ผลของนารินเจนินต่อการเพิ่มจำนวนของเฮอร์ปีส์ซิมเพล็กซ์ไวรัส

นางสาวขนิษฐา ชิตเพชร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยา ภาควิชาชีวเคมีและจุลชีววิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

EFFECT OF NARINGENIN ON THE REPLICATION OF HERPES SIMPLEX VIRUS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Microbiology
Department of Biochemistry and Microbiology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2010

Thesis Title	EFFECT OF NARINGENIN ON THE REPLICATION OF HERPES SIMPLEX VIRUS
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Field of Study	Microbiology
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ขนิษฐา ชิตเพชร: ผลของนารินเจนินต่อการเพิ่มจำนวนของเฮอร์ปีส์ซิมเพล็กซ์ไวรัส. (EFFECT OF NARINGENIN ON THE REPLICATION OF HERPES SIMPLEX VIRUS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.วิมลมาศ ลิปิพันธ์, 105 หน้า.

การศึกษาฤทธิ์ยับยั้งไวรัสเฮอร์ปีส์ซิมเพล็กซ์ทัยป์ 1 สายพันธุ์ KOS และทัยป์ 2 สายพันธุ์ Baylor 186 ของ naringenin ใน Vero cells โดยวิธี plaque reduction, inactivation และ prophylactic activity assay พบว่า naringenin มีฤทธิ์ยับยั้ง HSV-1 และ HSV-2 โดยค่าความเข้มข้นของสารที่ยับยั้งการเจริญของไวรัส 50% (IC_{50}) ของ naringenin ในการยับยั้ง HSV-1 เท่ากับ 46.64±7.06, 43.30±2.80 และ 36.08±1.86 µg/ml ตามลำดับ และมีค่า SI เท่ากับ 7.07, 7.61 และ 9.12 ตามลำดับ ค่า IC_{50} ของ naringenin ในการยับยั้ง HSV-2 เท่ากับ 48.27±5.14, 47.58±5.24 และ 39.39±1.68 µg/ml ตามลำดับโดยมีค่า SI เท่ากับ 6.83, 6.93 และ 8.36 ตามลำดับ การทดสอบความเป็นพิษต่อเซลล์ของ naringenin โดยวิธี trypan blue exclusion และ MTT reduction assay พบว่าความเข้มข้นของสารที่เป็นพิษต่อเซลล์เพาะเลี้ยง 50% (CC_{50}) ที่เวลา 24 ชั่วโมง จากการทดสอบทั้งสองวิธีมีค่ามากกว่า 500 µg/ml ค่า CC_{50} ที่เวลา 48 ชั่วโมง เท่ากับ 329.01±33.37 และ 378.71±25.25µg/ml และค่า CC_{50} ที่เวลา 72 ชั่วโมง เท่ากับ 305.37±18.36 และ 304.56±7.41 µg/ml ตามลำดับ นอกจากนี้ในการประเมินกลไกการออกฤทธิ์เบื้องต้นของ naringenin ในการยับยั้ง HSV-1 โดยใช้วิธี virus yield inhibition assay, required treatment period for inhibition of plaque formation, adsorption, penetration, virucidal และ combined effect of naringenin with acyclovir on plaque formation พบว่า naringenin มีฤทธิ์ยับยั้งการจับของ HSV บนผิวเซลล์เพาะเลี้ยง และมีฤทธิ์ทำลายอนุภาคของไวรัสโดยตรง นอกจากนี้การให้ naringenin ร่วมกับ acyclovir ให้ผลยับยั้ง HSV-1 แบบ synergistic ในการศึกษานี้แสดงให้เห็นว่า naringenin มีฤทธิ์ยับยั้งไวรัสเฮอร์ปีส์ซิมเพล็กซ์และอาจนำไปพัฒนาเป็นยารักษาโรคที่เกิดจากการติดเชื้อ HSV ได้ในอนาคต

ภาควิชา…..ชีวเคมีและจุลชีววิทยา….ลายมือชื่อนิสิต…….
สาขาวิชา….จุลชีววิทยา…….....ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก……
ปีการศึกษา…2553……..

517 6556733: MAJOR MICROBIOLOGY
KEYWORDS: HERPES SIMPLEX VIRUS/ NARINGENIN/ANTIVIRAL ACTIVITY

KHANIDTHA CHITPHET: EFFECT OF NARINGENIN ON THE REPLICATION OF HERPES SIMPLEX VIRUS. THESIS ADVISOR: ASSOCIATE PROFESSOR VIMOLMAS LIPIPUN, Ph. D., 105 pp.

Antiviral activities of naringenin against herpes simplex viruses type 1 (HSV-1) strain KOS and type 2 (HSV-2) strain Baylor 186 on Vero cells using plaque reduction, inactivation, and prophylactic assay were investigated. Naringenin exhibited anti-HSV activity in all assays. The 50% inhibitory concentrations (IC_{50}) of naringenin against HSV-1 were 46.64±7.06, 43.30±2.80, and 36.08±1.86 µg/ml, respectively, and the selective index (SI) were 7.07, 7.61, and 9.12, respectively. In the inhibition of HSV-2, IC_{50} values were 48.27±5.14, 47.58±5.24 and 39.39±1.68 µg/ml, respectively, and SI of 6.83, 6.93 and 8.36 were observed in naringenin treatment. The cytotoxic concentration of naringenin that inhibited cell growth 50 % (CC_{50}), in trypan blue exclusion and MTT reduction assays, were more than 500 µg/ml at 24 h, 329.01±33.37 and 378.71±25.25µg/ml at 48 h and 305.37±18.36 and 304.56±7.41 µg/ml at 72 h, respectively. In addition, possible mechanism of action of naringenin against HSV-1 was determined using virus yield inhibition assay, required treatment period for inhibition of plaque formation, adsorption, penetration, virucidal and combined effect of naringenin with acyclovir on plaque formation. The results indicated that naringenin inhibited virus adsorption to cell surface and did directly inactivate virus particle. Furthermore, the synergistic anti-herpes simplex effect of naringenin was observed when combined with acyclovir. This study indicated that naringenin showed anti-herpes simplex activity and was the promising anti-herpetic agent.

Department: Biochemistry and Microbiology Student's signature……
Field of study:…….Microbiology……...Advisor's signature……...
Academic year:…...2010……..

ACKNOWLEDGEMENTS

I wish to express my infinite gratitude and deep appreciation to my thesis advisor, Associate Professor Dr. Vimolmas Lipipun for her guidance, suggestion, kindness, encouragement, and valuable help throughout my research study. She always stands beside me to help, be consulted, and advise me in every way that would bring me success and great development.

I would like to express my sincere gratitude and deep appreciation to Associate Professor Dr. Nongluksna Sriubolmas, Associate Professor Sattaporn Sirotamarat and Associate Professor Dr. Uthai Sotanaphun for their kindness and helpful suggestion for the completion of this thesis.

I also would like to express my sincere thanks to all staffs in the Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for their kindness and valuable help during my study.

My financial support was partly provided by the teaching assistantship of the Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The budget of this research study was supported by a thesis supporting fund of the Graduate School, Chulalongkorn University.

I would like to express my grateful appreciation to Miss Pattaraporn Sasivimolphan and Mrs. Ariya Khanboon for their help and suggestion.

I wish to express my sincere appreciation to the Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for providing facilities during my study.

My sincere gratitude is also given to my friends for their great love, understanding, assistance, and encouragement throughout this graduate study.

Finally, special gratitude is extended to my dear parents, my sister, and my family for their love, understanding, helpful and continuous supports all my life.

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3-OSTs	=	3-*O*-sulfotransferase
ACV	=	acyclovir
AChE	=	acetylcholinesterase
AIDS	=	acquired immunodeficiency syndrome
AMP	=	adenosine monophosphate
BVDU	=	brivudin
°C	=	degree celcius
CAT	=	catalase
Cat	=	category
CC_{50}	=	50% cytotoxic concentration
CDV	=	cidoforvir
CO_2	=	carbondioxide
cm	=	centimeter
CMV	=	cytomegalovirus
CPE	=	cytopathic effect
Cu	=	copper
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxycytosine triphosphate
dGTP	=	deoxyguanosine 5'- triphosphate
dNTPs	=	deoxynucleoside triphosphate
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
ED_{50}	=	50% effective dose
EDTA	=	ethylene diamine tetra acetate
ELISA	=	enzyme-linked immunosorbent assay
FBS	=	fetal bovine serum
FCV	=	famcyclovir
g	=	gram
GCV	=	ganciclovir

GSH	=	glutathione
GSH-Px	=	glutathione peroxidase
GST	=	glutathione *S*-transferase
h	=	hour
H_2O	=	water
HDL	=	high density lipoprotein
HHV	=	human herpesvirus
HIV	=	human immunodeficiency virus
HPMP	=	3-hydroxy-2-phophonylmethoxypropyl
HPMPA	=	3-hydroxy-2-phophonylmethoxypropyladenine
HPMPC	=	3-hydroxy-2-phophonylmethoxypropylcytosine
HSV	=	herpes simplex virus
HveA	=	herpesvirus entry mediator
HveB	=	nectin-2
HveC	=	nectin-1
IC_{50}	=	50% inhibitory concentration
ICP	=	infected cell polypeptide
ICR	=	imprinting control region
ICSP	=	infected cell specific polypeptide
ICTV	=	International Conference for Taxonomy of Viruses
IDU	=	idoxuridine
i.v.	=	intravenous
kg	=	kilogram
l	=	liter
LATs	=	latency associated transcripts
LD_{50}	=	median lethal dose
m	=	meter
MEM	=	minimum essential medium
mg	=	milligram
mcg, µg	=	microgram
min	=	minute
ml	=	milliliter

mm	=	millimeter
mM	=	millimolar
MOI	=	multiplicity of infection
MTT	=	thaizoyl blue tetrazolium bromide
NA	=	naringenin
nm	=	nanometer
no.	=	number
PAA	=	phosphonoacetic acid
PBS	=	phosphate buffered saline
PCV	=	penciclovir
PFA	=	phosphonoformic acid (foscarnet)
PFU	=	plaque forming unit
RNA	=	ribonucleic acid
ROS	=	reactive oxygen species
rpm	=	round per minute
S.D.	=	standard deviation
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel eletrophoresis
SI	=	selective index
SOD	=	superoxide dismustase
$TCID_{50}$	=	50% tissue culture infective dose
TK	=	thymidine kinase
UL	=	long unique sequences
US	=	short unique sequences
VACV	=	valacyclovir
VP	=	virion polypeptide
VZV	=	varicella zoster virus

CHAPTER I

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are common pathogens for humans, and can cause serious infections in the immunocompromised patients and neonates. This will often result in painful, disablitating lesions and death in worst case (Whitley, 1995). HSV is causing a wide-spread spectrum of mild to severe disorders. These include acute primary and recurrent mucocutaneous disorders recognized as herpes labialis, eczema herpeticum, and genital herpes through herpes keratitis, herpes meningitis, and life-threatening herpes encephalitis in the otherwise healthy adult. Moreover, HSV infection in immunocompromised patients and neonates are usually more severe than in the normal host (Whitley, 1995). Among HSV-related pathologies, genital herpes is an important sexually transmitted disease (STD) caused by HSV-2, with the exception of a minority of cases caused by HSV-1 (Corey *et al*., 1983; Whitley, 2001). However, HSV-1 infection is acquired more frequently and earlier than HSV-2 infection. Moreover, HSV infections may also cause serious problems to infected individuals due to the following virus properties. Firstly, the virus establishes latent infections that can be periodically reactivated. Secondly, under certain circumstances, the virus can produce serious infections of the central nervous system including meningitis and acute necrotizing encephalitis; the viruses may produce fatal infections in patients with immune deficiencies (Whitley, 2001). Finally, the immediate-early genes of HSV-1 can activate genes of different viruses such as human immunodeficiency virus, varicella-zoster virus (Whitley, 2001). HSV-2 is also known as oncogenic virus which has the ability to alter cells into tumor cells (Whitley, 2001).

In Thailand, HSV infections have been often found in various populations. The serum samples from patients and normal healthy groups were detected for viral antibody by enzyme-linked immunosorbent assay (ELISA) (Thammaborvorn *et al*., 2007). It was reported that average percent prevalence (No of positive IgG/Total samples) of HSV infection between 1998 and 2004 was 66.44%, and average percent

incidence (No of positive IgM/Total samples) of infected cases of HSV was 5.24 %. The prevalence of HSV infection in the period 1998-2004 did not change much compared with previous study in 1993-1997. In contrast, the incidence of HSV infection increased from 1.05 % (1993-1997) to 5.24% (1998-2004) (Thammaborvorn *et al*., 2007).

In the treatment of herpes simplex virus infections, nucleoside analogs, acyclovir (ACV) and other nucleoside derivatives, penciclovir, valaciclovir, famciclovir and ganciclovir have been used clinically as anti-herpes simplex drugs and there are several alternative drugs with clinically relevant activity against HSV infection such as idoxuridine, vidarabine, trifluridine, and foscarnet, however, these drugs are possible toxic, mutagenic, and teratogenic to the host (Coen, 1991). Acyclovir (ACV) is most commonly used for the treatment of HSV infections. It has been reported that ACV in topical, oral, or intravenous forms was highly effective particularly on the first episode of HSV infection (Leung and Sacks, 2000). Nevertheless, an important problem for the use of ACV is the increase of HSV resistant strains (Shin *et al*., 2001) especially in immunocompromised patients. Resistance to ACV and related nucleoside analogues can occur by mutation in HSV thymidine kinase or rarely DNA polymerase (Khan *et al*., 2005). An antiviral drug inhibiting DNA polymerase of HSV, foscarnet, is often used to treat ACV-resistant virus and recommended for only severe infection. However, foscarnet may induce mutation in viral DNA polymerase gene when used upon prolonged period, and foscarnet-resistant viruses have been isolated (Bestman-Smith and Boivin, 2003). Consequently, these mutants are rarely resistant to drug combination with existing compounds. Therefore, there is a universal interest in the development and identification of efficacious new anti-HSV agents with no adverse effects. There are various methods reported in the current literature to study anti-herpetic activities of plant extracts or plant-derived molecules. Many *in vitro* and *in vivo* studies have been published on the antiviral activity of flavonoids (Minami *et al*., 2003).

For biological activity, antioxidants act against free radicals to protect cells. Free radicals play a major role in a number of biological processes such as the intracellular killing of bacteria by neutrophil granulocytes and certain cell signaling processes. However, they can cause cell damage, which may contribute to the

development of cardiovascular disease and cancer. Free radicals probably involved in the mechanisms of aging itself. The body has many mechanisms to decrease free radical that induced damage and repaired damage. Antioxidant enzymes play a key role in these defense mechanisms, such as glutathione peroxidase, glutathione reductase, catalase, thioredoxin reductase and superoxide dismutase. Furthermore, chemical antioxidants neutralize free radicals by accepting or donating an electron to eliminate the unpaired condition. Synthetic or natural antioxidants are widely used in nutrition and medicine and prepared foods for the effect to delay the natural aging process and to prevent certain diseases that result from cellular damage.

Naringenin is major flavonone that occur in various medicinal plants such as citrus fruits, grapefruits, tomato fruits and also presents in vegetables, tea and wine. Naringenin is present not only in the solid tissues but also found in the juice (Krause and Galensa, 1992; Susanti *et al*., 2007). Low concentrations of naringenin are found in tomatoes and tomato-based products. During processing to tomato ketchup naringenin chalcone, which is found in fresh tomatoes, especially tomato skin, is converted to naringenin (Krause and Galensa, 1992). The structure of naringenin is shown in Fig. 1.

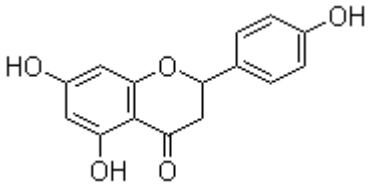

Figure 1. Structures of naringenin

Although antioxidant activity of naringenin is lower compared with many other polyphenols. Naringenin has been reported to regulate apolipoprotein B secretion by HepG2 cells on lipid metabolism, possibly through inhibition of cholesterol ester synthesis (Borradaile *et al*., 1999) and to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase and acyl coenzyme A: cholesterol O-acyltransferase in rats (Lee *et al*., 1999). Moreover, a decrease in plasma low-density lipoprotein levels and hepatic cholesterol levels in rabbits fed a high-cholesterol diet supplemented with dietary orange juice or dietary grapefruit juice has been reported. This report suggested that the reduction of LDL cholesterol and hepatic cholesterol induced by dietary citrus juice or grapefruit juice could be due to endogenous effects

of juice components, possibly flavonoids, particularly naringenin (Kurowska *et al*., 2000). High-density lipoprotein levels in hypercholesterolemic human subjects were also increased (Kurowska *et al*., 2000). There are many reports about anti-inflammatory actions (Manthey *et al*., 2001) and effects on sex hormone metabolism of naringenin (Ruh *et al*., 1995; Rosenberg *et al*., 1998; Déchaud *et al*., 1999; Yoon *et al*., 2001). Most studies concerned the possible role of naringenin in grapefruit juice-drug interactions (Fuhr, 1998; Bailey *et al*., 2000). Naringenin is an inhibitor of the enzyme cytochrome P-450 IIIA (CYP3A4) (Ghosal *et al*., 1996) and may be causing the interaction. Nevertheless, other constituents in grapefruit seem to be more important in this event. The cancer-preventive effects may be due to stimulation of DNA repair by naringenin which, by stimulating base excision repair processes, may prevent mutagenic changes in prostate cancer cells (Gao *et al*., 2006).

Since naringenin is available and can be isolated from various thai medical plants, the purpose of this study was to investigate the antiviral activities of naringenin against HSV-1 strain KOS and HSV-2 strain Baylor 186. The antiviral activity was evaluated by inactivation, plaque reduction and prophylaxis assay. Furthermore, preliminary mechanism studies of naringenin were performed through virucidal, post-binding, penetration virus growth inhibition, time of addition and combination with ACV assays, against HSV-1, to find its possible mode of antiviral activity.

The results from this study could provide the information on the *in vitro* anti-HSV-1 and HSV-2 activities of naringenin. Moreover, naringenin would be interesting candidate for the anti-HSV drug development in the future.

CHAPTER II

REVIEW OF LITERATURE

2.1 Herpes simplex virus

The name herpes comes from the Latin herpes which, in turn, comes from the Greek word herpein which means to creep or crawl along the skin. This reflects the creeping or spreading nature of the skin lesions caused by many herpes virus types (Beswick, 1962; Wildy, 1973). Herodotus was the first to draw an association between these cutaneous eruptions and fever, the association that has survived today. Galen indicated that HSV recurrences develop at the same anatomical site (Roizman, B. and Knipe, 2001).

2.2 Characteristics of herpes simplex virus

Herpes simplex viruses (HSV) are large enveloped viruses; 150-200 nm diameter; with a distinct virion structure characteristic of the viruses (Figure 2) which are classified in the genus *Simplexvirus* (White and Fenner, 1994; Taylor *et al*., 2002), in the subfamily *Alphaherpesvirinae*, and family *Herpesviridae* by the International Committee on the Taxonomy of Viruses (ICTV) (Van Regenmortel *et al*., 1991; Roizman and Knipe, 2001). These are classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily but not exclusively in sensory ganglia (Roizman and Pellet, 2001).

The HSV virion consists of four structural features (Wildy *et al*., 1960): (i) an electron-opaque core containing viral DNA, (ii) an icosahedral capsid surrounding the core, (iii) an amorphous tegument that surrounds the capsid, and (iv) an outer lipid bilayer envelope studded with viral glycoprotein spikes on its surface. The core contains the linear double strand DNA (dsDNA) genome wrapped as a toroid or spool in a liquid crystalline state (Zhou *et al*., 1999).

A small fraction of the HSV virion DNA possibly circular (Roizman and Knipe, 2001). The capsid is composed of 162 capsomers arranged in icosahedral symmetry. It encloses the DNA genome and consists of 6 external proteins pUL6, pUL18 (VP23), pUL19 (VP5), pUL25, pUL35 (VP26) and pUL38 (VP19C) (Roizman and Sears, 1993). The surrounding tegument contains 22 proteins that include pUL4, pUL11, pUL13, pUL14, pUL16, pUL17, pUL21, pUL36 (VP1/2), pUL37, pUL41, pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16), pUL49 (VP22), pUL51, pUL56, pUS2, pUS3, pUS10, pUS11, ICP0, and ICP4. The envelope contains 5 proteins, pUL20, pUL43, pUL45, pUL49A and pUS9 along with 11 glycoproteins, gpUL1 (gL), gpUL10 (gM), gpUL22 (gH), gpUL27 (gB), gpUL44 (gC), gpUL53 (gK), gpUS4 (gG), gpUS5 (gJ), gpUS6 (gD), gpUS7 (gI) and gpUS8 (gE) (Mettenleiter, 2004). The tegument, a term introduced by Roizman and Furlong to describe the space between the surface of the capsid and the undersurface of the envelope (Roizman and Furlong, 1974), has no distinctive structures in thin sections, but on negative staining, it may appear to be fibrous. Sometimes, the tegument is distributed asymmetrically, and its thickness may vary depending on the virion location within the infected cell; when the amount is variable, there is less of it in virions accumulating in the perinuclear space than in those accumulating in the cytoplasmic vacuoles. Some evidence introduces the amount of tegument is more likely to be determined by the virus than by the host. The thickness of tegument variability results in the variation in size of virions. Finally, the envelope consists of a lipid bilayer with approximately 12 different viral glycoproteins embedded in it called spikes. Primary studies on purified HSV virions suggested that they contain more than 30 distinct proteins, which were designated as virion polypeptides (VP). Of the approximately 30 known and another 10 suspected virion proteins, at least 12 are on the surface of the HSV virion and at least 10 are glycosylated. The viral glycoproteins on envelope surface are gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN which are important in HSV infection (Roizman, 1996; Roizman and Knipe, 2001). The glycoprotein B (gB) is required for viral entry and induces neutralizing antibody of host immune response, whereas glycoprotein C (gC) is involved in cell attachment and plays a role in blocking host immune response to infection.

Moreover, gC can protect infected cells from antibody-dependent cellular cytotoxicity because of having a C3b complement component receptor (Friedman *et al*., 2000; Lubinski *et al*., 2002). The glycoprotein D (gD) is required for post attachment of virus. The Glycoprotein E (gE) can bind with IgG *via* Fc receptor in gE-gI complex form. This Fc receptor protect both against immunologic attack by steric hindrance resulting from binding of normal IgG or from bipolar bridging of HSV antibody which can attach to gE/gI by its Fc end and simultaneously to another HSV glycoprotein by one Fab arm. Therefore, gC has an immune escape function. The glycoprotein G (gG) plays a role in egress and cell-to-cell spread, and glycoprotein H (gH) forms complex with gL. This complex is required for fusion of host membranes, and cell-to-cell spread. The glycoprotein J (gJ) can block apoptosis, whereas glycoprotein J (gJ) is important for viral exocytosis. However, the function of glycoprotein N (gN) is still unknown (Roizman and Knipe, 2001).

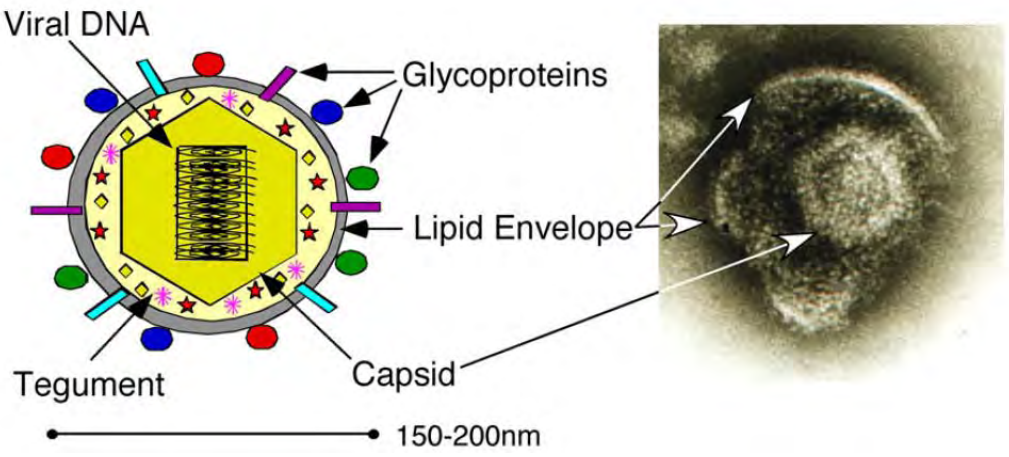

Figure 2. Structure of herpes simplex virus (Taylor *et al*., 2002).

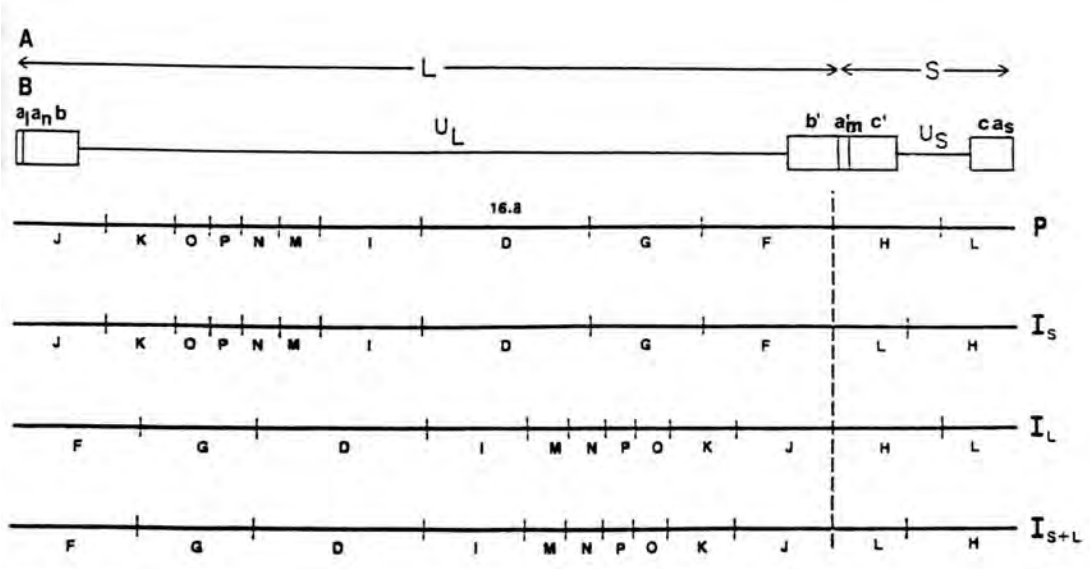

Figure 3. The arrangement of DNA sequences in HSV genome (Roizman and Knipe, 2001).

The HSV genome consists of 152 kilo base pairs (kbp), with a G+C content of 68.3% for HSV-1 and 69% for HSV-2 (Roizman, 1996). The homology between two types of HSV genome is about 50%. The HSV genome is composed of two covalently linked components, designated as Long (L) and Short (S), based upon their relative length (Figure 3). Each component consists of unique sequences regions (U_L or U_S) flanked by inverted repeat sequence (Whitley and Roizman, 2001). Most packaged HSV DNA is linear and double stranded. The ends of the genome may be held together or are in close proximity in as much as a small fraction of the packaged DNA appears to be circular and the bulk of the linear DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells. DNA extracted from HSV virions contains ribonucleotides, nicks, and gaps (Roizman and Knipe, 2001).

Most of the polypeptides specified by one virus type are antigenically related to the polypeptides of the other type. They are designated as either infected cell specific polypeptide (ICSPs) or infected cell polypeptides (ICPs). There are three groups of HSV proteins; α, β, and γ; which are synthesized in a sequential order. First,

six α-proteins such as ICP0, ICP4, ICP22, ICP27, ICP47, and U_S1.5. The α-polypeptides synthesis occurs very early after infection, about 2 to 4 hours post infection, but some α-proteins continue to be produced throughout the period of infection. Second, the β-polypeptides reach peak rate of synthesis approximately 5 to 7 hours after infection (Honess and Roizman, 1975; Roizman and Knipe, 2001), and are usually involved in viral DNA replication. These proteins are divided into two groups; β_1 and β_2. The β_1 proteins occur very soon after infection, exemplified by polypeptides ICP6; the large component of the viral ribonucleotide reductase (Roizman and Knipe, 2001); and ICP8; the major DNA binding protein (Conley *et al*., 1981). They are differentiated from α-proteins by their requirements for functional ICP4 protein and for their synthesis (Honess and Roizman, 1975; Roizman and Knipe, 2001). The β_2 proteins are synthesized later; they include the viral thymidine kinase (TK) and DNA polymerase. Most of the β proteins are responsible for viral nucleic acid metabolism and are the main target of antiviral chemotherapy (Whitley, 2001). The appearance of β gene products signals the onset of viral DNA synthesis. Lastly, the γ-proteins are primarily structural polypeptides, included viral glycoproteins, capsid, and some components of tegument. They are divided into two classes, γ_1 and γ_2. The γ_1 polypeptides are early synthesized in the absence of viral DNA replication. While the γ_2 polypeptides occur after the viral DNA replication has initiated. These γ-proteins also act as a main target for host immune response (Roizman and Knipe, 2001; Taylor *et al*., 2002). There are two ways, invasiveness and neurogrowth, resulting the virulence of HSV. Firstly, invasiveness is the capacity of virus to access a target organ, including nervous system tissues, from the portal of entry. The virus is necessary to multiply at peripheral sites to disseminate to the target organ. Secondly, neurogrowth is the viral ability to grow in nervous system tissue. Therefore, HSV virulence requires at least two distinct sets of viral function. The first are viral genes which are responsible for reach to and injury of cells. The second are viral genes and gene functions that turn off host responses to infection (Whitley and Roizman, 2001).

2.3 Multiplication of herpes simplex virus

Replication of HSV is a multistep process (Figure 4), including entry, viral gene expression, viral DNA synthesis, virion assembly, and egress of progeny virions (Figure 5). After the onset of infection, DNA is uncoated and transported to the host cell nucleus. Then, there is transcription of immediate-early genes, which encode for the regulatory proteins, and is followed by the expression of proteins encoded by early and then late genes. These proteins include enzymes necessary for viral replication and structural proteins. Assembly of the viral core and capsid takes place within the nucleus. Envelopment at the nuclear membrane and transport out of the nucleus occur through the endoplasmic reticulum and the Golgi apparatus. Glycosylation of the viral membrane occurs in the Golgi apparatus. Mature virions inside vesicles are transported to the outer membrane of the host cell. Release of progeny virus is accompanied by cell death. Replication for all herpesviruses is considered inefficient, with a high ratio of noninfectious to infectious viral particles (Whitley, 2001).

The initial virus attachment depends on the interaction of viral envelope gC, and, to a lesser extent gB, with the glycosaminoglycan moieties of heparin sulfate of cell surface (WuDunn and Spear, 1989; Herold *et al*., 1991; Shieh *et al*., 1992; Roizman and Knipe, 2001). Recent report suggested that gB also binds to cell surface independently of heparin sulfate and allows viral entry into cell (Bender *et al*., 2005). After the initial step, virion attachment is stabilized by binding to a coreceptor. This sequential step involves the interaction of gD with one of the several cellular molecules (Spear *et al*., 2000; Spear and Longnecker, 2003; Spear *et al*., 2006). There are many coreceptors, the first is a member of the tumor necrosis factor (TNF) receptor family originally called herpesvirus entry mediator (HVEM) but renamed HveA (Montgomery *et al*., 1996; Whitbeck *et al*., 1997). HveA is primarily found in lymphoid cells and also found in other cell types such as lung, liver, and kidney (Kwon *et al*., 1997). The second family belongs to the immunoglobulin (Ig) superfamily, including nectin-1or HveC (Geraghty *et al*., 1998) and nectin-2 or HveB (Warner *et al*., 1998). These cellular proteins were shown to act as intercellular adhesion molecules and to be localized at adhesion junctions (Aoki *et al*., 1997; Lopez *et al*., 1998; Takahashi *et al*., 1999; Satoh-Horikawa *et al*., 2000).

Nectin-1 is expressed in epithelial, fibroblast, neural, and hematopoietic cells. It mediates entry of all HSV-1 and HSV-2 strains (Cocchi *et al*., 2000), whereas, Nectin-2 is expressed in gall bladder, kidney, and testis. It mediates entry of HSV-2 selectively, but not for wild-type HSV-1 (Lopez *et al*., 2000). The third coreceptor is specific sites in heparin sulfate generated by certain isoforms of 3-*O*-sulfotransferases (3-OSTs) called 3-*O*-sulfated heparin sulfates. The 3-*O*-sulfated heparin sulfates generated by 3-OST isoform 2, 3A, 3B, 4, 5, and 6 can efficiently mediate entry of HSV-1 only. These coreceptors are broadly distributed on human tissue, primarily in heart, brain, lung, kidney, liver, skeletal muscle, and placenta (Shukla *et al*., 1999; Liu *et al*., 1999; Shworak *et al*., 1999; Xia *et al*., 2002; Tiwari *et al*., 2004, 2005; Xu *et al*., 2005; O'donnell, 2006). The last step in viral entry is the fusion of the HSV envelope with the plasma membrane of the host cell by an undetermined mechanism. Current evidence indicates that virus-cell fusion requires gD (Ligas and Johnson, 1988 Roizman and Knipe, 2001), gB (Sarmiento, 1979), and gH-gL heterodimer (Forrester *et al*., 1992; Perez-Romero *et al*., 2005). However, intact virions may also enter *via* endocytic pathway into some cell types at some conditions (Nicola and Straus, 2004; Milne *et al*., 2005; Nicola *et al*., 2005).

Following the fusion, viral nucleocapsid and tegument proteins are released into the host cell cytoplasm. The nucleocapsid and some tegument proteins, VP16 and VP1-2, are transported through the microtubules network to the nuclear pore (Sodeik *et al*., 1997) while other tegument proteins remain in the cytoplasm. The nucleocapsid releases its DNA into the nucleus at the pore, while leaving an empty capsid at the cytoplasmic side of the complex (Roizman and Knipe, 2001).

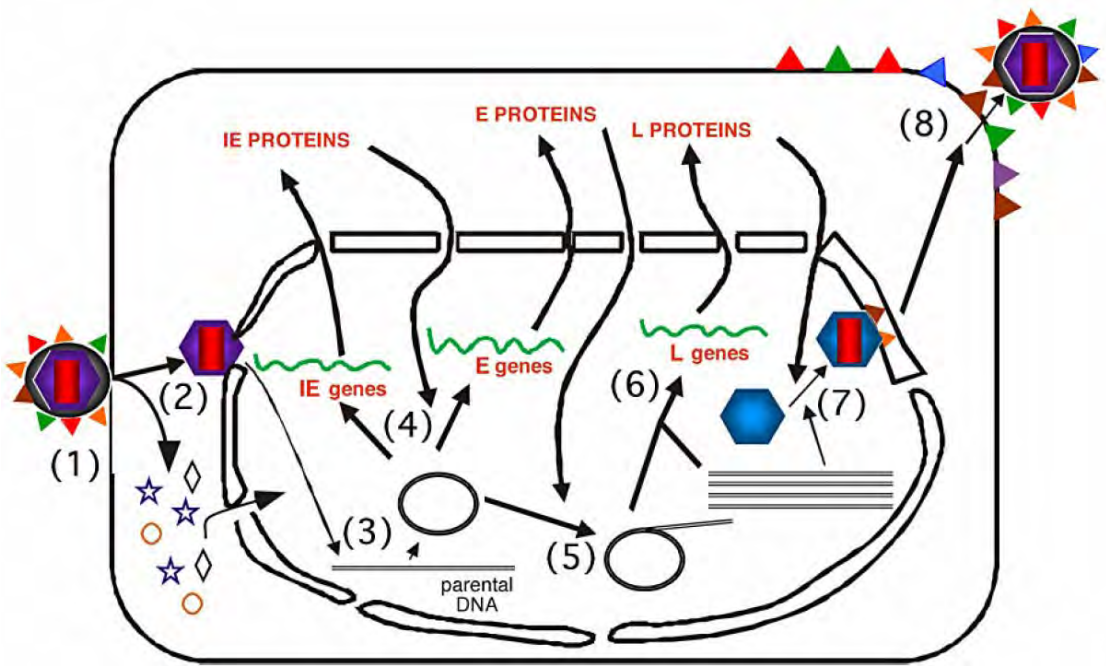

Figure 4. The cycle of productive HSV replication in a cell. The stages of HSV infection are: (1) Receptor binding and membrane fusion; (2) Release of the viral nucleocapsid and tegument into the cell cytoplasm and transport of the nucleocapsid to the nuclear pore; (3) Release of viral DNA into the nucleus; (4) Transcription and translation of the viral immediate early (IE) and early (E) genes; (5) viral DNA synthesis; (6) Transcription and translation of the viral late (L) genes; (7) capsid assembly and DNA packaging; and (8) egress of progeny virions (Taylor *et al.*, 2002).

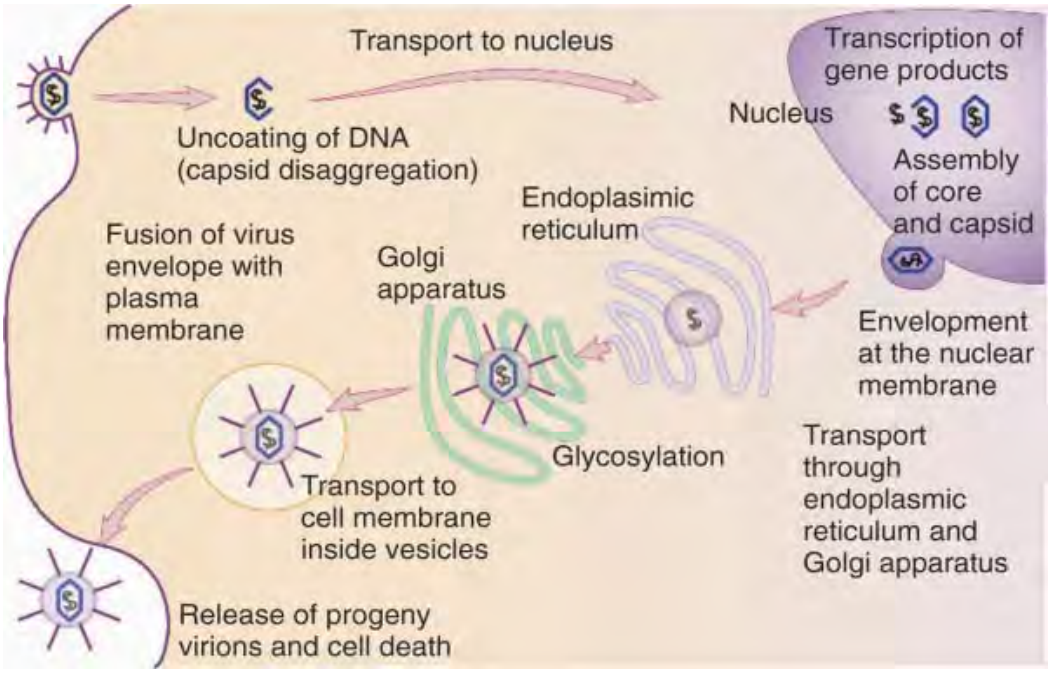

Figure 5. Herpes simplex virus replication (Whitley, 2001).

After infection, the incoming viral DNA circularizes rapidly in the absence of viral protein s ynthesis (Garber *et al*., 1993), then the HSV gene transcription occur.

Host RNA polymerase II is used for viral mRNAs synthesis. When cellular proteins are enough for the synthesis of viral transcripts, viral proteins are essential for the initiation and enhancement of transcription of certain genes. To produce the full length of viral gene products required for productive viral infection and replication, these proteins act in concert with an abundance of cellular proteins. The immediate early (IE) or α genes are first gene which transcribed during viral infection. Initiation of these genes transcription proceeds by recruitment of cellular transcriptional machinery to IE gene promoters that contain various host regulatory sequences. Prior HSV protein synthesis is not required for IE gene expression. However, an HSV protein carried in with the tegument, VP16, is necessary for enhance the expression of the α-proteins (Batterson and Roizman, 1983). The α proteins include various multifunctional proteins that play important roles in the regulation of later viral gene expression as well as in control of the host cell. Then expression of the early (E) or β

genes occurs. It requires at least 3 IE proteins; ICP0, ICP4, and ICP27, but this expression is not related with the onset of viral DNA synthesis (Uprichard and Knipe, 1996).

The β-proteins are commonly involved in viral DNA replication. Further synthesis of α proteins are blocked by these proteins and lead to transcription of γ genes, the third set of viral RNAs (Roizman and Knipe, 2001).

Prior to DNA replication, α proteins initiate the transcription including the E genes and a subset of the late (L) or γ genes, called early-late, leaky-late, or γ_1 genes. The synthesis of these proteins, during later in the infection cycle is not completely dependent on viral DNA replication. Nevertheless, their levels are significantly increased upon the initiation of DNA replication. Another subset of late genes; the true late or γ_2 genes; is transcribed only after the initiation of viral DNA replication. Viral DNA replication initiates at the origins of replication within the HSV genome in nucleus. The theta replication mechanism is believed to proceed initially (Figure 6). After its initiation, viral DNA synthesis begins and it is likely that a rolling-circle replication mechanism takes over to produce concatemeric molecules. Thus, most of the viral progeny DNA molecules that accumulate in the infected cell nucleus are head-to-tail concatemers. There are seven herpes genes which were considered as essential for viral DNA replication. These genes encode protein products that function as an origin binding protein (U_L9), a DNA binding protein ICP8 (U_L29), a helicase-primase complex (U_L5, U_L8, U_L52), and a DNA polymerase (U_L30, U_L42). Along with this, HSV expresses several other early viral gene products, for example, thymidine kinase, ribonucleotide reductase and uracil *N*-glycosylase. These proteins are required for nucleotide metabolism, viral DNA synthesis and repair in resting cells, particularly neurons. However, they are not necessary for viral replication (Roizman and Knipe, 2001).

Assembly of viral capsid requires synthesis of many late proteins, and occurs within the nucleus. These capsid proteins, such as VP5, VP19c, VP21, VP22a, VP24, and VP26, are synthesized in cytoplasm and transported into the nucleus. Empty capsid shells are loaded with viral DNA by a method that concurrently cleavages of HSV progeny DNA concatemers and packages genome-length monomers within the capsid (Roizman and Knipe, 2001). There is not clearly about the mechanism of

DNA cleavage and packaging but it is known to require site-specific breaks to the concatemers at definite distances from the packaging signal (Smiley *et al*., 1990; Roizman and Knipe, 2001).

After encapsidation of full-length viral genomic DNA molecules, the nucleocapsids are competent of budding through the inner nuclear membrane (Vlazny *et al*., 1982; Roizman and Knipe, 2001). Interactions between capsid and tegument proteins and between tegument proteins and viral glycoproteins promote this budding process. Nucleocapsids obtain some tegument proteins and a glycoprotein studded envelope upon budding through the inner nuclear membrane. The egress of the virion particle from the space between the inner and outer nuclear membranes to the exterior of the infected cell remains controversial. The virion egress has hypothesized in two general pathways.

A re-envelopment pathway proposed that enveloped particles fuse with the outer nuclear membrane, by which de-envelopment of the nucleocapsids and release free nucleocapsids into the cytoplasm (Roizman and Knipe, 2001). A luminal pathway proposed that the enveloped particles transit from the inner nuclear space through the cytoplasm in the the endoplasmic reticulum lumen or in vesicles to the golgi where virion glycoprotein completely mature. These matured virions are released from the cell by a normal secretory route (Enquist *et al*., 1999). Nevertheless, current evidences lead considerable support to the re-envelopment model as the main route of virion egress (Granzow *et al*., 2001; Skepper *et al*., 2001).

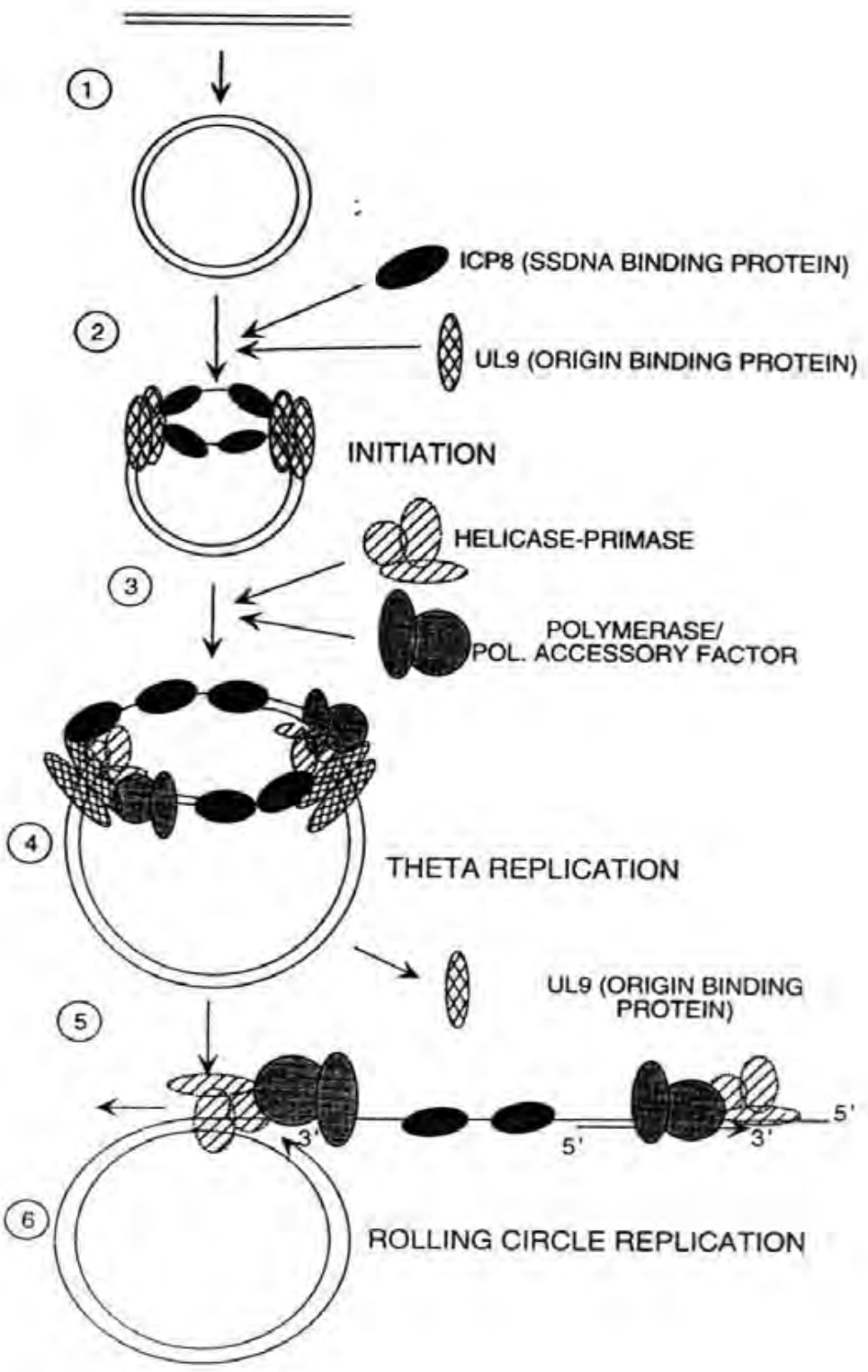

Figure 6. A model of HSV DNA replication (Roizman and Knipe, 2001).

2.4 Fate of the infected cells

The structural alterations in infected cells occur in numerous ways. First, the nucleolus becomes enlarged and eventually disaggregates. Meanwhile, host chromosomes become marginated, and subsequently in infection, the nucleus become distorted and multilobed. Second, viruses induce changes in appearance of cellular membranes. The duplication and folding of intracellular membranes are characteristic of late HSV infected cells (Roizman and Knipe, 2001). HSV can causes a cell-type-dependent fragmentation and dispersal of golgi vesicles throughout the cytoplasm (Campadelli-Fiume *et al*., 1993). Furthermore, viral proteins particurly glycoproteins are inserted into cellular membranes resulted in alterations of cellular membrane structure and antigenicity (Roizman and Knipe, 2001). Another character of HSV infected cells is Polykaryocytosis. HSV-1 and HSV-2 cause cells to round up and adhere to each other or fuse into polykaryocytes (Ruyechan *et al*., 1979; Roizman and Knipe, 2001). Third, rearrangements of the microtubular network are apparent too early in infection. Therefore, the microtubules at the junction of the network with the plasma membrane appear to be disrupted in infected cells (Ward *et al*., 1998). Eventually, the accumulation of viral replication proteins, viral DNA of progeny, and nucleocapsid components within nucleus (De Bruyn Kops and Knipe, 1988; Roizman and Knipe, 2001) may cause the constitution of intranuclear inclusion bodies in HSV-infected cells (Roizman and Knipe, 2001). These large eosinophilic intranuclear inclusion bodies usually are found both in HSV-infected tissues and in suitably stained cell cultures (White and Fenner, 1994).

HSV shuts down host cell RNA, DNA, and protein synthesis (Roizman and Knipe, 2001) because the virus can use many cellular proteins for its own protein synthesis (Taylor *et al*., 2002). Therefore, host DNA synthesis is shut off (Roizman and Roane, 1964), host protein synthesis declined quickly (Roizman and Knipe, 2001), and glycosylation of host proteins discontinues (Smiley *et al*., 1992). Moreover, in HSV infected cells, host macromolecular metabolism is altered in at least three ways. First, the virion-host shutoff (vhs) protein causes the degradation of mRNA display in infected cells early in infection (Zelus *et al*., 1996; Karr and Read, 1999). Subsequently, multiple viral genes are involved to obstruct cellular transcription (Preston and Newton, 1976) and translation (Roizman and Knipe, 2001)

to simplify viral gene transcription and translation. Consequently, ICP27 inhibits RNA maturation by redistributing host splicing factors (Sandri-Glodin *et al*., 1995; Sandri-Goldin and Hibbard, 1996). Therefore, HSV regulatory protein ICP24 inhibits host RNA splicing and leads to the reduction in cellular mRNA levels during infection (Hardwice and Sandri-Goldin, 1994). This result has little or no effect on viral RNA synthesis because very few viral transcripts are spliced. Another viral protein, ICP22 is required for alterations of host RNA polymerase II following infection (Spencer *et al*., 1997), possibly altering the ability of this complex to transcribe from the genome. Eventually, the virus selectively destabilizes (Advani *et al*., 2000) and degrades (Everett *et al*., 1994) a category of cellular proteins, particularly those involved in host cell cycle regulation (Advani *et al*., 2000; Roizman and Knipe, 2001).

2.5 Lytic and latent infection

The life cycle of HSV involves both lytic (productive) and latent (non-productive) infection. Upon HSV in introduced onto a mucosal surface, or at a break in the skin, and it replicates productively in epithelial cells at the site of inoculation and undergoes spreads through the tissue (Roizman and Knipe, 2001). As HSV spreads from the primary site of infection, the virus also infects sensory neurons (either trigeminal ganglia for HSV-1 or sacral ganglia for HSV-2.) by fusion with the neuronal membrane at the axonal termini, and the nucleocapsid is transported to the nucleus in the cell body in a ganglion. Viral DNA is released into the nucleus. Virus either replicates productively or establishes a latent infection in the neuronal cell nucleus. During latency, the HSV genome remains in the nucleus of the sensory neuron as circular, extra-chromosomal DNA. Lytic gene expression is repressed, but the latency-associated transcript (LAT) is expressed at high abundance, which helps to silence lytic gene expression. Clearly, in latent infections HSV does not cause lytic infection, as occurs in mucous membranes and still promotes neuronal survival during its latency. The virus remains in this state for the lifetime of host, or until the proper signal periodically reactivate the virus and new progeny viruses are produced. New components of the virion move by anterograde transport to both peripheral and central branches of the neuron. The signal and mechanisms involved in this process are not

known, but it appears that host immune status (Roizman and Knipe, 2001) and certain physical stresses, such as fever or exposure to ultraviolet light, can increase the chance of reactivation (Taylor *et al*., 2002).

2.6 Pathology and pathogenesis of herpes simplex virus infection

The pathogenesis of HSV-1 and HSV-2 infections in humans and animal models has some general similarities and some important differences. The site of primary infection depends on the way in which the patient acquires the virus. HSV-1 is primarily causes orolabial lesions, stromal keratitis, and occasionally encephalitis (Stanberry *et al*., 1997). It is often noted that HSV-1 usually causes infection at oropharyngeal tract, and is spread by respiratory droplets or by direct contact with infected saliva. HSV-2 primarily associated with genital infections but is also capable of necrotizing stromal keratitis, encephalitis, meningitis, and neonatal ophthalmic, and neurologic complications in infants surviving infection (Stanberry *et al*., 1997). It is usually sexually transmitted and replicates in the genital, perigenital, or anal skin sites. This reflects the mode of transmission rather than any intrinsic property of virus (Sack *et al*., 2004). HSV-2 is significantly more neurovirulent than HSV-1 by all routes of infection in animal models (Smith and Sutherland, 1986).

The pathogenesis of HSV infections is understood through knowledge of the events of replication, including virulence, and establishment of latency in both humans and animal models. The pathogenesis of human disease depends on intimate, personal contact a susceptible individual (namely, one who is seronegative) with someone excreting HSV. Exposure to HSV at mucosal surfaces or abraded skin sites permits viral entry and initiation of HSV replication in the cells of epidermis and dermis. Lesion induced in the skin and mucous membranes by HSV-1 and HSV-2 are the same and resemble those of varicella zoster virus (Roizman and Knipe, 2001)**.**

The pathologic changes induced by the replication of HSV are similar for both primary and recurrent infection but vary in the quantitative extent of cytopathology. These changes represent a combination of virally mediated cellular death and associated inflammatory response because HSV causes cytolytic infections. Changes induced by viral infection include ballooning of infected cells and the appearance of condensed chromatin within the nuclei of cells, followed by subsequent degeneration

of the cellular nuclei of cells, generally within parabasal and intermediate cells of the epithelium. Cell fusion appeared as multinucleated giant cells provides an efficient method for cell-to-cell spread for HSV, even in the presence of neutralizing antibody (Brooks *et al*., 2001).

With cell lysis, clear fluid (namely vesicular) containing large quantities of virus appears between the epidermis and dermal layer. The vesicular fluid contains cell debris, inflammatory cells, and multinucleated giant cells. There is an intense inflammatory response, usually in corium of the skin. With healing, the vesicular fluid becomes pustule with the recruitment of inflammatory cells, and then it scabs. Scarring is uncommon but has been found in some patients with often recurrent lesions. Vascular changes in the area of infection include perivascular cuffing and areas of hemorrhagic necrosis. These histopathologic findings become particularly prominent when the infection occurs in organs of the body other than skin, as is encountered with HSV encephalitis or disseminated neonatal HSV infection (Roizman and Knipe, 2001).

Initial HSV infection is usually subclinical. Both clinical acquisition and clinical acquisition are associated with sufficient replication of virus to permit infection of sensory or autonomic nerve ending. HSV is transported intraaxonally to the nerve cell bodies in ganglia. During the initial phase of infection, viral replication occurs in ganglia and contiguous neural tissue. Then, virus spreads to other mucosal skin through centrifugal migration of infectious virions via peripheral sensory nerves. This can explain the large surface area involved, the high frequency of new lesions distant from the initial crop of vesicles that is characteristic in primary genital or oral-labial HSV infection patients, and viral recovery from neural tissue distant from neurons innervating the inoculation site. Moreover, contiguous spread of locally inoculated virus may take place and allow mucosal extension of disease(Roizman and Knipe, 2001).

Because of infection with HSV-1 generally to oropharynx, initial replication of virus occurs in the oropharyngeal mucosa. The trigeminal ganglion becomes colonized and harbors latent virus. Acquisition of HSV-2 infection is usually the consequence of transmission by genital contact. Virus replicates in genital mucosa with seeding of the sacral ganglion. The mechanism controlling the reactivation of

HSV infection are unknown. However, after the establishment of latency as described above a recurrence of HSV infection is known as reactivated infection or recurrent infection. This form of infection leads to recurrent vesicular lesions of the skin such as herpes labialis or recurrent genital herpes (Roizman and Knipe, 2001). HSV reinfection with a different strain can occur but extremely uncommon in the normal host. It is called exogenous reinfection (Brook *et al*., 2001).

Primary HSV infections are usually asymptomatic. Only rarely virus can spread beyond the dorsal root ganglia, thereby becoming systemic. Such circumstances include multiorgan disease of pregnancy, disseminated neonatal HSV infection, and disseminated HSV infection in immunocompromised host. It is likely that the widespread organ is involved as a result of viremia in the host incapable of limiting viral replication to mucosal surfaces (Roizman and Knipe, 2001).

2.7 Immunity of herpes simplex virus infection

Host responses to infection with HSV influence the acquisition of disease, the severity of infection, resistance to the development of latency, the maintenance of latency, and the frequency of recurrences. Both antibody-mediated and cell-mediated immunity are clinically important. Immunocompromised patients with defects in cell-mediated immunity are more severe and more extensive HSV infections than those with deficits in humural immunity. The surface viral glycoproteins act as antigens recognized by antibodies mediating neutralization and immune-mediated cytolysis. During primary infection, IgM antibodies directed against envelope glycoprotein gB and gD appear transiently and are followed by IgG and IgA that persist over time. The more severe the primary infection or the more frequent the recurrences, the greater the level of antibody response. However, the pattern of antibody response does not correlate with the frequency of recurrent disease. However, cell-mediated immunity and nonspecific host defense mechanisms are also important in controlling both primary and recurrent infections of HSV. All these responses may influence on the acquisition of disease, the severity of infection, and the host resistance to subsequent HSV reactivation as described above (Roizman and Knipe, 2001).

In spite of the presence of both host HSV-specific humoral and cellular immunity, spontaneous reactivations can occur. This immunity plays a role in limiting

local viral replication, so that recurrent infections are less extensive and less severe. Accordingly, many recurrences are asymptomatic and detected only by viral shedding in secretions. When symptomatic recurrent infections occur, Episodes of recurrent HSV-1 infection are usually manifested as cold sores or fever blisters near the lip. More that 80% of the human population harbors HSV-1 in a latent form, but only a small portion experience recurrences (Roizman and Knipe, 2001).

Because of passive transfer of maternal antibodies in many newborns are lost during the first 6 months of life, whereas the period of greatest susceptibility to primary herpes infection occurs between 6 months and 2 years. Thus, the humoral immunity does not prevent either recurrences or exogenous reinfection. Therefore, transplacentally acquired antibodies from the mother are not totally protective against infection of newborns but with conflicting results (Kahlon and Whitley, 1988; Roizman and Knipe, 2001). HSV-1 antibodies begin to appear in early childhood and present in most persons by adolescence. Antibodies to HSV-2 rise during the age of sexual activity and adolescence (Roizman and Knipe, 2001).

2.8 Epidemiology of herpes simplex virus infection

All human herpesviruses probably diversified from a common ancestor a million years ago. There is a host-linked evolution of the human herpesviruses such that the mediation of the cospeciation of these human herpesviruses occurred through these species-specific latent infections (Umene and Sakaoka, 1999). HSV is endemic in virtually every human society throughout the world. Humans are the only natural reservoirs for HSV and there are close associations recognized between HSV-1 strains and historical human populations. HSV-1 is more likely to infect the trigeminal ganglia and HSV-2 the sacral ganglia, explaining the clinical manifestations of orofacial and genital herpes infections. Although HSV-1 and HSV-2 are usually transmitted by different routes and involve different areas of the body, there is a great deal of overlap between the epidemiology and clinical manifestations of infection. HSV-1 is probably constantly present in humans than other viruses. Primary HSV-1 infections usually occur in the young child, less than 5 years of age, and most often asymptomatic. With clinical illness, the most common sites of this virus infection are the mouth and lips. Gingivostomatitis usually is the clinical manifestation in young

children while pharyngitis is associated with HSV-1 primary infection in young adults (Whitley *et al*., 1998).

The spectrum of disease caused by HSV includes primary and recurrent infections of mucous membranes (e.g., gingivostomatitis, herpes labialis, and genital infections), neonatal and congenital HSV infection, eczema herpeticum in patients with underlying atopic dermatitis or visceral HSV infections in immunocompromised hosts, HSV encephalitis, and an association with erythema multiforme. Ocular complications include lid, conjunctival, corneal, intraocular infections, and retinitis. Patients with chemotherapy, organ or bone marrow transplant recipients, and patients with HIV infection can develop multiple and extensive lesions and visceral spread may occur in some cases (Safrin, 1992; Johnson and Polsky, 1993; Stewart *et al*., 1995). The different clinical manifestations of HSV infections appear related to host populations and the age of acquisition of the infection (Gorbach *et al*., 1998). The incubation period of primary HSV infection ranges from 1 to 28 days. HSV is infectious during asymptomatic shedding and for the 5 to 10 days it takes for the skin or mucous membrane lesions to heal (Wilhelmus, 1998). Latency with HSV is prevalent, with at least 33% of the world with clinically evident recurrent HSV infections (Whitley *et al*., 1998).

HSV-1 and HSV-2 may be shed asymptomatically at the time of primary, initial, or recurrent infection. Geographic location, socioeconomic status, and age influence HSV-1 prevalence. In developing countries, seroconversion happens early in life. By 5 years of age, approximately one third of children seroconverted and this frequency increased to 70% to 80% by early adolescence (Roizman and Knipe, 2001). Because HSV-1 is transmitted principally by contact with infected oral secretions or lesions, the incidence and prevalence are influenced by factors that affect the degree of exposure to these sources of infection, such as crowding, poor hygiene, and age. Seropositive children and adults, including those with no history of HSV labialis, periodically shed HSV in saliva and are the major source of transmitting HSV infections. The proportion of the population with positive serology for HSV-1 that sheds ranges from 1 to 10%, but it may be greater in children or in the first 2 years after the primary infection and is also markedly increased in the immunosuppressed patient (Gorbach *et al*., 1998). The risk of infection is directly proportional to the titer

of virus shed, which is usually more in a symptomatic patient. These studies indicated that the frequency of direct person-to-person contact, indicative of crowding encountered with lower socioeconomic status, appears to be the main mediator of HSV-1 infection. The largest reservoir of HSV-1 infections in the community is recurrent herpes labialis. The frequency of recurrent HSV-1 infection is approximately 33% in several studies. Recurrent infections may occur in the absence of clinical symptoms, but still have viral shedding. At any given times approximately 1% to 5% of normal adults will be excreting HSV (Roizman and Knipe, 2001).

HSV-2 is transmitted sexually by contact with infected genital secretions or mucocutaneous surfaces; acquisition is directly related to sexual activity. Most HSV-1 and HSV-2 transmission, nevertheless, occurs during periods of asymptomatic shedding in the absence of recognized signs or symptoms of disease. Only a minority of persons who are HSV-2-seropositive have a history of symptomatic disease. Most patients (70%) are not aware of infection with genital HSV; with education, however, 50% can be taught to identify the clinical infection (Benedetti *et al*., 1999). Virtually all persons who have symptomatic primary genital HSV-2 will experience both symptomatic recurrence and asymptomatic shedding. Although most genital HSV infections are caused by HSV-2, an increasing proportion is attributable to HSV-1 (Roizman and Knipe, 2001). Genital HSV-1 infections are usually less severe and less prone to recur than those caused by HSV-2 (Reeves *et al*., 1981). HSV-2 seroprevalence increases from about 20% to 30% at age 15-29 years to 35% to 60% at age 60 years (Fleming *et al*., 1997). There are many factors that affect the acquisition of HSV-2 infection such as sex, race, marital status, number of sexual partners, and place of residence. HSV-2 infection is more frequent in women than men and in African-American than whites. This infection prevalence is higher in cities than in suburbs. Importantly, the number of sexual partners greatly influences the acquisition of HSV infection (Roizman and Knipe, 2001) due to the highest prevalence of antibodies against HSV-2 among female prostitute (75%). As with HSV-1 infection of the mouth, HSV-2 primary and recurrent infection may be symptomatic or asymptomatic. Either situation also provides a reservoir of virus for transmission to susceptible persons. HSV-2 infection trends to recur more often than HSV-1 infection (Whitley and Roizman, 2001).

The clinical manifestations of primary HSV-1 infection range from completely asymptomatic to gingivostomatitis in young child and pharyngitis or tonsillitis in adult. Subsequent recovery from primary oropharyngeal infection, the individual maintains a chance of suffering from recurrent attacks of herpes labialis; known as fever blisters or cold sores. Recurrent oralabial lesions are leaded by a prodome of pain, burning, tingling, or itching which commonly for 6 hours. Vesicles,generally three to five, appear most commonly on the vermilion border of the lip. These lesions are totally recovered after 8 to 10 days. The frequency of recurrence varies among individuals (Whitley and Roizman, 2001).

Primary genital herpes resembles as macules and papules followed by vesicles, pustules, and ulcers (Whitley and Roizman, 2001) on penis in male, vulva in female, and perianal region in male homosexuals. Systemic complications in men are rare; whereas, aseptic meningitis and urinary retention in women are common. Recurrent genital herpes is characterized by a prodrome and localized irritation. It appears as three to five vesicles on genital. Genital HSV infection is rarely transferred from mother to fetus during pregnancy. It causes neonatal herpes. Moreover, HSV diseases can result in; skin, eye, or mouth infections; encephalitis with or without skin infections; or disseminated disease involved multiple organs (Whitley *et al*., 1981; Roizman and Knipe, 2001).

Primary herpes keratoconjunctivitis is associated with either unilateral or bilateral conjunctivitis while recurrent infection is generally unilateral. Eye infection with HSV is associated with photophobia, tearing, and eyelid edema accompanied by dendritic lesions (Whitley and Roizman, 2001; Roizman and Knipe, 2001). Visual activity is decrease in patients who have ulcers of the cornea. Geographic ulcers can develop and lead to blindness from repeat HSV infection (Whitley and Roizman, 2001).

Herpes encephalitis, commonly type 1 infection, is a rare manifestation of HSV; however, It is thought to be the general cause of sporadic fatal encephalitis. HSV can affect nervous system, causing significantly meningitis, myelitis, and radiculitis (Whitley and Roizman, 2001).

Immunocompromised patients with underlying disease, immunosuppressive therapy, or malnutrition are at increased risk for potentially lethal disseminated HSV

infection. These patients may develop progressive disease involving respiratory tract, commonly esophagus, or gastrointestinal tract. In addition to reactivation of latent HSV infections in these patients can occur at multiple sites and an average time of healing is over 6 weeks. Moreover, HSV disease in immunocompromised patients appear to occur more frequently and can promote resistance with requisite therapy. Lastly, HSV infection increases the risk for acquisition of HIV infection (Roizman and Knipe, 2001).

Genital HSV pose risks to both mother and fetus. Pregnant women may develop widely disseminate maternal disease after HSV infection (Roizman and Knipe, 2001) that led to life-threatening disease, such as hepatitis, thrombocytopenia, leucopenia, and encephalitis. Maternal primary or initial genital HSV infection is major risk to the fetus (Kulhanjian *et al*., 1992). Although primary infection before 20 weeks of gestation has been associated with spontaneous abortion, recurrent HSV infection is the most common form during gestation. Neonate infection can occur in uterus (approximate 5% of infections), intrapartum (approximate 80%), or postnatally infection (Whitley, 2001). Transmission of infection to the fetus is generally associated with the actual shedding of virus from infected maternal secretions in the mother's birth canal at the time of delivery. Cervical shedding of virus among pregnant women varies frequency. However, 70% of infants who develop neonatal disease are born to mothers who do not have a history of genital herpes and are asymptomatic at the time of delivery (Brooks *et al*., 2001). Neonatal HSV infection occurs about 1 in 3,000 to 1 in 5,000 deliveries per year and it is almost symptomatic and frequently lethal from disseminated infection particularly CNS infection. Mortality in the absence of therapy is more than 80% and all of few survivors have neurologic impairment (Roizman and Knipe, 2001).

2.9 Antiherpes virus agents

Over the past 40 years, antiviral agents for the treatment of infections due to herpes simplex virus (HSV) have been developed. Treatments are now available for primary (the initial infection with HSV) and recurrent disease. However, many, perhaps most, infections are asymptomatic or unrecognized, and thus individuals do not seek treatment (Koutsky *et al*., 1990; Ashley and Wald, 1999). Major factors to

consider in selecting treatment are the immune status of the host, the site of infection, and whether the infection is primary or recurrent.

There are three classes of anti-herpes virus drugs in current clinical use (Andrei *et al.*, 1995). The first category is pyrophosphate analogues such as phosphonoacetic acid (PAA) and phosphonoformic acid (PFA), known as foscarnet or trisodium phosphonoformate.

Foscarnet directly inhibits the viral DNA polymerase and does not require phosphorylation by viral TK (Field, 2001; Chilukuri and Rosen, 2003; Mofin and Thouvenot, 2003). This binding site involves in releasing the pyrophosphate product of DNA synthesis during DNA polymerization process. It is active against acyclovir-resistant, TK-deficient HSV isolates. Resistance to foscarnet is rare and arises via mutations in the viral DNA polymerase (Chilukuri and Rosen, 2003; Morfin and Thouvenot, 2003). According to very poor absorption after oral administration, foscarnet is available as an i.v. preparation and topical preparation. Its significant toxicities limit its HSV use for treatment of acyclovir-resistant infections. The most common side effect of foscarnet is renal toxicity, associated with tubular intestinal lesions (Snoeck, 2000).

The second category of anti-herpes virus drug is the nucleoside analogues that can be divided into two groups. One is a variety of purine nucleoside analogues including acyclovir (ACV), valacyclovir (VACV), penciclovir (PCV), and famcyclovir (FCV).

Acyclovir (ACV), 9-(2-hydroxyethoxymethyl) guanine or acycloguanosine, is a drug of choice for prophylaxis and treatment of HSV infections for many years. ACV specfically targets herpes virus-infected cells because the viral thymidine kinase (TK) phosphorylates acyclovir to its monophosphate form. Cellular kinases then perform subsequent phosphorylations, yielding biologically active ACV-triphosphate. ACV-triphosphate is a potent inhibitor of HSV DNA polymerase and is a competitive inhibitor of cellular deoxyguanosine triphosphate resulted in viral enzyme inactivation. The inactivation of viral DNA polymerase occurs by lacking the 3’-hydroxy group required for subsequent 5’ to 3’phosphodiester linkages to elongate viral DNA chain of ACV molecule; thereby, DNA chain termination appears once the drug enters the viral DNA (Figure 7).

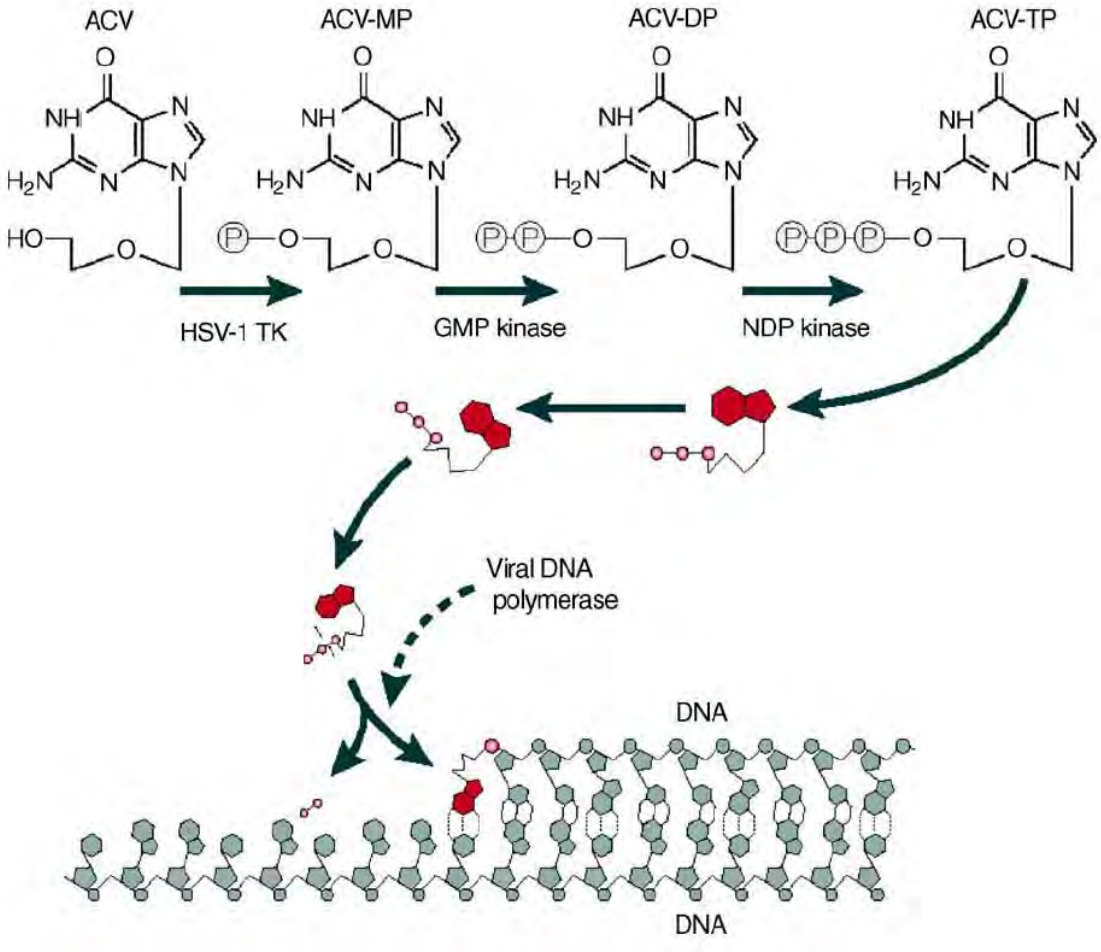

Figure 7. Mechanism of antiviral action of acyclovir (ACV) (De Clercq, 2004 a).

HSV develops resistance to acyclovir predominantly as a result of alterations in viral TKs and less frequently from mutations in the viral DNA polymerase (Kimberlin *et al*., 1995). ACV resistant mutants of HSV can be recovered from both *in vivo* and in cell cultures. The first case of clinical viral resistant strains was published in 1982 (Roizman and Knipe, 2001). To date, disease due to resistant viruses is almost exclusively a problem in the immunocompromised patients (Field, 2001; Shin *et al*., 2001; Bacon *et al*., 2003).

There are three kinds of ACV-resistant mutants. Firstly, thymidine kinase deficient virus is the group of mutants that lose thymidine kinase activity or fail to produce this enzyme (Hill *et al*., 1991). Secondly, thymidine kinase altered virus is

the group of mutant that can produce the enzyme but has altered thymidine kinase substrate specificity by point mutation. Thirdly, the mutation of some viruses occur by alteration of DNA polymerase activity (Roizman and Knipe, 2001). These mutations leading to resistance occur spontaneously during viral replication. Management of drug-resistant HSV infections depends on the mechanism of resistance of mutant strains.

Valacyclovir, the *L*-valine ester of acyclovir, is well absorbed (50%) and rapidly converted to acyclovir in the liver (Tyring *et al*., 2002). The mechanism of action of penciclovir is similar to that of acyclovir. Penciclovir triphosphate achieves higher intracellular concentrations and has a longer half-life in HSV-infected cells. However, it is 100-fold less potent in inhibiting viral DNA polymerase than acyclovir triphosphate. Famciclovir, a diacetyl ester prodrug of penciclovir, is well absorbed (70%) from the gastrointestinal tract (Cirelli *et al*., 1996).

Another group is a variety of pyrimidine nucleoside analogues including idoxuridine (IDU), trifluridine (TFT), and brivudin (BVDU). Similar to purine nucleoside analogues, these drugs require the specific phosphorylation to their monophosphte forms by viral enzyme; however, the monophosphate is converted to the diphosphate form by HSV or VZV encoded thymidine kinase (De Clercq, 2004a). Upon further phosphorylation by cellular kinases, nucleoside triphosphate can then interact with viral DNA polymerase, either as competitive inhibitor with natural substrate or as an alternative substrate incorporation into growing viral DNA chain and affection both stability and the DNA function. Idoxuridine and trifluridine in form of eye drops or ophthalmic cream have been used for HSV keratitis (De Clercq, 2005), while both oral and topical administration of brivudin can be used for herpes labialis, herpes keratitis, and herpes zoster (De Clercq, 2004b).

The third category of anti-herpes drug is independent from viral thymidine kinase for their activation. These drugs include the acyclic nucleoside phophonates, such as cidoforvir (HPMPC; 3-hydroxy-3-phosphonylmethoxypropyl-cytosine). Cidoforvir (CDV) has a potent and broad spectrum anti-DNA virus activity that includes all human herpes viruses (Naesens *et al*., 1997). CDV is an acyclic nucleoside 5’-monophosphate that is phosphorylated by host cell kinases to an active intracellular metabolite which selectively inhibits the viral DNA polymerase. Because

cidofovir is not dependent on viral TK for activation, it is also active against TK-deficient HSV isolates. Resistance to cidofovir is rare (Chilukuri and Rosen, 2003; Morfin and Thouvenot, 2003).

Vidarabine (Ara-A) is an adenine analog that is phosphorylated by cellular kinases to vidarabine triphosphate, which competitively inhibits viral, and to a lesser extent, cellular DNA polymerases. Docosanol (a saturated 22-carbon aliphatic alcohol) can inhibit fusion between the HSV envelope and the host cell plasma membrane, blocking viral entry (Pope *et al*., 1998). Docosanol is available for the treatment of recurrent herpes labialis.

The development of HSV vaccines has been challenging because the viruses establish latency and reactivations occur in the presence of humoral and cell-mediated immunity (Bernstein and Stanberry, 1999). Potential goals for HSV vaccines include:

(1) prevention of disease with or without partial protection against HSV infection;

(2) prevention of latent infection of the sensory ganglia;

(3) prevention of recurrences in individuals who are already latently infected with HSV.

The first vaccine enveloped was killed whole-virus HSV-1 and HSV-2 vaccines which licensed in Germany (Roizman and Knipe, 2001). However, many clinical studies showed the results of these vaccines efficacy differ widely. Inactivated vaccines may be some advantages for patients with recurrent infection, but long-term benefit could not be established, including the potential risks especially as a cofactor in the development of cervical carcinoma. Followed by a partially purified HSV-1 vaccine, glycoprotein HSV-1 and HSV-2 subunit vaccines, and recombinant glycoprotein vaccines. Some recombinant glycoprotein vaccines appear highly immunogenic and now few of them are testing in clinical trails, particularly recombinant HSV-2 vaccine. One is a recombinant HSV-2 glycoprotein D vaccine (Straus *et al*., 1993) and the other is glycoprotein B and glycoprotein D recombinant vaccine (Roizman and Knipe, 2001). However, these two vaccines require efficient adjuvant, complete Freund's adjuvant or lipophilic muramyl tripeptide, to demonstrate high antibody titer and protective effects against HSV-2 recurrent infection (Bernstein *et al*., 2005), or immune enhancer (Quenelle *et al*., 2006). The

HSV vaccines that have been studied to the greatest extent are subunit glycoprotein constructs. A recombinant truncated gD2 (glycoprotein D of HSV-2) combined with alum and the adjuvant MPL (3-de-O-acylated monophosphoryl lipid A) vaccine (GlaxoSmithKline) is being evaluated in phase III clinical trials for the prevention of genital herpes in women who are seronegative for both HSV-1 and HSV-2 (Stanberry *et al*., 2002). Alternative approaches for HSV vaccines include genetically attenuated or replication-impaired HSVs, DNA vaccines, and vectored vaccines (Roizman and Knipe, 2001).

2.10 Antiherpes virus activity of natural substances

There are various methods reported in the current literature to study anti-herpetic activities of plant extracts or plant-derived molecules. Many kinds of medicinal plants have a long history of use. There are several advantages, including often fewer side effects, better patient tolerance, local availability, relatively acceptance due to long time use, and less prone to the emergence of drug resistance strains. It has been reported that essential oils show anti-viral activity (Schnitzler *et al*., 2001; Minami *et al*., 2003).

Table 1. Antiherpetic activity of some medicinal plants.

Plant	Antiviral activity	References
Aglaia odorata	HSV-1	Lipipun *et al*., 2003
Artemisia arborescens	HSV-1 and HSV-2	Sinico *et al*., 2005
Cedrus libani	HSV-1	Loizzo *et al*., 2008
Clinacantus nutans	HSV-1 and HSV-2	Sangkitporn *et al*., 1995
Glyptopetalum sclerocarpum	HSV-1 and HSV-2	Sotanaphun *et al*., 1999

Table 1. Antiherpetic activity of medicinal plants (continues).

Plant	Antiviral activity	References
Grifola frondosa	HSV-1	Gu *et al*., 2007
Holoptelia integrifolia	HSV-1	Rajbhandari *et al*., 2001
Hyssopus officinalis	HSV-2	Koch *et al*., 2008
Matricaria recutita	HSV-2	Koch *et al*., 2008
Melissa officinalis	HSV-1 and HSV-2	Schnitzler *et al*., 2008
Mentha piperita	HSV-1 and HSV-2	Schuhmacher *et al*., 2003
Pelargonium sidoides	HSV-1 and HSV-2	Schnitzler *et al*., 2008
Salvia officinalis	HSV-1 and HSV-2	Schnitzler *et al*., 2008
Santolina insularis	HSV-1 and HSV-2	De Logu *et al*., 2000; Koch *et al*., 2008
Symphonia globulifera	HSV-1	Lopez *et al*., 2001
Thymus vulgaris	HSV-2	Koch *et al*., 2008
Ventilago denticulata	HSV-1	Lipipun *et al*., 2003
Vismia macrophylla	HSV-1	Lopez *et al*., 2001
Zingiber officinale	HSV-2	Koch *et al*., 2008

There are various kinds of natural options available for the prevention and treatment of herpes simplex virus infection. Natural remedies that show promise either for prophylaxis or treatment of HSV infection such as lysine, vitamin C, zinc, adenosine monophosphate, glutathione, copper, and medicinal plants.

Lysine, an essential amino acid, has been demonstrated to inhibit normal replication of HSV, interferes with cellular receptor function to inhibit HSV-1 binding and it is antagonist of the growth-promoting action of arginine on HSV replication in tissue. The proteins synthesized by HSV commonly contain more arginine than host cells, and arginine is important for HSV replication. Lysine acts as antagonist of arginine by various mechanisms. For instances, lysine is an antimetabolite of arginine. It increases arginine excretion by competing with arginine reabsorption at renal tubule, competes in arginine absorption at intestine, activates enzyme arginase to induces arginine degradation, and competes with arginine transportation into the cells. Lysine treatment, both oral and topical preparations, in orofacial or genital herpes can shorten the course and duration of the disease, decrease the severity of lesions, and decrease the frequency of recurrences (Betsy *et al*., 2005).

Zinc ions at concentration of 0.1 mM have been reported to almost completely inhibit replication of HSV-1 and HSV-2 *in vitro via* the selective inhibition of viral DNA polymerase. Most of topical preparations of zinc have been shown to be effective of cutaneous human HSV infections treatment, particularly zinc sulfate (Godfrey *et al*., 2001), or zinc monoglycerolate (Apisariyakulm *et al*., 1990). It has been showed that topically applies zinc can shorten the duration of HSV skin infection and probably prevent both spontaneous and sunlight-induced recurrent infections with causing a few adverse effects, for example, irritation and unpleasant dryness. Moreover, oral zinc administration also can reduce the duration and severity of HSV infection and may protect HSV recurrences induced by sun exposure (Godfrey *et al*., 2001); however, in order to prevent zinc-induced copper deficiency, long-term zinc supplementation should be accompanied by a copper supplement (Fosmire, 1990).

Coppers or cupric ions have been reported to completely inhibit HSV plaque formation when combined with ascorbic acid which is reducing agent (Sagripanti *et al*., 1997). Cu (II) ions bound with high affinity to viral DNA favoring guanosine residues (Sagripanti *et al*., 1991) and then produced oxidative base damage and gave the products including single and double DNA strand breakages as well as base modifications, mainly 8-OH-deoxyguanosine, and free radicals (Toyokuni and Sagripanti, 1996).

Vitamin C or ascorbic acid has been reported to inactivate HSV both *in vitro* and *in vivo* (Betanzos-Cabrera *et al*., 2004). These results suggested that vitamin C accelerated the healing of HSV lesions (Betanzos-Cabrera *et al*., 2004), decreased the mean time until remission of symptoms (Terezhalmy *et al*, 1978; Hovi *et al*., 1995), severity, and significantly reduced HSV yield after the first day of treatment (Hovi *et al*., 1995). The vitamin C treatment was most effective when initiated drug during the prodromal period and used in treatment of an acute infection (Betsy *et al*., 2005).

Resveratrol, 3, 5, 4'-trihydroxystilbene has been reported that it has an antioxidant activity (Stivala *et al*., 2001), anti-cancer activity (Jang *et al*., 1997), antimicrobial activity (Jeandet *et al*., 1995), and anti-HSV activity. *In vitro* study of anti-HSV activity of resveratrol showed that this substance effectively inhibits HSV by targeting immediate early events in HSV replication (Docherty *et al*., 1999). The mechanism of action of resveratrol is not clearly known, but it has been reported that resveratrol disrupt the cell cycle by inhibition cell cycle factors (Schang *et al*., 1998) or phosphorylation (Stewart *et al*., 1999). HSV requires cellular function associated with cell cycle progression in order to replicate (Hossain *et al*., 1997). Thus contributes to the inhibitory effects of this substance on HSV replication. Topical application of resveratrol effectively suppressed the development of HSV wild type and ACV resistant strain induced cutaneous lesion without dermal toxicity *in vivo* (Docherty *et al*., 2004).

Glutathione; γ-glutamyl-cysteinyl-glycine or GSH, an SH group containing tripeptide, is the most prominent intracellular low-molecular weight thiol found in eukaryotic cells. GSH acts as an intracellular water-soluble antioxidant and detoxifying agent (Droge *et al*., 1994). Many viral infections, including human immunodeficiency virus (HIV) (Kalebic *et al*., 1991), hepatitis C virus (Boya *et al*., 1999), parainfluenza-1, and HSV (Vogel *et al*., 2005) have been shown to associate with mark depletion of extra-cellular and intracellular GSH levels. It has been reported that exogenous GSH can induce a strong concentration-dependent inhibition of HIV, sendai virus, and HSV replication *in vitro*. Moreover, GSH has been suggested to use as a dietary supplement in HIV patients in clinical uses (Lyn, 2000). Most data indicated that GSH inhibit HSV replication by interfering with very late stages of HSV life cycle (Palamara *et al*., 1995).

Vitamin E or α-tocopheral protects cell membranes from oxygen free radical causing cellular damage. It has been reported as enhancer in the healing of wounds (Slater and Block, 1991; Martin, 1996). In clinical studies, topical application of vitamin E oil or capsule relieved pain, aided in the rapidly healing of oral herpetic lesions, reduced lesion development, and decreased severity of disease (Sheridan *et al*, 1997).

2.11 Naringenin

Naringenin is major flavonone that occur in various medicinal plants such as citrus fruits, grapefruits, tomato fruits and also presents in vegetables, tea and wine (Krause *et al*., 1992; Tomás-Barberán *et al*., 2000; Susanti *et al*., 2007). Low concentrations of naringenin are found in tomatoes and tomato-based products. During processing to tomato ketchup, naringenin chalcone, which is found in fresh tomatoes, especially tomato skin, is converted to naringenin (Krause *et al*., 1992). Although antioxidant activity of naringenin is lower compared with many other polyphenols. Naringenin has been reported to regulate apolipoprotein B secretion by HepG2 cells on lipid metabolism, possibly through inhibition of cholesterol ester synthesis (Borradaile *et al*., 1999) and to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase and acyl coenzyme A:cholesterol O-acyltransferase in rats (Lee *et al*., 1999). Moreover, a decrease in plasma low-density lipoprotein levels and hepatic cholesterol levels in rabbits fed a high-cholesterol diet has been reported (Kurowska *et al*., 2000). An increase of high-density lipoprotein levels in hypercholesterolemic human subjects was also (Kurowska *et al*., 2000). There are many reports about anti-inflammatory actions (Manthey *et al*., 2001) and effects on sex hormone metabolism of naringenin (Ruh *et al*., 1995; Rosenberg *et al*., 1998; Déchaud *et al*., 1999; Yoon *et al*., 2001). Most studies concern possible role of naringenin in grapefruit juice-drug interactions (Fuhr, 1998; Bailey *et al*., 2000). Naringenin is an inhibitor of the enzyme cytochrome P-450 IIIA (CYP3A4) (Ghosal *et al*., 1996) and may be causing the interaction. Nevertheless, other constituents in grapefruit seem to be more important in this event. The cancer-preventive effects may be due to stimulation of DNA repair by naringenin which, by stimulating base

excision repair processes, may prevent mutagenic changes in prostate cancer cells (Gao *et al*., 2006).

The cross-over study to investigate plasma concentrations of naringenin in human subjects consuming their habitual diets, and high or low in fruit and vegetables, which consisted of a 2 week baseline period and two 5 week intervention periods with a 3 week wash-out period in between. The high-vegetable diet provided various fruits and vegetables daily including on average one glass of orange juice, one-half orange and one-half mandarin. The high-vegetable diet contained naringenin 29 mg, whereas low-vegetable diet contained few fruit and vegetables and no citrus fruit. After the high-vegetable diet, naringenin is detectable in 22% of all samples. naringenin is bioavailable from the diet, but the plasma concentration of naringenin is poor biomarkers of intake (Erlund *et al*., 2002).

The anticarcinogenic properties of naringenin from the study about the effect of naringenin on cell proliferation of an HT-29 colon cancer cell line, which were cultured in 96-well tissue culture plates. The test group contained naringenin concentrations ranging from 0.02 to 2.85 mmol, whereas the control group did not contain naringenin. Cell proliferation was measured by colourimetric assay using the 2% WST-1 cell proliferation kit. Naringenin at concentrations greater than 0.71 mmol significantly inhibited cell proliferation. These results suggested that naringenin may be chemoprotective agents for colon cancer (Frydoonfar *et al*., 2002).

The safe and effective acetylcholinesterase (AChE) inhibitors in the treatment of Alzheimer's disease of naringenin, which was extracted from *Citrus junos*, was investigated. The results showed that this extract had a significant inhibitory effect on AChE *in vitro*, in a dose dependent manner. Furthermore, naringenin had anti-amnesic activity in in vivo study using ICR mice with amnesia induced by scopolamine (1 mg/kg body weight). Naringenin significantly improved scopolamine-induced amnesia, when oral administered to mice at 4.5 mg/kg and did not show any sign of toxicity during the experiment. These results suggested that naringenin may be an alternative agent against Alzheimer's disease (Heo *et al*., 2003).

The effect of naringenin on the acute nephrotoxicity induced by cisplatin (7 mg/kg, i.v.) was studied in the rat. Oral administration of naringenin (20 mg/kg/day) for 10 days, starting 5 days before cisplatin single i.v. injection, exhibited significant

protection of renal function ($p < 0.05$). Naringenin decreased the extent of cisplatin-induced nephrotoxicity, as demonstrated by significant reduction in serum urea, creatinine concentrations and body weight loss and also marked reduction in urinary fractional sodium excretion and glutathione *S*-transferase (GST) activity. Moreover, it decreased polyuria and increased creatinine clearance. Naringenin markedly prevented cisplatin-induced alterations in renal cortex and improved GST activity. In combined treatment group of cisplatin and naringenin, antioxidant enzymes namely superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) were significantly increased to 54.5, 30.3 and 35.6%, respectively compared to cisplatin treated group. Cisplatin-induced alterations in renal cortex lipid peroxides and GST activity were markedly improved by naringenin. Cisplatin-induced alterations in renal cortex antioxidant defense system were greatly prevented by naringenin. In cisplatin-naringenin combined treatment group, antioxidant enzymes namely superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) were significantly increased to 54.5, 30.3 and 35.6%, respectively compared to cisplatin treated group. Platinum renal content was not affected by naringenin treatment. The results showed the antioxidant potential of naringenin and provided further insight into the mechanisms of cisplatin-induced nephrotoxicity (Badary *et al*., 2005).

The effect of naringenin on tumor growth was investigated in various human cancer cell lines and sarcoma S-180-implanted mice. Cytotoxicity of naringenin in cell lines derived from cancer of the breast (MCF-7, MDA-MB-231), stomach (KATOIII, MKN-7), liver (HepG2, Hep3B, Huh7), cervix (Hela, Hela-TG), pancreas (PK-1), colon (Caco-2), and leukemia (HL-60, NALM-6, Jurkat, U937) was demonstrated. Naringenin-induced cytotoxicity was low in Caco-2, whereas, high in leukemia cells compared to the others. Naringenin induced apoptosis, with hypodiploid cells detected in both Caco-2 and HL-60 by flow cytometric analysis, by dose-dependent. *In vivo* study, naringenin dose-dependently inhibited tumor growth in sarcoma S-180-implanted mice, following intraperitoneal or peroral injection once a day for 5 days. Moreover, no significant toxicity was observed in naringenin-treated group (Kanno *et al*., 2005).

To determine the content of kaempferol and naringenin, antioxidant activity and cytotoxicity of methanol extracts from the leaves of *Melastoma decemfidum*. The concentration of naringenin and kaempferol was determined using Gas Chromatography. Then, the crude leaf extracts, single and combination of the two pure flavonoids were screened for antioxidant and cytotoxic activities against the human cervical cancer cell lines using DPPH (2, 2-diphenyl-1-picrylhydrazine) radical-scavenging assay and MTT assay, respectively. The results showed that the combination of flavonoids compound had the strongest antioxidant activity with IC_{50} value of 31.39 μg/ml and cytotoxicity against human cervical cancer cell lines with IC_{50} value of 43.73 μg/ml, whereas, the combination of pure compounds of naringenin and kaempferol had strongest antioxidant activity and cytotoxicity followed by the crude extracts, pure kaempferol and pure naringenin alone. In addition to, the relationship between cytotoxic properties and the antioxidant capacity of *M. decemfidum* leaf extracts were investigated. In common, the strongest antioxidant extracts were also the most cytotoxic. The antioxidant concentrations are less than the cytotoxic concentrations for every sample. Therefore, the compounds had antioxidant activity at non-cytotoxic concentrations. This study showed that the more cytotoxic the extracts, the better the antioxidant effect. Antioxidant activity and cytotoxicity were relative to the high content of kaempferol and naringenin in the methanolic leaf extracts of *M. decemfidum*. Furthermore, the crud extracts of the flowers of *Melastoma malabathricum* (Melastomataceae) had been identified as naringenin and then screened for their antioxidant and cytotoxic activities, carried out by the DPPH radical-scavenging assay and MTT assay against a MCF7 cell line, respectively. Naringenin was found to be active as radical-scavengers with IC_{50} values of 0.52 mM and was also found to be active in inhibiting cell proliferation of MCF7 with IC_{50} values of 0.28 μM (Susanti *et al*., 2007).

In experimental rats, oral administration of cadmium chloride (5 mg/kg/day) for 28 days significantly induced the renal damage which was phenomenon from the increased levels of serum urea, uric acid, creatinine with a significant ($p < 0.05$) decrease in creatinine clearance. Co-administration of naringenin (25 and 50 mg/kg/day) along with Cd resulted in a reversal of Cd-induced biochemical changes in kidney accompanied by a significant decrease in lipid peroxidation and an increase

of renal antioxidant defense system. The histopathological studies demonstrated that naringenin (50 mg/kg/day) decreased the toxicity of Cd and preserved the normal histological architecture of the renal tissue. It suggest that the nephroprotective potential of naringenin in Cd toxicity might be due to its antioxidant and metal chelating properties, which could be useful for achieving optimum effects in Cd-induced renal damage (Renugadevi *et al*., 2009).

Moreover, recent study showed that naringenin powerfully inhibited the assembly and secretion of apolipoprotein B100-containing lipoproteins in cultured hepatocytes. Moreover, naringenin could improve dyslipidemia and insulin resistance in a mouse model of the metabolic syndrome. The previous study used low-density lipoprotein receptor-null mice fed a high-fat diet (Western, TD96125) to examine that naringenin prevents atherosclerosis. The results from *in vivo* study showed that naringenin improved the dyslipidemia in Western-fed low-density lipoprotein receptor-null mice, leading to decreased atherosclerosis. They also suggested that a potential therapy for the hyperlipidemia and atherosclerosis associated with insulin resistance (Mulvihill *et al*., 2010).

The studies of toxicity of naringenin are scarce. A single, 2-g oral dose of naringin was administered to a human volunteer with no adverse effects (Booth *et al*., 1958; Wilcox *et al*., 1999). Pure compound of naringin at 500 mg oral dose, had also been administered to humans with no adverse responses (Ameer *et al*., 1996; Ishii *et al*., 1997). In *in vitro* study of cytotoxicity for naringenin in a number of cells lines, the IC_{50} (50% inhibitory concentration) for cell growth was > 1 mM for this compound in the human hepatoma cell line HepG2, the Macacus' rhesus monkey kidney cell line MA-104, and the human lung cancer cell line A549 (Hodgson *et al*., 1998). The results of these studies indicated that naringenin was relatively low toxicity in cell culture.

2.12 *In vitro* methods for antiviral test

There are various methods to determine the antiviral activity as well as the interpretation of the results have been virtually specific to each laboratory and are consequently not comparable to one another, so simple procedures and guidelines for evaluation antiviral or virucidal activity of compounds have been urgently needed. This is a series of assays through which the anti-HSV activity of candidate antiviral compound may be evaluated *in vitro*, the potency and the antiviral mechanism of the compound can be investigated.

HSV can grow well in various cell types to yield high titers of virus stocks. Cell lines usually are used to grow HSV and tested for the anti-HSV activity of compounds include BHK (baby hamster kidney cell line), RK13 (rabbit kidney cell line), Vero (monkey kidney cell line), and CV1 (monkey kidney cell line) (Harland and Brown, 1997). Antiviral compounds may block HSV replication cycle in different stages (Figure 8), thus virus replications in cell cultures may also be monitored by the detection of viral products, for example viral DNA, RNA, or glycoproteins (Dargan, 1998).

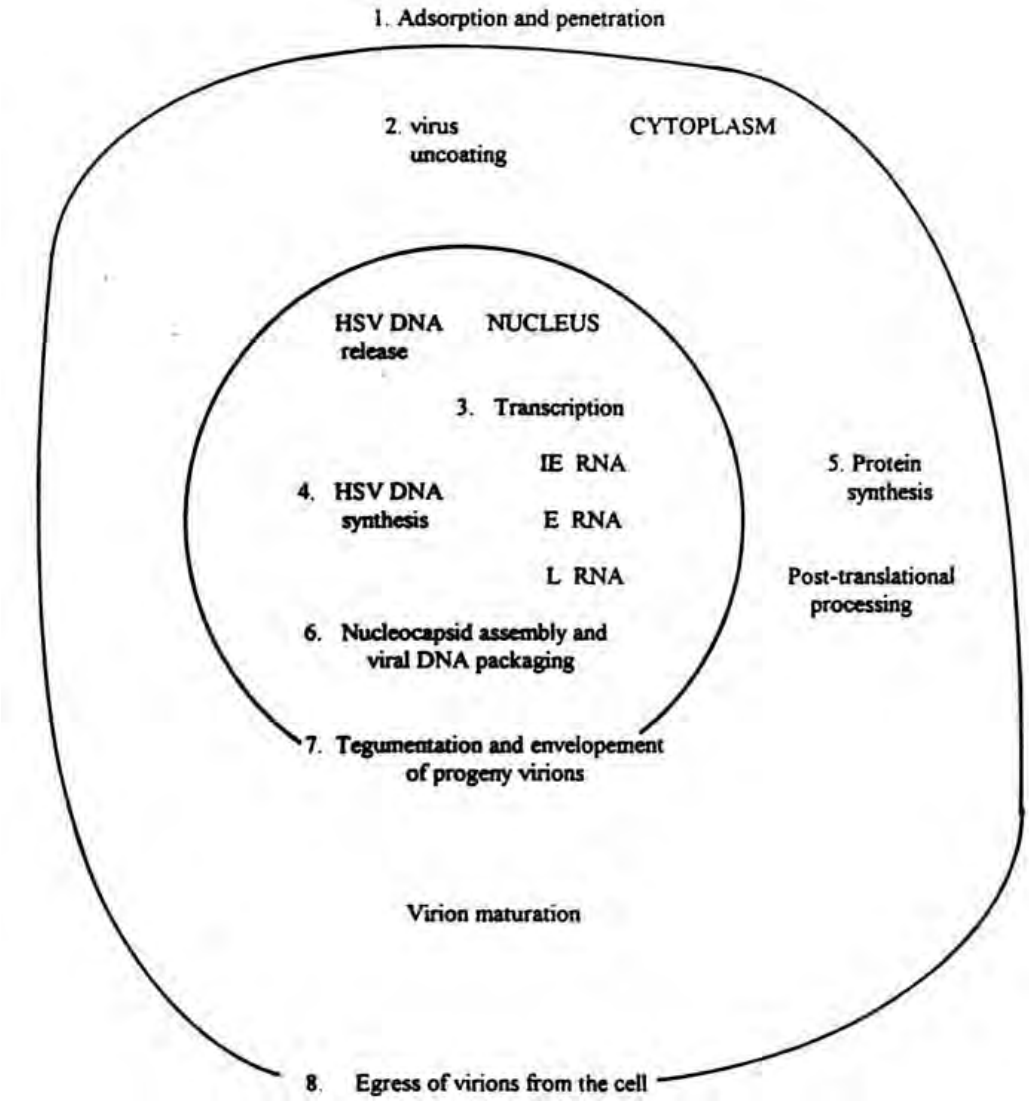

Figure 8. Stages in the HSV replication cycle that might be blocked by antiviral agents (Dargan, 1998).

Cytotoxicity tests define the upper limit concentration of test compounds which can be used in subsequent antiviral tests. The simplest cytotoxicity tests *in vitro* are the vital staining techniques. These methods are easy to perform, require little equipment, and give reliable results (Galt *et al*., 1990; Sydiskis *et al*., 1991). In these methods, cells are added with trypan blue or neutral red dyes. Trypan blue is excluded by live cells, but stains dead cells blue color (Anderson *et al*., 2003; Washman *et al*., 2003). Neutral red is taken up by live cells, staining them a brownish-red color, whereas dead cells remain colorless. In addition to vital-staining test, cell viability can

be confirmed by additional experiment, such as measuring cell ability to cleave tetrazolium salt by mitochondrial enzymes to give a color product and measuring incorporation of ^{3}H thymidine into cellular DNA. Absence of cytotoxicity in *in vitro* test does not mean that test compound does not have toxicity *in vivo*. Similarly, a moderate level of cytotoxicity in *in vitro* test may not necessarily exclude *in vivo* use of the compound (Konsula and Bariel, 2005).

The method most commonly used for evaluation of *in vitro* antiviral activities is based on the different abilities of viruses to replicate in culture cells in which test compounds appeared and determine effective dose (ED_{50}). As previously mentioned, HSV can cause cytopathic effects (CPE) or form plaques in cell cultures, thus ED_{50} is the dose of the test compound which inhibited CPE, reduced or inhibited plaque formation, and reduced virus yield or other viral functions by 50% (Vlietinck and Berghe, 1991).

Plaque reduction and inhibition assay are the most commonly assays used for antiviral assay (Liu *et al*., 2004). These assays use a constant number of viral particles and varying the non-toxic concentrations of test compound (Abou-Karam and Shier, 1990; Liu *et al*., 2004). Ordinarily, a monolayer of cultured cells is allowed to bind virus and then overlaid with semisolid medium (overlay medium) to prevents virus spreading from the area of originally infected cells. The test compound can be added into cell monolayer before or after virus adsorption is accomplished. The infected cultures are incubated for an appropriate time period, then they are fixed, stained with dye. The number of plaques, the areas of infected cells, are counted as plaque forming unit (PFU). By reference to the number of plaques observed in virus control or untreated control, ED_{50} is calculated and expressed (Liu *et al*., 2004).

The infectivity of virus could be examined by microscopic observations of characteristics of viral CPE (Yip *et al*., 1991) or by the dye uptake assay (Marchetti *et al*., 1996). The therapeutic index (TI) or selective index (SI) can be computed by the ratio of the maximum drug concentration at which 50% of the growth of normal cells is inhibited to the minimum drug concentration at which 50% of virus is inhibited.

Virus yield inhibition assay is usually used to measure antiviral activity. In this method cell monolayer is infected with virus, and various concentrations of test compound are added after virus adsorption. Following a cycle of viral replication, the

harvested cell cultures are disrupted by three cycles of freeze-thaw, supernatants are kept, and then virus yields are determined by plaque assay. Plotting the diminishing infectious virus yields provides drug dose-response curves that yielding more information than can be obtained from effective concentration (EC_{50}) value alone (Yoosook *et al*., 1999; Tenser *et al*., 2001; Washman *et al*., 2003).

To investigate the antiviral mechanism, the major goal is to identify the virus gene product that is the target of test compound. According to this concept, it is important to identify and isolate drug resistant virus mutants from treated cultures by various method, including single round selection, mapping the drug resistance gene, or DNA sequencing. However, if no drug-resistant variant can be isolated, it will be important to investigate the point in the virus replication at which drug-sensitive function operates.

Virucidal assay is the method to examine the eliminating infectivity when virus particles are mixed with various concentrations of test compound. Virus titers receive from the titration of this mixture solution in cell monolayer. Virucidal activity may be caused by disintegration of the entire virus particles, solubilization of the herpersvirus envelope, or the chemical modification, degradation, or masking of some essential envelope proteins. HSV particles treated with a virucidal agent are blocked at adsorption and/or penetration, the earliest stage of the virus replication cycle.

To investigate the stage of HSV adsorption to target cells, two methods have been used. The first method indirect measures virus adsorption by quantifying the decrease in infectivity of the inoculum applied to cell monolayers along with time (Hayashi *et al*., 1992). The second method quantified virus particles bound to cell surfaces by measuring the radioactivity associated with ^{35}S-methionine labeled virus particles accumulating with time. Both methods should be performed at 4ºC, since at this temperature HSV particles can attach to cell surfaces but cannot penetrate into target cells.

To investigate the effect of test compound on the rate of HSV entry into target cells, penetration assay have been used (Rosenthal *et al*., 1984). This assay depends on the observation that HSV binds to cells at 4ºC but does not penetrate until the temperature is increased. After virus attachment at 4ºC, the medium containing test compound are added to cell monolayer and then shift temperature to 37ºC to allow

virus penetration. Virus particles that have not yet penetrate the cells are inactivated, at various times after temperature up shift, by treating the infected cell cultures with low pH solution. The infectivity that becomes resistant to low pH inactivation with time is the rate of virus penetration.

The assays to examine the stage of HSV replication cycle inhibited by test compound at a point subsequent to virus entry include electron microscope studies, which show morphological changes during viral replication, or single step HSV growth experiments in which a single antiviral concentration of test compound is added to infected cell cultures at progressively later times throughout the virus replication cycle. If test compound inhibits protein function that is required in early stage of infection, late addition of test compound will have no inhibitory effect on infectious virus yield. On the other hand, if the target protein of antiviral agents has function in late infection, or throughout the replication cycle, the viral infectivity will decrease whenever the drug is present (Washman *et al*., 2003).

Currently, HSV-DNA synthesis has been studied by a dot-blot, Southern blotting method (Kuo *et al*., 2001; Evers *et al*., 2004). In the past, it used to separate of ^{3}H-thymidine labeled infected-cell DNA into viral and cellular fractions by cesium chloride gradient centrifugation (Dargen and Subak-Sharpe, 1986). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can be used to study effect of test substances on viral protein synthesis and post-translational processing. Changes in the quantities or the apparent molecular weights of HSV specified polypeptide bands on SDS-PAGE gels are effects of inhibition in HSV replication (Washman *et al*., 2003). Perhaps, these reductions resulted from test substances that inhibit viral gene transcription, viral mRNA translation, or reduce viral mRNA or protein products stability (Dargan, 1997). Other antiviral assays based on measurement of specialized function and viral products have been studied using currently advanced methods, for examples, flow cytometric analysis (Pavic *et al*., 1997), nucleic acid hybridization (Lin and Ricciardi, 2000), and enzyme-linked immunosorbent assay (ELISA) (Anderson *et al*., 2003).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell culture

A continuous cell line of African green monkey (*Cercopithecus aethiops*) kidney, called Vero cell ATCC CCL-81, purchased from ATCC, was propagated in MEM growth medium supplemented with 8% FBS and 1% antibiotic-antimycotic agents.

The monolayer culture of Vero cells in tissue culture flask, was washed once with phosphate buffer saline solution (PBS) and trypsin-EDTA was added for 2-3 minutes. When the cells were detached, trypsin-EDTA was discarded, and growth medium was added. The flask was tapped lightly and cell suspension was mixed thoroughly by using pipette. The viable cells were counted by trypan blue staining. The cell suspension was diluted in complete growth medium to an appropriate concentration and distributed into a new 25 cm^2 or 75 cm^2 tissue culture flask. Then, the cells were incubated at 37ºC in a humidified-5% CO_2 incubator until the cell monolayer was confluent.

3.1.2 Herpes simplex virus

HSV-1, strain KOS, and HSV-2, strain Baylor 186, were kindly provided by the Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University. A virus stock was prepared from Vero cell monolayer infected with the virus at multiplicity of infection (M.O.I.) of about 0.1 plaque forming unit per cell (PFU/cell) (Lipipun *et al*., 2000). After 1 h of viral adsorption at 37°C, the growth medium was added. The infected Vero cells were incubated in a humidified-5% CO_2 incubator at 37°C until the cell population showed cytopathogenic effect (CPE) more than 80%. Then, the cells were disrupted by repeatedly freeze-thawed three times at -70°C. The disrupted cell suspension was pelleted by centrifugation at 3,000 rpm for

10 minutes. The supernatant was aliquoted into microtubes and stored at -70°C as virus stock.

3.1.3 Naringenin

Naringenin (Sigma, Cat. No. N-5893, Lot 067K1084) was dissolved in DMSO and diluted to concentration of 1 mg/ml in growth medium (final concentration of DMSO was 0.5%) for stock solution. The Naringenin stock solution was distributed into small aliquots and stored at -20°C until used.

3.1.4 Acyclovir

Acyclovir (Sigma, Cat. No. A-4669, Lot 117F0756) was dissolved in DMSO and diluted to concentration of 500 µg/ml in growth medium (final concentration of DMSO was 0.5%) for stock solution. The ACV stock solution was aliquoted and stored at -20°C until used.

3.2 Method

3.2.1 Determination of viral titer

Titration of virus was performed by plaque assay. Serial ten-fold dilutions of virus stock in MEM were added onto Vero cell monolayer in a 96-well tissue culture plate (Nunc, Denmark) in amount of 25 µl/well in quadruplicate. The virus was allowed to adsorb for 1 h in a humidified-5% CO_2 incubator at 37°C. Then, 75 µl of overlay medium were added to each well and the plate was incubated for 2 to 3 days in a humidified-5% CO_2 incubator at 37°C. After the incubation period, the medium was discarded and the infected cells were fixed with 12% formalin in normal saline solution for 1 h and stained with 0.05% methylene blue solution for 1 h. The number of plaques was counted under an inverted microscope and the virus titer was calculated as plaque forming unit per milliliter (PFU/ml).

3.2.2 Cytotoxicity test

Cytotoxicity test was performed to determine the effects of naringenin on cell proliferation and viability by using trypan blue exclusion method and MTT reduction assay.

3.2.2.1 Trypan blue exclusion method

Cytotoxicity was determined by trypan blue exclusion method (modified from Hayashi *et al*., 1996; Liu *et al*., 2004). Vero cells were seeded in 96-well tissue culture plate at a cell concentration of $2x10^5$ cells per well in 100 µl of growth medium. After incubation of the cells in a humidified-5% CO_2 incubator at 37°C for 24 h, various concentrations of naringenin were added to the plate, and the incubation was continued for 24, 48 and 72 h then the cells were trypsinized. The number of cells in collected suspensions was determined in a hemocytometer after staining with an equal volume of trypan blue solution. Trypan blue is excluded by live cells, but stains dead cells blue. Mean value of the cell number was calculated. Results were expressed as the ratio between the number of viable cells in treated cultures and viable cells in the untreated control cultures. The graph between percent of cell viability and drug concentration was plotted. The 50% cytotoxicity concentration (CC_{50}) was defined as the concentration which caused 50% reduction in the number of viable cells.

3.2.2.2 MTT (Thiazolyl blue tetrazolium bromide) reduction assay

For cytotoxicity assay, MTT reduction method (modified from Washman *et al*. 2003; Liu *et al*., 2004) was used. Vero cells were seeded in 96-well tissue culture plate at a cell concentration of $2x10^5$ cells per well in 100 µl of growth medium. After incubation of the cells in a humidified-5% CO_2 incubator at 37°C for 24 h, various concentrations of naringenin were added to the plate, and the incubation was continued for 24, 48 and 72 h. Cell viability was examined based on the ability of the cells to cleave the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; (Sigma) by the mitochondrial enzyme succinate dehydrogenase to give a blue formazan crystal. MTT was dissolved in PBS at a

concentration of 5 mg/ml and sterilized by filtration to remove a small amount of insoluble residue. At the time indicated above, the MTT solution was added to each well (25µl/well) and the plates were incubated again in a humidified-5% CO_2 incubator at 37°C for 4 h. Then, 100 µl of acid-isopropanol were added to all wells and mixed thoroughly on plate shaker at 150 rpm to dissolve the dark blue crystals. After 20 min at room temperature to ensure that all crystals were dissolved, the plates were read on Bio Rad microplate reader (Model 3550), using a test wavelength of 595 nm and a reference wavelength of 655 nm. Optical density should be directly correlated with cell quantity. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration which caused 50% reduction in optical density compared with that of untreated control cultures.

CC_{50} values for both trypan blue exclusion method and MTT reduction method were calculated by regression analysis, $Y = aX + b$, whereas Y is the amount of cells (% of control); X is the concentration of the test substances; and a and b are constant values.

3.2.3 Anti-HSV-1 and HSV-2 activity of naringenin

The antiherpes simplex virus activities were investigated by using plaque reduction, inactivation and prophylactic activity assays.

3.2.3.1 Plaque reduction assay

To determine the activity of naringenin, or ACV (as positive control) against intracellular viral replication, plaque reduction or post-treatment assay was performed by the following method (modified form Hayashi *et al.*, 1996 and Gong *et al.*, 2004). Twenty-five microliters of virus were added onto Vero cell monolayer in 96-well tissue culture plates (virus 30 PFU/well). After 1 h of viral adsorption in a humidified-5% CO_2 incubator at 37°C, the overlay medium containing different concentration of naringenin (6.25-100 µg/ml) in appropriate concentration was added to the cultures. After further incubation in a humidified-5% CO_2 incubator at 37°C for 48 h, the infected cells were fixed with formalin, stained with methylene blue solution as

previously mentioned, and the number of plaques was counted as plaque forming unit (PFU).

3.2.3.2 Inactivation assay

To determine neutralizing activity of naringenin, or ACV (as positive control) to herpes simplex virus, inactivation assay was performed by the following method (modified form Hayashi *et al*., 1996). Twenty-five microliters of virus were incubated with 25 µl of sample (concentration 6.25-100 µg/ml) in a humidified-5% CO_2 incubator at 37°C for 1 h. This mixture was then added onto Vero cell monolayer in triplicated wells (virus 30 PFU/well) and incubated in humidified-5% CO_2 incubator at 37°C for another 1 h. The overlay medium containing different concentration of test substances in appropriate concentration was added to the cultures. The cultures were incubated in a humidified-5% CO_2 incubator at 37°C for 48 h. The cells were fixed, stained and the number of plaques was counted as previously mentioned.

3.2.3.3 Prophylactic activity assay

To determine the antiviral activity of naringenin against viral adsorption, viral penetration, or other events before virus entering into the cells, prophylactic activity assay or pre-treatment assay was performed by the following method (modified form Hayashi *et al*., 1996). Fifty microliters of naringenin or ACV (as positive control) were added onto Vero cell monolayer in triplicated wells and incubated in a humidified-5% CO_2 incubator at 37°C for 1 h. After each concentration of naringenin (6.25-100 µg/ml) was discarded, the cells were infected with 25 µl of HSV-1 or HSV-2 and incubated in a humidified-5% CO_2 incubator at 37°C for another 1 h. The overlay medium with or without naringenin was added to the cultures after unadsorbed virus was discarded. The cell cultures were incubated in a humidified-5% CO_2 incubator at 37°C for 48 h. The cells were fixed, stained and the number of plaques was counted as previously mentioned.

The antiviral activity of naringenin was tested and compared with that of ACV in term of 50% inhibitory concentration (IC_{50}). The IC_{50} was determined by equation

Y = aX + b, whereas Y is the number of plaques (% of control); X is the concentration of the test substances; and a and b are constant values.

3.2.4 Preliminary tests for the mechanism of action of naringenin

Preliminary tests for the mechanism of action of naringenin against HSV-1 were performed by the following methods.

3.2.4.1 Virus yield inhibition assay

The antiviral activity of naringenin was evaluated by varying the time of addition of substance to determine the effect of this substance on the growth of HSV-1 in virus yield inhibition assay (modified from Kurokawa *et al*., 1995; Yang *et al*., 2005). Briefly, confluent Vero cells monolayer in 25 cm^2 tissue culture flasks were infected with HSV-1 at 0.01 PFU/cell and allowed the viruses to be absorbed for 1 h in a humidified-5% CO_2 incubator at 37°C. Unadsorbed viruses were discarded and the infected cells were washed three times with PBS. The growth medium (as control) or MEM containing naringenin at concentration greater than the IC_{50} value was then added onto the cultures at various time points either 1 h before viral infection or after viral infection periods (1 and 3 h post infection). After carefully washing by PBS in every step, the infected cells were then maintained in MEM alone or MEM containing naringenin for 3 and/or 9 h at 37°C in a humidified-5% CO_2 incubator. Then the media were discarded, each infected cell monolayer was washed three times with PBS and replaced with fresh growth medium. The cultures were freeze-thawed three times, then the suspension was pooled and centrifuged at 3000 rpm for 15 min to release cell-associated viruses into supernatants. The supernatants were kept at -70°C until used in viral assay. Virus titers in the supernatants were determined by plaque assay which serial 10-fold dilutions of each supernatant were added onto Vero cell monolayer in 96-well tissue culture plates, as previously mentioned.

Total infectivity was quantified by PFU and expressed as percent of virus yield as compared to control.

3.2.4.2 Required treatment period for inhibition of plaque formation

To determine the effect of treatment period for inhibition of plaque formation by naringenin (modified from Chuanasa *et al*., 2008). Vero cells were seeded at a concentration of 6×10^5 cells/ml in 24-well tissue culture plates and grown at 37 ºC for 24 h. The cells were infected with HSV-1, 50 PFU/0.5 ml/well. After 1 h of viral adsorption, the cells were added with various concentration of substance in overlay medium. At each time of incubation of 3, 6, 12, 24 and 48 h at 37°C in a humidified-5% CO_2 incubator, the overlay medium supplemented with compound was removed. The cells were washed and replaced with the compound-free overlay medium and incubated thereafter up to 48 h, respectively.

3.2.4.3 Post-binding assay

The effect of naringenin on HSV attached to Vero cells was examined by following method (modified from Piret *et al*., 2002). Confluent Vero cells seeded in 24-well tissue culture plates were maintained at 4°C for few min. The cells were first incubated with 50-100 PFU of viruses at 4°C for 2 h to allow stable attachment of the viruses without fusion with cell membrane. After incubation, unbound viruses were removed, and cells were then washed with cold PBS (as control), low pH citrate buffer (as positive control), or PBS containing increasing concentrations of naringenin at 4°C for 1 min. Cells were washed once with PBS and overlay medium was added. After incubation in humidified-5% CO_2 atmosphere at 37°C for 48 h, cells were fixed, washed, and stained as described previously. The amount of viruses which had attached and penetrated into cells after temperature shift to 37°C was evaluated according to the number of PFU.

3.2.4.4 Penetration assay

The effects of naringenin on the rate of penetration of HSV into Vero cells were investigated (modified from Piret *et al*., 2002). Confluent Vero cells seeded in 24-well tissue culture plates were incubated with viruses 50-100 PFU of viruses at 4°C for 2 h of viral attachment. After removal of unbound viruses, the incubation temperature was shifted to 37°C to allow penetration of bounded virus into cells. At

selected times after the temperature shift (0, 15, 30, and 60 min), the cells were treated with PBS (as control) or PBS containing increasing concentrations of naringenin for 1 min. Then, the cells were added with overlay medium and incubated in a humidified-5% CO_2 atmosphere at 37°C for 48 h. The cells were then fixed, washed, and stained. The amount of viruses which had penetrated into the cells was evaluated following the determination of numbers of PFU.

3.2.4.5 Virucidal assay

The direct effect of naringenin on HSV-1 infectivity was evaluated by the following method (modified from Minami *et al*., 2003 and Yang *et al*., 2005). Briefly, different concentrations of naringenin were mixed thoroughly with 10^5-10^6 PFU/ml of virus. The mixture was then incubated in a humidified-5% CO_2 incubator at 37°C for 1 h. After incubation, serial 10-fold dilutions of mixture were added on Vero cell monolayer. The cells were incubated in a humidified-5% CO_2 incubator at 37°C for another 1 h for viral adsorption. The residual virus infectivity was determined by plaque assay as previously mentioned.

3.2.4.6 Combined effect of naringenin with acyclovir on plaque formation

The combined effect of naringenin with ACV was examined for anti-HSV-1 activity in the plaque reduction assay (Kurokawa *et al*., 1995; Suzuki *et al*.,2006). Triplicate cultures of Vero cells in 96-well tissue culture plates were infected with 30 PFU/well of HSV-1 (KOS) for 1 h. The cells were added with overlay medium containing various concentrations of naringenin and/or ACV, and then cultured at 37ºC for 48 h. The infected cells were fixed, stained and then the number of plaques was counted under a microscope. The inhibitory concentrations for 50% plaque reduction (IC_{50}) were determined from a curve relating the plaque formation (%) of untreated culture to the concentration of drugs.

The combined activity of naringenin with ACV was evaluated by constructing an isobologram (Kurokawa *et al*., 1995; Suzuki *et al*., 2006). Synergy and antagonism are defined as deviations from an additive effect, which results when two drugs

interact as if they were the same drug. Curves falling below the line of additivity indicate synergy, curves on the line indicate an additive reaction and curves above the line indicate an antagonistic reaction.

The IC_{50} was used to calculate the fractional inhibitory concentration (FIC) according to the formula that previously shown (Piret *et al*., 2002). The interaction between naringenin and ACV was interpreted according to the combined FIC index (FIC of naringenin plus FIC of ACV). When the combined FIC index is equal to 1, the combination is assumed to act in an additive manner; when it is < 1 the interaction is synergistic; and when the combined FIC index is > 1 the interaction is antagonistic (Cheng *et al*., 2009).

3.2.5. Statistical analysis

The data were expressed as mean ± S.D. The statistical significance of the difference between mean values was determined by Student's t-test. Data were considered different at a significance level of $p < 0.05$.

CHAPTER IV

RESULTS

4.1 Cytotoxicity of naringenin on Vero cells

In this study, cellular toxicity of naringenin and ACV was determined as CC_{50}. All CC_{50} values in this study were calculated by regression analysis (see appendix). The maximal concentration of DMSO that did not affect the cytotoxicity to Vero cells was 2% (Lipipun *et al*., 2003). In addition, the result of cytotoxicity of DMSO on Vero cells determined with MTT reduction assay was shown in Figure 9. The CC_{50} value of DMSO was 7.4 %. The maximum concentration of DMSO used in all antiviral tests was 0.5% which did not show any effect to cell growth.

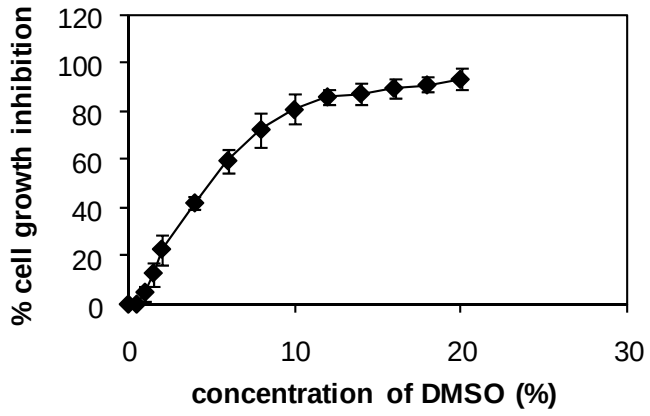

Figure 9. Cytotoxicity of DMSO on Vero cells determined by MTT reduction assay. Each point represented the percentage of cell growth inhibition compared with the untreated control Vero cells. Data were reported as the mean ± S.D. from three independent experiments.

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Cytotoxicity of naringenin was examined by trypan blue exclusion method and MTT reduction assay.

In trypan blue exclusion method, in which dead cells were stained blue while living cells remained clear, the total viable cell numbers in treated Vero cells as compared with untreated control Vero cells was shown in Figure 10. Naringenin exhibited CC_{50} values of > 500, 329.52±33.37 and 305.64±18.36 µg/ml for 24, 48 and 72 h incubation, respectively.

In MTT reduction assay, which measured cellular enzymes activity correlated with cell viability, showed the similar results of cytotoxicity of naringenin on Vero cells (Figure 11). The CC_{50} values of naringenin were > 500, 378.71±25.25 and 304.56±7.41 µg/ml for 24, 48 and 72 h incubation, respectively

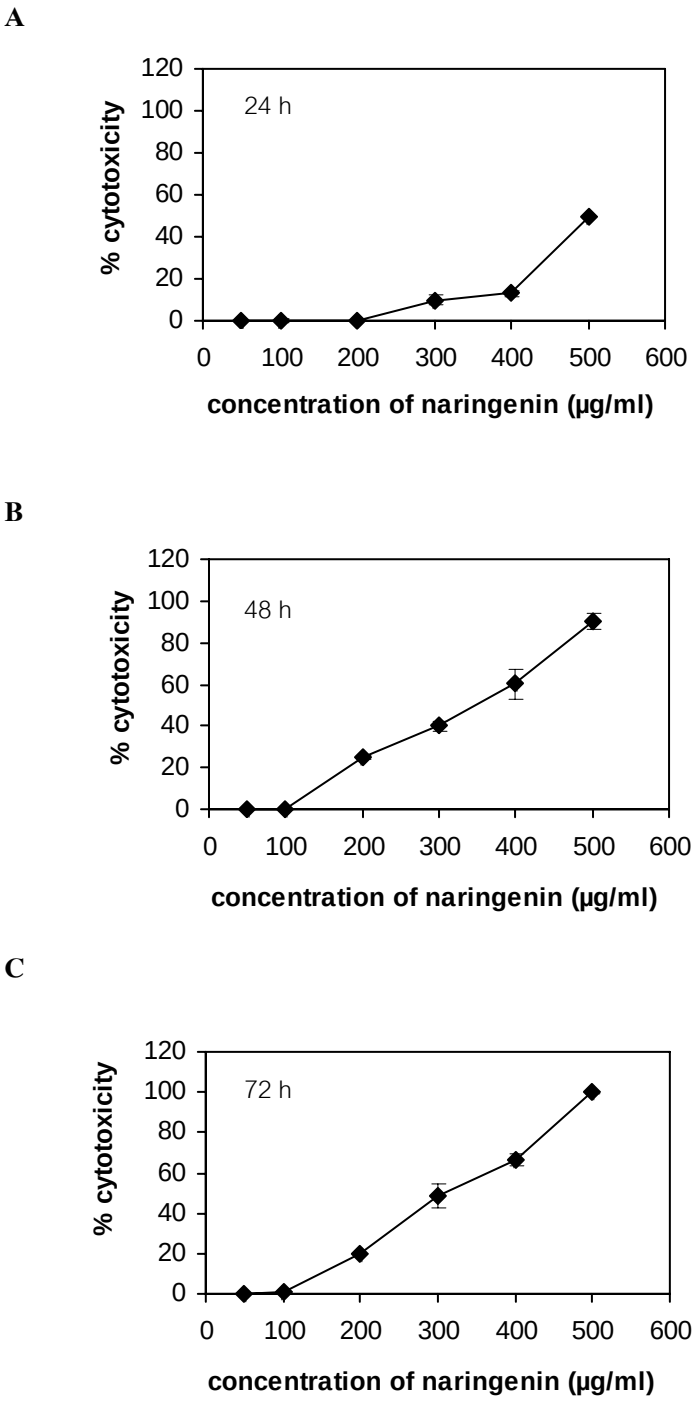


Figure 10. Cytotoxicity of naringenin determined by trypan blue exclusion method. At various times of incubation, 24 h (A), 48 h (B) and 72 h (C). Vero cells were incubated with various concentrations of test compounds for 24, 48 and 72 h. Then the cells were counted after staining with trypan blue. The data were reported as mean ± S.D. from at least three independent experiments.

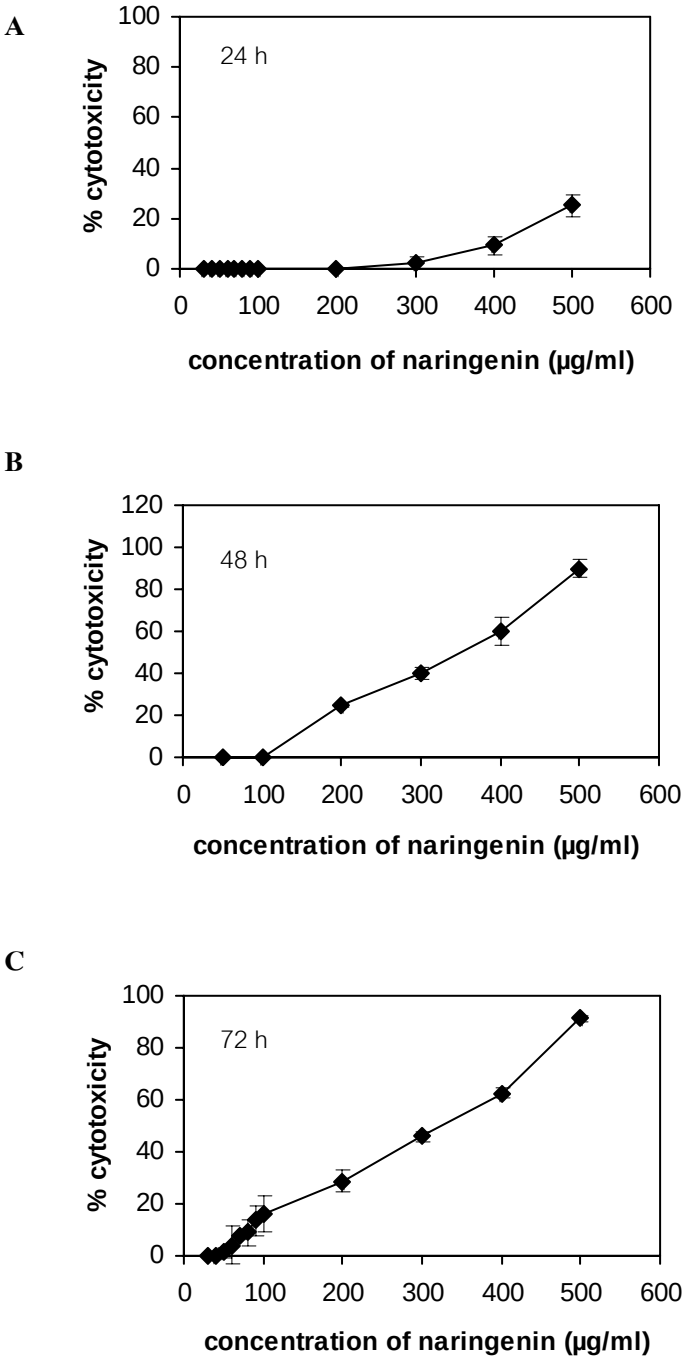


Figure 11. Cytotoxicity of naringenin determined by MTT reduction assay. At various times of incubation, 24 h (A), 48 h (B) and 72 h (C). The data were reported as mean ± S.D. from at least four independent experiments.

The CC_{50} values of naringenin on Vero cells were summarized in Table 1. The CC_{50} values of naringenin determined by the trypan blue exclusion method were similar to those obtained by the MTT reduction assay.

Table 2. Cytotoxicity of naringenin on Vero cells.

Incubation time of naringenin	CC_{50} (µg/ml) of naringenin[a]	
	Trypan blue exclusion method	MTT reduction assay
24 h	> 500	> 500
48 h	329.52±33.37	378.71±25.25
72 h	305.64±18.36	304.56± 7.41

[a]50% cytotoxic concentration (CC_{50}) represented concentration of compound required to reduce viability of the cells by 50%. The reported values were derived from at least three independent assays of both methods.

4.2 Anti-HSV-1 and HSV-2 activities of naringenin

Antiviral activities of naringenin and ACV (as a positive control) were determined as 50% inhibitory concentration (IC_{50}).

4.2.1 Effect on plaque reduction assay

The antiviral activities of naringenin and ACV (as positive control) obtained from plaque reduction assay or post-treatment were shown in Figure 12. The IC_{50} values of naringenin against HSV-1 and HSV-2 were 46.64±7.06 and 48.27±5.14 µg/ml, respectively. ACV inhibited HSV-1 and HSV-2 plaque formation on Vero cells with IC_{50} of 0.59±0.06 and 0.63±0.07 µg/ml, respectively. The results showed that at the same concentration the efficiency of plaque inhibition of all test compounds to HSV-1 infected cells was similar to HSV-2 infected cells. Naringenin at 100 µg/ml completely inhibited the HSV-1 and HSV-2 plaque formation.

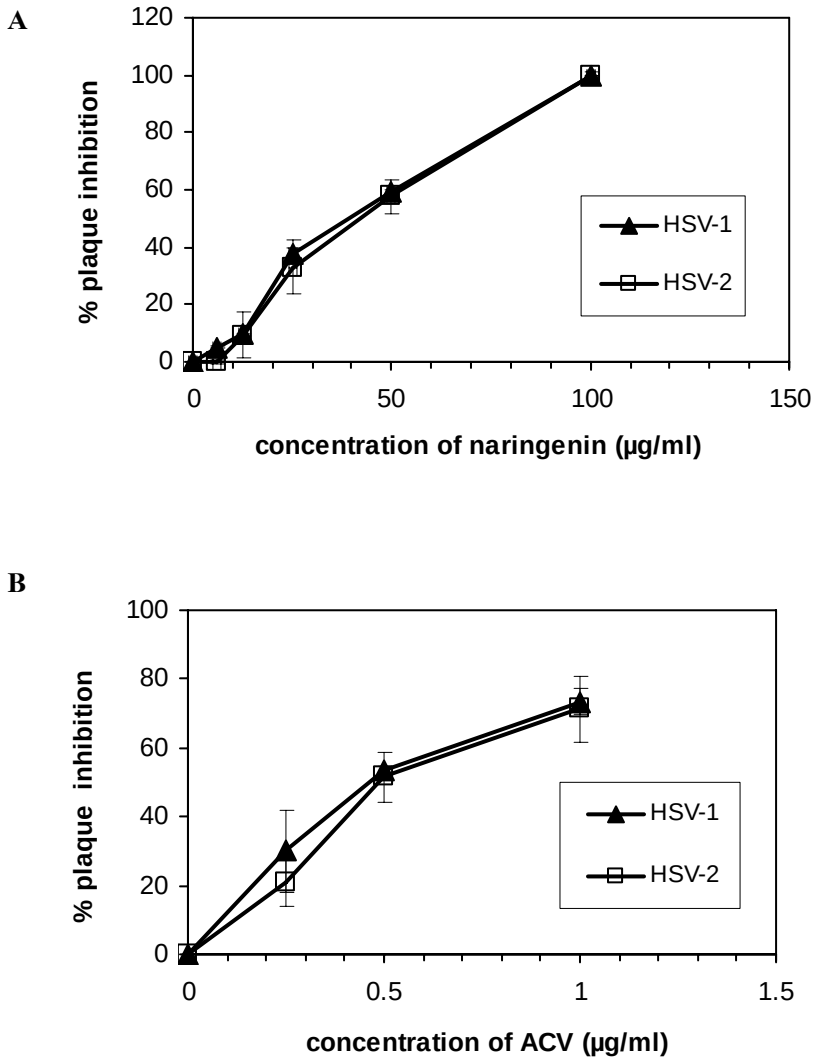


Figure 12. Anti-herpes simplex virus activity of naringenin (A) and ACV (B) determined by plaque reduction assay. The data were reported as mean ± S.D. from at least four independent experiments. Percent of plaque inhibition between treated and untreated cells after infection with concentration of naringenin more than 25 µg/ml was significantly different (p<0.05).

4.2.2 Effect on viral inactivation assay

Inactivation assay was used to investigate the inhibitory activity of naringenin to HSV-1 and HSV-2 on Vero cells, and ACV was used as a positive control. The anti-HSV-1 and anti-HSV-2 activities of each compound were shown in Figure 13. The IC_{50} values of naringenin against HSV-1 and HSV-2 were 43.30±2.80 and

47.58±5.24 µg/ml, respectively. ACV inhibited HSV-1 and HSV-2 plaque formation on Vero cells with IC_{50} of 0.49±0.06 and 0.57±0.03 µg/ml, respectively. The IC_{50} values of each compound required for inactivation of HSV-2 was similar to the value required for inactivation of HSV-1. When the concentration of naringenin was increased to 100 µg/ml, the inhibitory activities of these compounds were 100%.

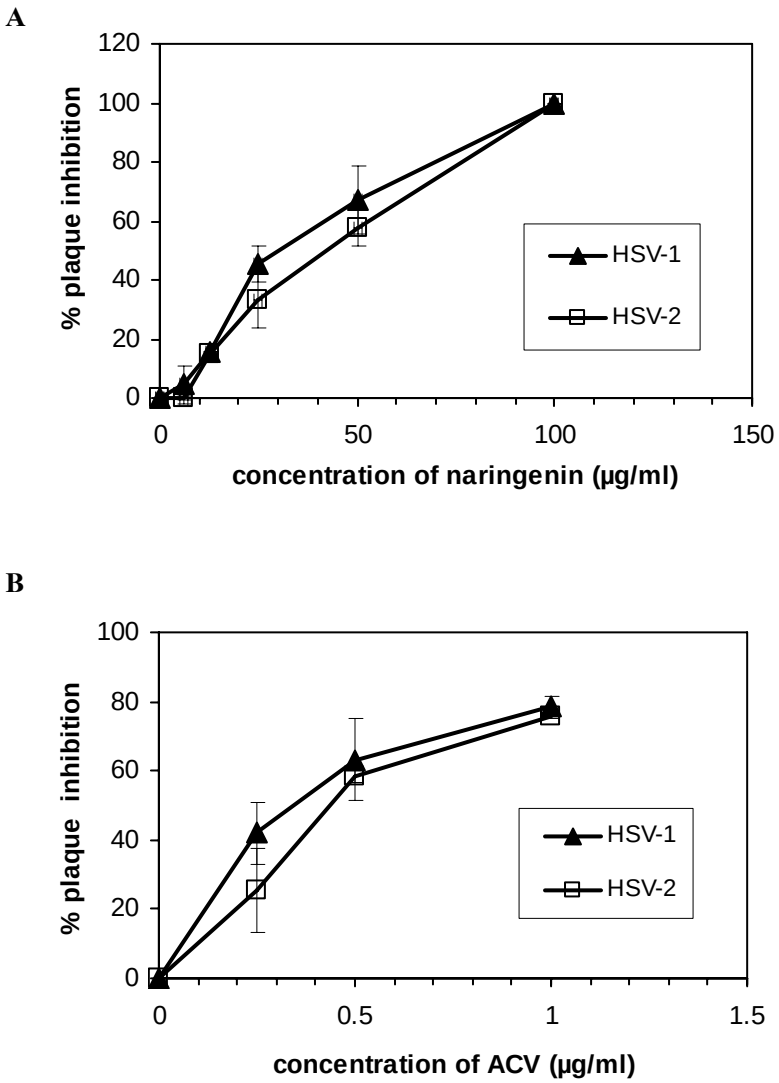


Figure 13. Anti- herpes simplex virus activity of naringenin (A) and ACV (B) determined by inactivation assay. The data were reported as mean ± S.D. derived from at least four independent experiments. Percent of plaque inhibition between treated and untreated cells after infection with concentration of naringenin more than 25 µg/ml was significantly different (p<0.05).

4.2.3 Effect on prophylactic activity assay

The results of viral inhibition were shown in Figure 14. In the inhibition of HSV on Vero cells, the IC_{50} values of naringenin against HSV-1 and HSV-2 were 36.08±1.86 and 39.39±1.68 µg/ml, respectively. ACV inhibited HSV-1 and HSV-2 plaque formation on Vero cells with IC_{50} of 0.50±0.09 and 0.56±0.02 µg/ml, respectively.

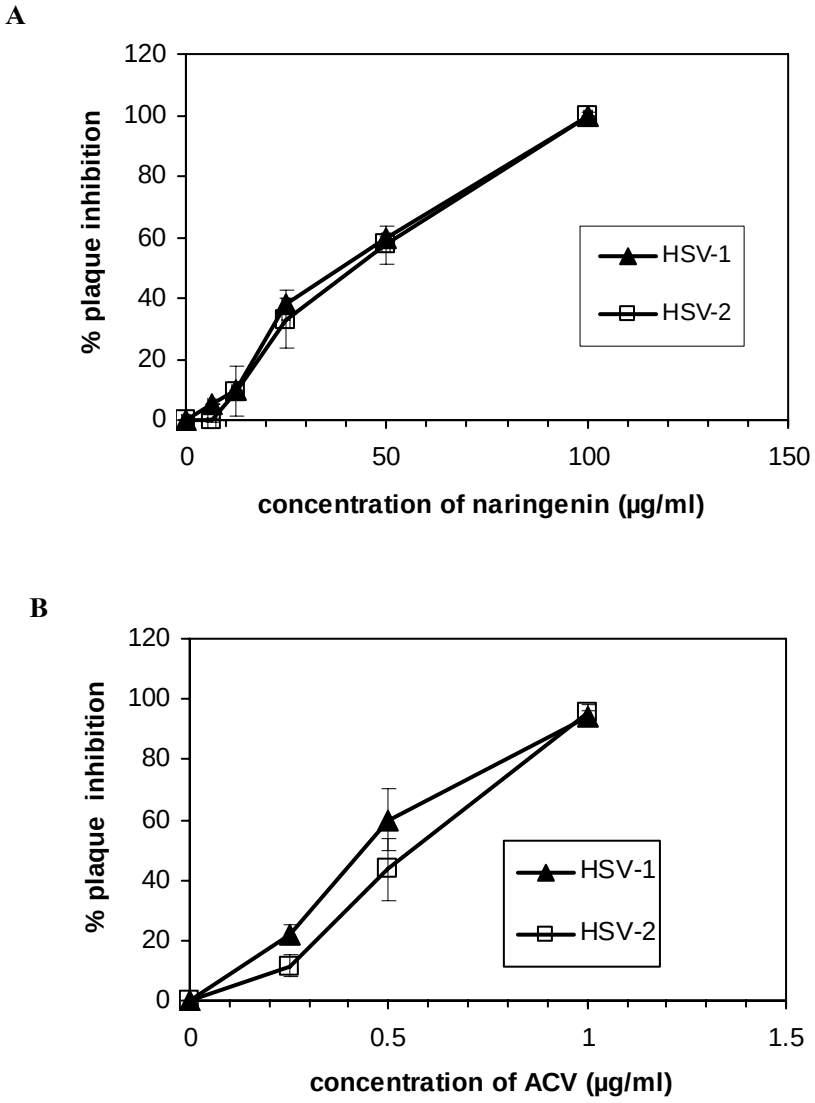


Figure 14. Anti-herpes simplex virus activity of naringenin (A) and ACV (B) determined by prophylactic activity assay. The data were reported as mean ± S.D. from at least four independent experiments. Percent of plaque inhibition between treated and untreated cells after infection with concentration of naringenin more than 6.25 µg/ml was significantly different (p<0.05).

The IC_{50} and SI (selective index) values against HSV-1 and HSV-2 of naringenin and ACV obtained from plaque reduction, inactivation and prophylactic activity assay on Vero cells were summarized in Table 3 and Table 4. In all cases, SI were more than 2 according to the CC_{50} of each drug higher than its IC_{50}. In this study, CC_{50} of ACV was not excluded; however CC_{50} was assumed that more than 1 mg/ml, which was no cytotoxicity concencentration in anti- herpes simplex virus activity. There were observed differences in the degree of viral inhibition depending on the assay used. The IC_{50} obtained from prophylactic test was less than the IC_{50} obtained from the plaque reduction assay ($p<0.05$).

Table 3. Antiviral activity of naringenin and ACV against HSV-1.

Compounds	HSV-1					
	Inactivation assay		Plaque reduction assay		Prophylactic activity assay	
	IC_{50} (µg/ml)	SI[a]	IC_{50} (µg/ml)	SI[a]	IC_{50} (µg/ml)	SI[a]
Naringenin	43.30±2.80	7.61	46.64±7.06	7.07	36.08±1.86	9.13
Acyclovir	0.49±0.06	>1000	0.59±0.06	>1000	0.50±0.09	>1000

[a]selective index of each drug calculated from CC_{50} / IC_{50} in each treatment and the results were determined from at least three independent experiments.

Table 4. Antiviral activity of naringenin and ACV against HSV-2.

Compounds	HSV-2					
	Inactivation assay		Plaque reduction assay		Prophylactic activity assay	
	IC_{50} (µg/ml)	SI[a]	IC_{50} (µg/ml)	SI[a]	IC_{50} (µg/ml)	SI[a]
Naringenin	47.58±5.24	6.93	48.27±5.14	6.83	39.39±1.68	8.36
Acyclovir	0.57±0.03	>1000	0.63±0.07	>1000	0.56±0.02	>1000

[a]selective index of each drug calculated from CC_{50} / IC_{50} in each treatment and the results were determined from at least three independent experiments.

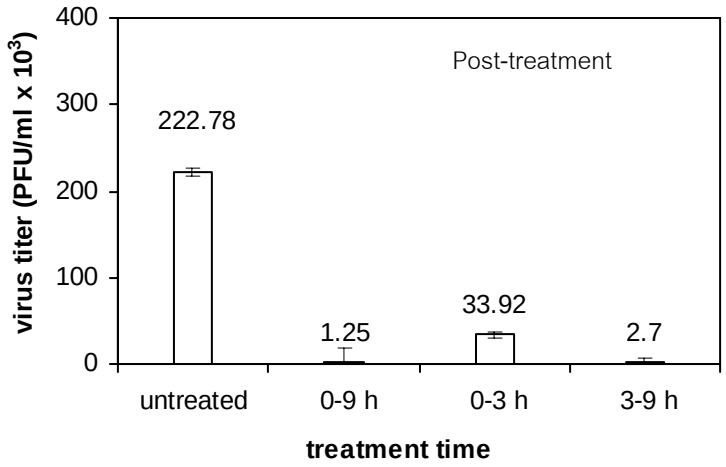
4.3 Mode of anti-HSV activity of naringenin

Preliminary tests for possible mechanisms of action of naringenin in anti-HSV-1 infection were performed using, virus yield inhibition assay, required treatment period for inhibition of plaque formation, post-binding assay, penetration assay**,** virucidal assay and combined activity with ACV.

4.3.1 Effect on virus yield inhibition assay

To study the inhibitory effect of naringenin on the stages of HSV-1 infection, a time of addition experiment was performed. Naringenin was added to Vero cells at various time points including before and after virus infection. The results showed that naringenin treatment both before and after virus inoculation potentially suppressed HSV-1 infection as displayed in Figure 15. When naringenin was added to the cells as 1 h pre-treatment at 37°C and then washed out before viral infection, the reduction of virus yield was more than 80% as compared with untreated control cells. Moreover, the addition of naringenin to pretreated cells at 1 and 3 h post infection exhibited HSV inhibitory activity higher than 98% for HSV-1 ($p<0.05$). The extent of inhibition of HSV-1 production was observed when the treatment with naringenin was started either 1 or 3 h after virus inoculation and removed at 3 and 9 h post infection, respectively. Indeed, the inhibition of HSV-1 replication was greater than that of naringenin added to the cells at 1 h after virus infection, and maintained in the culture until the end of the experiment. However, all three conditions in which naringenin was added to the cells after virus challenge resulted in reduction of HSV-1 infectivity by more than 50% as compared to untreated infected cells ($p<0.05$). The result indicated that naringenin affected all steps of HSV replication. In addition, it probably affected the susceptibility of the target cells to infection by HSV.

A



B

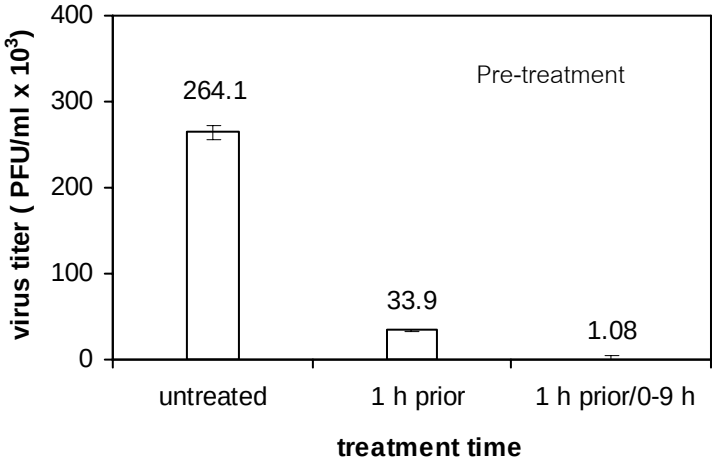


Figure 15. Effect of naringenin treatment on virus yield: (A) Post-treatment; cells were incubated with the media containing naringenin for 9 h and 3 h after viral adsorption period (0-9 h, 0-3 h), cells were also incubated with compound-free medium for 3 h after viral adsorption period and then the medium containing naringenin for 6 h (3-9 h). (B) Pre-treatment; cells were pretreated with naringenin for 1 h before viral adsorption period and were incubated with compound-free medium for 9 h (1 h prior), cells were pretreated with naringenin for 1 h before viral adsorption and were incubated with medium containing naringenin for 9 h (1 h prior/0-9 h). Infected cells incubated with compound-free medium (untreated) were

used as control. The data represented the average of the virus yield with the standard deviation from three independent experiments.

4.3.2 Required treatment period for inhibition of plaque formation

The period of time for incubation of the infected cells with naringenin did affect the inhibitory activity of the compound. Viral inhibition of 100% against HSV-1 were observed for the cells maintained in naringenin at 90 µg/ml for 24 and 48 h after infection ($p < 0.05$). Viral inhibition of 2.27 and 72.86% were indicated when the infected cells were maintained in the media with 90 µg/ml of naringenin for 6 and 12 h after HSV-1 infection, respectively. Viral inhibition of 0, 0, 3.23 and 3.66% against HSV-1 were observed for the cells maintained in naringenin at 30, 50, 70, 90 µg/ml for 6 h after infection, respectively. No inhibition of plaque formation was observed in HSV-1 infection for 3 h (Figure 16).

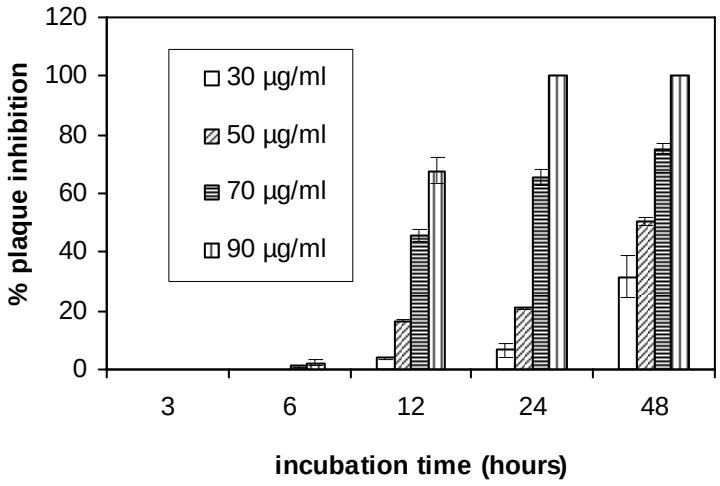


Figure 16. Effect of treatment period for inhibition of plaque formation by naringenin for anti-HSV-1 activity. Cells were incubated in overlaid medium containing 30-90 µg/ml of naringenin after 1 h of viral adsorption. At each time of incubation of 3, 6, 12, 24 and 48 h at 37 ◦C, the overlaid medium supplemented with compound was removed. The cells were washed and replaced with the compound-free overlaid medium and incubated thereafter for up to 48 h, respectively. The data represented the average of plaque formation with the standard deviation from three independent experiments.

4.3.3 Effect on viral adsorption

The effect of naringenin on adsorption of HSV-1 to Vero cells was analyzed by post-binding assay. The results were shown in Figure 17. Citrate buffer pH 4, used as a positive control, completely inhibited viral adsorption and no plaque was detected. Naringenin at 60-100 µg/ml significantly inhibited (p<0.05) plaque formation, approximately 20-40% compared to untreated control cells in viral adsorption assay.

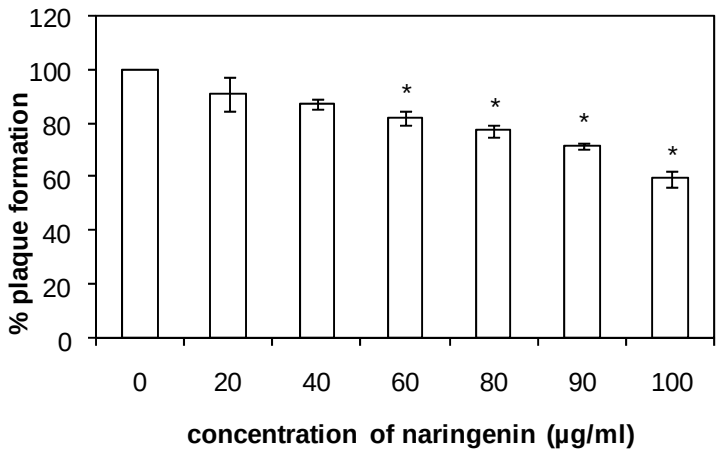


Figure 17. Effect of naringenin on HSV-1 adsorption. This effect was determined by post-binding assay. HSV-1 was attached to Vero cells and then incubated with naringenin at 4°C. The number of plaque was reported as mean ± S.D. from three independent experiments. Low pH citrate buffer was used as a positive control and no plaque was detected. * Plaque formation was significantly different from that of untreated control (p<0.05).

4.3.4 Effect on viral penetration

In penetration assay modified from Piret *et al*. (2002), Vero cells were infected with HSV-1 and incubated at 4°C. In this condition, virus could not penetrate into cells. At temperature of 37°C viral penetration was prevented by treating the infected cells with naringenin for different period of time. The result was shown in Figure 18. Naringenin at 60-100 µg/ml significantly inhibited (p<0.05) plaque formation, approximately 20-40% compared to untreated control cells in viral penetration assay. Percent plaque inhibitions were not significantly different (p>0.05) in various times of viral penetration and these values of percent plaque inhibitions (at 0 min) were similar to that of viral adsorption assay (Figure 17).

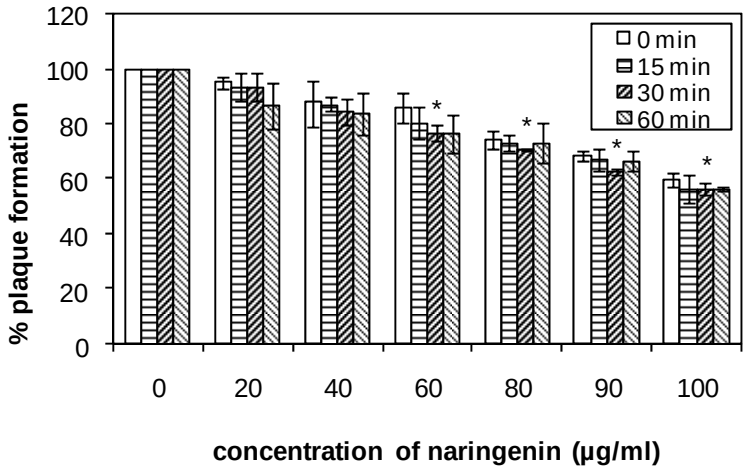


Figure 18. Effect of naringenin on HSV-1 penetration. After viral adsorption at 4°C, the temperature was shifted to 37°C for 0, 15, 30, and 60 minutes to allow penetration of bounded virus into treated and untreated control cells. The number of plaques was reported as mean ± S.D. from three independent experiments. * Plaque formation was significantly different from that of untreated control (p<0.05).

4.3.5 Virucidal activity

The virucidal activity of naringenin against HSV-1 was displayed in Table 5. The HSV-1 titers of cells treated with higher concentration of naringenin decreased

compared with untreated control cells in condition of 37°C, 1 h incubation. The results showed that concentration of naringenin at 90, 360 and 1440 µg/ml reduced HSV-1 titer by 73.27, 95.25 and 96.78%, respectively. This difference on viral titer was significant (p<0.05). It could be concluded that naringenin showed direct effect on HSV-1 infectivity.

Table 5. Virucidal activity[a] of naringenin against HSV-1.

Concentration of naringenin (µg/ml)	HSV-1 titer (x 10^5 PFU/ml)
0	24.5 ± 1.72
6.25	24.1 ± 2.30
12.5	22.8 ± 1.86
25	19.5 ± 0.77
50	18.0 ± 0.92
75	16.9 ± 1.40
90	6.5 ± 0.65[b]
360	1.2 ± 0.14[b]
1440	0.7 ± 0.14[b]

[a]Virucidal assay was performed by incubation of virus and naringenin at 37°C for 1 h. The residual virus infectivity was titrated by plaque assay and reported as mean ± S.D. of three independent experiments.

[b]HSV-1 titer was significantly lower than that of untreated control (p<0.05).

4.3.6 Combined effect of naringenin with acyclovir on plaque formation

Combination of ACV with naringenin was investigated for their anti-HSV activities. The concentrations used in this assay for both compounds were not cytotoxic. The combined effect of naringenin with ACV on plaque formation of HSV-1 in Vero cells was analyzed by isobologram and the percent plaque formation of each combination was expressed as the representative data of three independent experiments. The data were shown in Figure 19. The curve fell below the dotted line

of the additive effect at all concentrations indicating that the combination of acyclovir and naringenin exhibited synergism against HSV-1. Low concentrations of ACV could inhibit HSV-1 infection by the addition of naringenin. The IC_{50} value for naringenin could be reduced from 46.64±5.14 µg/ml of naringenin alone to 33.71±2.44, 20.75±2.11, 13.78±3.16 and 8.95±0.24 µg/ml of naringenin plus 0.1, 0.2, 0.3 and 0.4 µg/ml of ACV, respectively. The FICs of naringenin plus ACV were in the range 0.78–0.89, indicating that the combinatorial effect of naringenin plus ACV in inhibiting HSV-1 infection was synergistic (Table 6). None of these drug combinations exhibited cytotoxic effect against Vero cells at the concentrations used as assessed by cytotoxicity assay (data not shown).

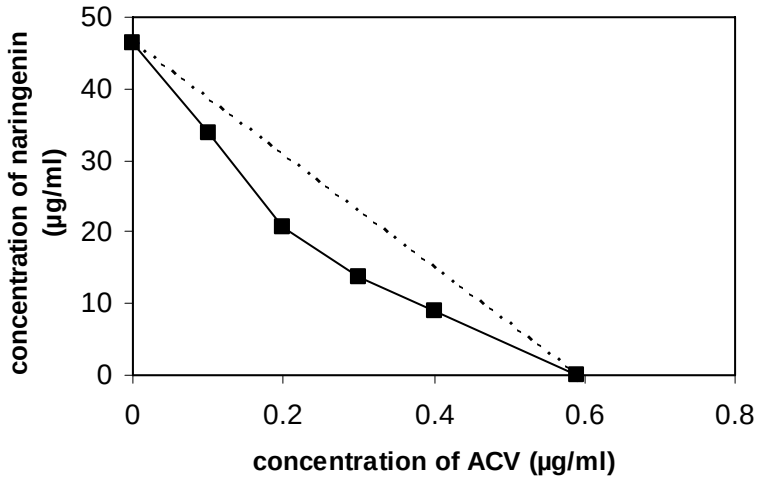


Figure 19. Combination of naringenin and acyclovir on HSV-1 plaque formation analyzed by isobologram. The figure showed data from three independent experiments using HSV-1. The dotted line indicated the theoretical additive activity. The measured line (■) was lower than the dotted line indicating synergy.

Table 6. Inhibitory effects of naringenin in combination with ACV against the infection of HSV-1 in Vero cells.

Compound(s)	Mean (IC_{50}±SD)[a]	$FIC_{naringenin}$+ FIC_{ACV}[b]	FIC index interpretation
Naringenin alone	46.64±7.06	-	-
ACV alone	0.59±0.06	-	-
Naringenin +0.1μg/ml ACV	33.71±2.44	0.89	synergistic
Naringenin +0.2μg/ml ACV	20.75±2.11	0.78	synergistic
Naringenin +0.3μg/ml ACV	13.78±3.16	0.80	synergistic
Naringenin +0.4μg/ml ACV	8.95±0.24	0.87	synergistic

[a]Results were based on three independent experiments.

[b]$FIC_{naringenin}$ and FIC_{ACV} were the fractional inhibitory concentrations (FICs) of naringenin and ACV, respectively.

CHAPTER V

DISCUSSION AND CONCLUSION

Many chemotherapeutic agents have been developed and used for HSV infections, most of these are nucleoside analogs with selective antiviral activity. Acyclovir (ACV) is the most common. However, resistance to ACV and the other nucleoside analogs has been reported (Coen, 1991; Galasso *et al*., 1997; Leung and Sacks, 2000; De Clercq, 2004a; Brady and Bernstein, 2004). Therefore, the development of novel compounds with alternative mechanisms of antiviral action is important. Naringenin, a potent antioxidant, has been used as a supplement therapy in several diseases associated with oxidative stress such as anti-inflamation (Manthey *et al*., 2001), anticancer agents (Guthrie and Carroll, 1998) and anti-atherogenic compounds (Mulvihill and Huff, 2010). In addition, many antioxidants including glutathione and ascorbate have shown anti-HSV activity (Betanzos-Cabrera *et al*., 1994: Palamara *et al*., 1995). Therefore, in this investigation naringenin was investigated for the antiviral activity against HSV-1 and HSV-2 infection *in vitro*.

In *in vitro* antiviral activity assay, an effective anti-HSV agent should be non-toxic to the cell culture at the effective concentration. Thus, cytotoxicity of naringenin was primarily investigated. Dimethylsulfoxide (DMSO) was used as solvent and diluted in medium for all test substances in this study. The maximum final concentration of DMSO in test solutions used in all antiviral assays was 0.5%, which did not show any toxicity to Vero cell cultures. No viral inhibition was observed for 0.5% DMSO in medium. The concentrations of all test substances using anti-HSV activity assays in plaque reduction assay were lower than CC_{50} and the selective indices were 7.07 for HSV-1 and 6.83 for HSV-2.

Acyclovir triphosphate, an active form of ACV in host cells treated with ACV, has significantly higher affinity to HSV DNA polymerase than to intracellular α-DNA polymerase. ACV has been proven to be safe in cell cultures, animal models, and humans (Kurokawa *et al*., 1995; Liu *et al*., 2004) with high selectivity. ACV is a

current standard treatment for HSV-1 and HSV-2 infection. Therefore, ACV was used as a positive control and used as a combined drug with naringenin in this study.

In Cytotoxicity test, naringenin affected Vero cell proliferation and resulted in reduction of cell viability. Trypan blue exclusion method and MTT reduction method similar 50% cytotoxic concentration (CC_{50}) for naringenin with the value of CC_{50}. However, several recent studies proved that both naringenin (pure compound) and naringenin in citrus juice did not show any serious side effect in either animals or humans in oral preparation (Ameer *et al*., 1996; Ishii *et al*., 1997; Wilcox *et al*., 1999).

Naringenin showed antiviral activities against HSV-1 and HSV-2 on Vero cells. Naringenin inhibited HSV-1 plaque formation with similar efficiency to HSV-2 when treated at the same concentration. In inactivation assay, the IC_{50} value of naringenin was lower than the IC_{50} values obtained from plaque reduction assay. The difference in IC_{50} value between the two methods might be related to the prolonged contact time of the test substances with the cells, since Vero cell monolayers were incubated with naringenin or ACV only after viral adsorption period in plaque reduction assay, while, in inactivation assay, the cell cultures were incubated with the test substances both during and after viral adsorption period as the result of mixing the virus and each test substance together before infection to the cells. Naringenin showed similar efficacy against HSV-2 compared with that of HSV-1 not only in plaque reduction assay and inactivation assay but also showed activity in prophylactic activity assay. In addition, naringenin showed more efficacy against HSV in prophylactic activity assay than plaque reduction assay ($p< 0.05$).

In virucidal assay, naringenin at concentration 90 µg/ml exhibited virucidal activity. This effect showed direct inactivation of HSV-1 of naringenin. It may be essential mechanism for the treatment of HSV-1 infection. Further studies of using this mechanism to develop preparation of naringenin should be considered.

The effect of treatment period for the antiviral activity of naringenin was studied and time-dependent activity of the compound on HSV-1 growth inhibition was demonstrated. A complete inhibitory effect was observed after 24-48 h incubation of the infected cells in the medium containing naringenin at 90 µg/ml. The decrease in

inhibitory effect was observed when the infected cells were incubated in medium containing naringenin at 30-70 µg /ml and/or incubated for 3 or 6 or 12 h.

Naringenin effectively reduced the virus yield when it was added for 3 and 9 h after 1 h of viral adsorption and for 6 h after 3 h of incubation of infected cells in compound-free medium. The compound was also effective in reducing virus yield when the cells were treated with the compound 1 h prior to viral infection and incubated further either in compound-free medium or compound-supplemented medium. However, the compound did show less efficacy in the cells pretreated with the compound 1 h before viral adsorption and further incubated with compound-free medium. It exhibited inhibitory activity with 1-h treatment prior to viral infection and further treatment in the early phase and late phase of viral replication.

Possible mechanisms of action of naringenin were confirmed by virus growth inhibition and pre-treatment testing. Naringenin at concentration of 90 µg/ml which was higher than its IC_{50} was used in virus yield inhibition assay (Kurokawa *et al*., 1995). In the assay of pre-incubation, naringenin was added to the Vero cells and then removed and washed before virus challenge, significant substantial inhibition of HSV-1 production was observed. Moreover, the addition of naringenin after viral adsorption period without pretreatment of the cells still had potential to reduce HSV infection. The inhibition of viral production when naringenin was added after viral challenge was less than when naringenin was added before viral challenge ($p < 0.05$), corresponding to the antiviral activity obtained from inactivation and plaque reduction assay. In addition, addition of naringenin at 3 hour post infection inhibited virus production to a higher extent than the addition at 1 hour post infection. All together, these results demonstrated that naringenin acted mainly by reducing the susceptibility of Vero cells to HSV-1 and HSV-2 infection and inhibiting HSV replication after the viruses had entered into the cells.

The mechanisms by which naringenin inhibited HSV-1 infection is unclear. However, the fact that change in intracellular redox status occurs both *in vivo* and *in vitro* in different kinds of viral infections such as influenza and parainfluenza (Hennet *et al*., 1992; Ciriolo *et al*., 1997) suggests that the impairment of redox status inside host cells is essential for the initiation and maintenance the replication of virus. Furthermore, decreasing in both extracellular and intracellular levels of total

glutathione, a cellular thiol antioxidant, which occurred at early time after HSV-1 infection of Vero cells has been reported as a major reason in the impairment of intracellular redox status (Palamara *et al*., 1995; Vogel *et al*., 2005). Previous studies represented that supplementation with exogenous glutathione could inhibit HSV-1 replication and replenish intracellular glutathione level (Palamara *et al*., 1995; Nucci *et al*., 2000). Glutathione; γ-glutamyl-cysteinyl-glycine or GSH, an SH group containing tripeptide, is the most prominent intracellular low-molecular weight thiol found in eukaryotic cells. GSH serves as an important intracellular water-soluble antioxidant and detoxifying agent (Droge *et al*., 1994). HSV infection has been shown to associate with mark depletion of extra-cellular and intracellular GSH levels; furthermore, it has been reported that exogenous GSH is able to induce a strong concentration-dependent inhibition of HIV, sendai virus, and HSV replication *in vitro* (Vogel *et al*., 2005). Although the mechanism of antiviral activity of GSH needs to be fully elucidated, all data indicated that GSH inhibit HSV replication by interfering with very late stages of HSV life cycle (Palamara *et al*., 1995).

Naringenin is major flavonone that occur in various medicinal plants such as citrus fruits, grapefruits, and tomato fruits and also found in tea and wine (Krause *et al*., 1992; Susanti *et al*., 2007). The studies of antioxidant activity of naringenin had been investigated. Crude extracts of the flowers of *Melastoma decemfidum* plants contained naringenin which determined antioxidant activity using DPPH radical-scavenging assay. The results showed that naringenin had strong antioxidant activity. Therefore, naringenin, a potent antioxidant, might prevent this impairment which is a primary phenomenon produced by viral infection (Susanti *et al*., 2007).

From the combined effect of naringenin with ACV in Vero cells (Figure 19 and Table 6), naringenin enhanced the anti-HSV-1 activity of ACV synergistically. The IC_{50} for ACV and naringenin, when combined with ACV, could be reduced to the IC_{50} when the drugs were used alone. ACV is a nucleoside analog that exhibits anti-herpetic activity after phosphorylation by viral TK. Acyclovir triphosphate then interferes with viral DNA polymerization through competitive inhibition with guanosine triphosphate and obligatory DNA chain termination (De Clercq, 2004a). The results showed that naringenin inhibited viral replication but the mechanisms of

action of naringenin was not clear. However, it suggested that naringenin may be the alternative agent combined with ACV to treat HSV-1 infection.

In conclusion, naringenin showed antiviral activity against HSV-1 infection in viral adsorption assay. The susceptibility of target cells to virus infection may be reduced. Percent plaque inhibitions were not significantly in various times of viral penetration. Naringenin inhibited virus production in the cells after infection in virus yield inhibition assay. Naringenin did inhibit virus by directly inactivating virus, or inhibiting virus attachment to cell receptors, or inhibiting virus penetration into the target cells. Naringenin exhibitted anti-HSV-1 and anti-HSV-2 activities at concentrations below its CC_{50}. Moreover, naringenin enhanced the anti-HSV-1 activity of ACV synergistically. Therefore, based on these favorable profiles, naringenin may have a potential use in preventive therapy and treatment therapy for HSV-1 and HSV-2 infection.

With regard to the effect of naringenin upon HSV protein synthesis and HSV mutation, the amount of viral glycoprotein should be evaluated. So, further researches are required to better characterize the clearly mechanisms of action of naringenin. Anti-HSV resistant strain should be investigated. Moreover, the effect of naringenin on the replication of other herpes viruses should be studied to provide effective therapy of HSV-related diseases.

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APPENDICES

APPENDIX A

Chemical agents and instruments

1. Chemical agents

Absolute methanol GR (E. Merck, Darmstadt, Germany)

Citric acid (E. Merck, Darmstadt, Germany)

Chloroform (Sigma, MO, U.S.A.)

Crystal violet (Fluka, Switzerland)

Dimethyl sulfoxide (DMSO) (Sigma, MO, U.S.A.)

Formaldehyde 38% w/v AR (Carlo Erba, Milano, Italy)

Hydrochloric acid (E. Merck, Darmstadt, Germany)

Methylcellulose (Sigma, MO, U.S.A.)

Potassium chloride (KCl) (May & Bayer, England)

Potassium dihydrogen phosphate (KH_2PO_4) (E. Merck, Lot. NO. 547A17873, Darmstadt, Germany)

Dipotassium hydrogen phosphate (K_2HPO_4) (E. Merck, Darmstadt, Germany)

Propan-2-ol (Fisher Scientific, U.K.)

Sodium bicarbonate ($NaHCO_3$) (E. Merck, Darmstadt, Germany)

Sodium chloride (NaCl) (E. Merck, Lot. No. K27736104021, Darmstadt, Germany)

Disodium hydrogen phosphate (Na_2HPO_4) (May & Bayer, Lot. No. 50028, England)

Thiazolyl blue tetrazolium bromide (MTT) (Sigma, Lot No. 085K5304, U.S.A.)

Tragacanth (Pharmaceutical chemicals, Denmark)

Trypsin-EDTA (1X) (Gibco, Canada)

2. Instruments

Analytical balance (Satorius, Germany)

Automatic pipet (Drummond Scientific, U.S.A.)

Automatic pipet P2-20/P20-200/P100-1000 (Socorex, Switzerland)

Centrifuge (Sigma, Germany)

Hemocytometer (Boeco, Germany)

Laminar air flow (Holten, U.S.A.)

Microplate reader model 3550 (Biorad, U.S.A.)

Multichannel automatic pipette, 8 channel (Socorex, Switzerland)

Orbital Shaker SO3 (Stuart scientific, Redhill, UK)

pH meter (Beckman, U.S.A.)

Refrigerator 4◦C (Sharp, Thailand)

Refrigerator 20◦C (Ariston, U.S.A.)

Refrigerator -80◦C (Forma Scientific, U.S.A.)

Ultrasonicator

Vortex mixer (Scientific, NY, U.S.A.)

Water bath (Thelco, U.S.A.

3. Laboratory supplies

Cryotubes (Nunc, Denmark)

Glasswares (Pyrex, U.S.A.)

Microcentrifuge tubes

Millipore filters 0.2 µm, Acrocap filter unit (Gelman Laboratory, U.S.A.)

Pipette tips (Nunc, Denmark)

Syringe filters, Acrodisc (Pall Corporation, U.S.A.)

Tissue culture flasks (Nunc, Denmark)

Tissue culture plates (Nunc, Denmark)

APPENDIX B

Medium and Reagents

1. Growth medium

Vero cells CCL-81 were grown and maintained in Eagle's minimum essential medium (MEM). Minimum essential medium with Eagle's salts and Glutamine (Gibco, U.S.A.) without sodium bicarbonate powder 9.5 g was dissolved in deionized distilled water. 2.2g/L of sodium bicarbonate (Sigma, U.S.A.) were added to the solution. This medium solution was mixed well and adjusted pH to 7.2-7.4 with 6N HCl. Then, the solution was adjusted volume to 1,000 ml by deionized distilled water. This solution was sterilized by filtration with 0.22 µm millipore filter membrane. Before use, this MEM solution was supplemented with 8% fetal bovine serum (FBS, Gibco, U.S.A.) and 1% antibiotic-antimycotic agents (Gibco, U.S.A) which contained 10,000 units/ml of penicillin G sodium, 10,000 µg/ml of streptomycin sulfate, and 25 µg/ml of amphotericin B as fungizone in 0.85% saline.

2. 0.05% Methylene blue in distilled water

Methylene blue	5 g
Distilled water	100 ml

3. 12% Formaline in normal saline solution

38% Formaldehyde	320 ml
0.85% Normal saline (NaCl) solution	680 ml

4. Phosphate Buffer Saline Solution (PBS)

NaCl	8.00 g
KCl	0.20 g
KH_2PO_4	0.20 g
Na_2HPO_4	1.15 g
Deionized distilled water to	1,000 ml

This solution is sterilized by autoclaving for 15 minutes at 121◦C, 15 lb/in^2

5. Low-pH Citrated buffer,pH 3.0

Sodium citrate	40 mM
Potassium chloride	10 mM
Sodium chloride	135 mM
Deionized distilled water to	1,000 ml

pH of this solution is adjusted to 3.0 and then the buffer is sterilized by autoclaving for 15 minutes at 121◦C, 15 lb/in^2

6. Plaque overlay medium

Solution A

2x MEM with 2% antibiotic-antimycotic agent

This solution was sterilized by filtration through 0.22 µm Millipore filter membrane

Solution B

Tragacanth (Pharmaceutical chemicals, Denmark)	2 g
Deionized distilled water	100 ml

This solution was sterilized by autoclaving for 15 minutes at 121◦C, 15 lb/in^2

The solution A and B were mixed well at a ratio of 1:1 and were supplemented with 2% fetal bovine serum (FBS, Gibco, U.S.A.) before use

7. MTT solution

Thiazolyl blue tetrazolium bromide (MTT)	500 mg
PBS	100 ml

This solution was sterilized by filtration to remove a small amount of insoluble residues present in some batches of MTT

8. Acid-isopropanol

HCl	1.23 ml
Isopropanol	1,000 ml

Calculation of median cytotoxic concentration (CC_{50}) and Inhibitory concentration (IC_{50}) by regression formula.

CC_{50} is the concentration of naringenin, or acyclovir which exhibit 50% cytotoxicity.

CC_{50} is calculated from the regression formula Y = aX + b, when Y is optical density or number of viable cells, X is the concentration of naringenin, or ACV in µg/ml, a is slope, and b is an intercept or the distance between X axis and the point where the regression line come across Y axis

IC_{50} is the concentration of naringenin, or ACV which can inhibit 50% of plaque formation or optical density reduction.

IC_{50} is calculated from the regression formula Y = aX + b, when Y is number of plaque or optical density, X is the concentration of naringenin, or ACV in µg/ml, a is slope, and b is an intercept or the distance between X axis and the point where the regression line come across Y axis

FIC is calculated from formula , FIC index = $FIC_{naringenin}$ + FIC_{ACV} = (IC_{50} of naringenin in combination/ IC_{50} of naringenin alone)+(IC_{50} of ACV in combination/ IC_{50} of ACV alone)

Determination of regression coefficient

Regression coefficient between concentration and percent inhibition or incubation time and percent inhibition was determined using excel program as following example

Concentration (µg/ml)	%inhibition	Correlation coefficient
6.25	0.00	$R^2 = 0.98$
12.5	15.20	
25	33.10	
50	57.57	
100	100.00	

BIOGRAPHY

Miss Khanidtha Chitphet was born on May 15, 1985 in Suratthani, Thailand. She received her Bachelor Degree of Science in Pharmacy (1st Class Honours) in 2008 from the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.