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Antiviral Activities of *Hedera helix* containing Hederasaponin B and *Phyllanthus urinaria* containing Corilagin against Enterovirus Infections

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Antiviral Activities of *Hedera helix* containing Hederasaponin B and *Phyllanthus urinaria* containing Corilagin against Enterovirus Infections

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Abstract

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Enteroviruses (EV) are the positive sense (+) single stranded RNA viruses in the picornaviridae family causing several diseases to mammalian including humans. Usually, human enteroviruses (HEV) infections cause various symptoms including the hand-foot-mouth disease (HFMD), common cold, myocarditis, acute hemorrhagic conjunctivitis, encephalitis, poliomyelitis, and aseptic meningitis, etc. Especially, some EVs including enterovirus71 (EV71)

and coxackievirus16 (CVA16) cause HFMD with neurological complications with death and many cases reported in many countries including big outbreaks. However, there are still no specially approved antiviral drugs and vaccines highly effective against HEV. Additionally, because many EVs cause diseases in children, the antiviral agents against HEVs should have the lower level of side effects than others. Based on these backgrounds, several materials including compounds from plant having been tested that could play a role in effective inhibition of viruses and virus-specific functions with lower level of side effects, and may avoid emergence of resistant mutant types in developing effective antiviral agents.

In the efforts of finding HEV inhibitors from plant origin materials, we identified hederasaponin B and corilagin from *Hedera helix* and *Phyllanthus urinaria*, respectively showed antiviral activities against HEV including EV71 and CVA16. Especially in the current studies, hederasaponin B showed antiviral acrivities against EV71 especially at subtype C3 and C4a. C3 and C4a subtypes are major causative agent of HFMD in Asian countries including Korea. Addiotionally, corilagin showed antiviral activities against EV71 and CVA16. CVA16 is second major type causing HFMD followed by EV71 in many countries including Korea.

Among EVs, Coxackievirus B3 (CVB3) is one of the major type causing myocarditis and pancreatitis at humans. CVB3 also has well established mouse infection model of pancreatitis. To identify broad use of antiviral agents against EVs, hederasaponin B and *Phyllanthus urinaria* extract were treated to identify the antiviral activities against CVB3 in vivo. The animal experiments were approved by the Institutional Animal Care and Use committees (IACUC) of the Korea Center for Disease Control and Prevention (KCDC). 5 week old female Balb/c mice were infected by CVB3 after administration of *Hedera helix* and *Phyllanthus urinaria* extracts. Body weight check and histological analysis of pancreara were carried out.

Based on these results, the extracts of *Hedera helix* and *Phyllanthus urinaria* can be suggested to the novel therapeutic agents against EVs infections with broad-spectrum antiviral activities.

Keywords: Enterovirus, Hand foot and Mouth Disease, Hederasaponin B, *Hedera Helix*, Corilagin, *Phyllanthus urinaria* Antiviral activity.

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List of Abbreviations

ACRONYM	FULL NAME
EV	Enterovirus
HEV	Human Enterovirus
CVA	Coxackievirus A
CVB	Coxackievirus B
CPE	Cytopathic Effect
HFMD	Hand, Foot and Mouth Disease
DNA	Deoxy ribonucleic acid
RNA	Ribo nucleic acid
PI	Post Infection
CMC	Carboxymethyl Cellulose
SRB	Sulforhodamine B
TI	Therapeutic Index
IC	Inhibitory Concentration
EC	Effective Concentration
CC	Cytotoxicity Concentraion
HPLC	High Performance Liquid Chromatography
EtOH	Ethanol
MeOH	Methanol
IP	Intra Peritoneal
IV	Intra Venous

ACRONYM	FULL NAME (cont.)
VP	Viral Protein
TCID	Tissue Culture Infectious Dose
CCID	Cell Culture Infectious Dose
MEM	Minimal Essential Medium
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
PBS	Phosphate Buffered Saline
UTR	Untranslated region
IRES	Internal Ribosom Entery Site
IACUC	Institutional Animal Care and Use committees

I. Literature Review

1. HEVs virion

HEVs are positive sense (+) single stranded RNA viruses and belong to the picornaviridae family. In the Picornaviridae family, there are 12 RNA viruses genera including Aphthovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Parechovirus, Teschovirus, Tremovirus, Avihepatovirus, Senecavirus, and Sapelovirus. However, the last 5 genera have not been reported to be related to human infections yet. HEVs virion is spherical and non-enveloped. The size of HEVs virion is about 30 nm in diameter. HEV is composed of a protein shell surrounding the naked RNA genome. The capsid consists of a densely-packed icosahedral arrangement of 60 protomers, each consisting of 4 polypeptides, VP1, VP2, VP3 and VP4. VP4 is located on the internal side of the capsid (Song, 2012) (Fig. 1).

2. HEVs genome

HEVs genome is positive-sense (+), single-stranded RNA viruses which replicate entirely in the cytoplasm (Follett *et al*., 1975). Non-segmented genome is composed of a single linear RNA molecule having length of about 7000 to 8500 nucleotides. The 5′-end of the genome contains a covalently conjugated 22 amino acid peptide, termed virion protein genome linked (VPg) (Cameron *et al.,* 2010) (Fig. 2).

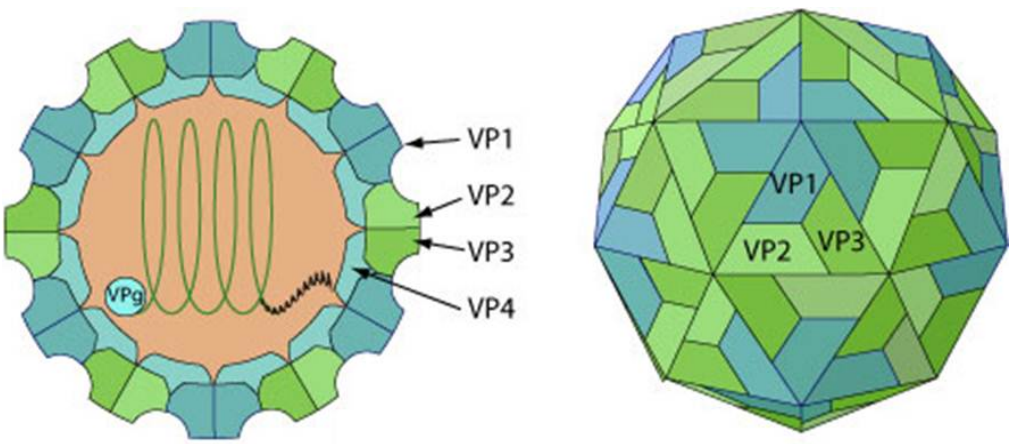

Fig. I. The brief structure of HEVs virion.

The schematic HEVs capsid shows the pseudoequivalent packing arrangement of VP1, VP2 and VP3. VP4 is on the interior of the capsid. The biologic promoter is not same as the icosahedral asymmetric subunit (Song, 2012).

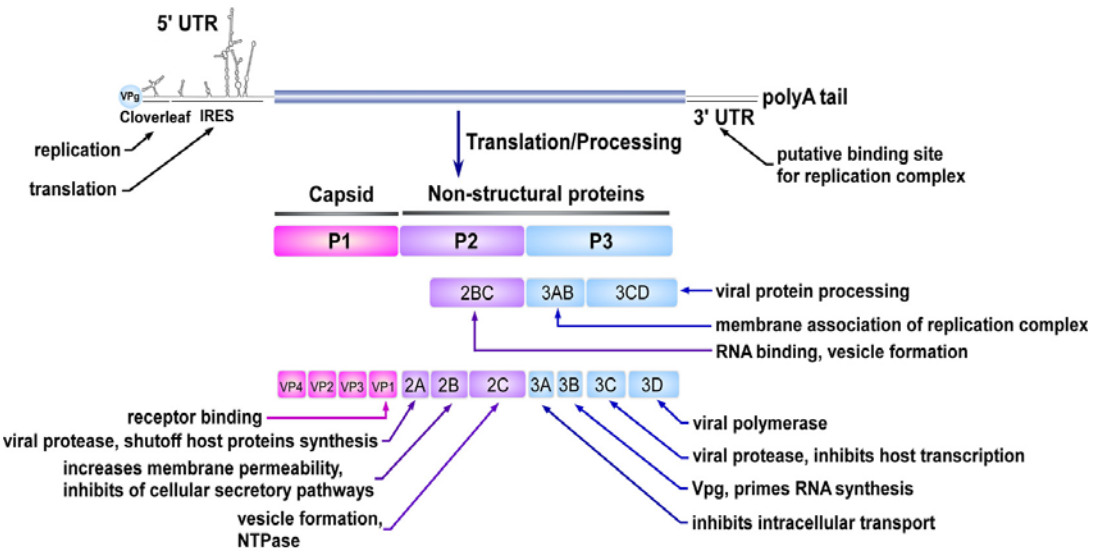

Fig. II. The schematic HEVs genome: the polyprotein products and their major functions.

A diagrammatic picture of the HEVs genome is above. The 11 mature polypeptides are shown with the three main cleavage intermediates (Song, 2012). The main biological functions are included for each polypeptide. UTR, untranslated region; IRES, internal ribosome entry site; VPg, viral protein genome-linked (Lin *et al*., 2009).

First, RNA is released from the protection of the viral capsid into the cytoplasm of the host cells. Right after that event, the translation initiation factors and ribosomes to the internal ribosomal entry site (IRES) of the 5′-

untranslated region (5′-UTR) are binded followed by the translation of virus proteins. Additionally, the RNA genome contains a single open reading frame (ORF) and a 3′-untranslated region (3′-UTR) at the 3′ terminus (Song, 2012). The translated product of the ORF is a large polyprotein, co- or post-translationally cleaved by translated viral proteases into P1, P2 and P3 regions. The P1 region is the part of the polyprotein which generates the four structural proteins (VP1, VP2, VP3 and VP4) (Huang *et al*., 2011; Nicklin *et al*., 1987). The first three viral proteins (VP1,VP2,VP3) reside at the outer surface of the viruses While the shorter VP4 is located completely at the inner surface of the capsids. The capsid proteins mediate the initiation of infection by binding to a receptor on the host membrane (Lin *et al*., 2009; He *et al*., 2000). Two viral proteases, 2A protease (2Apro) and 3C protease (3Cpro), are encoded by the non-structural protein coding region (Song, 2012). They are important for viral polyprotein processing (Strebel and Beck, 1986; Svitkin *et al*., 1979; Toyoda *et al*., 1986). 2Apro involves the dissociation of the P1 capsid polyprotein from the P2 and P3 non-structural polyproteins (Song, 2012). It is involved in the cleavage of the eukaryotic initiation factor 4G during an EV71 infection, (Kuo *et al*., 2002) which is important for host protein synthesis. 3Cpro is complicated in the proteolytic processing of the viral polyprotein and assists in the interaction of the 5′-UTR with the RNA-dependent RNA polymerase (3Dpol) for viral RNA replication (Lei *et al*., 2010). Viral protein 2B and its precursor 2BC have been suggested to be responsible for membranous alteration in infected cells (Aldabe *et al*., 1996; Van Kuppeveld *et al*., 1997; Doedens and Kirkegaard, 1995; Barco and Carrasco, 1995; Van Kuppeveld *et al*., 1996). Protein 3A, a membrane binding protein, plays a role in inhibiting cellular protein secretion and mediating presentation of membrane proteins during viral infection (Doedens and Kirkegaard, 1995; Doedens *et al*., 1997). According to the biochemical data, the

3AB protein plays role in a multifunctional protein. The hydrophobic domain in the 3A portion of the protein associates with membrane vesicles (Towner *et al*., 1996; Fujita *et al*., 2007). This interaction is believed to anchor the replication complex to the virus-induced vesicles. Recombinant 3AB interacts with HEVs 3D and 3CD in vitro (Hope *et al*., 1997). The membrane-associated 3AB protein binds directly to the polymerase precursor 3CD on the cloverleaf RNA of the HEVs, stimulating the protease activity of the 3CD, and may serve as an anchor for 3D polymerase in the RNA replication complexes (Xiang *et al*., 1995). Adding 3AB stimulated the activity of HEVs 3D polymerase in vitro (Plotch and Palant, 1995). Furthermore, 3AB has been demonstrated to function as a substrate for 3D polymerase in VPg uridylylation (Richards *et al*., 2006). The 3AB protein has been proposed to be delivered to the replication complexes for VPg uridylylation compared to 3B (the mature VPg) (Song, 2012). The HEVs 3B proteins (VPg) is small peptides, containing 21 to 23 amino acids, which are covalently linked with the 5' termini of picornavirus genome via a 5' tyrosyluridine bond in the conserved tyrosine residue in the VPg (Paul *et al*., 1998). The uridylylated VPg is utilized as a primer in both positive- and negative-strand RNA synthesis (Pettersson *et al*., 1978). The viral RNA-dependent RNA polymerase 3D is one of the major components of the viral RNA replication complex (Song, 2012). The purified poliovirus 3D polymerase from the complex exhibits elongation activity (Van Dyke and Flanegan, 1980).

3. The classification of HEV

HEVs comprise more than 80 immunologically-distinct serotypes known to be the infectious pathogen to humans (Song, 2012). The groupe is as follows:

polioviruses (PVs; 3 serotypes), echoviruses (ECV; 34 serotypes), coxsackieviruses A (CVAs; 24 serotypes), coxsackieviruses B (CVBs; 6 serotypes) and enterovirus types 68-116 (EVs ; 49 serotypes), mainly on the basis of their pathogenicity in humans and laboratory animals (Wikipedia). Altogether these HEV types fall into four genetically distinct species, HEV-A to HEV-D, within the Enterovirus genus (Hyypiä *et al*., 1997; Wolthers *et al*., 2008). The species HEV-A consists of 18 serotypes: CVA 2 to 8, 10, 12, 14 and 16, EV 71, 76, 89, 90, 91,92 and 114. The HEV-B serotypes consists of 60 serotypes: CVB 1 to 6, CVA 9, ECV 1 to 7, 9, 11 to 21, 24 to 27, 29 to 34, EV 69, 73 to75, 77 to 88, 93, 97, 98, 100, 101, 106, 107 and 110. The species HEV-C consists of 20 serotypes: PV 1 to 3, CVA 1, 11, 13, 17, 19 to 22, 24, EV 95, 96, 99, 102, 104, 105, 109, 113 and 116. The species HEV-D consists of four serotypes, EV 68, 70, 94 and EV 111 (Oberste *et al*., 1999).

3.1. Coxsackievirus B 1

Coxsackievirus B 1 (CVB 1) has been connected with outbreaks of diseases including pleurodynia and aseptic meningitis (Miwa and Sawatari, 1994; Joo *et al*., 2005; Ikeda *et al*., 1993; Chiou *et al*., 1998). Clinical symptoms of CVB1 are similar to other group B coxsackieviruses including aseptic meningitis, pleurodynia, myocarditis, meningoencephalitis and hand-foot and mouth disease. CVB 1 also cause systemic neonatal illness sometimes presenting as fulminant hepatitis with coagulopathy, a syndrome usually associated with echovirus 11 rather than with encephalomyocarditis syndrome typical for CVB 2-5 (Chiou *et al*., 1998; Modlin, 1986). CVB 1 has been reported with known serotype during 1970 and 2005 approximately 2.3% (Song, 2012). CVB 1 has an epidemic pattern in circulation, with increased activity occurring with irregular intervals and usually lasting several years (MMWR, CDC, 2010). The CVB 1 circulation

was started to increase since the 1990s. Two thirds of CVB 1 reports during the study period were from infants aged <1 year, and respiratory specimens were the most common source of its detection (Song, 2012). However, no fatal outcomes were reported in 104 known CVB 1 detections. Similar to CVB 4 and 5, the summer to autumn seasonal pattern in CVB 1 detections was more important than for the majority of other serotypes. Reports of CVB 1 had the least prominent male predominance of all serotypes (52.5%) (Song, 2012).

3.2. Coxsackievirus B 2

Clinical illnesses associated with coxsackievirus B 2 (CVB 2) include aseptic meningitis, myocarditis, and neonatal systemic illness (Thivierge and Delage, 1982; Smith, 1970; Alexander *et al*., 1993; Barson and Reiner, 1986). Outbreaks in settings such as football teams and summer camps have been reported (Alexander *et al*., 1993; Schiff, 1979). However, widespread community outbreaks are untypical. CVB 2 has been occupied about 5.2% in reported serotype cases during 1970 and 2005. The virus has an endemic pattern of circulation with year-to-year variability and has consistently appeared among the top 15 serotypes (Song, 2012). Two episodes showing unusually high activity of CVB 2 have been reported in 1976 and 1994. They became the most common reports, accounting for >15% of all reported enteroviruses. The percentage of children aged <1 year among reports of CVB 2 detections exceeded 60%, and CSF was the most common source of surveillance.

3.3. Coxsackievirus B 3

Coxsackievirus B 3 (CVB 3) has been reported as the common pathogen causing various diseases including myopericarditis, aseptic meningitis, neonatal systemic illness, meningoencephalitis in immunodeficient persons, herpangina,

and rash illnesses (Bendig *et al*., 2003; Fujioka *et al*., 2000; Spanakis *et al*., 2005). Outbreaks of neonatal infections and herpangina and a cluster of myocarditis cases in the context of community wide CVB 3 outbreak have been reported (Hosoya *et al*., 1997; Rossouw *et al*., 1991; Nakayama *et al*., 1989). CVB 3 accounted for about 3.9% of reports with known serotype. CVB 3 shows an epidemic circulatory pattern. The increases in its activity are of variable duration and extent and occur after variable periods of quiescence (Song, 2012). CVB 3 has coherently occurred among the 15 most common reported enteroviruses. However, CVB3 has never been the most commonly reported virus, with a highest ranking of second in 1980 and 1994. The proportion of children aged <1 year among reports of CVB 3 detections exceeded 60%, and respiratory specimens were the most common source of detection (Song, 2012). Among reported CVB3 cased, fatality was about 5.4%.

3.4. Coxsackievirus B 4

Outbreaks of coxsackievirus B4 (CVB 4) are very rare. Most common clinical syndromes associated with CVB 4 include aseptic meningitis, encephalitis, myopericarditis, neonatal infections, febrile rash illnesses, and respiratory manifestations (Cree *et al*., 2003; Niklasson *et al*., 1985; Porres *et al*., 1985). CVB 4 accounted for 4.2% of reports with known serotypes and has consistently appeared among the 15 most common enteroviruses, but it has never been the predominant serotype (Song, 2012). CVB 4 has an endemic circulatory pattern. About 60% of all CVB 4 detections came from young infants, and CSF was the most common source. Similar to coxsackievirus B5, the summer to automn seasonality in CVB 4 detections was more prominent than for the majority of other serotypes. CVB 4 had one of the highest proportions of reports with fatal outcome (9.8%), with a significantly higher risk of death when

compared with fatal outcomes among persons infected with any other enterovirus serotypes (Song, 2012).

3.5. Coxsackievirus B5

The major clinical presentations of coxsackievirus B5 (CVB 5) infections resemble to those observed for other group B coxsackieviruses including aseptic meningitis, meningoencephalitis, myopericarditis and neonatal systemic illness (encephalomyocarditis syndrome), and acute flaccid paralysis (AFP) also at polioviruses. Hand-foot and mouth disease (HFMD) and herpangina also have been reported, and a potential cause of type 1 diabetes has been introduced. Outbreaks, mostly of aseptic meningitis, are common in epidemic years (Stambos *et al*., 2005; Helin *et al*., 1968; Smith, 1970; Lindenbaum *et al*., 1975; Modlin, 1996; CDC, 1984; Kopecka *et al*., 1995; Graves *et al*., 1997). CVB 5 has a distinct epidemic circulatory pattern. The regular sharp increases every 3 to 6 years, which usually last for 1 year. The extent of the increase in activity varied, and smaller peaks sometimes were observed between major ones. CVB 5 consistently appeared among the most commonly reported enteroviruses and was the most commonly identified enterovirus seven times (1972, 1973, 1983, 1989, 1996, 2000, and 2005). About 50% of all CVB 5 detections came from young chindren and infants, and CSF was the most common source. The summer to autumn seasonality in CVB 5 detections was more prominent than for most other serotypes.

3.6. Coxsackievirus B 6

Coxsackievirus B 6 (CVB 6) was fist isolated and charcterised by Hammon *et al*.(1960). They have also recorded the association of this virus in two cases of aseptic meningitis. Clinical diagnoses of CVB 6 were encephalitis, meningitis,

rheumatic carditis, myocarditis, pneumonitis and upper respiratory infection (URI). CVB 6 has not so far been implicated in large outbreaks of illnesses (Madhavan *et al*., 1976).

3.7. Coxsackievirus A 16

Coxsackievirus A16 (CVA 16) is one of the most frequently appeared HEVs with various symptoms. The most common illness associated with CVA16 infection is hand-foot and mouth disease. More serious manifestations are rare, but cases of aseptic meningitis, fatal myocarditis, rhabdomyolysis with renal failure, and neonatal febrile illness have been described, and outbreaks have been reported (Chang *et al*., 1999; Portolani *et al*., 2004; Wang *et al*., 2004; Cooper *et al*., 1989; Ferson and Bell, 1991). During 1970 and 2005, CVA 16 accounted for 1.2% of reports with known serotype. The virus rarely appeared among the 15 most common enteroviruses and never ranked higher than sixth (Song, 2012). CVA 16 also has an endemic circulatory pattern. The reported overall levels of CVA 16 activity were higher during the 1970s and 1980s and have declined since 1990. The age group most commonly associated with CVA 16 detections was children aged 1 to 4 years, and specimens coded as "other" were the most frequent source, possibly referring to scrapings from hand, foot, and mouth disease lesions. The summer to autumn seasonality was present. Reported fatality was about 1.1% in known CVA 16 infections.

3.8. Enterovirus 71

Enterovirus 71(EV 71) is one of the most important HEVs infection recently. Many outbreak cases have been reported with high mortality especially at Asian countries. EV71 is main cause of HFMD with serious neurologic

compliactions (e.g., aseptic meningitis, polio-like paralysis, and bulbar encephalitis). Outbreaks of paralysis were reported in Europe during the 1960s and 1970s (Melnick, 1984). During the late 1990s and early 2000s, outbreaks of fatal bulbar encephalitis among young children occurred in the southeast Asian countries, usually in the context of EV 71-associated outbreaks of hand, foot, and mouth disease (Chan *et al*., 2000; Huang *et al*., 1999; Chang *et al*., 1999; Brown *et al*., 1999). Localized outbreaks of EV 71-associated illnesses have been documented in the United States since 1973 (Melnick, 1984) However, no detections were reported to NESS until 1983. During 1983 and 2005, a total of 270 cases of EV 71 were reported (Song, 2012). The overall temporal pattern of EV 71 reports was consistent with endemic circulation. An increasing trend in overall level of reported activity was documented, especially after the mid-1990s, but large-scale nationwide activity, similar to the ones in southeast Asia in the late 1990s and early 2000s, has not been observed. Young children before school age were the most common source of EV 71 infection. Urgently, it is necessary to develop antiviral agent and vaccine against EV71 because it can cause death in young children with neurological complications explained above.

4. Antiviral agents from plants

It is widely recognized that the utility of most antiviral agents is limited by the inherent toxicity of the compounds and the appearance of drug resistant virus following their continued use (Song, 2012). Traditionally, plants have long been used as folk remedies. Moreover, many plants have been collected by ethnobotanists and examined to identify possible sources for antiviral agents. Viruses respond differently to plant extracts and it has been suggested that natural products are preferable to synthetic compounds (Vanden Berghe *et al*., 1986; Vlietinck *et al*., 1991). A wide variety of active phytochemicals, including the flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, furyl compounds, alkaloids, polyines, thiophenes, proteins and peptides have been identified (Song, 2012).

Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and people who suffer from viral infection (Hudson, 1990; Venkateswaran *et al*., 1987; Thyagarajan *et al*., 1988; Thyagarajan *et al*., 1990). Research interests for antiviral agent development was started after the Second World War in Europe and in 1952 the Boots drug company at Nottingham, England, examined the action of 288 plants against influenza A virus in embryonated eggs. They found that 12 of them suppressed virus amplification (Chantrill *et al*., 1952). During the last 25 years, there have been numerous broad-based screening programmes initiated in different parts of the globe to evaluate the antiviral activity of medicinal plants for in vitro and in vivo assays. Canadian researchers in the 1970s reported antiviral activities against herpes simplex virus (HSV), poliovirus type 1, CVB 5 and echovirus 7 from grape, apple, strawberry and other fruit

juices (Konowalchuk and Speirs, 1976a; Konowalchuk and Speirs, 1976b; Konowalchuk and Speirs, 1978a; Konowalchuk and Speirs, 1978b).

One hundred British Columbian medicinal plants were screened for antiviral activity against seven viruses (McCutcheon *et al*., 1995). The extracts of Rosa nutkana and Amelanchier alnifolia showed high activities against an enteric corona virus. A root extract of Potentilla arguta and a branch tip extract of Sambucus racemosa entirely inhibited respiratory syncytial virus (RSV). Also, the extract of Ipomopsis aggregata demonstrated good activity against parainfluenza virus type 3. A Lomatium dissectum root extract completely inhibited the cytopathic effects of rotavirus. In addition to these, extracts prepared from Cardamine angulata, Conocephalum conicum, Lysichiton americanum, Polypodium glycyrrhiza and Verbascum thapsus exhibited antiviral activity against herpes virus type 1 (Song, 2012).

The human rotavirus (HRV), RSV and influenza A virus were susceptible to a liquid extract from Eleutherococcus senticosus roots. In contrast, the DNA viruses, adenovirus and HSV type 1 virus (HSV-1) were not inhibited by the same plant extract (Glatthaar-Saalmuller *et al*., 2001). In that study, it was concluded that the antiviral activity of Eleutherococcus senticosus extract is viral RNA dependant.

Related studies also showed that influenza RNA was inhibited by a water-soluble extract of Sanicula europaea (L.) (Turan *et al.,* 1996). In the follwed study, it was shown that an acidic fraction obtained from the crude extract of Sanicula europaea was the most active fraction to inhibit human parainfluenza virus type2 replication at the noncytotoxic concentrations (Karagoz *et al*., 1999).

Medicinal plants have a variety of chemical constituents having the ability to inhibit the replication cycle of various DNA or RNA viruses. Compounds from natural sources can be the possible sources to control viral infection. In this

reasons, many research groups in Asia, Far East, Europe, and America have given particular attention to find and develop antiviral agents from their native traditional plant medicines. Some typical examples of such medicines and their antiviral activities are introduced in Table I.

Table I. Summary of the mechanism of the most active antiviral compounds from plants (Jassim and Naji, 2003).

Class of compound	Mechanism virus target	Example of plant source
Furyl compounds: Furocoumarins, furanochromones	DNA and RNA genomes. Interaction require long–wave ultraviolet (UVA, 300–400nm)	Rutaceae and Apiaceae (Umbelliferae)
Alkaloids constitute: *β*-carbolines, fuanoquinolines, camptothecin, atropine, caffeine, indolizidines, swainsonine, castanospermine, colchicines, vinblastine	DNA and other Polynucleotides and virions proteins. In some interactions are enhanced by UVA	Rutaceae, *Camptotheca acuminate*, *Atropa belladona*, *Swainsona canescens, Astragalus lentiginosus, Castanospermum australe, Aglaia roxburghiana*
Polyacetylenes (polyines)	Membrane interaction. Phototoxic activity frequently requires UVA	Asteraceae, Apiaceae, *Campanulaceae* *Panax ginseng*, *Bidens* sp., *Chrysanthemum sibiricum*
Polysaccharides	Blocking virus binding	*Achyrocline flaccida, Bostrychia montagnei, Cedrela tubiflora, Prunella vulgaris, Sclerotium glucanicum, Stevia rebaudiana, Rhizophora mucronata*
Thiophenes	Membrane interaction.	*Aspilia, Chenactis douglasii,*

Table I. (Continued)

Class of compound	Mechanism virus target	Example of plant source
Thiophenes	Phototoxic activity frequently requires UVA.	*Dyssodia anthemidifolia, Eclipta alba, Eriophyllum lanatum*
Flavonoids: amentoflavone, theaflavin, iridoids, agathisflavone, phenylpropanoid glycosides, robustaflavone, morin, rhusflavanone, baicalin succedaneflavanone, chrysosplenol C, coumarins, galangin (3,5,7trihydroxyflavone),	Blocking RNA synthesis. Exhibited HIV-inhibitory activity	*Maclura cochinchinensis, Markhamia lutea, Monotes africanus, Pterocaulon sphacelatum, Rhus succedanea, Scutellaria baicalensis, Selaginella sinensis, Sophora moorcroftiana, Sophora tomentosa, Tephrosi* sp.
Terpenoids: sesquiterpene, triterpenoids (moronic acid, ursolic acid, maslinic acid and saponin)	Membrane-mediated mechanisms. Inhibition of viral DNA synthesis	*Acokanthera* sp., *Anagallis arvensis*, *Cannabis sativa, Geum japonicum, Glycyrrhiza glabra, Glycyrrhiza radix, Glyptopetalum sclerocarpum, Gymnema sylvestre, Maesa lanceolata, Olea europa, Quillaja saponaria, Rhus javanica, Strophanthus gratus*
Lignans Podophyllotoxin and related lignans (cyclolignanolides), such as the peltatins	Blocking virus replication	*Amanoa aff. Oblongifolia, Juniperus communis, Justicia procumbens, Podophyllum peltatum*
Dibenzocyclooctadiene lignans such as schizarin B and taiwanschirin D	Blocking HBV replication Blocking influenza virus type A replication	*Kadsura matsudai* *Rhinacanthus nasutus*
Rhinacanthin E and rhinacanthin F	Inhibition of viral RNA and DNA replication	*Aloe barbadensis, Aster scaber, Cassia angustifolia, Dianella longifolia, Euodia roxburghiana, Geum japonicum,*
Miscellaneous phenolic compounds:		

anthraquinone chrysophanic acid, caffic acid, eugeniin, hypericin, tannins (condensed polymers), proanthocyanidins, salicylates and quinines (naphthoquinones, naphthoquinones and anthraquinones in particular aloe emodin)	*Hamamelis virginiana,* *Hypericum* sp.*,* *Melissa officinalis,* *Phyllanthus myrtifolius,* *Phyllanthus urinaria,* *Punica granatum,* *Rhamnus frangula,* *Rhamnus purshianus,* *Rheum officinale,* *Rhinacanthus nasutus,* *Shepherdia argentea,* *Syzgium aromatica,* St. John's wort

Table I. (Continued)

Class of compound	Mechanism virus target	Example of plant source
Proteins and peptides	Interaction with ribosome	*Clerodendrum Inerme,*
1. Single chain ribosome-inactivating proteins	function in the infected cell and inhibited viral protein synthesis	*Dianthus caryophyllus,* *Gelonium multiflorum,* *Momordica charantia,*
Pokeweed antiviral proteins (PAP) (MRK29, MAP30 and GAP31)	Inactivate infective HIV and HIV-infected cells	*Phytolacca Americana,* *Saponaria officinalis,* *Trichosanthes kirilowii,*
Panaxagin	Inhibit the HIV reverse transcriptase	*Triticum aestivum* *Phytolacca Americana,*
Alpha- and beta-antifungal proteins	Inhibit the HIV reverse transcriptase	*Momordica charantia,* *Gelonium multiflorum* *Panax ginseng* *Vigna unguiculata*
2. Dimeric cytotoxins	Interaction with ribosome function in the infected cell and inhibit viral protein synthesis	*Ricinus communis,* *Abrus precatorius,* *Adenia digitata*
3. Lectins	Viral membrane interactions	*Canavalia ensiformis,* *Lens culinaris,* *Phaseolus vulgaris,* *Triticum vulgaris*
4. Antiviral factor	Mechanism of action is not known	*Nicotiana glutinosa*
5. Meliacine	Affect virus replicative cycle	*Melia azedarach*

5. Clinical use of antiviral agents

The current armamentarium for the chemotherapy of viral infections consists of 37 licensed antiviral drugs (Table II). For the control of human immunodeficiency virus (HIV) infections, 19 compounds have been formally approved: the nucleoside reverse transcriptase inhibitors (NRTIs) zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir and emtricitabine; the nucleotide reverse transcriptase inhibitor (NtRTI) tenofovir disoproxil fumarate; the non nucleoside reverse transcriptase inhibitors (Song, 2012).

(NNRTIs) nevirapine, delavirdine and efavirenz; the protease inhibitors saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir (combined with ritonavir at a 4/1 ratio) and atazanavir; and the viral entry inhibitor enfuvirtide. To control the chronic hepatitis B virus (HBV) infections, lamivudine as well as adefovir dipivoxil have been approved. Among the anti-herpesvirus agents, acyclovir, valaciclovir, penciclovir (when applied topically), famciclovir, idoxuridine and trifluridine (both applied topically) as well as brivudin are used in the treatment of herpes simplex virus (HSV) and/or varicella-zoster virus (VZV infections; and ganciclovir, valganciclovir, foscarnet, cidofovir and fomivirsen (the latter upon intravitreal injection) have proven useful in the treatment of cytomegalovirus (CMV) infections in immunosuppressed patients (i.e. AIDS patients with CMV retinitis). Following amantadine and rimantadine, the neuraminidase inhibitors zanamivir and oseltamivir have recently become available for the therapy (and prophylaxis) of influenza virus infections. Ribavirin has been used in the treatment of respiratory syncytial virus (RSV) infections as aerosol type, and the combination of ribavirin with (pegylated)

interferon-alpha has received increased acceptance for the treatment of hepatitis C virus (HCV) infections (De Clercq, 2004).

Table II. Antiviral drugs of current clinical use (Song, 2012).

Targets		Chemical Name	Activity spectrum	Mechanism of action
Anti-HIV	NRTIs	Zidovudine (AZT)	Type 1, 2	RT
		Dianosine (ddT)	Type 1, 2	RT
		Zalcitabinee (ddC)	Type 1, 2	RT
		Stuvudine (d4T)	Type 1, 2	RT
		Lamivudine (3TC)	Type 1, 2 HBV	RT
		Abacavir (ABC)	Type 1, 2	RT
		Emtricitabine ((-)-FTC)	HIV, HBV	Similar to that of 3TC
	NtRTIs	Tenofovir disoproxil (bis(POC)PMPA)	Type 1, 2 Other retroviruses HBV	RT
	NNRTIs	Nevirapine	Type 1	Allosteric "pocket" of RT
		Delavirdine	Type 1	RT
		Efavirenz	Type 1	Similar to that of nevirapine
	PIs	Saquinavir	Type 1, 2	Peptidomimetic inhibitor
		Ritonavir	Type 1, 2	As for saquinavir
		Indinavir	Type 1, 2	As for saquinavir
		Nelfinavir	Type 1, 2	As for saquinavir
		Amprenavir	Type 1, 2	As for saquinavir
		Lopinavir	Type 1, 2	As for saquinavir
		Atazanavir	Type 1, 2	As for saquinavir

Table II. (Continued)

Targets		Chemical Name	Activity spectrum	Mechanism of action
Anti-HIV	Viral Entry inhibitors	Enfuvirtide	Type 1	Virus-cell fusion inhibitor
Anti-HBV		Lamivudine (3TC)	Type 1, 2 HBV	RT
		Adefovir Dipovoxil	HBV, HIV ther retroviruses HSV, CMV	RT
Anti-HSV	HSV & VZV inhibitors	Emtricitabine	HIV, HBV	Similar to that of lamivudine
		Acyclovir (ACG)	Type 1, 2 VZV	Viral DNA polymerase
		Valaciclovir (VACV)	As for acyclovir	Similar to that of acyclovir
		Penciclovir (PCV)	Type 1, 2 VZV	Similar to that of acyclovir
		Famciclovir (FCV)	Type 1, 2 VZV	Similar to that of acyclovir
		Idoxuridine (IDU, IUdR)	Type 1, 2 VZV	Incorporated into DNA
		Trifluridine (TFT)	Type 1, 2 VZV	Inhibits conversion of dUMP to dTMP
		Brivudin (BVDU)	HSV1, VZV Other HSV	Viral DNA polymerase
Anti-IFA		Amantadine	IFA-A	Blocks M2 ion channel
		Rimantadine	IFA-A	As for mantadine
		Zanamivir	IFA-A,B	N-acetylneura-midase analogue
		Oseltivir	As for zanamivir	As for zanamivir
		Ribavirin	Various DNA & RNA viruses	IMP dehydrogenase

[a]NRTIs; Nucleoside reverse transcriptase inhibitors, NtRTIs; Nucleotide reverse transcriptase inhibitors, NNRTIs; Non-nucleoside reverse transcriptase inhibitors, Pis; Protease inhibitors, HIV; Human immunodeficiency virus, HBV; Hepatitis B virus, HSV; herpes simplex virus, VZV; varicella-zoster virus, CMV; cytomegalovirus, IFA; influenza virus, RSV; respiratory syncytial virus, HCV; hepatitis C virus, RT; reverse transcriptase.

II. General Introduction

Enterovirus71 (EV71) is a positive-stranded RNA virus that belongs to the enterovirus (EV) genus of the Picornaviridae family(McMinn, 2002). EV71 is classified in the genus enterovirus along with other polioviruses, coxsackievirus A (CVA) and coxsackievirus B (CVB), and echoviruses(E)(Hyypia *et al.*, 1997; Laine *et al.*, 2005; Poyry *et al.*, 1996). At species level, EV71 together with coxsackievirus (CV) A2–A8, A10, A12, A14 and A16 are members of HEV-A. The EV71 genome encodes a long polyprotein with a single open reading frame and includes three genomic regions designated P1, P2 and P3. The P1 region encodes four structural capsid proteins (VP4, VP2, VP3, and VP1), while the P2 and P3 regions encode seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Chan *et al.*, 2010; Huang *et al.*, 2010). EV71 genotypes are genetically classified into genogroupsA, B and C on the basis of the VP1 sequence analysis (Brown *et al.*, 1999).

EV71 is a causative agent of hand, foot and mouth disease (HFMD) and herpangina;and it can also cause severe neurological diseases, such as brainstem encephalitis and poliomyelitis-like paralysis(Chumakov *et al.*, 1979; McMinn, 2002; Wang *et al.*, 2003)**.** HFMD, mainly caused by EV71 and CVA16, commonly occurs in young children, particularly those less than 5 years of age

(Zhang *et al.*, 2011). CVA16-associated HFMD is milder than that caused by EV71, and has a much lower incidence of severe complications, including death(Chang *et al.*, 1999). In recent years, numerous large outbreaks of EV71-associated HFMD with high morbidity and mortality have occurred in eastern and southeastern Asian countries and regions. The big HFMD outbreaks with fatal neurological complications that have occurred since 2007 are mainly due to the subgenotype C4a of EV71 (Zhang *et al.*, 2011). Also, the subgenotype C3 was detected in Korea in 2000(Cardosa *et al.*, 2003). However, until now, neither a vaccine nor a therapeutic agent/method is available.

In the current study, novel antiviral activity of 30% EtOH extract of *Hedera helix* against EV71 C3 and EV71 C4a was reported, and a bioassay-guided isolation and identification of an active compound from *Hedera helix* against EV71 C3 and EV71 C4a were conducted. The antiviral activity of hederasaponin B and the extract and fractionates of *Hedera helix* against EV71 C3 and EV71 C4a is promising and urgently need to be evaluated in vivo for its potential capacity as the therapeutics of HFMD, since the 30% EtOH extract of *Hedera helix* is very safe medicine currently used for the treatment of bronchitis in children.

Corilagin is a polyphenol and a member of hydrolysable ellagitannins (Zhao L *et al*, 2008, Duan W *et al*, 2005). Corilagin was first isolated in 1951 from

Caesalpinia coriaria, and named after the original plant. There are several reports showing that corilagin, also known as β-1-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-D-glucose, could be isolated from Phyllanthus species, including Phyllanthus urinaria, Phyllanthus emblica, and Phyllanthus amarus (also known as Phyllanthus niruri) (Duan W. *et al*., 2005, JiKai *et al*., 2002, Patel JR *et al*., 2011). Corilagin can also be found in Terminalia catappa L., Nephelium lappaceum L., Alchornea glandulosa, Excoecaria agallocha L., and in the leaves of Punica granatum (Kinoshita *et al*., 2007, Li Y *et al*., 2012). Corilagin is also present in Terminalia chebula Retz with chebulagic acid and punicalagin (Park JH *et al*., 2011). Interestingly, chebulagic acid and punicalagin, which are structurally similar to corilagin and contain gallic acid and hexahydroxydiphenoyl (HHDP) ester moieties, have been showed to exhibit broad-spectrum antiviral activity against HSV-1, HCMV, HCV, and the measles virus (Satomi *et al*., 1993, Lin LT *et al*., 2011, Lin LT *et al*., 2013, Yang Y *et al*., 2012). Furthermore, recent studies have shown that chebulagic acid and geraniin exhibit antiviral activity in vitro and in vivo against human EV71 (Yang Y *et al*., 2012, Yang Y *et al*., 2013). However, although corilagin has been shown to exhibit anti-inflammatory effects in HSV-1-infected microglia (Guo *et al*., 2010), antiviral activity has not been reported against EV71 and CA16. Several reports suggest that *P. amarus* containing tannins including corilagin and geraniin

exhibit a high degree of antiviral activity against HIV infection (Notka *et al*., 2004). Previous reports have shown that corilagin also possesses antioxidant, hepatoprotective, thrombolytic, antiatherogenic, antihypertention, anticancer, and antihyperalgesic activities (Zhao *et al*., 2008, Duan W *et al*., 2005, Moreira *et al*., 2013, Kinoshita *et al*., 2007, Satomi *et al*., 1993, Thitilertdecha *et al*., 2010, Hau DK *et al*., 2010).

P. urinaria is a herb species of the Phyllanthaceae family, together with *P. amarus* (Yuandani *et al*., 2013). There are only a few reports that demonstrate the medical applications of Asian-originating *P. urinaria* compare to those of *P. amarus*, which commonly found in the hotter coastal area of India and widely spread throughout tropical and subtropical countries around the world. *P. amarus* is one of promising medical plants that have been widely evaluated in clinical trials based on its preclinical potential for the treatment of HIV, jaundice, hypertension, and diabetes (Peter JR *et al*., 2011). However, the antiviral activities of *P. urinaria* and *P. amarus* have not been tested against Enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16). Therefore, in this study we investigate the antiviral activity of *P. urinaria* and its major component corilagin against EV71 and CVA16, two major causative infectious viruses associated with hand, foot, and mouth disease (HFMD) in infants and young children (Mao Q *et al*., 2013, Tan CW *et al*., 2012).

Finally, we used replicon system of EVs to identity the antiviral mechanism of hederasaponin B and corilagin from *Hedera helix* and *Phyllanthus urinaria,* respectively. Rupintrivir was used as the positive control of antiviral agent against EVs. In luciperase assay, hederasaponin B, corilagin and rupintrivir were assessed at the concentration of 50,10,2 and 0.4μg/mL after transfection to vero cells.

III. Main text

Chapter 1

Antiviral activity of *Hedera helix* containing Hederasaponin B against Enterovirus 71 subgenotypes C3 and C4a

Abstract

Enterovirus 71 (EV71) is the predominant cause of hand, foot and mouth disease (HFMD). The antiviral activity of hederasaponin B from *Hedera helix* against EV71 subgenotypes C3 and C4a was evaluated in vero cells. In the current study, the antiviral activity of hederasaponin B against EV71 C3 and C4a was determined by cytopathic effect (CPE) reduction method and western blot assay. Our results demonstrated that hederasaponin B and 30% ethanol extract of *Hedera helix* containing hederasaponin B showed significant antiviral activity against EV71 subgenotypes C3 and C4a byreducing the formation of a visible CPE. Hederasaponin B also inhibited the viral VP2 protein expression,

suggesting the inhibition of viral capsid protein synthesis.These results suggest that hederasaponin B and *Hedera helix* extract containing hederasaponinB can be novel drug candidates with broad-spectrum antiviral activity against various subgenotypes of EV71

Key words : Enterovirus 71, Antiviral activity, Hederasaponin B, *Hedera helix*, Hand foot and mouth disease

1.1. Introduction

Enterovirus71 (EV71) is a positive-stranded RNA virus that belongs to the enterovirus (EV) genus of the Picornaviridae family(McMinn, 2002). EV71 is classified in the genus enterovirus along with other polioviruses, coxsackievirus A (CVA) and coxsackievirus B (CVB), and echoviruses(E)(Hyypia *et al.*, 1997; Laine *et al.*, 2005; Poyry *et al.*, 1996). At species level, EV71 together with coxsackievirus (CV) A2–A8, A10, A12, A14 and A16 are members of HEV-A. The EV71 genome encodes a long polyprotein with a single open reading frame and includes three genomic regions designated P1, P2 and P3. The P1 region encodes four structural capsid proteins (VP4, VP2, VP3, and VP1), while the P2 and P3 regions encode seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Chan *et al.*, 2010; Huang *et al.*, 2010). EV71 genotypes are genetically classified into genogroupsA, B and C on the basis of the VP1 sequence analyses (Brown *et al.*, 1999).

EV71 is a causative agent of hand, foot and mouth disease (HFMD) and herpangina;and it can also cause severe neurological diseases, such as brainstem encephalitis and poliomyelitis-like paralysis(Chumakov *et al.*, 1979; McMinn, 2002; Wang *et al.*, 2003)**.** HFMD, primarily caused by EV71 and CVA16, commonly occurs in young children, particularly those less than 5 years of age

(Zhang *et al.*, 2011). CVA16-associated HFMD is milder than that caused by EV71, and has a much lower incidence of severe complications, including death (Chang *et al.*, 1999). In recent years, numerous large outbreaks of EV71-associated HFMD with high morbidity and mortality have occurred in eastern and southeastern Asian countries and regions. The large HFMD outbreaks with fatal neurological complications that have occurred since 2007 are mainly due to the subgenotype C4a of EV71 (Zhang *et al.*, 2011). Also, the subgenotype C3 was detected in Korea in 2000 (Cardosa *et al.*, 2003). However, until currently, neither a vaccine nor a therapeutic treatment is available.

Hedera helix (English ivy, Common ivy) is an evergreen dioecious woody liana, one of the 15 species of the genus *Hedera*,Araliaceaefamily.The dry extract of *Hedera helix* is currently known to act as an anti-inflammatory(Gepdiremen *et al.*, 2005; Suleyman *et al.*, 2003), anti-bacterial,mucolyticand anti-spasmodic agent(Sieben *et al.*, 2009; Trute *et al.*, 1997). Also, *Hedera helix* extract has been claimed to exhibit in vitro bronchodilatatory effect on cell cultures (Sieben *et al.*, 2009; Trute *et al.*, 1997), and the pharmaceutical manufacturers declare the beneficial effect of ivy-based remedies in the treatment of cough symptoms during the course of acute and chronic bronchitis.However, to date, no detailed study has been carried out to assess the antiviral activity of *Hedera helix* against EV71.

In the current study, a novel antiviral activity was identified that 30% EtOH extract of *Hedera helix* has effect against EV71 C3 and EV71 C4a, and a bioassay-guided isolation and identification of an active compound from *Hedera helix* against EV71 C3 and EV71 C4a were conducted. The antiviral activity of hederasaponin B and the extract and fractionates of *Hedera helix* against EV71 C3 and EV71 C4a is promising and urgently need to be evaluated in vivo for its potential capacity as the therapeutics of HFMD, since the 30% EtOH extract of *Hedera helix* is very safe medicine currently used for the treatment of bronchitis in children.

1.2. Materials and Methods

Viruses and cell lines

EV71 C3 and EV71 C4a were obtained from the division of vaccine research in Korea Centers for Disease Control and Prevention (KCDC), and propagated in African green monkey kidney (vero) cells at 37 °C. Vero cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic solution. Antibiotic-antimycotic

solution, trypsin-EDTA, FBS, and MEM were supplied by Gibco BRL (Invitrogen Life Technologies, Karlsruhe, Germany). The tissue culture plates were purchased from Falcon (BD Biosciences, San Jose, CA, USA). Sulforhodamine B (SRB) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade.

Fractionation and isolation

For preparation of materials, 30% EtOH extract of *Hedera helix* was obtained from the Sampoong Corporation (Korea) in May 2011. 30% EtOH extract (500g) was suspended in water and then partitioned with EtOAc and n-BuOH, successively. Each soluble fraction was evaporated in vacuo to yield the residues of EtOAc (23 g), and n-BuOH (150 g) extracts, respectively. n-BuOH soluble fraction (100 g) was column chromatographed on a Diaion HP-20 (500 g, 10 x 50 cm) using stepwise-gradient with MeOH : H_2O (0:100, 20:80, 40:60, 60:40, 80:20, 100:0; each 1000 mL) to afford 6 fractions. Each extract and fraction was tested for SRB-based cytotoxicity and antiviral activity against EV71 C3 and C4a. For further purification, the active fraction was subjected to ODS column chromatography (300g, YMC-Gel ODS-A, 150 um, 5 x 50 cm) using isocratic

elution with MeOH : H_2O (70:30) to give a pure compound. The purified compound was identified as hederasaponin B by direct comparison with the authentic compound.

Antiviral activity assay

Assays of antiviral activity was evaluated by the SRB method using CPE reduction, recently reported (Choi *et al.*, 2009). One day before infection, vero cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells/well. Next day, medium was removed and then washed with 1× PBS. Subsequently, 0.09 mL of the diluted virus suspension containing 50% cell culture infective dose($CCID_{50}$) of the virus stock was added to produce appropriate CPE within 48h after infection, followed by the addition of 0.01 mL of medium supplemented with FBS containing anappropriate concentration of the compounds were added.The antiviral activity of each test material was determined with a 5-fold diluted concentration ranging from 0.4 to 50 μg/mL.Four wells were used as virus controls (virus-infected non-compound-treated cells) while Four wells were used as cell controls (non-infected non-compound-treated cells). The culture plates were incubated at 37°C in 5% CO_2

for 2 days until appropriate CPE was achieved. Subsequently, the 96-well plates were washed once with 1× PBS, and 100μL of cold (−20°C) 70% acetonewas added on each well and left standing for 30 min at −20°C. After the removal of 70%acetone, the plates were dried in a dry oven for 30 min, followed by the addition of 100 μL of 0.4%(w/v) SRB in 1% acetic acid solution to each well, and left standing at room temperature for 30 min. SRB was then removed, andthe plates were washed 5 times with 1% acetic acid beforeoven-drying. The plates were dried in a dry oven. After drying for 1 day, the morphology of the cells to observation the effect of compounds onEV71 C3 and EV71 C4a -induced CPE were observed under microscope at 0.4 ×10 magnification (Axiovert 10; Zeiss, Wetzlar, Germany), and images were recorded. Bound SRB was then solubilized with 100μL of 10 mM unbuffered Tris-base solution, and the plates were left on a table for 30 min. The absorbance was then read at 540 nm by using a VERSAmaxmicroplate reader (Molecular Devices, Palo Alto, CA, USA) with a reference absorbance at 620 nm. The results were then transformed into percentage of the controls, and the percent protection achieved by the test CFS in the EV71-infected cells was calculated using the following formula: $\{(OD_t)_{EV71}-(OD_c)_{EV71}\}\div\{(OD_c)_{mock}-(OD_c)_{EV71}\}\times100$ (expressed in %), where $(OD_t)_{EV71}$ is the optical density measured with a given CFS in EV71-infected cells; $(OD_c)_{EV71}$ is the optical density measured for the control untreated

EV71-infected cells; and $(OD_c)_{mock}$ is the optical density measured for control untreated mock-infected cells. Antiviral activity was presented as % of control. Ribavirin and DMSO were used for positive and negative control, respectively.

Cytotoxicity assay

Vero cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells/well. Next day, medium was removed and each well was washed by phosphate buffered saline (PBS). The 96-well plates were treated to compounds in maintenance medium for 48h at 37°C, in parallel with the virus-infected cell cultures. For each compounds, 3 wells were used as controls (non- compound-treated cells). After 48h of incubation, cytotoxicity was evaluated by SRB assay as previously described (Lin *et al.*, 1999).Cytotoxicity was presented as % of control.

Western blot analysis

Vero cells were plated onto 6-well culture plates at a density of 5×10^5cells/well 24 h before infection with EV71 C3 and EV71 C4a. EV71 C3 and EV71 C4a infected cells were treated with hederasaponin B and ribavirin at a concentration 50μg/mL for 48 h for detection of viral VP2protein. Mock-infected cells treated with 0.1% DMSO and EV71 infected cells treated with 0.1% DMSO were used as controls. Cells were lysed in ice-cold RIPA lysis buffer containing 50 mMTris-Hcl pH7.4, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1% SDS, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF, 5 mM sodium fluoride and 5 mM sodium orthovanadate.The preparation of sample protein (30 μg) boiled for 10 min at 100°C and separated in 12 % acrylamide gels run at 100 V for 1 h (for detection of VP1).The SeeBlue®Plus2prestained protein ladder (Invitrogen) was used as a molecular weight standard.The gels were transferred to a nitrocellulose membrane using the Invitrogen iBlot®Gel Transfer Device (Invitrogen, Carlsbad, CA) at 20V for 7 mins.

For detection of VP2, membranes were blocked with 5 % skim milk (Difco) dissolved in phosphate buffered saline–Tween 20 (PBST) overnight at 4°C on a shaker. The blots were washed three times with PBST before being incubated

with primary mouse anti-enterovirus 71 monoclonal antibody (Millipore) dissolved in 5% skim milk at a dilution of 1:1000.For the loading control, separate blots containing the same samples were incubated with primary α-Tubulin mouse monoclonal IgG_1 (SantacruzBiotechnology) dissolved in 5% skim milk at a dilution of 1:1,000.The blots were incubated with primary antibodies at room temperature on a shaker. The blots were then washedthree times with PBST for 10 min each time.This was followed by incubation with the secondary antibodies polyclonal goat anti-mouse IgG(H+L)–HRP (GenDEPOT) for 1 h at room temperature on shaker.Dilution of secondary antibody was done in 5% skim milk at a ratio of 1:5,000.Membranes were then rinsed three times with PBST for 10 min each time.Membranes were developed by the enhanced chemiluminescence (ECL) method using West-Q chemiluminescent substrate (GenDEPOT).

1.3. Results

Antiviral activity of 30% EtOH extract of *Hedera helix* against two major EV71 C3 and EV71 C4a

During the screening of antiviral activity of several medical plants extracts against EV71, we found that 30% EtOH extract of *Hedera helix,* which was widely used in clinic for the treatment of cough symptoms, also possessed significant antiviral activity against EV71 C3 (Table 1.1) and C4a (Table 1.2). To find the active antiviral compounds in 30% EtOH extract of *Hedera helix*, the extract which was further fractionated into EtOAc,n-BuOH, $CHCl_3$, and Hexane fraction, and we found that the antiviral activity was highly retained in n-BuOH fraction (data not shown). Thus, we decided toseparate the n-BuOH soluble fraction using a Diaion HP-20column and obtained 6 fractions after stepwise-gradient with MeOH : H_2O. Each fraction was tested for SRB-based cytotoxicity and antiviral activity against EV71 C3 (Table 1.1)and C4a (Table 1.2), and we found that the 40% and 60% of MeOH fractions showed significant antiviral activity.Afterfurther purification, we identified hederasaponin B as a major compound in the 40% and 60% of MeOH fractions (data not shown).

Antiviral activity and Cytotoxicity of hederasaponin B against EV71 C3 and EV71 C4a

Next, hederasaponin B from *Hedera helix* was further investigated for its antiviral activity against EV71 C3 and EV71 C4a. The antiviral assay demonstrated that hederasaponinB possessed antiviral activity against EV71 C3with an EC_{50} value of 24.77 µg/ml and against EV71 C4a with an EC_{50} value of 41.77 µg/ml (Table 1.3).In addition, it was not toxic to vero cells with a cell viability of about 100% at a concentration of 50 µg/ml.

The effect of hederasaponin B on EV71 C3 and EV71 C4a induced CPE

The effect of hederasaponin B on EV71 C3- and EV71 C4a-induced CPE was recorded by capturing images using a microscope (Axiovert 10; Zeiss, Wetzlar, Germany). In the absence of infection of vero cells with EV71 C3 and EV71 C4a, cells treated with vehicle (Fig. 1.1A) (Fig. 1.2A), 50 µg/ml hederasaponin B (Fig. 1.1B) (Fig. 1.2B), or ribavirin (Fig. 1.1C) (Fig. 1.2C) showed typical spread-out shapes with normal morphology. At this concentration, especially, no signs of

cytotoxicity of hederasaponin B were observed. Infection with EV71 C3 and EV71 C4a in the absence of hederasaponin B resulted in a severe CPE (Fig. 1.1D) (Fig. 1.2D). Addition of hederasaponin B to the infected vero cells inhibited the formation of a visible CPE (Fig. 1.1E) (Fig. 1.2E). However, the addition of ribavirin in vero cells infected with EV71 C3 or EV71 C4a could not prevent the CPE (Fig. 1.1F) (Fig. 1.2F). This indicates that the CPE of the viral infection is prevented by the presence of hederasaponin B.

Hederasaponin B affects viral VP2 protein synthesis

Viral VP2 proteins syntheses were compared between drug-treated and untreated infected cells. As shown in Figure 3, when cells were infected with virus and cultured in the absence of drugs until processed for western blot, virus VP2 protein could be detected in the untreated cells. The size of EV71 VP2 protein has been determined to be 34 kDa and α-Tubulin was used as a loading control in the experiment as well as to ensure that hederasaponin B used in this study did not affect the synthesis and expression of host cellular proteins.The western blot analysis also showed that the viral VP2 protein expression was decreased dramatically by hederasaponin B (50 μg/ml) at 48 h after infection by EV71 C3

(Fig. 1.3A) and EV71 C4a (Fig. 1.3B). However, ribavirin used as a positive control did not show cytotoxicity as well as antiviral activity in vitro and in western blot analysis, which is consistent with the previous reportsby Choi *et al*. (2010). Collectively, these resultssuggested that hederasaponin B possessed antiviral activity against EV71 C3 and C4a by inhibiting viral protein expression, and thus could be considered as an antiviral drug candidate for the treatment of HFMD.

1.4. Discussion

The current antiviral drug armamentarium comprises of almost 40 compounds that have been officially approved for clinical use including 19 drugs for treating human immunodeficiency virus (HIV) infection, 3 drugs for treating hepatitis B virus (HBV) infection, 7 drugs for treating herpes simplex virus (HSV) infection and varicella-zoster virus (VZV) infection, 2 drugs for treating respiratory syncytial virus (RSV) infection and hepatitis C virus (HCV) infection, 5 drugs for treating cytomegalovirus (CMV) infection and 4 drugs for treating influenza

virus infection. However, to date, there is no approved antiviral drug for the treatment of enterovirus infections (Park *et al.*, 2012).

Pleconaril is a potent anti-viral inhibitor of enteroviruses that is under evaluation for the treatment of diseases associated with picornavirusinfections(Pevear *et al.*, 1999). Pleconaril exerts its activity on capsid function by integrating with high affinity and specificity in the hydrophobic pocket of the virion. In 2007, Schering-Plough completed a phase II double-blind, placebo-controlled trial to study the effects of pleconaril nasal spray on common cold symptoms(De Palma *et al.*, 2008), but the US Food and Drug Administration has not approved pleconaril because of concerns of emergence of viral resistance and side effects in patients (Fleischer and Laessig, 2003). The relevance of pleconaril resistance was demonstrated in a study by Pevear *et al*. (2005). Other candidates having an antiviral effect against EV71 include WIN54954, SCH48973, rupintrivir, raoulic acid, and punicalagin. In addition, antiviral activity of betulin, betulinic acid, betulonic acid, chebulagicacidisolated from the fruits of *Terminaliachebula*, and that of matrineisolated from the root of Chinese Sophora herb plantshas been studied against enterovirus.Ribavirin has also been used to treat various DNA and RNA virus infections, although acquired resistance to it has been demonstrated in various virus populations and in some patients (Graci and Cameron, 2006).

Further, significant antiviral activity of ribavirin against EV71 in vero cells was not observed. Therefore, broad-spectrumantiviral compounds should be developed against various genogroups of enteroviruses in the future.

The present study describes the cytotoxicity and antiviral activity of hederasaponin B. Hederasaponin B was shown to exhibit anti-viral activity against EV71 C3 and EV71 C4a byreducing the formation of a visible CPE. In addition, the inhibitory effects of hederasaponin B and ribavirin against EV71 were analyzed by western blot assay. The expression of EV71 C3 and C4a VP proteinswas inhibited in the presence of 50 μg/ml of hederasaponin B. However, ribavirin did not show anyinhibitory effect against EV71 infection. Theseresults suggest that hederasaponin B could be abroad-spectrum antiviral compound that is effective against various EV71 subgenotypes. In addition, 30% EtOH extract of *Hedera helix,* which has been widely used for the treatment of acute and chronic obstructive pulmonary bronchitis due to its secretolytic and bronchiolytic effects in adults and children,also has a significant anti-viral activity againstEV71 C3 and C4a, thereby suggesting that it can be developed as an anti-viral drug for EV71 infection. It was also found that 40% and 60% MeOH fractions from 30% EtOH extract of *Hedera helix* also have significant anti-viral effects against EV71 C3 and C4a; and especially, hederasaponin B, one of the

major compounds of the 40% and 60% MeOH fractions showed a significant anti-EV71 activity.

In conclusion, hederasaponin B was shown to be effective against EV71. Also, anti-EV71 activity analysis with EV71 subgenotypes C3 and C4a did not reveal a subgenotype-specific activity pattern. Further studies will be required to explore the detailed antiviral mechanism of action of hederasaponin B. The researches focusing on suppression of enterovirus replication by hederasaponin B will be carried out because hederasaponin B is well known to be belonged to triterpenoidsaponins (Han *et al.*, 2013) and it has been demonstrated that triterpenoidsaponins inhibit viral nucleotide synthesis against herpes simplex virus type-1 in previous studies (Simoes *et al*., 1999)

Table 1.1 Antiviral activity of the extract and fractions of *Hedera helix* against EV71 C3.

Test material	CC_{50}[a]	EC_{50}[b]	TI[c]
Hedera Helix 30% EtOH extract	>50	6.58±0.11	7.59
100% H_2O	>50	ND[d]	-
20% MeOH	>50	25.23±4.93	1.98
40% MeOH	>50	7.92±1.44	6.31
60% MeOH	>50	2.75±1.05	18.18
80% MeOH	>50	38.22±4.13	1.31
100% MeOH	31.03	ND[d]	-

Results are presented as the mean EC_{50} values ±S.D obtained from three independent experiments carried out in triplicate.

[a]Concentration required to reduce cell growth by 50% (μg/ml).

[b]Concentration required to inhibit virus-induced CPE by 50% (μg/ml).

[c]Therapeutic index = CC_{50}/EC_{50}

[d]Not determined

Table 1.2 Antiviral activity of the extract and fractions of *Hedera helix* against EV71 C4a.

Test material	CC_{50}[a]	EC_{50}[b]	TI[c]
Hedera Helix 30% EtOH extract	>50	22.00±2.06	4.55
100% H_2O	>50	ND[d]	-
20% MeOH	>50	ND[d]	-
40% MeOH	>50	43.12±1.91	1.16
60% MeOH	>50	47.10±5.16	1.06
80% MeOH	>50	ND[d]	-
100% MeOH	31.03	ND[d]	-

Results are presented as the mean EC_{50} values ±S.D obtained from three independent experiments carried out in triplicate.

[a]Concentration required to reduce cell growth by 50% (μg/ml).

[b]Concentration required to inhibit virus-induced CPE by 50% (μg/ml).

[c]Therapeutic index = CC_{50}/EC_{50}

[d]Not determined

Table 1.3 Antiviral activity of hederasaponin B isolated from *Hedera helix* against EV71 C3 and C4a.

	EV71 C3			EV71 C4a		
Compound	CC_{50}[a]	EC_{50}[b]	TI[c]	CC_{50}[a]	EC_{50}[b]	TI[c]
Hederasaponin B	>50	24.77±12.56	2.02	>50	41.77±0.76	1.18
Ribavirin	>50	ND[d]	-	>50	ND[d]	-

Results are presented as the mean EC_{50} values ±S.D obtained from three independent experiments carried out in triplicate.

[a]Concentration required to reduce cell growth by 50% (µg/ml).

[b]Concentration required to inhibit virus-induced CPE by 50% (µg/ml).

[c] Therapeutic index = CC_{50}/EC_{50}

[d] Not determined

Figure legends

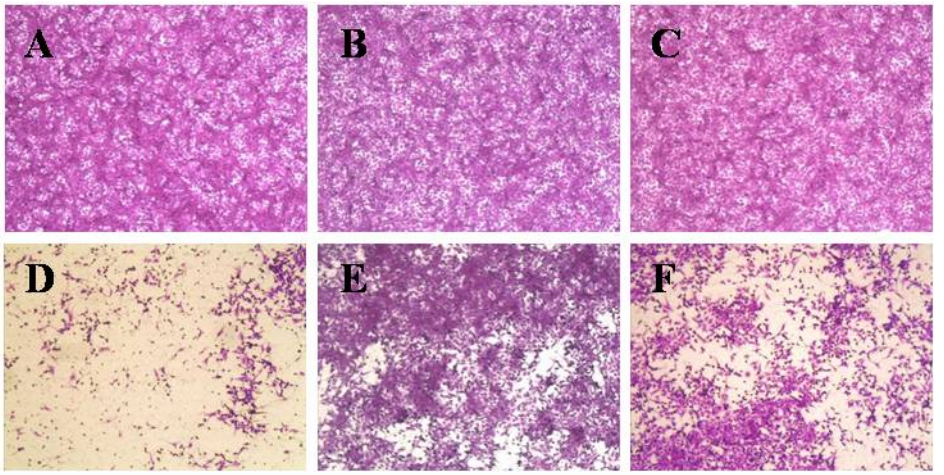

Figure 1.1 Effect of hederasaponin B on Enterovirus 71 (EV71) C3–induced cytopathic effect. The virus-infected vero cells were treated with ribavirin or 50 μg/mL hederasaponin B. After, cell viability was evaluated by sulforhodamine B (SRB) assay, and the morphology of cells was photographed using a microscope. (A) Non-infected cells; (B) Non-infected cells treated with hederasaponin B; (C) Non-infected cells treated with ribavirin; (D) EV71 C3-infected cells; (E) EV71 C3-infected cells treated with hederasaponin B;(F) EV71 C3-infected cells treated with ribavirin.

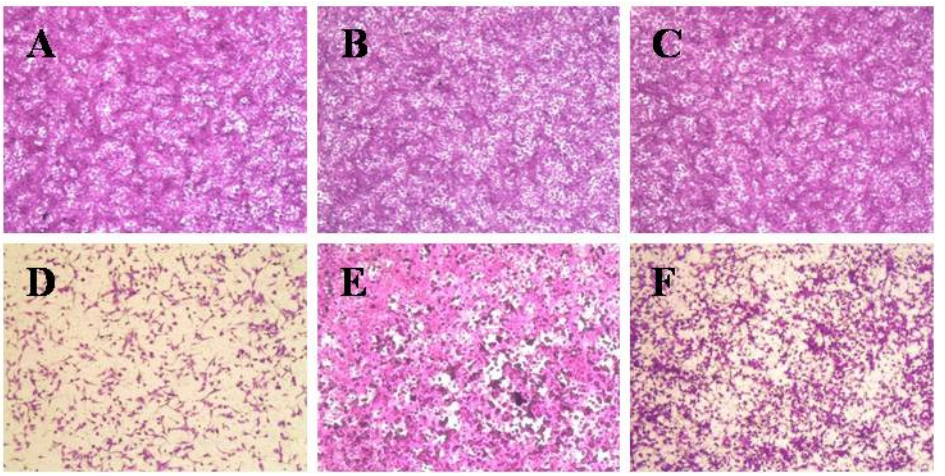

Figure 1.2 Effect of hederasaponin B on Enterovirus 71 (EV71) C4a–induced cytopathic effect. The virus-infected vero cells were treated with ribavirin or 50 μg/mL hederasaponin B. After, cell viability was evaluated by sulforhodamine B (SRB) assay, and the morphology of cells was photographed using a microscope. (A) Non-infected cells; (B) Non-infected cells treated with hederasaponin B; (C) Non-infected cells treated with ribavirin; (D) EV71 C4a-infected cells; (E) EV71 C4a -infected cells treated with hederasaponin B; (F) EV71 C4a-infected cells treated with ribavirin.

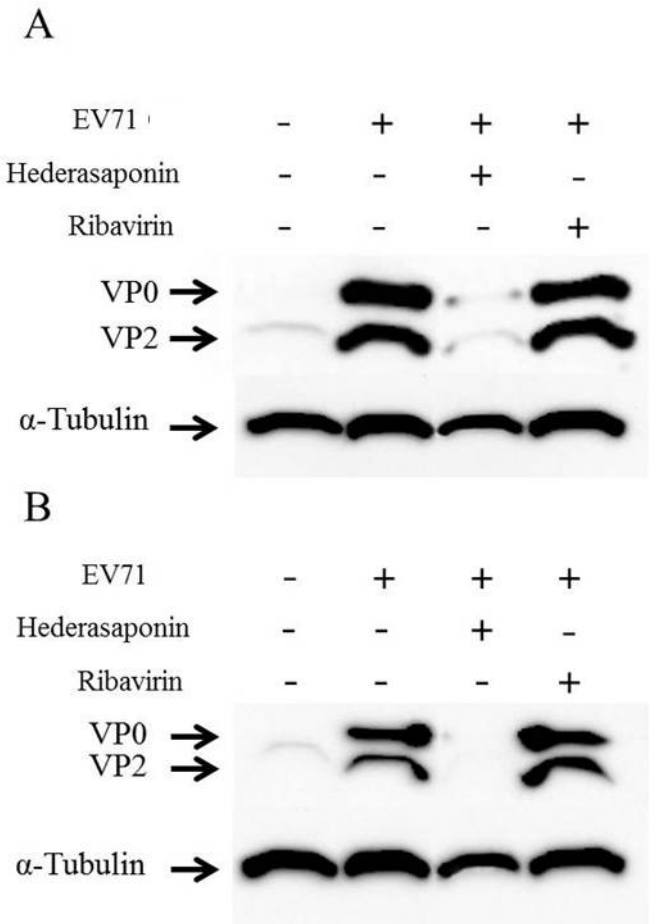

Figure 1.3 Effect of hederasaponin B on the VP2 expression. Western blot analyses were performed to determine the effect of hederasaponin B and ribavirin on the production of EV71 C3 and EV71 C4a VP2 proteins. The reduction in protein expression of EV71 C3 VP2 (a) and EV71 C4a VP2 (b) was identified after treatment with 50 μg/ml concentration of hederasaponin B or ribavirin for 48 h. α-tubulin was used as a loading control for each set of samples.

Chapter 2

Antiviral effects of *Phyllanthus urinaria* containing Corilagin against human enterovirus 71 and Coxsackievirus A16

Abstract

Human enterovirus 71 (EV71) and Coxsackievirus A16 (CA16) are major causative agents of hand, foot, and mouth disease (HFMD) especially in infants and children under 5 years of age. Despite recent outbreaks of HFMD, there is no approved therapeutics against EV71 and CA16 infection. Moreover, in a small percentage of cases, the disease progression can lead to serious complications of the central nervous system. In this study, it was investigated that the antiviral effect of corilagin and Phyllanthus urinaria extract, which contains corilagin as a major component, on EV71 and CA16 infection in vitro. Especially, in this study, it was indicate that corilagin reduces the cytotoxicity induced by EV71 or CA16 on Vero cells with and IC50 value of 5.6 g/ml and 32.33 g/ml, respectively. It

was confirmed that the presence of corilagin in EtOAc and BuOH fractions from *P. urinaria* extract and this correlated with antiviral activity of the fractions against EV71 or CA16. Future studies will be required to confirm the antiviral activity of corilagin and *P. urinaria* extract in vivo. Challenging a model with a lethal dose of viral infection will be required to test this. Collectively, our work provides potential candidates for the development of novel drugs to treat HFMD.

Key words : EV71, CVA16, Corilagin, *Phyllanthus urinaria*, HFMD

2.1. Introduction

Recently, it was found that corilagin, a hydrolysable tannin isolated from Ardisia splendens, exhibited significant antiviral activity against Coxsackievirus A16 (CVA16) infection. Since corilagin is a minor component of A. splendens, previous studies were utilized to identify another medicinal plant with corilagin as a major constituent. Then the investigations were carried out whether corilagin and these corilagin-containing medicinal plants exhibited antiviral activity against enterovirus 71 (EV71) and CVA16.

Corilagin is a polyphenol and a member of hydrolysable ellagitannins (Zhao L *et al,* 2008, Duan W *et al*, 2005). Corilagin was first isolated in 1951 from Caesalpinia coriaria, and named after the original plant. There are several reports showing that corilagin, also known as β-1-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-D-glucose, could be isolated from Phyllanthus species, including Phyllanthus urinaria, Phyllanthus emblica, and Phyllanthus amarus (also known as Phyllanthus niruri) (Duan W. *et al*., 2005, JiKai *et al*., 2002, Patel JR *et al*., 2011). Corilagin can also be found in Terminalia catappa L., Nephelium lappaceum L., Alchornea glandulosa, Excoecaria agallocha L., and in the leaves of Punica granatum (Kinoshita *et al*., 2007, Li Y *et al*., 2012).

Corilagin is also present in Terminalia chebula Retz with chebulagic acid and punicalagin (Park JH *et al*., 2011). Interestingly, chebulagic acid and punicalagin, which are structurally similar to corilagin and contain gallic acid and hexahydroxydiphenoyl (HHDP) ester moieties, have been showed to exhibit broad-spectrum antiviral activity against HSV-1, HCMV, HCV, and the measles virus (Satomi *et al*., 1993, Lin LT *et al*., 2011, Lin LT *et al*., 2013, Yang Y *et al*., 2012). Furthermore, recent studies have shown that chebulagic acid and geraniin exhibit antiviral activity in vitro and in vivo against human EV71 (Yang Y *et al*., 2012, Yang Y *et al*., 2013). However, although corilagin has been shown to exhibit anti-inflammatory effects in HSV-1-infected microglia (Guo *et al*., 2010), antiviral activity has not been reported against EV71 and CA16. Several reports suggest that *P. amarus* containing tannins including corilagin and geraniin exhibit a high degree of antiviral activity against HIV infection (Notka *et al*., 2004). Previous reports have shown that corilagin also possesses antioxidant, hepatoprotective, thrombolytic, antiatherogenic, antihypertention, anticancer, and antihyperalgesic activities (Zhao *et al*., 2008, Duan W *et al*., 2005, Moreira *et al*., 2013, Kinoshita *et al*., 2007, Satomi *et al*., 1993, Thitilertdecha *et al*., 2010, Hau DK *et al*., 2010).

P. urinaria is a herb species of the Phyllanthaceae family, together with *P. amarus* (Yuandani *et al*., 2013). There are only a few reports that demonstrate the medical applications of Asian-originating *P. urinaria* compare to those of *P. amarus*, which commonly found in the hotter coastal area of India and widely spread throughout tropical and subtropical countries around the world. *P. amarus* is one of promising medical plants that have been widely evaluated in clinical trials based on its preclinical potential for the treatment of HIV, jaundice, hypertension, and diabetes (Peter JR *et al*., 2011). However, the antiviral activities of *P. urinaria* and *P. amarus* have not been tested against Enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16). Therefore, in this study the investigation was carried out to identify the antiviral activities of *P. urinaria* and its major component corilagin against EV71 and CVA16, two major causative infectious viruses associated with hand, foot, and mouth disease (HFMD) in infants and young children (Mao Q *et al*., 2013, Tan CW *et al*., 2012).

In recent years, there has been an occasional circulation of CVA16 and EV71 in the Western Pacific region including Korea, Japan, and China. The most recent HFMD outbreak occurred in China during 2008 (Mao Q *et al*., 2013, Solomon T *et al*., 2010). Therefore, HFMD has become an important public health concern in this region. Although HFMD caused by CA16 and EV71 usually ends with

mild disease symptoms including blisters or ulcers on the hands, feet, and mouth and pharyngitis in infants and children under 5-years of age, there are rare cases that cause serious complications in the central nervous system (CNS) resulting in aseptic meningitis, encephalitis, and myocarditis 2008 (Mao Q *et al*., 2013, Solomon T *et al*., 2010). Importantly, there are no safe and effective vaccines or therapeutics to treat CA16 and EV71 infection (Mao Q *et al*., 2013, Cheng A *et al*., 2013, Lin SY *et al*., 2008). More importantly, recent studies suggest that co-infection with CA16 and EV71 can result in serious neurological complications in the CNS and might increase the possibility of genetic recombination, which can cause HFMD outbreak (Mao Q *et al*., 2013). Thus the development of antiviral agents like as corilagin that can simultaneously inhibit CVA16 and EV71, would be an ideal HFMD therapeutic.

2.2. Materials and Methods

Viruses and cell lines

The CA16 and EV71 viruses were obtained from the division of vaccine research of the Korea Center Disease control and prevention (KCDC), and were

propagated at 37 °C in Vero cells, which are kidney epithelial cells that originated from an African green monkey kidney. Vero cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic solution. Antibiotic-antimycotic solution, trypsin-EDTA, FBS, and MEM were purchased from Gibco BRL (Invitrogen Life Technologies, Karlsruhe, Germany). Tissue culture plates were purchased from Falcon (BD Biosciences, San Jose, CA, USA). Sulforhodamine B (SRB) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade.

Antiviral activity assay

Antiviral activity was assayed by employing the SRB method using cytopathic effect (CPE) reduction as recently reported (Choi HJ *et al*., 2009). One day prior to infection, 2×10^4 cells/well of Vero cells were seeded onto a 96-well culture plate. The next day, medium was removed and cells were washed with PBS. Subsequently, 0.09 mL of the diluted virus suspension containing a 50% cell culture infective dose (CCID50) of the virus was added to produce the appropriate CPE within 48 h after infection. Next, 0.01 mL of medium supplemented with FBS containing an appropriate concentration of the

compounds was added. The antiviral activity of each test material was determined using a 5-fold diluted concentration that ranged from 0.4 to 50 μg/mL. Four wells were used as viral controls (virus-infected non-compound-treated cells) while the other four wells were used as cell controls (non-infected non-compound-treated cells). The culture plates were incubated at 37 °C in 5% CO2 for 2 days until the appropriate CPE was achieved. Subsequently, the 96-well plates were washed once with PBS, and 100 μL of ice-cold 70% acetone was added to each well and incubated for 30 min at −20 °C. After removing the 70% acetone, plates were dried in a dry oven for 30 min, after addition of 100 μL of 0.4% (w/v) SRB in 1% acetic acid solution to each well for 30 min at room temperature. SRB was then removed, and the plates were washed 5 times with 1% acetic acid before oven-drying. The plates were then dried in a dry oven. After drying for 1 day, the cell morphology was observed using an Axiovert microscope (Axiovert 10; Zeiss, Wetzlar, Germany) to examine the effect of the compounds on EV71 and CA16-induced CPE. Bound SRB was then solubilized with 100 μL of 10 mM unbuffered Tris-base solution, and the plates were left on a table for 30 min. The absorbance was then read at 540 nm using a VERSA max microplate reader (Molecular Devices, Palo Alto, CA, USA) with a reference absorbance at 620 nm. To calculate the IC50 values, the results were then quantified as percentage of the controls and the IC50 values were graphically

obtained from the dose-response curves. The percent protection achieved by the test compound in the EV71 and CA16-infected cells was calculated using the following formula: {(ODt)EV71-(ODc)EV71}÷{(ODc)mock-(ODc)EV71}×100 (expressed in %) where (ODt)EV71 is the optical density measured with a given test compound in EV71-infected cells; (ODc)EV71 is the optical density measured for the control untreated EV71-infected cells; and (ODc)mock is the optical density measured for control untreated mock-infected cells. The antiviral activity was presented as % of control. Ribavirin (Duchefa) and DMSO were used as positive and negative control, respectively.

Cytotoxicity assay

Vero cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells/well. The next day, medium was removed and the cells were washed with PBS. The cells were treated with compounds in maintenance medium for 48 h at 37 °C, in parallel with the virus-infected cell cultures. For each compounds, 3 wells were used as controls and were not treated with the compounds. After 48 h of incubation, cytotoxicity was evaluated using the SRB assay as previously described (Choi HJ *et al*., 2009). Cytotoxicity is presented as % of control.

Preparation of corilagin and *P. urinaria* fractions

Corilagin was kindly donated by Prof. Seung Hyun Kim, College of Pharmacy, Yonsei University. The leaves and twigs of *P. urinaria* were purchased from Kyongdong Oriental Herbal Market (Seoul, Korea) in 2013 and voucher specimens (YEPU-1310) documenting these collections have been deposited at the College of Pharmacy, Yonsei University. The dried leaves and twigs of P. urinaria (600 g) were cut into pieces and extracted with methanol (MeOH) (3 × 2 L) in an ultrasonic apparatus at room temperature to yield 95.4 g of extract after evaporation of the solvent. The extract was suspended in H2O and successively partitioned with n-hexane (3.9 g), CHCl3 (6.4 g), ethylacetate (EtOAc) (21.4 g), n-butanol (n-BuOH) (39.2 g), and H2O (24.3 g) fractions after removal of the solvents in vacuo.

The EtOAc and n-BuOH fractions of P. urinaria were standardized based on corilagin, using HPLC-DAD. In brief, corilagin in these fractions was separated and quantified using the Agilent 1290 HPLC system and YMC-Hydrosphere C18 column (5 μm, 4.6 × 150 mm I.D.) by UV detection at 254 nm. The mobile phase was composed of (A) acetonitrile and (B) water with 0.1% phosphoric acid at a flow rate of 1 mL/min, with the following gradient elution: 0 min, 20% A, and 20 min, 40% A. Using this analytical condition, the calibration curve for

corilagin and the content of corilagin in the EtOAc and n-BuOH fractions of *P. urinaria* were determined (Fig. 2.1).

Statistical Analysis

To compare multiple groups, one-way ANOVA was carried out and followed by the Tukey post hoc test. Values of $p < 0.05$ were considered significant at a 95% confidence interval. The 'ns' stands for not significant between groups compared.

2.3. Results

Investigation of the antiviral activity of Corilagin against CVA16 and EV71

In our recent study, the antiviral activity of compounds isolated from Ardisia splendens was identified; one active compound was corilagin, also known as β-1-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-d-glucose, exhibited significant antiviral activity against CA16. Here, the antiviral activity of corilagin against CA16 was confirmed and the antiviral activity of corilagin towards EV71 was also assessed. CA16 and EV71 are two major causative agents of HFMD, and recent studies found that coinfection of CA16 and EV71 is critical for HFMD outbreak (Mao Q *et al*., 2013). The antiviral assays in this study demonstrate that corilagin possesses significant antiviral activity towards EV71 as well as CVA16 (Fig. 2.2). About 65% and 38% of Vero cells infected with CA16 survived if treated with 50μg/ml and 10 μg/ml of corilagin, respectively. In contrasts, less than 10% of cells were viable after CA16 infection, even after 50 μg/ml of ribavirin was treated (Fig. 2.2A). Similarly, 10 g/ml of corilagin significantly increased cell viability against EV71 infection by 63%, whereas ribavirin showed relatively weak antiviral activity against EV71 infection even at 50 μg/ml of concentration (Fig. 2.2B). For both CA16 and EV71 infections, it was confirmed that ribavirin exhibited only marginal antiviral activity of less than 50% at 50 μg/ml. The IC50 values of corilagin were 5.60 ± 1.07 μg/ml and 32.33 ± 0.42 μg/ml against EV71 and CA16, respectively (Table 2.1). Collectively, it

was identified that corilagin exhibits antiviral activity against CVA16 and found that corilagin can inhibit EV71 infection in Vero cells.

The effect of corilagin on CVA16- and EV71-induced cytotoxicity

To confirm the cytoprotective effects of corilagin against CVA16 and EV71 infection, the morphology of Vero cells treated with corilagin after CVA16 or EV71 infection was also examined. Uninfected and vehicle-treated Vero cells exhibit typical spread-out shapes and normal morphology (Fig. 2.3A and 2.4A). Treatment with 50 μg/mL corilagin (Fig. 2.3B and 2.4B) or ribavirin (Fig. 2.3C and 2.4C) did not alter the morphology and did not induce cytotoxicity in the cells. Vero cells infected with CVA16 or EV71 in the absence of corilagin exhibited severe CPE (Fig. 2.3D and 2.4D), whereas the addition of corilagin to infected Vero cells inhibited the formation of a visible CPE (Fig. 2.3E and 2.4E). In contrast, Vero cells treated with ribavirin after CA16 and EV71 infection only modestly prevented CPE (Fig. 2.3F and 2.4F). Thus, it was confirmed that corilagin could prevent CPE of Vero cells due to CA16 or EV71 viral-infection.

Corilagin is a major component of *P. urinaria*

Based on the previous reports suggesting the hepatoprotective activity of corilagin isolated from *P. urinaria* (Patel JR *et al*., 2011, Jikai L *et al*., 2002, Yuandani *et al*., 2013), the content of corilagin in the EtOAc and n-BuOH fractions of *P. urinaria* was determined. Using HPLC-DAD, we prepared a standard calibration curve for corilagin. Corilagin content in the EtOAc and n-BuOH fractions from *P. urinaria* were determined to be 8.7% and 5.6%, respectively. This suggests that corilagin is a major component of these fractions.

Corilagin from *P. urinaria* possesses significant antiviral activity against CVA16 and EV71

Previous reports have described the isolation of corilagin from *P. urinaria* (Jikai L *et al*., 2002, Yuandani *et al*. 2013). Other studies have shown that P. urinaria possessed broad-spectrum antiviral activity against HSV, EBV, HCMV, HCV, and the measles virus (Liu KC *et al*., 1999). Therefore, the antiviral activity of *P. urinaria*, which contains corilagin as a major component, against CVA16 and EV71 was investigated. The antiviral activity and cytotoxicity of methanol extract and organic fractions, including EtOAc and BuOH fractions,

were determined with a 5-fold diluted concentration ranging from 0.4 to 50 μg/mL. The methanol extract exhibited inhibitory effects greater than 80% against CVA16 (Fig. 2.5) and EV71 (Fig 2.6), with IC50 values of 45.9 ± 5.39 μg/mL and 32.4 ± 0.35 μg/mL, respectively (Table 2.2). The ethyl acetate and butanol fraction also showed significant antiviral activity of about 50% against CA16 at 50 μg/ml (Fig. 2.5), with IC50 values of 4.79 ± 0.81 μg/ml, 39.5 ± 6.51 μg/ml, respectively (Table 2.2). In addition, the antiviral activity of ethyl acetate and butanol fractions against EV71 exhibited about 51% and 95% antiviral protection, respectively, at 50 μg/ml (Fig. 2.6) and IC50 values were 43.8 ± 9.17μg/ml, 31.8 ± 2.29 μg/ml (Table 2.2). However, hexane and water fractions did not show any significant antiviral activity against CVA16 and EV71 (Table 2.2). These results correlate with the idea that corilagin mainly exists in the ethyl acetate and butanol fractions of P. urinaria extract. Collectively, it was showed that corilagin and the ethyl acetate and butanol fraction of *P. urinaria* MeOH extract contains corilagin as a major component and possesses significant antiviral activity against CVA16 and EV71 infections. These data suggest that this is a novel antiviral candidate to treat HFMD.

2.4. Discussion

In this study, vero cells (Lin LT *et al*., 2013, Yang Y *et al*., 2012) were used *to* determine that corilagin isolated from P. urinaria has an IC50 value of 5.6 μg/ml and the CC50 was 327 μg/ml (Table 2.1). This data suggests that corilagin also significantly inhibited EV71 replication in vitro. The antiviral activity of corilagin against CVA16 was also assessed; for this, we determined that the IC50 value was 32.33 μg/ml (Table 2.1). These data indicate that corilagin might also inhibit CA16 replication, in addition to EV71. Taken together, these data indicate that corilagin could be an effective antiviral agent to treat hand, foot, and mouth disease caused either by EV71 or CVA16, and might therefore prevent severe complications with neurological disease due to EV71 infection. Additional preclinical animal studies are required to evaluate the antiviral effect of corilagin in vivo; however, the lack of an appropriate animal model for EV71 infection might hinder such investigations.

Due to the lack of approved antiviral therapies for EV71 and CVA16 infection, the involvement of central nervous system and long-term neurological sequelae involving reduced cognitive function spurred the development of novel antiviral agents from medical plants. Medicinal plants contain a wide variety of active

phytochemicals that possess anciently developed mechanisms to prevent viral invasion. Among them, corilagin, an ellagitannin that contains hexahydroxydiphenoyl (HHDP) ester (Duan W *et al*., 2005, Lin LT *et al*., 2011) was focused. It was found that corilagin and *P. urinaria*, which contains corilagin as a major component, exhibited significant antiviral activity against EV71 and CVA16. Interestingly, antiviral activity of corilagin was much higher against EV71 than CVA16. In the case of CVA16, the antiviral effect of EtOAc fraction was higher than BuOH fraction, MeOH fraction, and corilagin. Considering this, there might be other compounds having synergistic antiviral effect with corilagin against CVA16 in the EtOAc fraction. However, in EV71 case, corilagin has the highest antiviral effect as compared to MeOH, EtOAc, and BuOH fraction. In addition to corilagin, other hydrolysable tannins that also contain a HHDP unit have been reported to inhibit HSV and HIV-1 infections. Moreover, structures containing a HHDP unit have been regarded as valuable pharmacophores for inhibiting HIV enzymatic activity against HIV-1 RT and integrase (Lin LT *et al*., 2011, Guo YJ *et al*., 2010, Notka F. *et al*., 2004). Further studies are required to elucidate the relationship between the structure and activity of ellagitannin and the importance of the HHDP unit. This information would help reveal the mechanism behind the antiviral activity of corilagin and other ellagintannins.

Although it could not be determined the mechanism of antiviral activity of corilagin, a previously report has shown that corilagin exhibit strong scavenging activity against free radicals and possess antioxidant activity (Kinoshita S. *et al*., 2007, Thitilertdecha *et al*.,2010, Chen Y *et al*., 2011). This antioxidant activity might be closely linked to the antiviral activity of these compounds. In addition, corilagin exhibited an anti-inflammatory effect in HSV-1 encephalitis and HSV-1-infected microglial cells by inhibiting the secretion of pro-inflammatory cytokines including TNF-alpha and IL-1β, as well as by inhibiting NO synthesis (Lin Lt *et al*., 2011, Guo YJ *et al*., 2010). Likewise, the inhibition of NF-kB nuclear translocation by corilagin was shown in the LPS-stimulated RAW264.7 macrophage cell line, and subsequent suppression of IL-1β, TNF-α, iNOS, and COX-2 expression was attributed to blocking NF-κB activation (Zhao *et al*., 2008, guo YJ *et al*., 2010). The antiviral activity of corilagin was tested in vitro using vero cells, which kidney epithelial cells originating from the African green monkey. Since vero cells are interferon-deficient, the compensatory NF-κB pathway might strongly influence any antiviral activity of corilagin. The role of NF-κB pathway on antiviral activity in the epithelium has long been considered to be a critical defense mechanism during primary viral infection. In this regard, despite of elevated level of TNF-α after EV71 infection, 2C Protein from EV71 suppresses TNF-α-mediated NF-κB activation by inhibiting IKKβ activation.

This suggests that EV71 infection efficiently blocks NF-κB activation even in the presence of enhanced TNF-α production during the progression of EV71-induced diseases (Zheng Z *et al*., 2011). Thus, NF-κB activation by corilagin might induce NF-κB-dependent apoptosis of primarily infected cells during the initial stage of infection and stop viral incubation that is required for propagation.

Although the antiviral activity of corilagin in vitro seems promising, it was already reported that the oral administration of corilagin did not induced significant biological activity in vivo (Park JH *et al*., 2011, Shiota *et al*., 2004). It might be due to the difficulty in the absorption and metabolism of corilagin by intestinal microflora. The incubation of tannins with anaerobic microflora contained in feces of animals led to hydrolysis of them into several metabolites including gallic acid and ellagic acid (Shiota *et al*., 2004). Thus, future in vivo studies are required to determine the antiviral activity of corilagin against EV71 and CVA16 using i.v. or i.p. administration. These administration methods might not result in the hydrolysis of corilagin.

In vivo toxicological studies with ellagitannins have shown that these compounds are very safe and only show minimal toxicity (Kinoshita S. *et al*., 2007, Lin LT *et al*., 2013, Chen Y *et al*., 2011). Although there are few reports that have utilized *P. urinaria*, *P. amarus* extracts have been evaluated in human

clinical studies to treat of HIV, jaundice, and hypertension based on promising preclinical results (Patel JR *et al*., 2011, Notka F *et al*., 2004, Yuandani *et al*., 2010). Several clinical studies using *P. amarus* have shown that there are no significant side effects or toxicity (Patel JR *et al*., 2011). Thus, it is presumed that corilagin and *P. amarus* might not be toxic; however, future in vivo studies are needed, to confirm the safety of corilagin.

Collectively, the antiviral activity of corilagin against EV71 and CVA16 was confirmed. Corilagin was selected based on previous bio assay-guided screening of compounds isolated from Ardisia splendens. In the current study, *P. urinaria* was decided to be used as the primary source of corilagin. In addition to corilagin, the EtOAc and BuOH fractionates from MeOH extract of *P. urinaria* also exhibited significant antiviral activity against EV71 and CVA16.

Table 2.1 Antiviral activity of corilagin against CA16 and EV71

	CA16			EV71		
Compound	CC_{50} [a]	IC_{50} [b]	TI [c]	CC_{50} [a]	IC_{50} [b]	TI [c]
Corilagin	107	32.33±0.42	3.30	327	5.60±1.07	58.39
Ribavirin	>50	ND [d]	-	>50	ND [d]	-

Results are presented as the mean IC_{50} values ± S.D obtained from three independent experiments carried out in triplicate.

[a] Concentration required to reduce cell growth by 50% (μg/mL).

[b] Concentration required to inhibit virus-induced CPE by 50% (μg/mL).

[c] Therapeutic index = CC_{50}/IC_{50}

[d] Not determined

Table 2.2 Antiviral activity of *P. urinaria* extracts against CA16 and EV71 in Vero cells.

	CA16			EV71		
Test material	CC_{50}[a]	IC_{50}[b]	TI[c]	CC_{50}[a]	IC_{50}[b]	TI[c]
Methanol	>50	45.9 ± 5.39	2.17	>50	32.4 ± 0.35	1.54
Hexane	>50	ND[d]	-	>50	ND[d]	-
Ethyl acetate	>50	4.79 ± 0.81	17.2	>50	48.3 ± 9.17	1.41
Butanol	>50	39.5 ± 6.51	2.53	>50	31.8 ± 2.29	1.57
Water	>50	ND[d]	-	>50	ND[d]	-
Ribavirin	>50	ND[d]	-	>50	ND[d]	-

Results are presented as the mean IC_{50} values ± S.D obtained from three independent experiments carried out in triplicate.

[a] Concentration required to reduce cell growth by 50% (μg/mL).

[b] Concentration required to inhibit virus-induced CPE by 50% (μg/mL).

[c] Therapeutic index = CC_{50}/IC_{50}

[d] Not determined

Figure legends

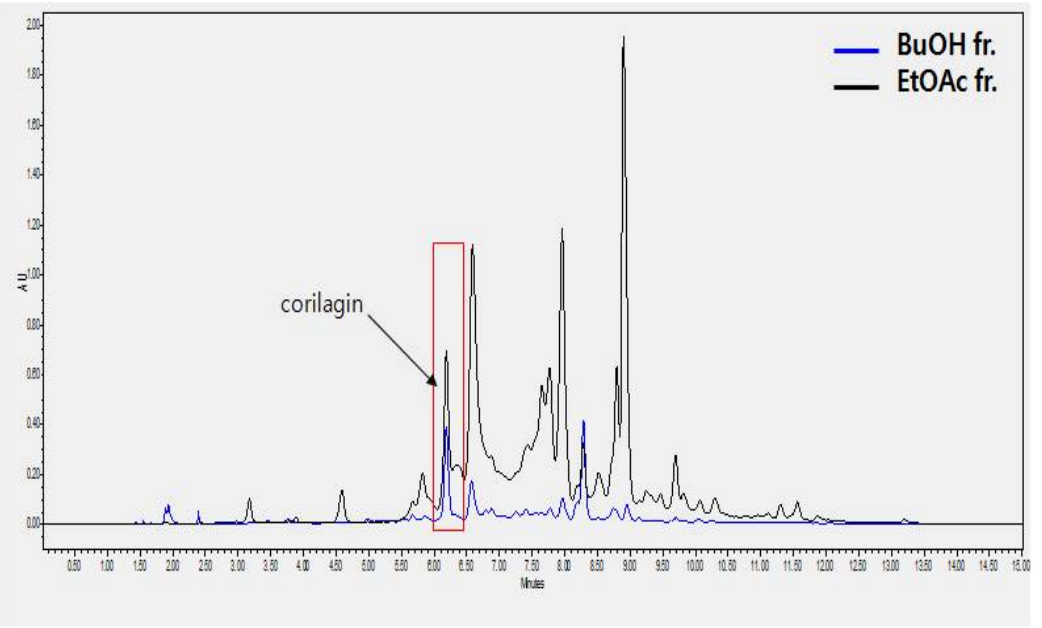

Figure 2.1 HPLC chromatograms of EtOAc and *n*-BuOH fractions of *P. urinaria.*
The existence of corilagin in the EtOAc and *n*-BuOH fractions of *P. urinaria* were assessed using HPLC-DAD as described in Materials and Methods.

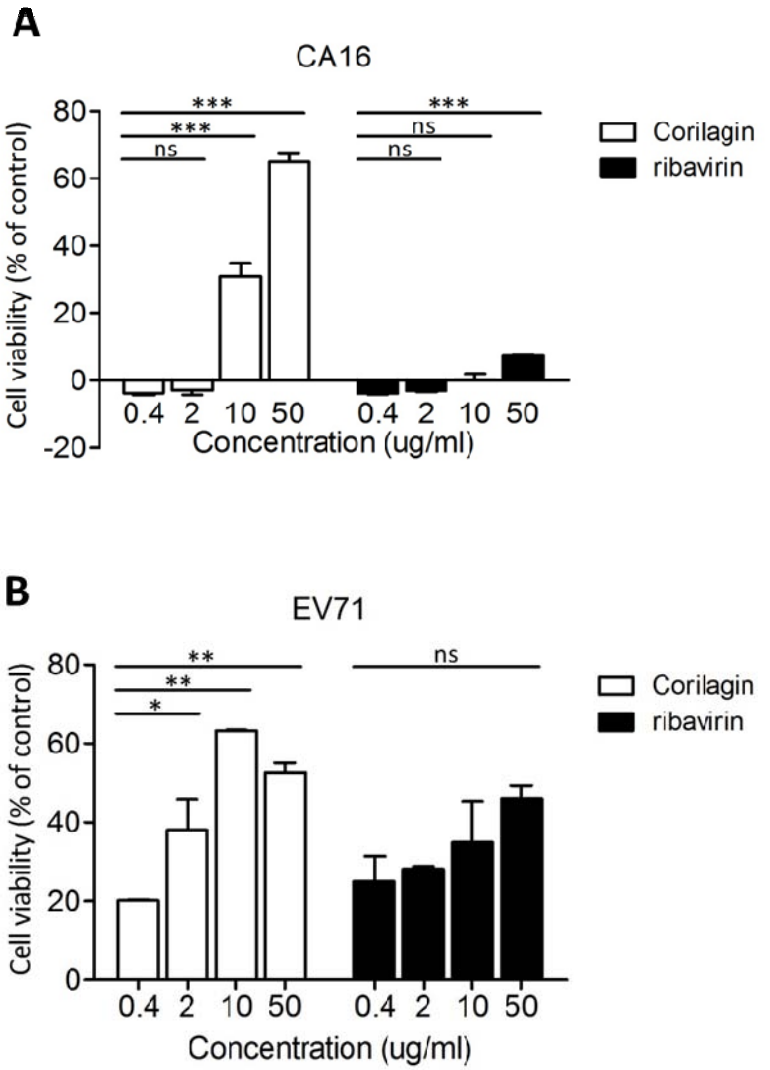

Figure 2.2 The antiviral activity of corilagin against CA16 and EV71.

Antiviral activity of corilagin against CA16 (A) and EV71 (B) in Vero cells. The indicated concentration of corilagin ranging from 0.4 to 50 μg/ml were added to Vero cells infected with 50% cell culture infective dose (CCID50). Cells were culture for 48 h and the antiviral activity was determined by CPE reduction assay. Results are presented as the mean ± S.D. of the percentage values obtained from three independent experiments carried out in triplicate.

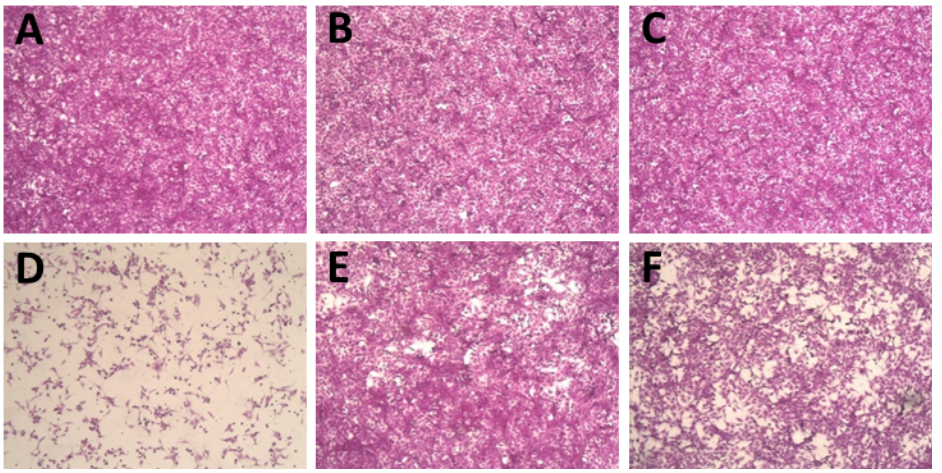

Figure 2.3 Effect of corilagin on CVA16–induced cytotoxicity. The CVA16-infected Vero cells were treated with 50 μg/mL of ribavirin or corilagin for 48 h. The viability of cells was evaluated by sulforhodamine B (SRB) assay, and the morphology of cells was imaged using a microscope. (A) Noninfected cells; (B) noninfected cells treated with corilagin; (C) noninfected cells treated with ribavirin; (D) CVA16-infected cells; (E) CVA16-infected cells treated with corilagin; (F) CVA16-infected cells treated with ribavirin.

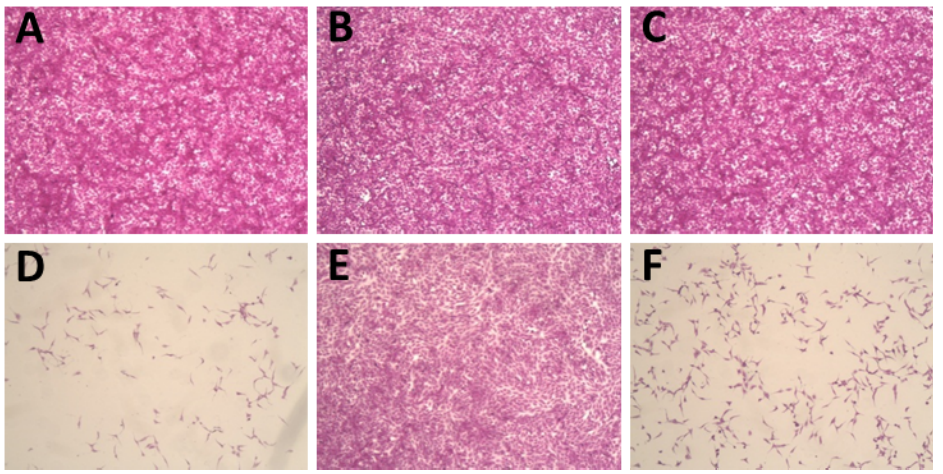

Figure 2.4 Effect of corilagin on EV71–induced cytotoxicity. The EV71-infected Vero cells were treated with 50 μg/mL of ribavirin or corilagin. After 48 h of incubation, cell viability was evaluated using the sulforhodamine B (SRB) assay, and the morphology of cells was imaged using a microscope. (A) Noninfected cells; (B) noninfected cells treated with corilagin; (C) noninfected cells treated with ribavirin; (D) EV71-infected cells; (E) EV71-infected cells treated with corilagin; (F) EV71-infected cells treated with ribavirin.

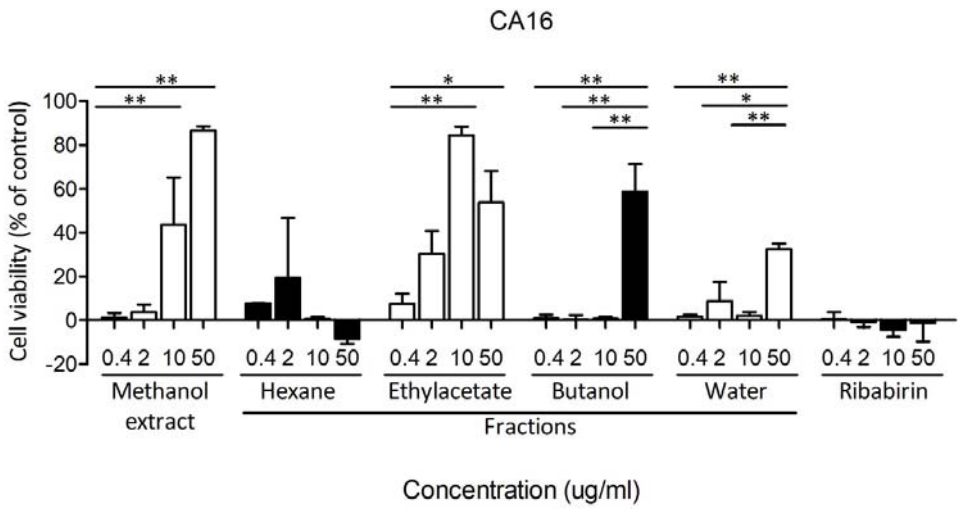

Figure 2.5 Antiviral activity of *P. urinaria* extracts against CVA16

Antiviral activity of organic fractions obtained from *P. urinaria* MeOH extract was assessed against CVA16 in Vero cells. The indicated concentrations of the organic fractions were introduced into CVA16-infected Vero cells with a 5-fold diluted concentration ranging from 0.4 to 50 μg/ml. After 48 h of incubation, the antiviral activity was investigated by a CPE reduction assay. Results are presented as the mean ± S.D of the percentage values obtained from three independent experiments carried out in triplicate.

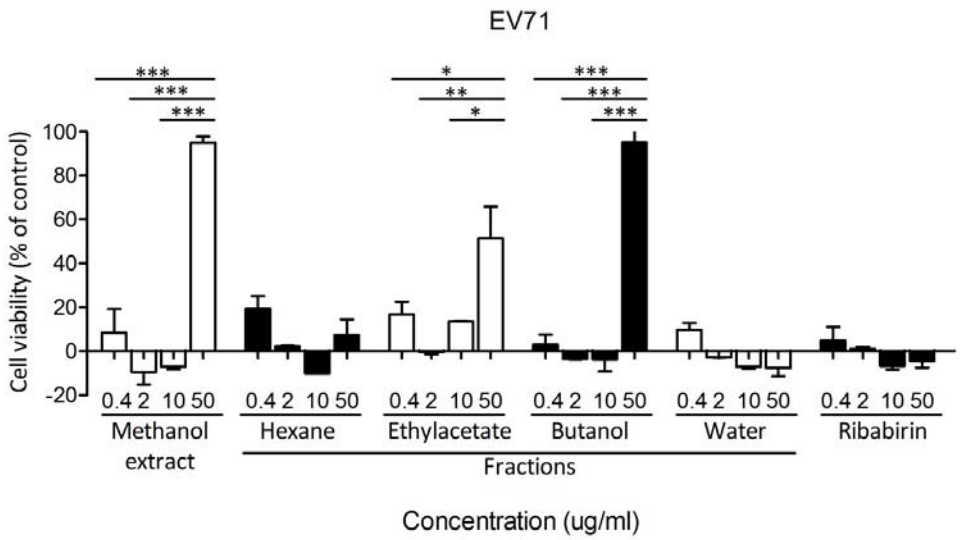

Figure 2.6 Antiviral activity of *P. urinaria* extracts against EV71

Antiviral activity of organic fractions obtained from *P. urinaria* MeOH extract was assessed against EV71 in Vero cells. The indicated concentrations of the organic fractions were treated into EV71-infected Vero cells with a 5-fold diluted concentration ranging from 0.4 to 50 μg/ml. After 48 h of incubation, the antiviral activity was investigated by CPE reduction assay. Results are presented as the mean ± S.D of the percentage values obtained from three independent experiments carried out in triplicate.

Chapter 3

Mechanism studies and In vivo Antiviral Activities of *Hedera Helix* and *Phyllanthus urinaria* against Enterovirus Infections

Abstract

Tha animal models of human enterovirus are not still well established. Animal models for EV71 are under developing and non human primate is the only one model for carrying out antiviral and vaccine studies. Among EVs, CVB3 is known to cause diseases including pancreatitis and myocarditis in mouse model. For broad uses of *Hedera Helix* and *Phyllanthus urinaria* against EVs infection, antiviral activities of two plants were investigated by CVB3 mice model. Wild-type inbred BALB/c mice were purchased from Charles River Laboratories (Orient Bio Inc., Sungnam, Korea). Mice were maintained under specific pathogen-free conditions in the experimental facility at KCDC. The experiments were approved by the Institutional Animal Care and Use committees of the

KCDC. The female 5-week-old BALB/c mice weighed from 14g to 16g were divided equally into four groups, control and intraperitoneally inoculated with 1 x 10^6 TCID50 of CVB3 in 150ul Vero cell lysate. Mice inoculated with vero cell lysate only without CVB3 infection was used as control. After anesthetized with ether, all mice were sacrificed on day 5 postinfection for histological analysis. Addionally, replicon system of EVs are werll established. EV71 and CVB3 replicon systems were used to reveal the antiviral mechanism of the agents.

Key words : Antiviral activity, Animal model, Enterovirus, *Hedera Helix*, *Phyllanthus urinaria*

3.1. Introduction

Coxsackieviruses, members of the genus Enterovirus of the family Picornaviridae, are amongst the smallest human pathogens, approximately 30 nm in diameter, and consist of an RNA molecule surrounded by a protein capsid (Heli Harvala *et al*. 2005). Coxsackievirus B3 (CVB3) is a single stranded, positive sense RNA virus that is one of the major etiological viral agents of human myocarditis and dilated cardiomyopathy (Iwona A *et al*., 2014). Especially, studies that show an association between coxsackievirus infection and acute pancreatitis in humans are given additional support by the extensive data from mouse studies demonstrating that CVB3 is tropic for the exocrine pancreas (Ramsingh AI., 1997). To identify the broad spectrum use of *Hedera helix* and *Phyllanthus urinaria*, the pancreatitis mice model by CVB3 infection was used and the antiviral activities of Hederasaponin B and *P. urinaria* extracts were evaluated.

The replicon system of EVs was used to identity the antiviral mechanism of hederasaponin B and corilagin from *Hedera helix* and *Phyllanthus urinaria,* respectively. Rupintrivir was used as the positive control of antiviral agent against EVs. In luciperase assay, hederasaponin B, corilagin and rupintrivir were

assessed at the concentration of 50,10,2 and 0.4μg/mL after transfection to vero cells.

3.2. Materials and Methods

Animal model

Wild-type inbred BALB/c mice were purchased from Charles River Laboratories (Orient Bio Inc., Sungnam, Korea). Mice were maintained under specific pathogen-free conditions in the experimental facility at KCDC. The experiments were approved by the Institutional Animal Care and Use committees (IACUC) of the KCDC. The female 5-week-old BALB/c mice weighed from 14g to 16g were divided equally into four groups, control and intraperitoneally inoculated with 1 x 10^6 TCID50 of CVB3 in 150ul Vero cell lysate. Mice inoculated with vero cell lysate only without CVB3 infection was used as control. After anesthetized with ether, all mice were sacrificed on day 5 postinfection for histological analysis.

Treatment of antiviral agents

Phyllanthus urinaria extract or hederasaponin B was administered to mice (5 mice per group). *Phyllanthus urinaria* extract (50mg/kg/dose) or hederasaponin B (10mg/kg/dose), dissolved in 0.5% carboxymethyl cellulose (CMC) and PBS in 3% ethanol (EtOH), was orally and intraperitoneally administered once daily for 4 days after CVB3 infection. BALB/c mice were intraperitoneally injected with 1 x 10^6 TCID50 of CVB3.

Construction of Enterovirus replicon

To identify the antiviral mechanism of hederasaponin B and corilagin against EV71 and CVB3, replicon system (pRIBFluc-EV71) was used in this study. Replicon system is contructed to P1 capside coding region of EVs be replaced by firefly luciferase gene to identify the effects of only replication and translation in cells (Fig.3.1a). To carry out the replicon experiment, pRiBFuc-EV71 and p53CB3Luc plasmid were kindly donated by Dr. Cho at KRIBB in Ochang, Chungbuk (Fig.3.1b). Using this replicon system, target agents could be identified to have antiviral activities against EVs by suppression of replication of viruses.

pRIB Fluc-EV71 plasmid was linearized by MluI enzyme and in vitro transcription was carried out by T7 RNA polymerase (promega RiboMAX kit). Right after 2ug trasfection of transcripted RNAto vero cell, compounds were treated for 8 hrs by x-treme. Finally, luciferase activities were assessed by one-glo.

Replicon assay.

Replicons used for this study were p53CB3/T7-LUC and pRIBFluc-EV71 wt(kindly provided by Korea research institute of biosience and biotechnology) (van Kuppeveld FJ1 *et al*., 1995). The replicon used contains the CVB3 and EV71 cDNA in which the P1 capsid coding region is replaced by the firefly luciferase gene (Wessels E1 *et al* 2005). The plasmid was linearized, purified, and transcribed in vitro by T7 RNA polymerase, and transcript RNAs were transfected into vero cells. After transfection, the cells were cultured in the presence or absence of drugs at 37°C. At 8 h posttransfection, the cells were lysed and analyzed for firefly luciferase activity.

3.3. Results

Antiviral activities of hederasaponin B and corilagin using replicon system against EV71 and CVB3

The effect of hederasaponin B and coliragin on RNA replication and translation were studied directly by use of a subgenomic replicon p53CB3/T7-Luc and pRIBFluc-EV71 wt, in which the P1 region has been replaced with the firefly luciferase gene (Fig. 3.2). The level of luciferase activity in cells is a measure of viral RNA replication and translation. This result showed that, 10 ug/ml coliragin strongly inhibits firefly luciferase accumulation, demonstrating that coliragin hinders viral RNA replication or translation, while 10 ug/ml hederasaponin B, a general inhibitor against viral RNA replication or translation.

Hederasaponin B and corilagin were treated at the concentration of 50, 10, 2, 0.4 ug/ml for 8hrs right after 2ug trasfection of transcripted RNA to vero cells using x-treme. Then, using one-glo, luciferase activities were evaluated. In this experiment, rupintrivir known as 3C protease inhibitor of EVs were used as positive control. Rupintrivir was shown to suppress the viral translation and transcription in cells perfectly. Coliragin showed over 50% suppression effect at the concentration of 10 ug/ml. However, hederasaponin B showed almost no

antiviral activity in the replicon system (Fig.3.5 and 3.7). However, in identification of cell toxicity, both hederasaponin B and corilagin showed high safety in use (Fig.3.6 and 3.8).

Effects of antiviral agent against CVB3 infection in vivo

Firstly, the body weight changes of CVB3-infected mice were assessed to monitor the pancreatic infection of CVB3. CVB3-infected mice lost their weight after CVB3 infection, and which was not recovered within 5 days postinfection (Fig. 3.9a). The treatment of CVB3-infected mice with *Phyllanthus urinaria* extract and hederasaponin B did not significantly attenuate the CVB3-induced body weight loss (Fig. 3.9a).

For the pathological analysis, histology sections were prepared from pancreata of mice which were infected with CVB3 and treated with vehicle, *Phyllanthus urinaria* extract or hederasaponin B. Uninfected pancreata of mice were histologically normal (Fig. 3.9b), and following 5 days after CVB3 infection, the pancreata of mice showed almost complete ablation of acini. However, in CVB3-infected mice, which were also administered with *Phyllanthus urinaria* extract or

hederasaponin B showed reduced pathology, though still have some acini (Fig. 3.9b). Collectively, these results suggest that *Phyllanthus urinaria* extract and hederasaponin B exhibited antiviral activity against CVB3 in vivo, and partially prevented pancreatic infection of CVB3.

3.4. Discussion

In this study, the antiviral mechanisms of hederasaponin B and corilagin against EV71 and CVB3 infection were identified using replicon system. Only corilagin block the EV71 and CVB3 replication in cells. To remove the possibility of luciperase ativity result by cell toxicity, celltiter-glo assay was carried out and both corilagin and hederasaponin B showed no vero cell toxicities. Resultly, corilagin was identified to play a role at the viral replication.

Addtionall, for revealing antiviral mechanism of hederasaponin B, IRES dual reporter system will be applicated furtherly. The animal experiments of CVB3 were carried out through checking the body weight and histology of pancreas. The data of body weight were not significantly different because the group only administered CVB3 without antiviral agent showed low less of body weight. *P. urinary* extract was used instead of corilagin in vivo assay because it was difficult to obtain the pure compound of corilagin. In the further study, the effect

of the corilagin will be tested at the mice model to identify the antiviral activity in vivo. However, pancreatic acini were influenced by treatment of hederasaponin B and *P. urinary* extract (Fig. 3.9). Further study will be carried out after setting up of EV71 mice models to identify the effects of corialgin and hederasaponin B in vivo.

Figure legends

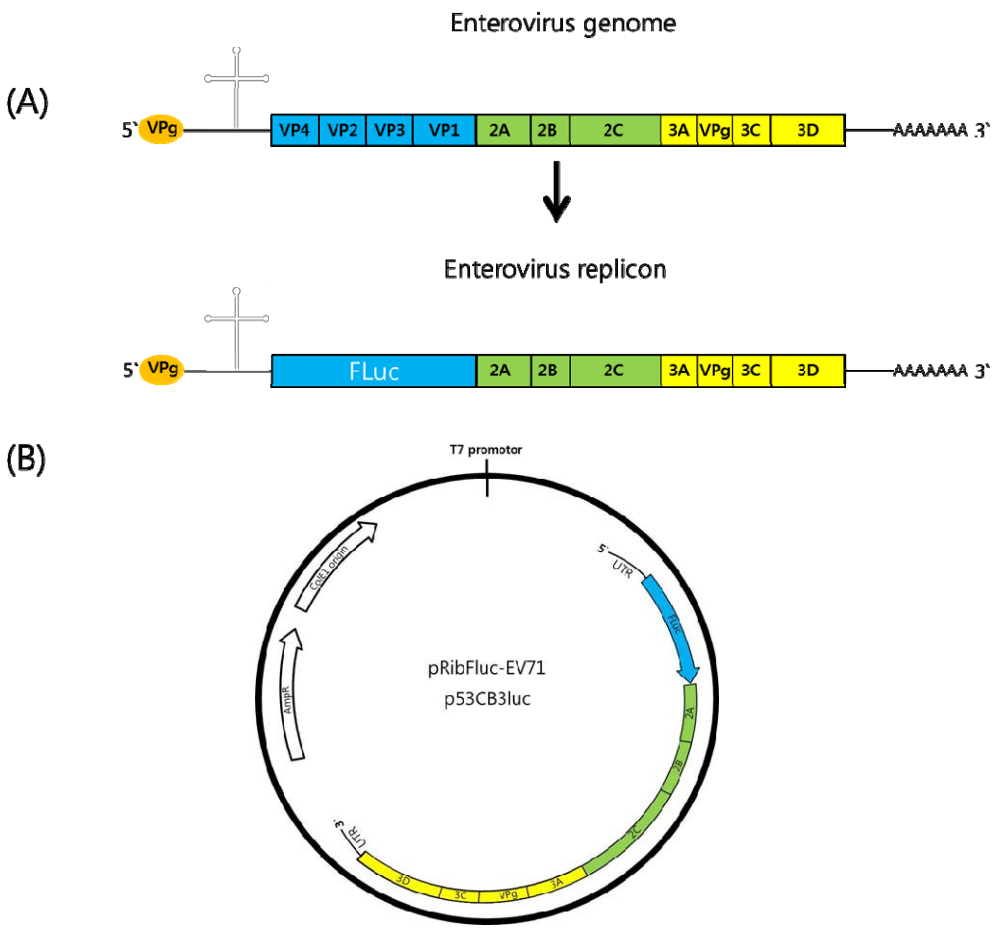

Figure 3.1 Construction of replicon system of enterovirus

Enterovirus P1 capside coding region is replaced byfirefly luciferase gene. Viral replication and translation can be observed clearly (A). Replicon pRiBFuc-EV71and p53CB3Luc plasmid for replicon asssay (B)

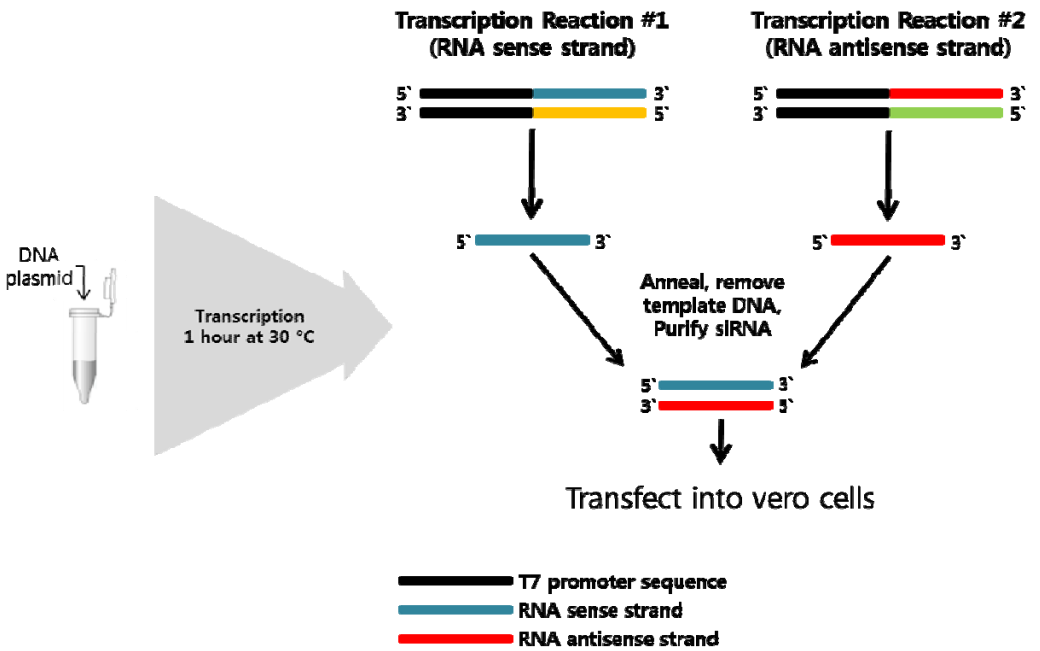

Figure 3.2 In vitro transcription of pRIBFluc-EV71and p53CB3luc plasmid by T7 RNA polymerase (promega RiboMAX kit)

T7 RNA polymerase was treated in pRIBFluc-EV71and p53CB3luc plasmid at 30°C for 1 hr. Then, 2ug of viral mRNA was transfected in vero cell using x-treme and incubated at 37°C incubator for 24 hrs.

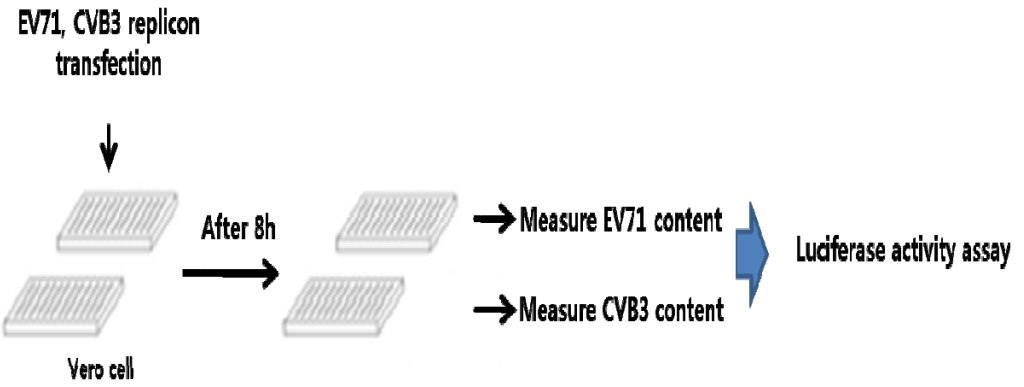

Figure 3.3 Concept of luciperase assay of EV71 and CVB3 in vero cells

Viral mRNAtransfected vero cells were seeded at 96 well plate. Each compound was treated for 8hrs and 100ul of ONE-glo was treated to each well for 10 mins. Firefly luciferase activity was estimated using microplate reder.

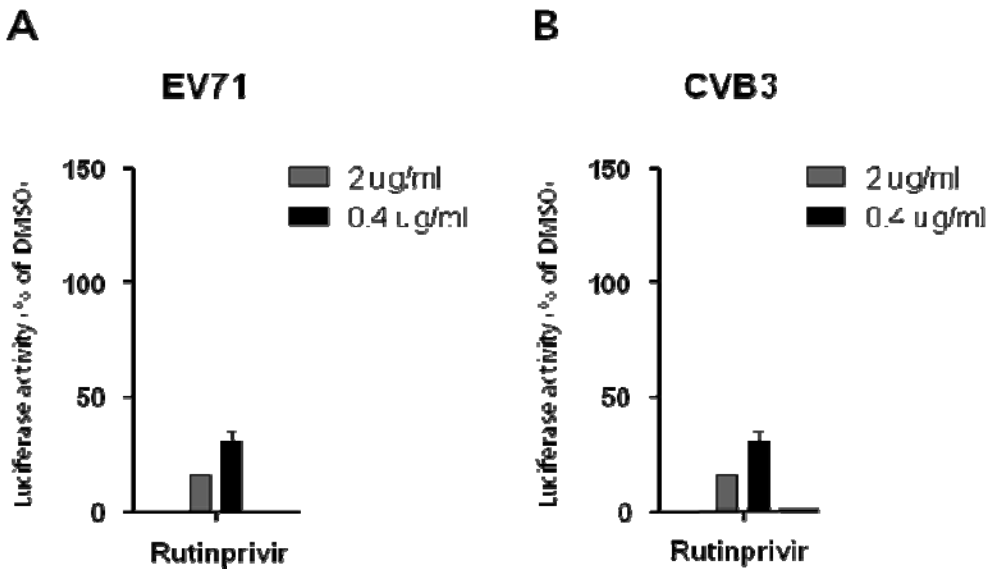

Figure 3.4 Effects of rupintrivir against EV71 and CVB3 replicon

Rupintrivir was used as positive control (entrerovirus 3C protease inhibitor). Rupintrivir suppressed viral replication. Rupintrivir showed effect at the concentration of 2, 0.4 ug/ml by reduction of over 50% viral replication. .

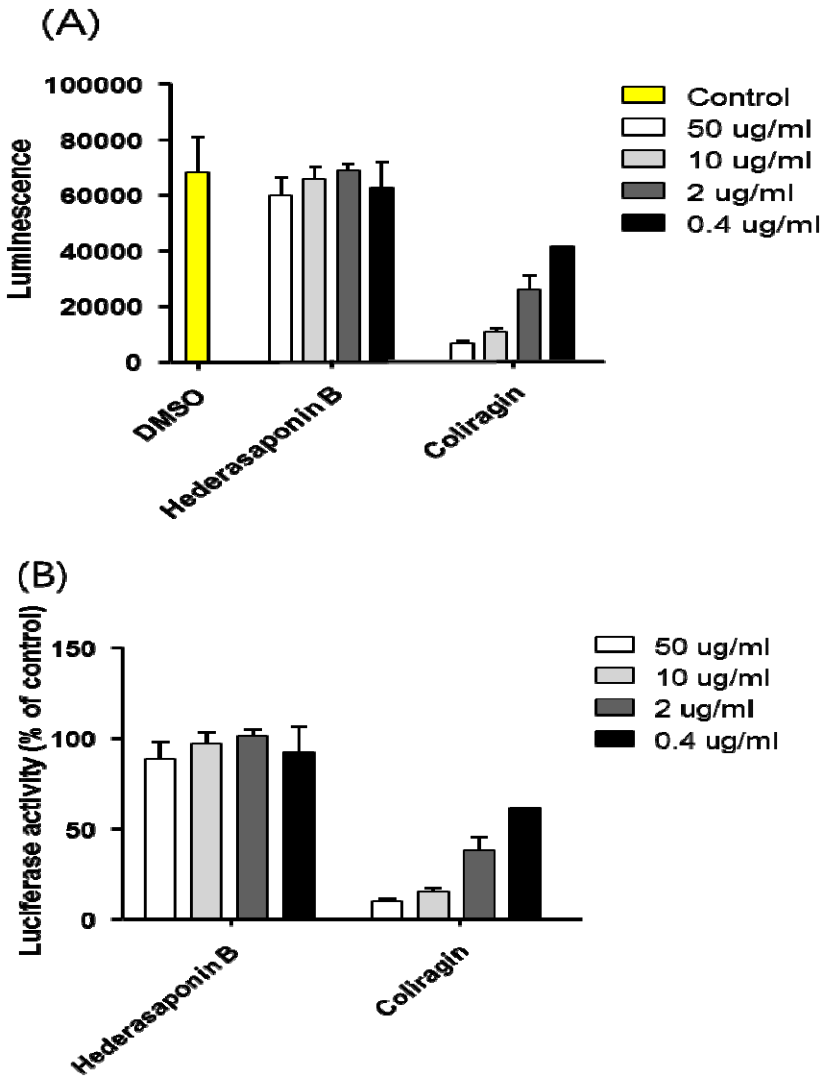

Figure 3.5 Effects of Hederasaponin B and Corilagin to EV71 replicon

Luciferase activities were estimated after treatment of hederasaponin B and corilagin in EV71 ransfected vero cell. Corilagin showed effectiveness against EV71 replicon dose dependently compared to hederasaponin B.

(A) Luminescense data of antiviral agents against EV71 replicon

(B) Luciferase activities data antiviral agents against EV71 replicon

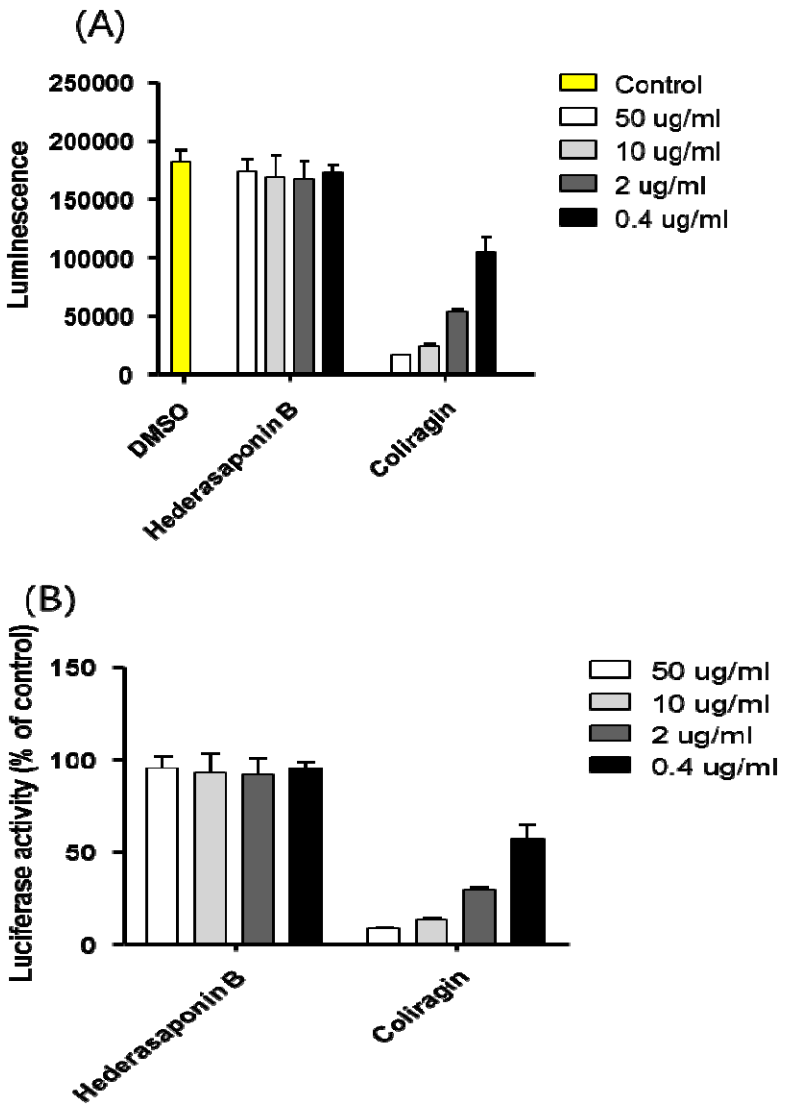

Figure 3.6 Effects of Hederasaponin B and Corilagin to CVB3 replicon

Luciferase activities were estimated after treatment of hederasaponin B and corilagin in CVB3 ransfected vero cell. Corilagin showed effectiveness against CVB3 replicon dose dependently compared to hederasaponin B.

(A) Luminescense data of antiviral agents against CVB3 replicon

(B) Luciferase activities data antiviral agents against CVB3 replicon

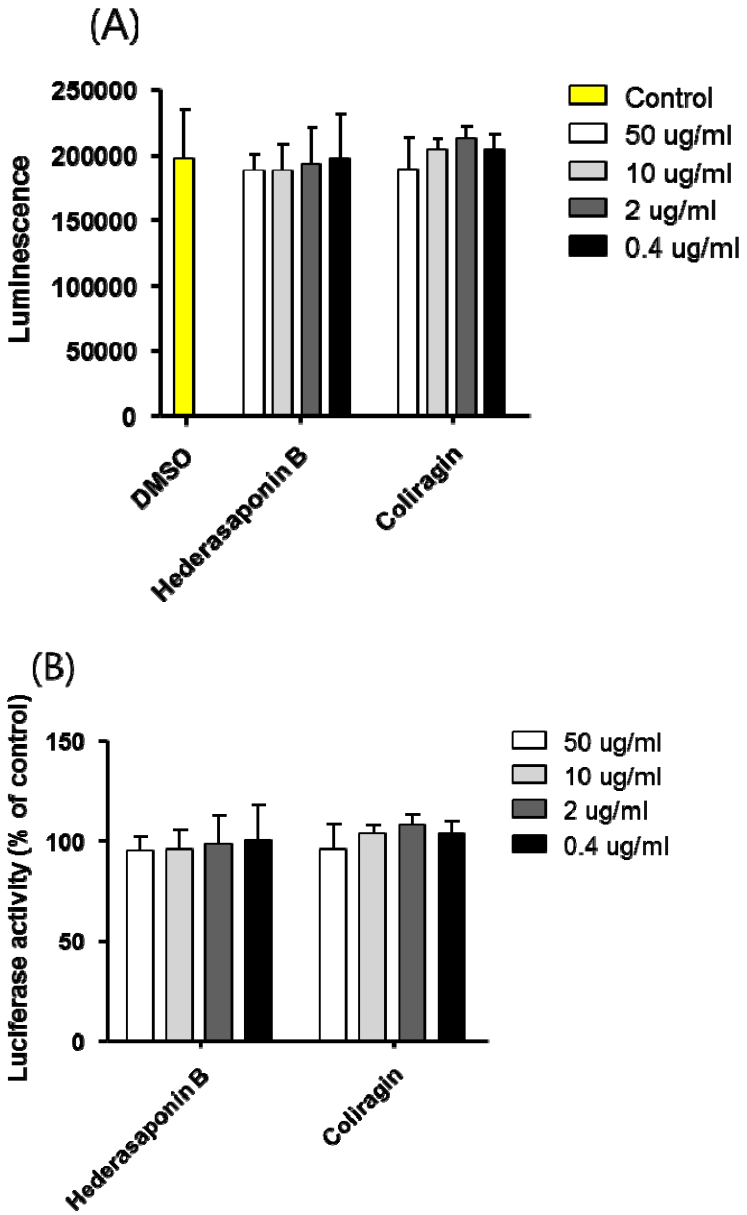

Figure 3.7 Cytotoxicities of antiviral agents in EV71 replicon system

No cytotoxicity was found in uses of two compounds (vector tranfection only).

(A) Luminescense data of antiviral agents in EV71 replicon system

(B) Luciferase activities data antiviral agents in EV71 replicon system

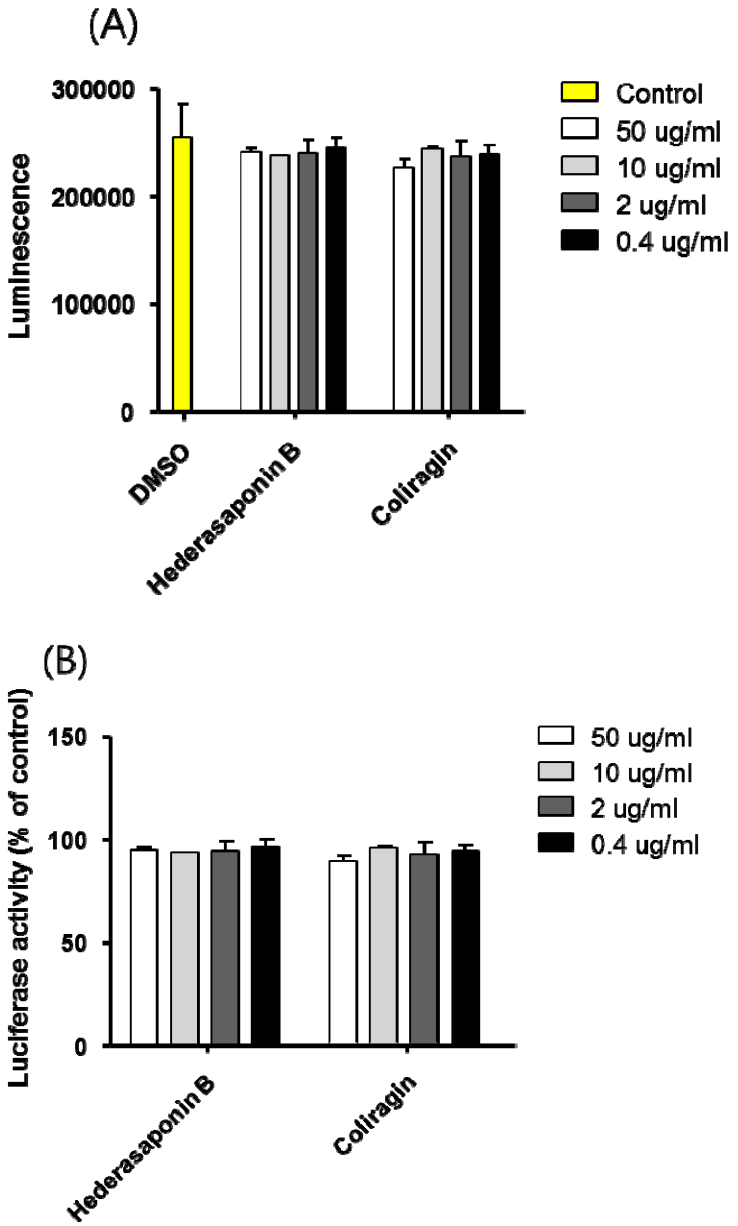

Figure 3.8 Cytotoxicities of antiviral agents in CVB3 replicon system

No cytotoxicity was found in uses of two compounds (vector tranfection only).

(A) Luminescense data of antiviral agents in CVB3 replicon system

(B) Luciferase activities data antiviral agents in CVB3 replicon system

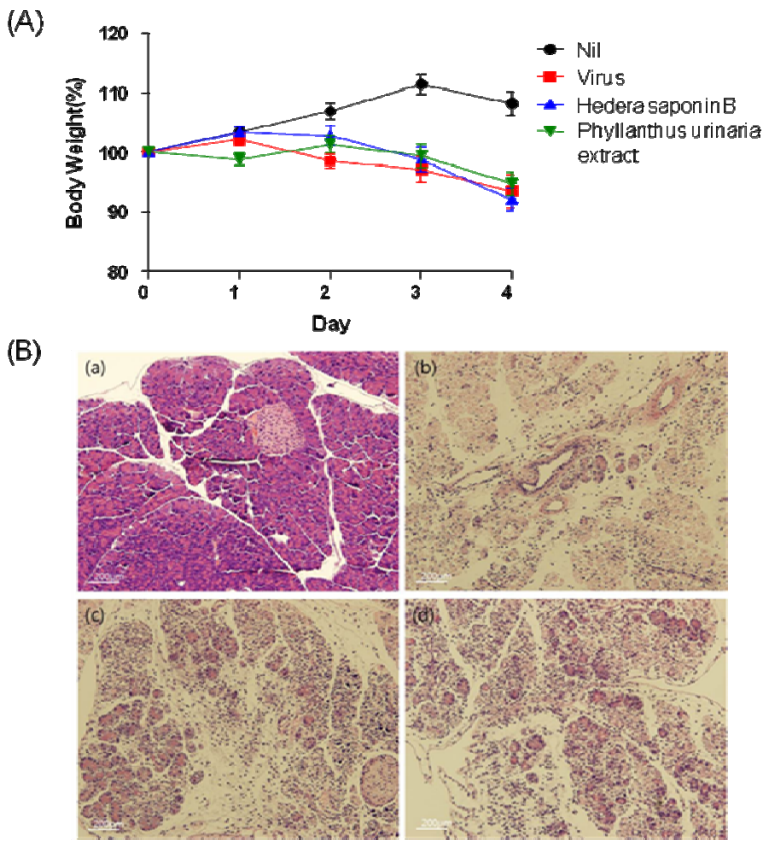

Figure 3.9 Morbidity and histological analysis by *Phyllanthus urinaria* extract or hederasaponin B administration with CVB3 infections. *Phyllanthus urinaria* extract or hederasaponin B conditioned groups of BALB/c mice were infectedwith a 1 x 10^6 TCID50 of CVB3 and then assessed for morbidity as measured by weight loss (A). On day 5 post CVB3 infection, histology was analyzed form pancreata of mice. (Ba) noninfected mice; (Bb) CVB3-infected mice; (Bc) CVB3-infected mice treated with *Phyllanthus urinaria* extract; (Bd) CVB3-infected mice treated with hederasaponin B.

General Conclusions

In this study, hederasaponin B and corilagin from *Hedera Helix* and *Phyllanthus urinaria*, respectively showed the antiviral activities against enterovirus infection especially HFMD.

First, hederasaponin B from *Hedera Helix* showed antiviral effectiveness against EV71 which is main cause of HFMD with neurological complications including death in children. Also, hederasaponin B was identified to have antiviral acitivities against EV71 subgenotype C3 and C4a causing outbreaks in Asian countries.

Additionally, the antiviral activity of corilagin from *Phyllanthus urinaria* against EV71 and CVA16 was identified. CVA16 is the second main causative agent associated with HFMD by enterivurus infection. Corilagin has effectiveness to both EV71 and CVA16.

To reveal the antiviral mechanism of each compound, replicon system was used. In this experiment, corilagin was identified to inhibit the viral replication of HEV including EV71 and CVB. However, hederasaponin B was not and further study as IRES dual reporter system will be carried out to identify the antiviral mechanism against HEV.

Finally, for the identification of effectiveness of two compounds in vivo, animal studies using mice model were carried out. CVB3 mice model was used because it is well established animal model in HEV. Both hederasaponin B and corilagin showed effectiveness in mice pancreatic acini. It is expected that both compounds can be used as broad spectrum use as anti HEV therapy.

In conclusion, this study suggested that hederasaponin B and corilagin will be novel therapeutic agents against enterovirus infections including severe cases with neurological complications. Futher study will be required to reveal more detail mechanism of antiviral activities and broad spectrum uses to other enterovirus infection of two agents.

국 문 초 록

엔테로바이러스 감염증 치료제 개발을 위한 아이비엽과 진주초의 항바이러스 효과 연구

여 상 구

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엔테로바이러스는 picornaviridae family 에 속하는 positive-sense single-stranded RNA virus 이다. 엔테로바이러스는 인간을 포함한 포유류에게서 다양한 질환을 일으키며 일반적으로 인간에게 감염되는 엔테로바이러스는 수족구병, 감기증상, 심근염, 급성 출혈성 결막염, 무균성 뇌수막염 등의 질환을 일으킨다. 특히, 최근 보고에 의하면 엔테로바이러스

71 형과 콕사키바이러스 16 형 등은 신경증상을 동반하는 중증 수족구병을 유발하여 소아를 사망에 이르게 할 수도 있다. 하지만 이러한 상황에도 불구하고 아직까지 개발이 완료된 효과적인 치료제나 백신이 없는 상황이다. 이러한 배경을 근거로, 현재 다양한 치료제 개발이 진행중이다. 본 연구에서는 아이비엽에서 추출한 hederasaponin B 와 여우구슬에서 추출한 corilagin 을 이용하여 엔테로바이러스에 대한 항바이러스 효과를 확인하였다. Hederasaponin B 의 경우 최근 한국을 포함한 동남아시아 지역에서 가장 심각한 중증 수족구병의 원인체인 엔테로바이러스 71 C3 와 C4a 형에 대한 항바이러스 효과를 확인하였으며 corilagin 은 수족구병의 주요 원인체인 엔테로바이러스 71 형과 CVA16 에 효과가 있는 것을 확인하였다. 또한 엔테로바이러스 replicon 을 이용한 실험 수행 결과 corilagin 의 경우 바이러스 replication 을 억제하는 것을 확인할 수 있었다.

엔테로바이러스의 경우 실험동물 현재 가능한 실험동물 모델이 극히 제한적이며 이중 CVB3(콕사키바이러스 B 형)에 대한 마우스 모델이 가장 잘 확립되어 있으며 췌장염을 유발한다. 이에 본 연구에서 발견한 항바이러스 물질이 다양한 범위의 엔테로바이러스에 적용될 수 있는지 여부를 확인하기 위해 이를 이용한 실험을 진행하였다.

본 실험은 질병관리본부 실험동물윤리위원회의 승인하에 이루어졌으며 5 주령 female Balb/c 마우스에서의 Hederasaponin B 와 corilagin 의 엔테로바이러스에 대한 항바이러스 효과를 확인하였다. 이러한 연구결과를 바탕으로 아이비엽과 여우구슬 추출물이 효과적인 수족구유발 엔테로바이러스에 대한 항바이러스 물질로 사용이 가능하며 향후 다양한 엔테로바이러스 감염증에도 확대 적용할 수 있을 것으로 예상된다.

키워드 : 엔테로바이러스, Hederasaponin B, 아이비엽, Corilagin, 진주초(여우구슬), 항바이러스활성

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References

Brown, B. A., Oberste, M. S., Alexander, J. P., Jr., Kennett, M. L. and Pallansch, M. A. (1999). Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J Virol73, 9969-9975.

Cardosa, M. J., Perera, D., Brown, B. A., Cheon, D., Chan, H. M., Chan, K. P., Cho, H. and McMinn, P. (2003). Molecular epidemiology of human enterovirus 71 strains and recent outbreaks in the Asia-Pacific region: comparative analysis of the VP1 and VP4 genes. Emerg Infect Dis9, 461-468.

Chan, Y. F., Sam, I. C. and AbuBakar, S. (2010). Phylogenetic designation of enterovirus 71 genotypes and subgenotypes using complete genome sequences. Infect Genet Evol10, 404-412.

Chang, L. Y., Lin, T. Y., Huang, Y. C., Tsao, K. C., Shih, S. R., Kuo, M. L., Ning, H. C., Chung, P. W. and Kang, C. M. (1999). Comparison of

enterovirus 71 and coxsackie-virus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. Pediatr Infect Dis J18, 1092-1096.

Choi, H. J., Kim, J. H., Lee, C. H., Ahn, Y. J., Song, J. H., Baek, S. H. and Kwon, D. H. (2009). Antiviral activity of quercetin 7-rhamnoside against porcine epidemic diarrhea virus. Antiviral Res81, 77-81.

Chumakov, M., Voroshilova, M., Shindarov, L., Lavrova, I., Gracheva, L., Koroleva, G., Vasilenko, S., Brodvarova, I., Nikolova, M., Gyurova, S., Gacheva, M., Mitov, G., Ninov, N., Tsylka, E., Robinson, I., Frolova, M., Bashkirtsev, V., Martiyanova, L. and Rodin, V. (1979). Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Arch Virol60, 329-340.

De Palma, A. M., Vliegen, I., De Clercq, E. and Neyts, J. (2008). Selective inhibitors of picornavirus replication. Med Res Rev28, 823-884.

Fleischer, R. and Laessig, K. (2003). Safety and efficacy evaluation of pleconaril for treatment of the common cold. Clin Infect Dis37, 1722.

Gepdiremen, A., Mshvildadze, V., Suleyman, H. and Elias, R. (2005). Acute anti-inflammatory activity of four saponins isolated from ivy: alpha-hederin, hederasaponin-C, hederacolchiside-E and hederacolchiside-F in carrageenan-induced rat paw edema. Phytomedicine12, 440-444.

Graci, J. D. and Cameron, C. E. (2006). Mechanisms of action of ribavirin against distinct viruses. Rev Med Virol16, 37-48.

Han, L. T., Fang, Y., Li, M. M., Yang, H. B. and Huang, F. (2013). The Antitumor Effects of Triterpenoid Saponins from the Anemone flaccida and the Underlying Mechanism. Evidence-based complementary and alternative medicine : eCAM2013, 517931.

Huang, Y. P., Lin, T. L., Hsu, L. C., Chen, Y. J., Tseng, Y. H., Hsu, C. C., Fan,

W. B., Yang, J. Y., Chang, F. Y. and Wu, H. S. (2010). Genetic diversity and C2-like subgenogroup strains of enterovirus 71, Taiwan, 2008. Virol J7, 277.

Hyypia, T., Hovi, T., Knowles, N. J. and Stanway, G. (1997). Classification of enteroviruses based on molecular and biological properties. J Gen Virol78 (Pt 1), 1-11.

Laine, P., Savolainen, C., Blomqvist, S. and Hovi, T. (2005). Phylogenetic analysis of human rhinovirus capsid protein VP1 and 2A protease coding sequences confirms shared genus-like relationships with human enteroviruses. J Gen Virol86, 697-706.

Lin, Z. X., Hoult, J. R. and Raman, A. (1999). Sulphorhodamine B assay for measuring proliferation of a pigmented melanocyte cell line and its application to the evaluation of crude drugs used in the treatment of vitiligo. J Ethnopharmacol66, 141-150.

McMinn, P. C. (2002). An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol Rev26, 91-107.

Park, K. S., Choi, Y. J. and Park, J. S. (2012). Enterovirus infection in Korean children and anti-enteroviral potential candidate agents. Korean J Pediatr55, 359-366.

Pevear, D. C., Tull, T. M., Seipel, M. E. and Groarke, J. M. (1999). Activity of pleconaril against enteroviruses. Antimicrob Agents Chemother43, 2109-2115.

Poyry, T., Kinnunen, L., Hyypia, T., Brown, B., Horsnell, C., Hovi, T. and Stanway, G. (1996). Genetic and phylogenetic clustering of enteroviruses. J Gen Virol77 (Pt 8), 1699-1717.

Sieben, A., Prenner, L., Sorkalla, T., Wolf, A., Jakobs, D., Runkel, F. and Haberlein, H. (2009). Alpha-hederin, but not hederacoside C and

hederagenin from Hedera helix, affects the binding behavior, dynamics, and regulation of beta 2-adrenergic receptors. Biochemistry48, 3477-3482.

Simoes, C. M., Amoros, M. and Girre, L. (1999). Mechanism of antiviral activity of triterpenoid saponins. Phytotherapy research : PTR13, 323-328.

Suleyman, H., Mshvildadze, V., Gepdiremen, A. and Elias, R. (2003). Acute and chronic antiinflammatory profile of the ivy plant, Hedera helix, in rats. Phytomedicine10, 370-374.

Trute, A., Gross, J., Mutschler, E. and Nahrstedt, A. (1997). In vitro antispasmodic compounds of the dry extract obtained from Hedera helix. Planta Med63, 125-129.

Wang, S. M., Lei, H. Y., Huang, K. J., Wu, J. M., Wang, J. R., Yu, C. K., Su, I. J. and Liu, C. C. (2003). Pathogenesis of enterovirus 71 brainstem encephalitis in pediatric patients: roles of cytokines and cellular immune activation in

patients with pulmonary edema. J Infect Dis188, 564-570.

Jae-Hyung Song. Isolation and Antiviral Activity of Manassantin B, Saucerneol, Saucernetin-8 from Saururus chinensis Baill against Human Enteroviruses. 2012. PhD dgree. Wonkwang Univ. Korea.

Zhang, Y., Wang, J., Guo, W., Wang, H., Zhu, S., Wang, D., Bai, R., Li, X., Yan, D., Zhu, Z., Tan, X., An, H., Xu, A. and Xu, W. (2011). Emergence and transmission pathways of rapidly evolving evolutionary branch C4a strains of human enterovirus 71 in the Central Plain of China. PLoS One6, e27895.

Zhao L, Zhang SL, Tao JY, Pang R, Jin F, Guo YJ, Dong JH, Ye P, Zhao HY, Zheng GH: Preliminary exploration on anti-inflammatory mechanism of corilagin (beta-1-o-galloyl-3,6-(r)-hexahydroxydiphenoyl-d-glucose) in vitro. International immunopharmacology (2008) 8(7):1059-1064.

Duan W, Yu Y, Zhang L: Antiatherogenic effects of phyllanthus emblica

associated with corilagin and its analogue. Yakugaku zasshi : Journal of the Pharmaceutical Society of Japan (2005) 125(7):587-591.

Moreira J, Klein-Junior LC, Cechinel Filho V, de Campos Buzzi F: Anti-hyperalgesic activity of corilagin, a tannin isolated from phyllanthus niruri l. (euphorbiaceae). Journal of ethnopharmacology (2013) 146(1):318-323.

Patel JR, Tripathi P, Sharma V, Chauhan NS, Dixit VK: Phyllanthus amarus: Ethnomedicinal uses, phytochemistry and pharmacology: A review. Journal of ethnopharmacology (2011) 138(2):286-313.

Liu KC, Lin MT, Lee SS, Chiou JF, Ren S, Lien EJ: Antiviral tannins from two phyllanthus species. Planta Med (1999) 65(1):43-46.

Jikai L, Yue H, Henkel T, Weber K: One step purification of corilagin and ellagic acid from phyllanthus urinaria using high-speed countercurrent chromatography. Phytochem Anal (2002) 13(1):1-3.

Kinoshita S, Inoue Y, Nakama S, Ichiba T, Aniya Y: Antioxidant and hepatoprotective actions of medicinal herb, terminalia catappa l. From okinawa island and its tannin corilagin. Phytomedicine : international journal of phytotherapy and phytopharmacology (2007) 14(11):755-762.

Li Y, Yu S, Liu D, Proksch P, Lin W: Inhibitory effects of polyphenols toward hcv from the mangrove plant excoecaria agallocha l. Bioorganic & medicinal chemistry letters (2012) 22(2):1099-1102.

Satomi H, Umemura K, Ueno A, Hatano T, Okuda T, Noro T: Carbonic anhydrase inhibitors from the pericarps of punica granatum l. Biological & pharmaceutical bulletin (1993) 16(8):787-790.

Thitilertdecha N, Teerawutgulrag A, Kilburn JD, Rakariyatham N: Identification of major phenolic compounds from nephelium lappaceum l. And their antioxidant activities. Molecules (Basel, Switzerland) (2010) 15(3):1453-1465.

Park JH, Joo HS, Yoo KY, Shin BN, Kim IH, Lee CH, Choi JH, Byun K, Lee B, Lim SS, Kim MJ *et al*: Extract from terminalia chebula seeds protect against experimental ischemic neuronal damage via maintaining sods and bdnf levels. Neurochemical research (2011) 36(11):2043-2050.

Lin LT, Chen TY, Chung CY, Noyce RS, Grindley TB, McCormick C, Lin TC, Wang GH, Lin CC, Richardson CD: Hydrolyzable tannins (chebulagic acid and punicalagin) target viral glycoprotein-glycosaminoglycan interactions to inhibit herpes simplex virus 1 entry and cell-to-cell spread. Journal of virology (2011) 85(9):4386-4398.

Lin LT, Chen TY, Lin SC, Chung CY, Lin TC, Wang GH, Anderson R, Lin CC, Richardson CD: Broad-spectrum antiviral activity of chebulagic acid and punicalagin against viruses that use glycosaminoglycans for entry. BMC microbiology (2013) 13(187.

Yang Y, Xiu J, Zhang L, Qin C, Liu J: Antiviral activity of punicalagin toward human enterovirus 71 in vitro and in vivo. Phytomedicine : international

journal of phytotherapy and phytopharmacology (2012) 20(1):67-70.

Yang Y, Xiu J, Liu J, Zhang L, Li X, Xu Y, Qin C: Chebulagic acid, a hydrolysable tannin, exhibited antiviral activity in vitro and in vivo against human enterovirus 71. International journal of molecular sciences (2013) 14(5):9618-9627.

Yang Y, Zhang L, Fan X, Qin C, Liu J: Antiviral effect of geraniin on human enterovirus 71 in vitro and in vivo. Bioorganic & medicinal chemistry letters (2012) 22(6):2209-2211.

Guo YJ, Zhao L, Li XF, Mei YW, Zhang SL, Tao JY, Zhou Y, Dong JH: Effect of corilagin on anti-inflammation in hsv-1 encephalitis and hsv-1 infected microglias. European journal of pharmacology (2010) 635(1-3):79-86.

Notka F, Meier G, Wagner R: Concerted inhibitory activities of phyllanthus amarus on hiv replication in vitro and ex vivo. Antiviral research (2004) 64(2):93-102.

Hau DK, Zhu GY, Leung AK, Wong RS, Cheng GY, Lai PB, Tong SW, Lau FY, Chan KW, Wong WY, Lam KH *et al*: In vivo anti-tumour activity of corilagin on hep3b hepatocellular carcinoma. Phytomedicine : international journal of phytotherapy and phytopharmacology (2010) 18(1):11-15.

Yuandani, Ilangkovan M, Jantan I, Mohamad HF, Husain K, Abdul Razak AF: Inhibitory effects of standardized extracts of phyllanthus amarus and phyllanthus urinaria and their marker compounds on phagocytic activity of human neutrophils. Evid Based Complement Alternat Med (2013) 2013(603634.

Mao Q, Wang Y, Yao X, Bian L, Wu X, Xu M, Liang Z: Coxsackievirus a16: Epidemiology, diagnosis, and vaccine. Human vaccines & immunotherapeutics (2013) 10(2).

Tan CW, Chan YF, Sim KM, Tan EL, Poh CL: Inhibition of enterovirus 71 (ev-71) infections by a novel antiviral peptide derived from ev-71 capsid protein vp1. PloS one (2012) 7(5):e34589.

Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH: Virology, epidemiology, pathogenesis, and control of enterovirus 71. The Lancet infectious diseases (2010) 10(11):778-790.

Cheng A, Fung CP, Liu CC, Lin YT, Tsai HY, Chang SC, Chou AH, Chang JY, Jiang RH, Hsieh YC, Su IJ *et al*: A phase i, randomized, open-label study to evaluate the safety and immunogenicity of an enterovirus 71 vaccine. Vaccine (2013) 31(20):2471-2476.

Lin SY, Wang CC, Lu YL, Wu WC, Hou WC: Antioxidant, anti-semicarbazide-sensitive amine oxidase, and anti-hypertensive activities of geraniin isolated from Phyllanthus urinaria. Food Chem Toxicol (2008) 46(7):2485-2492

Choi HJ, Kim JH, Lee CH, Ahn YJ, Song JH, Baek SH, Kwon DH: Antiviral activity of quercetin 7-rhamnoside against porcine epidemic diarrhea virus. Antiviral research (2009) 81(1):77-81.

Choi HJ, Song JH, Park KS, Kwon DH: Inhibitory effects of quercetin 3-rhamnoside on influenza a virus replication. Eur J Pharm Sci (2009) 37(3-4):329-333.

Chen Y, Chen C: Corilagin prevents tert-butyl hydroperoxide-induced oxidative stress injury in cultured n9 murine microglia cells. Neurochemistry international (2011) 59(2):290-296.

Zheng Z, Li H, Zhang Z, Meng J, Mao D, Bai B, Lu B, Mao P, Hu Q, Wang H: Enterovirus 71 2c protein inhibits tnf-alpha-mediated activation of nf-kappab by suppressing ikappab kinase beta phosphorylation. Journal of immunology (Baltimore, Md : 1950) (2011) 187(5):2202-2212.

Shiota S, Shimizu M, Sugiyama J, Morita Y, Mizushima T, Tsuchiya T: Mechanisms of action of corilagin and tellimagrandin i that remarkably potentiate the activity of beta-lactams against methicillin-resistant staphylococcus aureus. Microbiology and immunology (2004) 48(1):67-73.

Buskiewicz IA, Koenig A, Roberts B, Russell J, Shi C, Lee SH, Jung JU, Huber SA, Budd RC c-FLIP-Short Reduces Type I Interferon Production and Increases Viremia with Coxsackievirus B3. PLoS One. (2014)9:9(5)

Heli Harvala, Hannu Kalimo, Jeffrey Bergelson, Glyn Stanway and Timo Hyypiä. Tissue tropism of recombinant coxsackieviruses in an adult mouse model. J. of General Virology. (2005) 86(7):1897-907

Ramsingh AI. Coxsackieviruses and pancreatitis. Front Biosci. (1997)16(2):53-62

Van Kuppeveld FJ1, Galama JM, Zoll J, Melchers WJ. Genetic analysis of a hydrophobic domain of coxsackie B3 virus protein 2B: a moderate degree of hydrophobicity is required for a cis-acting function in viral RNA synthesis. J Virol. 1995 Dec;69(12):7782-90.

Wessels E1, Duijsings D, Notebaart RA, Melchers WJ, van Kuppeveld FJ. A proline-rich region in the coxsackievirus 3A protein is required for the

protein to inhibit endoplasmic reticulum-to-golgi transport. J Virol. 2005 Apr;79(8):5163-73.

Nonpolio Enterovirus and Human Parechovirus Surveillance --- United States, 2006—2008 Weekly, MMWR, CDC, December 10, 2010 / 59(48);1577-1580