**A THESIS FOR THE DEGREE OF MASTER OF SCIENCE**

# Antiviral Activity of Constituents Identified in Propolis against Human Rhinovirus and their Possible Mode of Action

프로폴리스 유래성분의 **human rhinovirus**에 대한
항바이러스 활성 및 작용 기구 연구

**By**

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**UNDER THE DIRECTION ADVISER YOUNG JOON AHN**
**SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL**
**OF SEOUL NATIONAL UNIVERSITY**

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**APPROVED AS A QUALIFIED DISSERTATION OF MIN JUNG KWON**
**FOR THE DEGREE OF MASTER OF SCIENCE**
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# Antiviral Activity of Constituents Identified in Propolis against Human Rhinovirus and their Possible Mode of Action

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## ABSTRACT

HRV is the most frequent causative agents of mild upper respiratory tract infections, including common colds, and accounts for about 30-50% of all cases. They are usually a self-limited illness confined to the upper respiratory tract. However, HRV can also infect the lower respiratory tract symptoms, such as pneumonia and wheezing in children and chronic obstructive pulmonary disease (COPD) in adults. There have been difficulties to provide efficient treatments or vaccination because of over 100 serotypes. Current common cold medicine on sale is only efficient to reduce the symptoms. Accordingly, drug misuse and abuse has been raised and antihistamines and antibiotics usages have caused many side effects. Its socio-economic importance in terms of employee absenteeism, physician visits, and medication costs is also considerable. Therefore, safe

and efficient on diverse serotypes treatment development is demanded significantly for anti-HRV agents. This study aimed to investigate antiviral activity of propolis involved in bee products and its constituents and elucidate their possible mode of action.

An assessment was made of the cytotoxic and antiviral activity of the propolis and another previously known propolis constituents toward HeLa cell (ATCC CCL–2) and three HRV serotypes (HRV-2 ATCC VR-1112AS/GP, HRV-3 ATCC VR-1113, and HRV-4 ATCC VR–1114AS/GP), respectively, using a Sulforhodamine B bioassay. The Brazilian ethanolic extract was sequentially partitioned into different organic solvents and chloroform soluble fraction and ethyl acetate soluble fraction showed remarkable antiviral activities. Respective fractions were separated and identified by various chromatographic techniques. Kaempferol ($CC_{50,}$ 65.0; $IC_{50}$, 3.7; TI, 17.57) from chloroform soluble fraction showed significantly high antiviral activity compared with currently used antiviral agents with broad spectrum, ribavirin ($CC_{50,}$ > 100; $IC_{50}$, 74.7; TI, > 1.3), even with high cytotoxicity. *(E)*-ρ-coumaric acid ($CC_{50,}$ > 100; $IC_{50}$, 74.6; TI, > 1.3) from ethyl acetate soluble fraction and ribavirin did not differ significantly in the antiviral activity from each other. As the result of investigation on possible mode of action, kaempferol and *(E)*-p-coumaric acid did not alter the infectivity of virus particles (HRV-3). Time-course addition experiments of two constituents were also carried out. 10 μg/mL kaempferol affects the initial stage of HRV infections (0-6 h) and 100 μg/mL (*E*)-*p*-coumaric acid showed its antiviral activity when added just after the virus inoculation and very early stage after the virus inoculation (0-1 h). Inhibition of RNA replication levels of HRV-3 was investigated by real-time RT PCR. At the presence of kaempferol and (*E*)-*p*-coumaric acid, the RNA replication levels of HRV were reduced. Also expression of

RNA levels of rhinovirus receptors (ICAM-1) and inflammatory cytokines (IL-6) were inhibited by two constituents. As the result of antiviral activity of previously known propolis constituents, High antiviral activity was also observed with chrysin, quercetin, acacetin, luteoloin (4.1-5.8 μg $mL^{-1}$). Caffeic acid, protocatechuic acid, fisetin, galangin, and ferulic acid showed moderate antiviral activity compared with ribaivrin (9.4-48.3 μg $mL^{-1}$). Among the quercetin, chrysin, caffeic acid, and protocatechuic acid, only protocatechuic acid decreased the virus infectivity through interaction with virus particles.

In conclusion, global efforts to reduce the level of antibiotics justify further studies on propolis-derived materials containing the compounds described as potential antiviral products or lead molecules for the prevention or eradication from humans from diseases by HRV.

**Key words**: Human rhinovirus, Natural antiviral agents, propolis, kaempferol, (*E*)-*p*-coumaric acid

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# Ⅰ. Introduction

Human rhinoviruses (HRVs) are known to be one of the most frequent causative agents of mild upper respiratory tract infections, or common colds (Savolainen et al., 2003; Heikkinen and Jarvinen, 2003). They are usually a self-limited illness confined to the upper respiratory tract. HRV can also infect the lower respiratory tract symptoms, such as pneumonia and wheezing in children and chronic obstructive pulmonary disease (COPD) in adults (Collinson et al., 1996; Pitkäranta et al., 1998). In addition, the frequent appearance of HRV infections and their economic importance in terms of employee absenteeism, physician visits, and medication costs make it a subject of primary importance. (Gaudernak et al., 2002; Rotbart, 2002.; Heikkinen et al., 2003).

In spite of the medical significance of the common cold and its complications, attempts to develop effective treatments or vaccination have been relatively limited and unsuccessful because of a number of serotypes (Turner, 2001). In practice, symptomatic treatment of the common cold is still a useful approach, although there are not many treatment protocols for which the efficacy and tolerability have been documented in placebo-controlled trials. Prevention, using vaccination, from respiratory viruses has so far had little success because of absence of a suitable common antigen cross the wide range of HRV serotypes (Heikkinen et al., 2003; Papi and Contoli, 2011). As the results of no medicine and vaccination, drug misuse and abuse has been raised and antihistamines

and antibiotics usages have caused many side effects, although they are not effective against viruses (Gonzales et al., 2001). OECD Health Data 2010 Report said that antibiotic use in Korea is the highest among surveyed nations and 51 percent Koreans who answered the survey thought that antibiotics were effective for common cold symptoms (OECD, 2010). Its rash administrations can trigger antibiotic-resistant disease. As an example, ribavirin has been used as chemoprophylaxis for treatment of various DNA and RNA viruses including HRV, but it also concerned about virus-acquired resistance to ribavirin (Graci and Cameron, 2006). There is, therefore, a critical need for the development of new improved anti-HRV agents with novel target sites because a commercial vaccine is still not available.

Natural compounds extracted from plants or plant-derived materials have been suggested as alternative sources for anti-HRV products. This approach is appealing, in part, because they constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe and often act at multiple and novel target sites, thereby reducing the potential for resistance (Raskin et al., 2002). Much effort has been focused on plant preparations and their constituents as potential sources of commercial antiviral products for prevention or eradication of HRV. There are some criteria to HRV-induced cold treatments and prevention: 1) broad spectrum activity because of the high number of HRV serotypes, 2) early administration in infection to demonstrate a good antiviral effect because of the fast infection kinetics, 3) safety because of the broad application by millions of people, 4) low risk of resistance development (Patick, 2006; Rollinger and Schmidtke, 2010). In the screening of plant-

derived materials for anti-HRV activity, ethanol extract of propolis was shown to have antiviral activity against HRV-4 ATCC VR–1114AS/GP. No information has been obtained concerning the potential of propolis-derived materials to control HRV, although pharmacological actions of propolis have been well described by Castaldo and Cappaso (2002).

In this study, an assessment is made of the cytotoxic and anti-HRV activities of the constituents from Brazilian propolis and another 12 known compounds of propolis toward HeLa cell (ATCC CCL-2) and three HRV serotypes (HRV-2 ATCC VR-1112AS/GP, HRV-3 ATCC VR-1113, and HRV-4 ATCC VR–1114AS/GP), respectively. The cytotoxic and antiviral activities of the test compounds were compared with those of ribavirin with broad spectrum. The possible modes of action of the test compounds also were elucidated.

# II. Literature review

## 1. Rhinovirus

HRV is the most frequent causative agents of mild upper respiratory tract infections, including common colds, and accounts for about 50% of all cases (Mäkelä et al., 1998; Heikkinen et al., 2003). The hallmark symptoms of common colds are nasal stuffiness and discharge, sneezing, sore throat, and cough. However, 80% of asthma exacerbations in school-aged children and half of all asthma exacerbations in adults are associated with viral upper respiratory infection, and the majority of viruses isolated are HRV (Arruda, et al., 1997; Johnston et al., 1995; Gern et al., 1999; Yamada and Sasaki, 2003). Acute otitis media, sinusitis, and cystic fibrosis in children are also major clinical illnesses induced by rhinovirus. Furthermore, rhinovirus can infect the lower respiratory tract symptoms, such as pneumonia and wheezing in children and COPD in adults (Collinson et al., 1996; Pitkäranta et al., 1998). The common cold is a considerable economic burden on society in terms of visits to doctors and other health-care providers, treatments, and absences from work, school, or day care (Turner, 2002; Heikkinen et al., 2003; Rollinger and Schmidtke, 2010). The market of common colds medicine was reported to be 80 billion won in Republic of Korea in 2005. (http:// http://weekly.khan.co.kr/khnm.html?mode=view&artid=13217&code=115)

The incidence of HRV infections peaks during autumn, with a subsequent smaller outbreak in the spring. In the transmission of viruses that cause upper respiratory infections, there are three major mechanisms: 1) hand contact with secretion that contain

the virus, either directly from an infected person or indirectly from environmental surfaces, 2) small-particle aerosols lingering in the air for an extended time, or 3) direct hit by large-particle aerosols from an infected person. However, viral contamination of the hand contacts is the primary route of HRV transmission (Gwaltney et al., 1978; Heikkinen et al., 2003).

HRVs are single-stranded, positive-sense RNA enteroviruses. The name reflects the primary site of infection, upper respiratory tract. They were assigned to the family Picornaviridae because HRV are nonenveloped, icosahedral viruses of small size with a diameter of about 30 nm (*pico* means small in Latin). They have been cataloged primarily by capsid serotyping relative to a historical repository of about 100 strains, obtained from clinical specimens. HRVs are classified by their use of either intercellular adhesionmolecule-1 (ICAM-1) or low-density lipoprotein receptor (LDLR) as their receptor for cell entry (Greve et al., 1989; Hofer et al., 1994; Palmenberg et al., 2009). The anti-viral drug sensitivity pattern, the existence of a dimorphic binding site, can also be used to parse the strains into two groups called antiviral groups A and B (Andries et al., 1990). Currently, virus classification is primarily based on phylogenetic relationships. The partial sequences of viral capsid-coding regions, noncoding regions and a limited number of regions, and a limited number of complete genomes have resulted in a division of the original strains into two species: HRV-A and HRV-B (Horsnell et al., 1995; Ledford et al., 2004). In 2009, new phylogenetic analysis with whole genome sequences of HRV was investigated and it represents HRV-C and -D as third and fourth species, respectively (Palmenberg et al., 2009).

Infectious virions consist of an icosahedral protein shell (capsid) that surrounds and protects the genome, a single positive-stranded RNA molecule of approximately 7400 nucleotides. The capsid consists of 60 copies of each of the four capsid protein VP1-4 (Rollinger and Schmidtke, 2010). The HRV genome begins with a short peptide (VPg) covalently coupled to the 5'-non-coding region (5'-NCR), followed by the capsid-coding region, P1. The P2 and P3 regions encode for the nonstructural proteins that include two viral proteases (2A and 3C) and the RNA-dependent RNA polymerase. These regions are followed by a short 3'-non-coding region and a poly-A tail. The replication of HRVs happens in the cytoplasm of the host cell. After entering the cell, the genome is translated firstly and then copied to a "negative strand", an RNA molecule with a complementary nucleotide sequence. The latter is subsequently used as a template for synthesizing a large number of copies of the viral genome, which are partly used as mRNAs for viral protein synthesis and partly incorporated as genomes into the progeny virus particles (Savolainen et al., 2003).

The pathogenesis of HRV infections has been clarified through observation from experimentally induced infections in normal volunteers (Bardin et al., 1996; Gern et al., 1997). Rhinovirus infection begins with the deposition of viruses in the anterior nasal mucosa or in the eye, from where they get to the nose via the lacrimal duct. The viruses are then transported to the posterior nasopharynx by mucociliary action. In the adenoid area, the viruses gain entrance to epithelial cells by binding to specific receptors on the cells. Once inside the epithelial cell, the virus starts to replicate rapidly. Progeny viruses can be detected within 8-10 h after intranasal inoculation of rhinoviruses (Harris and Gwaltney, 1996). The infectious dose of rhinovirus is small, and up to 95% of individuals

without antibodies against the specific viral serotype are infected after intranasal challenge (Hendley and Gwaltney, 1988; Gwaltney and Hayden, 1992). The shedding of rhinoviruses peaks on the second day after intranasal inoculation and thereafter decreases rapidly, but small amounts of viruses can be discovered in nasal secretions for up to 3 weeks after infection (Winther et al., 1986; Gustafson et al., 1996). HRV infection triggers vasodilation and increased vascular permeability in the nasal mucosa, leading to nasal obstruction and rhinorrhoea. The mechanism is still unknown because no histopathological changes were observed in nasal biopsy specimens from infected person. This led to the suggestion that clinical symptoms are primarily caused by the inflammatory response of the host to the virus infection and not by the cytopathic effect (CPE) of HRV (Winther et al., 1986). Experimental infection was also used to examine the causation between rhinovirus infection and lower respiratory infection such as asthma exacerbations. Its mechanisms are still unknown and only immunological response is investigated (James et al., 1999; Yamada et al., 2003). Immunological investigations suggest that cytokines, including interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), IL-8, IL-6, and IL-11 were found in nasal secretions of patients with colds. The cytokines concentration indicates the severity of symptoms in upper and lower HRV-induced respiratory tract disease (Noah et al., 1995; Einarsson et al., 1996; Zhu et al., 1996).

**2. Anti-HRV agents derived from natural products**

Nature provides an astonishing pool of secondary metabolites from plants, microorganisms, protozoan, insects, and other animal sources. One assumes that

secondary metabolites evolved as reaction to their target receptors related to defense, protection, attraction, and signaling. These adaption progresses have enriched not only the metabolites' structural diversity but have also optimized drug-like metabolic traits likely to have favorable pharmacokinetic properties (Samiulla et al., 2005; Zheng et al., 2005). Most of natural sources remedies are described in ethnopharmacological sources or handed down for generations. A clear advantage of the application of these products is their absent or relatively low toxicity due to a usually long-term trial especially on self-limited illness, common cold. They also may attribute in a beneficial way for the treatment of common cold by reducing symptom severity and duration due to their immune-modulating, antioxidative, and antiinflammatory properties. Beside these commonly observed bioactivities of natural products, multi component mixtures like botanicals often show overlapping symptomatic effect as well as synergistic and/or additive properties (Rollinger and Schmidtke, 2010). Also, these natural products can be improved by repeated structure modification in the hope that the change being made will result in increased potency, selectivity, duration of action, bioavailability, and reduced toxicity (Vlietinck and Berghe, 1991).

Newman and Cragg (2007) analyzed the number of drugs approved between 1981 and 2006 and circumstantiated that especially the anti-infective area is strongly dependent on natural products and structures derived from natural scaffolds. The anti-infective including the antiviral vaccines are with 22.8% or 230 launched drugs by far the major category with only about 30% being synthetic in origin. From 1981 to 2006, 78 vaccines and antiviral drugs have been approved. Excluding the high number of vaccines and biological, most of the small antiviral molecules are based on nucleoside structures or on

peptidomimetics: only 16.7% are classified as totally synthetic drugs. However, till now, neither a synthetic nor a naturally derived anti-HRV drug substance has been approved for the treatment or prevention of HRV infections (Rollinger and Schmidtke, 2010).

Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and humans who suffer from viral infection (Venkateswaran, 1987; Thyagarajan et al., 1988, 1990; Hudson, 1990). The Boots drug company, Nottingham, England, examined the action of 288 plants against influenza A virus in embryonated eggs in 1952. They found that 12 of them suppressed virus amplification (Chantrill et al., 1952). Intensive research and development efforts in the field of natural products revealed a variety of active phytochemicals, including the flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, furyl compounds, alkaloids, polyines, thiophenes, proteins, and peptides against human rhinovirus. Some volatile essential oils of commonly used culinary herbs, spices, and herbal teas have also exhibited high level of antiviral activity (Jassim and Naji, 2003). (Table 1)

**3. Rhinovirus chemotherapy**

Antiviral research as chemotherapeutic approaches have been intensely spanning several decades. Comprehensive reviews on small molecule inhibitors that target specific virus functions including virus attachment, uncoating, virus RNA replication, and viral protein synthesis and processing have been extensively detailed. Antiviral agents directed toward virus, capsid uncoating, and 3C protease as well as agents that indirectly impair viral replication are the best studied. (Table 2)

### 3.1. Options to prevent virus attachment and/or uncoating

Inhibition of virus attachment and/or uncoating interrupts the viral life cycle at its beginning and prevents HRV infections. Options to prevent these early steps of the viral life cycle include (i) virus neutralization by HRV-specific antibodies, (ii) receptor blockade by antibodies directed against the cellular receptors ICAM-1 or LDL, (iii) soluble receptor molecules, or (iv) compound interacting with the viral capsid.

Application of HRV-specific antibodies is thought to be no promising approach for prevention or therapy of HRV infections because of a number of serotypes (Rollinger and Schmidtke, 2010). However, antibodies directed against the cellular receptor or soluble receptor molecules of major or minor group HRV could inhibit 90 and 10% of HRV serotypes, respectively. Therefore, the strategy to prevent virus-receptor interaction by receptor antibodies or soluble receptor molecules has been evaluated *in vitro* and *in vivo* (Colonno et al., 1986; Hodits et al., 1995). Hayden et al. (1988) studied the prophylactic effectiveness and safety of intranasally administered rhinovirus murine ICAM-1 antibody and reported no toxicity and no enough effectiveness in overall infection or illness rates. A new strategy was the creation of multivalent Fab fusion proteins against ICAM-1. CFY196 exhibited a better avidity and *in vitro* potency against HRV over conventional MAbs. CFY196 is under development as nasal spray with the name of ColdSol (Fang and Yu, 2004).

Antagonism of virus-receptor interaction was considered as another promising way to prevent HRV attachment to host cells. Soluble forms of fully or truncated ICAM-1 (Marlin et al., 1990; Crump et al., 1994; Ohlin et al., 1994), and LDL or VLDL-receptor concatemers exhibited antiviral activity against major and minor group HRV, respectively,

in cell culture (Marlovits et al., 1998; Moser et al., 2005; Nicodemou et al., 2005). Soluble forms of ICAM-1 compete with receptor binding sites on the virus capsid, hinder an early infection event such as entry or uncoating, or directly inactivate HRV due to the formation of empty capsid. (Marlin et al., 1990; Greve et al., 1991; Arruda et al., 1992; Martin et al., 1993). A soluble LDL receptor fragment neutralizes viral infectivity by aggregation (Marlovits et al., 1988). Concatemers of the third ligand binding module of the VLDL-receptor do not lead to viral aggregation but block the receptor binding sites and possibly inhibit viral uncoating by cross-linking the viral capsid subunits via multi-module binding (Moser et al., 2005; Rollinger and Schmidtke, 2010).

The HRV capsid consists of a densely-packed icosahedral arrangement of 60 promoters, each consisting of 4 proteins (VP1, VP2, VP3, and VP4). VP1, VP2, and VP3 are located on the viral surface while VP4, located on the inner surface, remains in close association with the RNA core and functions as an anchor to the virus capsid (Doryen Bubeck et al., 2005; Stephen B. Greenberg. 2003). Elucidation of the solved, three-dimensional crystal structure of HRV reveals a canyon formed by the junction of VP1 and VP3 (Rossmann et al., 1985). Beneath this canyon, within VP1, lies a pore that leads to a hydrophobic pocket that is occupied by a pocket factor proposed to be a fatty acid. Numerous molecules from diverse chemical classes have been described with *in vitro* antiviral activity against HRV and that bind in this region (Carrasco, 1994; Shih, 2004). Antiviral activity is related to the ability to prevent viral attachment and/or uncoating presumably by preventing capsid destabilization and subsequent release of RNA into the cytoplasm. *In vitro*, capsid-binders typically are active against most but not all HRV serotypes and possess a wide range of susceptibilities. This variability in susceptibility

has served as the basis for one method of classification of HRV serotypes into two different groups, A and B (Andries et al., 1990). The first capsid-binders that were synthesized and studied clinically include a series of oxazolinyl isozazoles, the so-called WIN compounds from Sterling Winthrop Pharmaceutical group. First inhibitors originated from juvenile hormone mimetics that demonstrated some activity against HRV-1A. Determination of the X-ray structure of HRV-14 helped to understand the compounds' binding sites at the virus capsid (Rossmann et al., 1985). Subsequent X-ray studies of HRV-WIN compound complexes revealed the location and nature of binding sites and provided information concerning interactions within these sites (Smith et al., 1986; Rossmann, 1989; Kim et al., 1993). This knowledge was used for optimization and design of new compounds (Powers et al., 1982). Optimized WIN compounds, for example disoxaril (WIN 51711) and pleconaril (WIN 63843), were developed. The first broad-spectrum disoxaril was tested in clinical trials and demonstrated *in vitro* antiviral activity against both enteroviruses and HRV (Otto et al., 1985). Subsequent synthetic chemistry efforts led to the discovery of pleconaril, an orally bioavailable 5-methyl-oxadiazole analogue with potent broad-spectrum activity against a wide variety of enterovirus and HRV serotypes and clinical isolates (Pevear et al., 1999; Kaiser et al., 2000). However, based on drug interaction, marginal treatment effect, and possibility of transmission of resistant viruses, the FDA did not approve the applied oral administration of pleconaril for the treatment of common cold. The molecular mechanism of drug interaction of orally given pleconaril was shown to be based on hepatic cytochrome P450 3A activation (Ma et al., 2006). To reduce adverse effects, Shering-Plough under license of ViroPharma completed a phase II clinical trial with an intranasal formulation of pleconaril for the

potential treatment of common cold in high-risk populations in 2007. The clinically effective capsid-binder pirodavir was published against broad-spectrum of HRV and new analogue BTA-798 improved oral bioavailability also clinically tested. (Andries et al., 1992; Barnard et al., 2004). Although intensive research leading to the discovery of potent anti-HRV capsid-binders has been conducted, no agent permitted for prevention and/or therapy of rhinovirus-induced diseases yet.

### 3.2. Development of protease inhibitors

2A protease and 3C protease represent potential anti-HRV targets because of their primary role for viral polyprotein processing and the high conservation of critical amino acids (Binford et al., 2005). The proteolytic activity of 2A protease of HRV-14 was specifically inhibited by two elastase-specific inhibitors and an antiviral peptide representing a derivative of the caspase inhibitor zVAD.fmk (Molla et al., 1993; Deszcz et al., 2004; Deszcz et al., 2006). Homophthalimides (e.g. LY353349) were described as inhibitors of 2A protease as well as 3C protease. In contrast to protease 3C, no structure-activity relationship studies have been reported for 2A protease. Moreover, protease 2A accomplished one cleavage in HRV polyprotein while protease 3C performs all other cleavages. After elucidation of the crystal structure of 3C protease, computer modeling of structural features of protease inhibitors became possible (Kuo et al., 2009). In addition, structure-based design used to develop mechanism-based inhibitors of the 3C protease with broad and potent antiviral activity against multiple HRV serotypes (Matthews et al., 1999; Alisi et al., 2008; Apicella et al., 2008; Maugeri et al., 2008). Structure-activity studies were performed to optimize protease inhibitors and resulted in the identification

of a highly active anti-HRV compounds AG7088 (rupintrivir) (Dragovich et al., 1988, 2002). Rupintrivir has a broad spectrum of HRV and inhibition of HRV replication is strongly correlated with reduction in the level of IL-6 and IL-8 release into cell supernatant. It suggested rupintrivir also diminished symptoms severity. (Patick et al., 1999; Zalman et al., 2000) The pharmacokinetics and safety studies demonstrated that intranasal rupintrivir, administered as single doses of 4 and 8 mg in every 3 hr, six times per day, for 7 days, was safe and well tolerated (Hsyu et al, 2002). The prevention and treatment of experimentally induced rhinovirus colds in healthy volunteers were conducted to assess rupintrivir nasal spray (2% solution) (Hayden et al., 2003). Rupintrivir prophylaxis reduced the proportion of subjects with positive viral culture and viral titers but did not decrease the frequency of colds. Clinical development was terminated because rupintrivir did not act in a subsequent natural infection study in patients. (*E*)-(*S*)-4-((*S*)-2-3-[(5-methyl-isoxazole-3-carbonyl)-amino]-2-oxo-2H-pyridin-1-yl-pent-4-ynoylamino)-5-((*S*)-2-oxo-pyrrolidin-3-yl)-pent-2-enoic acid ethyl ester, an orally bioavailable inhibitor of HRV 3C protease, was developed. It is an irreversible inhibitor that forms a covalent bond with the 3C protease active site cysteine and demonstrated an antiviral activity against all HRV and related picornaviruses tested (Patick et al., 2005). Currently, no further clinical development is planned.

### 3.3. Inhibition of viral RNA synthesis

The blocking of viral RNA synthesis during replication represents another site for chemotherapeutic interdiction. RNA of rhinovirus can be targeted in a sequence-specific manner by deoxyribozymes, morpholino oligomers, and small interfering ribonucleic

acids (Phipps et al., 2004; Schubert et al., 2004; Stone et al., 2008). Also, 2-furylmercury chloride, flavonoids (e.g. 3-methylquercetin), and pyrrolidine dithiocarbamate interfere with rhinoviral RNA synthesis and inhibit HRV replication in cell culture-based assays (Van Hoof et al., 1984; Gaudernak et al., 2002; Verheyden et al., 2004; Krenn et al., 2005). The nucleoside analog ribavirin inhibits a broad spectrum of RNA and DNA viruses (Clercq et al., 1991; Andersen et al., 1992). The cellular inosine monophosphate dehydrogenase that controls de novo synthesis of purine nucleosides indicates the principal target in the mode of action of ribavirin (Clercq, 2009). When ribavirin is incorporate into picornavirus RNA, it pairs equally well with either uracil or cytosine inducing mutations that can be lethal to RNA viruses (Crotty et al., 2000). Further identified mode of action for ribavirin include inhibition of genomic RNA capping, enhancement of host T-cell-mediated immunity against viral infections through helping to switch the host T-cell phenotype from type 2 to type 1 (Reyes, 2001). Another compound with potent anti-HRV activity *in vitro* is enviroxime, a benzimidazole derivative (DeLong and Reed, 1980). It inhibits viral positive strand RNA synthesis (Ninomiya et al., 1985). In particular, the 3A protein, which is involved in the initiation of positive strand RNA synthesis, was implicated as likely target of drug activity (Heinz and Vance, 1995; 1996). However, results from another study suggest that enviroxime targets a complex of proteins and/or cellular factors and that the exact mode of action remains to be proved (Brown-Augsburger et al., 1999). Also, efforts to overcome poor bioavailability and side effects of the compound are demanded greatly. None of these compounds was tested in clinical studies.

**Table1. Reported antiviral activities of natural compounds**

| Target | Natural compound | HRV tested | Author |
|---|---|---|---|
| Capsid binder | Farnesiferol B | HRV-2 | Rollinger et al., 2008 |
| | Farnesiferol C | HRV-2 | Rollinger et al., 2008 |
| | 6,7,8-TC[a] | HRV-2 | Rollinger et al., 2009 |
| | Flavan | HRV-1B | Bauer et al., 1981 |
| | Arborinine | HRV-2 | Rollinger et al., 2009 |
| 3C protease | (+)-Thysanone | HRV-14 | Singh et al., 1991 |
| | 2-Methoxy-stypandrone | HRV-14 | Singh et al., 2001 |
| | 9,10-Penanthra-quinone | HRV-14 | Singh et al., 2001 |
| Viral replication | Quercetin 3-methylether | HRV-15 | Van Hoof et al., 1993 |
| | DTMF[b] | 20 HRV serotypes | Ishitsuka et al., 1982 |
| | Glaucine | HRV-14 | Spasova et al., 2008 |
| | Oxoglaucine | HRV-14 | Spasova et al., 2008 |
| | *o*-Coumaroylamide[c] | HRV-14 | Spasova et al., 2008 |
| | *p*-Coumaroylamide[d] | HRV-14 | Spasova et al., 2008 |
| | Axillarin | HRV-2 | Tsuchiya et al., 1985 |
| - | Raoulic acid | HRV-2, -3 | Choi et al., 2010a |
| | Gallic aicd | HRV-2, -3 | Choi et al., 2010b |

[a] 6,7,8-Trimethoxy-coumarin.

[b] 4'5- Dihydroxy-3,3',7-trimethoxyflavone.

[c] *o*-Coumaroylamide of 3 aminomethylglaucine.

[d] *p*-Coumaroylamide of 3 aminomethylglaucine.

**Table 2. Synthetic antiviral drugs of current clinical use**

| Target | Drug | HRV tested | Author |
|---|---|---|---|
| Capsid binder | Disoxaril | 35 serotypes | Otto et al., 1985 |
| | Pleconaril | All serotypes | Ledford et al., 2004 |
| | Pirodavir | All serotypes | Andries et al., 1992 |
| | Oxime ether 14 | 16 serotypes | Watson et al., 2003 |
| | DDOPMI[a] | HRV-2 | Kuz'min et al., 2007 |
| | SCH 38057 | 6 serotypes | Rozhon et al., 1993 |
| | Ro 09-0881 | 12 serotypes | Ninomiya et al., 1993 |
| | BW 863C | HRV-1B | Baucer et al., 1981 |
| | Isoflavan | HRV-1B | Conti et al., 1988 |
| | MDL 20,957 | 32 serotypes | Kenny et al., 1986 |
| | MDL-860 | 90 serotypes | Powers et al., 1982 |
| 2A and 3C Proteinase | Homophthalimides | HRV-2, HRV-14 | Wang et al., 1988 |
| 3C Proteinase | Rupintrivir | 48 serotypes | Patick et al., 1999 |
| | Compound[b] | 35 serotypes | Patick et al., 2005 |
| RNA synthesis | 2-Furylmercury chloride | 17 serotypes | Verheyden et al., 2004 |
| | Ribavirin | HRV-2, HRV-14 | Andersen et al., 1992 |
| | Enviroxime | 15 serotypes | Wikel et al., 1980 |

[a] 5-{3-[(2',4'-Difluoro-3,5-dimethylbiphenyl-4-yl)oxy]propyl}-3-methylisoxazole.

[b] (*E*)-(*S*)-4-((*S*)-2-3-[(5-methyl-isoxazole-3-carbonyl)-amino]-2-oxo-2H-pyridin-1-yl-pent-4-ynoylamino)-5-((*S*)-2-oxo-pyrrolidin-3-yl)-pent-2-enoic acid ethyl ester.

## 4. Propolis

### 4.1. Characteristics of propolis

Propolis is one of the few natural remedies that has maintained its popularity over long period of time. It is a strongly adhesive, resinous substance collected, transformed, and used by honeybees. Honeybees collect the resin from the cracks in the bark of trees and leaf buds. This resin is masticated, salivary enzymes added, and the partially digested material is mixed with beeswax and used in the hive (Ghisalberti, 1979; Marcucci, 1995). The precise composition of raw propolis varies with the source. In general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% various other substances, including organic debris (Monti et al., 1983; Cirasino et al., 1987). Up to now, more than 180 constituents, mainly polyphenols, have been identified as constituents of propolis. By far the largest groups of the constituents isolated are flavonoid pigments, which are ubiquitous in the plant kingdom. Therefore, the same flavones have been isolated from different samples of propolis and the series of flavonoids isolated from proplis correlate reasonably well with those present in the plants from which honeybees collect proplis. The substances identified in propolis are familiar constituents of food, food additives, and/or generally recognized as safe (GRAS) substances (Burdock, 1998).

The word propolis is derived from the Greek *pro*-, for or in defence, and *polis*-, the city, that is, defense of the city (Ghisalberti E. L. 1979). It thus implies a product involved in the defense of the bee community. Bees use propolis for diverse purposes, among them

to seal openings in the hive. In addition to avoiding the entrance of intruders, this contributes to maintaining the hive inner temperature at 35℃. The walls of the comb hexagonal cells contain a mixture of beeswax and propolis. Propolis not only hardens the cell walls but also contributes to the attainment of an internal aseptic environment. The entrance to the hive is also lined internally with propolis. Evidence that leaves no doubt as to the anti-microbial properties of propolis comes from another use of the product in the hive: bees cover the carcasses of intruders that were killed and are too heavy to be thrown off the hive with propolis. The process comes close to an embalming effect, because the dead bodies dry out without undergoing putrefaction (Ghisalberti E. L. 1979; Salatino et al., 2005). This is obviously important to protect the hive from a widespread bacterial infection.

### 4.2. Historical and current use of propolis

There is a long historical use of propolis in human, at least to 300 BC and its use contributes now in home remedies and personal goods. Egyptians knew the antiputrefactive properties of propolis and employed it to embalm cadavers (Ghisalberti E. L., 1979). The drug was used as an antiseptic and cicatrizant in wound treatment and as mouth disinfectant, with these uses being perpetuated in the Middle Ages and among Arab physicians. Propolis was also recognized by other peoples unrelated to Old World civilization: Incas employed propolis as an antipyretic agent, and the London pharmacopoeias of the seventeenth century listed propolis as an official drug. Between the 17th and 20th century, the drug became very popular in Europe on account of its antibacterial activity. (Castaldo and Cpasso, 2002; Sforcin and Bankova, 2011)

Modern herbalists benefited from the antibacterial, antifungal, antiviral, hepatoprotective, and antiinflammatory properties, to improve the body's natural resistance to infections and to treat gastroduodenal ulcers. Applied externally, propolis relieves various types of dermatitis caused by bacteria and fungi (Castaldo et al., 2002).

Propolis has gained wide acceptance in popular medicine in diverse parts of the world. Such popularity has stimulated the commercial use of propolis in food and beverages, as well as everyday products such as soaps and toothpastes. The broad interest in propolis over the world has encouraged intensive research in propolis chemistry and biological activities. (M. C. Marcucci, 1995; Vassya S. Bankova et al., 2000; Bankova, 2005; Kumazawa et al., 2008)

### 4.3. Pharmacological properties

Propolis presents plenty of biological and pharmacological properties, such as antibacterial, antifungal, antitumor, immunomodulatory, antioxidant, antiviral, and antiparasite activites (Burdock, 1998). Specifically, antimicrobial activity of propolis and its extracts has been elucidated against various bacterial strains, yeast and fungi, and protozoa. (Dobrowolski et al., 1991; Ikeno et al., 1991; Bankova et al., 1995; DeCastro and Higashi, 1995; Digrak et al., 1995) Also, there have been a great number of publications considering the antitumor action of propolis and its constituents. The antitumoral effect of ethanol extract of propolis was demonstrated in mature mice bearing Ehrlich carcinoma and it was due to the flavonoids inhibiting DNA synthesis (Scheller et

al., 1989). Its constituents, particularly caffeic acid phenethyl ester (CAPE) and chrysin, have been identified with chemopreventive and antitumoral properties in the apoptotic process and proliferation of cancer cells. It has been suggested that propolis, CAPE, and chrysin may inhibit tumor cell progression and may be useful as potential chemotherapeutic or chemopreventive anticancer drugs (Sawicka et al., 2012). Propolis constituents, such as chrysin, kaempferol, isopentyl ferulate and quercetin, have antiviral activity against several herpes viruses, poliovirus, influenza virus A/Hong Kong, and HIV (Burdock, 1998; Búfalo et al., 2009).

# Ⅲ. Materials & Methods

## 1. Apparatus

$^{1}H$ and $^{13}C$ NMR spectra were in recorded in $CD_3OD$ on a Bruker AM-500 spectrometer (Rheinspettem, Germany) at 600 and 150 MHz (TMS as an internal standard), respectively and chemical shifts were given in δ (ppm). Mass spectra were obtained in methanol with JEOL GSX 400 spectrometer (Tokyo). Silica gel 60 (0.063-0.200 mm, Merck, Darmastadt, Germany) was used for column chromatography. Pre-coated silica gel plates (Silica gel 60 $F_{254}$, 0.20 mm, Merck) were used for analytical thin layer chromatography (TLC). A high performance liquid chromatography (HPLC) (Agilent 1200, Santa Clara, CA) and medium-pressure liquid chromatography (MPLC) (Isolera one Biotage®, Uppsala, Sweden) was used for isolation of active principles.

## 2. Chemicals

The ribavirin (commercially available antiviral agent), and 13 natural compounds used in this study. Ribavirin was purchased from Tokyo Chemical Industry (Tokyo, Japan). Quercetin, fisetin, galangin, chrysin, protocatechuic acid, ferulic acid, acacetin, luteolin, nerolidol, caffeic acid, benzoic acid, (-)-ρ-anisic acid and (-)-epigallocatechin gallate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibiotic-antimycotic and sulforhodamine B (SRB) were supplied by Gibco BRL (Grand Island, NY, USA) and Sigma-Aldrich, respectively. All other chemicals were of reagent grade.

### 3. Sample preparation

Propolis derived from Brazil, Jeju and Chungju were obtained from Rural Development Administration (Korea). They were finely powdered using a blender. Each 200 g sample was extracted twice 1000 mL with 80% ethanol at room temperature for 2 days and filtered (Whatman No. 2). The combined filtrate was concentrated to dryness by rotary evaporation at 40℃ to yield 57.97 g, 58.81 g and 61.89 g, respectively.

### 4. Extraction and isolation of active principles from Brazilian propolis

The Brazilian ethanolic extract (20 g) was sequentially partitioned into hexane (0.07 g), chloroform (13.58 g), ethyl acetate (1.64 g), butanol (1.72 g) and water (2.99 g) portions for subsequent bioassay (Fig. 1). The organic solvent fractions were concentrated by dryness by rotary evaporation at 40℃, and water fraction was concentrated at 48℃.

Due to its potent antiviral activity, the chloroform fraction and the ethyl acetate fraction were isolated respectively. Chloroform fraction (20 g) was chromatographed by MPLC (Biotage, Isolera One, Sweden) with SNAP 340 g silica (Biotage) column and eluted with a gradient of chloroform and methanol [100:0 (1500 mL), 98:2 (3500 mL), 95:5 (4000 mL), 89:11 (3500 mL), 80:20 (2000 mL), 70:30 (1000 mL) by volume] and finally with methanol (1000 mL) to provide 87 fractions (each about 180 mL). Column fractions were monitored by TLC on silica gel plates with chloroform and methanol (95:5 by volume). Fractions with similar $R_f$ values on the TLC plates were pooled and spots were detected by spraying with 5% sulfuric acid and the heating on a hot plate. TLC

analysis methods to determine fractions were used same way which described above. TLC pattern gave 6 fractions named C1-C6. The active fraction C2 (7.65 g) was separated by MPLC with SNAP 340 g silica (Biotage) column and eluted with a gradient of chloroform and methanol [100:0 (300 mL), 99:1 (350 mL), 98:2 (550 mL), 95:5 (1000 mL), 90:10 (1000 mL), 85:15 (550 mL), 70:30 (700 mL) by volume] and finally with methanol (1000 mL) to provide 25 fractions (each about 180 mL). The fractions were monitored by TLC analysis with chloroform and methanol (95:5). The following active fraction C22 (1.86 g) was also separated by MPLC with SNAP 100 g silica (Biotage) column and eluted with a gradient of chloroform and methanol [99:1 (150 mL), 98:2 (150 mL), 97:3 (300 mL), 95:5 (1000 mL), 91:9 (1000 mL), 90:10 (300 mL), 80:20 (100 mL), by volume] and finally with methanol (500 mL) to 150 fractions. The TLC analysis was conducted with chloroform and methanol (95:5). The active fraction C223 (1.28 g) was separated by MPLC with SNAP 100 g silica (Biotage) column and eluted with a gradient of chloroform and methanol [99:1 (150 mL), 98:2 (150 mL), 97:3 (300 mL), 95:5 (1000 mL), 91:9 (1000 mL), 90:10 (300 mL), 80:20 (100 mL), by volume] and finally with methanol (500 mL) to 150 fractions. The TLC analysis with chloroform and methanol (95:5) were conducted and the following fraction C2233 (0.43 g) was chromatographed on a silica gel column and successively eluted with a stepwise gradient of chloroform and methanol [100:0 (1600 mL), 99:1 (700 mL), 98:2 (200 mL), 95:5 (600 mL), 90:10 (150 mL), 80:20 (150 mL), 0:100 (500 mL) by volume] to provide 39 fractions. Column fractions were monitored by TLC analysis with chloroform and methanol (9:1). As the result of TLC analysis, C22335 (0.13 g) was chromatographed on a silica gel column lastly and eluted with a gradient of hexane and acetone [100:0 (300 mL), 90:10 (200 mL),

80:20 (200 mL), 70:30 (200 mL), 50:50 (400 mL), 30:70 (200 mL), 20:80 (200 mL), 10:90 (200 mL) by volume] to provide 24 fractions. TLC analysis was conducted with hexane and acetone (6:4). As the result of TLC analysis, the active fraction C223355 (26.3 mg) was pooled and purified by HPLC with C18 reverse phase column (4.6 x 250 mm, 5u) using a mobile phase of water comprised 0.3% TFA and acetonitrile at 280 nm in flowrate 0.8 mL/min. It was eluted with a gradient of the solvents. [65:35 (20 min), 0:100 (30 min), 65:35 (35 min)] Finally, a potent active principle 1 (11.5 mg) was isolated at the retention time of 3.343 min (Fig. 2, Fig. 3). For the identification of compound 1, spectroscopic analysis was performed.

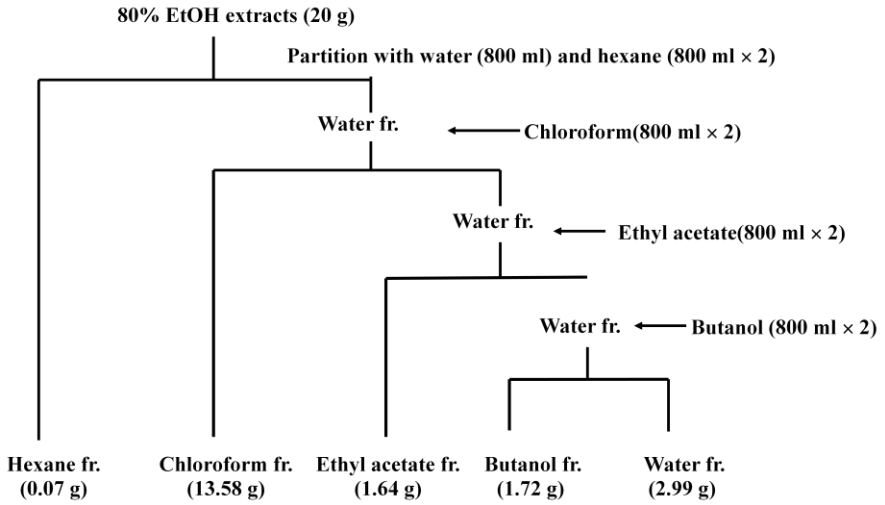


**Fig. 1. Solvent fraction procedures of ethanol extract from Brazil propolis**

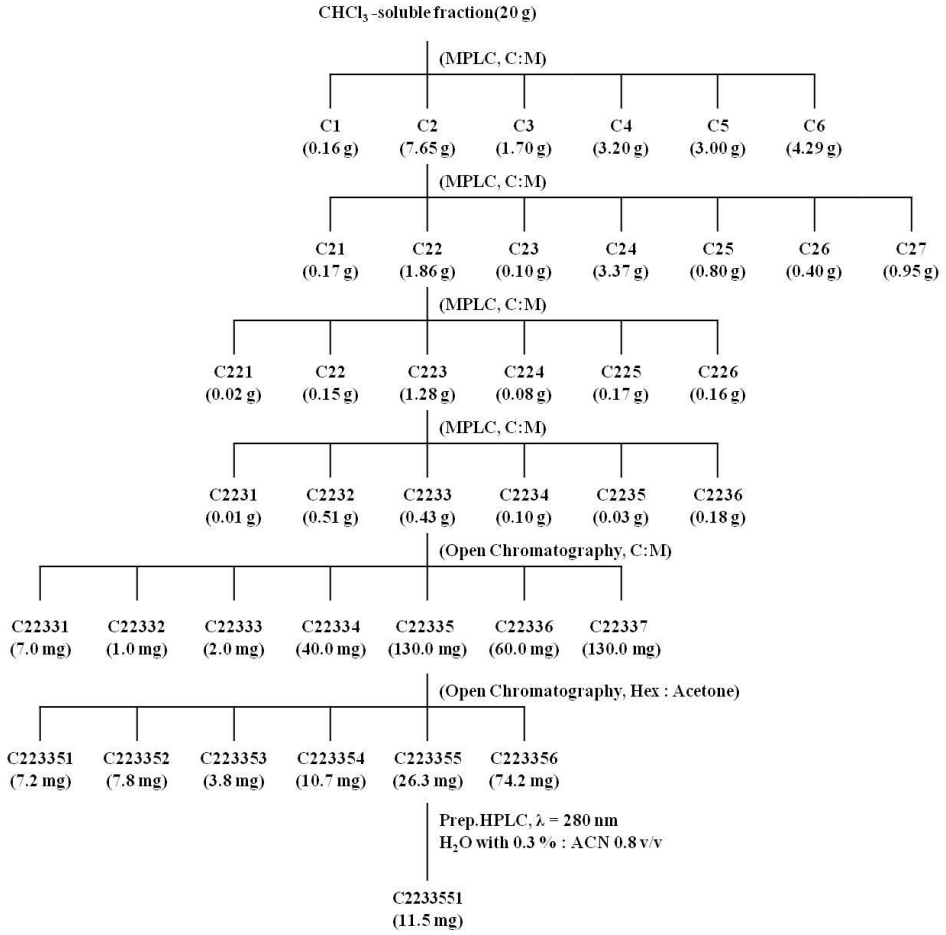


**Fig. 2. Isolation procedure of antiviral principle from chloroform fraction**

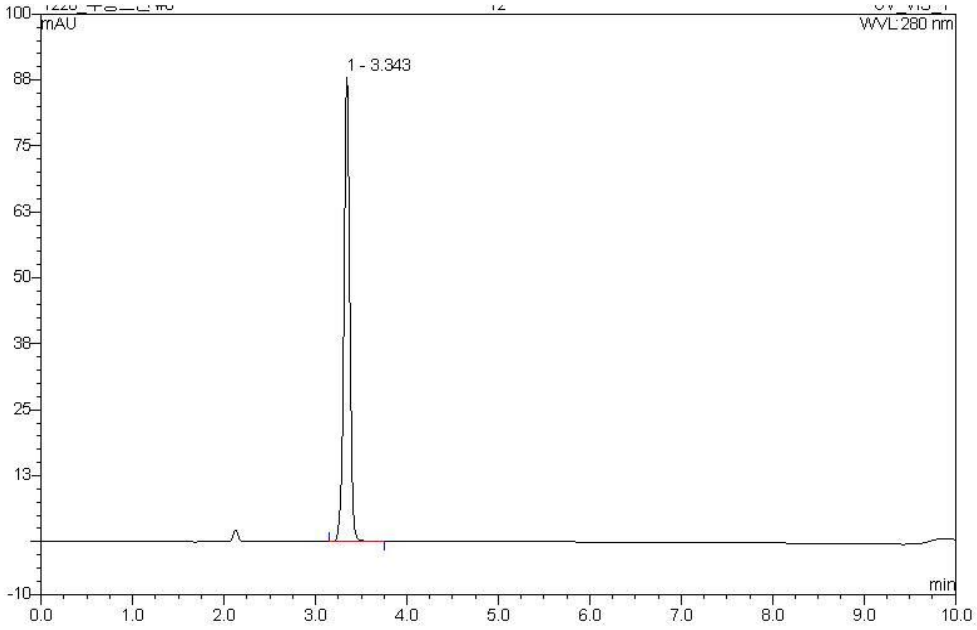


**Fig. 3. HPLC chromatogram of the active isolate 1**

The ethyl acetate fraction (3 g) was chromatographed by MPLC (Biotage, Isolera One, Sweden) with SNAP 100 g silica (Biotage) column and eluted with a gradient of chloroform and methanol [100:0 (500 mL), 99:1 (500 mL), 98:2 (1000 mL), 95:5 (1500 mL), 91:9 (1000 mL), 80:20 (1000 mL) by volume] and finally with methanol (500 mL) to provide 250 fractions (each about 22 mL). Column fractions were monitored by thin-layer chromatography (TLC) on silica gel plates (silica gel 60 $F_{254}$, Merck) with chloroform and methanol (95:5 by volume). Fractions with similar $R_f$ values on the TLC plates were pooled. Spots were detected by spraying with 5% sulfuric acid and the heating on a hot plate. As a results of TLC pattern totally six fractions were received which named as E1 to E6. The bioactive fraction, E4 was separated into chloroform soluble (E41, 0.25 g) and nonsoluble fractions (E42, 0.39 g). The bioactive fraction, E41 was washed with hexane and chloroform several times to purify. The purified E41 (crystal)

was separated by preparative HPLC with C18 reverse phase column (4.6 mm i.d. × 150 mm) using a mobile phase of methanol and water (7:3 by volume), at 280nm in flow rate 1 mL/min. Finally a potent active principle 2 (0.17 g) was isolated at the retention time of 11.490 min (Fig. 4, Fig. 5). For the identification of compound 2, spectroscopic analysis was performed.

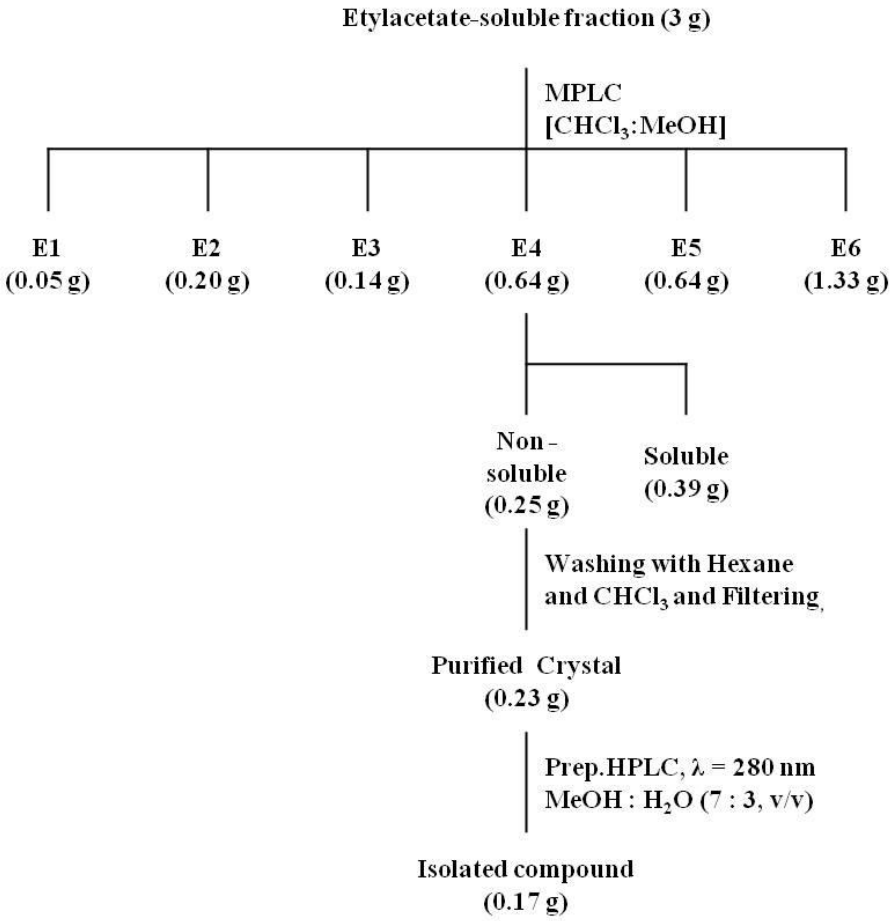


**Fig. 4. Isolation procedure of antiviral principle from ethylacetate fraction**

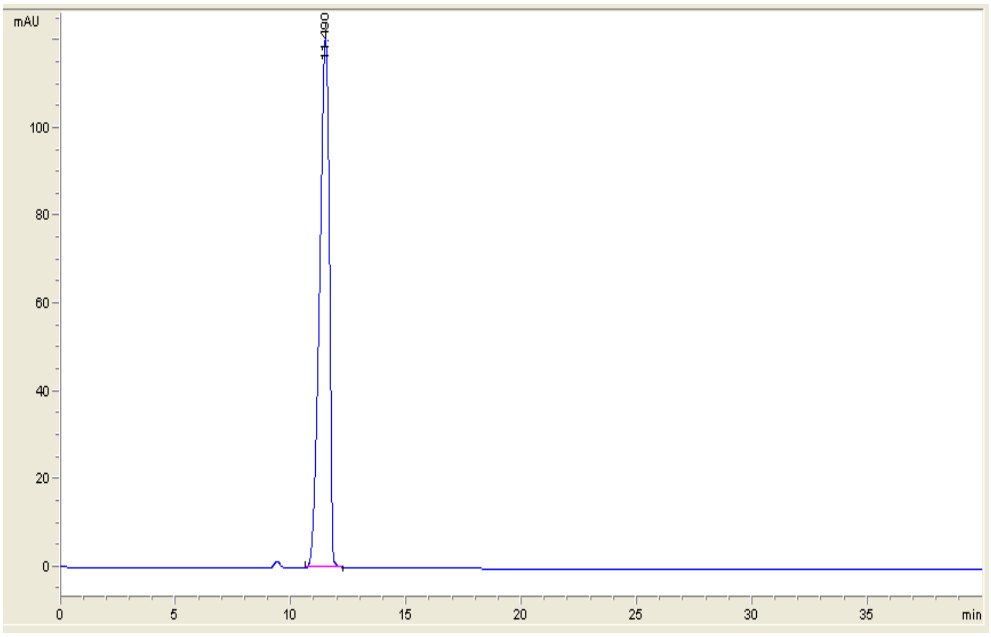


**Fig. 5. HPLC chromatogram of active isolate 2**

## 5. Cell line, HRV serotypes and materials

HeLa ATCC CCL–2 (a human cervix epithelial cell line) were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The cell line was maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum containing 1% mixture of antibiotic-antimycotic solution and incubated in a humidified incubator with 5% $CO_2$ at $37^0$C. HRV 4 serotype (HRV 4 ATCC VR-1114AS/GP**)** was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and HRV 2 and HRV 3 serotype were kindly gifted by Dr. Hwa Jung Choi (National Medicines Research Centre, Korea Research Institute of Bioscience & Biotechnology, Republic Korea).

Phosphate buffered saline **(**PBS) (pH 7.4), RIPA buffer, and mammalian cell protease inhibitor cocktail were purchased from Sigma-Aldrich., *bovine Serum Albumin (*BSA) from Biotechnology Santa Cruz, CA, and ribavirin from TCI, America. Mixture of antibiotic-antimycotic solution, minimum essential medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco, Invitrogen™. Superscript First – Strand Synthesis System for RT-PCR were purchased from Invitrogen, Carlsbad, CA, USA. Maxima SYBR Green/ROX qPCR Master Mix (2X) was obtained from Thermo Scientific, CA, USA.

### 7. Cytotoxic assay

HeLa cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% mixture of antibiotic-antimycotic solution and 30 mM $MgCl_2$. HeLa cells grown to confluence in 96-well microplates were exposed to several different concentrations of the test materials form 0.1 μg/mL to 100 μg/mL. The culture plates were incubated for 2 days in a 37℃ incubator with a humidified atmosphere of 5% $CO_2$. The cytotoxicity of test materials to the human cell lines was evaluated using a SRB assay (Vichai and Kirtikara, 2006). 96-well plates were washed one time with phosphate buffered saline (PBS, pH 7.3), 100 μL of cold 70% acetone (-20℃) were added to the top of each well and left for about 30 min at -20℃. After acetone was removed, 96-well plates were left to dry totally. In the SRB assays, 100 μL of 0.057% (w/v) SRB in 1% acetic acid solution were added to each well and left at room temperature for 30 min.

Then SRB was removed and the plates were washed five times with 1% acetic acid before oven drying. The 96-well plates were then left to dry at oven. Bound SRB was solubilized with 100 μL of 10 mM unbuffered tris-base solution and plates were left on a table for at least 30 min. The OD values were recorded using Versa Max microplate reader (Molecular Devices, CA, USA) at a 560 nm subtracting the background measurement at 620 nm. Cytotoxicity was exposed as 50% cytotoxicity concentration ($CC_{50}$) of the compound that reduced the viability of cells to 50% compared with the control wells.

## 8. HRV production

Three serotypes, HRV -2, -3 and -4 were amplified in HeLa cells incubated in MEM with 10% fetal bovine serum and 1% antibiotic-antimycotic solution supplemented 30 mM $MgCl_2$. After 48 hours of incubation at $37^0$C, the viruses were released from cells by three cycles of freeze/thaw at $-80^0$C/$25^0$C. Cell debris was removed by centrifugation 2000 rpm and the supernatant was aliquoted and frozen at $-80^0$C.

### 9. Virus titration

Virus titration was determined on HeLa cell lines depending on the viruses using 10 serial 2-fold-dilution steps and was evaluated by the SRB assay after viral infection. Control cells were incubated without the viruses. $TCID_{50}$ (50% tissue culture infection dose) value was used as a proper virus concentration.

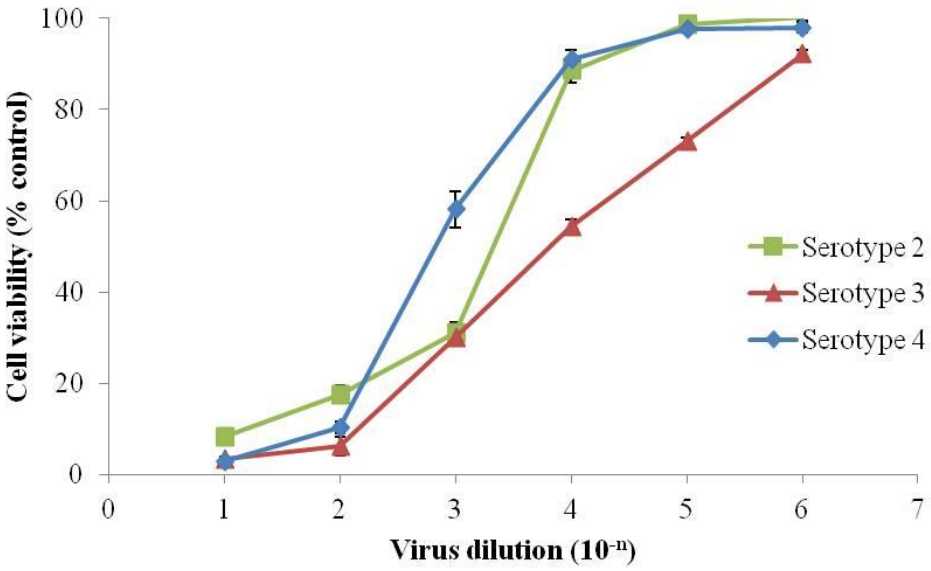


**Fig. 6. Titration curve of HRV.**

**10. Antiviral assays**

Cell monolayers were grown in 96-well microtiter plates. Several different concentrations of test compounds which show non-toxicity to the test human cell lines (lower than $CC_{50}$) were checked for antiviral properties. A proper virus dose ($TCID_{50}$) of each virus serotype was added to each well and incubated in the same incubation above. Test wells contained treated infected cells, while controls consisted of untreated infected, treated uninfected and untreated uninfected cells. Ribavirin and (-)-epigallocatechin gallate served as a positive control in the antiviral tests. After 2 days, viral cytopathogenic effect (CPE) was recorded. Cell viability was evaluated by the SRB assay. The 50% antiviral effective concentration, i.e. 50% inhibitory concentration ($IC_{50}$) of the viral

effect is expressed as the concentration that achieves 50% protection of treated infected cells from HRV induced destruction. The percent protection is calculated as Protection % = $\frac{A-B}{C-B}$ x 100**,** where A, B and C are the OD values of treated infected, untreated infected, and untreated uninfected cells, respectively.

**11. Effects of the active constituents on the infectivity of HRV particles**

To test effects of propolis constituents on the infectivity of HRV particles, HRV was pre-treated for 1 h at 4$^{o}$C with test components at proper concentrations. HeLa cells were infected with the pretreated or unpretreated HRVs for 1 h at 37$^{o}$C. Unbound viruses were removed by washing the test wells twice with PBS, and then cells were incubated in fresh medium supplemented with or without the test constituents for 2 days at 37$^{o}$C. Antiviral activity was determined by SRB assay after two-day incubations.

**12. Time course of compound addition**

The time-of-addition effect of active constituents was examined according to previously described procedures (Choi *et al*., 2010b). HeLa cells were seeded onto 96-well culture plates. After washing with 1X PBS buffer, each 100 μg/mL of (*E*)-ρ-coumaric acid, 10 μg/mL kaempferol were then added onto the cells at either before (−1 h), during (0 h) or after (1, 2, 4, 6 h) the period of HRV 3 infection. For the treatment −1h,

after 1 h treatment with the test compounds, cells were washed off and infected with *the HRV in compound-free medium.* After 2 days, antiviral activity was carried out as above described. Ribavirin 100 μg/mL was used as positive control.

### 3.8. Real-time RT-PCR analysis

To evaluate the level of gene expression, real-time RT-PCR with SYBR Green dye was applied. After treatments of 100 μg/ml (*E*)-ρ-coumaric acid and 10 μg/ml kaempferol for 2 days, the total RNA extraction from the mock and infected cultures was achieved with the RNeasy kit following the manufacturer's instructions. Contaminating genomic DNA was removed using RQ1 RNase-free DNAase. Amount of total RNA (1 μg) was converted to cDNA using Superscript First – Strand Synthesis System for RT-PCR. The cDNA products were stored in aliquots at −80 ◦C until needed. Perform five log10-fold dilutions of cDNA for each RNA to determine PCR efficiency (100 ng - 10 pg per reaction). Quantitative RT-PCR was conducted in 96-well plates using Applied Biosystem StepOne-plus real-time PCR system (Life Technology); A volume of each reaction mixture (20 μL) consisted of 10 μL Maxima SYBR Green/ROX qPCR Master Mix (2X), 2 μL of forward and reverse primers (5 pmol of each), 1μL cDNA (8 ng), and 7 μL water. Oligonucleotide PCR primer pairs are listed in Table 3. The reaction conditions were as follows: 50℃ for 2 minutes, 95℃ for 10 minutes, and then 50 cycles of 95℃ for 15 seconds and either 60$^{o}$C (β2-microglobulin (B2M), IL-6, ICAM-1, HRV3) for 30 seconds. All samples were run in duplicate. Experiments were performed three times. The relative level of mRNA expression of specific gene was calculated based on $2^{-\Delta\Delta C}{}_{T}$ method, and

normalized to mRNA level for the housekeeping gene B2M; $\Delta\Delta C_T = (C_{T.Target} - C_{T\beta 2\text{-microglobulin}})$; $C_T$ representing the threshold cycle.

**Table 3. Primers used for real-time RT-PCR**

| Gene name | RefSeq ID | Forward primer and Reverse primer | cDNA amplicon size | Citation |
|---|---|---|---|---|
| B2M | AF072097 | CTCCGTGGCCTTAGCTGTG<br>TTTGGAGTACGCTGGATAGCCT | 68 | Iwasaki *et al*., 2004 |
| ICAM-1 | NM_000201.2 | ACCTCCCCACCCACATACATTT<br>GGCATAGCTTGGGCATATTCC | 96 | Viemann *et al*., 2004 |
| IL-6 | M14584 | GACCCAACCACAAATGCCA<br>GTCATGTCCTGCAGCCACTG | 68 | Iwasaki *et al*., 2004 |
| HRV 3 | DQ473490.1 | CGGCCCCTGAATGCGGCTAA<br>GAAACACGGACACCCAAAGTA | 115 | Kares, 2004 |

# III. Results

### 1. Antiviral activity of propolis against HRV

The antiviral activity of propolis produced in Brazil, Jeju, and Chungju against HRV-2 and -4 was evaluated using a SRB assay (Table 4). Potencies varied according to origin and serotypes. Based on $IC_{50}$ values, the ethanolic extract was proved to have antiviral activity against two serotypes. The other two extracts exhibited no antiviral activity.

Cytotoxic effects of the test propolis extracts on HeLa cell line are likewise compared. Strong cytotoxicity was observed with ethanolic extracts of propolis produced in Jeju and Chungju. Moderate cytotoxicity was obtained from Brazilian extracts.

Based on TI values, Brazilian propolis extract was observed as potential antiviral source.

**Table 4. Antiviral and cytotoxic activity of propolis derived from different origin**

| | | HRV-2 | | HRV-4 | |
|---|---|---|---|---|---|
| Origin | $CC_{50}$[a] (μg/mL) | $IC_{50}$[b] (μg/mL) | TI[c] | $IC_{50}$[b] (μg/mL) | TI[c] |
| Brazil | 19.5 ± 1.39 | 12.6 ± 3.02 | 1.55 | 15.4 ± 3.59 | 1.26 |
| Jeju | 5.3 ± 0.13 | > 100 | — | > 100 | — |
| Chungju | 6.8 ± 0.22 | > 100 | — | > 100 | — |

[a] Concentration of test materials producing 50% inhibition of virus-induced cytopathic effects.

[b] The 50% cytotoxic concentration for HeLa cells.

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

**2. Isolation and identification of active principles from Brazilian propolis**

Fractions obtained from the solvent hydrolyzable of the ethanolic extract of Brazilian propolis were tested against HRV-4 by a SRB assay (Table 5). As judged by $IC_{50}$ values, the hexane-, chloroform- and ethyl acetate-soluble fractions showed the most pronounced antiviral activity. Moderate and weak activity was produced by the water- and butanol-soluble fractions, respectively. Of the test fractions, the chloroform-soluble fraction possessed the highest TI value. Therefore, the chloroform-soluble fraction was used to identify peak activity fractions for the next step in the purification.

**Table 5. Antiviral activity of fractions obtained from the solvent hydrolyzable of the ethanolic extract of Brazilian propolis materials against HRV-4 using a SRB assay**

| Test material | $CC_{50}$[a] (μg/mL) | $IC_{50}$[b] (μg/mL) | TI[c] |
|---|---|---|---|
| Hexane-soluble fraction | 52.7 | 4.9 | 10.6 |
| Chloroform-soluble fraction | 82.3 | 5.0 | 16.5 |
| Ethyl acetate-soluble fraction | > 100 | 5.2 | > 1.3 |
| Butanol-soluble fraction | > 100 | 78.4 | > 1.2 |
| Water-soluble fraction | > 100 | 26.7 | > 3.8 |

[a] Concentration of test materials producing 50% inhibition of virus-induced cytopathic effects.

[b] The 50% cytotoxic concentration for HeLa cells.

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

The yield, cytotoxicity, antiviral activity, and SI values of each subfraction derived from chloroform- and ethyl acetate-soluble fractions are given in Tables 6 and 7.

**Table 6. Antiviral activity of each subfraction from chloroform-soluble fraction against HRV-4**

| Fractions | Yield (g) | $CC_{50}$[a] (μg/mL) | $IC_{50}$[b] (μg/mL) | TI[c] |
|---|---|---|---|---|
| C1 | 0.16 | 36.1 | > 100 | — |
| C2 | 7.65 | 19.6 | 0.95 | 20.6 |
| C3 | 1.70 | 14.2 | > 100 | — |
| C4 | 3.20 | 52.2 | > 100 | — |
| C5 | 3.00 | 41.4 | > 100 | — |
| C6 | 4.29 | 70.1 | > 100 | — |
| C21 | 0.17 | 50.0. | 7.0 | 7.1 |
| C22 | 1.86 | 42.2 | 0.8 | 52.8. |
| C23 | 0.10 | 16.14 | > 100 | — |
| C24 | 3.37 | 28.9 | > 100 | — |
| C25 | 0.80 | 46.6 | 0.9 | 51.7 |
| C26 | 0.40 | 44.7 | > 100 | — |
| C27 | 0.95 | 43.4 | > 100 | — |
| C221 | 0.02 | 37.3 | 23.0 | 1.6 |
| C222 | 0.15 | 52.2 | > 100 | — |
| C223 | 1.28 | 31.2 | 1.0 | 30.9 |
| C224 | 0.08 | 53.0 | 13.1 | 4.0 |

**Table 6. (Continued)**

| Fractions | Yield (g) | $CC_{50}$[a] (μg/mL) | $IC_{50}$[b] (μg/mL) | TI[c] |
|---|---|---|---|---|
| C225 | 0.17 | 37.7 | 16.5 | 2.3 |
| C226 | 0.16 | 57.6 | 14.1 | 4.1 |
| C2231 | 0.01 | 42.5 | > 100 | — |
| C2232 | 0.51 | 17.9 | 0.7 | 25.6 |
| C2233 | 0.43 | 22.2 | 0.7 | 30.5 |
| C2234 | 0.10 | 51.0 | 1.8 | 28.5 |
| C2235 | 0.03 | 40.5 | > 100 | — |
| C2236 | 0.18 | 30.5 | > 100 | — |
| C22331 | 0.007 | 25.3 | > 100 | — |
| C22332 | 0.001 | — | — | — |
| C22333 | 0.002 | — | — | — |
| C22334 | 0.040 | 6.1 | 0.6 | 10.2 |
| C22335 | 0.130 | 6.4 | 0.5 | 12.8 |
| C22336 | 0.060 | 21.5 | 5.8 | 3.7 |
| C22337 | 0.130 | > 10 | > 10 | — |
| C223351 | 7.2[d] | 25.3 | > 10 | — |
| C223352 | 7.8[d] | 8.0 | > 10 | — |
| C223353 | 3.8[d] | 6.4 | > 10 | — |
| C223354 | 10.7[d] | 21.5 | 4.7 | 4.6 |
| C223355 | 26.3[d] | 52.6 | 3.7 | 14.2 |

**Table 6. (Continued)**

| Fractions | Yield (g) | $CC_{50}$[a] (μg/mL) | $IC_{50}$[b] (μg/mL) | TI[c] |
|---|---|---|---|---|
| C223356 | 74.2[d] | > 10 | > 10 | — |
| C2233551 | 11.5[d] | 65.0 | 3.7 | 17.6 |

[a] Concentration of test materials producing 50% inhibition of virus-induced cytopathic effects.

[b] The 50% cytotoxic concentration for HeLa cells.

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

[d] Unit, mg.

**Table 7. Antiviral activity of each subfraction from ethyl acetate-soluble fraction against HRV-4**

| Fractions | Yield (g) | $CC_{50}$[a] (μg/mL) | $IC_{50}$[b] (μg/mL) | TI[c] |
|---|---|---|---|---|
| E1 | 0.05 | > 100 | 37.5 | > 2.7 |
| E2 | 0.20 | 5.0 | > 100 | — |
| E3 | 0.14 | 79.2 | 61.2 | 1.3 |
| E4 | 0.64 | > 500 | 72.3 | > 6.9 |
| E5 | 0.64 | 50.6 | 33.0 | 1.5 |
| E6 | 1.33 | > 100 | > 100 | — |
| E41 | 0.25 | > 500 | 75.1 | > 6.6 |
| E42 | 0.39 | > 100 | 81.4 | > 1.3 |

[a] Concentration of compounds producing 50% inhibition of virus-induced cytopathic effects.

[b] The 50% cytotoxic concentration for HeLa cells.

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

SRB bioassay-guided fractionation of the chloroform-soluble fraction afforded an active principle identified by spectroscopic analysis including EI-MS (Fig. 7), $^{1}$H NMR (Fig. 8), and $^{13}$C NMR (Fig. 9). The active principle (compound 1) was kaempferol (3, 5, 7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one) (Fig. 10). EI-MS revealed a molecular ion at *m/z* 286 $[M]^{+}$. The interpretations of proton and carbon signals of compound 1 were largely consistent with those of Hadizadeh *et al.* (2003) and Markham *et al.* (1978).

Kaempferol: yellow powder. EI-MS (70 eV), *m/z* (relative intensity): 286 $[M]^{+}$, 257 (1), 213 (3), 184 (1), 143 (4), 121 (12), 93 (10), 69 (100, base peak), 65 (10). $^{1}$H NMR ($CD_3OD$, 600MHz) and $^{13}$C NMR ($CD_3OD$, 150MHz): See Table 8.

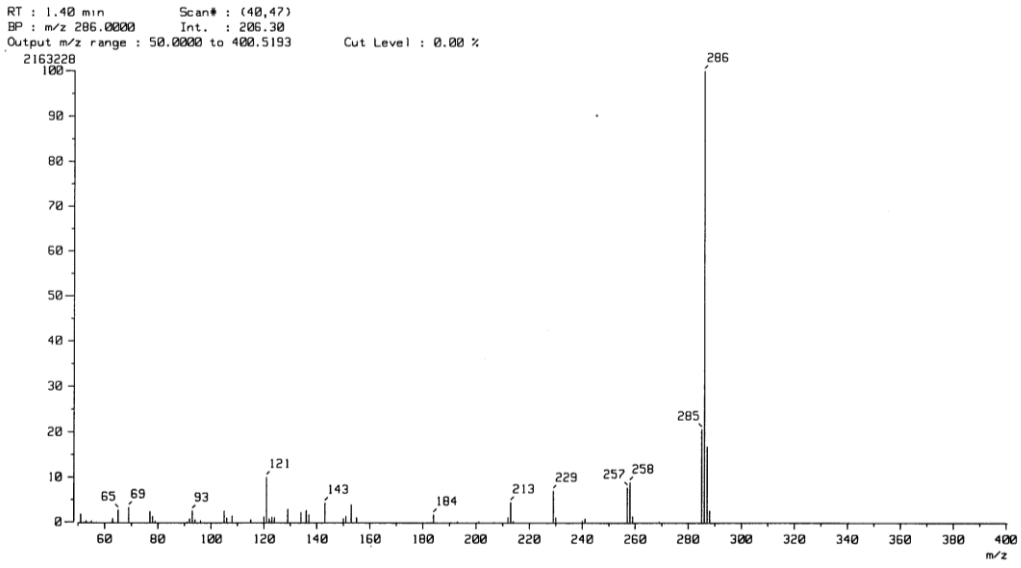


**Fig. 7. Mass spectrum of compound 1**

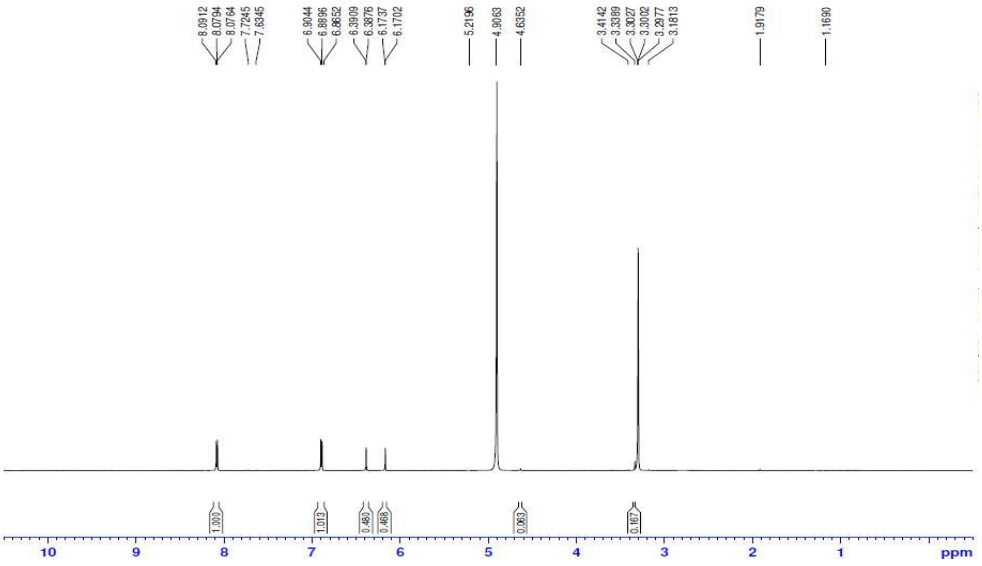


**Fig. 8. $^{1}$H NMR spectrum of compound 1**

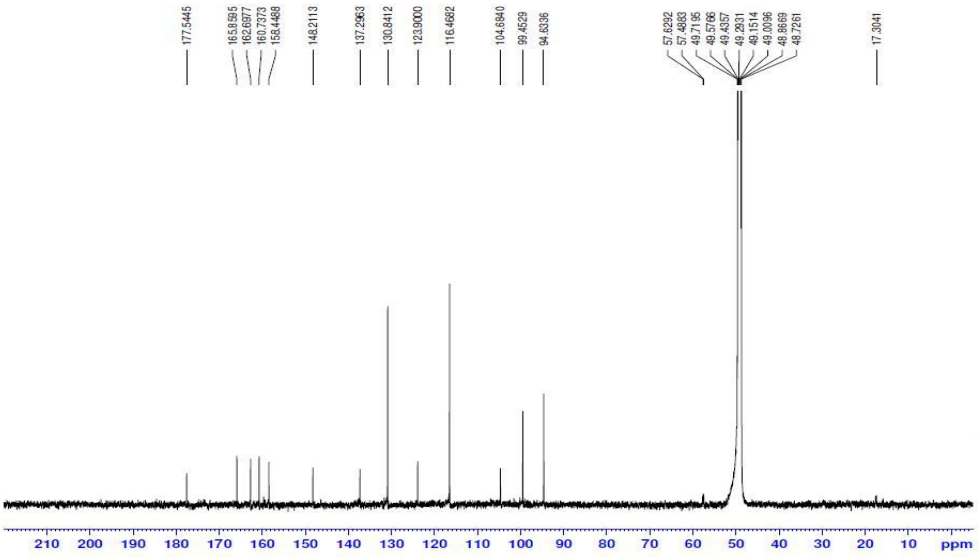


**Fig. 9. $^{13}$C NMR spectrum of compound 1**

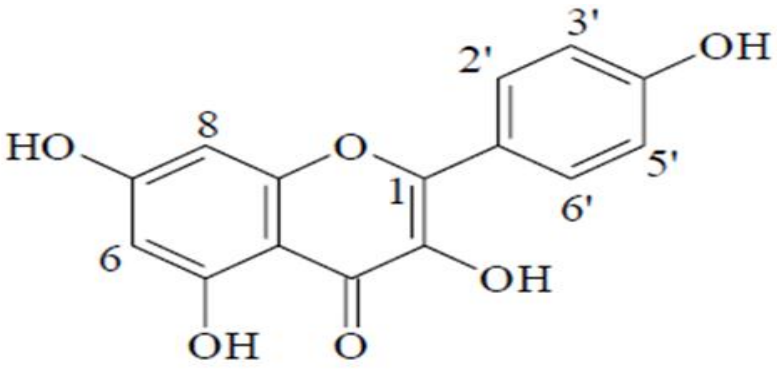


**Fig. 10. Structure of kaempferol**

**Table 8. $^{1}H$ and $^{13}C$ NMR spectral data for compound 1**

| Position | Partial structure | $\delta_C$ (ppm) | $\delta_H$ (ppm) | $\delta_C$ (ppm) (DMSO-$d_6$, 25.15MHz) Markham (1978) | $\delta_H$ (ppm) ($CD_3OD$, 300 MHz) Hadizadeh *et al* (2003) |
|---|---|---|---|---|---|
| 1 | C | 158.4 | | 156.2 | |
| 2 | C | 137.2 | | 135.6 | |
| 3 | C | 177.5 | | 175.9 | |
| 4 | C | 104.7 | | 103.1 | |
| 5 | C | 162.7 | | 163.9 | |
| 6 | CH | 99.5 | 6.17 d ($J$ = 2.2 Hz) | 98.2 | 6.27 d ($J$ = 1.9 Hz) |
| 7 | C | 165.6 | | | |

**Table 8. (Continued)**

| Position | Partial structure | $\delta_C$ (ppm) | $\delta_H$ (ppm) | $\delta_C$ (ppm) (DMSO-$d_6$, 25.15MHz) Markham (1978) | $\delta_H$ (ppm) ($CD_3OD$, 300 MHz) Hadizadeh *et al* (2003) |
|---|---|---|---|---|---|
| 8 | CH | 94.6 | 6.39 d (*J* = 6.8 Hz) | 93.5 | 6.46 d (*J* = 1.9 Hz) |
| 9 | C | 160.7 | | 160.7 | |
| 1’ | C | 123.9 | | 121.7 | |
| 2’ | CH | 130.8 | 8.07 d (*J* = 8.9 Hz) | 129.5 | 8.15 d (*J* = 8.9 Hz) |
| 3’ | CH | 116.5 | 6.86 d (*J* = 8.9 Hz) | 115.4 | 6.99 d (*J* = 8.9 Hz) |
| 4’ | C | 148.2 | | 146.8 | |
| 5’ | CH | 116.5 | 6.88 d (*J* = 8.9 Hz) | 115.4 | 6.99 d (*J* = 8.9 Hz) |
| 6’ | CH | 130.8 | 8.07 d (*J* = 8.9 Hz) | 129.5 | 8.15 d (*J* = 8.9 Hz) |

Bioassay-guided fractionation of the ethyl acetate-soluble fraction afforded an active principle identified by spectroscopic analysis including EI-MS (Fig. 11), $^1$H NMR (Fig. 12) and $^{13}$C NMR (Fig. 13). The active principle (compound **2**) was (*E*)-*p*-coumaric acid (Fig. 14). EI-MS revealed a molecular ion at *m/z* 164 $[M]^+$. The interpretations of proton

and carbon signals of compound **2** were largely consistent with Swisłocka *et al*. (2012) and Pitchai *et al*. (2012).

(*E*)-*p*-coumaric acid (compound **2**): white powder. EI-MS, *m/z* (relative intensity): 164 $[M]^+$ (100), 147 (47), 119 (36), 107 (13), 91 (26), 73 (10), 65 (12.7). $^1$H NMR ($CDCl_3$, 600 MHz) and $^{13}$C NMR ($CDCl_3$, 150 MHz): See Table 9.

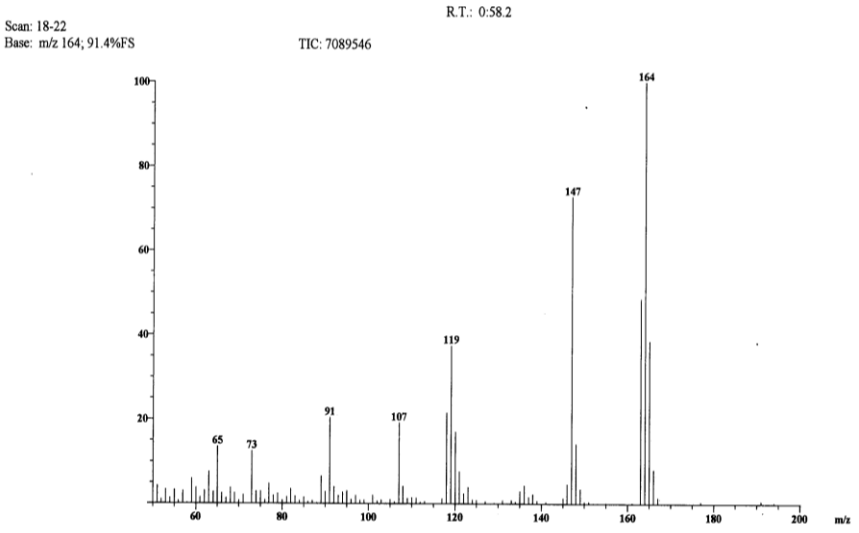


**Fig. 11. Mass spectrum of compound 2**

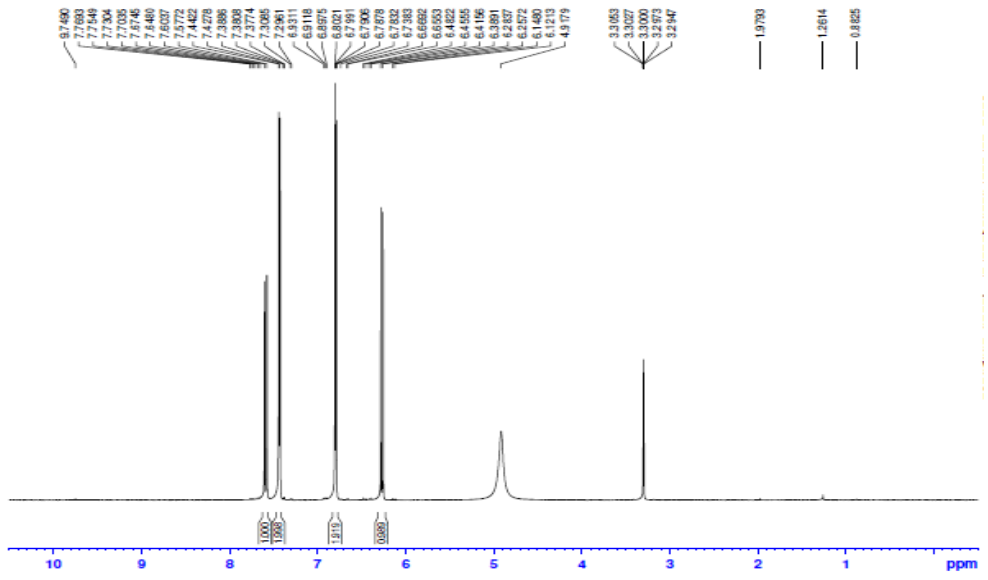

**Fig. 12. $^{1}$H NMR spectrum of compound 2**

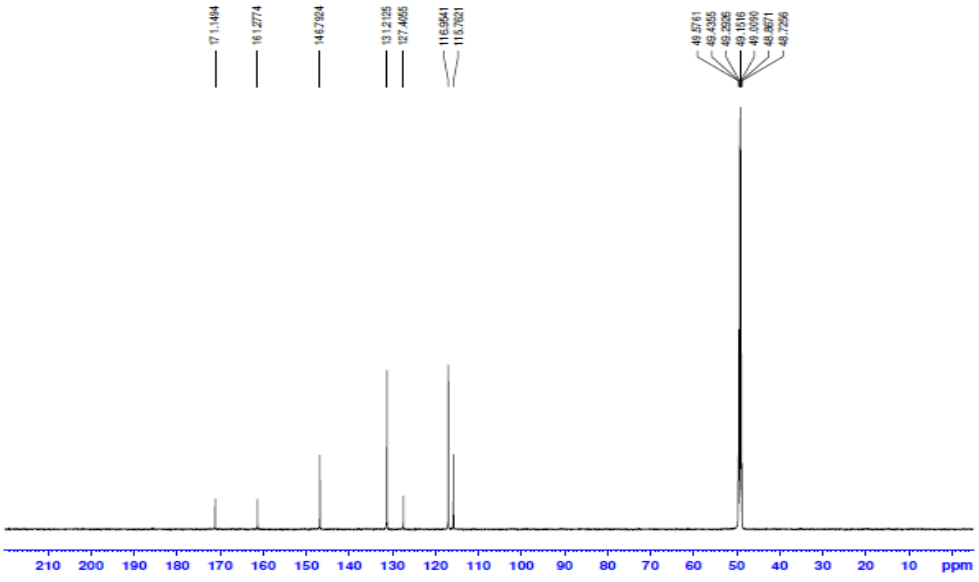

**Fig. 13. $^{13}$C NMR spectrum of compound 2**

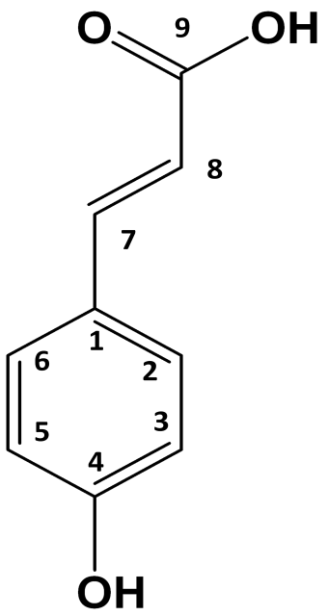


**Fig. 14. Structure of (*E*)-*p*-coumaric acid**

**Table 9. $^{1}$H and $^{13}$C NMR spectral data for compound 2**

| Position | Partial structure | $\delta_C$ (ppm) | $\delta_H$ (ppm) | $\delta_C$ (ppm) ($CDCl_3$, 500 MHz) Pitchai *et al.* (2012) | $\delta_H$ (ppm) (DMSO, 300 MHz) Swisłocka *et al.* (2012) |
|---|---|---|---|---|---|
| 1 | CH | 127.4 | — | 125.6 | 12.1 |
| 2 | CH | 131.2 | 7.44 d ($J$ = 8.6 Hz) | 129.4 | 7.49 |
| 3 | CH | 115.8 | 6.79 ($J$ = 6.8 Hz) | 114.4 | 6.79 |

**Table 9. (Continued)**

| Position | Partial structure | $\delta_C$ (ppm) | $\delta_H$ (ppm) | $\delta_C$ (ppm) ($CDCl_3$, 500 MHz) Pitchai *et al.* (2012) | $\delta_H$ (ppm) (DMSO, 300 MHz) Swisłocka *et al.* (2012) |
|---|---|---|---|---|---|
| 4 | CH | 161.3 | 9.75 | 159 | 9.96 |
| 5 | CH | 115.8 | 6.79 (*J* = 6.8 Hz) | 114.4 | 6.79 |
| 6 | CH | 131.2 | 7.44 (*J* = 8.6 Hz) | 129.4 | 7.49 |
| 7 | CH | 146.8 | 6.28 (*J* = 16.0 Hz) | 144.1 | 6.29 |
| 8 | CH | 117.0 | 7.58 (*J* = 16.0 Hz) | 115.0 | 7.52 |
| 9 | C | 171.1 | | 166.8 | |

**3. Cytotoxic and antiviral activity of test compounds**

The cytotoxicity of 14 test compounds (Fig. 15) and ribavirin on HeLa cells was determined (Table 10). Based on $CC_{50}$ values, potent cytotoxicity was observed with kaempferol (65.0 μg/mL) and luteolin (96.7 μg/mL). No cytotoxicity ($CC_{50}$, > 100 μg/mL) was observed with the other 12 compounds and ribavirin.

The antiviral activity of 14 test compounds and ribavirin against HRV-2 was evaluated using a SRB assay (Table 10). As judged by $IC_{50}$ values, kaempferol (2.1 μg/mL) was the most toxic compound, followed by chrysin (4.4 μg/mL) and galangin (5.4 μg/mL). $IC_{50}$ of quercetin, luteolin, fisetin, and caffeic acid is between 10.3 and 12.1 μg/mL. Low antiviral activity was produced by ferulic acid, acacetin, EGCG, and (*E*)-*p*-coumaric acid ($IC_{50}$, 34.0-62.2 μg/mL). These compounds were more pronounced

antiviral activity than ribavirin ($IC_{50}$, 74.1 μg/mL). The other four compounds exhibited no activity ($IC_{50}$, > 100 μg/mL). High TI (> 8.3) was observed with kaempferol, chrysin, galangin, quercetin, luteolin, fisetin, and caffeic acid, compared with ribavirin (TI, > 1.3).

The antiviral effects of all compounds on HRV-3 were likewise compared (Table 11). Based on $IC_{50}$ values, kaempferol (3.4 μg/mL) was the most toxic compound, followed by chrysin, quercetin, and luteolin (4.1-5.8 μg/mL). $IC_{50}$ of caffeic acid, protocatechuic acid, and fisetin is between 9.4 and 19.2 μg/mL. Low antiviral activity was obtained from acacetin, galangin, ferulic acid, and (*E*)-*p*-coumaric acid ($IC_{50}$, 30.6-74.6 μg/mL). The antiviral activity of these compounds was higher than that of ribavirin. The other four compounds exhibited no activity ($IC_{50}$, > 100 μg/mL). High TI (> 8.5) was produced by kaempferol, chrysin, quercetin, luteolin, caffeic acid, and protocatechuic acid, compared with ribavirin (TI, > 1.3).

Against HRV-4 (Table 12), protocatechuic acid ($IC_{50}$, > 3.3 μg/mL) and kaempferol (3.7 μg/mL) exhibited the strongest antiviral activity, followed by luteolin, quercetin, and chrysin ($IC_{50}$, 4.2-6.2 μg/mL). $IC_{50}$ of caffeic acid and fisetin was between 11.9 and 19.0 μg/mL. Galangin, acacetin, and ferulic acid exhibited low antiviral activity ($IC_{50}$, 23.8-45.1 μg/mL). These compounds were more toxic than ribavirin. Very low to no activity was observed with the other five compounds. High TI (> 8.4) was obtained from kaempferol, luteolin, quercetin, chrysin, and caffeic acid.

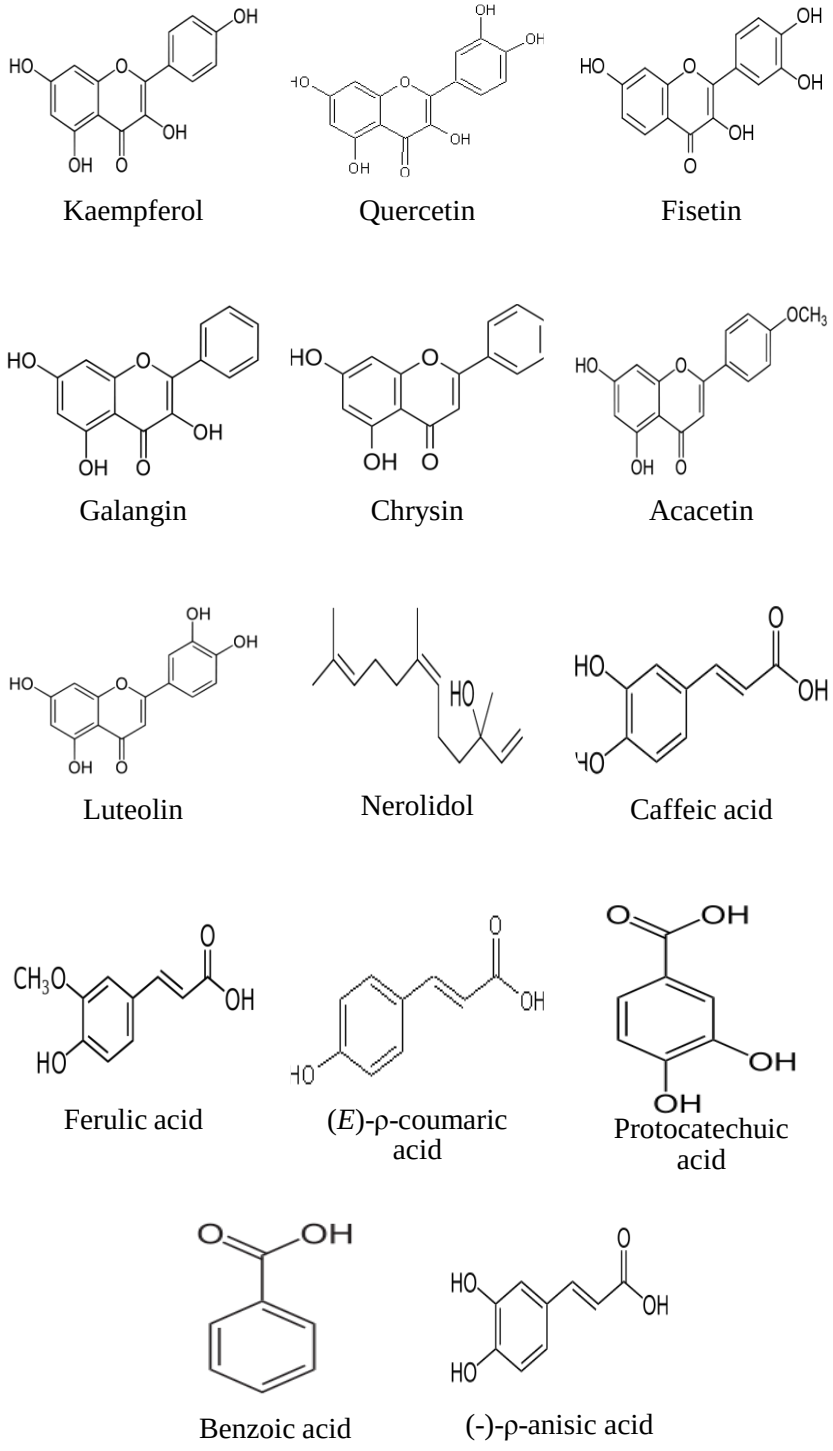


**Fig. 15. The structure of kwon propolis compound**

**Table 10. Antiviral and cytotoxic activity of 15 test compounds and antiviral drug ribavirin against HRV-2 using SRB assay**

| Class[a] | Test compound | $CC_{50}$[b] (μg/mL) | $IC_{50}$[c] (μg/mL) | TI[d] |
|---|---|---|---|---|
| FL | Kaempferol[e] | 65.0 ± 0.12 | 2.1 ± 1.30 | 30.9 |
| | Quercetin | > 100 | 10.3 ± 6.12 | > 9.7 |
| | Fisetin | > 100 | 11.7 ± 4.35 | > 8.6 |
| | Galangin | > 100 | 5.4 ± 4.18 | > 18.7 |
| FE | Chrysin | > 100 | 4.4 ± 2.50 | > 22.7 |
| | Acacetin | > 100 | 46.4 ± 5.39 | > 2.1 |
| | Luteolin | 96.7 ± 0.06 | 10.7 ± 0.60 | > 9.0 |
| ST | Nerolidol | > 100 | > 100 | — |
| PP | Caffeic acid | > 100 | 12.1 ± 1.06 | > 8.3 |
| | Ferulic acid | > 100 | 34.0 ± 5.65 | > 2.9 |
| | (*E*)-*p*-Coumaric acid[e] | > 100 | 62.2 ± 1.27 | > 1.6 |
| BA | Protocatechuic acid | > 100 | > 100 | — |
| | Benzoic acid | > 100 | > 100 | — |
| | (*E*)-*p*-Anisic acid | > 100 | > 100 | — |
| — | EGCG[f] | > 100 | 62.0 ± 5.22 | 1.5 |
| — | Ribavirin | > 100 | 74.1 ± 6.98 | > 1.3 |

[a] FL, flavonol; FE, flavonone; ST, sesquiterpenoid; PP, phenyl propanoid; BA, benzoic acid.

[b] Concentration of compounds producing 50% inhibition of virus-induced cytopathic effects.

[c] The 50% cytotoxic concentration for HeLa cells.

[d] Therapeutic index = $CC_{50}/IC_{50}$.

[e] Compound identified in this study. The other compounds were reported by Marcucci.

[f] (−)-Epigallocatechin gallate.

**Table 11. Antiviral and cytotoxic activity of 15 test compounds and antiviral drug ribavirin against HRV-3 using SRB assay**

| Class[a] | Test compound | $IC_{50}$[c] (μg/mL) | TI[d] |
|---|---|---|---|
| FL | Kaempferol | 3.4 ± 0.12 | 19.1 |
| | Quercetin | 4.7 ± 0.69 | > 21.3 |
| | Fisetin | 19.2 ± 1.00 | > 5.2 |
| | Galangin | 31.4 ± 0.23 | > 3.18 |
| FE | Chrysin | 4.1 ± 1.22 | > 24.3 |
| | Acacetin | 30.6 ± 5.2 | > 3.3 |
| | Luteolin | 5.8 ± 0.75 | > 17.2 |
| ST | Nerolidol | > 100 | — |
| PP | Caffeic acid | 9.4 ± 0.47 | > 10.6 |
| | Ferulic acid | 48.3 ± 4.33 | > 2.1 |
| | (*E*)-*p*-Coumaric acid | 74.6 ± 2.14 | > 1.3 |
| BA | Protocatechuic acid | 11.8 ± 1.78 | > 8.5 |
| | Benzoic acid | > 100 | — |
| | (*E*)-*p*-Anisic acid | > 100 | — |
| — | EGCG[e] | > 100 | — |
| — | Ribavirin | 74.7 ± 0.54 | > 1.3 |

[a] FL, flavonol; FE, flavonone; ST, sesquiterpenoid; PP, phenyl propanoid; BA, benzoic acid.

[b] Concentration of compounds producing 50% inhibition of virus-inducedcytopathic effects.

[c] The 50% cytotoxic concentration for HeLa cells.

[d] Therapeutic index = $CC_{50}/IC_{50}$.

[e] Compound identified in this study. The other compounds were reported by Marcucci.

[f] (−)-Epigallocatechin gallate abbreviation.

**Table 12. Antiviral and cytotoxic activity of 15 test compounds and antiviral drug ribavirin against HRV-4 using SRB assay**

| Class[a] | Test compound | $IC_{50}$[c] (μg/mL) | TI[d] |
|---|---|---|---|
| FL | Kaempferol[e] | 3.7 ± 0.33 | 17.6 |
| | Quercetin | 5.5 ± 1.25 | > 18.2 |
| | Fisetin | 19.0 ± 3.80 | > 5.3 |
| | Galangin | 23.8 ± 7.76 | > 4.2 |
| FE | Chrysin | 6.2 ± 1.34 | > 16.1 |
| | Acacetin | 29.1 ± 1.02 | > 3.4 |
| | Luteolin | 4.2 ± 2.25 | > 23.8 |
| ST | Nerolidol | > 100 | — |
| PP | Caffeic acid | 11.9 ± 2.78 | > 8.4 |
| | Ferulic acid | 45.1 ± 0.98 | > 2.2 |
| | (*E*)-*p*-Coumaric acid[e] | 99.2 ± 8.36 | > 1.0 |
| BA | Protocatechuic acid | 3.3 ± 1.84 | > 30.3 |
| | Benzoic acid | > 100 | — |
| | (*E*)-*p*-Anisic acid | > 100 | — |
| — | EGCG[f] | > 100 | — |
| — | Ribavirin | 79.1 ± 7.61 | > 1.3 |

[a] FL, flavonol; FE, flavonone; ST, sesquiterpenoid; PP, phenyl propanoid; BA, benzoic acid.

[b] Concentration of compounds producing 50% inhibition of virus-induced cytopathic effects.

[c] The 50% cytotoxic concentration for HeLa cells.

[d] Therapeutic index = $CC_{50}/IC_{50}$.

[e] Compound identified in this study. The other compounds were reported by Marcucci

[f] (−)-Epigallocatechin gallate abbreviation.

**4. Effect of test compounds on the infectivity of HRV-3 particles**

The effects on the infectivity of HRV particles of (*E*)-*p*-coumaric acid, kaempferol, quercetin, chrysin, protocatehuic aicd, and caffeic acid were compared with those of ribaivirin (Fig. 16). Based on their cytotoxicity and antiviral activity, kaempferol and the other compounds were treated with 10 and 100 μg/mL, respectively. The antiviral activity of preincubation with (*E*)-*p*-coumaric acid, kaempferol, quercetin, caffeic acid, chrysin, protocatechuic acid, and ribaivirin resulted in 3.4, 4.1, 6.3, 7.0, 7.4, 40.4, and 6.4%, respectively. However, protocatehuic acid interacted with the particle of HRV-3, as pre-exposure of the virus to the compound have changed the infectivity of HRV particles. Continuous presence of (*E*)-*p*-coumaric acid, kaempferol, quercetin, caffeic acid, chrysin, and ribaivirin during infection led to a significant increase in the antiviral activity. Similar results were produced by the control infections. This finding indicates that all of the compounds (except for protocatechuic acid and ribavirin) do not interact with the particles of HRV-3, thus no effects on virus infectivity.

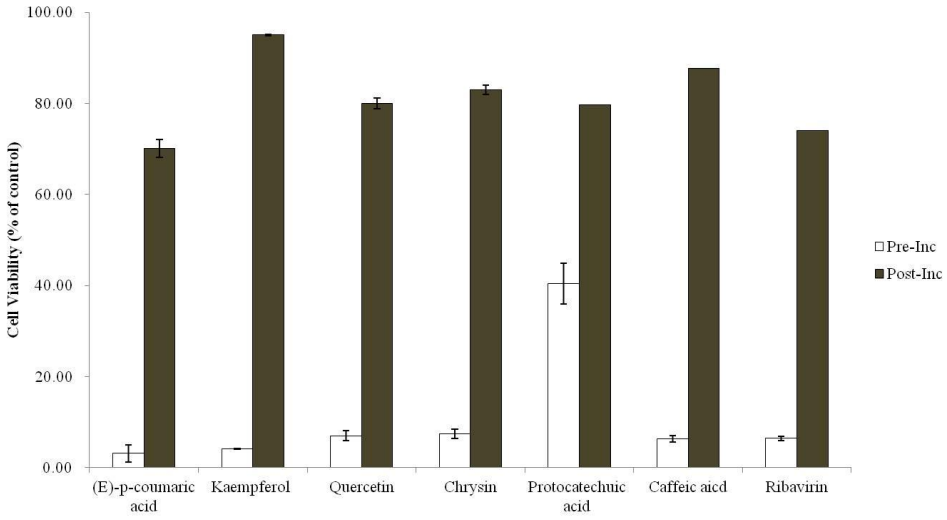


**Fig. 16. Effects of (*E*)-*p*-coumaric acid, kaempferol, quercetin, chrysin, protocatehuic aicd, and caffeic acid on the infectivity of HRV-3 particles. Kaempferol and the other compounds were treated with 10 and 100 μg/mL, respectively**

**5. Time course of test compound addition**

Time-course addition experiments of two test constituents were carried out to determine the stages at which the constituents exerted its inhibitory effects (Fig. 17). In addition of 100 μg/mL (*E*)-*p*-coumaric acid simultaneously with HRV-3, it showed its antiviral activity when added just after the virus inoculation (0 h) and very early stage after the virus inoculation (1 h). The inhibitory rate of (*E*)-*p*-coumaric acid declined to 50% or less except during the virus inoculation (0 h). The 10 μg/mL kaempferol suppressed HRV infection when added just after the virus inoculation (0 h) and also after the virus inoculation (1-6 h). The inhibitory rate of kaempferol declined to 30% or less when added at either prior (-1 h) or post infection (12 h). This observation indicates that kamepferol

affects the initial stage of HRV infection-3. Ribavirin also showed similar results.

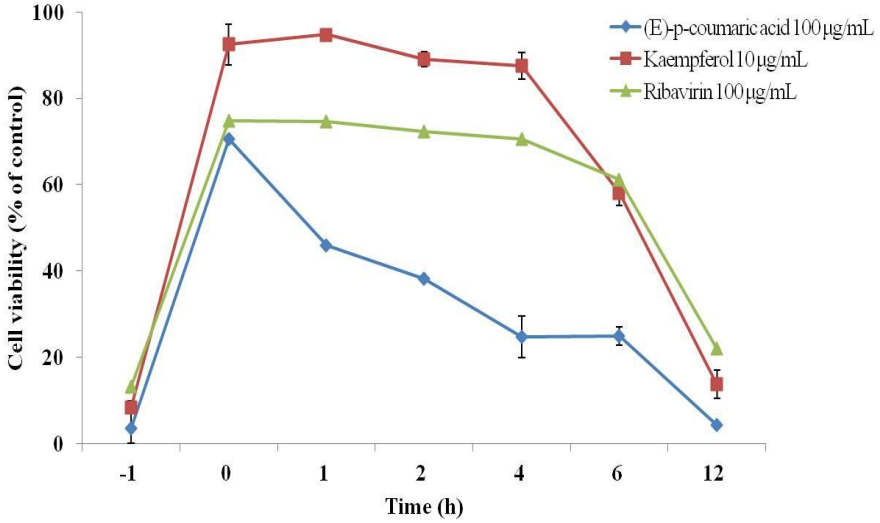


**Fig. 17. Time-of-addition effect of 100 μg/mL (*E*)-*p*-coumaric acid, 10 μg/mL kaempferol, and 100 μg/mL ribavirin on HRV-3 replication in HeLa cells**

**6. Effects of kaempferol and (*E*)-*p*-coumaric acid on the level of HRV replication**

The RNA replication levels of HRV-3 on the HeLa cells were remarkably inhibited in the cell cultures treated with kaempferol or (*E*)-*p*-coumaric acid (Fig. 18). At the presences of 10 μg/mL kaempferol or 100 μg/mL (*E*)-*p*-coumaric acid on the HeLa cell cultures infected with HRV-3, the RNA replication levels of the HRV-3 were reduced by 212.9 or 2.88 folds, respectively, compared with the replication levels in the cell cultures without compound treatment.

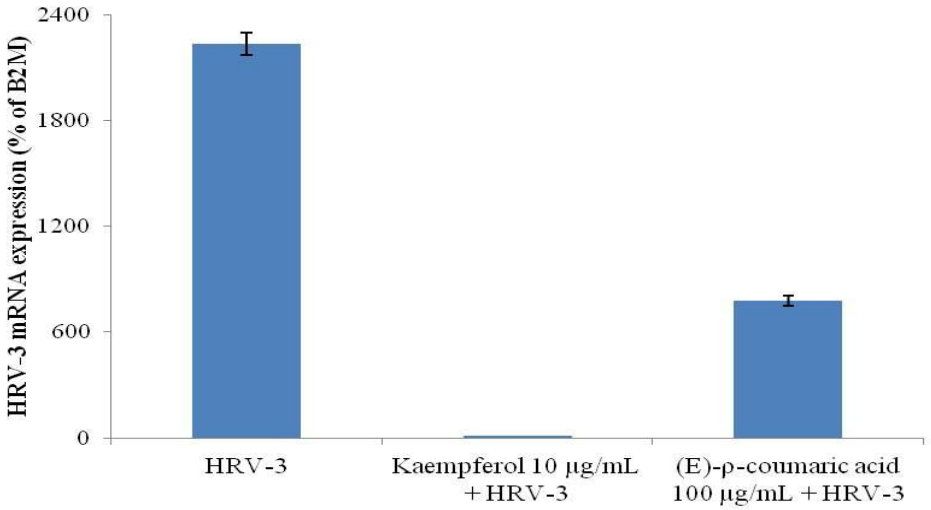


**Fig. 18. Replication of HRV-3 on HeLa cells 2 days after infection in the presence of 10 μg/mL kaempferol or 100 μg/mL (*E*)-*p*-coumaric acid detected by real-time RT-PCR. HRV RNA expressions were normalized to the constitutive expression of β2-microglobulin (B2M) mRNA**

**7. Effects of kaempferol and (*E*)-*p*-coumaric acid on ICAM-1 expression**

mRNA expressions of ICAM-1 on HeLa cells 2 days after infection in the presence of 10 μg/mL kaempferol or 100 μg/mL (*E*)-*p*-coumaric acid were investigated using real-time RT-PCR (Fig. 19). HRV-3 infections increased ICAM-1 mRNA expression, but these increases were reduced by kaempferol or (*E*)-*p*-coumaric acid.

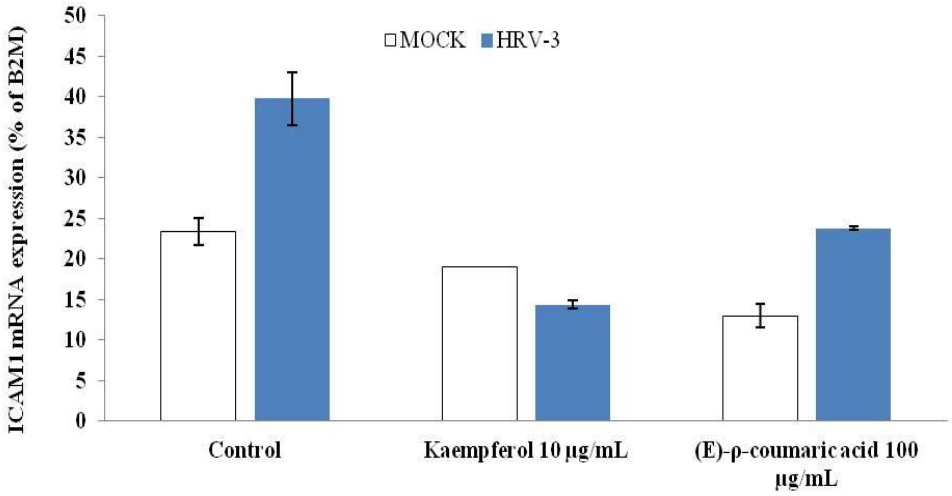


**Fig. 19. mRNA expressions of ICAM-1 on HeLa cells infected with or without HRV-3 2 days after infection in the presence of 10 μg/mL kaempferol or 100 μg/mL (*E*)-*p*-coumaric acid**

**8. Effects of kaempferol and (*E*)-ρ-coumaric acid on IL-6 expression**

mRNA expressions in infected and non-infected HeLa cells after 2-day treatments with kaempferol or (*E*)-*p*-coumaric acid were examined using real-time RT PCR (Fig. 20). In the nontreated cell cultures, HRV-3 evoked increase in cytokine IL-6 mRNA expression levels in HeLa cells, however the expression levels were significantly reduced in the treated cell cultures with kaempferol. (*E*)-*p*-Coumaric acid was effective in inhibiting the viral induction of IL-6.

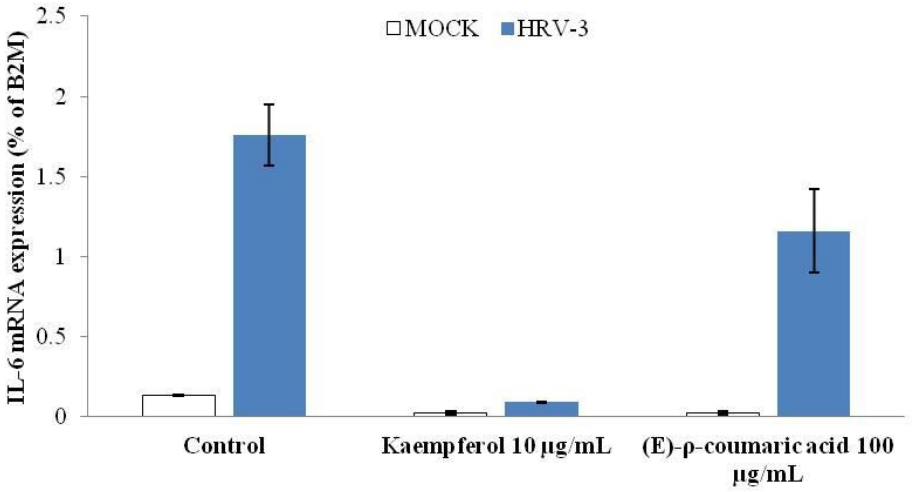


**Fig. 20. mRNA expression of IL-6 on HeLa cells infected with or without HRV-3 in the presence of 10 g/mL kaempferol or 100 g/mL (*E*)-*p*-coumaric acid detected by real-time RT-PCR**

# Ⅳ. Discussion

Natural compounds extracted from plants or plant-derived materials have been suggested as alternative sources for anti-HRV products. This approach is appealing, in part, because they constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe and often act at multiple and novel target sites, thereby reducing the potential for resistance (Raskin et al., 2002). In additiona, propolis and its constituents have been studied on their bioactive capacities and their antiviral activity against a number of viruses such as herpes viruses, poliovirus, influenza virus A/Hong Kong, and HIV (Burdock, 1998; Búfalo, et al., 2009). In this study with propolis from different origin against HRV, potent antiviral activity against HRV was observed in Brazil propolis.

The differential responses of various viruses are affected by extrinsic and intrinsic factors such as the plant species, the parts of the plants, the solvents used for extraction, the geographical location where the plants were grown, and the application methods (Jassim and Naji, 2003). In this study, antiviral responses according to geographical locations were studied. The composition of propolis depends on the geographic patterns and plant sources significantly (Salatino A., et al., 2011). In Brazil propolis from the southeast region and Amazon contains chiefly phenylated phenylpropanoids (Bankova et al., 1996) and polyprenylated benzophenonesm (Ishida et al., 2011), respectively. Geranyl flavonones have been reported for propolis from the Pacific region, such as Taiwan

(Huang et al., 2007) and Okinawa (Kumazawa et al., 2008). Propolis from eastern Mediterranean regions, such as Greece, Crete and Turkey, may contain predominantly diterpenes (Silici and Kutluca, 2005). The variation in the chemical composition of propolis from different origin also caused the diverse biological activities (Salatino A., et al., 2011).

There are few data from researches of the antiviral effects of propolis and its constituents. In virological studies conducted with extracts obtained with various solvents, some fractions affected the production of influenza viruses A and B, vaccinia virus and Newcastle disease virus in different biological testing systems (Maksimova-Todorova, 1985). The effect of propolis on several DNA and RNA viruses, including herpes simplex type 1, an acylclovir resistant mutant, herpes simplex type 2, adenovirus type2, vesicular stomatitis virus and poliovirus type 2 were investigated. Propolis reduced the titer of herpes simplex virus and exerted a virucidal action on the herpes simplex and vesicular stomatitis virus (Amoros et al., 1992a; 1992b). One of propolis constituents, isopentyl ferulate, significantly inhibited the infectious activity of influenza virus A (Hong Kong strain) (Serkedjieva et al., 1992). Similar results were found when the in vitro activity of 3-methylbut-2-enyl caffeate identified in propolis was investigated against herpes simplex virus type 1 (Amoros et al., 1994). Current study showed that constituents of propolis had remarkable antiviral activity against HRV. However, there has been no much studied except the clinic evaluation of propolis with common cold infection groups by Szmeja et al in 1989.

In the present study, ethanol extract from the Brazilian propolis exhibited potent antiviral activity against HRV. The bioactive constituents were identified as the kaempferol and (*E*)-ρ-coumaric acid. Kaempferol is a flavonoid widely distributed in the plant kingdom. There are over two thousand articles in PubMed reporting the isolation and/or biological properties of this compound (Calderón-Montaño et al., 2011). However, this study is the first research about its antiviral activity against HRV and its activity also exhibited significantly higher than commercial antiviral agents, ribavirin. Kaempferol, a constituent from *Consolida oliveriana* (Marin et al., 2009), *Celastrus tatarinovii* (Rzadkowska-Bodalska et al., 1975), *Echites hirsute* (Chien et al., 1979), *Lilium candidum* (Vachalkova et al., 2000), *Rhamnus procumbens* (Goel et al., 1988) etc. It has been reported on various activities; antioxidant activity in vitro and in vivo (Sanz et al., 1994; Hibatallah et al., 1999; Kampkotter et al., 2007), anti-inflammatory activity (Della et al., 1988; Toker et al., 2004; Kim et al., 2010), anticancer activity as inducing apoptosis (Brusselmans et al., 2005; Kang et al., 2009) and inhibiting angiogenesis (Schindler et al., 2006; Ahn et al., 2009), antimicrobial activity (Cai and Wu, 1996; Kataoka et al., 2001; Martitni et al., 2004; Lin et al., 2008) , antidiabetic activity (Basnet et al., 1993; Lee et al., 2009), anti-osteoporotic activity (Wattel et al., 2003) and antiviral activity against herpes simplex virus (Debiaggi et al., 1990; Lyu et al., 2005), influenza virus (Jeong et al., 2009) and human immunodeficiency virus (Mahmood et al., 1996; Min et al., 2001). Separated and identified (*E*)-ρ-coumaric acid had potent antiviral activity against HRV similar with ribavirin. (*E*)-ρ-coumaric acid was firstly isolated from *Fructus mume* and identified to be one of the main components (Ou et al., 2011). This compound was also found in *Gueldenstaedtia stenophylla* (Wang et al., 2012), American cranberry fruit (Zuo et al.,

2002), *Smallanthus sonchifolius* (Neves and da Silva, 2007), *Vanilla planifolia* (Sinha et al., 2007) etc. It has various activities; antimicrobial activity (Herald and Davidson, 1983; Cho et al., 1998; Maddox et al., 2010), antioxidant activity (Castelluccio et al., 1995; Laranjinha et al., 1995; Zhang et al., 2000), immunomodulatory and anti-inflammatory properties *in vivo* (Luceri et al., 2004; Pragasam et al., 2012), protective effect against UVB-induced cell damage (Lodovici et al., 2003), antiviral activity against infectious bursal disease virus (Ou et al., 2011).

Elucidation of the mode of action of antiviral natural products and agents plays a role for virus control because it may give useful information on the most appropriate scaffolds, delivery means, and resistance management. The mode of action of two identified compounds was investigated in this study. Firstly, both two constituents did not interact with virus particles and inhibit virus absorption to the HeLa cells. In 2010, Choi proved that gallic acid from *Woodfordia fruticosa* Kurz did directly interact or activate with HRV particles. It indicated gallic acid had the mode of action derived from the inhibition of virus absorption. Among the 12 propolis constituents, only protocatechuic acid did interact or activate with HRV particles. Of time course study, gallic acid only had effect at early stage of viral replication after infection (only during inoculation, 0h). (*E*)-ρ-coumaric acid (0h) presented similar activity with gallic acid, however, kaempferol (0, 1, 2, 4, and 6h) presented relatively continuous activity at early stage of viral replication. Kamepferol showed similar aspect with commercial antiviral agent, ribavirin (0, 1, 2, 4, and 6h).

Many studies demonstrated that HRV infection upregulates ICAM-1 expression on airway epithelial cells, thus facilitating further viral attachment and entry (Papi et al., 2002). Therefore, inhibit the production of ICAM-1, which is crucial in developing new anti-HRV agents (Jang et al., 2006). The current experiments presented that HRV-3 induced the forms of ICAM-1 expressed in HeLa cells and presences of kaempferol and (*E*)-ρ-coumaric acid in the infected cell cultures have brought about the decline of mRNA expression of ICAM-1. Currently used antibiotics, erythromycin, macrolide, and clarithromycin, also reported that declined the expression of ICAM-1 (Khair et al., 1995; Jang et al., 2006).

HRV infection induces the production of several inflammatory mediators, such as kinins, leukotrienes, histamine, IL -1, -6, and -8, tumour necrosis factor, and RANTES (regulated by activation normal T cell expressed and secreted) in the nasal secretions (Noah *et al*, 1995; Zhu *et al*., 1996; Turner *et al*., 1998; Heikkinen and Jarvinen, 2003; Newcomb, 2007, Bochkov *et al*., 2010). The concentrations of IL -6 and -8 in nasal secretions correlate with the severity of the symptoms in patients with colds (Noah *et al* 1995; Zhu *et al* 1996; Turner *et al*., 1998). Cytokines such as TNF-α, IL-1β, chemokines such as IL-8, IL-6, lipid mediators and peptides induce up-regulation expression of the adhesion molecules (Panés *et al*., 1999; Yasuda *et al*., 2006)**.** Rhinovirus infections have been reported to be responsible for triggering exacerbations of asthma through inducing gene expression of these cytokines in asthmatic subjects (Bochkov *et al*., 2010). This present study assessed that kaempferol and (*E*)-ρ-coumaric acid were effective in inhibiting the viral inductions of cytokines, IL-6.

Results of this study indicate that Brazilian propolis constituents could be useful as antiviral agents for HRV. For practical use of the Brazilian propolis and its constituents as novel anti-HRV agents to proceed, further research is indispensable. Though current study only observed the ICAM-1 expression as mRNA level, protein level of ICAM-1 expression is primarily needed to assure. Also, investigation of various inflammatory mediators expression will help to prove that propolis constituents have effect on common colds and HRV induced disease. Above this, safety issues for human health, various and specific mode of action, and effective formulations for improving the antiviral potency and stability in human gastrointestinal tract is demanded.

# Ⅴ. Literature cited

**Ahn M. R., Kunimasa K., Kumazawa S., Nakayama T., Kaji K., Uto Y., Hori H., Nagasawa H., and Ohta T.** 2009. Correlation between antiangiogenic activity and antioxidant activity of various components from propolis. Molecular nutrition and food research. 53: 643-651.

**Alisi M. A., Apicella C., Cellai L., Dragone P., Fioravanzo E., Florio S., Furlotti G., Mangano G., Ombrato R., Luisi R., Pompei R., Rincicotti V., Russo V., Vitiello M., and Cazzolla N.** 2008. New anti-viral drugs for the treatment of the common cold. Bioorganic and medicinal chemistry. 16: 3091–3107.

**Amoros M., Lurton E., Boustie J., Girre L., Sauvager F., and Cormier M.** 1994. Comparison of the anti-herpes simplex virus activities of propolis and 3-methylbut-2-enyl caffeate. Journal of natural products. 57: 644-647.

**Amoros M., Sauvager F., Girre L., and Cormier M.** 1992a. *In vitro* antiviral activity of propolis. Apidologie 23: 231-240.

**Amoros M., Simões C. M. O., Girre L., Sauvager F., and Cormier M.** 1992b. Synergistic effect of flavones and flavonols against Herpes Simplex virus Type1 in cell culture. Comparison with the antiviral activity of propolis. Journal of natural products. 55: 1732-1740.

**Andersen D. O., Murray B. K., Robins R. K., and North J. A.** 1992. In vitro antiviral activity of ribavirin against picornaviruses. Antiviral chemistry and chemotherapy 6: 361–37.

**Andries K., Dewindt B., Snoeks J., Wouters L., Moereels H., Lewi P. J., and Janssen P. A.** 1990. Two groups of rhinoviruses revealed by a panel of antiviral compounds present sequence divergence and differential pathogenicity. Journal of virology. 64: 1117-1123.

**Andries K., Dewindt B., Snoeks J., Willebrords R., van Eemeren K., and Stokbroekx R., and Janssen P. A.** 1990. In vitro activity of pirodavir (R77975), a substituted phenoxy-pyridazinamine with broad-spectrum antipicornaviral activity. Antimicrobial agents and chemotherapy. 36: 100-107.

**Andries K., Dewindt B., Snoeks J., Willebrords R., van Eemeren K., Stokbroekx R., and Janssen P. A.** 1992. In vitro activity of pirodavir (R 77975), a substituted phenoxy-pyridazinamine with broad-spectrum antipicornaviral activity. Antimicrobial agents and chemotherapy 36: 100–107.

**Apicella C., Cellai L., Dragone P., Fioravanzo E., Florio S., Furlotti G., Mangano G., Ombrato R., Luisi R., Pompei R., Rincicotti V., Russo V., Vitiello M., and Cazzolla N.** 2008. New anti-viral drugs for the treatment of the common cold. Bioorganic and medicinal chemistry. 16: 3091–3107.

**Arruda E., Pitkäranta A., Witek T. J. Jr, Doyle C. A., and Hayden F. G.** 1997. Frequency and natural history of rhinovirus infections in adults during autumn. Journal of clinical microbiology. 35: 2864-2868.

**Arruda E., Crump C. E., Marlin S. D., Merluzzi V. J., and Hayden F. G.** 1992. *In vitro* studies of the antirhinovirus activity of soluble intercellular adhesion molecule-1. Antimicrobial agents and chemotherapy. 36: 1186–1191.

**Bankova S. V, de Castro L. S, and Marucci C. M.** 2000. Propolis: recent advances in chemistry and plant origin. Apidologie 31: 3-15.

**Bankova S. V.** 2005. Recent trends and important developments in propolis research. Evidence-based complementary and alternative medicine. 2: 29-32.

**Bankova V., Christov R., Kujumgiev A., Marcucci, M. C. and Popov S.** 1995. Chemical composition and antibacterial activity of Brazilian propolis. Zeitschriftfu È r Naturforschung Section C. Biosciences. 50: 167-172.

**Bardin P. G., Sanderson G., Robinson B. S., Holgate S. T., and Tyrrell D. A.** 1996. Experimental rhinovirus infection in volunteers. European respiratory journal. 9: 2250-2255.

**Barnard D. L., Hubbard V. D., Smee D. F., Sidwell R. W., Watson K. G., Tucker S. P., and Reece P. A.** 2004. In vitro activity of expanded-spectrum pyridazinyl oxime ethers related to pirodavir: novel capsid-binding inhibitors with potent antipicornavirus activity. Antimicrobial agents and chemotherapy 48: 1766–1772.

**Basnet P., Kadota S., Terashima S., Shimizu M., and Namba T. 1993.** Two new 2-arylbenzofuran derivatives from hypoglycemic activitybearing fractions of Morus insignis. Chemical and pharmaceutical bulletin. 41: 1238-1243.

**Bauer D. J., Selway J. W., Batchelor J. F., Tisdale M., Caldwell I. C., and Young D. A.** 1981. 4′,6-Dichloroflavan (BW683C), a new anti-rhinovirus compound. Nature. 292: 369–370.

**Binford S. L., Maldonado F., Brothers M. A., Weady P. T., Zalman L. S., Meador 3rd J. W., Matthews D. A., and Patick A. K.** 2005. Conservation of amino acids in human rhinovirus 3C protease correlates with broad-spectrum antiviral activity of rupintrivir, a novel human rhinovirus 3C protease inhibitor. Antimicrobial agents and chemotherapy. 49: 619–626.

**Bochkov Y. A., Hanson K. M., Keles S., Brockman-Schneider R. A., Jarjour N. N. and Gern J. E.** 2009. Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma. Mucosal immunology. 3: 69–80.

**Brown-Augsburger P., Vance L. M., Malcolm S. K., Hsiung H., Smith D. P., and Heinz B. A.** 1999. Evidence that enviroxime targets multiple components of the rhinovirus 14 replication complex. Archives of virology. 144: 1569–1585.

**Brusselmans K., Vrolix R., Verhoeven G., and Swinnen J. V.** 2005. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. Journal of biological chemistry. 280: 5636-5645.

**Bubeck D., Filman J. D., Cheng N., Steven C. A., Hogle M. J., and Belnap M. D.** 2005. The structure of the poliovirus 135s cell entry intermediate at 10-angstrom resolution reveals the location of an externalized polypeptide that binds to membranes. Journal of virology. 70: 7745-7755.

**Búfalo M. C., Figueiredo A. S., Sousa J. P. B., Candeias, J. M. G., Bastos J. K., Sforcin J. M.** 2009. Anti-poliovirus activity of Baccharis dracunculifolia and propolis by cell viability determination and real-time PCR. Journal of applied microbiology. 107: 1669-1680.

**Burdock G. A.** 1998. Review of the biological properties and toxicity of bee proplis. Food and chemical toxicology. 36: 347-363.

**Cai L., and Wu C. D.** 1996. Compounds from Syzygium aromaticum possessing growth inhibitory activity against oral pathogens. Journal of natural prodducts. 59: 987-990.

**Calderón-Montaño J. M., Burgos-Morón E., Pérez-Guerrero C., and López-Lázaro M.** 2011. A review on the dietary flavonoid kaempferol. Mini-reviews in medicinal chemistry. 11: 298-344.

**Carrasco L.** Picornavirus inhibitors. 1994. Pharmacological therapy. 64: 215-290.

**Castaldo S., and Cpasso F.** 2002. Proplis, an old remedy used in modern medicine. Fitoterapia. 73 Supplement 1 S1-S6.

**Castelluccio C., Paganga G., Melikian N., Bolwell G. P., Pridham J., Sampson J., and Rice-Evans C.** 1995. Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants. FEBS Letters. 368: 188–192.

**Chantrill B. H., Coulthard C. E., Dickinson L., Inkley G. W., Morris W., and Pyle A. H.** 1952. The action of plant extracts on a bacteriophage of *Pseudomonas pyocyanea* and on influenza A virus. Journal of general microbiology 6: 74–84.

**Chien M. M., Svoboda G. H., Schiff P. L., Jr. Slatkin D. J., and Knapp J. E.** 1979. Chemical constituents of Echites hirsuta (Apocynaceae). Journal of pharmaceutical sciences. 68: 247-249.

**Cho J. Y., Moon J. H., Seong K. Y., and Park K. H.** 1998. Antimicrobial activity of 4-hydroxybenzoic acid and trans 4-hydroxycinnamic acid isolated and identified from rice hull. Bioscience, biotechnology, and biochemistry. 62: 2273-2276.

**Choi H. J., Lim C. H., Song J. H., Baek S. H., and Kwon D. H.** 2009. Antiviral activity of raoulic acid from Raoulia australis against Picornaviruses. Phytomedicine. 16: 35–39.

**Choi H. J., Song J. H., Bhatt L. R., and Baek S. H.** 2010. Anti-human rhinovirus activity of gallic acid possessing antioxidant capacity. Phytotherapy research. 24: 1292-1296.

**Choi H. J., Song J. H., Bhatt L. R., and Baek S. H.** 2010. Anti-human rhinovirus activity of gallic aicd possessing antioxidant capacity. Phytotherapy research. 24: 1292-1296.

**Cirasino L., Pisati A. and Fasani F.** 1987. Contact dermatitis from propolis. Contact Dermatitis. 16: 110-111.

**Clercq E. D., Murase J., and Marquez V. E.** 1991. Broad-spectrum antiviral and cytocidal activity of cyclopentenylcytosine, a carbocyclic nucleoside targeted at CTP synthetase. Biochemical pharmacology. 41: 1821–1829.

**Clercq E. D.** 2004. Antiviral drugs in current clinical use. Journal of clinical virology. 30: 115-133.

**Clercq E. D.** 2008. Antivirals: current state of the art. Future virology. 3: 393-405.

**Clercq E. D.** 2009. Another ten stories in antiviral drug discovery (part C): “Old” and “new” antivirals, strategies, and perspectives. Medicinal research reviews. 29: 611–645.

**Clercq E. D.** 2010. Antiviral therapy: quo vadis? Future medicinal chemistry. 2: 1049-1053.

**Collinson J., Nicholson K. G., Cancio E., Ashman J., Ireland D. C., Hammersley V., Kent J., and O'Callaghan C.** 1996. Effects of upper respiratory tract infections in patients with cystic fibrosis. Thorax. 51: 1115-1122.

**Colonno R. J., Callahan P. L., and Long W. J.** 1986. Isolation of a monoclonal antibody that blocks attachment of the major group of human rhinoviruses. Journal of virology. 57: 7-12.

**Conti C., Orsi N., and Stein M. L.** 1988. Effect of isoflavans and isoflavenes on rhinovirus 1B and its replication in HeLa cells. Antiviral research. 10: 117–127.

**Crotty S., Maag D., Arnold J. J., Zhong W., Lau J. Y., Hong Z., Andino R., and, Cameron C. E.** 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nature medicine. 6: 1375–1379.

**Crump C. E., Arruda E, and Hayden F. G.** 1994. Comparative antirhinoviral activities of soluble intercellular adhesion molecule-1 (sICAM-1) and chimeric ICAM-1/immunoglobulin A molecule. Antimicrobial agents and chemothearapy 38:1425–1427.

**Debiaggi M., Tateo F., Pagani L., Luini M., and Romero E.** 1990. Effects of propolis flavonoids on virus infectivity and replication. Microbiologica. 13: 207-213.

**DeCastro S. L., and Higashi K. O.** 1995. Effect of different formulations of propolis on mice infected with Trypanosoma cruzi. Journal of ethnopharmacology. 46: 55-58.

**Della L. R., Ragazzi E., Tubaro A., Fassina G., and Vertua R.** 1988. Antiinflammatory activity of benzopyrones that are inhibitors of cyclo- and lipo-oxygenase. Pharmacological research communications. 20: Suppl 5, 91-94.

**DeLong D. C., and Reed S. E.** 1980. Inhibition of rhinovirus replication in in organ culture by a potential antiviral drug. The journal of infectious disease. 141: 87–91.

**Deszcz L., Cencic R., Sousa C., Kuechler E., and Skern T.** 2006. An antiviral peptide inhibitor that is active against picornavirus 2A proteinases but not cellular caspases. Journal of virology. 80: 9619–9627.

**Deszcz L., Seipelt J., Vassilieva E., Roetzer A., and Kuechler E.** 2004. Antiviral activity of caspase inhibitors: Effect on picornaviral 2A proteinase. FEBS letters. 560: 51–55.

**Digrak M., Yilmaz O., and Ozcelik S.** 1995. In vitro anti-microbial effect of propolis collected in Elazig region. Turkish journal of biology. 19: 249-257.

**Dobrowolski J. W., Vohora S. B., Sharma K., Shah S. A., Naqvi S. A. H. and Dandiya P. C.** 1991. Antibacterial, antifungal, antiamoebic, antiinfammatory and antipyretic studies on propolis bee products. Journal of Ethnopharmacology 35: 77-82.

**Dragovich P. S., Prins T. J., Zhou R., Brown E. L., Maldonado F. C., Fuhrman S. A., Zalman L. S., Tuntland T., Lee C. A., Patick A. K., Matthews D. A., Hendrickson T. F., Kosa M. B., Liu B., Batugo M. R., Gleeson J. P., Sakata S. K., Chen L., Guzman C., Meador 3rd J. W., Ferre A., and Worland S. T.** 2002. Structure-based design, synthesis, and biological evaluation of irreversible human rhinovirus 3C protease inhibitors. 6. Structure-activity studies of orally bioavailable, 2-pyridone-containing peptidomimetics. Journal of medicinal chemistry. 45: 1607–1623.

**Dragovich P. S., Webber S. E., Babine R. E., Fuhrman S. A., Patick A. K., Matthews D. A., Lee C. A., Reich S. H., Prins T. J., Marakovits J. T., Littlefield E. S., Zhou R., Tikhe J., Ford C. E., Wallace M. B., Meador 3rd J. W., Ferre R. A., Brown E. L., Binford S. L., Harr J. E., DeLisle D. M., and Worland S. T.** 1998. Structure-based design, synthesis, and biological evaluation of irreversible

human rhinovirus 3C protease inhibitors. 1. Michael acceptor structure-activity studies. Journal of medicinal chemistry. 41: 2806–2818

**Einarsson O., Geba G. P., Zhu Z., Landry M., and Elias J. A.** 1996. Interleukin-11: stimulation in vivo and in vitro by respiratoy viruses and induction of airways hyperresponsiveness. Journal of clinical investigation. 97: 915-924.

**Fang F., and Yu M.** 2004. Viral receptor blockage by multivalent recombinant antibody fusion proteins: inhibiting human rhinovirus (HRV) infection with CFY196. Journal of antimicrobial chemotherapy 53: 23–25.

**Gaudernak E., Seipelt J., Triendl A., Grassauer A., and Kuechler E.** 2002. Antiviral effects of pyrrolidine dithiocarbamate on human rhinoviruses. Journal of virology. 76: 6004–6015.

**Gern J. E., Galagan D. M., Jarjour N. N., Dick E. C., and Busse W. W.** 1997. Detection of rhinovirus RNA in lower airway cells during experimentally induced infection. American Journal of Respiratory and Critical Care Medicine. 155: 1159-1161.

**Gern J. E.,and Busse W. W.** 1999. Association of rhinovirus infections with asthma. Clinical microbioloty. 12: 9-18.

**Ghisalberti E. L. 1979.** Propolis: a review. Bee World 60: 59-84.

**Goel R. K., Pandey V. B., Dwivedi S. P, and Rao Y. V.** 1988. Antiinflammatory and antiulcer effects of kaempferol, a flavone, isolated from Rhamnus procumbens. Indian journal of experimental biology. 26: 121-124.

**Gonzales R., Malone C. D., Maselli J. H., and Sande M. A.** 2001. Excessive antibiotic use for acute respiratory infections in the United States. Clinical infectious disease. 33: 757-762.

**Graci J. D. and Cameron C. E.** 2006. Mechanisms of action of ribavirin against distinct viruses. Reviews in medical virology 16: 37-48.

**Greenberg B. S.** 2003. Respiratory consequences of rhinovirus infection. Archives of internal medicine. 163: 278-284.

**Greve J. M., Davis G., Meyer A. M., Forte C. P., Yost S. C., Marlor C. W., Kamarck M. E., and McClelland A.** 1989. The major human rhinovirus receptor is ICAM-1. Cell. 56: 839-847.

**Greve J. M., Forte C. P., Marlor C. W., Meyer A. M., Hoover-Litty H., Wunderlich D., and McClelland A.** 1991. Mechanisms of receptor-mediated rhinovirus neutralization defined by two soluble forms of ICAM-1. Journal of virology 65*:* 6015–6023.

**Gustafson L. M., Proud D., Hendley O., Hayden F. G., and Gwaltney J. M. Jr.** 1996. Oral prednisone therapy in experimental rhinovirus infections. Journal of allergy and clinical immunology. 97: 1009-1014.

**Gwaltney J. M. Jr, Moskalski P. B., and Hendley J. O.** 1978. Hand-to-hand transmission of rhinovirus colds. Annals of internal medicine. 156: 442-448.

**Gwaltney J. M. Jr. and Hayden F. G.** 1992. Psychological stress and the common cold. New england journal of medicine. 326: 644-645.

**Hadizadeh F., Khalili N., Hosseinzadeh H., and Khair-Aldine R.** 2003. Kaempferol from saffron petals. Iranian Journal of pharmaceutical research. 2: 251-252.

**Harris J. M. 2nd and Gwaltney J. M. Jr.** 1996. Incubation periods of experimental rhinovirus infection and illness. Clinical infectious disease. 23: 1287-1290.

**Hayden F. G., Turner R. B., Gwaltney J. M., Chi-Burris K., Gersten M., Hsyu P., Patick A. K., Smith 3rd G. J., and Zalman L. S.** 2003. Phase II, randomized, double-blind, placebo-controlled studies of ruprintrivir nasal spray 2-percent suspension for prevention and treatment of experimentally induced rhinovirus colds in healthy volunteers. Antimicrobial agents and chemotherapy. 47: 3907–3916.

**Hayden F. G., Gwaltney J. M. Jr., and Colonno R. J.** 1988. Modification of experimental rhinovirus colds by receptor blockade. Antiviral research. 9: 233-247.

**Heikkinen T., and Jarvinen A.** 2003. The common cold. The lancet. 361: 51-59.

**Heinz B. A., and Vance L. M.** 1995. The antiviral compound enviroxime targets the 3A coding region of rhinovirus and poliovirus. Journal of virology. 69: 4189–4197.

**Heinz B. A., and Vance L. M.** 1996. Sequence determinants of 3A-mediated resistance to enviroxime in rhinoviruses and enteroviruses. Journal of virology. 7: 4854–4857.

**Hendley J. O. and Gwaltney J. M. Jr.** 1988. Mechanisms of transmission of rhinovirus infections. Epidemiologic reviews. 10: 243-258.

**Herald P. J., and Davidson P. M.** 1983. Antibacterial activity of selected hydroxycinnamic acids. Journal of food science. 48: 1378-1379.

**Hibatallah J., Carduner C., and Poelman M. C.** In-vivo and in-vitro assessment of the free-radical-scavenger activity of Ginkgo flavone glycosides at high concentration. The Journal of pharmacy and pharmacology. 1999. 51: 1435-1440.

**Hodits R. A., Nimpf J., Pfistermueller D. M., Hiesberger T., Schneider W. J., Vaughan T. J., Johnson K. S., Haumer M., Kuechler E., Winter G., and Blaas D.** 1995. An antibody fragment from a phage display library competes for ligand binding to the low density lipoprotein receptor family and inhibits rhinovirus infection. The journal of biological chemistry. 270: 24078-2408.

**Hofer F., Gruenberger M., Kowalski H., Machat H., Huettinger M., Kuechler E., and Blaas D.** 1994. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. PNAS. 91: 1839-1842.

**Horsnell C., Gama R. E., Hughes P. J., and Stanway G.** 1995. Molecular relationships between 21 human rhinovirus serotypes. Journal of general virology. 76: 2549-2555.

**Hsyu P. H., Pithavala Y. K., Gersten M., Penning C. A., and Kerr B. M.** 2002. Pharmacokinetics and safety of an antirhinoviral agent, ruprintrivir, in healthy volunteers. Antimicrobial agents and chemotherapy. 46: 392–397.

Hudson J. B. 1990. Antiviral compounds from plants Boca Raton, Ann Arbor, Boston: CRC Press.

**Ikeno K., Ikeno T., and Miyazawa C.** 1991. Effects of propolis on dental caries in rats. Caries Research. 25: 347-351.

**Ishitsuka H., Ninomiya Y. T., Ohsawa C., Fujiu M., and Suhara Y. 1**982. Direct and specific inactivation of rhinovirus by chalcone Ro 09-0410. Antimicrobial agents and chemotherapy. 22: 617–621.

**Iwasaki A., and Medzhitov R.** 2004. Toll-like receptor control of the adaptive immune responses. Nature immunology. 5: 987–995.

**Jang Y. J., Kwon H. J., and Lee B. J.** 2006. Effect of clarithromycin on rhinovirus-16 infection in A549 Cells. The European respiratory journal. 27: 12-19.

**Jassim. S.A.A., and Naji. M. A.** 2003. Novel antiviral agents: a medicinal plant perspective. Journal of applied microbiology. 95: 412-427.

**Jeong H. J., Ryu Y. B., Park S. J., Kim J. H., Kwon H. J., Kim J. H., Park K. H., Rho M. C., and Lee W. S.** 2009. Neuraminidase inhibitory activities of flavonols isolated from Rhodiola rosea roots and their in vitro anti-influenza viral activities. Bioorganic and medicinal chemistry. 17: 6816-6823.

**Johnston S. L., Pattemore P. K., Sanderson G., Smith S., Lampe F., Josephs L., Symington P., O'Toole S., Myint S. H., Tyrrell D. A., Holgate S. T.** 1995. Community study of role of viral infections in exacerbations of asthma in 9-11-year-old children. British Medical Journal. 310: 1225-1229.

**Kampkotter A., Gombitang N. C., Zurawski R. F., Timpel C., Chovolou Y., Watjen W., and Kahl R.** 2007. Effects of the flavonoids kaempferol and fisetin on thermotolerance, oxidative stress and FoxO transcription factor DAF-16 in the model organism Caenorhabditis elegans. Archives of toxicology. 81: 849-58.

**Kang G. Y., Lee E. R., Kim J. H., Jung J. W., Lim J., Kim S. K., Cho S. G., and Kim K. P.** 2009. Downregulation of PLK-1 expression in kaempferol-induced apoptosis of MCF-7 cells. European journal of pharmacology. 611: 17-21.

**Kares S., Lönnrot M., Vuorinen P., Oikarinen S., Taurianen S., and Hyöty H.** 2004. Real-time PCR for rapid diagnosis of entero- and rhinovirus infections using Light Cycler. Journal of clinical virology. 29: 99–104.

**Kataoka M., Hirata K., Kunikata T., Ushio S., Iwaki K., Ohashi K., Ikeda M., and Kurimoto M.** 2001. Antibacterial action of tryptanthrin and kaempferol, isolated from the indigo plant (Polygonum tinctorium Lour.), against Helicobacter pylori-infected Mongolian gerbils. Journal of gastroenterology. 36: 5-9.

**Kenny M. T., Dulworth J. K., Bargar T. M., Torney H. L., Graham M. C., and Manelli A. M.** 1986. In vitro antiviral activity of the 6-substituted 2-(3′,4′-dichlorophenoxy)-2H-pyrano[2,3-b]pyridines MDL 20,610, MDL 20,646, and MDL 20,957. Antimicrobial agents and chemotherapy. 30: 516–518.

**Khair O. A., Devalia J. L., Abdelazia M. M., Sapsford R. J., and Davi R. J.** 1995. Effect of erythromycin on Hemophilus influenza endotoxin-induced release of IL-6, IL-8 and sICAM-1 by cultured human bronchial epithelial cells. European respiratory journal. 8: 1451-1456.

**Kim J. M., Lee E. K., Kim D. H., Yu B. P., and Chung H. Y.** 2010. Kaempferol modulates pro-inflammatory NF-kappaB activation by suppressing advanced glycation endproducts-induced NADPH oxidase. Age. 32: 197-208.

**Kim K. H., Willingmann P., Gong Z. X., Kremer M. J., Chapman M. S., Minor I., Oliveira M. A., Rossmann M. G., Andries K., and Diana G. D.** 1993. A comparison of the anti-rhinoviral drug binding pocket in HRV14 and HRV1A. Journal of molecular biology. 230: 206–227.

**Krenn B. M., Holzer B., Gaudernak E., Triendl A., van Kuppeveld F. J., and Seipelt J.** 2005. Inhibition of polyprotein processing and RNA replication of human

rhinovirus by pyrrolidine dithiocarbamate involves metal ions. Journal of virology. 79: 13892–13899.

**Kumazawa S., Nakamura J., Murase M., Miyagawa M., Ahn M. R., and Fukumoto S.** 2008. Plant origin of Okinawan propolis: honeybee behavior observation and phytochemical analysis. 95: 781-786.

**Kuo C. J., Liu H. G., Lo Y. K., Seong C. M., Lee K. I, Jung Y. S., and Liang P. H.** 2009. Individual and common inhibitors of coronavirus and picornavirus main proteases. FEBS Letters. 583: 549–555.

**Kuz'min V. E., Artemenko A. G., Muratov E. N., Volineckaya I. L., Makarov V. A., Riabova O. B., Wutzler P., and Schmidtke M.** 2007. Quantitative structure–activity relationship studies of [(biphenyloxy)propyl]isoxazole derivatives. Inhibitors of human rhinovirus 2 replication. Journal of medicinal chemistry. 50: 4205–4213.

**Laurent K., Crump C. E., and Hayden F. G.** 2000. In vitro activity of pleconaril and AG7088 against selected serotypes and clinical isolates of human rhinoviruses. Antiviral research. 47: 215-220).

**Ledford R. M., Patel N. R., Demenczuk T. M., Watanyar A., Herbertz T., Collett M. S., Pevear D. C.** 2004. VP1 sequencing of all human rhinovirus serotypes: Insights into genus phylogeny and susceptibility to antiviral capsid-binding compounds. Journal of virology. 78: 3663-3674.

**Lee M. J., Rao Y. K., Chen K., Lee Y. C., and Tzeng Y. M.** 2009. Effect of flavonol glycosides from Cinnamomum osmophloeum leaves on adiponectin secretion and phosphorylation of insulin receptor-beta in 3T3-L1 adipocytes. Journal of ethnopharmacolgu. 126: 79-85.

**Lin R. D., Chin Y. P., Hou W. C., and Lee M. H.** 2008. The effects of antibiotics combined with natural polyphenols against clinical methicillin-resistant Staphylococcus aureus (MRSA). Planta Medica. 74: 840-846.

**Lodovici M., Caldini S., Morbidelli L., Akpan V., Ziche M., and Dolara P.** 2009. Protective effect of 4-coumaric acid from UVB ray damage in the rabbit eye. Toxicology. 255 (1-2): 1-5.

**Luceri C., Guglielmi F., Lodovici M., Giannini L., Messerini L., and Dolara P.** 2004. Plant phenolic 4-coumaric acid protects against intestinal inflammation in rats. Scandinavian journal of gastroenterology. 39: 1128-1133.

**Lyu S. Y., Rhim, J. Y., and Park W. B.** 2005. Antiherpetic activities of flavonoids against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in vitro. Archives of pharmacal research. 28: 1293-1301.

**Maddox C. E., Laur L. M., and Tian L.** 2010. Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa*. Current microbiology. 60: 53-58.

**Mahmood N., Piacente S., Pizza C., Burke A., Khan A. I., and Hay A. J.** 1996. The anti-HIV activity and mechanisms of action of pure compounds isolated from Rosa damascena. Biochemical and biophysical research communications. 229: 73-79.

**Mäkelä M. J., Puhakka T., Ruuskanen O., Leinonen M., Saikku P., Kimpimäki M., Blomqvist S., Hyypiä T., and Arstila P.** 1998. Viruses and bacteria in the etiology of the common cold. Journal of clinical microbiology 36: 539-542.

**Maksimova-Todorova V., Manolova N., Gegova G., Serkedzhieva Iu, and Uzunov S.** 1985. Antiviral action of some fractions isolated from propolis. Acta microbiologica Bulgarica. 17: 79-84.

**Marcucci M. C.** 1995. Propolis: Chemical composition, biological properties and therapeutic activity. Apidologie. 26: 83-99

**Marin C., Boutaleb-Charki S., Diaz, J. G., Huertas O., Rosales M. J., Perez-Cordon G., Guitierrez-Sanchez R., and Sanchez-Moreno M.** 2009. Antileishmaniasis activity of flavonoids from Consolida oliveriana. Journal of natural products. 72. 1069-74.

**Markham K. R., Ternai B., Stanley R., Geiger H., and Marby T. J.** 1978. Carbon-13 NMR studies of flavonoids—III: Naturally occurring flavonoid glycosides and their acylated derivatives. Tetrahedron. 34: 1389-1397.

**Marlin S. D., Staunton D. E., Springer T. A., Stratowa C., Sommergruber W., and Merluzzi V. J.** 1990. A soluble form of intercellular adhesion molecule-1 inhibits rhinovirus infection. Nature. 344: 70–72.

**Marlovits T. C., Zechmeister T., Gruenberger M., Ronacher B., Schwihla H., and Blaas D.** 1988. Recombinant soluble low density lipoprotein receptor fragment inhibits minor group rhinovirus infection *in vitro*. FASEB Journal. 12: 695–703.

**Marlovits T. C., Abrahamsberg C., and Blaas D.** 1998. Soluble LDL minireceptors. Minimal structure requirements for recognition of minor group human rhinovirus. Journal of biological chemistry. 273*:* 33835–33840.

**Martin S., Casasnovas J. M., Staunton D. E., and Springer T. A.** 1993. Efficient neutralization and disruption of rhinovirus by chimeric ICAM-1/immunoglobulin molecules. Journal of virology. 67: 3561–3568.

**Martini N. D., Katerere D. R., and Eloff J. N.** 2004. Biological activity of five antibacterial flavonoids from Combretum erythrophyllum (Combretaceae). Journal of ethnopharmacology. 93: 207-212.

**Matthews D. A., Dragovich P. S., Webber S. E., Fuhrman S. A., Patick A. K., Zalman L. S., Hendrickson T. F., Love R. A., Prins T. J., Marakovits J. T., Zhou R., Tikhe J., Ford C. E., Meador J. W., Ferre R. A., Brown E. L., Binford S. L., Brothers M. A., Delisle D. M., and Worland S. T.** 1999. Structure-assisted design of mechanism-based irreversible inhibitors of human rhinovirus 3C protease with potent antiviral activity against multiple rhinovirus serotypes. PNAS. 96: 11000–11007,

**Maugeri C., Alisi M. A., Apicella C., Cellai L., Dragone P., Fioravanzo E., Florio S., Furlotti G., Mangano G., Ombrato R., Luisi R., Pompei R, Rncicotti V., Russo V., Vitiello M., and Cazzolla N.** 2008. New anti-viral drugs for the treatment of the common cold. Bioorganic and medicinal chemistry. 16: 3091–3107.

**Min B. S., Tomiyama M., Ma C. M., Nakamura N., and Hattori M.** 2001. Kaempferol acetylrhamnosides from the rhizome of Dryopteris crassirhizoma and their inhibitory effects on three different activities of human immunodeficiency virus-1 reverse transcriptase. Chemical and pharmaceutical bulletin. 49: 546-550.

**Molla A., Hellen C. U., and Wimmer E.** 1993. Inhibition of proteolytic activity of poliovirus and rhinovirus 2A proteinases by elastase-specific inhibitors. Jorurnal of virology. 67: 4688–4695.

**Monti M., Berti E., Carminati G. and Cusini M.** 1983. Occupational and cosmetic dermatitis from propolis. Contact Dermatitis 9: 163.

**Moser R., Snyers L., Wruss J., Angulo J., Peters H., Peters T., and Blaas D.** 2005. Neutralization of a common cold virus by concatemers of the third ligand binding module of the VLDL-receptor strongly depends on the number of modules. Virology. 338*:* 259–269.

**Neves V. A., and da Silva M. A.** 2007. Polyphenol oxidase from yacon roots (*Smallanthus sonchifolius*). Journal of agricultural and food chemistry. 55: 2424-2430.

**Newcomb D. C., Sajjan U. S., Nagarkar D. R., Goldsmith A. M., Bentley J. K., and Hershenson M. B.** 2007. Cooperative effects of rhinovirus and TNF-alpha on airway epithelial cell chemokine expression. American journal of physiology. Lung cellular and molecular physiology. 293: L1021–L1028.

**Newman D. J. and Gordon C. M.** 2007. Natural products as sources of new drugs over the last 25 years. Journal of natural products. 70: 461-477.

**Nicodemou A., Petsch M., Konecsni T., Kremser L., Kenndler E., Casasnovas J. M., and Blaas D.** 2005. Rhinovirus-stabilizing activity of artificial VLDL-receptor variants defines a new mechanism for virus neutralization by soluble receptors. FEBS Letters. 579*:* 5507–5511.

**Ninomiya Y., Aoyama M., Umeda I., Suhara Y., and Ishitsuka H.** 1985. Comparative studies on the modes of action of the antirhinovirus agents Ro 09-0410, Ro 09-0179, RMI-15,731, 4′,6-dichloroflavan, and enviroxime. Antimicrobial agents and chemotherapy. 27: 595–599.

**Ninomiya Y., Shimma N., and Ishitsuka H.** 1990. Comparative studies on the antirhinovirus activity and the mode of action of the rhinovirus capsid-binding agents, chalcone amides. Antiviral research. 13: 61–74.

**Noah T. L., Henderson F. W., Wortman I. A., Devlin R. B., Handy J., Koren H. S., Becker S.** 1995. Nasal cytokine production in viral acute upper respiratory infection of childhood. Journal of infectious disease. 171: 584-592.

**Ohlin A., Hoover-Litty H., Sanderson G., Paessens A., Johnston S. L., Holgate S. T., Huguenel E., and Greve J. M.** 1994. Spectrum of activity of soluble intercellular adhesion molecule-1 against rhinovirus reference strains and field isolates. Antimicrobial agents and chemotherapy. 38: 1413–1415.

**Otto M. J., Fox M. P., Fancher M. J., Kuhrt M. F., Diana G. D., and McKinlay M. A.** 1985. In vitro activity of WIN 51711, a new broad-spectrum antipicornavirus drug. Antimicrobial agents and chemotherapy. 27: 883–886.

**Ou C., Pu Z., Li S., Pan Q., Hou N., Zhang F., and He C.** 2011. Effects of 4-hydroxycinnamic acid on chickens infected with infectious bursal disease virus. Journla of animal and veterinary advances. 10: 2292-2296.

**Palmenberg A. C., Spiro D., Kuzmickas R., Wang S., Djikeng A., Rathe J. A., Fraser-Liggett C. M., and Liggett S. B.** 2009. Sequencing and analyses of all known human rhinovirus genomes reveal structure and evolution. Science. 324: 55-59.

**Panés J., Perry M., and Granger D. N.** 1999. Leukocyte-endothelial cell adhesion: avenues for therapeutic intervention. British journal of pharmacology. 126: 537–550.

**Papi A., Papadopoulos N. G., Stanciu L. A., Bellettato C. M., Pinamonti S., Degitz K., Holgate S. T., and Johnston S. L.** 2002. Reducing agents inhibit rhinovirus-induced up-regulation of the rhinovirus receptor intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells. FASEB journal. 16: 1934–1936.

**Patick A. K., Brothers M. A., Maldonado F., Binford S., Maldonado O., Fuhrman S., Petersen A., Smith 3rd G. J., Zalman L. S., Burns-Naas L. A., and Tran J. Q.** 2005. In vitro antiviral activity and single-dose pharmacokinetics in humans of a novel, orally bioavailable inhibitor of human rhinovirus 3C protease. Antimicrobial agents and chemotherapy. 49: 2267–2275.

**Patick A. K., Binford S. L., Brothers M. A., Jackson R. L., Ford C. E., Diem M. D., Maldonado F., Dragovich P. S., Zhou R., Prins T. J., Fuhrman S. A., Meador J. W., Zalman L. S., Matthews D. A., and, Worland S. T.** 1999. In vitro antiviral activity of AG7088, a potent inhibitor of human rhinovirus 3C protease. Antimicrobial agents and chemotherapy. 43: 2444–2450.

**Patick A. K.** 2006. Rhinovirus chemotherapy. Antiviral research. 71: 391-396.

**Pevear D. C., Tull T. M., Seipel ME, and Groarke J. M.** 1999. Activity of pleconaril against enteroviruses. Antimicrobial agents and chemotherapy. 43: 2109-2115.

**Phipps K. M., Martinez A., Lu J., Heinz B. A., Zhao G.** 2004. Small interfering RNA molecules as potential anti-human rhinovirus agents: In vitro potency, specificity, and mechanism. Antiviral resarch. 61: 49–55.

**Pitchai P., Ulagi R., Mohan P., and Gengan R. M.** 2012. A novel alkaloid, four alkaloid precursors and a coumarin from feronia limonia. Indian journal of chemistry. 51B: 1171-1775.

**Pitkäranta A., Virolainen A., Jero J., Arruda E., and Hayden F. G.** 1998. Detection of rhinovirus, respiratory syncytial virus, and coronavirus infections in acute otitis media by reverse transcriptase polymerase chain reaction. Pediatrics. 102: 291-295.

**Powers R. D., Gwaltney J. M. Jr., and Hayden F. G.** 1982. Activity of 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile (MDL-860) against picornaviruses in vitro. Antimicrobial agents and chemotherapy. 22: 639–642.

**Pragasam S. J., Venkatesan V., and Rasool M.** 2012. Immunomodulatory and anti-inflammatory effect of p-coumaric acid, a common dietary polyphenol on experimental inflammation in rats. Inflammation. Epub ahead of print.

**Raskin I., Ribnicky. D. M., Komarnyts S., Ilic N., Poulev A., Borisjuk N., Brinker A., Moreno D. A., Ripoll C., Yakoby N., O'Neal J. M., Cornwell T., Pastor I., Fridlender B.** 2002. Plants and human health in the twenty-first century. Trends Biotechnology. 20: 522-531.

**Reyes G. R.** 2001. Ribavirin: Recent insights into antiviral mechanisms of action. Current opinion in drug discovery and development. 4:651–656.

**Rollinger J. M., Steindl T. M., Schuster D., Kirchmair J., Anrain K., Ellmerer E. P., Langer T., Stuppner H., Wutzler P., and Schmidtke M.** 2008. Structure-based virtual screening for the discovery of natural inhibitors for human rhinovirus coat protein. Journal of medicinal chemistry. 51: 842–851.

**Rollinger J. M., Schuster D., Danzl B., Schwaiger S., Markt P., Schmidtke M., Gertsch J., Raduner S., Wolber G., Langer T., and Stuppner H.** 2009. In silico target fishing for rationalized ligand discovery exemplified on constituents of Ruta graveolens L. Planta Medica 75: 195–204.

**Rollinger J. M. and Schmidtke M.** 2010. The human rhinovirus: Human pathological impact, mechanisms of antirhinoviral agents, and strategies for their discovery. Medicinal research reviews 31: 42-92.

**Rossmann M. G., Arnold E., Erickson J. W., Frankedberger E. A., Griffith J. P., Hecht H. J., Johnson J. E., Kamer G., Luo M., and Mosser A. G.** 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature. 317: 145-153.

**Rossmann M. G.** 1989. The structure of antiviral agents that inhibit uncoating when complexed with viral capsids. Antiviral resarch. 11: 3–13.

**Rotbart H. A.** 2002. Treatment of picornavirus infections. Antiviral research 53: 83-98.

**Rozhon E., Cox S., Buontempo P., O'Connell J., Slater W., De Martino J., Schwartz J., Miller G., Arnold E., Zhang A., Morrow C., Jablonski S., Pinto P., Versace R., Duelfer T., and Girijaavallabhan V.** 1993. SCH 38057: A picornavirus capsid-binding molecule with antiviral activity after the initial stage of viral uncoating. *Antiviral research.* 21: 15–35.

**Rzadkowska-Bodalska H., Olechnowicz-Stepien W., and Rzepka A.** 1975. The components of ethereal and methanolic extracts from the fruits of Celastrus tatarinovii (Rupr.). Polish journal of pharmacology and pharmacy. 1975. 27: 335-338.

**Salatino A., Weinstein T. E., Negri G., and Message D.** 2005. Origin and chemical variation of Brazilian propolis. Evidence-based complementary and alternative medicine : eCAM. 2: 33-38.

**Samiulla D. S., Vaidyanathan V. V., Arun P. C., Balan G., Blaze M., Bondre S., Chandrasekhar G., Gadakh A., Kumar R., Kharvi G., Kim H. -O., Kumar S., Malikayil J. A., Moger M., Mone M. K., Nagarjuna P., Ogbu C., Pendhalkar D., Raja Rao A. V. S., Venkateshwar Rao G., Sarma V. K., Shaik S., Sharma G. V. R., Singh S., Sreedhar C., Sonawane R., Timmanna U., and Hardy L. W.**

2005. Rational selection of structurally diverse natural product scaffolds with favorable ADME properties for drug discovery. Molecular diversity. 9: 131-139.

**Sanz M. J., Ferrandiz M. L., Cejudo M., Terencio M. C., Gil B., Bustos G., and Ubeda A., Gunasegaran R., and Alcaraz M. J.** 1994. Influence of a series of natural flavonoids on free radical generating systems and oxidative stress. Xenobiotica. 24: 689-99.

**Savolainen C., Blomqvist S., and Hovi T.** 2003. Human rhinoviruses. Paediatric respiratory reviews. 4: 91-98.

**Sawicka D., Car H., Borawska H. M., Niklinski J.** 2012. The anticancer activity of propolis. Folia histochemica et cytobiologica. 50: 25-37.

**Scheller S., Krol W., Swiacik J., Owczarek S., Gabrys S., and Shani J.** 1989. Antitumoral property of ethanolic extract of propolis in mice-bearing Ehrlich carcinoma, as compared to bleomycin. Zeitschrift fuÈr Naturforschung C. 44: 1063-1065.

**Schindler R., and Mentlein R.** 2006. Flavonoids and vitamin E reduce the release of the angiogenic peptide vascular endothelial growth factor from human tumor cells. The Journal of nutrition. 136: 1477-1482.

**Schubert S., Furste J. P., Werk D., Grunert H. P., Zeichhardt H., Erdmann V. A., and Kurreck J.** 2004. Gaining target access for deoxyribozymes. Journal of molecular biology. 339: 355–363.

**Serkedjieva J., Manolova N., and Bankova V.** 1992. Antiinfluenza virus effect of some propolis constituents virus effect of some propolis constituents and their analogues (esters of substituted cinnamic acids). Journal of natural products. 55: 294-297.

**Sforcin M. J., and Bankova V.** 2011. Propolis: Is there a potential for development of new drugs? Journal of ethnopharmacology. 133: 253-260.

**Shih S. R., Chen S. J., Hakimelahi G. H., Liu H. J., Tseng C. T., Shia K. S.** 2004. Selective human enterovirus and rhinovirus inhibitors: An overview of capsid-binding and protease-inhibiting molecules. Medicinal research reviews. 24: 449-474.

**Silici S., and Kutluca S.** 2005. Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. Journal of ethnopharmacology. 99: 69-73.

**Singh S. B., Cordingley M. G., Ball R. G., Smith J. L., Dombrowski A. W., and Goetz M. A.** 1991. Structure and stereochemistry of thysanone: A novel human rhinovirus 3C-protease inhibitor from Thysanophora penicilloides. Tetrahedron Letters. 32: 5279–5282.

**Singh S. B., Graham P. L., Reamer R. A., and Cordingley M. G.** 2001. Discovery, total synthesis, HRV 3C-protease inhibitory activity, and structure-activity relationships of 2-methoxystypandrone and its analogues. Bioorganic and medicinal chemistry. 11: 3143–3146.

**Sinha A. K., Verma S. C., and Sharma U. K.** 2007. Development and validation of an RP-HPLC method for quantitative determination of vanillin and related phenolic compounds in *Vanilla planifolia*. Journal of separation science. 30: 15-20.

**Smith T. J., Kremer M. J., Luo M., Vriend G., Arnold E., Kamer G., Rossmann M. G., McKinlay M. A., Diana G. D., and Otto M. J.** 1986. The site of attachment in human rhinovirus 14 for antiviral agents that inhibit uncoating. Science. 233: 1286–1293.

**Spasova M., Philipov S., Nikolaeva-Glomb L., Galabov A. S., Milkova T.** 2008. Cinnamoyl-and hydroxycinnamoyl amides of glaucine and their antioxidative and antiviral activities. Bioorganic and medicinal chemistry. 16: 7457–7461.

**Stone J. K., Rijnbrand R., Stein D. A., Ma Y., Yang Y., Iversen P. L., and Andino R.** 2008. A morpholino oligomer targeting highly conserved internal ribosome entry site sequence is able to inhibit multiple species of picornavirus. Antimicrobial agents and chemotherapy. 52: 1970–1981.

**Swisłocka R., Kowczyk-Sadowy M., Kalinowska M., and Lewandowski W.** 2012. Spectroscopic (FT-IR, FT-Raman, $^{1}$H and $^{13}$CNMR) and theoretical studies of p-coumaric acid and alkali metal p-coumarates. Spectroscopy. 27: 35-48.

**Szmeja Z., Kulczyński B., Sosnowski Z., and Konopacki K.** 1989. Therapeutic value of flavonoids in rhinovirus infections. Otolaryngologia polska. 43: 180-184.

**Thyagarajan S. P., Jayaram S., and Valliammai T. 1990. *Phyllanthus amarus* and hepatitis B (Letter). The Lancet 336: 49–50.**

**Thyagarajan S. P., Subramanian S., Thiranapasundaru T., Venkateswaran P. S., and Blumberg B. S.** 1988. Effect of *Phyllanthus amarus* on chronic carriers of hepatitis B virus. The Lancet. 2: 764–766.

**Toker G., Kupeli E., Memisoglu M., and Yesilada E.** 2004. Flavonoids with antinociceptive and anti-inflammatory activities from the leaves of Tilia argentea (silver linden). Journal of ethnopharmacology. 95: 393-397.

**Tsuchiya Y., Shimizu M., Hiyama Y., Itoh K., Hashimoto Y., Nakayama M., Horie T., and Morita N.** 1985. Antiviral activity of natural occurring flavonoids in vitro. Chemical and pharmaceutical bulletin. 33: 3881–3886.

**Turner R. B.** 2001. The treatment of rhinovirus infections: progress and potential. Antiviral research. 49: 1-14.

**Vachalkova A., Eisenreichova E., Haladova M., Mucaji P., Jozova B., and Novotny L.** 2000. Potential carcinogenic and inhibitory activity of compounds isolated from Lilium candidum L. Neoplasma. 47: 313-318.

**Van Hoof L., Vanden Berghe D. A., Hatfield G. M., and Vlietinck A. J.** 1984. Plant antiviral agents; V. 3-Methoxyflavones as potent inhibitors of viral-induced block of cell synthesis. Planta Medica. 50: 513–517.

**Venkateswaran P. S., Millman I., and Blumberg B. S.** 1987. Effects of an extract from *Phyllanthus niruri* on hepatitis B and Woodchuck hepatitis viruses: *in vitro* and *in vivo* studies. PNAS. 84: 274–278.

**Verheyden B., Andries K., and Rombaut B.** 2004. Mode of action of 2-furylmercury chloride, an anti-rhinovirus compound. Antiviral Research. 61: 189–194.

**Vichai V., and Kirtikara K.** 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nautre protocols 1: 1112-1116.

**Viemann D., Goebeler M., Schmid S., Klimmek K., Sorg C., Ludwig S., and Roth J.** 2004. Trancriptional profiling of IKK2/NF-kappa B- and p38 MAP kinase-dependent gen expression in TNF-alpha-stimulated primary human endothelial cells. Blood. 103: 3365–3373.

**Vlietinck A. J. and Berghe V. A. D.** 1991. Can ethnopharmacology contribute to the development of antiviral drugs? Journal of ethnopharmacology. 32: 141-153.

**Wagner E. K. and Hewlett M. J.** 1999. Basic Virology. Malden, Ma, USA: Blackwell Science.

**Wang G., Wang G., Lin B., Bai Y., and Qin M.** 2012. Chemical constituents from Gueldenstaedita stenophylla. China journal of Chinese materia medica. 37: 1412-1416.

**Wang Q. M., Johnson R. B., Jungheim L. N., Cohen J. D., and Villarreal E. C.** 1988. Dual inhibition of human rhinovirus 2A and 3C proteases by homophthalimides. Antimicrobial agents and chemotherapy. 42: 916–920.

**Watson K. G., Brown R. N., Cameron R., Chalmers D. K., Hamilton S., Jin B., Krippner G. Y., Luttick A., McConnell D. B., Reece P. A., Ryan J., Stanislawski P. C., Tucker S. P., Wu W. Y., Barnard D. L., and Sidwell R. W.** 2003. An orally bioavailable oxime ether capsid binder with potent activity against human rhinovirus. Journal of medicinal chemistry. 46: 3181–3184.

**Wattel A., Kamel S., Mentaverri R., Lorget F., Prouillet C., Petit J. P., Fardelonne P., and Brazier M.** 2003. Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. Biochemical pharmacology. 65: 35-42.

**Wikel J. H., Paget C. J., DeLong D. C., Nelson J. D., Wu C. Y., Paschal J. W., Dinner A., Templeton R. J., Chaney M. O., Jones N. D., and Chamberlin J. W.** 1980. Synthesis of syn and anti isomers of 6-[[(hydroxyimino)phenyl]methyl]-1-[(1-methylethyl)sulfonyl]-1H-benzimidaz ol-2-amine. Inhibitors of rhinovirus multiplication. Journal of medicinal chemistry. 23: 368–372.

**Winther B., Gwaltney J. M. Jr., Mygind N., Turner R. B., and Hendley J. O.** 1986. Sites of rhinovirus recovery after point inoculation of the upper airway. Journal of the American medical association. 256: 1763-1767.

**Yamada M., and Sasaki H.** 2003. Rhinovirus and Asthma. Viral Immunology. 16: 99-109.

**Yasuda H., Yamaya M., Sasaki T., Inoue D., Nakayama K., Yamada M., Asada M., Yoshida M., Suzuki T., Nishimura H., Sasaki H.** 2006. Carbocisteine inhibits rhinovirus infection in human tracheal epithelial cells. European respiratory jorunal. 28: 51–58.

**Zalman L. S., Brothers M. A., Dragovich P. S., Zhou R., Prins T. J., Worland S. T., and Patick A. K.** 2000. Inhibition of human rhinovirus-induced cytokine production by AG7088, a human rhinovirus 3C protease inhibitor. Antimicrobial agents and chemotherapy. 44: 1236–1241.

**Zang L. Y., Cosma G., Gardner H., Shi X., Castranova V., and Vallyathan V.** 2000. Effect of antioxidant protection by p-coumaric acid on low-density lipoprotein cholesterol oxidation. American journal of physiology. Cell physiology. 279: C954-960.

**Zhu Z., Tang W., Ray A., Wu Y., Einarsson O., Landry M. L., Gwaltney J. M. Jr., and Elias J. A.** 1996. Rhinovirus stimulation of interleukin-6 in vivo and in vitro. Evidence for nuclear factor kappa B-dependent transcriptional activation. Journal of clinical investigation. 97: 421-430.

**Zuo Y., Wang C., and Zhan J.** 2002. Separation, Characterization, and quantitation of benzoic and phenolic antioxidants in American cranberry fruit by GC−MS. Journal of agricultural and food chemistry. 50: 3789-3794.

# 프로폴리스 유래성분의 human rhinovirus에 대한 항바이러스 활성 및 작용 기구 연구

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## 초 록

Human rhinovirus (HRV)는 감기와 같은 상부호흡기 질병에서 가장 큰 요인을 차지하는 바이러스로 감기 원인 바이러스 중 30-50%를 차지한다. 하부호흡기에 감염될 경우 폐렴, 급성 천식, 만성 폐쇄성 폐질환, 천명 등을 일으키기도 해 그 심각성은 상당하다 할 수 있다. 더욱이 HRV의 100여 가지 혈청형(serotypes)은 백신 및 치료제 등의 개발을 어렵게 해 효과적인 감기약은 현재로선 전무한 실정이다. 시판되는 감기약의 대부분은 증상을 완화시키는 수준에 그쳐 이에 따른 약물의 오남용 및 항생제 저항성 등의 문제가 대두되고 있으며 감기환자의 결석, 결근, 치료비용 등 HRV 유발 질병의 사회적 비용 또한 상당하다. 이에 안전하고 다양한 혈청형에 효과를 보이는 감기 치료제를 찾고자 하는 노력이 더욱 커지고 있다. 본 연구에서는 항 HRV 활성을 보이는 여러 천연물 중 양봉산물의 하나인 프로폴리스 및 그 구성물질의 항바이러스성을 탐색하고 작용 기작을 규명하고자 하는

실험을 수행하였다.

실험에 이용된 Sulforhodamine B 검정법의 기주 세포로써 HeLa cell을 사용하였고 HRV-2, -3, -4, 세 가지 혈청형을 적용하여 넓은 범위의 혈청형에 효과를 보이는 프로폴리스 조성물을 찾고자 하였다. 브라질산 프로폴리스를 선정하여 용매별 순차 분획을 통해 클로로포름층, 에틸아세테이트 층에서 HRV에 대한 높은 항바이러스성을 확인하였고 이후 다양한 크로마토그래픽 방법을 사용하여 분리하였다. 클로로포름 층에서 분리한 kaempferol ($CC_{50,}$ 65.0; $IC_{50}$, 3.7; TI, 17.57) 은 시판되는 항바이러스제인 ribavirin ($CC_{50,}$ > 100; $IC_{50}$, 74.7; TI, > 1.3)에 비해 높은 세포독성을 보였으나 HRV 혈청형 3에 대해 훨씬 뛰어난 활성을 보였다. 또한 에틸아세테이트 층에서 분리, 동정한 (*E*)-ρ-coumaric acid ($CC_{50,}$ > 100; $IC_{50}$, 74.6; TI, > 1.3)는 ribavirin과 비슷한 수준의 활성을 HRV 혈청형 3에 보였다. 혈청형 2, 4에 대해서도 검정한 결과 두 물질의 활성에는 큰 차이가 없는 것으로 나타났다. (*E*)-ρ-coumaric acid와 kaempferol의 작용 기작을 규명하기 위한 실험을 수행한 결과 두 물질 모두 HRV 입자의 전염성에는 영향을 끼치지 않으며 (*E*)-ρ-coumaric acid의 경우 바이러스 접종과 동시에 처리한 경우에만 항바이러스성을 나타내고 접종 전이나 접종 후에는 활성이 50%이하로 떨어지는 것을 확인하였다. Kaempferol은 바이러스 접종 후 6시간까지 50% 이상의 효과를 보이는 것으로 나타났다. 또한 Real-time RT-PCR을 통해 두 분리물질이 모두 바이러스 복제를 억제하며 라이노바이러스 수용체(ICAM-1)와 염증성 시토카인(IL-6) mRNA 발현을 억제하는 것을 검증하였다. 기존에 알려진 프로폴리스 구성 물질 12개에 대해 HRV-3 검정을 실행한 결과, chrysin, quercetin, acacetin, luteolin이 4.1-5.8 μg $mL^{-1}$ 범위의 활성을 보였다. Caffeic acid,

protocatechuic acid, fisetin, galangin, ferulic acid 등도 (9.4-48.3 μg $mL^{-1}$) ribavirin에 비해 높은 효과를 보였다. Quercetin, chrysin, caffeic acid, protocatechuic acid 중에서 protocatechuic acid만이 바이러스 입자와 상호작용하여 감염성을 저하시키는 것으로 나타났다.

이상의 결과를 바탕으로 본 논문의 연구는 브라질산 프로폴리스에 함유된 활성본체를 밝혀냈다는데 그 의의가 있고, 구성 물질에 대한 생물검정과 작용 기작 규명을 통해 항바이러스제로써 가능성을 탐색하고 프로폴리스의 새로운 생리활성을 밝혀내어 농업적, 산업적으로 그 활용 가능성이 높다고 판단되며 추가적 연구가 요구된다.

**검색어:** 인간라이노바이러스, 천연항바이러스제, 프로폴리스, kaempferol, (*E*)-ρ-coumaric acid

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