacterial, anthelminthic, antifungal and antiviral. Previous studies of *C. nutans* antiviral activities were reported in fish and shrimp viruses. Ethanol extract of *C. nutans* had pre-infection antiviral activity against infectious hematopoietic necrosis virus (IHNV) and Oncorhynchus Masou virus (OMV) in fish cell line (Direkbusarakom et al., 1996). OMV or salmonid herpes virus 2 (SalHV2) is also known to be classified in the family Alloherpesviridae. The alcohol extracts from leaves of *C. nutans* was also effective

against yellow-head Rhabdovirus (YRV) in black tiger shrimp (*Penaeus monodon*). The result showed virucidal effect of *C. nutans* against the virus before injected into shrimp but there was no protective effect when feeding shrimp with the extract for a week before infection (Direkbusarakom et al., 1998). *In vitro* study of *C. nutans* extract against Cyprinid Herpesvirus 3 (CyHV-3) was tested in Koi fin cell line (KFC). The results showed antiviral activity of the extract against virus, both, before and after infection. The antiviral mechanism of extract in viral replication was not cleared. It was suspected to be effected to envelope of virus. Electron microscopic study showed defects on envelope of viral virion that was treated with *C. nutans* extract (Haetrakul, 2009).

CHAPTER III
MATERIALS AND METHODS

The experiments were divided into 3 phases

Phase 1: Extractions of *Clinacanthus nutans* (Burm.f.) Lindau

Phase 2: *In vitro* antiviral activities of *C. nutans* extracts against Cyprinid herpesvirus 3

Phase 3: *In vivo* antiviral activities of *C. nutans* extract against Cyprinid herpesvirus 3

3.1 Phase I: Herbal extractions

3.1.1 Plant material

Fresh aerial parts of *Clinacanthus nutans* (Burm.f.) Lindau was collected from medicinal herb plantation in Chiang Rai province during October to December 2013. The plants was washed thoroughly and dried before ground by using mixer grinder (Fig.1).

3.1.2 Extractions

C. nutans weight 9.8 kg was wrapped and soaked in 95% ethanol 36 liters at room temperature for 3 days (Fig.2). This method was repeated 3 times and the solutions were mixed together before filtration. The extract solution was filtered through the filter sheet and the filtrates were concentrated on a rotary evaporator to give dark green thick oil which was a crude extract. 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). The dichloromethane fraction was dark green thick oil and the n-butanol fraction was waxy brown. The dichloromethane fraction and n-butanol fraction were crude compounds. The solvent of each fraction was removed by evaporation in rotary evaporator. The extracts were then air dried or freeze dried and kept in -20°C freezer until used.

Figure 1 Plant material after grinding by using mixer grinder

Figure 2 The plant material was wrapped and soaked in 95% ethanol

3.2 Phase 2: *In vitro* antiviral activities of *C. nutans* extracts against Cyprinid Herpesvirus 3

The experiments were composed of:

3.2.1 Cell line and virus stock preparation

3.2.2 Viral assay

3.2.3 Cytotoxicity of *C. nutans* crude extract and 2 fractions of crude compound

3.2.4 Pre-infection antiviral activity of the extracts against CyHV-3

3.2.5 Post-infection antiviral activity of the extracts against CyHV-3

3.2.6 Statistical analysis

3.2.1 Cell and virus preparation

Koi Fin Cell line (KFC) (Fig.3) 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) (Sigma®) and 20% Leibovitz (L-15) medium supplemented with 10% fetal calf serum and antibiotics. Cells were cultured in T-75 flasks and incubated at 24°C. KFC were sub-cultured every 3 days until reach 95% confluent for 3 passages before using in the experiments.

Figure 3 Koi fin cell line at 95% confluent

Cyprinid Herpesvirus 3 (CyHV-3) was reactivated from - 80°C freezer. It was isolated from CyHV-3 infected koi that was confirmed by nested PCR (IQ2000TM KHV Detection System, Farming IntelliGene Tech.Corp®) at 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 ($TCID_{50}$) by using probit analysis with IBM SPSS statistics version 22 (New York, USA). CyHV-3 stocks were aliquoted into 1 ml and stored at -80°C until used.

3.2.2 Viral assay

Koi Fin Cell line was seeded with L-15-4% medium in 96-well plates. Cells were incubated at 24°C for 24 hours. The CyHV-3 was prepared into 10-fold serial dilution with L-15-2% for 5 concentrations. After 24 hours incubation, L-15-4% was removed from cell line in 96-well plate. Each dilution, 50 µl of virus was added for 8 wells. The virus was incubated with cell line at 24°C for 1 hour. Viral inoculum was removed and replaced with L-15-2% at 100 µl per well. The wells of control group were added with L-15-2%, 50 µl per well. Infected cells were incubated at 24°C in low temperature incubator for 14 days and were observed for cytopathic effect (CPE) daily. The 50% tissue culture infected dose ($TCID_{50}$) was calculated by using probit analysis with IBM SPSS statistics version 22 (New York, USA).

3.2.3 Cytotoxicity assay of *C. nutans* extracts

The cytotoxicity assay was performed to find the concentrations of *C. nutans* extracts that were not toxic to cells which were used in antiviral activity tests. The crude extract, dichloromethane fraction and n-butanol fraction were dissolved with dimethyl sulfoxide (DMSO) for preparing stock solutions. The concentration of crude extract stock solution was 500 mg/ml. The concentration of dichloromethane fraction stock solution was 1,000 mg/ml. The concentration of n-butanol fraction stock solution was 100 mg/ml. All extracts and DMSO were tested for their toxicity in cell line. Koi fin cell was seeded in 24-well plate and incubated at 24°C for 24 hours. The crude extract of *C. nutans* at 2,000, 1,000, 500, 100 and 50 µg/ml,

dichloromethane fraction at 1,000, 500, 100, 50 and 10 µg/ml, n-butanol fraction at 2,000, 1,500, 1,000, 500, 100 and 50 µg/ml were prepared from stock solutions. Each concentration of extracts was added at 500 µl per well in 6 wells. Control groups were added with 500µl of L-15-2% medium and DMSO at 0.1%, 0.5% and 1%, then incubated at 24ºC for 72 hours. Viable cells were counted by dying the cells with 1% trypan blue. Cell viability was then calculated to compare percent cell viability between experimental groups and control group by using chi square with IBM SPSS statistics version 22 (New York, USA). The results were considered statistically significant if the P value less than 0.05. The concentration that reduces the number of viable cell by 50% was evaluated as 50% cytotoxicity concentration (CC_{50}) by using doses-response curve between doses and percentages of survival cells and calculated by regression analysis

3.2.4 Pre-infection antiviral activity test

To compare the efficacy between crude extract, dichloromethane fraction and n-butanol fraction of *C. nutans* against CyHV-3 before infection in cell line. The extracts were prepared at sub toxic concentration to cell. The crude extract, dichloromethane fraction and n-butanol fraction were prepared at 1,000 µg/ml, 400 µg/ml and 1,000 µg/ml, respectively. All extracts were diluted as serial dilutions with L-15-2% for 5 concentrations. The crude extract was tested at 1,000, 500, 250, 125 and 62.5 µg/ml. The dichloromethane fraction was tested at 400, 200, 100, 50 and 25 µg/ml. The n-butanol fraction was tested at 1,000, 500, 250, 125 and 62.5 µg/ml. CyHV-3 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. After incubation, the mixtures were diluted with L-15-2% and prepared to 10-fold serial dilution for 5 concentrations. Cell medium was removed from 96-well plate and replaced with the mixtures, 50 µl per well in 8 wells for each concentration. All plates were incubated at 24ºC and cytopathic effect (CPE) was observed and recorded daily for 14 days. The control group 1 (positive control) was added with 10-fold serial dilution (5 concentrations) of virus without the extracts. The control group 2 (negative control) was added with L-15-2% without virus and extracts. The control

group 3 was added with the mixture between virus and DMSO at 0.1%. The end point dilution ($TCID_{50}$) of each extract at each period of contact time was calculated and tests were done in triplicates.

3.2.5 Post-infection antiviral activity test

To compare the efficacy between crude extract, dichloromethane fraction and n-butanol fraction of *C. nutans* against CyHV-3 after infected in cell line. CyHV-3 was prepared as 10-fold serial dilution for 5 concentrations with L-15-2% then added 50 µl of the virus per well in 8 wells on a 96-well plate. The infected cell was incubated at 24ºC for 1 hour then removed the inocula. The extracts were prepared in serial dilutions for 5 concentrations. The crude extract was tested at 1,000, 500, 250, 125 and 62.5 µg/ml. The dichloromethane fraction was tested at 400, 200, 100, 50 and 25 µg/ml. The n-butanol fraction was tested at 1,000, 500, 250, 125 and 62.5 µg/ml. The extracts were added at 100 µl in 8 wells per concentrations then incubated at 24ºC. CPE was observed and recorded daily for 14 days. The control group 1 (positive control) was added with 10-fold serial dilution of 5 concentrations of virus without the extracts. The control group 2 (negative control) was added with L-15-2% without virus and extracts. The control group 3 was added with DMSO 0.1%. The end point dilution ($TCID_{50}$) of each extract was calculated and tests were done in triplicates.

3.2.6 Statistical analysis

The end point dilution of virus in the antiviral activity experiments were calculated by using probit analysis with IBM SPSS statistics version 22 (New York, USA). The concentration that reduces viral titer over than 1 log10 which was 90% when compared to the control group was the effective concentration that inhibited viral replications. The extract that gave the best antiviral activities from phase 2 was selected to be used in phase 3 experiment.

3.3 Phase 3: *In vivo* antiviral activities of *C. nutans* extract against Cyprinid herpesvirus 3

To study the efficacy of *C. nutans* extract against Cyprinid Herpesvirus 3 in fish. The experiments were composed of:

3.3.1 Animal

3.3.2 Herbal extract

3.3.3 Toxicity test of *C. nutans* extract in koi

3.3.4 Evaluate the 50% Lethal dose (LD_{50}) of virus in koi

3.3.5 Pre-infection antiviral activity of *C. nutans* against Cyprinid Herpesvirus 3

3.3.6 Post-infection antiviral activity of *C. nutans* against Cyprinid Herpesvirus 3

3.3.7 Statistical analysis

3.3.8 Latent infection test

3.3.1 Animal

Koi carps (*Cyprinus carpio* koi) were obtained from a commercial fish farm in Thailand that was tested free from CyHV-3. The average length was 10 – 12 cm and average weight was 10 – 15 g. Fifteen koi carps were sampled randomly and tested negative to CyHV-3 by PCR. The quarantine process was done for 30 days at 28 – 30ºC. Fifty percent of water was changed daily. Koi carps were treated with 100% formalin at 25 ppm every 3 days for 3 treatments in order to exclude external parasites. Koi carps were screened for non-external parasites and no sign of bacterial infection before experiment**.** Koi carps were moved into 60 L tank of water with filtration system, at the density of 10 fish per tank and were kept at 24°C for 1 week before experiments. Koi carps were fed at 2% body weight with koi pellet commercial food once daily. Basic water quality parameters were recorded before and during the experiments. All carcasses from the experiment were burnt at the contagious carcass elimination facility. The tanks, equipment and water were treated with Sodium hypochlorite (Clorox®) at 250 mg/L for 30 minutes (Kasai et al., 2005).

3.3.2 Herbal extract

The *C. nutans* crude extract presented the highest antiviral activity from the results in phase 2 experiment was selected to be evaluated in the following experiments.

3.3.3 Toxicity test of *C. nutans* ethanol crude extract in koi

Ten koi carps were placed into a 60 L tanks, 3 replications for each concentration at 22 – 24°C. *C. nutans* crude extract was prepared in 5 different concentrations which were 5,000, 2,500, 1,000, 500 and 50 µg/ml. The extract was added every 72 hours for 3 times after 50% water change. Fish behavior and mortality were observed for 30 days. The control group 1 consisted of non-treated fish. The control group 2 was added with DMSO 0.1%. Each group contained 10 fish in each tank with 3 replications. The concentration of extract that killed 50% of fish was considered as the 50% lethal concentration (LC_{50}) by using dose-response curve between the concentrations of extract and fish mortality then calculated by regression analysis. Whole blood was taken in heparin tube and sent for biochemical analysis.

3.3.4 Evaluate the 50% lethal dose (LD_{50}) of virus in koi

CyHV-3 was prepared in 10-fold serial dilutions for 5 concentrations. Koi carps were sedated with clove oil prior to viral infection. Each dilution of virus was dropped at 50 µl onto each side of gill. Koi carps were wrapped in wet towel for 5 minutes before released back into the tank (Yasumoto et al., 2006). Koi carps were transferred to 60 L tanks, 10 fish per tank for 3 replications per concentration. The negative control was the group that koi carps were dropped with 50 µl of L-15-2% medium onto each side of gill, 10 fish per tank for 3 replications. Fish behavior and mortality were observed for 30 days at 22-24°C. Dead koi carps were checked for CyHV-3 by PCR to confirm that fish deaths were caused by CyHV-3 infection. The 50% lethal dose (LD_{50}) was determined by using dose-response curve between viral concentrations and fish mortality then calculated by regression analysis.

3.3.5 Pre-infection antiviral activity of *C. nutans* ethanol crude extract against Cyprinid Herpesvirus 3

CyHV-3 at the 50% lethal concentration was used in the experiment. *C. nutans* crude extract at the sub-toxic concentration was used at the highest concentration in the experiment. The extract was prepared by serial dilution for 4 concentrations which were 5,000, 1,000, 500 and 100 µg/ml. The virus was mixed with each concentration of extract then incubated at 24ºC for 1, 2, 3 and 4 hours. Koi carps were sedated with clove oil before dropping 50 µl of the mixture onto each side of the gill. Koi carps were wrapped in wet towel for 5 minutes before moving into 60 L tanks, 10 fish per tank for 3 replications. Koi carps in control group 1 (positive control) were dropped with 50 µl of CyHV-3 virus at the 50% lethal concentration (LD_{50}) onto each side of the gill, 10 fish per tank for 3 replications. Koi carps in control group 2 (negative control) were dropped with 50 µl of L-15-2% onto each side of the gill, 10 fish per tank for 3 replications. Koi carps in control group 3 were dropped with a mixture between 0.5% DMSO and CyHV-3 at 50 µl onto each side of the gill. Koi carps were moved into tanks, 10 fish per tank for 3 replications. Fish behavior and mortalities were observed daily for 30 days. The percent cumulative mortality in each group was calculated. Dead koi carps were checked for CyHV-3 by PCR to confirm that the deaths were caused by CyHV-3 infection. The samples of survival fish from experimental groups were collected and checked for viral latent infection.

3.3.6 Post-infection antiviral activity of *C. nutans* ethanol crude extract against Cyprinid Herpesvirus 3

Koi carps were infected with virus by sedation with clove oil before dropping 50 µl of virus at the concentration of LD_{50} onto both sides of the gill. Koi carps were wrapped in wet towel for 5 minutes before moving into a 60 L tanks, 10 fish per tank for 3 replications. *C. nutans* crude extract at the highest non-toxic concentration to fish was used in the experiment. The extract was prepared in serial dilution with L-15-2% for 4 concentrations which were 2,500, 1,000, 500 and

100 µg/ml. After inoculation for 0, 24 and 72 hours, each concentration of extract was added into the tanks and the treatment was repeated every 3 days for 3 treatments. Koi carps in control group 1 (positive control) were sedated with clove oil before dropping 50 µl of CyHV-3 virus at the 50% lethal concentration onto each side of the gill. Koi carps were wrapped in wet towel for 5 minutes then move into tanks, 10 fish per tank for 3 replications. Koi carps in control group 2 (negative control) were sedated with clove oil before dropping 50 µl of L-15-2% onto each side of the gill. Koi carps were wrapped in wet towel for 5 minutes then move into the tanks, 10 fish per tank for 3 replications. Koi carps in control group 3 were sedated with clove oil before dropping 50 µl of CyHV-3 virus at the 50% lethal concentration onto each side of the gill and DMSO at 0.25% concentration was added in to the tanks every 3 days for 3 times. Fish behavior and mortalities were observed daily for 30 days. The percent cumulative mortality in each group was calculated. Dead koi carps were checked for CyHV-3 by PCR to confirm that the deaths were caused by CyHV-3 infection. Dead koi carps were sent for histopathology examination. The samples of survival fish in experimental groups were collected to check for viral latent infection.

3.3.7 Statistical analysis

The data of fish mortalities in various concentrations of extract and times were analyzed by Chi square with IBM SPSS statistics version 22 (New York, USA). The results were considered statistically significant if the P value less than 0.05. The concentration that reduced fish mortality by 50% was considered as 50% effective dose (ED_{50}). ED_{50} was determined by using dose-response curve between crude extract concentrations and fish mortalities then calculated by regression analysis.

3.3.8 Latent infection

The survival koi carps from pre and post antiviral activity tests were moved into the tanks with average temperature was between 28 - 30°C. After 60 days at the non-permissive temperature, koi carps were moved back to the tanks with the average temperature was between 21 – 23 °C. Koi carps were kept for 30 days.

The gill biopsy samples and mucus samples were checked for CyHV-3 infection by PCR. Naïve koi carps were put into the tanks in order to perform co-habitation infection for 15 days. Stress induction was done by putting them into the net out of water for 30 seconds, twice (Bergmann and Kempter, 2011). After 7 days, gill samples and mucus samples were sent to check for CyHV-3 infection by PCR.

3.3.9 Histopathology

Dead koi carps were necropsy. The sample of gills, skin, heart, liver, spleen, kidney, intestine and brain were fixed in 10% formalin and processed for histopathological examination. Tissue sections were stained with hematoxylin and eosin (H&E).

CHAPTER IV
RESULT

4.1 Plant extraction

Dried plant materials weight 9.8 Kg were wrapped and soaked in 95% ethanol 36 L. After triple cycles of extraction, all solutions were mixed and filtered through filter paper and the filtrates were concentrated on a rotary evaporator to give dark green thick oil which was crude extract (3,764 g). The crude extract was partitioned between water and dichloromethane and the water layer then was extracted with n-butanol. The dichloromethane fraction was dark green thick oil (192.6 g) and the n-butanol fraction was brown wax (178.63 g).

4.2 *In vitro* antiviral activities of *C. nutans* extracts against Cyprinid herpesvirus 3

4.2.1 Cytotoxicity assay of *C. nutans* extracts

The toxic concentrations of extracts to KFC were determined by comparing % cell viability between experimental groups and control group. Crude extract starting from 1,000 µg/ml (Table 2), dichloromethane fraction at 100 µg/ml (Table 3) and n-butanol fraction at 1,000 µg/ml (Table 4) were significantly different from the control group ($P < 0.05$), 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 by using probit analysis with IBM SPSS statistics version 22 (New York, USA).

Table 2 Viability of KFC from ethanol crude extract cytotoxicity test (percent)

Crude extract	Rep. 1 (percent; n = 6)	Rep. 2 (percent; n = 6)	Rep. 3 (percent; n = 6)	Mean ± SD
Control	97.50 ± 1.52	97.33 ± 1.50	97.83 ± 1.17	97.56 ± 1.34
50 µg/ml	96.83 ± 1.17	95.83 ± 2.32	98.00 ± 0.63	96.89 ± 1.71
100 µg/ml	96.67 ± 1.75	95.67 ± 1.37	97.50 ± 1.22	96.61 ± 1.50
500 µg/ml	96.33 ± 1.75	95.33 ± 2.25	97.17 ± 0.98	96.28 ± 1.81
1,000 µg/ml	81.17 ± 4.21	84.67 ± 2.66	89.50 ± 1.64	85.11 ± 4.90*
2,000 µg/ml	33.00 ± 3.69	34.33 ± 4.59	30.00 ± 4.38	32.09 ± 2.04*
DMSO 1%	96.50 ± 1.52	96.17 ± 1.47	96.83 ± 1.31	96.50 ± 1.42*
DMSO 0.5 %	97.33 ± 1.51	97.00 ± 1.10	97.50 ± 0.84	97.28 ± 1.13*
DMSO 0.1%	98.00± 1.26	96.50 ± 1.05	97.50 ± 1.05	97.33 ± 1.24*

* Significantly different from control ($P < 0.05$)

Table 3 Viability of KFC from dichloromethane fraction cytotoxicity test (percent)

Dichloromethane fraction	Rep. 1 (percent; n = 6)	Rep. 2 (percent; n = 6)	Rep. 3 (percent; n = 6)	Mean ± SD
Control	98.33 ± 1.21	96.83 ± 2.04	97.50 ± 1.05	97.56 ± 1.54
10 µg/ml	98.00 ± 0.89	96.33 ± 1.37	97.67 ± 1.03	97.33 ± 1.28
50 µg/ml	97.17 ± 1.72	96.67 ± 1.51	97.83 ± 0.75	97.22 ± 1.40
100 µg/ml	87.83 ± 1.47	86.67 ± 1.63	86.00 ± 2.19	86.83 ± 1.86*
500 µg/ml	64.50 ± 2.88	63.33 ± 2.16	63.83 ± 3.66	63.89 ± 2.83*
1000 µg/ml	0.00	0.00	0.00	0.00*

* Significantly different from control ($P < 0.05$)

Table 4 Viability of KFC from n-butanol fraction cytotoxicity test (percent)

N-butanol fraction	Rep. 1 (percent; n = 6)	Rep. 2 (percent; n = 6)	Rep. 3 (percent; n = 6)	Mean ± SD
Control	97.67 ± 1.21	97.00 ± 0.89	97.50 ± 1.50	97.39 ± 1.04
50 µg/ml	97.83 ± 1.17	96.00 ± 0.89	97.17 ± 1.47	97.00 ± 1.37
100 µg/ml	97.33 ± 1.21	96.83 ± 1.32	97.17 ± 1.47	97.11 ± 1.28
500 µg/ml	96.83 ± 1.47	96.50 ± 1.22	97.00 ± 1.67	96.78 ± 1.40
1000 µg/ml	77.50 ± 1.05	76.17 ± 2.40	77.33 ± 1.63	77.00 ± 1.78*
1,500 µg/ml	64.67 ± 2.58	65.17 ± 1.72	65.00 ± 1.79	64.94 ± 1.95*
2,000 µg/ml	0.00	0.00	0.00	0.00*

* Significantly different from control ($P < 0.05$)

4.2.2 Viral assay

Infected cell was checked for CPE every day until 14 days. Cytopathic effect was observed in cell cytoplasm started on 6 days post inoculation. All cells in each well showed CPE on day 14. No CPE was found in negative control group. On day 14, viral titer was calculated by using probit analysis with IBM SPSS statistics version 22 (New York, USA). The end point dilution of viral titer was 2.38×10^4 $TCID_{50}$/ml.

4.2.3 Pre-infection antiviral activity

The result showed only ethanol crude extract had pre-infection antiviral activity against CyHV-3 infection in KFC. The highest concentration of dichloromethane fraction and n-butanol fractions at sub-toxic concentrations were not effective to inhibit virus before entering to cell. The direct antiviral activity effect of *C. nutans* ethanol crude extract against CyHV-3 at incubation period of 1, 2, 3 and 4 hours were shown in Figures 4 - 7. The effective concentrations of ethanol crude extract that significantly reduce virus titer (> 1 log 10) were started from 250 µg/ml at 1, 2, 3 and 4 hours pre-infection. The viral titers of CyHV-3 in effective concentration at 1, 2, 3 and 4 hour were 1.59×10^3, 1.32×10^3, 1.02×10^3 and 1.26×10^3 $TCID_{50}$/ml,

respectively. The control group titers were 1.75 x 10^4, 1.39 x 10^4, 1.15 x10^4 and 1.35 x 10^4 $TCID_{50}$/ml at 1, 2, 3 and 4 hours, respectively.

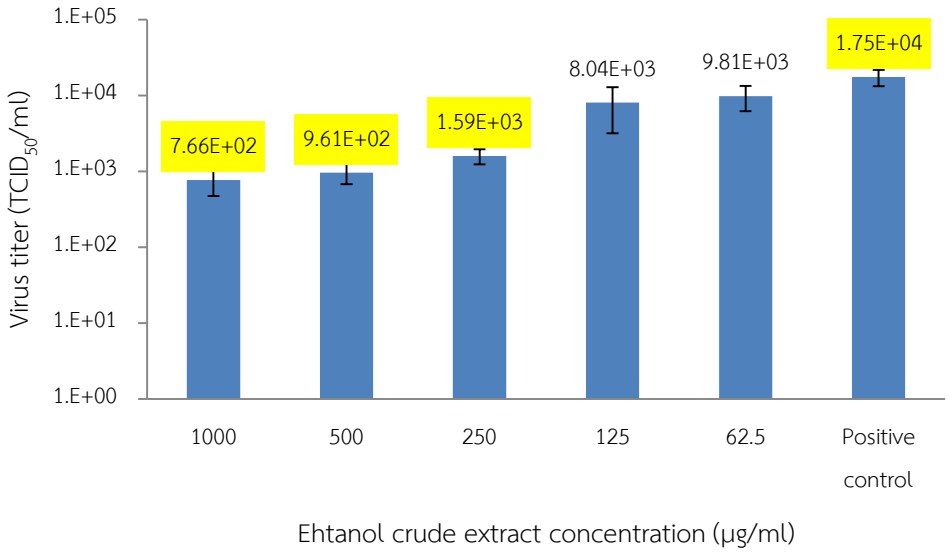

Figure 4 *In vitro* pre-infection antiviral assay of ethanol crude extract at 1 hour

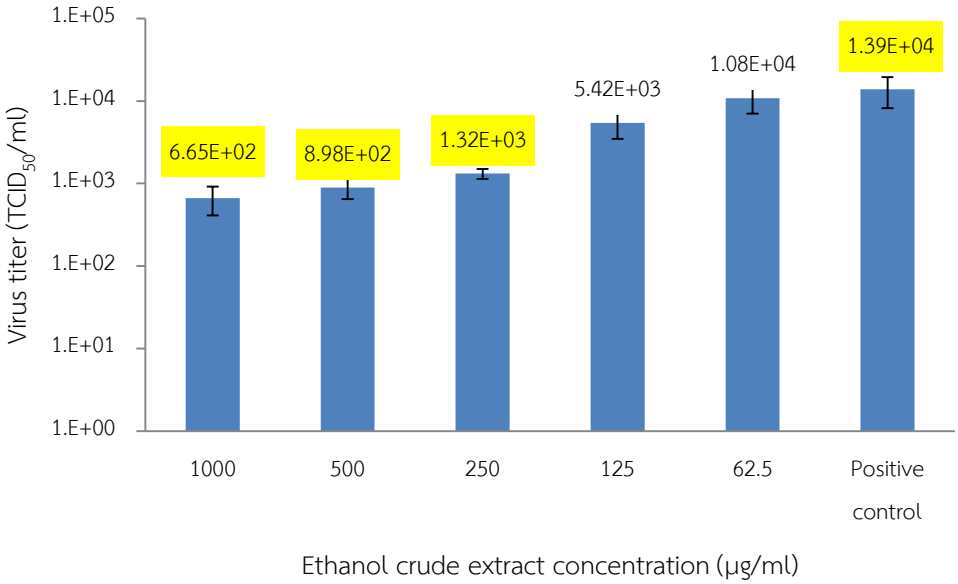

Figure 5 *In vitro* pre-infection antiviral assay of ethanol crude extract at 2 hour

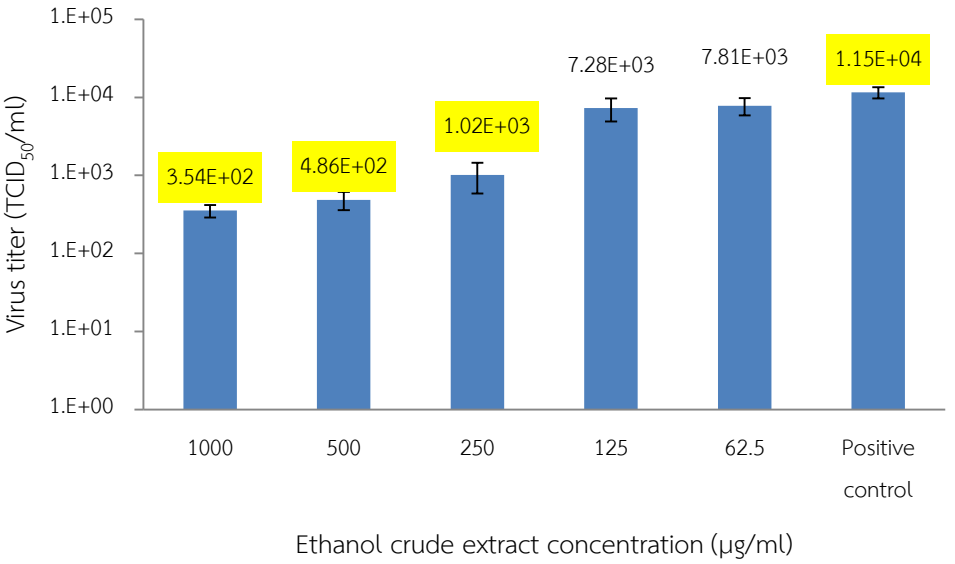

Figure 6 *In vitro* pre-infection antiviral assay of ethanol crude extract at 3 hour

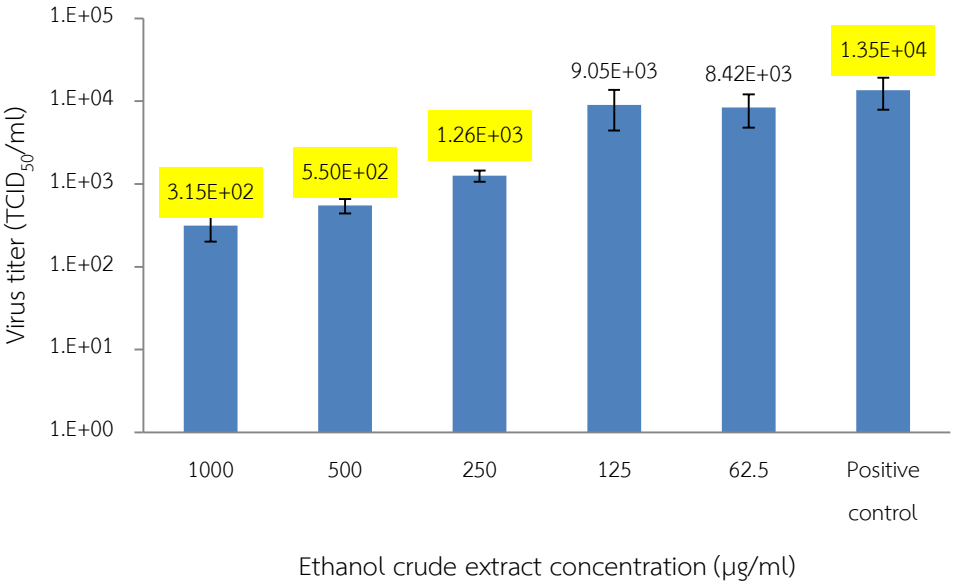

Figure 7 *In vitro* pre-infection antiviral assay of ethanol crude extract at 4 hour

4.2.4 Post-infection antiviral activity

The results showed that only ethanol crude extract was significantly effective in reducing viral concentration more than 1 log 10 (Fig.8). The viral titer in control group was 1.46 x 10^4 $TCID_{50}$/ml while in the effective group was 1.40 x 10^3 $TCID_{50}$/ml. The effective end-point concentration was starting from 500 µg/ml while the lower concentrations did not significantly reduce viral concentration when compared with the control group. Dichloromethane fraction and n-butanol fractions at non-toxic concentrations were not effective in inhibiting viral replication post-infection.

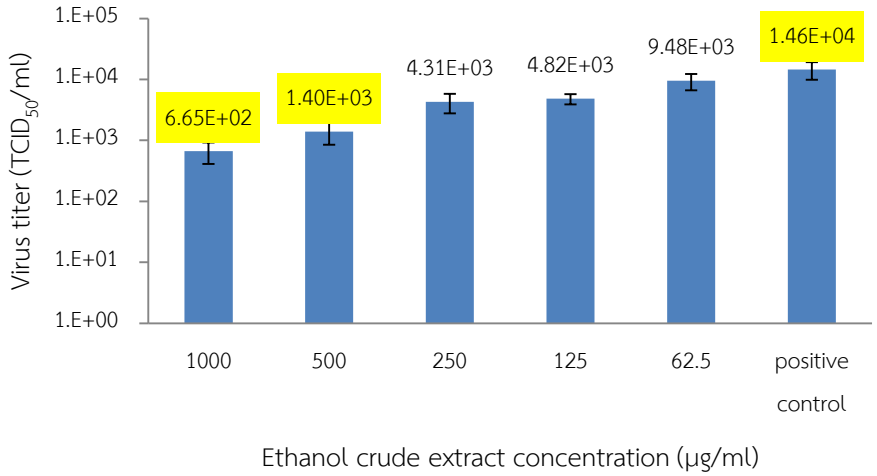

Figure 8 *In vitro* post infection antiviral assay of ethanol crude extract

From the result showed in phase 2 experiment, crude extract expressed the highest efficacy against viral infection in cell line. It was selected to be used in the following experiments.

4.3 *In vivo* antiviral activities of *C. nutans* extract against Cyprinid herpesvirus 3

4.3.1 Toxicity test of *C. nutans* ethanol crude extract in koi

C. nutans crude extract was tested at the concentration of 5,000, 2,500, 1,000, 500 and 50 µg/ml for their toxicity in fish. Fish in treatment group at 5,000 µg/ml showed less appetite comparing to other groups during treatment

period. The leftover food was approximately 25-30% in all 3 tanks. The water was greenish and fine sediment was seen. Some fish showed sign of grasping near water surface. All dead koi carps were noticed to have less weight and less body condition score by comparing the pre-treatment record. The first mortality was observed on day 14 post infection. Fish mortality in the group with 5,000 µg/ml was 13.33% at the end of experiment. Dead koi carps were noticed to have greenish pin point spots on their gills filaments. Fish in other groups did not show abnormal behavior or mortality. Since the result of mortality showed below 50% in the group at the highest concentration, the 50% lethal concentration (LC_{50}) value was estimated to be greater than the highest concentration which was higher than 5,000 µg/ml.

Water qualities were evaluated before adding the extract and on day 7, 14, 21 and 30. The tests were done by using water quality test kits (The department of biochemistry, faculty of veterinary science, Chulalongkorn university) The water hardness, alkalinity and pH of experimental groups at 5,000 µg/ml and 2,500 µg/ml were differed from control group on day 7 and 14 but within the normal range of water quality for freshwater fish (Appendix D).

Blood samples were taken from 6 koi carps in each herbal concentration. Blood samples were tested for percent pack cell volume (%PCV) or hematocrit, Alanine Aminotransferase (ALT), Aspatate Aminotransferase (AST), Alkaline Phosphatase (ALP), Blood Urea Nitrogen (BUN) and Creatinine by using the IDEXX VetTest® Chemistry Analyzer (DKSH company, Thailand). The average results showed in Table 5 with the normal reference in koi.

Table 5 Blood biochemical results from fish in toxicity test of *C. nutans* ethanol crude extract

Day 30	Control (n = 6)	5000 µg/ml (n = 6)	2500 µg/ml (n = 6)	1000 µg/ml (n = 6)	500 µg/ml (n = 6)	50 µg/ml (n = 6)	Normal range (Carpenter , 2013)
Hematocrit (%)	28.33 ± 3.01	27.67 ± 2.16	25.17 ± 2.32	26.5 ± 2.17	27 ± 2.35	28 ± 3.58	35 (24 - 43)
	18.67 ± 4.08	20.67 ± 6.02	19.17 ± 4.45	19 ± 4.56	20.67 ± 4.32	16.67 ± 3.01	31 (9 - 98)
AST (U/L)	174 ± 30.81	188 ± 48.08	169.17 ± 34.24	171.17 ± 31.86	167.33 ± 38.67	155.67 ± 34.02	121 (40 - 381)
ALP (U/L)	21.83 ± 6.55	23.33 ± 5.89	22.5 ± 5.75	19.33 ± 4.18	22.36 ± 2.94	22.5 ± 5.17	12 (4 - 56)
BUN (mg/dl)	2.12 ± 0.18	2.17 ± 0.27	2.1 ± 0.19	2.06 ± 0.1	2.09 ± 0.14	2.03 ± 0.06	2 (0.2 - 5)
Creatinine (mg/dl)	0.28 ± 0.15	0.29 ± 0.18	0.27 ± 0.12	0.28 ± 0.07	0.26 ± 0.08	0.25 ± 0.09	N/A

4.3.2 Evaluate the 50% lethal dose (LD_{50}) of virus in koi

The first mortality was observed on day 3 post infection in the groups that were infected with stock virus and at the group with dilution 10^{-1}. Mortalities in other groups were started on day 5-6 post infection and peak on day 10-12 post infection. All koi carps at the highest concentration group died on day 8 post infection. Total % cumulative mortality in the group exposed to viral dilution at 10^{0}, 10^{-1} and 10^{-2} were 100%. Total % cumulative mortality in the group exposed to viral dilution at 10^{-3}, 10^{-4}, 10^{-5} were 77.78%, 63.89% and 2.78%, respectively (Fig. 9). LD_{50} was $10^{2.77}$ $TCID_{50}$/100 µl calculated by probit analysis using IBM SPSS statistics version 22 (New York, USA).

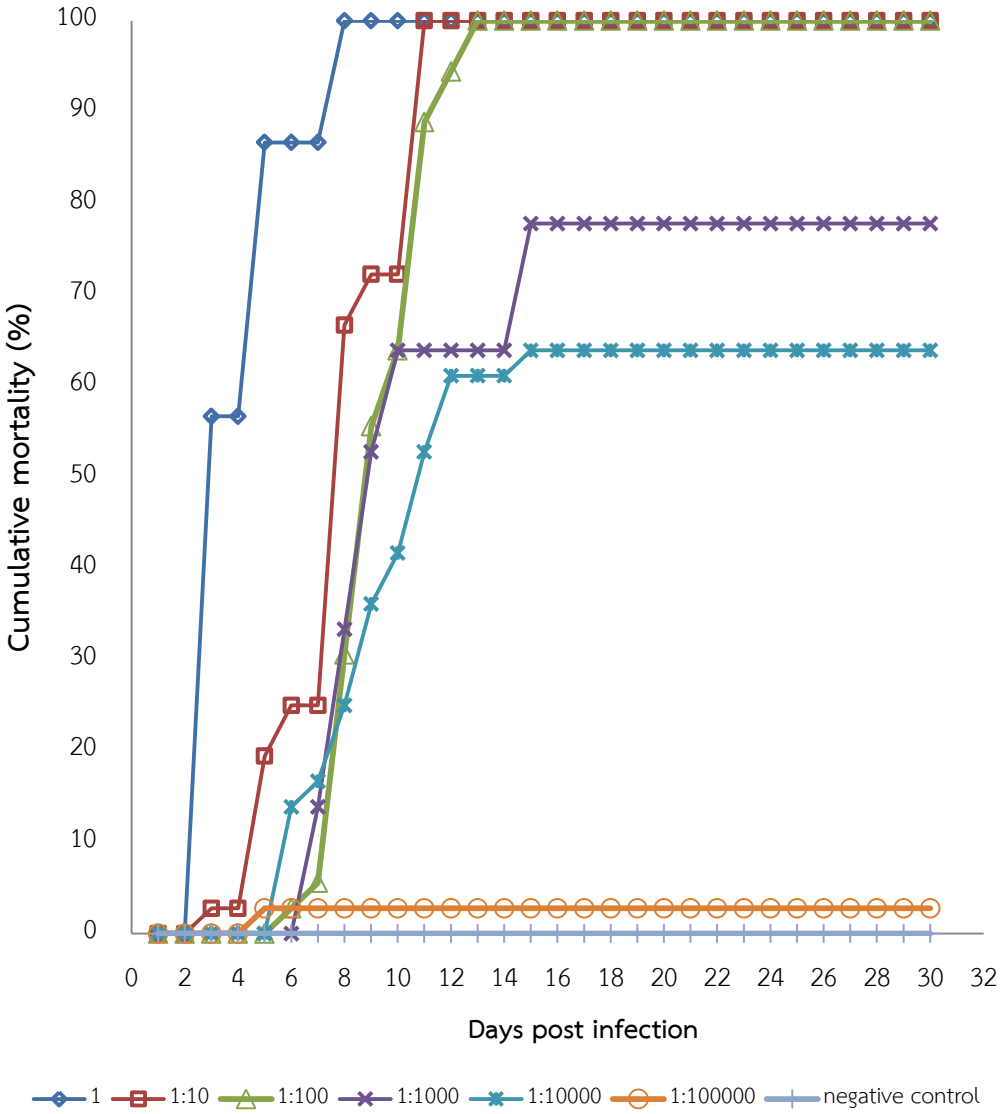

Figure 9 Percent cumulative mortality of koi carps that were challenged with CyHV-3

4.3.3 Pre-infection antiviral activity of *C. nutans* ethanol crude extract against Cyprinid Herpesvirus 3

C. nutans extract at 5,000, 1,000, 500 and 100 µg/ml concentrations were mixed with CyHV-3 stock virus at 5.02 x 10^2 $TCID_{50}$/ml. The mixtures were left at 24°C for 1, 2, 3 and 4 hours. There was no mortality nor abnormal behavior of fish in the group at 5,000 µg/ml concentration at 1, 2, 3 and 4 hours until end of the experiment. First mortality in other groups was observed on day 6 – 9 post infection

and peak on day 14 – 18 post infection. The percent cumulative mortalities of fish in 1,000 µg/ml groups were decreased significantly compared to positive control groups at 1, 2, 3 and 4 hours ($P < 0.05$) (Table 6 and Fig. 10 - 13). The 50% effective doses (ED_{50}) were 991.59 µg/ml, 782.72 µg/ml, 749.05 µg/ml and 707.78 µg/ml at 1, 2, 3 and 4 hours, respectively by using probit analysis with IBM SPSS statistics version 22 (New York, USA). The therapeutic index (LD_{50}/ED_{50}) of *C. nutans* ethanol crude extract against CyHV-3 in koi pre-infection were at least 5.04, 6.39, 6.68 and 7.06 at 1, 2, 3 and 4 hours, respectively. Fish exhibited abnormal behaviors such as less appetite and weakness a few days before death. Gill necrosis, sunken eyes, hyphema and herpetic lesions were evidenced in fish that died during peak mortality (Fig.14 – 15). Koi carps with neurological signs were seen on the third week after infection and a few days before death. All dead koi carps were tested to be positive to CyHV-3 by PCR.

Table 6 Pre-infection: average percent cumulative mortality in each concentration of *C. nutans* ethanol crude extract at different incubation period (1, 2, 3 and 4 hours)

Incubation period	Crude extract 5000 µg/ml (percent; n = 3)	Crude extract 1000 µg/ml (percent; n = 3)	Crude extract 500 µg/ml (percent; n = 3)	Crude extract 100 µg/ml (percent; n = 3)	Positive control (percent; n = 3)
1 hour	0*	43.33* ± 5.77	80.00 ± 10.00	83.33 ± 5.77	83.33 ± 5.77
2 hours	0*	36.67* ± 11.55	70.00 ± 0.00	76.67 ± 5.77	80.00 ± 10.00
3 hours	0*	33.33* ± 5.77	66.67 ± 15.28	73.33 ± 11.55	76.67 ± 11.55
4 hours	0*	30.00* ± 10.00	73.33 ± 5.77	73.33 ± 15.28	76.67 ± 5.77

*: Significantly different from positive control ($P < 0.05$)

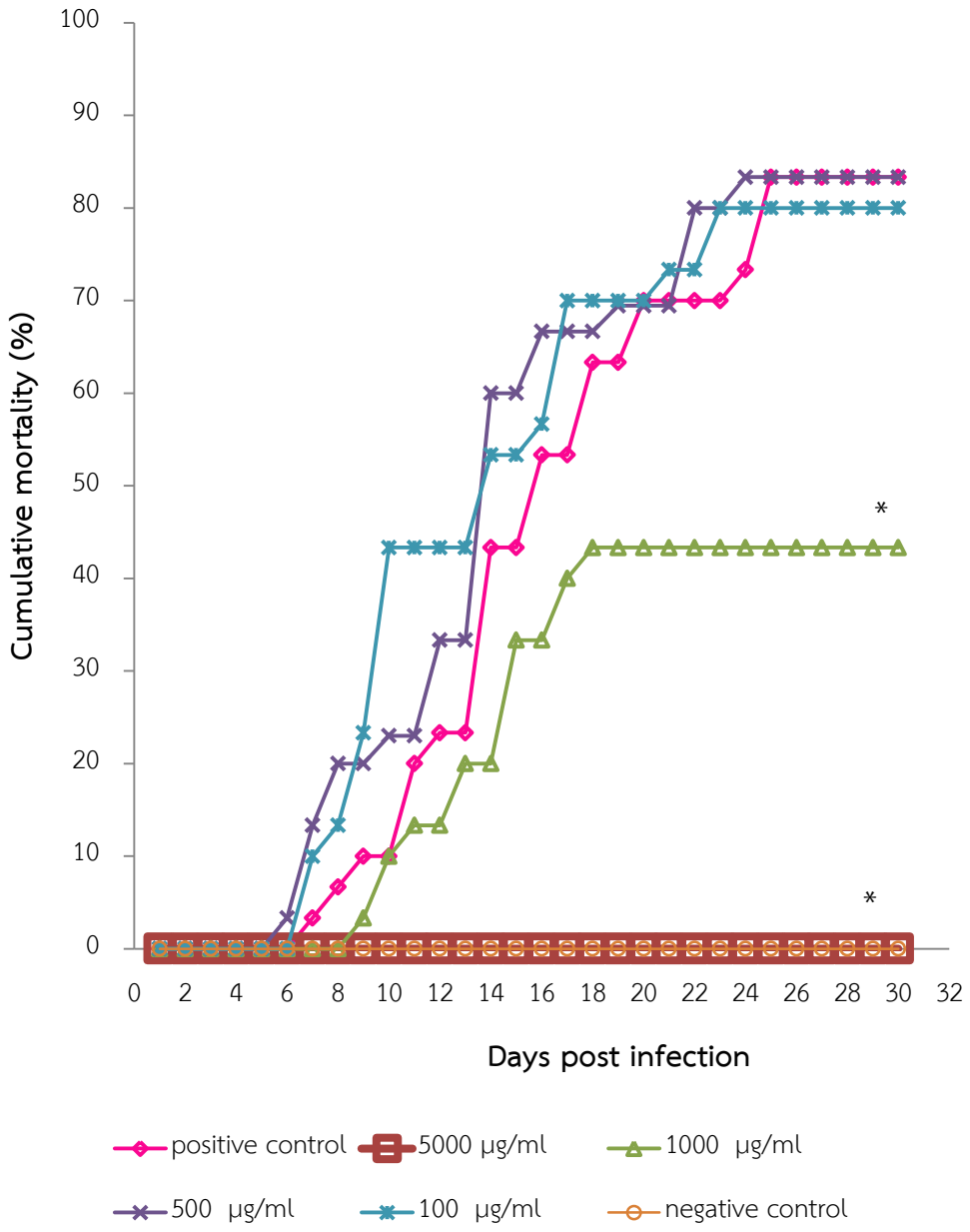

Figure 10 Cumulative mortalities of fish in pre-infection antiviral activity test of *C. nutans* ethanol crude extract at 1 hour (n = 30)

*: Significantly different from positive control ($P < 0.05$)

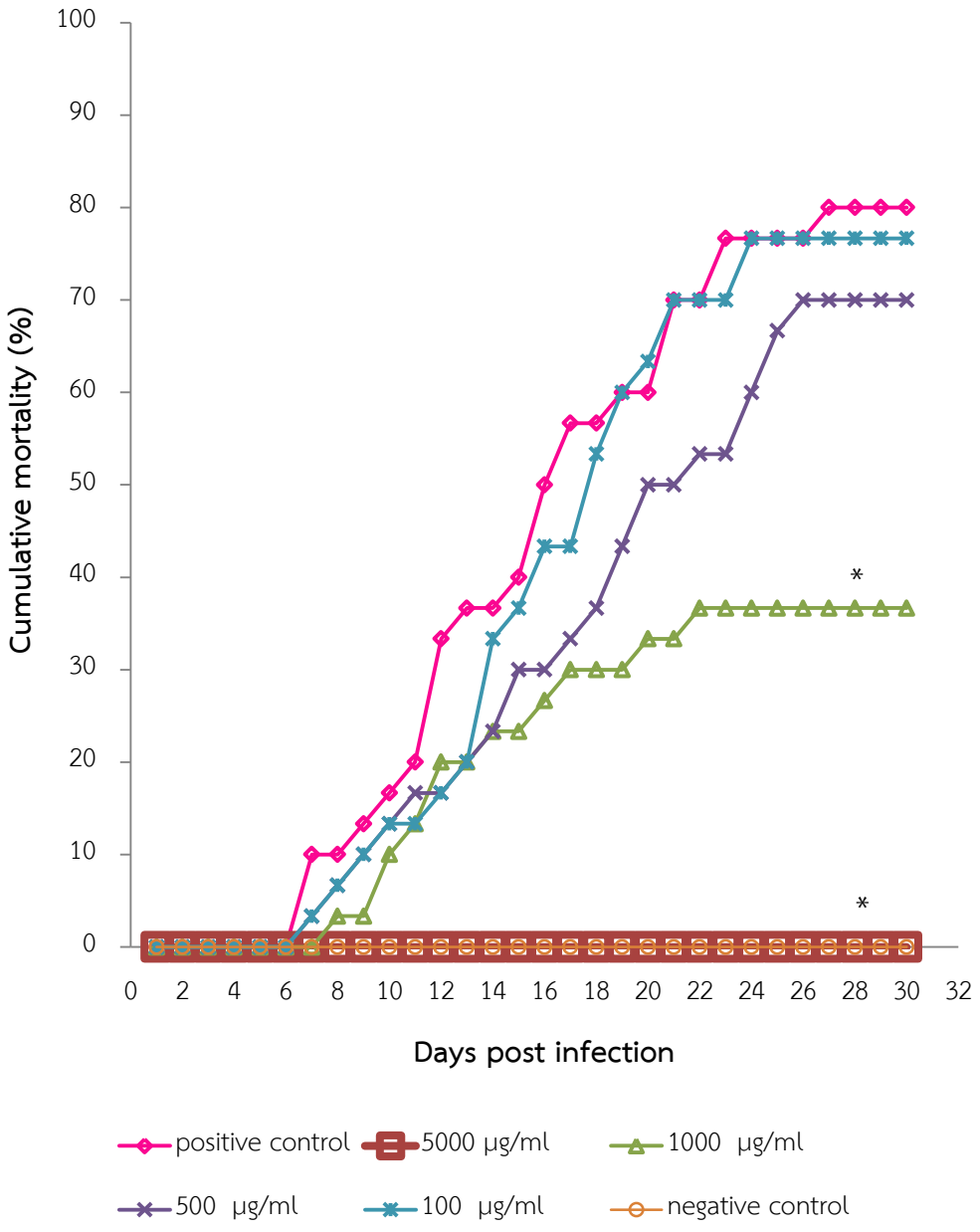

Figure 11 Cumulative mortalities of fish in pre-infection antiviral activity test of *C. nutans* ethanol crude extract at 2 hour (n = 30)

*: Significantly different from positive control ($P < 0.05$)

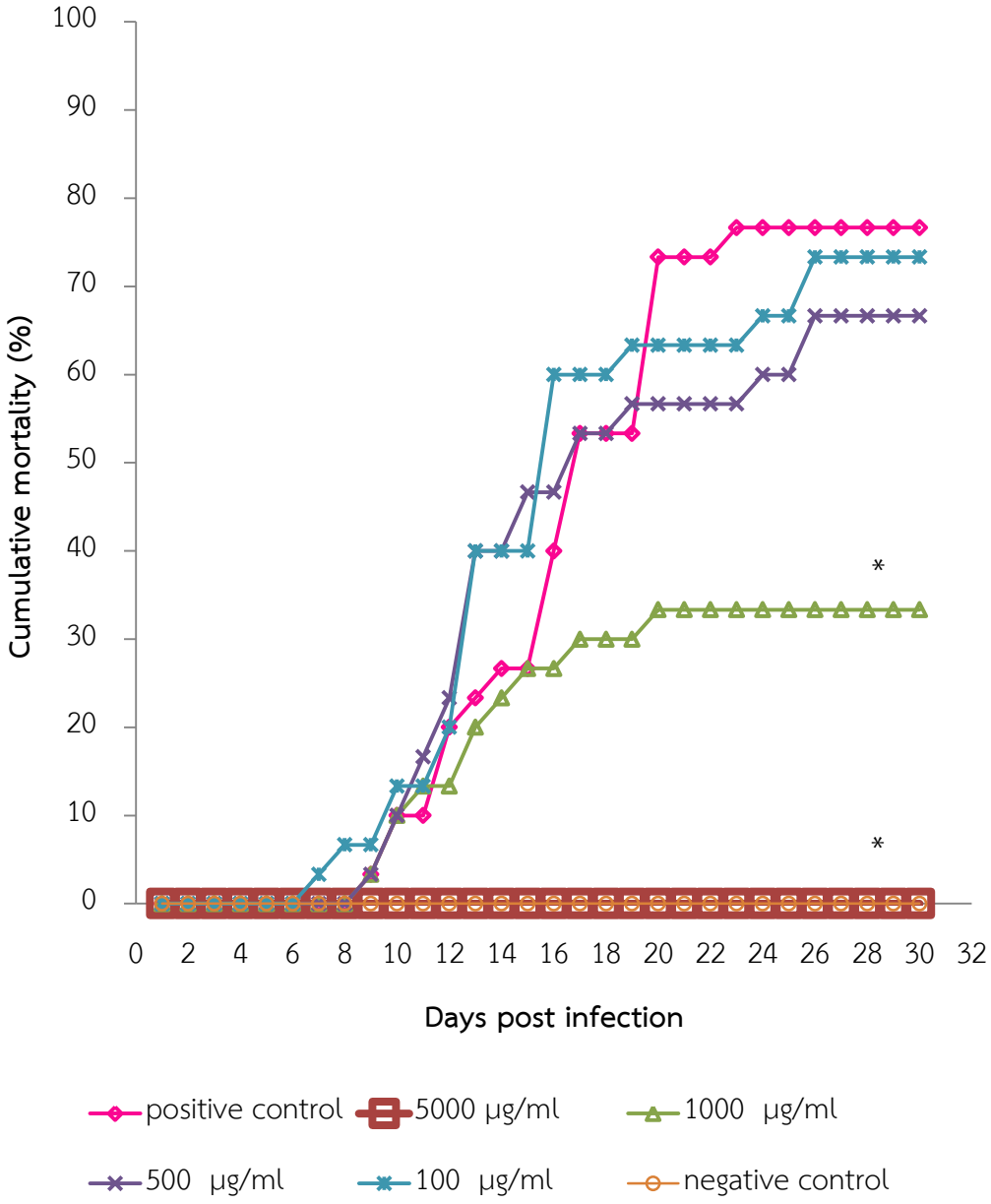

Figure 12 Cumulative mortalities of fish in pre-infection antiviral activity test of *C. nutans* ethanol crude extract at 3 hour (n = 30)

*: Significantly different from positive control ($P < 0.05$)

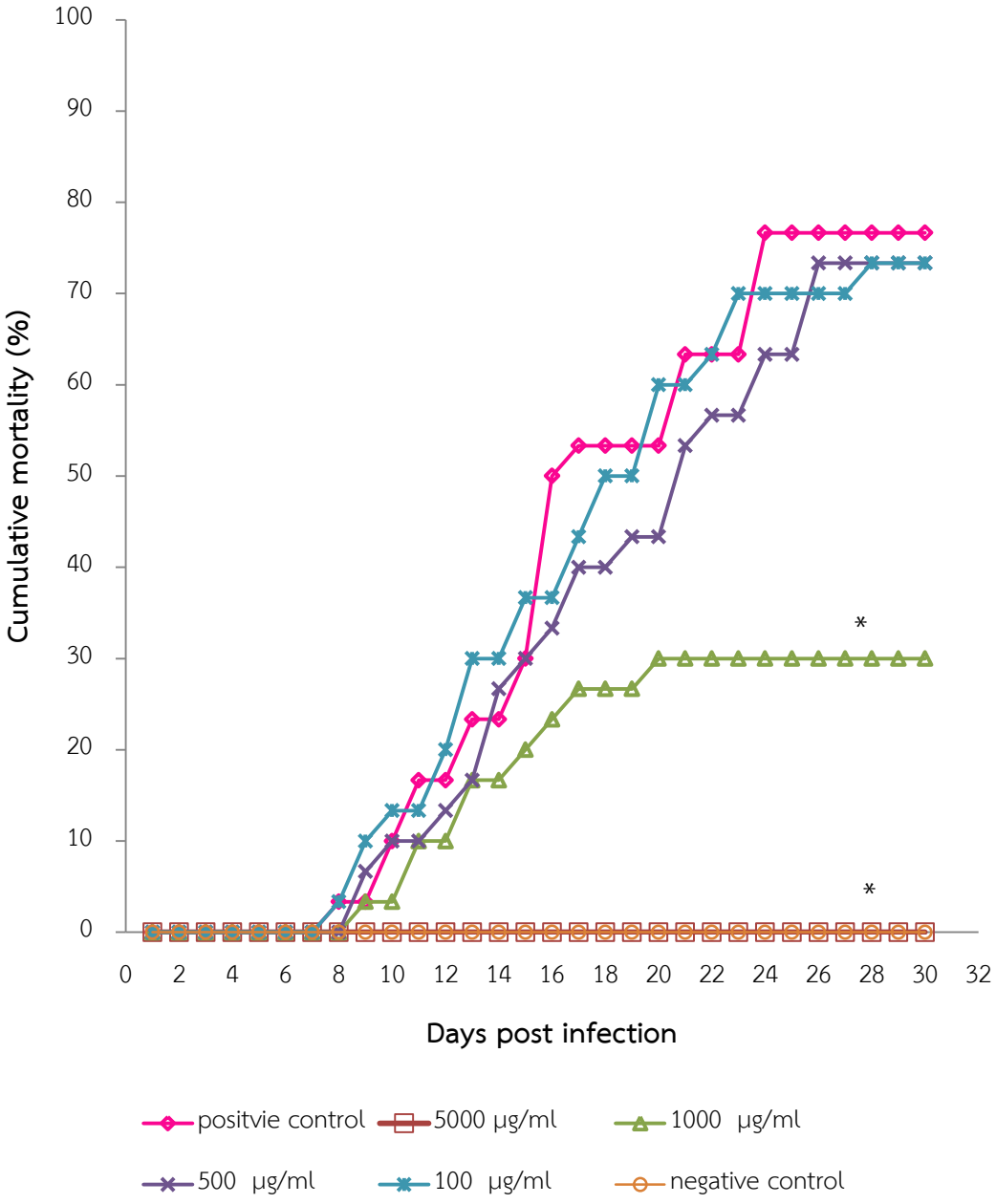

Figure 13 Cumulative mortalities of fish in pre-infection antiviral activity test of *C. nutans* ethanol crude extract at 4 hour (n = 30)

*: Significantly different from positive control ($P < 0.05$)

Figure 14 Sunken eye, gill erosion and necrosis of dead fish from pre-infection antiviral activity test of *C. nutans* ethanol crude extract

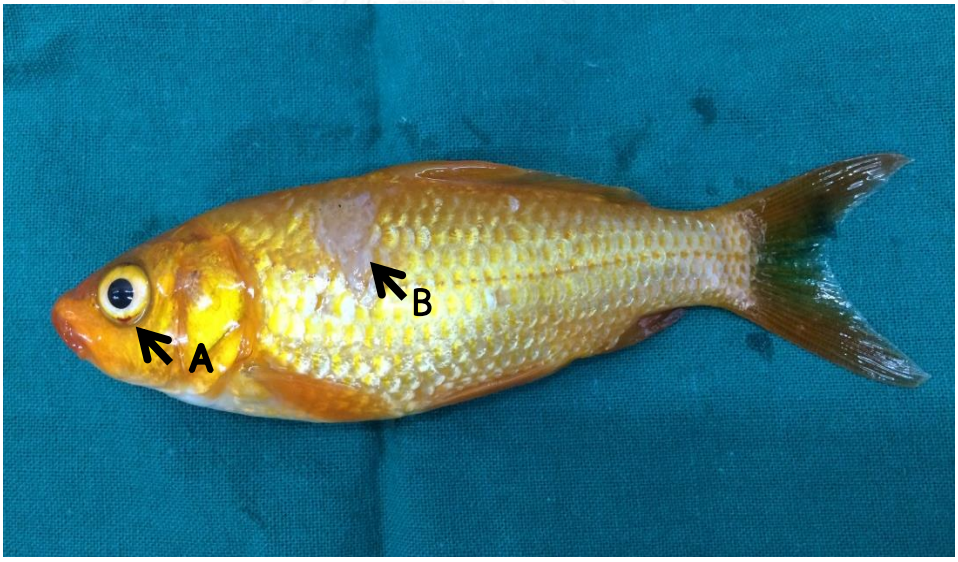

Figure 15 Hyphema (head arrow A) and herpetic lesion on skin (head arrow B) of dead fish from pre-infection antiviral activity test of *C. nutans* ethanol crude extract

4.3.4 Post-infection antiviral activity of *C. nutans* ethanol crude extract against Cyprinid Herpesvirus 3

Koi carps were infected with CyHV-3 stock virus at 5.02 x 10^2 $TCID_{50}$/ml and treated with *C. nutans* crude extract by bath at 2,500, 1,000, 500 and 100 µg/ml. The extracts were added at 0, 24 and 72 hour post infection. Treatments were repeated every 3 days for 3 times. The mortality of each group showed in Figure 16 – 18. The % cumulative mortalities of koi carps in group 2,500 µg/ml were decreased significantly when compared to positive control group at 0 and 24 hours ($P < 0.05$) (Table 7). The 50% effective dose (ED_{50}) were 2,050.88 µg/ml and 2,337.74 µg/ml at 0 and 24 hours, respectively by using probit analysis with IBM SPSS statistics version 22 (New York, USA). Fish in group treated with concentration 2,500 µg/ml showed delay mortalities compared to other concentrations and control even in the group at 72 hours. The therapeutic index (LD_{50}/ED_{50}) of *C. nutans* ethanol crude extract against CyHV-3 post infection were at least 2.44 and 2.14, at 0 and 24 hours, respectively. Fish exhibited abnormal behaviors such as less appetite and weakness a few days before death. Gill necrosis, sunken eyes, hyphema and herpetic lesions were found in fish that died during peak mortality. Koi carps with neurological signs were found in the third weeks after infection and few days before death. All dead koi carps were tested positive to CyHV-3 by PCR.

Table 7 Post infection: average percent cumulative mortality in each concentration of *C. nutans* ethanol crude extract at each treatment period of time

Treatment period	*C. nutans* 2,500 µg/ml (n = 3)	*C. nutans* 1,000 µg/ml (n = 3)	*C. nutans* 500 µg/ml (n = 3)	*C. nutans* 100 µg/ml (n = 3)	Positive control (n = 3)
0 hour	40 ± 10*	80 ± 10	83.33 ± 5.77	83.33 ± 5.77	86.67±15.28
24 hours	46.67* ± 5.77	73.33 ± 11.55	80 ± 10	83.33 ± 11.55	86.67 ± 15.28
72 hours	73.33 ± 5.77	83.33 ± 15.28	83.33 ± 5.77	90 ± 8.33	86.67 ± 15.28

*: Significantly different from control ($P < 0.05$)

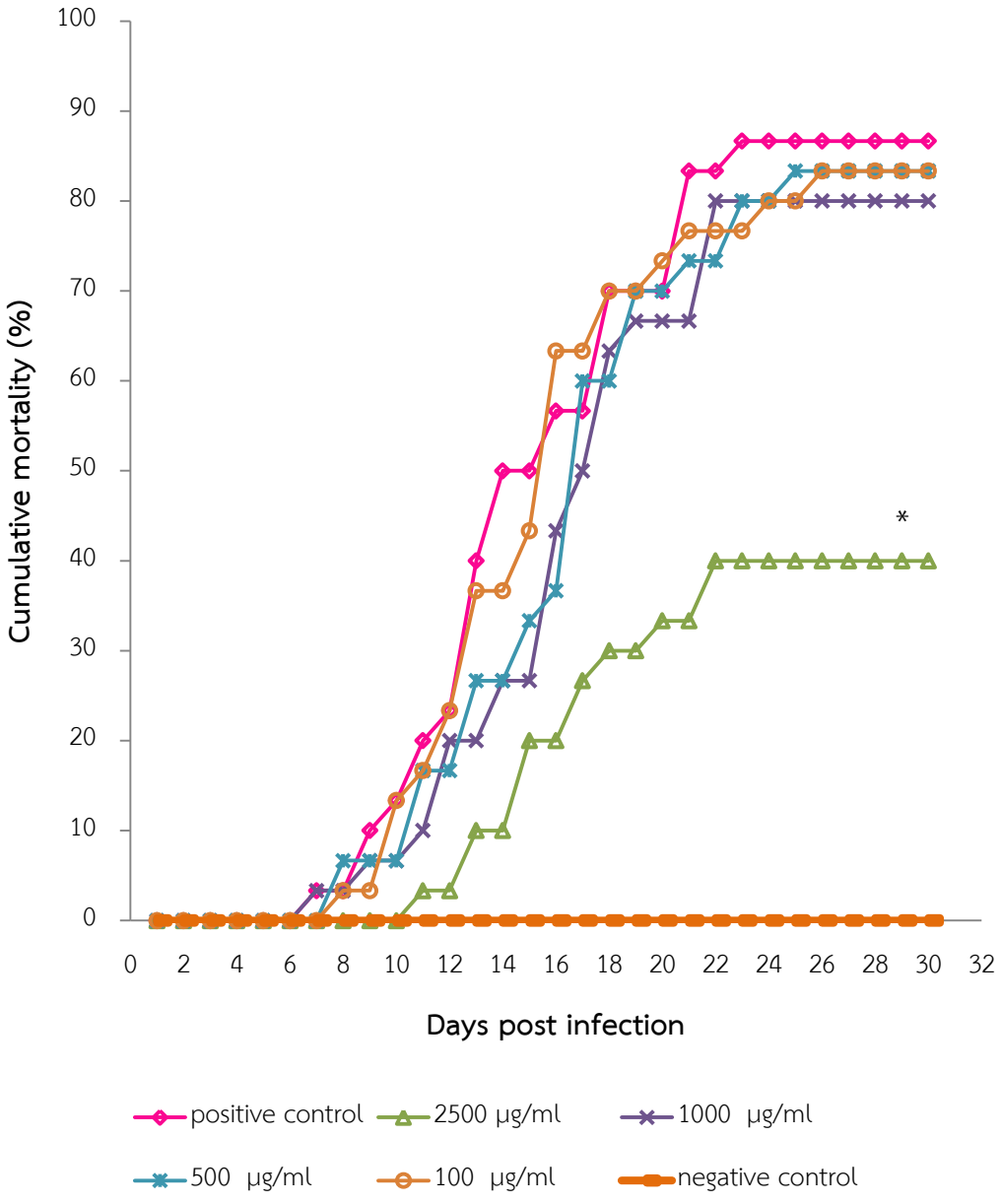

Figure 16 Percent cumulative mortalities of fish in post-infection antiviral activity test of *C. nutans* ethanol crude extract at 0 hour post infection (n=30)

*: Significantly different from positive control ($P < 0.05$)

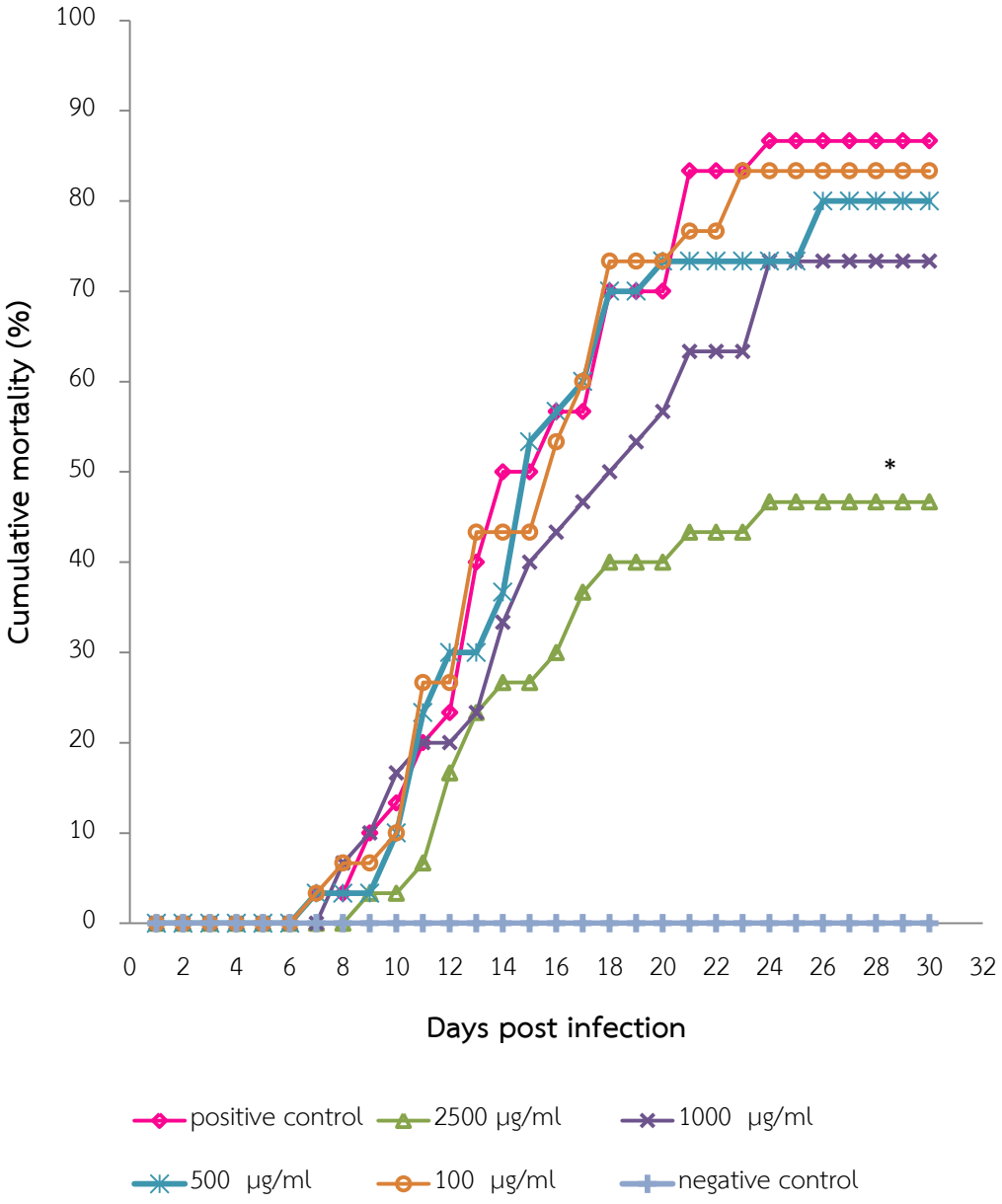

Figure 17 Percent cumulative mortalities of fish in post-infection antiviral activity test of *C. nutans* ethanol crude extract at 24 hour post infection (n=30)

*: Significantly different from positive control ($P < 0.05$)

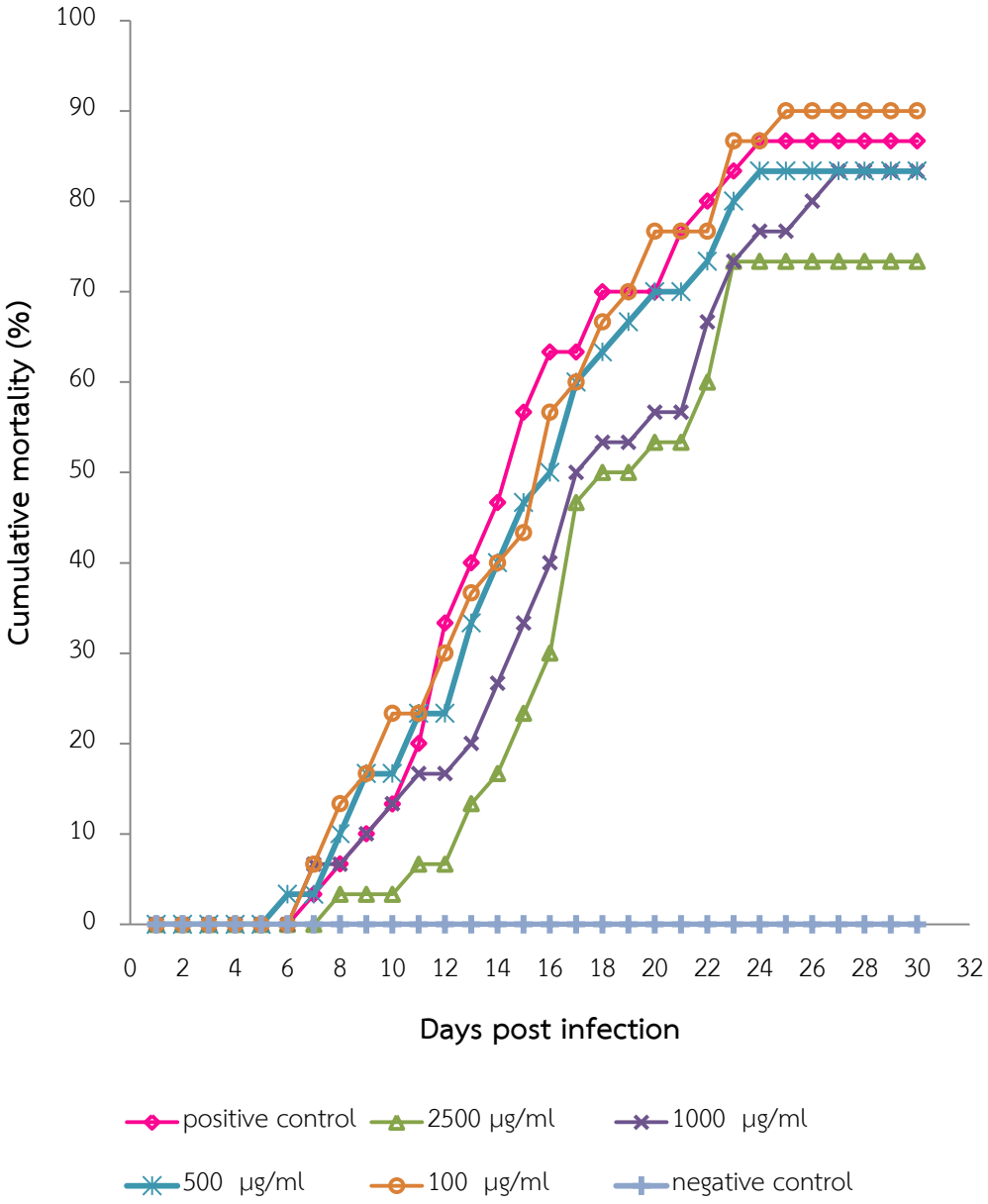

Figure 18 Percent cumulative mortalities of fish in post-infection antiviral activity test of *C. nutans* ethanol crude extract at 72 hour post infection (n=30)

4.4 Latent infection

Survived koi carps were moved into the tanks at temperature between 28 - 30℃. The amount of koi carps using in this experiment as show in Table 8. All koi carps were healthy and had normal appetite with no abnormal signs of infection. After 60 days, they were moved back to the temperature between 21 – 23 ℃ and were kept for 30 days. Gill biopsy samples and mucus sample were negative to CyHV-3 infection by PCR. Koi carps did not show any sign of abnormality or sign of viral infection. After 15 days of co-habitation with naïve koi carps, the stress induced method was performed. After 7 days, gill samples and mucus were sent to check for CyHV-3 by PCR and all samples gave negative result to CyHV-3 infection (Table 8).

Table 8 The amount of koi carps from pre and post antiviral activity tests using in latent infection experiment and result after stress was induced

Experiment	Amount	PCR result	Fish mortality
Pre-infection			
1 hour 5,000 µg/ml	10	negative	0
2 hour 1,000 µg/ml	10	negative	0
3 hour 500 µg/ml	5	negative	0
Post infection			
0 H 2,500 µg/ml	5	negative	0
0 H 1,000 µg/ml	3	negative	0

4.5 Histopathology

Dead koi carps were sent for histopathological examination. The results showed viral intranuclear inclusions in gill tissue samples (Fig 19) and intestinal epithelium (Fig 20). Intranuclear inclusion bodies were seen as basophilic material within the nucleus with marginal hyperchromatosis on the inner nuclear membrane. Gill lesions were branchitis, granulomatous, multifocal to coalescing, mild with occasional lamellar blunting and fusion. Skin from fish with herpetic lesion showed dermatitis, necrotizing, granulocytic and histiocytic, diffuse, marked with necrotizing

vasculitis, scale loss, frequent intranuclear viral inclusions and myocytic atrophy and degeneration (Fig 21). Intestinal sample showed enteritis, granulomatous and granulocytic, subepithelial, multifocal, marked with epithelial intranuclear viral inclusions. Heart myocarditis was noticed from sample in later stage of infection. Liver sample was found thrombosis, multifocal, moderate with necrotizing vasculitis, and multifocal, hepatocellular degeneration and necrosis. Spleen also showed lesions of histiocytosis, multifocal to coalescing, moderate with hemosiderosis and erythrophagocytosis. Fish, with neurological sign ie. swirling, had histopathological lesions in brain tissue with multifocal, mild neuronal degeneration and neuropil edema.

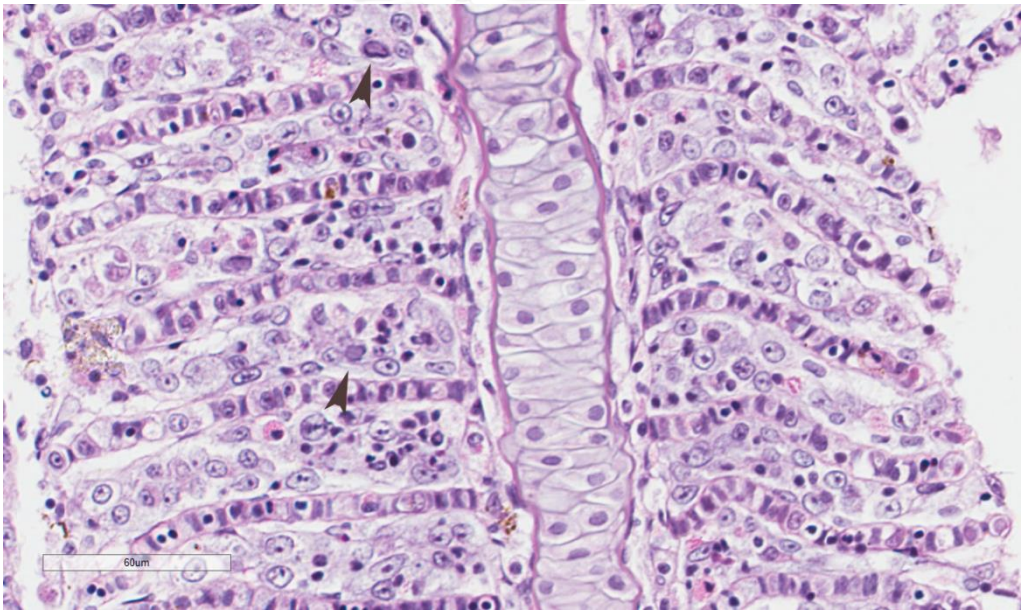

Figure 19 Intranuclear inclusion bodies in gill lamellae (head arrows) (400x) of dead fish from post infection antiviral activity test of *C. nutans* ethanol crude extract

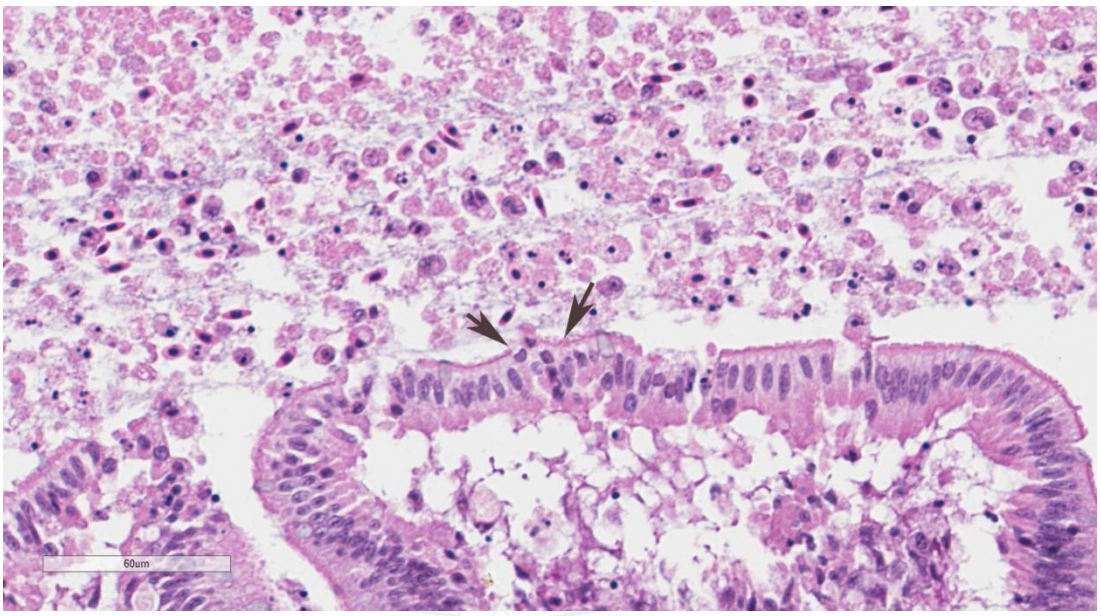

Figure 20 Intestine degeneration with intranuclear inclusions (head arrows) (400x) of dead fish from post infection antiviral activity test of *C. nutans* ethanol crude extract

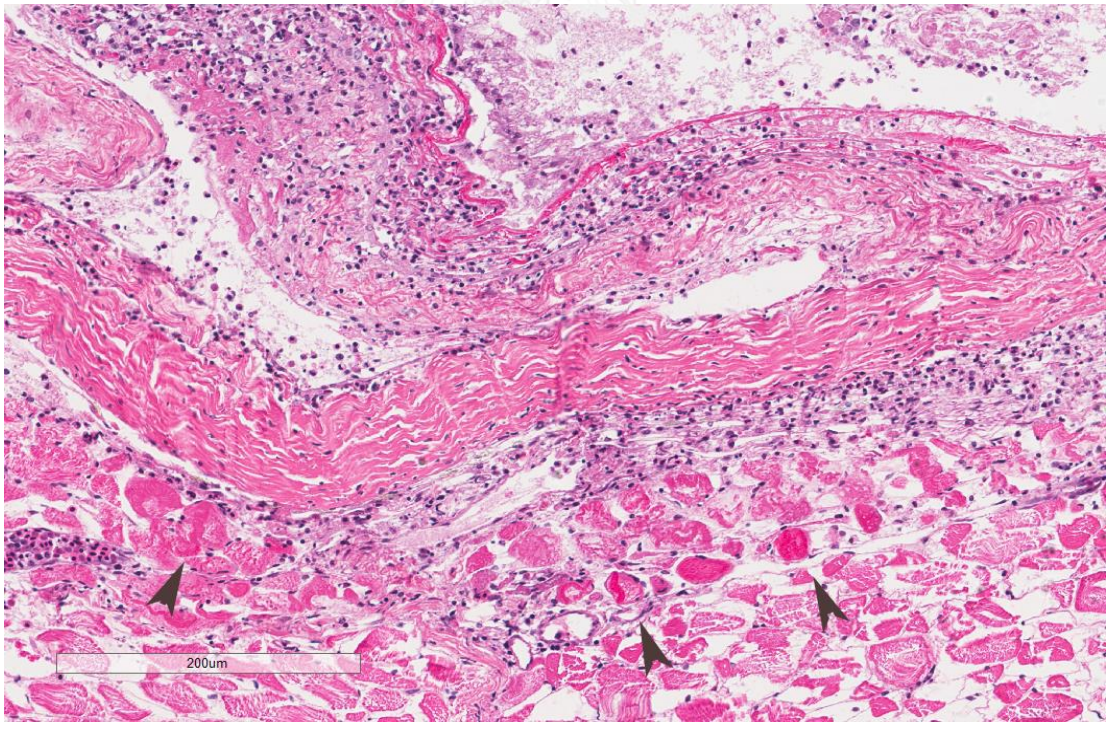

Figure 21 Rhabdomyocytis with degeneration and necrosis (head arrows) (100x) of dead fish from post infection antiviral activity test of *C. nutans* ethanol crude extract

CHAPTER V
DISCUSSION

5.1 Herbal extraction

Herbal medicinal plants are believed to be an important source of substances for alternative treatment. Many plants have been studied for their phytochemical compositions and biochemical activities. The development of extraction methods was reported to improve quality of extracted products (Huie, 2002). Solvents used for plant extraction and extraction time played an important effect on the amount of by product from plant extraction (Lapornik et al., 2005). The different polarities of solvent are considered for choosing in the extraction process. In this study, the ethanol which is a polar soluble solvent was chosen for *Clinacanthus nutans* (Burm.f.) Lindau (*C. nutans*) extraction. The ethanol extract was concentrated in a rotary evaporator to give greenish wax deposited on the side of the flask. All most all of the greenish wax contained chlorophyll and lipid components (Harborne, 1998). Dichloromethane and n-butanol were chosen to separate more purification compounds from the ethanol crude extract by their dissolve properties. N-butanol is moderate polar soluble solvent and dichloromethane is low polar soluble solvent (Snyder, 1974). *C. nutans* is one of the herbal plants that have been used for alternative treatment in human for a long time. Phytochemical analysis of *C. nutans* was reported to contain alkaloids, flavonoid (Satayavivad et al., 1996), chlorophyll (Sakdarat et al., 2009), cerebrosides (Tuntiwachwuttikul et al., 2004), C-Glycoside (Teshima et al., 1997; Chelyn et al., 2014), phenolic compounds (Kosai et al., 2016) and sulfur containing compounds (Tu et al., 2014). The crude extract and pure compounds were proven to be effective against HSV-1, HSV-2, Varicella zoster and anti-inflammatory effect both in experiment and clinical use (Jayavasu et al., 1992; Thawaranantha et al., 1992; Sangkitporn et al., 1995; Yoosook et al., 1999; Tuntiwachwuttikul et al., 2004; Jayavasu et al., 2013).

5.2 *In vitro* antiviral activities of *C. nutans* extracts against Cyprinid Herpesvirus 3

In this study, only the ethanol crude extract showed anti-CyHV-3 activities while other two fractions did not inhibit CyHV-3 at the concentration that was non-toxic to cell line. The CC_{50} of crude extract was 1,701.57 µg/ml, dichloromethane fraction was 522.47 µg/ml and n-butanol fraction was 1,797.98 µg/ml. The sensitivity of cell line to substances or chemicals is different among each cell type. KFC might be too sensitive to the two fractions especially to dichloromethane fraction that showed toxicity to cell at low concentration. In other studies, the CC_{50} of *C. nutans* extract against HSV-2 in human vero cell was 6,670 ug/ml (Yoosook et al., 1999). The study of *C. nutans* extract against HSV-1 in vero cell also showed CC_{50} at 6,210 ug/ml (Sakdarat et al., 2009). Cytotoxicity of *C. nutans* in CHSE-214 cell line in antiviral activities study against fish pathogens showed CC_{50} at 2,124 µg/ml (Direkbusarakom et al., 1996). This study showed higher susceptibility of cell line to *C. nutans* extracts in KFC.

Pre and post infection antiviral activity of *C. nutans* crude extract were effective both *in vitro* and *in vivo*. Ethanol extracts of *C. nutans* were reported to have antiviral activities against herpes simplex virus (HSV) before infection in human cell line (Jayavasu et al., 1992; Tuntiwachwuttikul et al., 2004). Vachirayonstien et al. (2010) studied the extracellular inhibitory effect of alcohol extract from *C. nutans* leaves against HSV-2 in cell culture. The result showed *C. nutans* extracts inhibited the virus before entering cells but had very low or no intracellular antiviral activity. The direct effect of *C. nutans* ethanol 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 but not after infection (Direkbusarakom et al., 1996). In contrast with our study, *C. nutans* show antiviral activity pre and post infection to CyHV-3 in KFC. Pre-infection antiviral activities at 1, 2, 3 and 4 hours showed efficiency of crude extract against CyHV-3 directly before entering to cell. Effective concentrations that could reduce viral titer more than 1 log 10 pre infection were at 250, 500 and 1,000 µg/ml. Post infection antiviral activity against CyHV-3 showed the effective concentration at 500 and 1,000

µg/ml. Some studies also reported post infection antiviral activity of *C. nutans* against viral infection in cell line. This effect was reported in pre-treatment and post-treatment antiviral activity by chloroform crude extract and two glycoglycerolipid compounds isolated from *C. nutans*. The result showed high efficacy of extract against HSV-1 and HSV-2 post infection greater than pre-treatment activity in cell line (Pongmuangmul et al., 2016). The galactosyl diglycerides isolated from *C. nutans* and synthetic monoglycosyl diglycerides also had an anti-HSV activities post-infection (Satakhun, 2001).

5.3 *In vivo* antiviral activities of *C. nutans* extract against Cyprinid herpesvirus 3

Toxicity of *C. nutans* crude extract in koi was > 5,000 µg/ml. It caused 13.33% mortality at the highest dosage. The fine sediment from extract was noticed in this experimental group. This sediment could be blocked into the gill and interrupted oxygen exchange process. This mortality possibly caused by hypoxia. The water parameters and blood results were increased in some values but were not higher than the normal range. The cause of death should be from mechanical toxicity more than chemical toxicity.

The pre and post antiviral activity against CyHV-3 infection in koi exhibited the effective results. The *C. nutans* crude extract showed direct antiviral activity against CyHV-3 in koi. The concentration of 5,000 µg/ml showed explicit result without any mortality of fish in this group. The concentrations that could significantly reduce fish mortality when compared to the control group were at 1,000 and 5,000 µg/ml in all periods of contact time. However, the longer contact time between herbal extract and the virus, the longer delay of fish mortality onset. It also delayed fish clinical signs which were noticed after 4-5 dpi., while in previous studies, fish showed clinical signs around 2 to 3 dpi (Hedrick et al., 2000; Gilad et al., 2002). The 50% effective dose (ED_{50}) were 991.59 µg/ml, 782.72 µg/ml, 749.05 µg/ml and 707.80 µg/ml at 1, 2, 3 and 4 hours, respectively. These were approximately half of EC_{50} in cell line.

Post infection antiviral activity of *C. nutans* crude extract showed efficiency to decrease mortality in infected fish. The time of treatment after infection also plays an important role in decreasing fish mortalities. The treatment groups that the extract was added right after infection and at 24 hours post infection significantly had reduced mortality at the concentration of 2,500 µg/ml ($P < 0.05$). While the results showed no difference in fish mortality when the extract was added at 72 hour post infection. The ED_{50} at 0 hour was 2,050.88 µg/ml and at 24 hour was 2,337.74 µg/ml which were higher than ED_{50} of pre-infection antiviral activity experiment. The results indicated that the extract could interfere in some step of viral replication at early stage of infection before 24 hours but not after 72 hours. These results were possibility due to rapid spread of viral infection in fish. The previous report showed an early detection of viral DNA from gill and skin since 8 hours post infection (Monaghan et al., 2015b). The virus was also reported to be detected in kidney and blood after 1 day post infection (Pikarsky et al., 2004) which indicated that the step of viremia had started. The excrement samples from infected fish were reported to be positive for CyHV-3 as early as 5 - 7 days (Dishon et al., 2005). The clinical signs of infected fish from the experiments started around 2 – 3 days before mortalities had occur which also in the same pattern as previous reports (Hedrick et al., 2000; Perelberg et al., 2003). Fish exhibited lethargy, appetite loss, sunken eyes, hypersecretion of mucus and gill necrosis followed by white patches on the skin, skin hyperemia, hyphema, abnormal swimming and grasping similar to previous reports (Hedrick et al., 2000; Gilad et al., 2002). Most of fish died after one day of abnormal swimming which indicated neurological damage that appeared in the late stage of infection. Fish in all treatment groups developed clinical signs later than the control group. This indicated that the extract could reduce viral concentration in water and slower down viral dissemination rate. There was no evidence of study in *C. nutans* antiviral mechanism after infection. The mechanism of extract for viral inhibition before infection was also unclear. It was suspected *C. nutans* caused defect to viral envelope. The electron microscopic study of infected cell line that was treated with *C. nutans* alcohol extract showed destruction of CyHV-3 envelope (Haetrakul, 2009). Direkbusarakom et al. (1998) also suspected the viral inactivation of *C. nutans* in

Yellow-headed virus in shrimp might occur by the reaction between the extract and viral envelope. Because the non-enveloped virus such as IPNV in fish was not inactivated by *C. nutans* while other enveloped viruses were affected (Direkbusarakom et al., 1996).

5.4 Latent infection

Survived fish from pre and post antiviral activity test were induced for viral reactivation. The temperature stress at 21 – 23°C was performed after fish had been kept in non-permissive temperature (28 - 30°C) for 60 days. Fish had normal behavior without any sign of viral infection for 30 days after temperature stress was induced. In contrast to the study of Eide et al. (2011), fish latency could be reactivated by temperature stress between 17-23°C. CyHV-3 DNA was able to be detected in gill swabs as early as day 2 post-temperature stress and peaked between days 8 and 12 by using real-time PCR. After temperature stress had failed. Naïve fish were put in to tanks in order to make co-habitation infection as described by Pikarsky et al. (2004). All fish did not show any abnormal sign after 15 days of co-habitation. Stress was induced by putting into net out of water for 30 seconds, twice (Bergmann and Kempter, 2011). After 7 days, gill sample and mucus were sent to check for CyHV-3 by PCR and all samples were negative to CyHV-3 infection. Although this seems to be able to conclude that *C. nutans* crude extract is effective against viral infection and no latent infection was found. However, Herpesviruses are characterized by their ability to have latency in host. It is possible that *C. nutans* crude extract was effectively reduce CyHV-3 infection in fish to a non-infective level. Thus, fish in this study were tested to be negative by PCR in latency period. This might be due to limitation of detection method because of low copies numbers of viral genome are expressed during latent infection (Dishon et al., 2007; Eide et al., 2011; Reed et al., 2014). CyHV-3 was reported to become latent in peripheral white blood cells and various tissues (Eide et al., 2011). The type of white blood cells latently infected with CyHV-3 was reported to be B cell (Reed et al., 2014). Viral DNA could be detected from various organs in fish after 2 months with no clinical sign and no gross lesion

from necropsy by real time PCR (Eide et al., 2011). Recent studies were focus on target gene for CyHV-3 latency detection. Reed et al. (2014) suggested that ORF-6 in B cells may have a conserved function that can be detected during viral latency period. The new cell line names CCF-K14 was developed from fibroblastic cells from caudal fin. Quantitative PCR was used to detect low copy numbers of the ORF89 gene in CCF-K 14 cell line when temperature was shift from 25°C to 15°C and 35°C (Imajoh et al., 2015). From all these information, the negative result from latent reactivation is possible from non-appropriated diagnostic method and tissue samples. Or it is also possible that *C. nutans* could provide protective measure against CyHV-3 in koi carps. Further study is recommended to check the viral virulence and existence in these treated fish.

5.5 Histopathology

Histopathological findings from CyHV-3 infected samples in pre and post infection antiviral activity test showed typical lesions of CyHV-3 infection as described by Miyazaki et al. (2008). Viral inclusions were found in gill samples and intestinal epitheliums. Acute inflammations of gill lamellae were obvious which possible due to viral infection. The epithelial cells of the gill lamellae were swollen and exhibited nuclear degeneration and intranuclear inclusion bodies were observed. In dead fish, gill lesions showed lamellar fusion and clubbing of the gill filaments, necrotic cells were seen at the tips of fused lamellae, which also found from samples in this study. Other organs were affected by viral infection. Fish with neurological signs that died in the 4th week post infection was classified as latter period of infection (Pikarsky et al., 2004). Koi carps were unable to control their swimming direction and showed swirling. The typical lesion of CyHV-3 infection such as herpetic skin lesions and gill necrosis were obvious in fish at latter stage of infection. Skin lesion showed dermatitis, necrotizing, granulocytic and histiocytic, diffuse, marked with necrotizing vasculitis, myocytic atrophy and degeneration. Intranuclear viral inclusions were found in epithelial cells. The erosive skin lesions were suspected to be necrosis of epidermal cells caused by dissemination of CyHV-3

in the water environment (Miyazaki et al., 2008). Heart, liver, kidney, spleen and brain were affected as a direct result of viremia. They were reported to detect intranuclear inclusion bodies in various organs from infected fish. Gill epithelium, renal tubular epithelial cells in the kidney, hepatocytes, splenocytes, epidermal cells and nerve cell have been described as major target cells of CyHV-3 infection (Hedrick et al., 2000; Perelberg et al., 2003; Gilad et al., 2004; Miyazaki et al., 2008). But viral inclusion was evidenced only in gill, intestine and skin lesions from this study.

5.6 Conclusion

Clinacanthus nutans (Burm.f.) Lindau is an herbal medicinal plant which can be use as alternative treatment in fish. The crude extract from aerial part of the plant expressed antiviral activity against Cyprinid herpesvirus -3 in koi carp and in Koi Fin Cell line. The capabilities were found both during pre and post infections. The therapeutic index (LD_{50}/ED_{50}) of *C. nutans* extract against CyHV-3 in koi pre-infection were at least 5.04, 6.39, 6.68 and 7.06 at 1, 2, 3 and 4 hours, respectively. The therapeutic index post infections were at least 2.44 and 2.14, at 0 and 24 hours, respectively. These results showed possibility to use *C. nutans* crude extract for CyHV-3 treatment and prevention. Environment was an important factor as viral reservoir. The virus was reported to be able to transmit into fish environment via fish excretion (Dishon et al., 2005). CyHV-3 titer was reported to be detectable in water of contaminated rivers and lakes by concentrating viral particles using ultracentrifugation followed by quantitative real-time PCR (qPCR) (Honjo et al., 2010). The viral DNA was reported to be detected by quantitative real time PCR from planktons in the lake where the spread of the disease was reported (Minamoto et al., 2011). Soil and pond sediment was also suspected to be a reservoir for CyHV-3. Because the amount of viral DNA in natural sediment was detected a lot higher than in the water from the same location by using real time PCR (Honjo et al., 2012). The virus was reported to have infectivity in water at least 4 hours but not at 21 hours without host (Perelberg et al., 2003). The pre-infection antiviral activity of *C. nutans* crude extract from this study showed remarkable effectiveness against CyHV-3 at the incubation time of 1, 2, 3 and 4 hours. The highest concentration of

the crude extract at 5,000 µg/ml could decrease viral titer down to the level that caused no mortality in fish. However, the crude extract at this concentration was tested to be sub-toxic to fish. It could be used as water or pond treatment. It is possible to be applied to use as disinfectant for utensils and equipment. The lower dose at 2,500 µg/ml was tested to be effective against CyHV-3 post infection. Beside the antiviral activity, *C. nutans* extract also showed other therapeutic properties. Biochemical activities of *C. nutans* were reported to have antioxidants property (Yong et al., 2013), Immunomodulatory effects (Sriwanthana et al., 1996) and anti-inflammatory and analgesic effects (Arullappan et al., 2014). Teshima et al. (1997) studied the n-butanol soluble fraction from alcohol extract of *C. nutans* from stems and leaves. The fraction was isolated by column chromatography to give flavone C-glycoside compounds which showed anti-inflammatory activities (Satayavivad et al., 1996). Phenolic contents from ethanol leaves extract of *C. nutans* also had an anti-inflammatory and analgesic activity in cell line (Thongrakard and Tencomnao, 2010). 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). 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 gave significant results in reducing viral titer after entering into the cells and also decreasing fish mortality. The possibility of inhibition may occur during viral replication after infection. Some monoglycerides showed antiviral activity against virus by destruction of viral envelopes (Thormar et al., 1994). The electron microscopic study of infected cell line that was treated with *C. nutans* alcohol extract also showed destruction of CyHV-3 envelope in koi fin cell line (Haetrakul, 2009).

The results from this study showed that *C. nutans* crude extract expressed antiviral activity against CyHV-3 in cell line and koi carps. These can be applied as therapeutic agent in common carp and koi aquacultures.

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APPENDIX

Appendix A Temporary inhibited announcement of carp imported into Thailand by The Department of Fisheries

ประกาศกรมประมง

เรื่อง การงดอนุญาตให้นำเข้าปลาตระกูลคาร์ฟ (Carp fish)

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ตามที่ได้มีรายงานข่าวการแพร่ระบาดของโรค Herpesvirus ในปลาตระกูลคาร์ฟที่ประเทศญี่ปุ่น พบว่าการระบาดมีความรุนแรง และขยายพื้นที่ไปเป็นบริเวณกว้าง ตั้งแต่กลางเดือนตุลาคม 2546 เป็นต้นมา สร้างความเสียหายให้บ่อเลี้ยงปลาตระกูลคาร์ฟ (Carp fish) อย่างหนัก ซึ่งอาจแพร่กระจายไปยังประเทศใกล้เคียง และยังไม่สามารถควบคุมการระบาดของโรคได้

เพื่อป้องกันมิให้เกิดการแพร่ระบาดของโรคดังกล่าวจากการนำเข้า กรมประมงจึงของดการออกหนังสืออนุญาตให้นำเข้าปลาตระกูลคาร์ฟ (Carp fish) ไว้เป็นการชั่วคราวจนกว่าจะมีคำสั่งเปลี่ยนแปลง

สำหรับผู้ที่ได้รับอนุญาตให้นำเข้าปลาดังกล่าวแล้ว ก่อนประกาศกรมประมงฉบับนี้จะมีผลบังคับใช้ ขอให้กักกันพื้นที่ปลาที่นำเข้าไว้เป็นเวลา 15 วัน และสุ่มตัวอย่างปลาตระกูลคาร์ฟ (Carp fish) ที่นำเข้ามาตรวจที่สถาบันวิจัยสุขภาพสัตว์น้ำจืด สำนักวิจัยและพัฒนาประมงน้ำจืด หากมีข้อสงสัยสอบถามรายละเอียดเพิ่มเติมได้ที่หมายเลขโทรศัพท์ 0 2579 4122 หรือ 0 2579 6803 หรือ 0 2561 4689 ในวันและเวลาราชการโดยด่วน

จึงประกาศมาเพื่อทราบโดยทั่วกัน

ประกาศ ณ วันที่ 18 พฤศจิกายน พ.ศ. 2546

สิทธิ บุญยรัตผลิน
(นายสิทธิ บุญยรัตผลิน)
อธิบดีกรมประมง

Appendix B Carp imported permit into Thailand by The Department of Fisheries

ประกาศกรมประมง

เรื่อง การอนุญาตให้นำปลาตระกูลคาร์พเข้ามาในราชอาณาจักร

ภายใต้ระบบการกักกันโรค

ตามที่ได้มีประกาศกรมประมง เรื่อง การงดอนุญาตให้นำเข้าปลาตระกูลคาร์พ (Carp fish) ลงวันที่ 18 พฤศจิกายน พ.ศ. 2546 ให้งดการออกหนังสืออนุญาตให้นำเข้าปลาตระกูลคาร์พ (Carp fish) ไว้เป็นการชั่วคราว เนื่องจากมีการแพร่ระบาดของโรคบางชนิดนั้น

เนื่องจากประเทศที่เคยมีการเกิดโรคเคเอชวี (KHV diseaseหรือ Koi herpesvirus disease)ในปลาคาร์พ ได้พัฒนาระบบการควบคุมและการตรวจรับรองการปลอดโรคดังกล่าวแล้ว เห็นควรให้มีการออกหนังสืออนุญาตให้นำปลาตระกูลคาร์พเข้ามาในราชอาณาจักรได้ แต่ต้องดำเนินการภายใต้ระบบการกักกันโรค เพื่อความปลอดภัยและป้องกันมิให้มีการนำโรคเข้ามาระบาดในประเทศไทย กรมประมงจึงออกประกาศดังนี้

ข้อ 1 ให้ยกเลิกประกาศกรมประมง เรื่อง การงดอนุญาตให้นำเข้าปลาตระกูลคาร์พ (Carp fish) ลงวันที่ 18 พฤศจิกายน พ.ศ. 2546

ข้อ 2 กรมประมงจะพิจารณาออกหนังสืออนุญาตให้นำปลาตระกูลคาร์พ (Carp fish) เข้ามาในราชอาณาจักรเพื่อการเพาะเลี้ยงได้ภายใต้ระบบการกักกันโรค โดยผู้ประสงค์จะขอหนังสืออนุญาตดังกล่าวต้องปฏิบัติตามเงื่อนไขที่กำหนดไว้ท้ายประกาศนี้

จึงประกาศมาเพื่อทราบโดยทั่วกัน

ประกาศ ณ วันที่ 30 กันยายน พ.ศ. 2547

สิทธิ บุญยรัตผลิน

(นายสิทธิ บุญยรัตผลิน)

อธิบดีกรมประมง

Appendix C Cell viability from *in vitro* cytotoxicity of *C. nutans* extracts

1. Alive cells from cytotoxicity of *C. nutans* ethanol crude extract

	Negative control	50 (µg/ml)	100 (µg/ml)	500 (µg/ml)	1,000 (µg/ml)	2,000 (µg/ml)
Rep. 1 (n = 100)	95	97	94	96	78	34
	98	96	98	93	80	29
	97	96	97	97	90	29
	99	96	97	97	81	38
	97	99	96	98	74	32
	99	97	98	97	84	36
Rep. 2 (n = 100)	100	93	96	93	88	40
	97	94	96	94	84	35
	96	98	94	93	88	32
	97	96	95	97	83	34
	98	99	95	97	82	38
	96	95	98	98	83	27
Rep. 3 (n = 100)	99	98	96	97	89	25
	96	98	98	97	92	28
	99	99	99	96	90	37
	97	98	96	97	89	33
	98	97	98	99	90	30
	98	98	98	97	87	27

2. Alive cells from cytotoxicity of *C. nutans* dichloromethane fraction

	Negative control	10 (µg/ml)	50 (µg/ml)	100 (µg/ml)	500 (µg/ml)	1,000 (µg/ml)
Rep. 1 (n = 100)	100	98	97	88	63	0
	97	97	97	87	70	0
	97	97	96	89	65	0
	98	98	98	86	64	0
	99	99	100	90	63	0
	99	99	95	87	62	0
Rep. 2 (n = 100)	94	95	94	88	60	0
	100	98	98	85	65	0
	97	95	97	87	62	0
	96	96	96	86	66	0
	98	98	98	89	63	0
	96	96	97	85	64	0
Rep. 3 (n = 100)	97	97	98	82	67	0
	97	98	98	87	63	0
	98	98	97	85	62	0
	99	99	98	87	68	0
	96	96	97	88	58	0
	98	98	99	87	65	0

3. Alive cells from cytotoxicity of *C. nutans* n-butanol fraction

	Negative control	50 (µg/ml)	100 (µg/ml)	500 (µg/ml)	1,000 (µg/ml)	1,500 (µg/ml)	2,000 (µg/ml)
Rep. 1 (n = 100)	97	99	96	98	78	65	0
	98	98	98	97	76	67	0
	99	98	98	95	79	68	0
	96	99	99	96	77	63	0
	97	97	97	96	77	61	0
	99	96	96	99	78	64	0
Rep. 2 (n = 100)	98	95	97	96	75	65	0
	96	97	97	96	78	66	0
	96	96	99	98	74	65	0
	98	97	95	98	80	64	0
	97	96	96	96	76	68	0
	97	95	97	95	74	63	0
Rep. 3 (n = 100)	98	98	97	95	76	65	0
	99	95	99	95	77	64	0
	96	98	96	98	78	68	0
	97	96	98	97	79	66	0
	98	97	98	99	79	64	0
	97	99	95	98	75	63	0

Appendix D Water quality from *in vivo* toxicity test of *C. nutans* ethanol crude extract

1. Water quality before the experiment

Parameters	Negative control (n = 3)	5,000 µg/ml (n = 3)	2,500 µg/ml (n = 3)	1,000 µg/ml (n = 3)	500 µg/ml (n = 3)	50 µg/ml (n = 3)	Normal range (Lloyd, 2001)
pH	7, 7.3, 7	7.3, 7.3,7	7,7,7	7.3,7,7	7.3,7.3,7	7,7.3,7	6.5 – 8.5
Ammonia (mg/l)	0, 0.1, 0.1	0, 0, 0.25	0.1, 0, 0.1	0.1, 0.1, 0.1	0, 0.1, 0.1	0.25, 0, 0.1	0.2 – 0.5
Nitrite (mg/l)	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	< 0.2
Alkalinity (mg/l)	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	> 100
Hardness (mg/l)	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	50 - 400

2. Water quality on day 7

Parameters	Negative control (n = 3)	5,000 µg/ml (n = 3)	2,500 µg/ml (n = 3)	1,000 µg/ml (n = 3)	500 µg/ml (n = 3)	50 µg/ml (n = 3)	Normal range (Lloyd, 2001)
pH	7.3, 7.3, 7	7.6, 7.6, 7.6	7.6,7.6, 7.6	7.3,7.6, 7.6	7.3,7.3, 7.6	7.3,7.3, 7.3	6.5 – 8.5
Ammonia (mg/l)	0, 0.1, 0.25	0.1, 0, 0.1	0.1, 0.25, 0.1	0.1, 0.1, 0	0.1, 0.25, 0.25	0.25, 0.1, 0.1	0.2 – 0.5
Nitrite (mg/l)	0.1, 0.05, 0.05	0.1, 0.1, 0.05	0.2, 0.05, 0.1	0.1, 0, 0.1	0.25, 0.1, 0.05	0.1, 0.05, 0	< 0.2
Alkalinity (mg/l)	100, 100, 100	160, 180, 160	200, 100, 160	160, 100, 100	100, 100, 200	160, 100, 100	> 100
Hardness (mg/l)	100, 100, 100	200, 200, 200	200, 200, 200	200, 200, 200	200, 200, 200	200, 200, 200	50 - 400

3. Water quality on day 14

Parameters	Negative control (n = 3)	5,000 µg/ml (n = 3)	2,500 µg/ml (n = 3)	1,000 µg/ml (n = 3)	500 µg/ml (n = 3)	50 µg/ml (n = 3)	Normal range (Lloyd, 2001)
pH	7, 7.3, 7.3	7.6, 7.6, 7.6	7.6, 7.6, 7.6	7.6, 7.3, 7.6	7.6, 7.6, 7.6	7.3, 7.3, 7	6.5 – 8.5
Ammonia (mg/l)	0, 0.1, 0.1	0, 0, 0.25	0.1, 0, 0.1	0.1, 0.1, 0.1	0, 0.1, 0.1	0.25, 0, 0.1	0.2 – 0.5
Nitrite (mg/l)	0.05, 0.1, 0.05	0, 0.05, 0	0.05, 0.05, 0	0.1, 0, 0.05	0.05, 0, 0.05	0.1, 0,05 0.2	< 0.2
Alkalinity (mg/l)	100, 100, 100	100, 160, 200	100, 100, 160	200, 100, 100	100, 100, 100	160, 100, 100	> 100
Hardness (mg/l)	100, 100, 100	200, 200, 200	200, 200, 200	200, 200, 200	200, 200, 200	200, 200, 200	50 - 400

4. Water quality on day 21

Parameters	Negative control (n = 3)	5,000 µg/ml (n = 3)	2,500 µg/ml (n = 3)	1,000 µg/ml (n = 3)	500 µg/ml (n = 3)	50 µg/ml (n = 3)	Normal range (Lloyd, 2001)
pH	7.3, 7, 7.3	7.3, 7.3, 7	7.6, 7, 7.3	7.3, 7.3, 7	7.3, 7.3, 7	7 ,7.3, 7.3	6.5 – 8.5
Ammonia (mg/l)	0, 0.1, 0.25	0, 0.1, 0.25	0.25, 0, 0.1	0, 0.1, 0.1	025, 0.1, 0	0.25, 0, 0.1	0.2 – 0.5
Nitrite (mg/l)	0.1, 0, 0.05	0.1, 0, 0.1	0, 0.05, 0	0, 0.05, 0.05	0, 0, 0.05	0, 0.1, 0	< 0.2
Alkalinity (mg/l)	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	> 100
Hardness (mg/l)	100, 100, 100	100, 200, 100	100, 100, 200	100, 100, 100	100, 100, 100	100, 100, 100	50 - 400

5. Water quality on day 30

Parameters	Negative control (n = 3)	5,000 µg/ml (n = 3)	2,500 µg/ml (n = 3)	1,000 µg/ml (n = 3)	500 µg/ml (n = 3)	50 µg/ml (n = 3)	Normal range (Lloyd, 2001)
pH	7.3, 7.3, 7	7.3, 7.3,7.6	7.3, 7.3, 7	7.3, 7, 7.6	7.3,7.3, 7.3	7, 7.3, 7.3	6.5 – 8.5
Ammonia (mg/l)	0.1, 0.1, 0.25	0, 0.1, 0.25	0.1, 0.25, 0.25	0.1, 0.25, 0.1	0.25, 0.1, 0.1	0.25, 0.25, 0.1	0.2 – 0.5
Nitrite (mg/l)	0.1, 0.05, 0.05	0, 0.05, 0.1	0.1, 0, 0.05	0, 0.05, 0	0.1, 0.1, 0	0.25, 0, 0.1	< 0.2
Alkalinity (mg/l)	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	> 100
Hardness (mg/l)	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	100, 100, 100	50 - 400

Appendix E Fish mortality data from *in vivo* pre-infection antiviral activity test

Table 1 Fish mortality data from *in vivo* pre-infection antiviral activity test at 1 hour incubation period

Concentration (µg/ml)	Tank 1 (n = 10)	Tank 2 (n = 10)	Tank 3 (n = 10)
5,000	0	0	0
1,000	4	5	4
500	9	8	7
100	8	9	8
Positive control	8	8	9

Table 2 Fish mortality data from *in vivo* pre-infection antiviral activity test at 2 hour incubation period

Concentration (µg/ml)	Tank 1 (n = 10)	Tank 2 (n = 10)	Tank 3 (n = 10)
5,000	0	0	0
1,000	3	3	5
500	7	7	7
100	8	7	8
Positive control	9	8	7

Table 3 Fish mortality data from *in vivo* pre-infection antiviral activity test at 3 hour incubation period

Concentration (µg/ml)	Tank 1 (n = 10)	Tank 2 (n = 10)	Tank 3 (n = 10)
5,000	0	0	0
1,000	3	4	3
500	5	8	7
100	8	6	8
Positive control	7	7	9

Table 4 Fish mortality data from *in vivo* pre-infection antiviral activity test at 4 hour incubation period

Concentration (µg/ml)	Tank 1 (n = 10)	Tank 2 (n = 10)	Tank 3 (n = 10)
5,000	0	0	0
1,000	2	3	4
500	8	7	7
100	6	7	9
Positive control	8	8	7

Appendix F Fish mortality data from *in vivo* post infection antiviral activity test

Table 1 Fish mortality data from *in vivo* post infection antiviral activity test at 0 hour treatment period after infection

Concentration (µg/ml)	Tank 1 (n = 10)	Tank 2 (n = 10)	Tank 3 (n = 10)
2,500	4	5	3
1,000	8	9	7
500	9	8	8
100	8	8	9
Positive control	9	7	10

Table 2 Fish mortality data from *in vivo* post infection antiviral activity test at 24 hour treatment period after infection

Concentration (µg/ml)	Tank 1 (n = 10)	Tank 2 (n = 10)	Tank 3 (n = 10)
2,500	5	5	4
1,000	8	8	6
500	7	9	8
100	9	9	7
Positive control	9	7	10

Table 3 Fish mortality data from *in vivo* post infection antiviral activity test at 72 hour treatment period after infection

Concentration (µg/ml)	Tank 1 (n = 10)	Tank 2 (n = 10)	Tank 3 (n = 10)
2,500	7	8	7
1,000	10	8	7
500	9	8	8
100	10	8	9
Positive control	9	7	10

VITA

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