Antiviral Activity of the Medicinal Plants, *Adina pilulifera*, *Narcissus tazetta* and *Wikstroemia indica*, against Respiratory Syncytial Virus

HO, Wing Shan

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Philosophy
in
Biology

September 2008

Thesis committee:

Prof. Samuel S.M. Sun (Supervisor)
Prof. Paul K.S. Chan (Committee Member)
Prof. M.C. Fung (Chairman)
Prof. N.K. Mak (External Examiner)

Acknowledgements

I would like to express my immense gratitude to my supervisors, Prof. Vincent E.C. Ooi and Prof. Samuel S.M. Sun, who have given me guidance, patience and support throughout my M.Phil study.

My gratitude is also extended to Prof. Paul K.S. Chan, Prof. M. C. Fung and Prof. N.K. Mak for being the members of my thesis committee and sparing their valuable time to assess my progress.

I would like to thanks Prof. Y.L. Li and Dr. Linda S.M. Ooi for their teaching of research techniques and precious guidance. I would also like to thanks Ms. J.Y. Xue for her teaching, assistance and sharing.

I am grateful to have the Institute of Chinese Medicine of CUHK provides me with the laboratory and equipment for my virus research. I would also like to express my sincere thanks to Ms. S.N. Lim, Dr. Lawrence C.M. Chiu and labmates for their advice, help and sharing.

Especially, I would like to give my heartfelt thanks to my family and friends for their care, support and patience throughout these years.

Abstract of thesis entitled:

Antiviral Activity of the Medicinal Plants, *Adina pilulifera*, *Narcissus tazetta* and *Wikstroemia indica*, against Respiratory Syncytial Virus

Submitted by HO, Wing Shan

for the degree of Master of Philosophy in Biology

at The Chinese University of Hong Kong in September 2008

Human respiratory syncytial virus (RSV) is the leading cause of severe respiratory tract infection in infants, children, the elderly and immunocompromised patients, commonly resulted in bronchiolitis and/or pneumonia. The estimated global annual infection and mortality figures for RSV are 64 million and 160,000 respectively. Up till now, there is still no licensed vaccine nor safe and effective antiviral strategy available against RSV. The development of new therapy to the disease is urgently needed. Due to the great chemical diversity, secondary plant metabolites serve as a major source of potential antiviral agents. In this study, two purified plant compounds, seven commercially available phytochemicals, as well as the water and ethanol extracts of nine medicinal plants were screened for their anti-RSV activity by observing their inhibition on RSV-induced cytopathic effect and their cytotoxicity were examined by MTT assay. A purified compound from *Wikstroemia indica*, designated as daphnoretin, and a lectin purified from *Narcissus tazetta* var. *chinensis*, designated as NTL, as well as the ethanol extract of *Adina*

pilulifera possess potent antiviral activity and low cytotoxicity. Subsequently the ethanol extract of *A. pilulifera* was further fractionated and the ethyl acetate fraction was found as the most potent anti-RSV fraction. The half maximal inhibition concentration (IC_{50}) of the ethyl acetate fraction of *A. pilulifera* and daphnoretin were 2.22 and 5.87 μg/ml, respectively, as studied by plaque reduction assay.

Antiviral activity of NTL (13 kDa) and the other two *N. tazetta* proteins were also investigated. The two mannose-binding proteins NTM2 (15.2 kDa) and NTM3 (6.7 kDa) were isolated from *Narcissus tazetta* cultivar by ammonium sulfate precipitation, mannose-agarose affinity chromatography and fast protein liquid chromatography. N-terminal amino acid sequence of NTM2 revealed considerable homology to NTL. From the *in vitro* antiviral study, NTL was found less cytotoxic and being the most potent protein with an IC_{50} value of 2.30 μg/ml in plaque reduction assay.

Mechanistic studies showed that daphnoretin, NTL and the ethyl acetate fraction of *A. pilulifera* exerted different modes of antiviral action. When the samples were added during different phases of RSV infection cycle, daphnoretin could slightly inhibit early viral infection but mainly on the later events. NTL inhibited both early and later events of the infection cycle, while the ethyl acetate fraction of *A. pilulifera* inhibited the events of later phase.

In conclusion, the three candidates were shown for the first time that they possess anti-RSV activity and have different modes of action. This study might provide scientific information for the future development of the three candidates as potential anti-RSV drugs.

摘要

呼吸道合胞病毒(RSV)是引致嬰兒、幼童、長者及缺乏免疫力的人呼吸道嚴重感染的主要原因之一，並可導致支氣管炎及肺炎。據估計，全球每年約有六千四百萬人受感染，十六萬人因而死亡。迄今仍沒有有效對付 RSV 的疫苗或低本高效的藥物，新的藥物仍極待開發。因此具有化學多樣性的植物次級代謝物質可成爲新型抗病毒藥物的主要來源。

本實驗對九種中草藥的水提取物及乙醇提取物和其他從植物中分離得到的單體化合物進行了抗 RSV 活性的篩選，采用四甲基偶氮唑鹽(MTT)比色法測定了它們對人喉上皮樣癌細胞(HEp-2)的細胞毒性，並采用細胞病變抑制法(CPE)和空斑法測定了它們的抗 RSV 活性。 實驗結果表明：1)水團花(*Adina pilulifera*)乙醇提取物、從了哥王(*Wikstroemia indica*)乙酸乙酯萃取部位分離得到的西瑞香素(daphnoretin)單體化合物、以及中國水仙(*Narcissus tazetta* var. *chinensis*)中的一個凝集素 NTL 具有較低細胞毒性和具有抗 RSV 活性。2)水團花乙醇提取物經萃取分離，其乙酸乙酯萃取部位具有較高抗 RSV 活性(半數抑制濃度(IC_{50})爲 2.22 μg/ml，選擇性指數(SI)爲 86.16。) 3)西瑞香素的 IC_{50} 爲 5.87 μg/ml，SI 爲 28.17。

此外，本實驗也對 NTL(13 kDa)與其他水仙蛋白進行了抗 RSV 活性的篩選。從一個水仙栽培品系(*Narcissus tazetta* cultivar)中，經硫酸胺沉澱法、甘露糖瓊脂親和層析法及快速蛋白液相層析法分離得到兩個甘露糖結合蛋白 NTM2(15.2 kDa)和 NTM3(6.3 kDa)。NTM2 與 NTL 具有某程度相似的氨基酸 N 末端序列。對 NTM2、NTM3 及 NTL 進行測試，發現 NTL 具有較低的細胞毒性和較高的抗 RSV 活性，其 IC_{50} 爲 2.30 μg/ml，SI 爲 141.36。

采用空斑法研究西瑞香素、NTL 和水團花乙酸乙酯萃取部位對不同的 RSV 感染週期的影響，發現它們具有不同的抗病毒機制：1)西瑞香素主要抑制 RSV 較後期的感染活動，也對前期的感染活動有輕微的抑制。2)NTL 可以同時有效抑

制 RSV 前期及後期的感染活動。3)水團花乙酸乙酯萃取部位只能抑制較後期的 RSV 感染活動。

通過以上的研究，我們首次發現西瑞香素、NTL 和水團花乙酸乙酯萃取部位具有顯著的抗 RSV 活性和較低的細胞毒性，並且它們具有不同的抗病毒機制。這爲進一步開發新型抗 RSV 藥物提供了科學數據。

Table of Contents

Chapter Two: Screening of medicinal plants and phytochemicals for antiviral activity against RSV

Chapter Three: Mechanistic studies of anti-RSV actions of various fractions of *Adina pilulifera*, and daphnoretin, a purified compound from *Wikstroemia indica*

Chapter Four: Antiviral activity of *Narcissus tazetta* proteins

List of Figures

List of Tables

List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
APA	*Allium porrum* agglutinin
AUA	*Allium ursinum* agglutinin
BCA	Bicinchoninic acid assay
CA	*Cymbidium* agglutinin
CC_{50}	50 % cytotoxic concentration
ConA	*Canavalia ensiformis* agglutinin
Cox B3	Coxsackie B3 virus
CPE	Cytopathic effect
CS-B	Chondroitin sulfate B
CT	Clinical trial
CTL	Cytotoxic T lymphocytes
DEN-2	Dengue virus type-2
DMSO	Dimethyl sulfoxide
EHA	*Epipactis helleborine* agglutinin
EMEM	Eagle's minimum essential medium
ERK	Extracellular signal-related kinase
F protein	Fusion protein
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
FPLC	Fast protein liquid chromatography
G protein	Attachment protein
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
GNA	*Galanthus nivalis* agglutinin
HBA	*Hevea brasiliensis* agglutinin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HEp-2	Human larynx epidermoid carcinoma cell line
HHA	*Hippeastrum* hybrid agglutinin
HIV	Human immunodeficiency virus

HS	Heparan sulfate
HSV	Herpes simplex virus
IC_{50}	50 % inhibition concentration
IFN	Interferon
IL	Interleukin
L protein	Large protein
LA	Live attenuated
LCA	*Lens culinaris* agglutinin
LOA	*Listera ovata* agglutinin
M protein	Matrix protein
mAb	Monoclonal antibody
Man	Mannose
MHA	*Myrianthus holstii* agglutinin
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N protein	Nucleoprotein
NF-κB	Nuclear factor kappa B
NK	Natural killer
NPA	*Narcissus pseudonarcissus* agglutinin
NS protein	Nonstructural protein
NTL	*Narcissus tazetta* lectin
OD	Optical density
P protein	Phosphoprotein
PAP	Pokeweed antiviral protein
PBS	Phosphate-buffered saline
PFP	Purified F protein
pfu	Plaque forming unit
PKC	Protein kinase C
PM	Plasma membrane
poly(A)	Polyadenylated
PSA	*Pisum sativum* agglutinin
PT	Preclinical trial
PVDF	Polyvinylidene difluoride
RIPs	Ribosome-inactivating proteins
RSV	Respiratory syncytial virus
RSV-IGIV	RSV immune globulin intravenous
SARS	Severe acute respiratory syndrome
SARS-CoV	SARS-associated coronavirus

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH protein	Small hydrophobic protein
SI	Selectivity index
siRNA	Short interfering RNA
SV	Subunit vaccine
$TCID_{50}$	50 % tissue culture infectious dose
TNF	Tumor necrosis factor
UDA	*Urtica dioica* agglutinin
VB	RSV budding
VF	Filamentous RSV
VFA	*Vicia faba* agglutinin
VSV	Vesicular stomatitis virus

Chapter 1

General Introduction

1.1 Respiratory Syncytial Virus (RSV)

Human respiratory syncytial virus (RSV) is the leading cause of serious upper and lower respiratory tract infection in infants, children, older adults and people of all ages with compromised respiratory, cardiac, or immune systems. It is a ubiquitous pathogen in all human populations, and the seasonality of RSV outbreak varies from place to place, which occurs during the winter and spring months in Europe, North America, and countries with temperate climates, while in the tropical countries it may occur in rainy season. RSV infects up to 65 % of infants in the first year of life, and virtually all infants are infected by 2 years of age. Bronchiolitis and/or pneumonia are the most common results of first infections, and approximately 80 % of childhood bronchiolitis cases and 50 % of infant pneumonia are due to RSV infection (Black, 2003). The estimated global annual infection and mortality figures for RSV are 64 million and 160,000 respectively (World Health Organization, 2008).

1.2 RSV biology

RSV was first isolated in 1956 from a chimpanzee with mild upper respiratory signs and was named "Chimpanzee Coryza Agent" (Morris *et al.*, 1956). In the following year, antigenically identical viruses were isolated from two infants both with lower respiratory disease (Chanock *et al.*, 1957). The virus was renamed Respiratory Syncytial Virus in recognition of its characteristic cytopathogenic effect (syncytium formation) in tissue culture (Chanock & Finberg, 1957).

RSV is classified in the genus Pneumovirus of the *Paramyxoviridae* family. The RSV virion is typically spherical with diameter of 150 to 200 nm (Figure 1.1), or it can exist in filamentous form with up to 400 nm in length (Figure 1.2). The determinants of these two morphological forms and the roles of such particles in virus transmission and pathogenicity are not completely understood (Gower *et al.*, 2005).

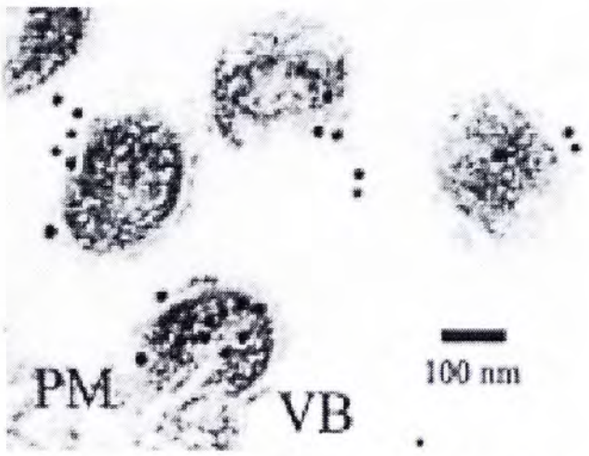


Figure 1.1 Transmission electron microscopic photo of spherical RSV budding (VB) from plasma membrane (PM) (Adapted from Brown *et al.*, 2002).

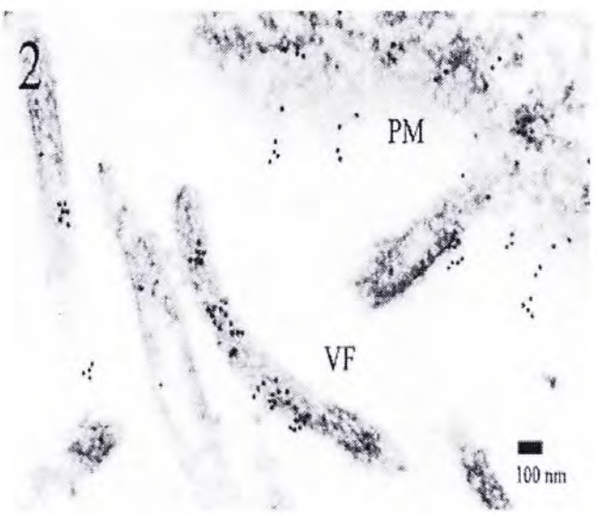


Figure 1.2 Transmission electron microscopic photo of filamentous RSV (VF) budding from plasma membrane (PM) (Adapted from Brown *et al.*, 2002).

RSV virion is surrounded by a lipid envelope derived from the plasma membrane of the host cell during budding. Its genome is composed of single-stranded, negative-sense ribonucleic acid (RNA) of 15,200 nucleotides containing 10 genes which encode for 11 proteins (Figure 1.3 and Table 1.1). The fusion (F), attachment (G), and small hydrophobic (SH) proteins are the three integral membrane proteins on the envelope. The F, G and nonstructural proteins (NS1 and NS2) interact with the matrix (M) protein layer on the internal surface of the envelope. The helical nucleocapsid, which is associated with the nucleoprotein (N), phosphoprotein (P) and large (L) proteins, is located inside the M protein layer (Figure 1.4).

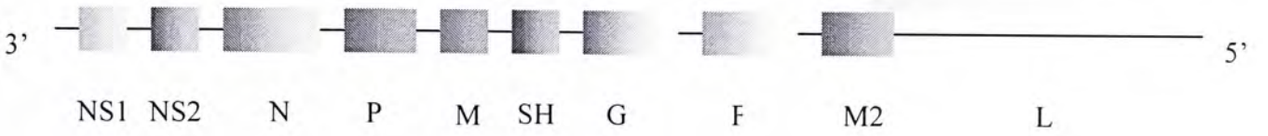


Figure 1.3 Genomic structure of RSV (Modified from Easton *et al.*, 2004).

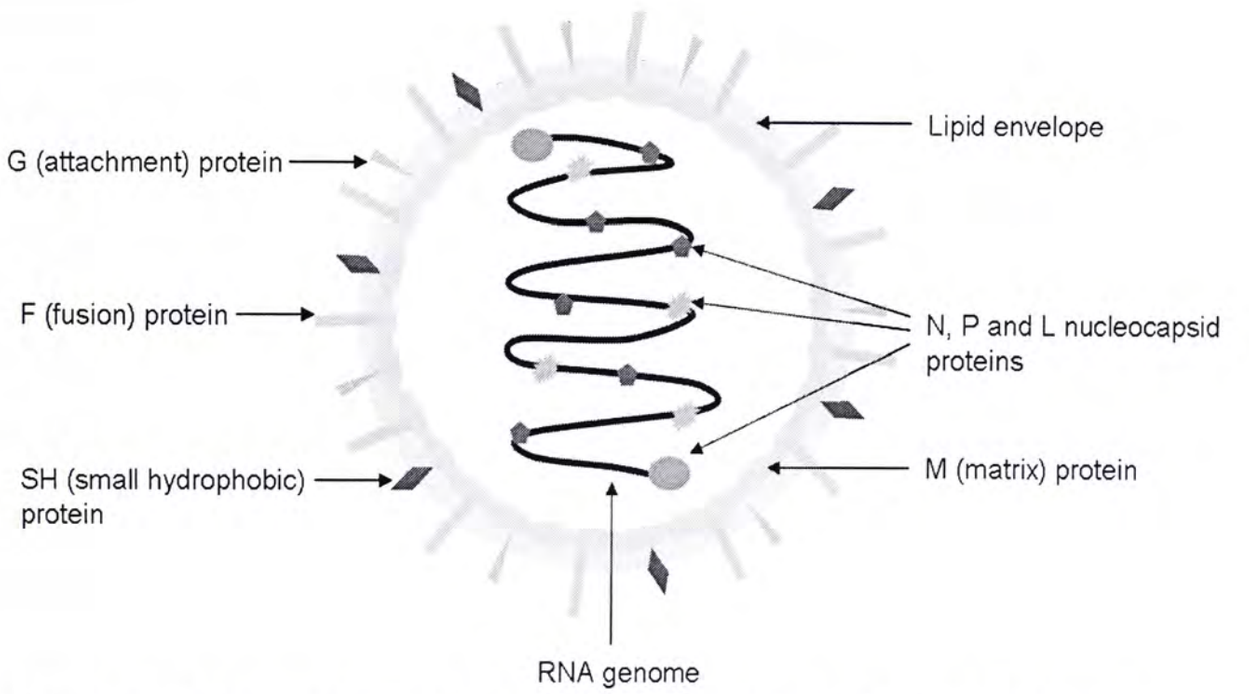


Figure 1.4 Respiratory syncytial viral structure (Modified from Hacking & Hull, 2002).

Table 1.1 RSV genes, proteins and protein functions (Modified from Hacking & Hull, 2002).

Genes	Proteins	Functions
NS1	NS1	Anti-interferon α and β activity
NS2	NS2	
N	N	Essential for transcriptional activity
P	P	Essential for transcriptional activity
M	M	Viral assembly
SH	SH	Function unknown
G	G	Viral attachment to host cell
F	F	Viral entry and syncytia formation
M2	M2-1	Transcription elongation factor
	M2-2	Viral transcription regulation
L	L	RNA polymerase

The replication cycle of RSV begins with the attachment of RSV to host cell surface, which is believed to be mediated by the interaction between a viral protein and a cellular receptor. The G protein of RSV was identified as the viral attachment protein when G protein-specific polyclonal antibody blocked adsorption of RSV to the surface of target cells (Levine *et al.*, 1987). However, a study found that G protein is not essential for cell attachment, but acts as an accessory protein which increases the efficiency of the process (Teng & Collins, 1998). Moreover, the cellular receptor for G protein has not been identified, although there is abundant evidence that RSV binds to cellular glycosaminoglycans (GAGs) (Bourgeois *et al.*, 1998; Teng *et al.*, 2001). GAGs are unbranched polysaccharide chains associated with most mammalian cells. The GAG types that appear to be important for RSV infection in HEp-2 cells are heparan sulfate (HS) and chondroitin sulfate B (CS-B) (Hallak *et al.*, 2000). The interaction between G protein with GAGs facilitates the viral entry, but alternative pathways may also be involved (Crim *et al.*, 2007).

After successful attachment, fusion of the viral envelope or infected cell membrane with the plasma membrane of uninfected cells can be carried out in the presence of F protein, and the process is enhanced by the G protein. Nucleocapsid is then released into the cytoplasm, and transcription of the viral messenger RNA (mRNA) is carried out by the L protein, which is an RNA polymerase, together with

the N and P proteins, which are essential for the L protein to carry out its function. The virus genome contains one polymerase entry site (leader or promoter sequence) at the 3' end of the genome. The polymerase complex travels along the genome until it reaches the conserved start sequence of each gene and initiates transcription. During the process, methylation of the immature mRNA occurs, probably also through the action of L protein, to produce a cap structure that ensures translation on host ribosomes. Transcription of a gene terminate when the polymerase reaches a conserved stop sequence that contains a short run of U residues, and thus a polyadenylated (poly(A)) tail is added to the mRNA (Easton *et al.*, 2004). Only a portion of polymerase (about 50%) can progress to the next gene and starts transcription, and dissociated polymerase can only reinitiate transcription by translocation to the 3' leader sequence. As a result, mRNA transcripts of genes more proximal to the 3' leader sequence are more abundant. The termination and reinitiation process is poorly understood, but it has been shown that the M2-1 protein is essential for full-length virus mRNA production (Fearns & Collins, 1999).

After viral products have been generated, replication of the viral genome can be carried out, which also involves the action of the L, N, and P proteins. The polymerase binds to the 3'end promoter sequence of the RNA genome and initiates RNA synthesis. Once initiated, the polymerase is committed to continue to the end of

the template to produce an antigenome. As the 5' end of the RNA genome contains an antisense copy of the promoter sequence, the 3' end of the antigenome possesses the promoter sequence for the next round of replication to generate the negative-sense single-stranded RNA for progeny virus genomes.

After considerable amount of progeny RSV genomes are produced, the viral assembly occurs. The M2-2 proteins accumulated during infection may involve in switching the virus from replication to assembly (Ahmadian *et al.*, 1999). New nucleocapsids are formed when N, P, L and M2-1 protiens are associated with the new RNA genomes, and then through the M2-1 proteins interact with M proteins, which have been associated with G proteins on cell membrane. M proteins function by rendering the nucleocapsid transcriptionally inactive before packaging and promote the association of the nucleocapsid complex with the region of cell membrane that will become the envelope after budding, which appears to be the reverse process of penetration. Besides, viral particles are transmitted directly from cell to cell without exiting from the cell membrane, via the cell fusion process mediated by F protein and RhoA (Pastey *et al.*, 2000).

1.3 RSV strains

Naturally occurring variants of RSV fall into two antigenic groups, A and B, which are identified on the basis of their differential reactivity with monoclonal antibodies and later confirmed on the basis of gene sequence showing particular divergence within the sequence of the G gene (Garcia-Barreno *et al.*, 1989). One strain tends to dominate during an individual epidemic in an individual location, although sometimes both strains can be isolated from different patients in the same area (Hall, 2001).

Several subtypes of the two strains have been identified on the basis of the variability within the G protein (Cane, 2001; Hall, 2001). Antibodies developed in patients against RSV are specific to the G protein of the particular strain involved in infection. It is speculated that the variability within the G proteins among various RSV strains and subtypes hampers the effectiveness of the host immune protection against infections by another RSV strain. It also makes the production of an effective vaccine against RSV quite difficult (Wilson *et al.*, 2000; Cane, 2001; Hall, 2001).

A number of studies suggested that strain A may results in more virulent infections than strain B. In a 2-year prospective analysis of 157 hospitalized infants, it was found that strain A RSV infections were associated with greater illness severity than strain B infections (McConnochie *et al.*, 1990). In the study, objective clinical

findings and laboratory measurements were used to derive and validate a severity index to quantitate illness severity. Another larger scale study, which involved 265 hospitalized infants assessed over a 3-year period, showed similar results (Walsh *et al.*, 1997). Among infants without underlying medical conditions, strain B RSV infection rarely required ventilatory support, in contrast to strain A infections, and had significantly lower severity indices.

1.4 RSV pathogenesis and host antiviral responses

RSV is transmitted through close contact with infected individuals or direct contact with infectious secretions. The incubation period is 2-8 days after initial contact. Shedding of virus begins within a day after infection, often before the onset of major symptoms. Shedding of RSV appears to correlate roughly with the age of the infected individual, the severity of the infection, and whether the infected person is immunocompromised (Black, 2003).

RSV induces direct cytopathic effect on lung epithelial cells, leading to the impairment of specialized functions such as cillial motility and sometimes epithelial destruction. Peribronchiolar mononuclear cell infiltration accompanied by submucosal edema and mucus secretion are commonly observed. These resulted in bronchiolar obstruction with patchy atelectasis and areas of compensatory emphysema (Openshaw,

2005). There are also many linger effects reported after RSV infection, including acute otitis media and persistent wheezing due to airway hyperreactivity that may contribute to the development of asthma (Black, 2003; Martinez, 2003).

Multicellular organisms have various strategies against viral infection. The first line of defense is cell-mediated immune response that involves the generation of cytokines such as interleukins (IL) and tumor necrosis factor (TNF), which activate and recruit macrophages, natural killer (NK) cells, and neutrophils that clear the infected cells by phagocytosis as well as by inducing cell lysis. The second line of defense is the humoral immune response that involves cytotoxic T lymphocytes (CTL), antibodies, and numerous cytokines. However, serious damage or death can occur before adequate immune responses arise, thus a third level of defense exists — induction of interferons (IFNs) by the presence of viral nucleic acids (Roulston *et al.*, 1999). These immune responses, however, lead to the enhancement of disease severity, which may be relevant not only to childhood bronchiolitis, but also to obstructive lung diseases in adults.

While virus-specific immune responses are largely responsible for protection against RSV-associated lower respiratory tract infections as well as recovery from infection, immune responses to RSV are short-lived, so reinfection can occur throughout life (Maggon & Barik, 2004). The severity of the disease generally

decreases with subsequent reinfections, and although reinfections are common, their frequencies decrease with age.

1.5 Prevention of RSV infection

1.5.1 Vaccines

Although the importance of RSV as a respiratory pathogen has been recognized for over 40 years, a vaccine is not yet available because of several problems inherent in RSV vaccine development. RSV has an RNA genome, and all RNA genomes accumulate mutations at a high rate due to the lack of replicational proof-reading mechanisms, which presents a significant challenge in designing a reliable vaccine against various RSV strains (Domingo & Holland, 1997). RSV-host interactions involve a large and complex network of signaling pathways that must play important roles in the manifestation of the RSV disease, but are only beginning to be unraveled. The immunopathology of RSV is also complex (Hacking & Hull, 2002), including the relatively unique phenomenon of vaccine-enhanced disease. An experimental formalin-inactivated RSV vaccine (Pfizer Lot 100) tested in infants in the mid 1960s was immunogenic but failed to offer protection against RSV infection. The immunized infants experienced a more severe disease in subsequent RSV infection, with two of the vaccinated infants died (Kim *et al.*, 1969). In fact,

immunologic immaturity of infants, the declining immune system of the elderly and the compromised immune system of some high risk patients are the major issues regarding the development of RSV vaccine. It is thus reasonable to develop a vaccine for pregnant women in the last trimester of pregnancy in order to pass a large maternal antibody load to infants, or produce a vaccine that would augment antibody production in the elderly or immunocompromised patients who has been infected by RSV previously.

A number of vaccines in fact have been progressed in the development to animal models and toxicology studies and some to clinical trials. Two main types of vaccines have been formulated and investigated, including live attenuated virus primarily for young infants (Murphy & Collins, 2002), and RSV subunit vaccines for people who have already experienced primary RSV infection, such as the elderly and older RSV-seropositive children with chronic cardiac or pulmonary disease. Table 1.2 shows the RSV vaccines under development.

Table 1.2 RSV vaccines under development (Maggon & Barik, 2004).

Product	Characteristics	Target population	Company/Agent	Status/Phase
LA	Cpts 248/404	Infants	Wyeth; NIAID	CT II
LA	A2cpts 248/404-SH	Infants	Wyeth; NIAID	CT II
LA	RB/HPIV3-RSV-A/B	Infants	MedImmune	PT
LA	RA2, M2-2	Infants	NIAID	PT
LA	2B33F	Infants	NIAID	PT
SV	PFP1	Elderly	Wyeth	CT I
SV	PFP2	Elderly	Wyeth	CT II
SV	PFP3	Elderly	Wyeth	CT II
SV	BBG2Na	Elderly	Pierre Fabre Medicament	CT III
SV	FG487808	Elderly	GSK	CT I
SV	F+G	Elderly	Aventis	CT II

LA: live attenuated; SV, subunit vaccines; CT, clinical trial; PT, preclinical trial.

Live attenuated RSV vaccine would mimic natural infection, and could be applied intranasally, thereby making it more suitable to young infants. Several strategies have been employed for the development of the vaccine, including the creation of host range mutants, cold passaged (*cp*) mutants, and temperature-sensitive (*ts*) mutants. The *cp* and *ts* mutants could only replicate at temperature below 37 °C, thus their growth would be inhibited at normal body temperature. The weakened strains are expected to be avirulent and causing limited infection or illness. However, clinical trials of these vaccine candidates were initially discouraging. They are either under-attenuated (cpRSV and RSVts-1) or over-attenuated (RSVts-2). Later on some *cpts* viruses (produced under combined conditions used for producing the *cp* and *ts* mutants) were shown immunogenic and phenotypically stable (Polack & Karron, 2004).

The F and G protein of RSV can induce production of neutralizing and protective antibodies. As less antigen variation is present in the F protein than in the G protein of RSV within the same subgroup, and the F protein contains conserved domains between both subgroup A and B, the F protein has been the focus of trials of various subunit vaccines. Purified F protein (PFP) has shown the greatest promise. PFPs were evaluated in phase I-II clinical trials with demonstrated safety and immunogenicity. Besides the PFP vaccines, recombinant vaccines consisting of F, G

and M subunits has also entered the Phase I clinical trials (Polack & Karron, 2004).

1.5.2 Passive anti-RSV antibodies

Another approach to achieve immunoprotection against RSV is the use of passive antibodies. Two forms of passive immunoprophylaxis are available for prescription, including RSV immune globulin intravenous (RSV-IGIV, RespiGam®) and palivizumab (Synagis®).

RSV-IGIV is a polyclonal concentrated neutralizing antibody produced from the sera of adult humans. A randomized, prospective study of administration of RSV-IGIV to high-risk infants showed significant reductions in the rate and severity of lower respiratory tract infection, the need for hospitalization and days spent in intensive care (Hemming *et al.*, 1987; Rodriguez *et al.*, 1997; Simoes *et al.*, 1998). It is given by a 2-4 hours intravenous infusion and costs about US$5000 per infusion (Maggon & Barik, 2004). However, the use of RSV-IGIV has several limitations. It is a blood-derived product so there is a danger of transmission of infectious pathogens. Besides, it interferes with the measles-mump-rubella vaccine and varicella vaccine. Side effects of RSV-IGIV include fever, rash, hypotension and, in rare cases, anaphylaxis.

Palivizumab is a humanized anti-F monoclonal antibody (mAb) composed of

human (95 %) and murine (5 %) antibody sequences. It received approval from the United States Food and Drug Administration (FDA) in 1998 and is now approved in over 50 countries as a standard for prevention of RSV in high-risk infants such as premature and those with chronic lung and congenital heart diseases. Palivizumab neutralizes a broad range of RSV strains in animal models, and in a phase III clinical study in 1502 high-risk infants, it reduced the incidence of hospitalizations by 55 % (The IMpact-RSV Study Group, 1998). Palivizumab is given as an intramuscular injection, and only monthly injections are required during the RSV seasons, making it more convenient than RSV-IGIV. An estimated 253,808 infants have received more than 1 million doses of palivizumab prophylaxis in the 4 years followed FDA approval, and postmarketing safety surveillance confirmed the pre-licensure safety profile of palivizumab used in more than 250,000 infants (Romero, 2003). Although palivizumab is expensive, up to US$4000 per RSV season (Stevens & Hall, 2004), it is considered as cost-effective in some studies (Elhassan *et al.*, 2006; Nuijten *et al.*, 2007).

Further modification of palivizumab generates a more potent second-generation mAb, motavizumab (Numax^{TM}), which is currently under evaluation in Phase III clinical trials. Besides, more recently, a third generation mAb, Numax-YTE, has been generated and aimed to extend the serum half-life in humans.

It is hoped that the antibody can reduce the frequency of dosing (Wu *et al.*, 2008). Table 1.3 shows the anti-RSV antibodies that are either in market or under active development.

Table 1.3 Anti-RSV antibodies in market and under development (Maggon & Barik, 2004).

Product	Characteristics	Company	Sales 2003 / status
RespiGam® (RSV-IGIV)	Immunoglobulin	MedImmune	Negligible
Synagis® (Palivizumab)	Human anti-F glycoprotein	MedImmune	$849 M
Felvizumab (RSHZ19)	Human anti-F glycoprotein	GSK Scotgen	Phase III completed
HNK20 (MoAb)	Immunoglobulin	CSL/Oravax Acambis	Phase III completed
Numax™ (Motavizumab)	Monoclonal antibody	MedImmune	Phase III
R19	Monoclonal antibody	Epicyte	Preclinical

1.6 Treatment for RSV infections

Attempts to develop effective therapy for RSV infections have been ongoing for as long as the virus has been recognized. However, no specific anti-RSV drug has been discovered yet. In clinical management, supportive therapies, including hospitalization, supplemental oxygen delivery, nasopharyngeal suctioning, and in the most severe cases, intubation and mechanical ventilation are employed. Other strategies include the use of bronchodilators, corticosteroids, and ribavirin.

1.6.1 Ribavirin (Virasole®)

Aerosolized ribavirin is the only antiviral preparation approved by FDA for treating severe lower respiratory tract RSV infections in the high risk hospitalized infants and young children, and it is a synthetic compound structurally related to ribonucleoside guanosine with broad spectrum antiviral activity against many DNA and RNA viruses. It inhibits viral multiplication by several mechanisms, including inhibition of viral polymerase, inhibition of 5' cap formation of mRNA, inhibition of inositol monophosphate dehydrogenase, and immune-modulating action (Maggon & Barik, 2004; Bosch *et al.*, 2007). The effect of ribavirin has been reviewed in many studies, but the results are inconsistent and contradictory. Administration of ribavirin is very labor intensive and possesses some hazards to caregivers (Black, 2003). Also,

the drug is expensive and its cost-effectiveness must be evaluated (Maggon & Barik, 2004). In 1996, the American Academy of Pediatrics recommended that ribavirin should be used after deliberation by physicians for children with substantial comorbidities (such as cardiopulmonary disease or immunosuppressive disease or therapy) or those with exceptionally severe RSV infection (Black, 2003).

1.6.2 Other antiviral strategies

Due to the absence of available vaccine or other immunoprophylatic agents, and the uncertain beneficial effect of ribavirin on the improvement of RSV disease in hospitalized patients, new strategies for therapeutic intervention during moderate to severe RSV disease are clearly needed. Antiviral compounds targeting different stages of viral cycle can be used according to various stages of disease. Antiviral drug that targets viral attachment and/or fusion can restrict early viral infections or further spreading of the virus. Viral replication inhibitors, such as ribavirin, can be used at a later time after initial infection and limit viral titre in patients. These antivirals have higher potential to be developed as successful antiviral agents for clinical use. A variety of antiviral compounds have been identified and tested for their ability to impair RSV replication *in vitro* and *in vivo*.

1.6.2.1 Attachment inhibitors

The attachment of RSV to target cells is largely mediated by the G protein. The process also requires the presence of GAGs on cell surface. Molecules that interfere with RSV to bind to the cell surface GAGs can inhibit the attachment process. Large polyanionic molecules such as heparin, sulphated polysaccharides like dextran sulphate, and sulphated polymers such as polyvinylalcohol sulfate are able to compete with the binding of RSV to cell surface GAGs and thereby inhibit viral replication (Hosoya *et al.*, 1991; Krusat & Streckert, 1997). Another molecule that has similar anti-RSV action as the polyanions is the RhoA derived peptide comprising amino acids 80 to 94 of the intracellular GTPase RhoA. The inhibition of RSV by this peptide is dependent on both the net charge and multimerization of the peptide. Successful inhibition largely occurs to RSV with G protein, suggesting that RSV G protein is a primary target of peptide binding (Budge *et al.*, 2004).

Some heteropolyanions called polyoxometalates which contain early transitional metal ions could cluster with the surrounding oxygen atoms, and they are shown to have *in vitro* anti-RSV activity (Barnard *et al.*, 1997; Shigeta *et al.*, 2003). Among them, HS-058 appears to inhibit RSV adsorption as well as syncytium formation (Shigeta *et al.*, 2006).

1.6.2.2 Fusion inhibitors

Entry of RSV to host cells involve fusion of the virus envelop to host cell membrane. This process is largely mediated by the F protein, which also involves in the formation of syncytia after RSV replication to allow virus spreading through cell to cell fusion.

RSV-specific fusion inhibitors, such as peptides derived from different domains of the RSV F protein, block RSV-induced syncytium formation at concentrations as low as 0.015 μM (Lambert *et al.*, 1996). Some small molecules that inhibit the fusion processes of RSV have been reported, including CL387626, RFI-641, BMS-433771, JNJ2408068 (also known as R-170591), RD3-0028, and VP-14637. All these molecules have been shown to have inhibitory effects on early virus-cell fusion. It was reported that CL387626, a disulfonated stilbene, significantly inhibited pulmonary replication of RSV when administered intranasally 4 or 5 days prior to virus challenge, but it failed to significantly inhibit pulmonary RSV replication when administered after virus challenge (Wyde *et al.*, 1998). So the compound is prophylactic, but prolonged prophylactic activity was observed in the study, indicating that the drug was not readily degraded from the lungs and this is hazardous to the host. Further modification of the drug yielded RFI-641, which is more potent and no persistency of antiviral activity in lung tissues observed. Both the

intranasal and aerosol administration of RFI-641 in *in vivo* studies showed reduction of the viral load (Huntley *et al.*, 2002; Weiss *et al.*, 2003). BMS-433771 is a benzimidazole derivative which is orally bioavailable, and presumably interferes with the functional interaction of the C- and N-terminal heptad repeats in the F protein by binding tightly in the pocket formed in the N-terminal heptad repeat region (Cianci *et al.*, 2005). JNJ2408068, another benzimidazole derivative, can protect cotton rats from pulmonary RSV infection by aerosol exposure for 15 minutes, one day prior or one day after virus inoculation (Wyde *et al.*, 2003). However, the drug shows long tissue retention times in several species of experimental animals (rat, dog, and monkey). The drug has been further modified to form molecules that have shorter half-lives in the lung tissues, but shows a drop in the RSV inhibitory effect. Thus, further optimization of those molecules and more *in vivo* tests are needed (Bonfanti *et al.*, 2007). RD3-0028 is a compound with a benzodithiin structure. It is shown to inhibit late stage RSV replication cycle events and is not directly virucidal (Sudo *et al.*, 2001). Pharmacokinetics of RD3-0028 was tested in rats by aerosol treatment, which might be a useful route for delivering the drug to RSV-infected patients (Sudo *et al.*, 2002). The triphenol compound VP-14637 inhibits RSV through a similar mechanism to that of JNJ2408068, which involves an interaction between the compound and a transient conformation of the RSV F protein (Douglas *et al.*, 2003;

Douglas *et al.*, 2005). The drug also exhibits good selective antiviral efficacy both *in vitro* and *in vivo* (Wyde *et al.*, 2005).

1.6.2.3 Replication inhibitors

RNA polymerase of RSV comprised of at least five viral components including the genomic RNA and the L, N, P, and M2-1 proteins. Multiple protein-protein and protein-RNA interactions mediate the assembly and activity of the RNA polymerase. Thus, it is a complex that is rich in potential antiviral targets. An assay that specifically measures the synthesis of RSV transcripts *in vitro* through capture of the mRNA poly(A) tails was developed. By using this assay, a novel RSV RNA polymerase inhibitor (Compound A) was discovered (Mason *et al.*, 2004). Compound A causes a general impairment of mRNA synthesis by RNA polymerase as the synthesis of all major RSV mRNA species are equally inhibited. Several derivatives of Compound A that have improved anti-RSV potency *in vitro* also exhibit activity in a mouse mode (Liuzzi *et al.*, 2005). This class of inhibitors may block synthesis of RSV mRNAs by inhibiting guanylylation of viral transcripts because short transcripts produce in the presence of inhibitor *in vitro* do not contain a 5'cap, and thus the synthesis of full-length RSV transcripts is prevented.

Another compound, YM-53403, potently inhibited the replication of RSV

strains belonging to both A and B subgroups *in vitro* (Sudo *et al.*, 2005). Time-dependent drug addition test showed that the compound inhibited RSV life cycle at around 8 hours post-infection. Moreover, a single point mutation in the L protein was found in two YM-53403-resistant viruses, further suggesting that early transcription and/or replication of RSV genome may be inhibited.

There are also other methods of regulation of viral gene expression. One such approach is the use of antisense drugs, which are synthetic oligo deoxynucleotides that are 15-20 nucleotide long and able to bind to small segments of the target mRNA that have the complementary sequence to the oligo nucleotide. Such binding may lead to a direct inhibition of translation if the antisense oligo is designed against the translational regulatory sequences of the mRNA (such as ribosome-binding site), or lead to degradation of the targeted mRNA by RNase. It was reported antisense oligos targeted against RSV NS1 and NS2 proteins diminished the expression of these two proteins (Maggon & Barik, 2004). The use of antisense oligos for viral translation inhibition was tested by two companies, but the results were not promising. On the other hand, antisense oligos can be coupled to 2',5'-oligoA, which recruits cellular 2',5'-oligoA-dependent RNase L that degrades the target viral RNA (Torrence, 1999). These oligos have shown potent inhibition of RSV replication *in vitro* (Adah *et al.*, 2001), and *in vivo* using an African green monkey model of RSV infection (Leaman

et al., 2002). Concomitant use of ribavirin and 2',5'-oligoA-coupled antisense oligos was reported to lead to a reduction in RSV replication *in vitro*, even both were used at suboptimal concentrations (Xu *et al.*, 2004). There are no publications describing antisense therapy in human clinical trials. Further improvements are needed to overcome some technical problems with antisense oligos, such as drug delivery to target cells, stability of the drug and cellular toxicity.

RNA interference is another gene-expression-mediated antiviral strategy. The mechanism involves an evolutionary conserved RNA metabolism pathway found in all eukaryotes, in which sequence-specific double-stranded RNA (short interfering RNA; siRNA) lead to specific degradation of target RNA and thus gene expression is regulated. The antiviral effect of RNA interference was first demonstrated against RSV (Bitko & Barik, 2001), which has been subsequently reproduced against other viruses. For RSV, this approach has been used to target the P and NS1 genes. siRNA designed to abolish either the P or NS1 gene led to inhibition of RSV replication *in vitro* and lowered viral titres in lung tissues in animal models by intranasal application. Airway reactivity was also decreased (Zhang *et al.*, 2004; Bitko *et al.*, 2005). However, siRNA may show various degrees of nonspecific, off-target effects at high concentrations, and the limited delivery of siRNA to susceptible or infected host remains a major challenge to the use of this approach as RSV therapeutic options.

1.6.2.4 Ethnobotanic medicines

Over the centuries, medicinal plants have been widely used for the treatment of various diseases and formed the basis of traditional medicaments in China, India, Africa as well as many other civilizations. It is speculated that only about 6 % of the 250,000 plant species on earth have been screened for biological activities and evaluated as a source of drugs (Verpoorte, 2000). Thus there is a great potential in finding more plants as a source of medicinal agents. The aims of using plants as sources of therapeutic agents are 1) to isolate bioactive compounds that can be used directly as drugs, e.g. morphine, taxol; 2) to identify bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce new entities of higher activity and lower toxicity, e.g. oxycodon (based on morphine), taxotere (based on taxol); 3) to use as pharmacologic tools, e.g. mescaline; 4) to use the whole plant or part of it as a herbal remedy, e.g. *Ginkgo biloba*, *Echinacea*. Approaches to selecting plants for drug discovery programs include random selection followed by chemical screening and/or by one or more biologic assays, follow-up of biologic activity reports or ethnomedical uses of plants, as well as the use of databases such as the NAPRALERT database that contains information on 43,879 higher plant species covering ethnomedical, chemical, and pharmacologic uses (Fabricant & Farnsworth, 2001).

Although numerous compounds have been tested against different viruses, in the market there are only 37 licensed antiviral drugs available (Chattopadhyay & Naik, 2007). The problems of side effects, viral latency, resistance to currently available antiviral drugs, as well as rapid spread and emergence of viral diseases like acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) and severe acute respiratory syndrome (SARS) caused by SARS-associated coronavirus (SARS-CoV) urged intensive screening of more potential antiviral candidates. Besides small molecules from medicinal chemistry, natural products are major sources of novel therapeutic agents for viral diseases. Chemical diversity of secondary plant metabolites resulted from plant evolution may be even more superior to that found in synthetic combinatorial chemical libraries. The interest in developing medicinal plants as antiviral agents has begun decades ago. The Boots drug company (Nottingham, England) screened 288 plants for anti-influenza activity and 12 of them were found able to suppress virus amplification (Chantrill *et al.*, 1952). Later on more screening programmes were launched to evaluate the antiviral activity of medicinal plants for *in vitro* and *in vivo* assays. Table 1.4 shows some recent experimental results of medicinal plants that exhibits antiviral activities against various viruses.

Table 1.4 Partial list of viruses that may be inhibited by medicinal plants (Modified from Mukhtar *et al.*, 2008).

Virus	Medicinal plant used	Antiviral effects	References
Herpes simplex virus (HSV)	*Phyllanthus urinaria* L.	1346TOGDG and geraniin from the plant inhibited HSV-1 and HSV-2, respectively	Yang *et al.*, 2007
Influenza virus	Elderberry extract	A randomized, double-blinded placebo-controlled study revealed that elderberry extract seems to offer an efficient, safe and cost-effective treatment for influenza	Zakay-Rones *et al.*, 2004
Hepatitis B virus (HBV)	*Boehmeria nivea* L.	A root extract reduced HBV production in an *in vitro* and *in vivo* model	Huang *et al.*, 2006
Severe acute respiratory syndrome-associated coronavirus (SARS-CoV)	*Lycoris radiate*	Lycorine isolated from the plant possesses anti-SARS-CoV activity	Li *et al.*, 2005a
Human immunodeficiency virus (HIV)	*Phyllanthus amarus* Schum. & Thonn.	Inhibited HIV replication both *in vitro* and *in vivo*	Notka *et al.*, 2004
Poliovirus	*Guazuma ulmifolia* Lam.	Inhibited poliovirus replication and blocked the synthesis of viral antigens in infected cell cultures	Felipe *et al.*, 2006
Dengue virus type-2 (DEN-2)	*Azadirachta indica* Juss.	Aqueous extract of the leaves inhibited DEN-2 both *in vitro* and *in vivo*	Parida *et al.*, 2002
Vesicular stomatitis virus (VSV)	*Trichilia glabra* L.	Leaves extract inhibited VSV	Cella *et al.*, 2004
Human adenovirus type 1	Black soybean extract	Inhibited the virus in a dose-dependent manner	Yamai *et al.*, 2003

The basic step for evaluation of the therapeutic potential of a medicinal plant is to prepare crude cellular lysate of the plant matrix followed by the extraction of various components that might have potential medicinal value or might be used as lead compounds for further synthesis of novel related compounds. The common class of antiviral compounds present in medicinal plants include phenolics and polyphenols, coumarins, flavonoids, terpenoids and essential oils, quinones, tannins, lignans, alkaloids, lectins, polypeptides, and sugar-containing compounds. Some of these are reviewed in the following section.

1.6.2.4.1 Anti-RSV medicinal plant components

1.6.2.4.1.1 Phenolics and polyphenols

Polyphenols extracted from *Blumea laciniata*, *Elephantopus scaber, Laggera pterodonta, Mussaenda pubescens, Schefflera heptaphylla* and *Scutellaria indica* inhibit RSV with the mean concentration of 50 % inhibition (IC_{50}) of 12.5 to 32 μg/ml, and selective indices (SI) ranging from 11.2 to 40 (Li *et al.*, 2004). Moreover, three known phenolic compounds, (-)-(R)-nyasol, its derivative, as well as broussonin A were isolated from the rhizomes of *Anemarrhena asphodeloides*, and their activities against the A2 strain of RSV were being higher than that of ribavirin (Bae *et al.*, 2007).

A biphenolic depsides 3,5-dicaffeoylquinic acid (Figure 1.5) were isolated from *Flos lonicerae* (Ojwang *et al.*, 2005) and was shown effective to inhibit at least four RSV subtype A and one RSV subtype B at a low micromolar level without significant cytotoxicity observed. The compound was able to reduce RSV titers by ~1 log at dose 12.5 and 25mg/kg/day, and by >2 log at 100mg/kg/day in a cotton rat model. The compound was also isolated from *Schefflera heptaphylla* (Li *et al.*, 2005b), and it was found that antiviral action of the compound was specific against RSV but not effective against influenza A, coxsackie B3, and herpes simplex type one (HSV-1) viruses. Besides, it appeared that the compound could inhibit RSV fusion.

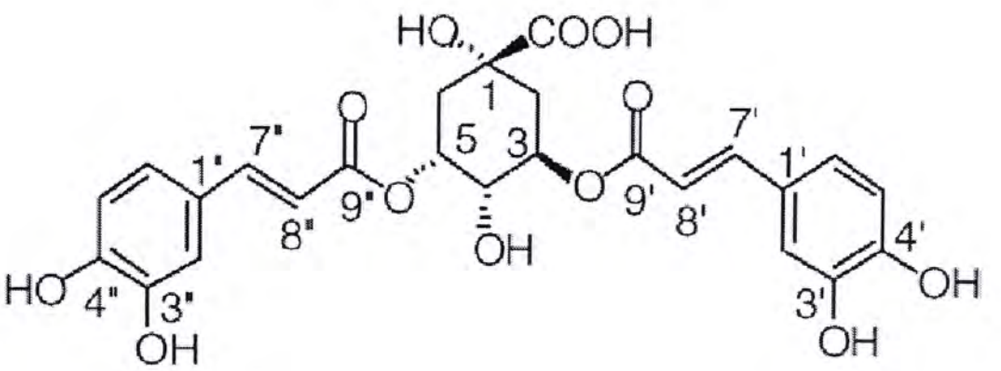


Figure 1.5 Structure of 3,5-dicaffeoylquinic acid (Ojwang *et al.*, 2005).

1.6.2.4.1.2 Flavonoids

It has been reported that some flavonoids such as catechin, quercetin, and hesperetin can inhibit replication of some viruses, including RSV (Kaul *et al.*, 1985).

Moreover, two new iridoid glycosides and three phenylpropanoid glycosides, namely luteoside A, luteoside B and luteoside C, from *Barleria prionitis* and the root of *Markhamia lutea* respectively, have potent *in vitro* anti-RSV activity (Chen *et al.*, 1998; Kernan *et al.*, 1998). In another study, five groups of biflavonoids (amentoflavone, agathisflavone, robustaflavone, rhusflavanone and succedaneflavanone) were isolated from *Rhus succedanea* and *Garcinia multiflora*, which exhibit various effects against herpes viruses and respiratory viruses, including RSV (Liu *et al.*, 1999).

SP-303, a flavonoid polymer isolated from the latex of *Croton lechleri*, has antiviral activity against RSV in cotton rats (Wyde *et al.*, 1993) and in primate model (Soike *et al.*, 1992). The study of the mode of action of SP-303 shows that RSV penetration was inhibited, suggesting that SP-303 might be able to bind to specific RSV proteins, which may sterically hinder RSV penetration (Barnard *et al.*, 1993).

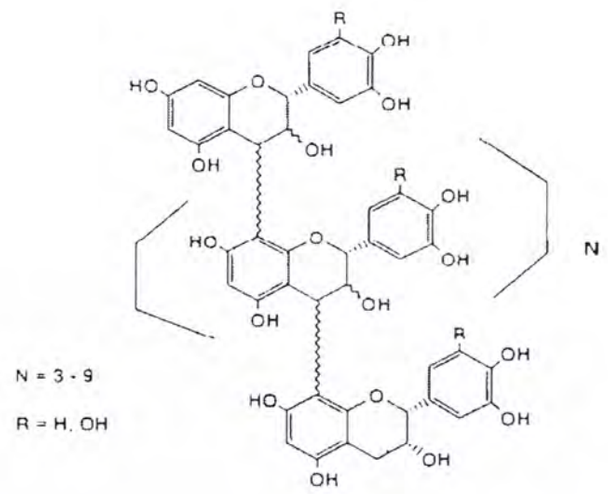


Figure 1.6 Proposed structure of SP-303 (Adapted from Wyde *et al.*, 1993).

1.6.2.4.1.3 Terpenoids and essential oils

Essential oils are components of fragrance of plants and are phenolic compounds. The oils that are highly enriched in isoprene are called terpenes, and when contain additional elements like oxygen, they are called terpenoids. Two highly active pure triterpenoids purified from an extract of the long leafstalk of the compound leaf of *Schefflera heptaphylla* exhibited antiviral activities against RSV, influenza A, coxsackie B3, and herpes simplex virus type 1 viruses (Li *et al.*, 2007). Dysoxylins A to D, belonging to the tetranortriterpenoid family, were isolated from *Dysoxylum gaudichaudianum* and were found to exhibit potent antiviral activity against RSV *in vitro* with EC_{50} (50 % effective concentration) ranged from 1 to 4 μg/ml (Chen *et al.*, 2007).

1.6.2.4.1.4 Lectins

1.6.2.4.1.4.1 General introduction to lectins

Lectins are a class of proteins that bind carbohydrates but do not biochemically modify the carbohydrates to which they bind. They are separated from immunoglobulins though lectins also possess apparent specificity of binding. Another characteristic of lectins is that they agglutinate cells and precipitate polysaccharides and glycoproteins. Despite hundreds of monosaccharides can be

found in nature, the large majority of lectins recognize just a few monosaccharides, mainly mannose, glucose, galactose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylneuraminic acid, as well as numerous oligosaccharides composed of these monosaccharides. Lectins are structurally diverse. They are oligomeric proteins composed of subunits, one or more of which carries a sugar binding site. Lectins occur in all classes and families of plants, animals and microorganisms, although not necessarily in every genus or species (Sharon & Lis, 2003).

1.6.2.4.1.4.2 Historical aspects of lectins

Lectin was discovered by Hermann Stillmark in 1888 during the search for toxic principle in castor beans (*Ricinus communis*) and the initial detection of the agglutinating effect of castor bean extracts to blood cells from different animals. Using the very primitive isolation methods available at that time, a hemagglutinating powder was obtained, which was named "ricin". It was later discovered that "ricin" was a mixture of a weakly agglutinating protein toxin (still known as ricin) and a nontoxic agglutinin (*Ricinus communis* agglutinin, RCA). In 1919, James B. Sumner isolated the pure lectin concanavalin A from jack beans (*Canavalia ensiformis*). In 1936, he discovered that in addition to the agglutinating activity, concanavalin A

could also precipitate glycogen from solution. Besides, hemagglutination by concanavalin A was inhibited by sucrose, suggesting that there might have a reaction of the plant protein with carbohydrates on the red cell surfaces. Systematic screening of plant extracts for agglutinating activities led to a breakthrough in hematology. In the late 1940s, it was reported that certain seeds contain agglutinins specific for human blood group antigens. The chemical nature of the blood group determinants as oligosaccharides was then delineated (Rüdiger & Gabius, 2001). Over the past few decades, many researches have been carried out to study the occurrence of lectins in various organisms. Their structures, biosynthesis, functions in nature, genetics, and applications in biology and medicine have also been under intense investigation.

1.6.2.4.1.4.3 Applications of lectins

Lectins are widely employed in research for diverse purposes. In the past, lectins were used for blood typing, fractionation of lymphocytes and bone marrow cells for bone marrow transplantation, and for chromosome analysis in human cytogenetics. Nowadays, they are invaluable tools for the detection, isolation and characterization of glycoproteins and other glycoconjugates, for cell and tissue histochemistry and for examination of cell surface changes during physiological and pathological processes such as cell division and cancer development.

In fact, some researches show the antiviral activities of plant lectins. Many enveloped viruses carry carbohydrate-containing proteins on their surface. As the mediators of the receptor binding and membrane fusion of the virus with the host cell, these glycoproteins are important to the infection process. Therefore, they are attractive therapeutic targets for the development of novel antiviral therapies. With the ability of lectins to specifically bind to carbohydrates, scientists are interested in testing various lectins against many viruses such as HIV, HSV, influenza virus, SARS-CoV and RSV (Balzarini *et al.*, 1992; Luo *et al.*, 2007; van der Meer *et al.*, 2007). In case of RSV, it is found that the post-adsorption events of RSV in HeLa cells can be inhibitied by plant lectins from *Listera ovata* and *Urtica dioica* (Balzarini *et al.*, 1992). Moreover, the mannose-binding lectin isolated from *Smilax glabra* is able to inhibit RSV-induced cytopathic effect on HEp-2 cells (Ooi *et al.*, 2004).

1.7 Objectives of the project

The development of RSV vaccine and the search for new antivirals against RSV is still ongoing. There is still a great potential in finding novel and potent antiviral agents from plants. For RSV, such researches may not be as many as those for the more life-threatening diseases such as AIDS, influenza, hepatitis B, and SARS. Therefore, the objectives of the present study are:

1) To screen various medicinal plant extracts and purified phytochemicals for anti-RSV activity,

2) To study the mode of antiviral action of the selected potential anti-RSV compounds.

Chapter 2

Screening of medicinal plants and phytochemicals for antiviral activity against RSV

2.1 Introduction

With the absence of available vaccine and specific anti-RSV drug, RSV infections could only be prevented by the use of expensive passive antibodies or treated by the non-specific, broad spectrum antiviral drug ribavirin as well as other supportive cares. Therefore, there is an urgent need for finding novel potent anti-RSV agents. Plants have formed the basis for traditional medicine system that have been used in many countries for thousands of years. Natural products from plants have provided one of the most important sources of lead compounds for the pharmaceutical industry and nearly 40 % of modern drugs are derived from natural sources (Jassim & Naji, 2003). At present, only a small fraction of the available plant species have been screened for their biological activities and evaluated phytochemically, and even less have been screened for their antiviral potential. Thus there is a great potential in finding anti-RSV agents from plants.

Previously, some *in vitro* studies were carried out to screen some plants for

their anti-RSV activities. In one study, certain traditional Argentine medicinal plants for treating infectious diseases (*Polygonum punctatum*, *Myrcianthes cisplatensis* and *Lithraea molleoides*) were shown to be able to inhibit RSV with median effective dose range from 78 to 120 μg/ml and maximal non-cytotoxic concentration greater than 250 μg/ml (Kott *et al.*, 1999). In another study, the extracts of 44 traditional Chinese medicinal herbs which had been orally administered for the treatment of respiratory infectious diseases were evaluated for their anti-RSV activities (Ma *et al.*, 2002), and among these, 25 herbs show potent antiviral activities against RSV with IC_{50} values ranging from 6.3 to 52.1 μg/ml and SI values ranging from 4.0 to 32.1. Two of the plants *Sophora flavescens* and *Scutellaria baicalensis* were further studied for their antiviral components, and subsequently eleven and four compounds were isolated, respectively. Among the compounds from *S. flavescens*, anagyrine, oxymatrine and sophoranol showed potent anti-RSV activities with same IC_{50} value of 10.4 μg/ml and SI values of 24, 12, and 24, respectively. Wogonin and oroxylin A from *S. baicalensis* were shown to have IC_{50} values of 7.4 and 14.5 μg/ml, and SI values of 16.1 and 4.0 respectively.

Previously in our laboratory, twenty one medicinal herbs traditionally used in southern China for the treatment of skin and respiratory diseases were also screened and six aqueous extracts from *Blumea laciniata*, *Elephantopus scaber*,

Laggera pterodonta, *Mussaenda pubescens*, *Schefflera heptaphylla* and *Scutellaria indica* exhibit anti-RSV activity with IC_{50} values lower than 50 μg/ml and SI values higher than 10 (Li *et al.*, 2004). The SI value of *S. heptaphylla* was found higher than that of ribavirin, and so isolation of active compounds were conducted and two caffeoylquinic acids were found to possess potent anti-RSV activity, that may resulted from inhibition of virus-cell and cell-cell fusion (Li *et al.*, 2005b).

As previously many medicinal plants used for treating respiratory diseases were screened for their anti-RSV activity by another research group of our laboratory, in this study for my M.Phil degree programme, some others medicinal plants that have been used for treating other diseases were screened for their *in vitro* anti-RSV activities. These include *Adina pilulifera* (Lam.) Franch. ex Drake (水團花), *Adina rubella* Hance (水楊梅), *Arctium lappa* L. (牛蒡), *Ardisia chinensis* Benth (小紫金牛), *Polygala aureocauda* Dunn (黃花倒水蓮), *Polygonatum odoratum* (Mill.) Druce (玉竹), *Pandanus amaryllifolius* Roxb. (香蘭葉), *Serissa serissoides* (DC.) Druce (白馬骨), and *Wikstroemia indica* (L.) C. A. Mey (了哥王).

Adina pilulifera roots have been used for treating dysentery, enteritis, dropsy, eczema and ulcers (Shi & Min, 2003). *Adina rubella* is a Chinese folk medicinal plant used as an antibacterial and anti-cough agent. Its ethanol extract was also reported to inhibit some tumors (Fang *et al.*, 1995).

Arctium lappa is also known as burdock, which is originated in Japan. It has been used as folk medicine as it possess antibacterial, fungicide, diuretic, antioxidative, and anxiolytic activities. It was also found able to inhibit platelet aggregation and HIV actions (Gentil *et al.*, 2006).

Ardisia chinensis is a shrub that is traditionally used as medicinal plant among the Yao minority in Southern China for the treatment of icterohepatitis. It is also used in remedies for tuberculosis, emptysis, orchitis and trauma. Recent researches shown that the aqueous extract of the plant could inhibit duck hepatitis B virus *in vitro*, and a polysaccharide of the plant exhibited a significant antiviral effect against Coxsackie B3 (Cox B3) virus, a virus that can cause a wide variety of human diseases, including fatal myocarditis, pericarditis, dilated cardio-myopathy, aseptic meningitis and acquired diabetes (Su *et al.*, 2006).

Polygala aureocauda have been used as folk medicine in Southern China. The root of *P. aureocauda* has been shown to possess antiviral, hypolipidemic and antioxidant activities (Li *et al.*, 2008). *Polygonatum odoratum* has been one of the herbs traditionally used in Korea and China as a treatment of type II diabetes mellitus, hypertension, and cardiovascular diseases (Choi & Park, 2002).

Pandanus amaryllifolius is a plant grown in Southeast Asia that has scented leaves, which are used as a food flavoring and in traditional medicine in the

Malaysia, the Philippines, Thailand, and Indonesia. Some chemical constituents of *Pandanus* species have been used as remedy for toothache and rhematism, and as diuretic and cardiotonic (Takayama *et al*, 2002). 4-hydroxybenzoic acid has been isolated from the hot water extract of the root of this plant and was shown to have hypoglycemic activity (Peungvicha *et al.*, 1998).

Serissa serissoides is used among different minorities of China in treating dyspepsia and hepatitis. Water soluble extract of the plant had marked inhibition effects on HBeAg and HBsAg expressed by 2.2.15 cells (Chen *et al.*, 1999). Its methanol extract was also found to have anti-tumor activity (Zhang & Sun, 2006). *Wikstroemia indica* root is used as a folk remedy for arthritis, tuberculosis, syphilis and pertussis (Ko *et al.*, 1993). Besides, daphnoretin (7-hydroxy-6-methoxy-3,7'-dicoumaryl ether) had been isolated from the plant (Chen *et al.*, 1981).

In addition to the medicinal plant extracts, two purified plant compounds including daphnoretin and *Narcissus tazetta* lectin (NTL), five flavonoids including quercetin, naringin, morin, baicalein and luteolin, as well as two well known purified phytochemicals including cinnamic acid and gallic acid were also made available for antiviral assays.

Screening of potential antiviral agents involves the use of rapid and simple

procedures to measure both antiviral activities as well as cytotoxicity in cell cultures. For antiviral assays, target-specific or non-target specific screening approaches can be employed. Target-specific approaches are biochemical assays of ligand-target interactions designed to focus on specific viral or cellular molecules, proteins or enzymatic activities. These cell-free assays can be developed to high throughput screening with increased screening capacity in a limited amount of time. High throughput screening involves highly automated and computerized operations to handle sample preparation, assay procedures and data processing. Various technologies are available for high throughput screening, including fluorescence-based assays, nuclear-magnetic resonance, affinity chromatography, and DNA microarray (Liu *et al.*, 2004).

Non-target specific approaches, on the other hand, screen for multiple targets in the context of a natural infection, which may reveals novel targets and structures not captured in target-specific assays. In the current RSV study, as the virus induces the formation of syncytia in infected cell cultures, antiviral activity can also be monitored by observation of this cytopathic effect. This non-target specific method is rather qualitative, and so the results are more reproducible. Moreover, the test is relatively less labour intensive. However, as this is a cell-based assay, it is limited to certain virus strain/target cell combinations that result in highly cytopathic

infections. Besides, such assay usually limited to stable cell lines that can be easily manipulated and propagated, and more resources and time have to be spent on keeping the cell line. The status of the cells used in antiviral assay may greatly affect viral infection, and false-positive results may be obtained if the cells are in unfavorable condition for successful viral infection or replication. To overcome the problem, stable cell lines should be used and virus control and cell control should be included in antiviral assays.

Appropriate virus titre should be used in antiviral screening assays. Virus infectivity assays measure the number of infectious particles in a viral suspension. This is done either by plaquing viruses on monolayer cell cultures so as to enumerate the number of plaques which originate from a single infectious virus particle (plaque forming unit; pfu), or by determining the highest dilution of virus suspensions which produce a cytopathic effect (CPE) in 50 % of the inoculated cell cultures (50 % tissue culture infectious dose, $TCID_{50}$).

Another main concern of antiviral agents is toxicity. In cell culture system, cytotoxicity can be assessed by different features of viable or non-viable cells, such as the state of metabolic activities. A rapid colorimetric assay for the measurements of cell growth and metabolic activity was described in 1983, which was called MTT assay (Mosmann, 1983). The assay is based on the reduction of a yellow

water-soluble substrate 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) which change into a water insoluble blue formazan by the active mitochondrial dehydrogenases in living cells, thus the amount of formazan produced reflects in proportion to the number of viable cells. The result is found to be reliable over a wide range of drug concentrations.

In the present study, anti-RSV potential of the samples were determined by CPE reduction assay. Their cytotoxicity were studied by MTT assay. The candidates that inhibited RSV at low concentrations and with low cytotoxicities were selected for further study on their modes of antiviral action.

2.2 Materials and Methods

2.2.1 Medicinal plants and phytochemicals

Dried and chopped samples of *A. chinensis*, *A. pilulifera*, *A. rubella*, *P. aureocauda*, *P. odoratum* and *S. serissoides* were obtained from the specialized herbal shop in Guangzhou, China. Fresh *A. lappa* root and *P. amaryllifolius* were purchased from local stores in Hong Kong. Various fractions of *P. aureocauda* and *W. indica*, as well as a purified compound from *W. indica*, designated as daphnoretin, were kindly provided by Ms. J.Y. Xue of the Department of Chemistry, Jinan University, Guangzhou, China. Voucher specimens were deposited in the Department of Biology, the Chinese University of Hong Kong, Shatin, Hong Kong, China.

Ribavirin, morin, baicalein, luteolin and gallic acid were purchased from Sigma. Naringin was purchased from Fluka and quercetin was purchased from Indofine Chemical Company Limited. Cinnamic acid was kindly provided by Professor Y.L. Li of the Institute of Traditional Chinese Medicine & Natural Products, Jinan University, Guangzhou, China. The purified lectin from the bulbs of *Narcissus tazetta* var. *chinensis*, designated as NTL, was kindly provided by Dr. Linda S.M. Ooi of the Department of Biology, the Chinese University of Hong Kong, Shatin, Hong Kong, China.

2.2.2 Plant extracts preparation

2.2.2.1 Aqueous extracts

For *A. pilulifera*, 200 g of samples were decocted in 2 L of distilled water for 1 h. For *A. chinensis*, *A. rubella*, *P. aureocauda*, *S. serissoides*, 20 g of samples were decocted in 200 ml of distilled water for 1 h. For *P. odoratum*, 50 g of samples were boiled in 300 ml of distilled water for 1 h. For the fresh *A. lappa* root and *P. amaryllifolius*, the plants were washed, air-dried and chopped into small pieces. 75 g of each sample were subsequently boiled in 200 ml distilled water for 1 h. The decoctions were then filtered with filter papers and the residues were decocted again. The aqueous extracts from two successive extractions were collected, combined and concentrated *in vacuo* at 60 °C, and then lyophilized as crude aqueous extracts.

2.2.2.2 Ethanol extracts

Except *P. aureocauda* and *W. indica*, ethanol extracts of the plants were prepared with the same procedures as those for aqueous extracts except that 95 % ethanol were used instead of distilled water. The samples were boiled in a reflux system at 50 - 60 °C using round bottom flasks and heating mantle. The extracts were concentrated at 40 - 50 °C *in vacuo*. Ethanol crude extracts and solvent fractions of *P. aureocauda* and *W. indica* were prepared with the assistance of Ms. J.Y. Xue (Xue, 2007). Briefly, 10 kg of each plant were immersed in 95 % ethanol 3

times, each for 15 days. The extracts were collected, concentrated *in vacuo* and lyophilized. To prepare solvent fractions, 1 kg of dried ethanol extract was dissolved in 3 L distilled water and partitioned sequentially with petroleum ether (3 L × 4 times), ethyl acetate (3 L × 4 times), and butanol (3 L × 4 times). The residue left after butanol partition was the residue water fraction. Different fractions were collected, concentrated and dried, respectively.

2.2.3 Cell and virus

HEp-2 cell (Human larynx epidermoid carcinoma cell line) and RSV (Long strain) were purchased from the American Type Culture Collection (ATCC). The cells were cultured in the growth medium comprised Eagle's minimum essential medium (EMEM, Invitrogen) supplemented with 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 24 mg gentamycin sulphate, 0.29 g glutamine (all from Sigma) and 10 % fetal bovine serum (FBS, Hyclone) and the cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere.

For RSV culture, 1.75×10^4 HEp-2 cells in 100 µl growth medium were seeded into each well of 96-well tissue culture plate and incubated for 24 h. After removing the growth medium, RSV stock was diluted using EMEM supplemented with 1 % FBS (maintenance medium) and added to the subconfluent HEp-2 cells (100 µl/well). The plate was observed daily under microscope for any cytopathic

effect (CPE) induced by the virus. When maximal syncytia formation was observed, the supernatant was collected and centrifuged at 4 °C, 2000 *g* for 10 min to remove cell debris. Aliquots of the supernatant were frozen at -80 °C until use.

2.2.4 Endpoint titration of RSV infectivity

Titration of virus infectivity was performed with a 50% tissue culture infectious dose ($TCID_{50}$) method. HEp-2 cells were seeded in 96-well plate (2.5×10^4 cells/well) and cultured with the growth medium (100 μl/well) for 24 h at 37 °C in 5% CO_2. When the cells became almost confluent in each well, the medium was aspirated and then 100 μl of virus suspension at each dilution after serial 10-fold dilution using the maintenance medium was added. The virus-inoculated cell cultures were further incubated for 4-6 days and CPE of virus were observed daily under microscope. The $TCID_{50}$ value was calculated by the Reed and Muench formula (Reed & Muench, 1938), which reflects the dose of virus that produce a cytopathic effect in 50% of the cultures inoculated.

2.2.5 Cytotoxicity test

Various ethanol extracts, ribavirin, phytochemicals, *P. aureocauda* and *W. indica* fractions were dissolved in dimethyl sulfoxide (DMSO) to make the stocks (40 - 160 mg/ml). They were diluted to appropriate test concentrations with the growth medium to make final DMSO concentration not to exceed 0.5%. Aqueous

extracts were dissolved in distilled water and filtered through 0.22 μm syringe filters to make the stocks (40 mg/ml) and subsequently diluted to test concentrations with the growth medium.

For cytotoxicity test, 0.625×10^4 HEp-2 cells were seeded in 96-well plates and cultured with 100 μl of growth medium for 24 h at 37 °C in 5% CO_2. Then 100 μl of two-fold diluted samples were added to the treatment wells. For each cell control well, 100 μl of the growth medium without the sample were added. The plates were further incubated for 48 h. The medium in each well was removed and then 100 μl growth medium and 10 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) at 5 mg/ml in phosphate-buffered saline (PBS) were added to each well. The plates were incubated for 4 h for the formation of blue formazan. After removing the supernatant, the blue formazan were solubilized by adding 100 μl DMSO and the optical density (OD) was measured at 570 nm with a microplate reader. The cytotoxic effects of different samples on the HEp-2 cells were expressed as percentage inhibition on cell metabolic activity and viability, which was calculated by the following equation:

$$\left(1 - \frac{\text{Average OD of treatment wells}}{\text{Average OD of control wells}}\right) \times 100\ \%$$

The percentage inhibition calculated was plotted against the corresponding

concentration of sample tested. The concentration of sample with half maximal inhibition on the growth and survival of HEp-2 cells (CC_{50}) was analyzed by the GraphPad Prism 4.0 software.

2.2.6 Antiviral assay

CPE reduction assay was adopted for screening the antiviral activities of different samples in this study. Samples were serially two-fold diluted with the maintenance medium. In brief, 2.5×10^4 HEp-2 cells in 100 µl growth medium were seeded into each well of 96-well plates and incubated for 24 h. When the cells were confluent, the growth medium was removed and 100 µl of RSV suspension at 100 $TCID_{50}$ and 100 µl diluted samples (both in maintenance medium) were added simultaneously to the treatment wells. For the virus control wells, RSV and the maintenance medium without the sample were added. For the cell control wells, 200 µl of the maintenance medium without the sample were added. The plates were incubated for 4-6 days for CPE development and CPE in each wells were then observed and scored. The percentage of cells showing CPE was scored and expressed as follow: no CPE = -; 1-25 % = 1+; 26-50 % = 2+; 51-75 % = 3+; 76-100 % = 4+. The concentration that reduced 50 % of CPE in respect to virus control was defined as 50 % inhibition concentration (IC_{50}) for this assay. The selective index (SI) was calculated from the ratio CC_{50}/ IC_{50} (Cos *et al.*, 2006).

2.3 Results

The extracts from 9 medicinal plants and 9 phytochemicals were studied for their inhibition on RSV-induced cytopathic effects (Table 2.1). Some of them did not show any inhibitory effects, including aqueous extracts of *A. lappa*, *A. chinensis*, *P. amaryllifolius*, *P. aureocauda*, *P. odoratum* and *S. serissoides,* as well as ethanol extracts of *A. lappa*, *P. amaryllifolius* and *P. odoratum*. Many of them possessed slight inhibitory activities, such as the aqueous extract of *A. pilulifera*, the ethanol extract of *A. rubella* and *A. chinensis*, the butanol and residue water fractions of *P. aureocauda*, as well as purified phytochemicals such as naringin, morin, gallic acid, cinnamic acid and baicalein.

On the other hand, the aqueous extract of *A. rubella*, the ethanol extracts of *P. aureocauda* and *S. serissoides*, the petroleum ether and butanol fractions of *W. indica* possessed moderate anti-RSV CPE activities. Quercetin and luteolin were found too cytotoxic, though they inhibited RSV-induced CPE at low concentrations.

The compounds with potent anti-RSV activities include the ethyl acetate fraction of *W. indica* (IC_{50} < 3.91 μg/ml; SI > 64.1), ethanol extract of *A. pilulifera* (IC_{50} ~15.6 μg/ml; SI > 32.0), daphnoretin (IC_{50} ~7.81 μg/ml; SI = 21.1), and NTL (IC_{50} ~3.13 μg/ml; SI = 104). The positive control drug ribavirin has an IC_{50} value of about 3.05 μg/ml (Figure 2.1) and SI value 21.4.

Table 2.1 Anti-RSV activity of medicinal plant extracts and phytochemicals

Tested samples	Part used	Test concentration nearest to IC_{50} (μg/ml)	CC_{50} (μg/ml)	Selectivity Index (SI)[a]	Maximum inhibition (%)	Optimal dosage (μg/ml)
Medicinal plant extracts:						
***Adina pilulifera* (Lam.) Franch. ex Drake:**	Whole plant					
Aqueous extract		125	>500	>4.00	50	125
Ethanol extract		15.6	>500	>32.0	100	31.3
***Adina rubella* Hance:**	Root					
Aqueous extract		62.5	>250	>4.00	>75	125
Ethanol extract		125	>250	>2.00	>75	250
***Arctium lappa* L.:**	Root					
Aqueous extract		>250	>250	-	-	-
Ethanol extract		>250	>250	-	-	-
***Ardisia chinensis* Benth:**	Whole plant					
Aqueous extract		>250	>250	-	-	-
Ethanol extract		125	>250	>2.00	100	250
***Pandanus amaryllifolius* Roxb.:**	Leaf					
Aqueous extract		>250	>250	-	-	-
Ethanol extract		250	>250	>1.00	50	250

(Table 2.1 continued)

***Polygala aureocauda* Dunn:**	Whole plant					
Aqueous extract		>250	>250	-	-	-
Ethanol extract		>62.5	>250	-	-	-
Petroleum ether fraction		>31.3	96.9	<3.10	100	62.5
Ethyl acetate fraction		>62.5	141	<2.25	<25	62.5
Butanol fraction		>125	>250	-	<25	125
Residue water fraction		>125	>250	-	-	-
***Polygonatum odoratum* (Mill.) Druce:**	Rhizome					
Aqueous extract		>250	>250	-	-	-
Ethanol extract		>250	>250	-	-	-
***Serissa serissoides* (DC.) Druce:**	Whole plant					
Aqueous extract		>250	>250	-	-	-
Ethanol extract		31.3	>250	>8.00	>75	31.3
***Wikstroemia indica* (L.) C. A. Mey:**	Root					
Ethanol extract		15.6	>250	>16.0	>75	62.5
Petroleum ether fraction		7.81	100	12.8	>75	31.3
Ethyl acetate fraction		<3.91	250	>64.1	100	31.3
Butanol fraction		31.3	>250	>7.78	>75	125

(Table 2.1 continued)

Purified phytochemicals:						
Quercetin	-	3.13#	4.56#	1.46	>75	3.13#
Naringin	-	>100#	>250#	-	-	-
Morin	-	100#	209#	2.09	50	100#
Baicalein	-	6.25#	24.3#	3.89	50	6.25#
Luteolin	-	6.25#	2.49#	0.398	50	6.25#
Gallic acid	-	>100#	>250#	-	25	100#
Cinnamic acid	-	>100#	>250#	-	25	100#
Daphnoretin	-	7.81	165	21.1	>75	31.3
NTL	-	3.13	325	104	100	12.5
Ribavirin *	-	3.05	65.2	21.4	100	12.2

IC_{50}: 50 % inhibition concentration; concentration that reduced 50 % of cytopathic effect in respect to virus control.

CC_{50}: 50 % cytotoxic concentration.

[a]: Selectivity index calculated based on the ratio of CC_{50} to the test concentration nearest to IC_{50}.

#: Concentration in μM.

*: Positive control drug.

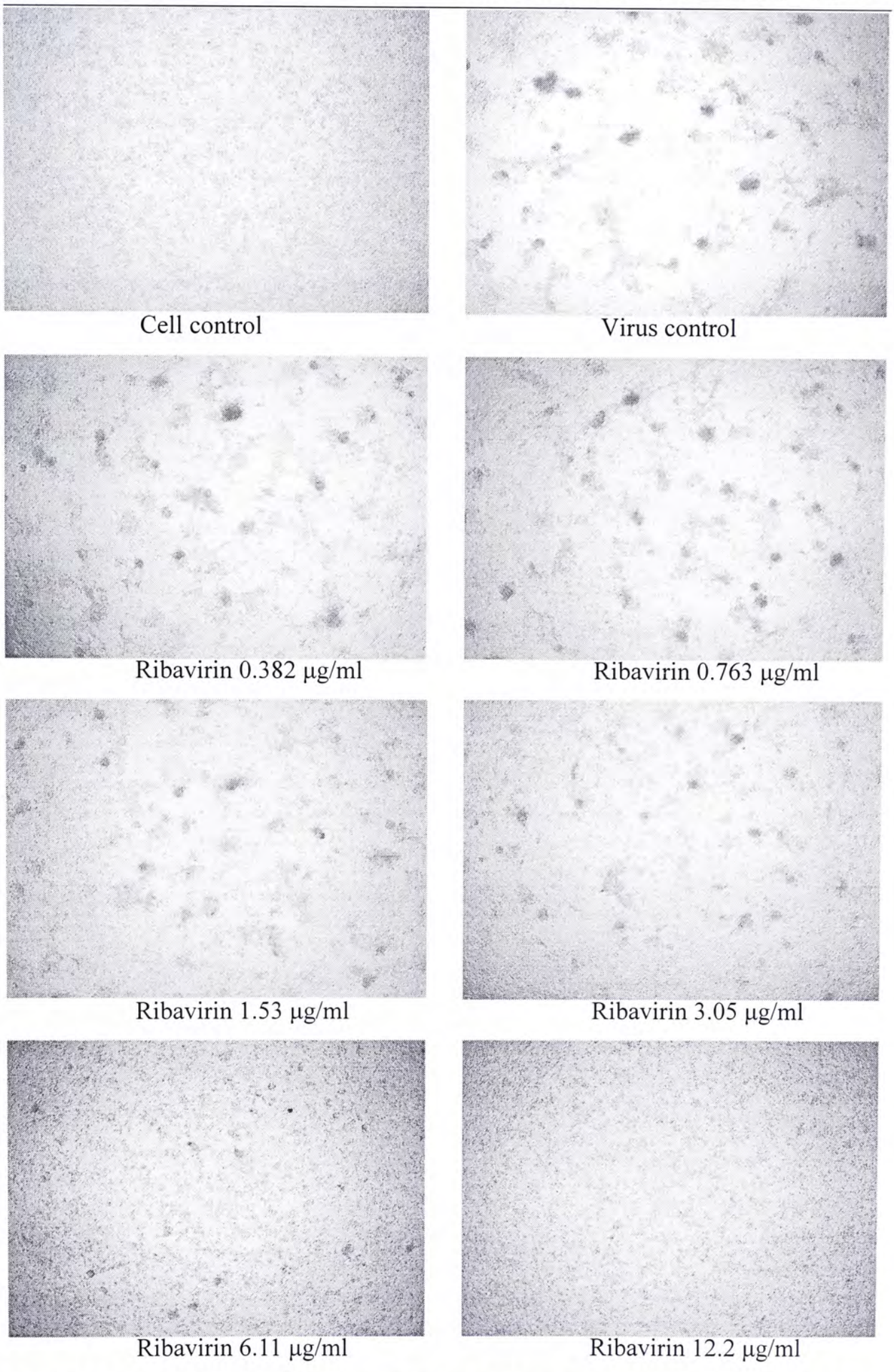


Figure 2.1. Microscopic photos showing CPE reduction by the positive control drug ribavirin in RSV-infected HEp-2 cells (magnification: 100×).

2.4 Discussion

In this study, CPE reduction assay was employed in the screening of the anti-RSV activity of medicinal plant constituents. Though this method is relatively qualitative and it is difficult to determine the exact percentage of inhibition at each tested concentration, it could still provide information about whether or not test compounds at a particular concentration could inhibit RSV-induced syncytium formation in a less expensive, less labour intensive and high throughput condition as compared to the other screening methods. The test concentrations that could almost inhibit 50 % syncytium formation compared to that of the virus control (i.e. close to the IC_{50} value) could then be estimated. Among the extracts or fractions from the 9 medicinal plant studied, the ethanol extract of *A. pilulifera* and the ethyl acetate fraction of *W. indica* possess the greatest anti-RSV activity with IC_{50} values of about 15.6 μg/ml and less than 3.91 μg/ml, respectively. Moreover, daphnoretin, the purified dicoumarin from the ethyl acetate fraction of *W. indica*, also exhibits antiviral activity with IC_{50} value of about 7.81 μg/ml and the compound may contribute to the anti-RSV action of the ethyl acetate fraction of *W. indica*.

On the other hand, a purified plant lectin, five commercially available plant flavonoids and two well-known phytochemicals were also studied for their anti-RSV activity. NTL, a lectin isolated from the bulb of *N. tazetta*, potently

inhibited RSV with IC_{50} value of about 3.13 μg/ml and with low cytotoxicity (CC_{50} = 325 μg/ml). For the other phytochemicals, only baicalein, quercetin and luteolin possess anti-RSV activities at relatively lower concentrations. Baicalein was previously isolated from *Scutellaria baicalensis* (Ma *et al.,* 2002) and assessed for its anti-RSV activity with IC_{50} value of 20.8 μg/ml and CC_{50} value of 250 μg/ml, but the commercially available baicalein used in this study has a higher cytotoxicity. This may be due to the difference of chemical source or assay method used, as a confluent cell culture was used in the previous study while a growing cell culture was employed in this study. Quercetin was reported to inhibit both the infection and replication of RSV (Kaul *et al.,* 1985), but there is no information about its cytotoxicity. In the present study, baicalein, quercetin and luteolin were found relatively toxic at low concentrations, so they were not considered as good candidates as antiviral drugs.

Daphnoretin, NTL and the ethanol extract of *A. pilulifera* have *in vitro* anti-RSV activity and low cytotoxicity, with SI values about 21.2, about 104 and higher than 32, respectively. Therefore, in the next chapter, the ethanol extract of *A. pilulifera* was further fractionated and the mode of antiviral action of the most active fraction, together with daphnoretin, were studied. In Chapter 4, the activity of NTL as well as other proteins of *N. tazetta* were further investigated.

Chapter 3

Mechanistic studies of anti-RSV actions of various fractions of *Adina pilulifera*, and daphnoretin, a purified compound from *Wikstroemia indica*

3.1 Introduction

Adina pilulifera is a shrub with small leaves, growing on wet soil. It is used for treating dysentery, enteritis, dropsy eczema, ulcers, and helps to disperse stasis and swelling (Shi & Min, 2003; Zhu & Min, 2005).

The chemical compositions of *A. pilulifera* have been reported in some studies (Table 3.1). However, there is still no reports on the bioactivity of the fractions and compounds extracted from this plant. In the previous chapter, the ethanol extract of *A. pilulifera* was found to possess inhibitory effect on RSV-induced cytopathic effect and low cytotoxicity on HEp-2 cells. Therefore, further fractionation of the extract was performed and the active fraction was tested for its mode of anti-RSV action in the present study.

Besides *A. pilulifera*, the mode of anti-RSV action of daphnoretin, which was isolated from *Wikstroemia indica*, was also studied.

Daphnoretin (7-hydroxyl-6-methoxy-3',7'-dicoumarylether) was first

isolated from *W. indica* in 1979 (Kato *et al.*, 1979). It was found to possess antifungal activity by inducing morphological deformation of *Pyricularia oryzae* mycelia with minimum morphological deformation concentration values of 68.4 ± 1.3 μM (Hu *et al.*, 2000). The compound caused 97 % inhibition of growth of Ehrlich ascites carcinoma cells in an *in vivo* assay (Lee *et al.*, 1981). Daphnoretin significantly inhibits some major sites in the DNA synthetic pathway, such as dihydrofolate reductase, orotidine monophosphate decarboxylase, thymidylate monophosphate kinase, and ribonucleotide reductase. Reduction of *in vitro* oxidative phosphorylation processes and inhibition of acid hydrolytic enzymes are also observed in the presence of daphnoretin (Hall *et al.*, 1982).

Daphnoretin also possesses the characteristic as an protein kinase C (PKC) activator (Ko *et al.*, 1993). It induces respiratory burst in rat neutrophils that may be resulted from direct activation of PKC (Wang *et al.*, 1995). Respiratory burst of activated neutrophils leads to the productions of reactive oxygen species, which are generally believed to be responsible for the antimicrobial effect of neutrophils. Furthermore, daphnoretin shows strong suppressive effect on hepatitis B surface antigen (HBsAg) expression in human hepatoma Hep3B cells and induces translocation of PKC from the cytosol to the membrane and down-regulates intracellular PKC levels. It is thus suggested that daphnoretin exerts its antiviral effect

similarly to a phorbol ester in regulating gene expression of HBsAg on Hep3B cells through the PKC pathway (Chen *et al.*, 1996).

Table 3.1 Chemical composition of *A. pilulifera*

Chemical	Reference
From root	
Naucleoside	
β-sitosterol	Zhu & Min, 2005
Noreugenin	
Palmitic acid	
Quinovic acid	
3β-*O*-β-$_D$-xylopyranosyl(1→3)-α-$_L$-rhamnopyranosyl-pyrocincholic acid 28-*O*-β-$_D$-glucopyranosyl(1→6)-β-$_D$-glucopyranosyl ester	
3β-*O*-β-$_D$-xylopyranosyl(1→3)-α-$_L$-rhamnopyranosyl-cincholic acid 28-*O*-β-$_D$-glucopyranosyl ester	
3-oxours-12-ene-27,28-dioic acid	
3β-*O*-β-$_D$-fucopyranosyl-quinovic acid	
3β-*O*-β-$_D$-quinovopyranosyl-quinovic acid	
3β-*O*-α-$_L$-rhamnopyranosyl-quinovic acid	
3β-*O*-β-$_D$-glucopyranosyl-quinovic acid	
Quinovic acid(28→1)-*O*-β-$_D$-glucopyranoside	
3β-*O*-β-$_D$-xylopyranosyl(1→3)-α-$_L$-rhamnopyranosyl-quinovic acid	
3β-*O*-β-$_D$-glucopyranosyl-quinovic acid 28-*O*-β-$_D$-glucopyranosyl ester	Shi & Min, 2003
Loganin	
3β-*O*-β-$_D$-fucopyranosyl-quinovic acid 28-*O*-β-$_D$-glucopyranosyl ester	
3β-*O*-β-$_D$-quinovopyranosyl-quinovic acid 28-*O*-β-$_D$-glucopyranosyl ester	
3β-*O*-β-$_D$-xylopyranosyl(1→3)-α-$_L$-rhamnopyranosyl-quinovic acid 28-*O*-β-$_D$-glucopyranosyl ester	
3β-*O*-β-$_D$-glucopyranosyl-(1→2)-β-$_D$-glucopyranosyl-quinovic acid 28-*O*-β-$_D$-glucopyranosyl ester	
7α-morroniside	
7β-morroniside	
3α,5α-tetrahydrodeoxycordifoline	

(Table 3.1 continued)

From stem	
Quinovic acid Morolic acid Betulinic acid Cincholic acid	Zhu & Min, 2005
From leaf	
Saponins β-sitosterol Stigmasterol	Zhu & Min, 2005
From whole plant	
Ethyl acetate fraction Sarracenin 2-methyl-5,7-dihydroxychromone *Butanol fraction* Morroniside	Xue *et al.*, 2007

3.2 Materials and Methods

3.2.1 Fractionation of *A. pilulifera* ethanol extract

Ethanol extract of *A. pilulifera* was prepared by the procedure as described in Section 2.2.2.2. The ethanol extract (31.53g) was dissolved in 50 ml distilled water. An equal volume of petroleum ether was then added and mixed vigorously. After mixing, the mixture was left undisturbed until two layers of solvent were formed. The petroleum ether layer was collected and fresh petroleum ether was added with the extraction procedure repeated four times until no color is observed in the solvent layer. All the collected solvents were pooled and evaporated to form the petroleum ether fraction. The aqueous layer was partitioned sequentially with ethyl acetate and butanol with the same procedure. The aqueous layer left after the partition became the residue water fraction. Different fractions were collected, concentrated and freeze-dried.

3.2.2 Cell and virus

The method of HEp-2 cells and RSV culture were described in Section 2.2.3.

3.2.3 Cytotoxicity test

Cytotoxicity of the four fractions on growing HEp-2 cells was determined by MTT assay. Different fractions were dissolved in DMSO to make the stocks of 160

mg/ml and detail procedure was described in Section 2.2.5.

3.2.4 Endpoint titration of RSV by $TCID_{50}$ method

RSV titre was scored by $TCID_{50}$ determination as described in Section 2.2.4.

3.2.5 Antiviral study by CPE reduction assay

Different fractions were dissolved in DMSO to make the stocks of 80 mg/ml. RSV at 100 $TCID_{50}$ and serially two-fold diluted samples were added to HEp-2 cells, CPE were observed under a microscope daily. Detail procedure was described in Section 2.2.6.

3.2.6 Endpoint titration of RSV by plaque assay

HEp-2 cells were cultured with the growth medium in 12-well plate at 37 °C in a humidified 5 % CO_2 atmosphere for 24 h (3×10^5 cells/well). When confluent cell monolayers were formed, the growth medium was removed and RSV suspension at each of the serial ten-fold dilutions using maintenance medium was added to the cells (200 µl/well). The plate was then re-incubated for 2 h with intermittent shaking at 15 min intervals. After that RSV suspensions were removed and the wells were rinsed twice with PBS (1 ml/well) to remove unbound RSV. Then the cells were overlaid with 1 % agarose (Invitrogen) in the maintenance medium (1 ml/well). After the agarose solidified, a further 1 ml maintenance medium was added. The plate was

incubated for 3 days for plaque formation, followed by fixation of cells with 10 % formalin and stained with 1 % crystal violet. The number of plaques was counted under light microscope after air-drying of the plate. The titre of RSV was expressed as the number of plaque forming units per ml (pfu/ml).

3.2.7 Antiviral study by plaque reduction assay

The ethyl acetate fraction of *A. pilulifera*, together with daphnoretin, were studied for their half maximal inhibitory concentrations on RSV-induced plaque formation. Ribavirin was used as the positive control drug. The three samples were dissolved in DMSO to make 80 mg/ml stocks. HEp-2 cells were seeded in 12-well plates (3×10^5 cells/well) and incubated for 24 h. After removing the growth medium, 100 μl of RSV at about 80 pfu and 100 μl two-fold diluted samples (both in the maintenance medium) were added to each well. For the virus control wells, 200 μl RSV at final 80 pfu were added. The plates were further incubated for 2 h with intermittent shaking. After that, RSV and the samples were discarded and the cells were rinsed with PBS twice. The cells were overlaid with 1 % agarose in the maintenance medium (1 ml/well). After the agarose solidified, 1 ml sample or 1 ml maintenance medium was added to the corresponding wells and virus control wells respectively. The plates were then incubated for 3 days to allow the formation of plaques. Cells were fixed and stained, and the number of plaques were counted under

light microscope. The percentage of inhibition of RSV plaques by the samples were calculated by:

$$\left(1-\frac{\text{Average no. of plaques in treatment wells}}{\text{Average no. of plaques in virus control wells}}\right)\times 100\ \%$$

The percentage of inhibition calculated were plotted against the corresponding concentration of samples and analyzed by the GraphPad Prism 4.0 software. The half maximal inhibitory concentration on RSV-induced plaque formation by each sample was defined as the IC_{50} for this assay.

3.2.8 Mode of antiviral action study

The ethyl acetate fraction of *A. pilulifera*, daphnoretin, ribavirin, and RSV were added to HEp-2 cells with different time of addition, as described in Zhu *et al.*, 2006, so the samples were present under different conditions: 1) during the early infection phase (the first 2 h post-infection) only; 2) during the whole infection phase (samples present throughout the experiment), or 3) during the later infection phase only (samples only present after 2 h of infection). HEp-2 cells at 3×10^5 cells/well were seeded in 12-well plates and incubated for 24 h. After removing the growth medium, 100 μl of RSV at about 80 pfu and 100 μl samples were added to each well with a final concentration of 7.81 μg/ml for ethyl acetate fraction and daphnoretin, and 3.9 μg/ml for ribavirin. For virus control wells and the wells of condition 3, 200

μl RSV at final 80 pfu were added. After further incubation for 2 hours, unbound RSV and samples were washed away by rinsing the cells with PBS twice. The cells were overlaid with 1 % agarose in the maintenance medium (1 ml/well). After the agarose solidified, 1 ml of sample at double concentration (15.625 μg/ml for ethyl acetate fraction and daphnoretin, and 7.81 μg/ml for ribavirin) was added to each well of conditions 2 and 3. For each of the virus control wells and the wells of condition 1, 1 ml maintenance medium was added. After plaque development, the number of plaques was counted and the percentage of plaques formed under different conditions compared to the virus control were calculated by:

$$\left(\frac{\text{Average no. of plaques in treatment wells}}{\text{Average no. of plaques in virus control wells}} \right) \times 100\,\%$$

The results were analyzed with one-way ANOVA followed by Tukey's multiple comparison test by the GraphPad Prism 4.0 software.

3.3 Results

A. pilulifera ethanol extract (31.5 g) was first partitioned with different organic solvents from low to high polarity. The fractions were concentrated and dried, and about 2.40 g petroleum ether fraction, 3.27 g ethyl acetate fraction, 11.1 g butanol fraction, and 8.28 g residue water fraction were obtained, respectively. The fractions were tested for their anti-RSV activity by CPE assay and their cytotoxicity were tested by MTT assay. Except the residue water fraction, the other three fractions possess anti-RSV activity with IC_{50} values of about 7.81, < 7.81 and 31.3 μg/ml for the petroleum ether, ethyl acetate and butanol fraction respectively. However, the petroleum ether fraction was also found cytotoxic with CC_{50} value of 122 μg/ml and among the three fractions, the ethyl acetate fraction has the highest SI value (Figure 3.1). Therefore, this fraction was chosen for further study.

In the next study, the IC_{50} values of ribavirin, daphnoretin and the ethyl acetate fraction of *A. pilulifera* were determined by plaque reduction assay, which were 2.18, 5.87 and 2.22 μg/ml respectively. Their SI values, calculated with the IC_{50} values determined by plaque reduction assay, were 30.0, 28.2 and 86.2 respectively (Table 3.2).

Their modes of antiviral action were studied by the addition of samples during different phases of the virus infection cycle. Significant reductions on plaque

formation were observed when the three samples were present during the whole infection cycle. Also, for ribavirin and the ethyl acetate fraction, similarly significant reduction on plaque number were also observed when the drug was only present during the later infection phase. For daphnoretin, a slight inhibitory effect was observed when the sample was only present during the early infection phase, while a more significant inhibition was observed when the sample was only added during the later infection phase (Figure 3.2).

Table 3.2 CC_{50}, IC_{50} and SI of ribavirin, daphnoretin and *A. pilulifera* ethyl acetate fraction.

	CC_{50} (μg/ml)	**#IC_{50} (μg/ml)**	**SI**
Ribavirin*	65.2	2.18	30.0
Daphnoretin	165	5.87	28.2
Ethyl acetate fraction of *A. pilulifera*	191	2.22	86.2

1) #: IC_{50} - concentration that leads to half maximal inhibition on number of plaques compared to virus control
2) SI: selectivity index (CC_{50}/IC_{50})
3) CC_{50} and IC_{50} were calculated from data obtained from three independent experiments with the generation of dose-response curves by GraphPad Prism 4.0 software.
4) *: Ribavirin is the positive control drug.

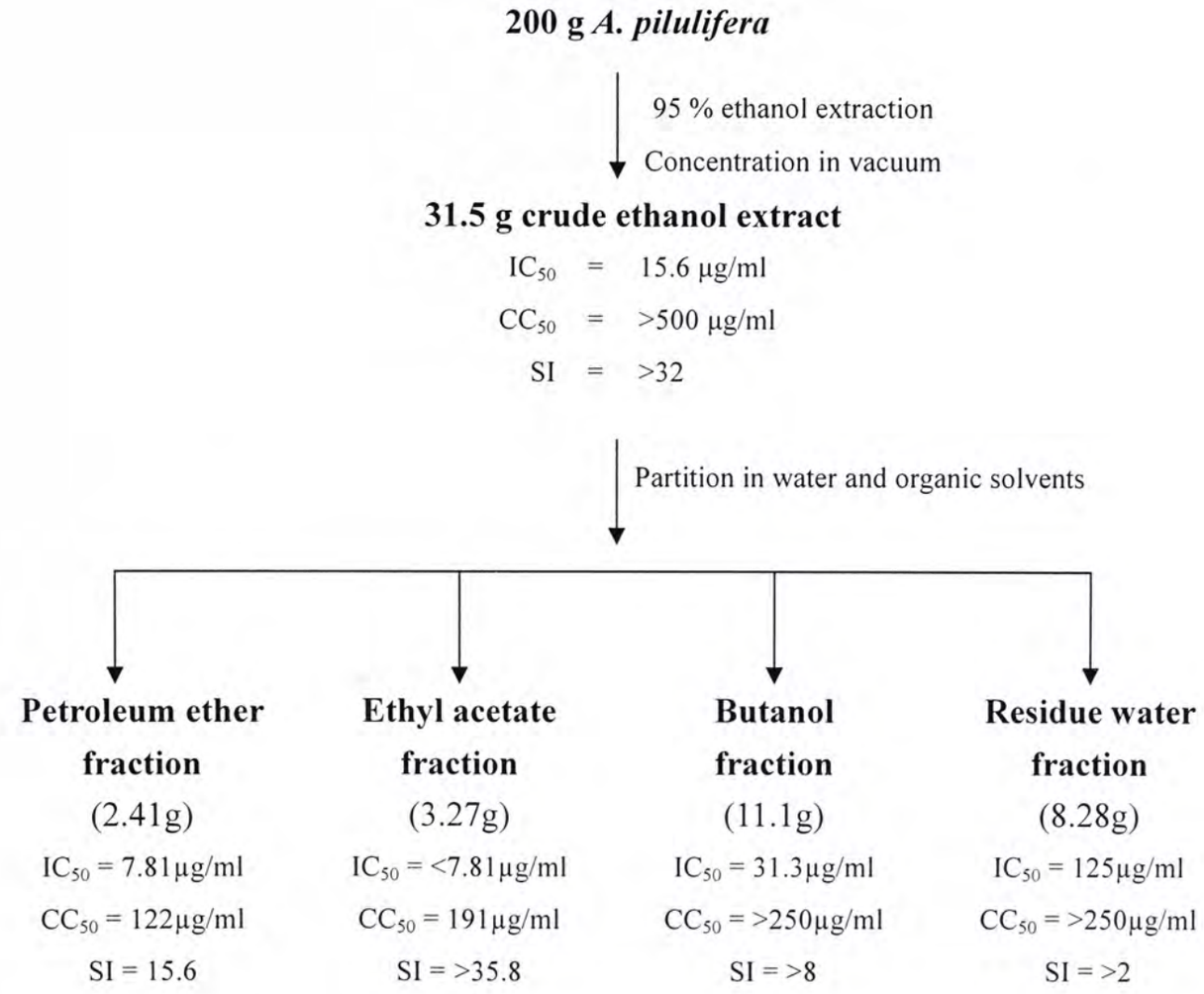
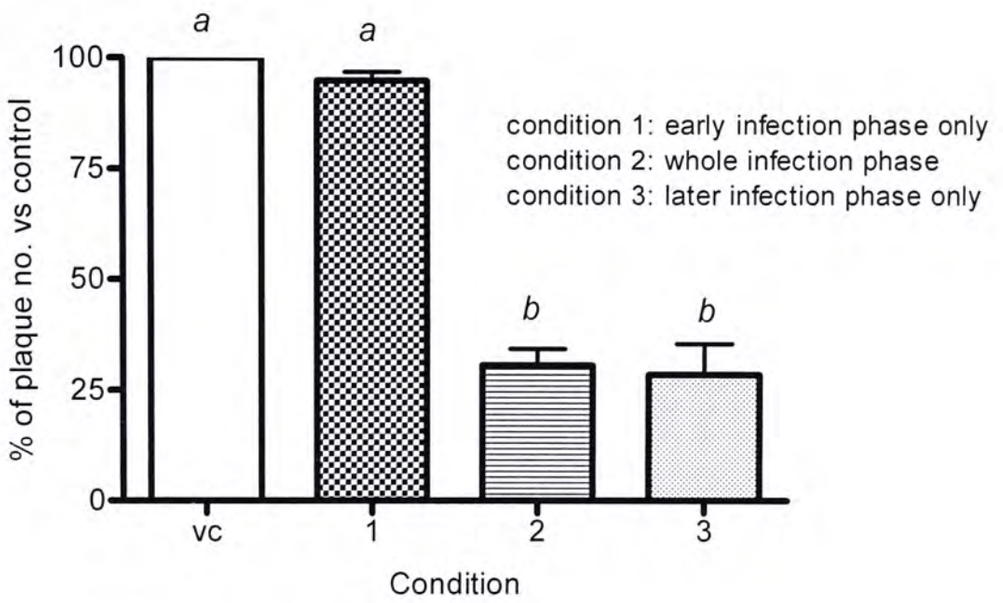


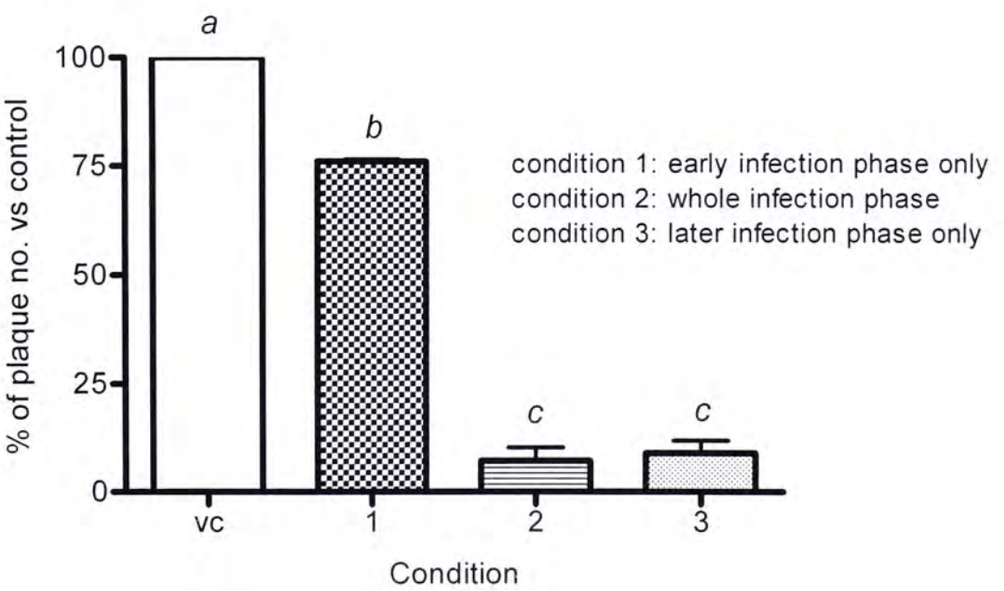
Figure 3.1 Fractionation of *A.pilulifera*.

The values of IC_{50} refer to the concentrations nearest to the actual IC_{50} (i.e. 50 % reduction of cytopathic effect in respect to virus control in CPE assay).

A Ribavirin



B Daphnoretin



C Ethyl acetate fraction

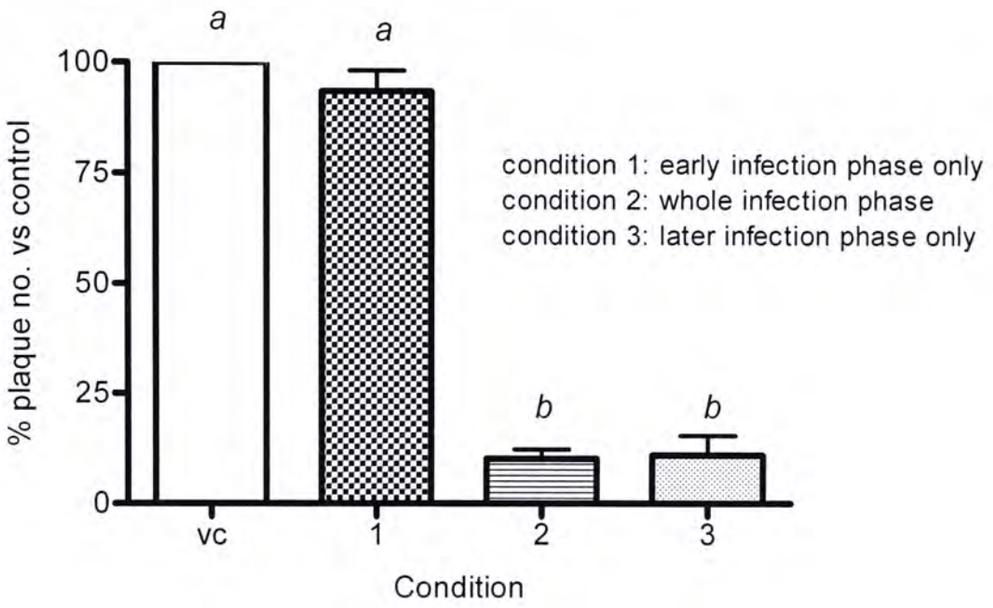


Figure 3.2 Mode of antiviral action study by plaque reduction assay.
HEp-2 cells were infected by RSV at about 80 pfu and A) 3.9 μg/ml ribavirin, B) 7.81 μg/ml daphnoretin, or C) 7.81 μg/ml ethyl acetate fraction of *A. pilulifera* were added during the early infection phase only (i.e. the first 2 h only), during the whole infection phase (i.e. samples present throughout the experiment), or during the later infection phase only (i.e. after the first 2 h). The percentage of number of plaques formed under different conditions compared to the virus control (VC) were calculated and analyzed by one-way ANOVA followed by Tukey's multiple comparison post-test, $P<0.001$. In each graph, the bars marked by the same letter have no significant difference. Results were expressed as mean ± S.D. obtained from three to four independent experiments with duplicate wells for each condition.

3.4 Discussion

Compounds with different polarities can be separated by fractionation of the ethanol extract of *A. pilulifera* using different organic solvents from low to high polarity, and the fractions showed different degree of inhibition on RSV. The residue water fraction, though not highly cytotoxic, could only inhibit RSV at a relatively high concentration (IC_{50} ~ 125 μg/ml). The other three fractions possess better inhibitory effect on RSV-induced CPE with the petroleum ether fraction being the most cytotoxic (CC_{50} = 122 μg/ml). The SI values of the ethyl acetate fraction (>35.8) was comparable to that of the ethanol extract (>32), while the other two fractions have lower SI values. Therefore, more in-depth studies of the ethyl acetate fraction, together with daphnoretin and ribavirin, were carried out. First, their IC_{50} values were studied with plaque reduction assay. This assay is quantitative, and the number of plaques formed reflects the number of infectious virions present. However, the assay is rather labour intensive, thus it is not suitable for high-throughput screening. The IC_{50} values obtained were used for calculation of the SI values, and it was found that SI value of daphnoretin was comparable to that of ribavirin (28.2 and 30.0 respectively) while the ethyl acetate fraction had a much higher SI value (SI = 86.2) than both ribavirin and the original ethanol extract.

Second, in order to determine which stage(s) of the RSV replication cycle

was inhibited by these samples (i.e. their modes of action), time-of-addition studies were carried out. The growth kinetics of RSV in cell cultures were studied in the 1960s, which found that the virus required 1 to 2 hours for adsorption and penetration, and viral antigen appears in the cytoplasm 9 to 10 hours after infection. At 10 to 14 hours, the first mature virus appears, and the virus growth cycle in HEp-2 cells is 45 - 50 hours (Bennett & Hamre, 1962; Jordan, 1962; Kisch *et al.*, 1962; Levine & Hamilton, 1969). It was also reported that approximately 60-90 % of RSV adsorb to cells in 30 minutes at 37 ^{o}C and over 95 % by 10-12 hours. Adsorbed viruses penetrate the cells within 45 minutes through virus-cell fusion (Levine & Hamilton, 1969; Stott & Taylor, 1985). Therefore, in usual RSV assays, the time allowed for virus adsorption and fusion is about 2 hours post-infection. In our time-of-addition studies, the test samples were either present for only 2 hours (condition 1: during the early infection phase only), present during the whole time course of plaque reduction assay (condition 2: during the whole infection phase), or present only after the first 2 hours of infection (condition 3: the later infection phase only). In order to achieve a more apparent difference between condition 2 and the virus control, concentrations of the test samples added (3.9 μg/ml for ribavirin; 7.81 μg/ml for both daphnoretin and the ethyl acetate fraction) were greater than their IC_{50} values. The results showed that for ribavirin and the *A. pilulifera* ethyl acetate fraction, no significant reductions on

plaque numbers were observed when the samples were present only in the early infection phase. On the other hand, significant decreases in the number of plaques were observed even when the samples were added after viral attachment and fusion. The decreases were comparable to that when the samples were present throughout the whole RSV infection cycle. The result of ribavirin was similar to that reported previously (Hruska *et al.*, 1980), which stated that ribavirin was still able to inhibit RSV plaque formation when added as late as 12 hours post-infection. Although the actual underlying mechanism of its inhibitory effect is still unknown, the actions and mechanisms of ribavirin on other viruses were reported, including the reduction of intracellular guanosine triphosphate pools, inhibition of capping of viral transcripts or suppression of humoral and cellular immune responses (Leyssen *et al.*, 2008). The result of the ethyl acetate fraction of *A. pilulifera* indicated that its mode of action is not likely by inhibition of viral attachment or fusion, but by inhibition of the later events of the virus replication cycle (such as replication, viral assembly or viral release) or by modulation of host cell's biological events to achieve the antiviral purpose.

For daphnoretin, about 25 % reduction in number of plaque was observed when the compound was present during the early infection phase only, and about 80 % reduction was observed when the compound was added after viral attachment and

fusion (i.e. 2 h post-infection), which showed a similar level of reduction as that when the compound was always present. Thus daphnoretin might be able to slightly inhibit viral attachment and/or fusion, and mainly inhibit the later viral development and spreading. Daphnoretin was reported as a PKC activator (Ko *et al.*, 1993; Wang *et al.*, 1995; Chen *et al.*, 1996). PKC is a large superfamily of calcium and phospholipids-dependent serine/threonine kinases with many isotypes. PKC has been implicated in the control of various cellular signals required for activation, proliferation, differentiation and survival. Upon stimulation and activation, translocations of PKC from cytosol to plasma membrane or other subcellular compartments occur. Different physiological stimuli induce selective translocation of PKC isotypes to distinct subcellular compartments (Shirai & Saito, 2002). It was found that PKC is involved in the RSV-induced nuclear factor kappa B (NF-κB; cellular transcription factor) and extracellular signal-related kinase (ERK) activation, which are involved in the release of the inflammatory mediator interleukin 8 and 15 (Bitko & Barik, 1998; Monick *et al.*, 2001; Ennaciri *et al.*, 2007) and hence immune response elicitation. Interestingly, the activation and translocation of one of the PKC isotype, PKCα, to cell membrane was observed upon RSV contact and to facilitate RSV fusion (San-Juan-Vergara *et al.*, 2004). Other PKC isotypes are not involved and the translocated PKCα were found colocalized with the attached RSV on plasma

membrane. However, it is unknown how PKCα is activated and how activated PKC facilitates RSV fusion, although it is speculated that there may be a relationship between PKCα-related caveolae formation and RSV-caveola binding.

On the other hand, daphnoretin activates PKC by direct binding to its regulatory domain, induces its translocation to the plasma membrane and lead to down-regulation of intracellular PKC level (Wang *et al.,* 1995; Chen *et al.,* 1996), although the isotype of PKC involved was not mentioned. It is unknown whether the inhibition of RSV plaque formation by daphnoretin in our study is related to its PKC activator role. But as the early infection phase of RSV is slightly inhibited by daphnoretin, it might be related to the reduction of the intracellular PKC pool by daphnoretin and thus less PKC were available for RSV-induced activation and translocation, thus affected RSV fusion. Daphnoretin is also able to inhibit RSV at the later infection phase, which may be due to direct inhibition on RSV replication process or modulation of cellular events in the host cell that interfere other events of the RSV replication cycle.

In next chapter, the antiviral activity of *Narcissus tazetta* proteins and the mode of antiviral action of the most potent protein were studied.

Chapter 4

Antiviral activity of *Narcissus tazetta* proteins

4.1 Introduction

Many plant proteins have antiviral activities against human viruses. For example, meliacine isolated from *Melia azedarach* is able to inhibit HSV-1 *in vitro* and *in vivo* by inhibiting specific infected-cell polypeptides produced in later phase in the virus infection cycle. Some of these polypeptides are involved in DNA synthesis and in the assembly of nucleocapsids, thus the production of viral DNA and maturation of the virus are affected (Alché *et al.*, 2002). A xylanase isolated from the roots of *Panax notoginseng* has suppressive effect on HIV-1 reverse transcriptase (Lam & Ng, 2002).

Besides, some ribosome-inactivating proteins (RIPs), such as pokeweed antiviral protein (PAP) from *Phytolacca americana*, trichosanthin from *Trichosanthes kirilowii*, MAP30 and MRK29 from *Momordica charantia*, as well as GAP31 from *Gelonium multiflorum*, are able to inhibit different viruses (Chattopadhyay & Naik, 2007). PAP can inhibit human cytomegalovirus (HCMV), HSV, HIV and influenza virus by specific depurination of both adenines and

guanines from viral and ribosomal RNA, and it also causes damage to the viral nucleic acid (Parikh & Tumer, 2004). Trichosanthin is able to inhibit HIV with other mechanism independent of ribosome inactivation. It also possesses anti-HSV activity, and this activity is substantially potentiated when it is used together with acyclovir or interferon (Zheng *et al.*, 2001). MAP30 and GAP31 inhibit the action of HIV-1 integrase, and also inhibit supercoil of HIV-1 DNA (Lee-Huang *et al.*, 1995). The two RIPs also inhibit HSV (Bourinbaiar & Lee-Huang, 1996). Furthermore, crude MRK29 causes 50 % inhibition on HIV-1 reverse transcriptase, and partially purified MRK29 is able to cause 82 % inhibition of HIV-1 p24 production in infected cells (Jiratchariyakul *et al.*, 2001).

Another group of antiviral proteins is plant lectins. Lectins are carbohydrate-binding proteins of non-immune origin that specifically bind to different carbohydrates without biochemical modification of their substrates. They are found in microorganisms, plants and animals. Among them, plant lectins have been detected in more than a thousand species of plants and several hundreds have been isolated. They are different in molecular structures and biochemical properties, and have been found in various vegetative tissues, and their levels in these tissues are variable and exhibit seasonal changes (Peumans & Van Damme, 1995). Some of the plant lectins and their carbohydrate specificities are listed in Table 4.1.

Table 4.1 Examples of plant lectins.

Plant	Lectin abbreviation	Carbohydrate specificity
Orchidaceae		
Listera ovata	LOA	α(1,3)Man
Epipactis helleborine	EHA	Man
Cymbidium hybrid	CHA	Man
Amaryllidaceae		
Galanthus nivalis	GNA	α(1,3)Man
Hippeastrum hybrid	HHA	α(1,3)-α(1,6)Man
Narcissus pseudonarcissus	NPA	α(1,6)Man
Alliaceae		
Allium porrum	APA	Man
Allium ursinum	AUA	Man
Pandanaceae		
Pandanus amaryllifolius	Pandanin	Man
Moraceae		
Artocarpus integrifolia	Jacalin	Galα(1,6)/Galβ(1,3)GalNAc
Fabaceae		
Canavalia ensiformis	ConA	Man>Glc>GlcNAc
Pisum sativum	PSA	Man>Glc/GlcNAc
Lens culinaris	LCA	Man>Glc>GlcNAc
Vicia faba	VFA	Man>Glc/GlcNAc
Lathyrus odoratus	-	Man>Glc>GlcNAc
Urticaceae		
Urtica dioica	UDA	GlcNAc oligomers
Cecropiaceae		
Myrianthus holstii	MHA	GlcNAc
Euphorbiaceae		
Hevea brasiliensis	HBA	GlcNAc

Gal: galactose; **GalNAc**: N-acetylgalactosamine; **Glc:** glucose; **GlcNAc:** N-acetylglucosamine; **Man:** mannose. The > symbol indicates a higher preference. (Adapted from Balzarini, 2007a).

Plant lectins play important biological roles both within and outside the plant. In leguminous plants, lectin may involve in symbiosis between nitrogen-fixing bacteria and the plants. In many other plants, lectins are usually associated with storage tissues such as seeds, tubers, bulbs, corms, rhizomes, root stocks and bark. Many of these lectins have been shown to also exhibit behaviour similar to other storage proteins. Their biosynthesis, subcellular localization, and developmental regulation are similar to other storage proteins. During seed germination or depletion of vegetative tissues during growth, lectins are degraded and appear to be important sources of nitrogen for the development process (Komath *et al.*, 2006).

Besides, a majority of plant lectins are involved in defense. Plants have to rely on constitutive or inducible accumulation of toxic low molecular weight secondary metabolites or proteins as defense strategies. These toxins may possess broad spectrum or specific activities. Lectins are capable to recognize and specifically bind to glycoconjugates present on the external surface of microorganisms, such as chitin in the fungal cell wall, or on the luminal surface of the gastrointestinal tract of plant predators like insects and animals. Some extremely cytotoxic proteins such as ricin, abrin and modecin are non-selective toxin for all eukaryotic organisms. These toxins inactivate all eukaryotic ribosomes by cleavage of the *N*-glycosidic bond of the 28S rRNA adenosine residue. A lectin of *Urtica*

dioica has potent antifungal properties (Broekaert *et al.*, 1989). It appears to affect the synthesis of the fungal cell wall (Van Parijs *et al.*, 1992). Some plant lectins have anti-insect properties, for example, the mannose-binding snowdrop (*Galanthus nivalis*) lectin is toxic for some chewing and sucking insects. It is not only active when included in an artificial diet for the insects but also when the insects were fed with transgenic plants expressing the lectin gene (Hogervorst *et al.*, 2006). The antinutritional effect of toxic lectins is not limited to insects, but also to animals and human. Oral acute toxicity of lectins on human includes nausea, bloating, vomiting and diarrhea. Experimental animals that fed on diet containing these lectins resulted in loss of appetite, decreased body weight and eventually death. Toxic plant lectins can affect gut epithelial cell turnover, damage the luminal membranes of the epithelium, interfere with nutrient adsorption and digestion, stimulate bacterial flora shifts and modulate the immune state of the digestive tract (Vasconcelos & Oliveira, 2004). Consequently, seeds and vegetative tissues that contain such antinutritive lectins are unpalatable and will be avoided, and this is beneficial for the plant species.

Lectins have been applied in different aspects of bioscience and biomedicine. They are used in detection, isolation and structural studies of glycoprotiens. They are also employed for studying membrane structures and the

changes of glycoconjugates during cell differentiation, growth and development. Moreover, lectins are useful in mapping neuronal pathways, cell identification and separation, as well as identification of microorganisms. Clinical uses of lectins include blood typing, preparation of chromosome maps, used as mitogens for testing of immune state of patients, and purging human bone marrow for transplantation (Lis & Sharon, 1998). In recent years, lectins have been tested for their toxicities against different cancer cells (Valentiner *et al.,* 2003; Heinrich *et al.,* 2005). Mistletoe lectins, for example, inhibit acute lymphoblastic leukemia both *in vitro* and *in vivo* (Seifert *et al.,* 2008). Some lectins have been used as carriers that are conjugated with chemotherapy drugs. The specificity of the lectins helps delivering the drugs to target sites (Bies *et al.,* 2004).

Many lectins possess antiviral properties against human enveloped viruses. Glycans on the viral envelope often have a crucial role in the entry of virus into the host cell and/or efficient transmission. Antiviral lectins can bind to the glycans of the viral envelope glycoproteins, thereby inhibiting the normal functions of the glycans. The antiviral activities of various lectins have been demonstrated mainly in HIV researches, which were based on different HIV strains and target cell types (Balzarini, 2007b). Lectins activities against HCMV, HCV, influenza virus, HSV, poxvirus, RSV and SARS-CoV were also published (Ooi *et al.,* 2004; Bertaux *et al.,*

2007; Favacho *et al.*, 2007; Kaur *et al.*, 2007; Keyaerts *et al.*, 2007; Luo *et al.*, 2007).

Plant proteins or lectins are indeed potential sources of novel anti-RSV agents. In this study, some *Narcissus tazetta* proteins were assessed for their anti-RSV activity. *N. tazetta* is a monocotyledonous perennial ornamental plant of the family Amaryllidaceae. The medicinal plant is used externally for parotitis, mastitis, boils and abscesses (Ooi *et al.*, 1998). Active substances, including pseudolycorine, lycorine, and tazettine, are present in the extract of *N. tazetta* var. *chinensis* (Liu *et al.*, 2006). In our laboratory, mannose-binding lectins have been isolated from leaves and bulbs of the plant (Ooi *et al.*, 1998; Ooi *et al.*, 2000). In the present study, anti-RSV activity of the *Narcissus tazetta* lectin (NTL) previously purified from *N. tazetta* var. *chinensis* as well as proteins isolated from another cultivar of *N. tazetta* were examined.

4.2 Materials and Methods

4.2.1 Crude proteins extraction from *Narcissus tazetta* cultivar

Bulbs of the *N. tazetta* cultivar were bought from mainland China. After cleaning and removal of roots and dried scale leaves, the bulbs were cut into small pieces and homogenized in 0.2 M NaCl. The homogenate was stored in 4 ^{o}C overnight, which was then centrifuged at 22,095 *g* for 30 minutes to remove debris. Ammonium sulfate at 356 g/L were added to the supernatant to a 80 % saturation. The precipitated proteins were collected after centrifugation and dialyzed extensively against distilled water to remove the salts. Crude protein powder was obtained after freeze-drying of the dialyzed proteins.

4.2.2 Separation of proteins with affinity column

The crude protein powder was dissolved in 20 mM MES buffer of pH 6.2 and fractionated on a D-mannose-agarose (Sigma) column. Unadsorbed proteins (the M1 fraction) were eluted with MES buffer, and adsorbed proteins were desorbed with MES buffer containing 0.2 M mannose to give the M2 fraction. The M3 fraction was obtained after washing the column with MES buffer containing 0.5 M NaCl. The fractions were desalted and mannose was removed by dialysis and the proteins were lyophilized.

4.2.3 Gel filtration of protein fractions on Superose column

Proteins with different molecular mass in the M2 and M3 fractions were separated by the Superose 12 HR 10/300 column on the fast protein liquid chromatography (FPLC) system (Pharmacia). The column was first calibrated with different molecular weight standards including bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.5 kDa), aprotinin (6.5 kDa), and cytidine (246 Da). The M2 or M3 fractions were dissolved in 0.1 M ammonium bicarbonate buffer, pH 8.0 and injected into the FPLC system. Proteins were eluted with the same buffer at flow rate of 0.4 ml/min and collected at 0.4 or 0.8 ml/tube, which were then desalted with PD-10 desalting columns (GE Healthcare) and lyophilized.

4.2.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profiles of crude protein extract and various fractions were studied by SDS-PAGE. Proteins were mixed with loading dye (1:1) with 2 % sodium dodecyl sulfate (SDS) and 2 % β-mercaptoethanol and denatured for 5 minutes at 95 ^{o}C. A low range SigmaMarker^{TM} (Sigma) and the proteins were then resolved using 15 % SDS-polyacrylamide gels. After electrophoresis the gels were stained with Coomassie Brilliant Blue R-250 for 1 h and subsequently destained with 10 % acetic acid and 40 % methanol.

4.2.5 Electroblotting and N-terminal amino acid sequence analysis

After SDS-PAGE, the proteins on the gel were transferred onto polyvinylidene difluoride (PVDF) membrane using Dunn's buffer containing sodium carbonate, sodium hydrogencarbonate and SDS. The membrane was then stained with Coomassie Brilliant Blue R-250 until blue bands were observed, and then destained with methanol. The membrane was air-dried and the desire bands were cut out for N-terminal amino acid sequence determination using an HP G1000A Edman degradation unit and HP-1000 HPLC system. The amino acid sequence analysis was done by Ms. Jessie Lee of the Department of Biology, The Chinese University of Hong Kong.

4.2.6 Protein concentration determination

Concentrations of proteins were determined by the standard bicinchoninic acid assay (BCA) kit (Pierce). A standard curve was prepared using 50 μl of 0, 25, 50, 100, 200 and 400 μg/ml bovine serum albumin, and 50 μl of protein samples were used for the test. 1ml BCA working reagent, containing 50 parts of reagent A and 1 part of reagent B (provided), was then added to each tube and incubated for 30 minutes at 37 ^{o}C. After incubation, all the tubes were cooled to room temperature. Absorbance was measured at 562 nm using a BioPhotometer (Eppendorf). Protein concentrations of the samples were determined from the standard curve.

4.2.7 Isolation and purification of *N. tazetta* lectin (NTL)

NTL was isolated as described in a previous study (Ooi *et al.*, 2000) and kindly provided by Dr. Linda Ooi. In brief, after homogenization of the bulbs of *N. tazetta* var. *chinensis* in 0.2 M NaCl and precipitation with ammonium sulfate at 30 – 80 % saturation, crude proteins were subjected sequentially to diethylamino ethanol-cellulose (Sigma) affinity column, mannose-agarose (Sigma) column, and Superose FPLC column (Pharmacia). Purified NTL (the DEAE P2M2GF fraction described in Ooi *et al.*, 2000) was obtained after desalting the proteins with PD-10 and analyzed by SDS-PAGE, and the N-terminal amino acid sequence was also determined. Haemagglutination activity of NTL was examined by mixing a serial two-fold dilution of the lectin with a 2 % suspension of rabbit erythrocytes in PBS. Haemagglutination inhibition tests were performed by mixing different sugar samples with NTL before adding rabbit erythrocyte suspension and the minimal concentration of the sugar that completely inhibited haemagglutination was calculated. The presence of carbohydrates in the lectin was tested by Fuchsin-sulfite staining after electrophoresis and electroblotting. The molecular mass of the purified NTL was determined to be about 26 kDa by gel filtration and 13 kDa by SDS-PAGE, and the NTL is therefore a mannose-binding, non-glycoprotein dimer with a molecular subunit of 13 kDa.

4.2.8 Antiviral activities of *N. tazetta* proteins and NTL

4.2.8.1 Cell and virus

The method of HEp-2 cells and RSV culture were described in Section 2.2.3.

4.2.8.2 Cytotoxicity test

Cytotoxicities of the proteins on growing HEp-2 cells were determined by MTT assay. Different proteins were dissolved in Milli-Q water and filtered through 0.22 μm syringe filters to make the stocks of 200 μg/ml and detail procedures were described in Section 2.2.5.

4.2.8.3 Endpoint titration of RSV by $TCID_{50}$ method

RSV titre was scored by $TCID_{50}$ determination as described in Section 2.2.4.

4.2.8.4 Antiviral study by CPE reduction assay

RSV at 100 $TCID_{50}$ and serially two-fold diluted proteins were added to HEp-2 cells and CPE were observed daily. Detail procedures were described in Section 2.2.6.

4.2.8.5 Endpoint titration of RSV by plaque assay

Detail procedures were described in Section 3.2.6.

4.2.8.6 Antiviral study by plaque reduction assay

RSV plaque reduction was used to determine the IC_{50} of NTL. Detail procedures were described in Section 3.2.7.

4.2.8.7 Mode of antiviral action study

NTL at a final concentration of 10 μg/ml was used for the assay. Detail method was described in Section 3.2.8.

4.3 Results

An amount of 1.66 g of crude protein was extracted from 1.25 kg of *N. tazetta* cultivar bulbs (Figure 4.1 C & D). The crude protein was first fractionated with mannose-agarose column (Figure 4.2), and adsorbed proteins were eluted with buffer that contain 0.2 M mannose (the M2 fraction, 9.92 mg) and subsequently with 0.5 M NaCl (the M3 fraction, 14.36 mg).

The two fractions were then subjected to FPLC, and the Superose 12 FPLC column was calibrated with various molecular weight standards to estimate their retention time (Table 4.2) and to generate a standard curve (Figure 4.3). For the M2 fraction, its FPLC fractions eluted at about 37.36 minute (an average of 4 experiments) were collected, pooled and designated as NTM2 (Figure 4.4), which has a molecular weight of 15.2 kDa as calculated with the FPLC standard curve. SDS-PAGE of NTM2 revealed a prominent band near the 14.2 kDa marker (Figure 4.7). For the M3 fraction, FPLC fractions eluted at about 40.38 minute (an average of 4 experiments) were pooled and designated as NTM3 (Figure 4.5), which is 6.7 kDa as calculated with the FPLC standard curve and showed a prominent band near the 6.5 kDa marker after SDS-PAGE (Figure 4.7). The extraction scheme of the proteins was showed in Figure 4.6.

The N-terminal amino acid sequence of NTM2 and NTM3 were analyzed

(Table 4.3). As there were not enough proteins prepared and blotted on membrane, only the first 20 and 5 amino acid residues of NTM2 and NTM3 were determined respectively. The first 20 N-terminal amino acid sequence of NTM2 shared a 45 % homology with that of NTL (Table 4.3), which was isolated from *N. tazetta* var. *chinensis* (Figure 4.1 A & B). NTL is a mannose-binding, non-glycoprotein dimer with a molecular subunit of 13 kDa (Ooi *et al.*, 2000). SDS-PAGE analysis of NTL revealed a protein band below the 14.2 kDa marker (Figure 4.7).

The proteins were tested for their anti-RSV activity by CPE assay and their cytotoxicity were tested by MTT assay (Table 4.4). NTM2 and NTM3 showed mild anti-RSV activity (IC_{50} value of about 25 and 50 μg/ml, respectively) and were relatively cytotoxic to HEp-2 cells (CC_{50} value of about 76.9 and 67.8 μg/ml, respectively). On the other hand, NTL inhibited RSV plaque formation (IC_{50} = 2.30μg/ml) and exhibited low cytotoxicity (CC_{50} = 325 μg/ml), and has a SI value 141 (Figure 4.8 & Table 4.5). Therefore, its mode of action was studied. Significant reductions on plaque formation were observed when the NTL was present during the whole infection cycle. Also, NTL was able to reduce plaque formation when it was either only present during the early infection phase or added during the later infection phase (Figure 4.9).

Table 4.2 Analysis of molecular weight standards by FPLC.

Molecular weight standard	MW (kDa)	Retention time (min)	Relative retention time
Bovine serum albumin	66.0	30.2	0.586
Ovalbumin	45.0	33.2	0.643
Carbonic anhydrase	29.0	35.8	0.693
Cytochrome *c*	12.5	38.6	0.747
Aprotinin	6.50	42.2	0.818
Cytidine	0.246	51.6	1.00

Table 4.3 Amino acid residues at the N-terminus of NTM2, NTM3 and NTL.

Protein	N-terminal sequence
NTM2	GNILTSGQSLLTTQSLLYYY.....
NTM3	GNNIH.....
NTL	DNILYSGETLYSGQFLNYGDYRFIMQADDNIPLYD.....

The N-terminal sequence of NTL was determined by Ooi *et al.*, 2000. Identical amino acid residues were underlined. "....." denoted that the amino acid sequence was not analyzed further.

Table 4.4 CPE reduction and cytotoxicity of NTM2, NTM3 and NTL.

Protein	Test concentration nearest to IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI[a]
NTM2	25.0	76.9	3.08
NTM3	50.0	67.8	1.36
NTL	3.13	325	104

IC_{50}: 50 % inhibition concentration; concentration that reduced 50 % of cytopathic effect in respect to virus control.

[a]: Selectivity index calculated based on the ratio of CC_{50} to the test concentration nearest to IC_{50}.

Table 4.5 IC_{50}, CC_{50} and SI values of NTL as determined by plaque reduction assay.

IC_{50}[#] (μg/ml)	CC_{50} (μg/ml)	SI
2.30	325	141

1) #: IC_{50} - concentration that leads to half maximal inhibition on number of plaques compared to virus control
2) SI: selectivity index (CC_{50}/IC_{50}).
3) CC_{50} and IC_{50} were calculated from data obtained from three independent experiments with the generation of dose-response curves by GraphPad Prism 4.0 software.

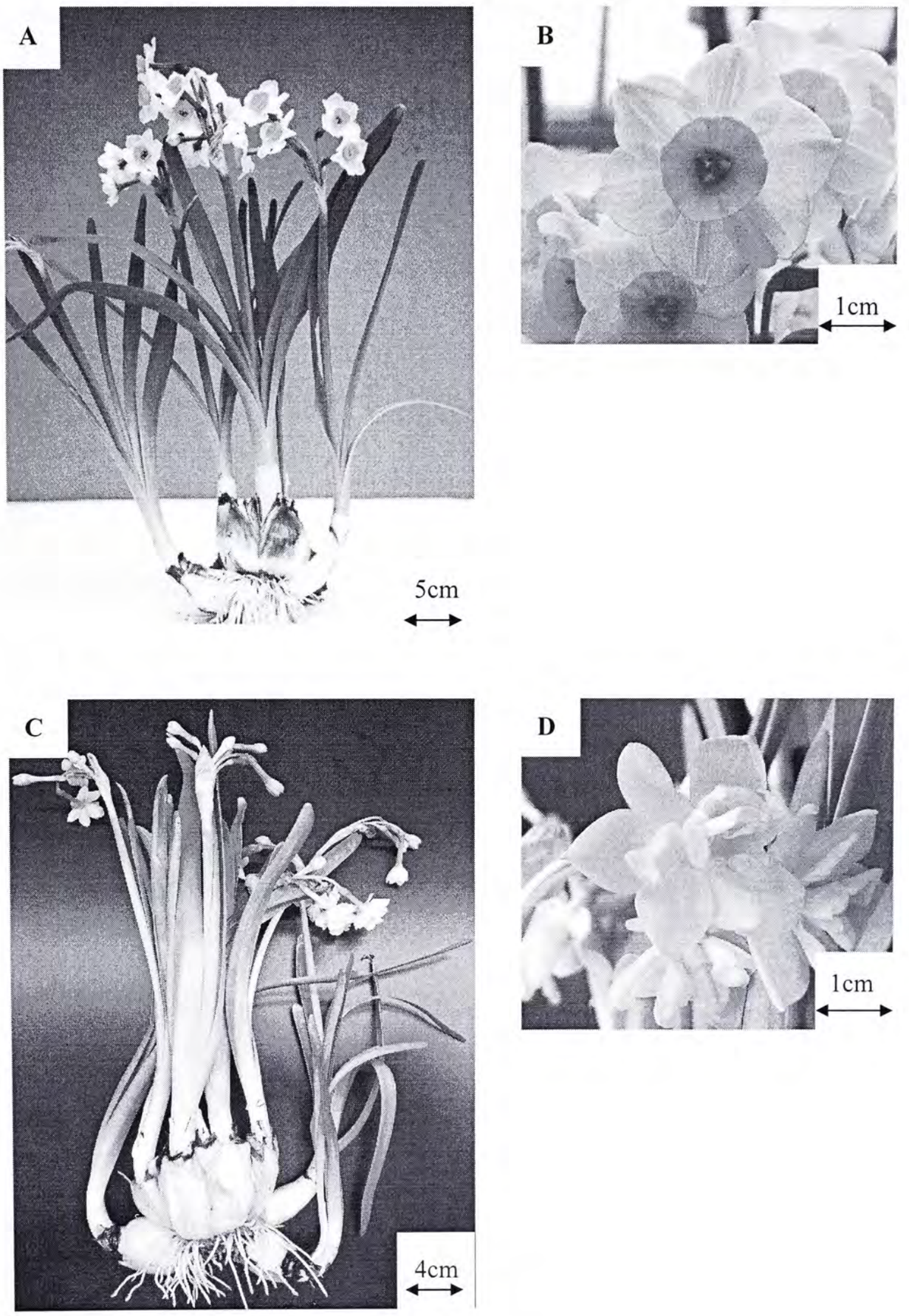


Figure 4.1 ***Narcissus tazetta.***
A) Whole plant & B) flowers of *N. tazetta* var. *chinensis*. C) Whole plant & D) flowers of *N. tazetta* cultivar.

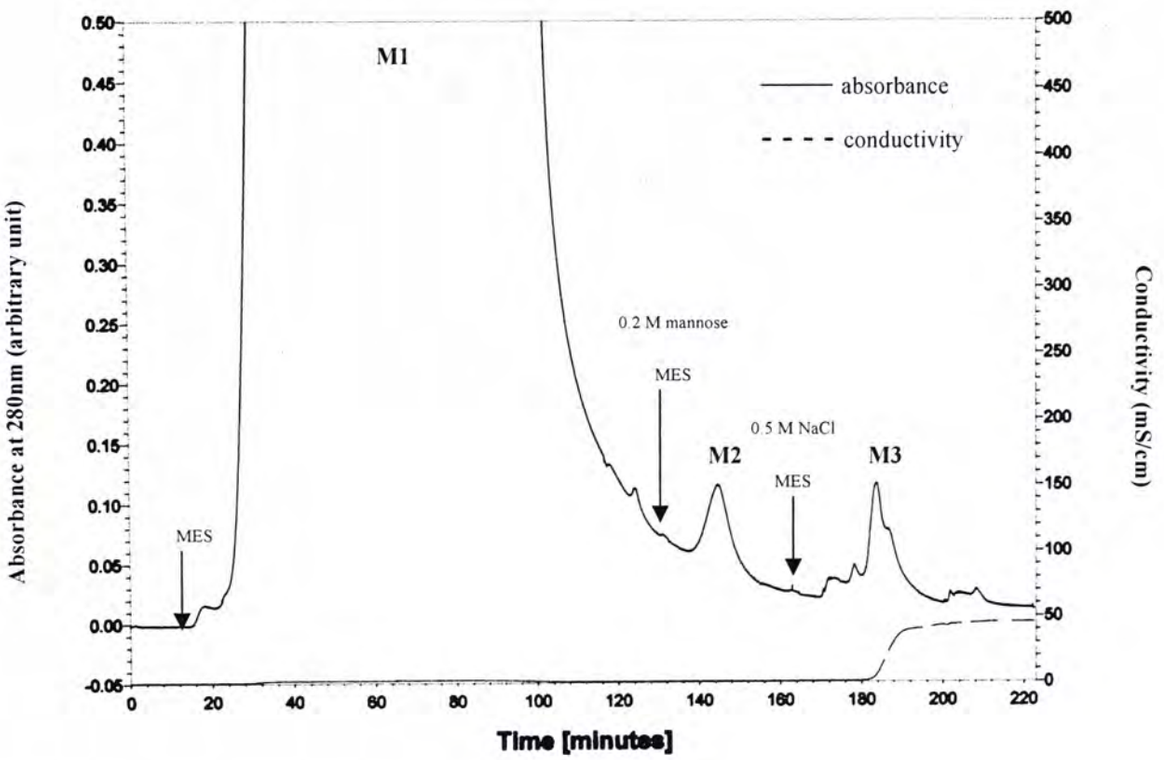


Figure 4.2 Affinity chromatography of *N. tazetta* cultivar crude protein extract on mannose-agarose column.

The mannose-agarose column was equilibrated with MES buffer (20mM, pH 6.2). The unadsorbed material (M1) was eluted with the same buffer, and the adsorbed proteins were eluted with 0.2M mannose in MES (M2) or 0.5M NaCl in MES (M3). The arrows indicate the time at which buffer was changed.

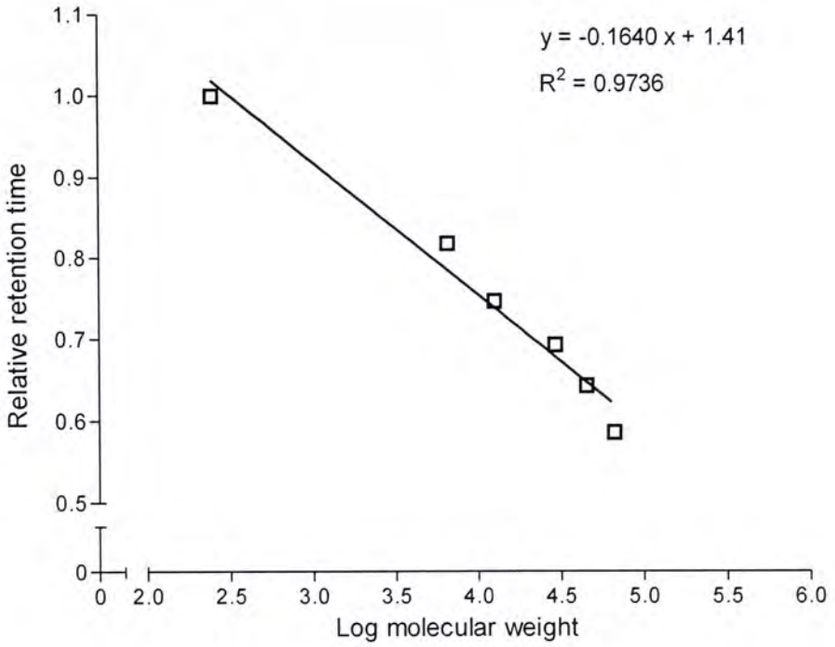


Figure 4.3 Analysis of molecular weight standards by Superose 12 FPLC column.

Superose 12 HR 10/300 column was equilibrated with 0.1M NH_4HCO_3 buffer, and calibrated with bovine serum albumin, ovalbumin, carbonic anhydrase, cytochrome *c*, aprotinin and cytidine. Relative retention time is the ratio of retention time of different standards to that of cytidine.

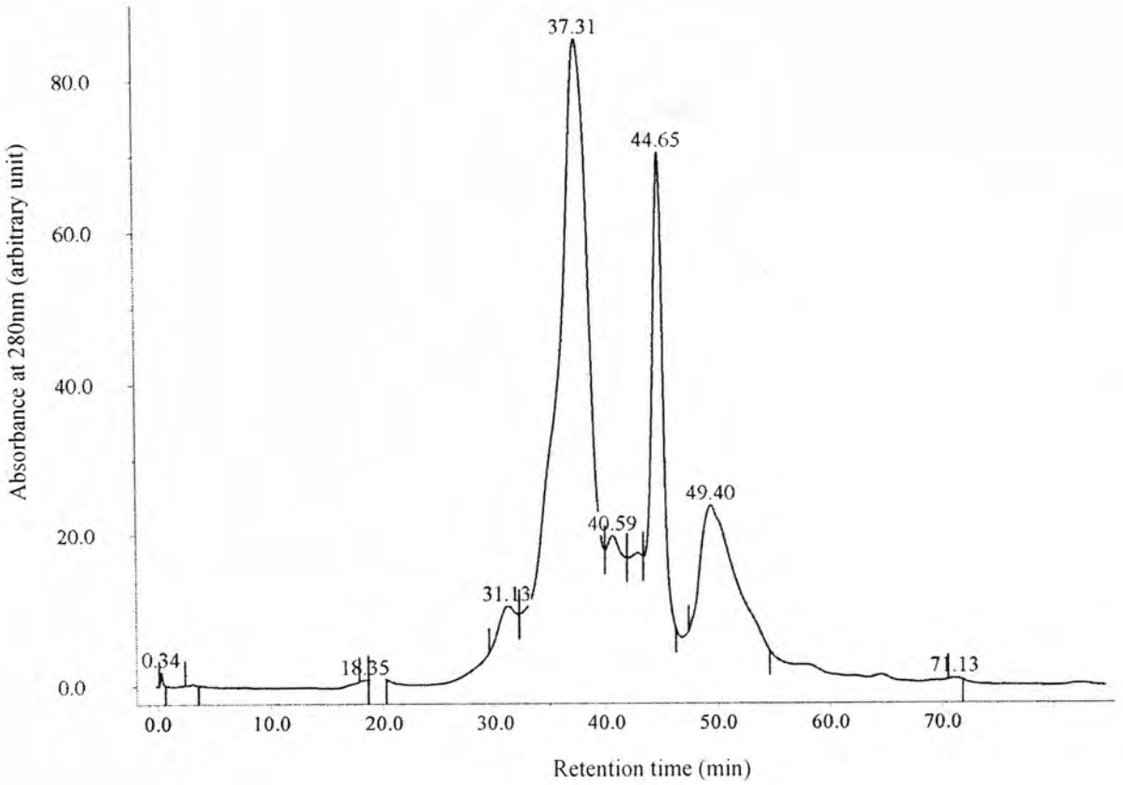


Figure 4.4 Gel filtration of the M2 fraction of *N. tazetta* cultivar by FPLC.
The M2 fraction was dissolved in 0.1M NH_4HCO_3 and eluted with the same buffer at a flow rate 0.4 ml/min. Fractions were collected and analyzed by SDS-PAGE and the fractions eluted around the first prominent peak (37.31 min) were pooled and designated as NTM2.

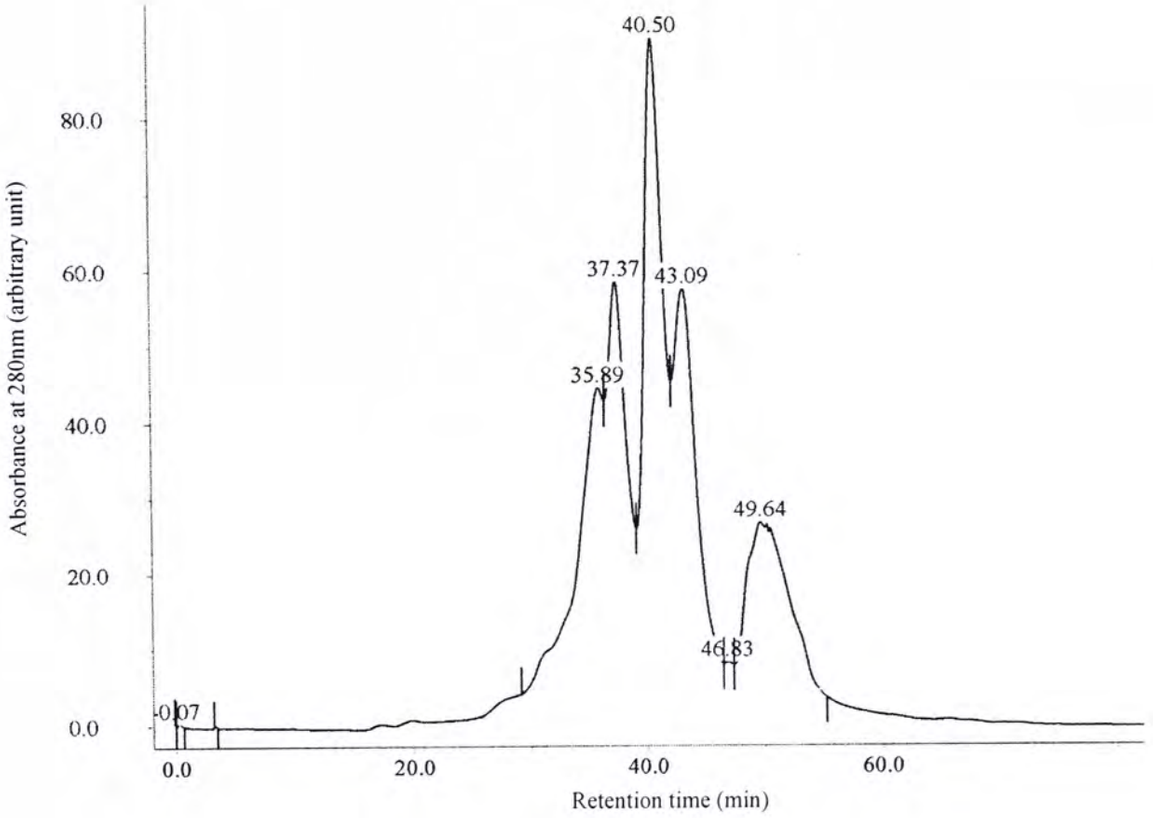


Figure 4.5 Gel filtration of the M3 fraction of *N. tazetta* cultivar by FPLC.
The M3 fraction was dissolved in 0.1M NH_4HCO_3 and eluted with the same buffer at a flow rate 0.4 ml/min. Fractions were collected and analyzed by SDS-PAGE and the fractions eluted around the highest peak (40.5 min) were pooled and designated as NTM3.

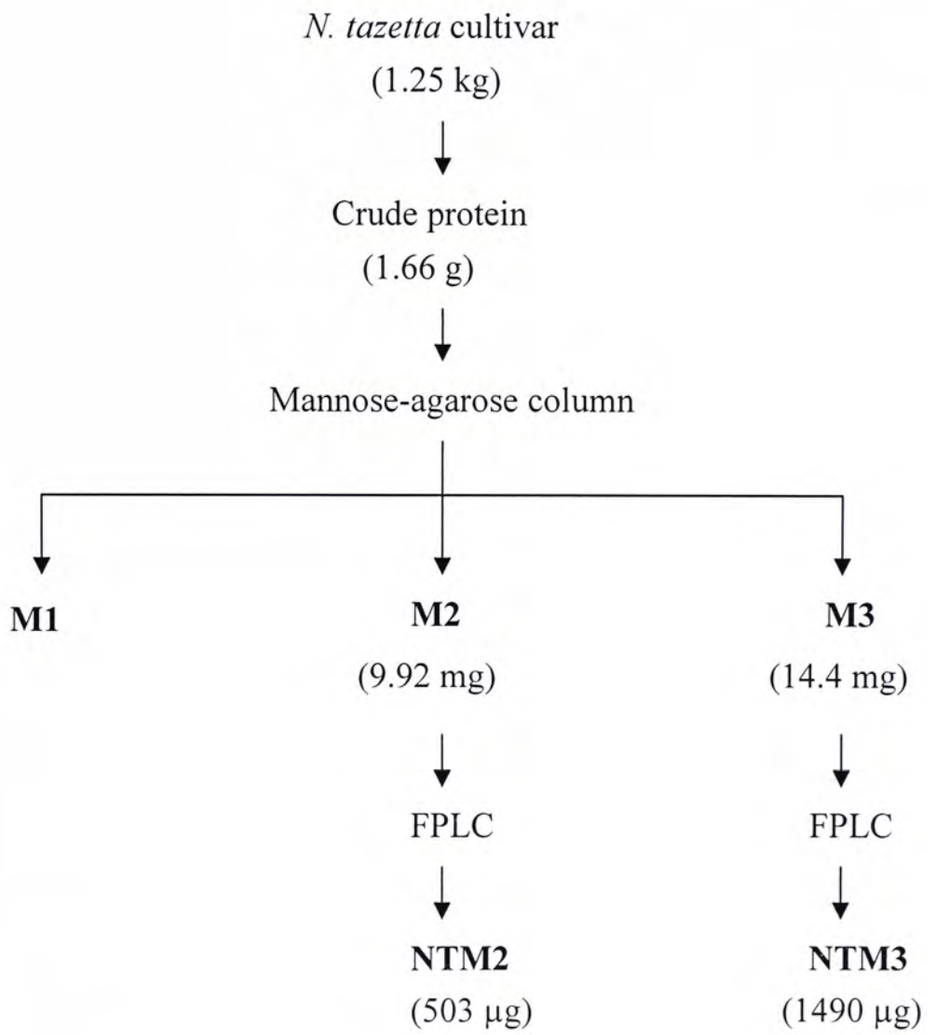


Figure 4.6 Isolation scheme of *N. tazetta* cultivar protein.

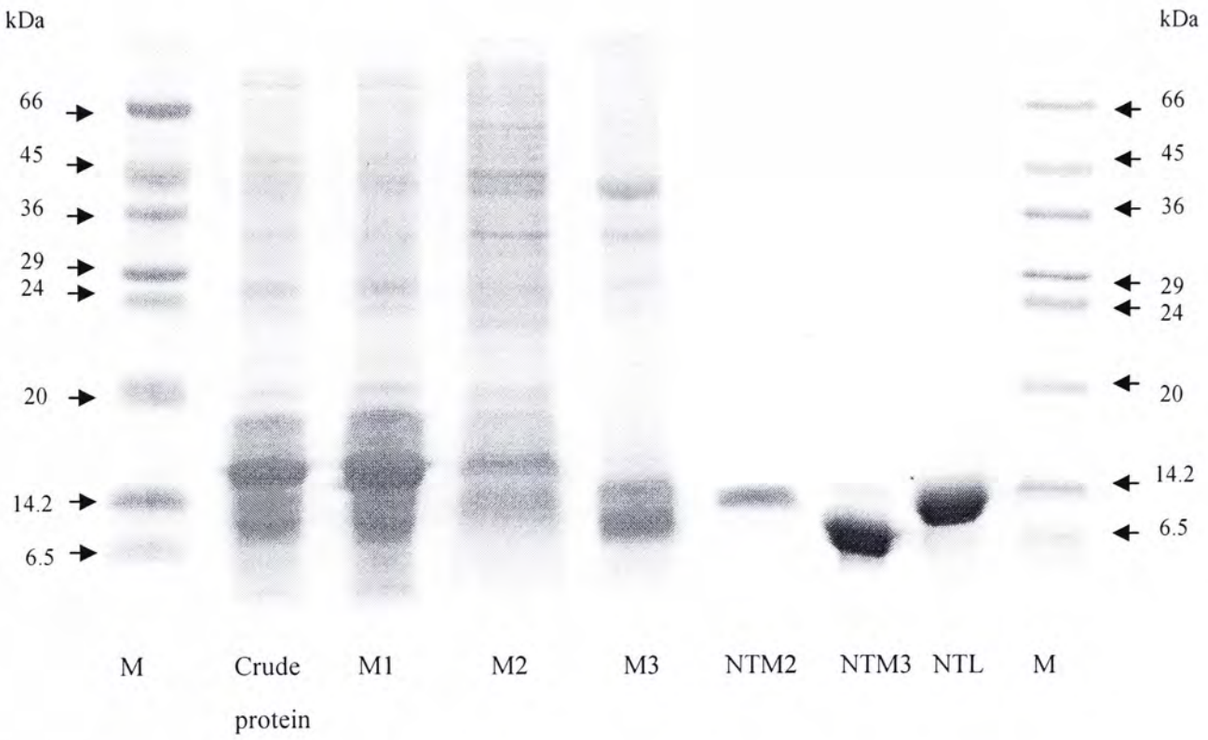


Figure 4.7 SDS-PAGE of NTL and *N. tazetta* cultivar crude and fractionated proteins.

Proteins were separated on 15 % gel and stained by Coomassie Brilliant Blue R-250. M: Low range SigmaMarker^{TM}.

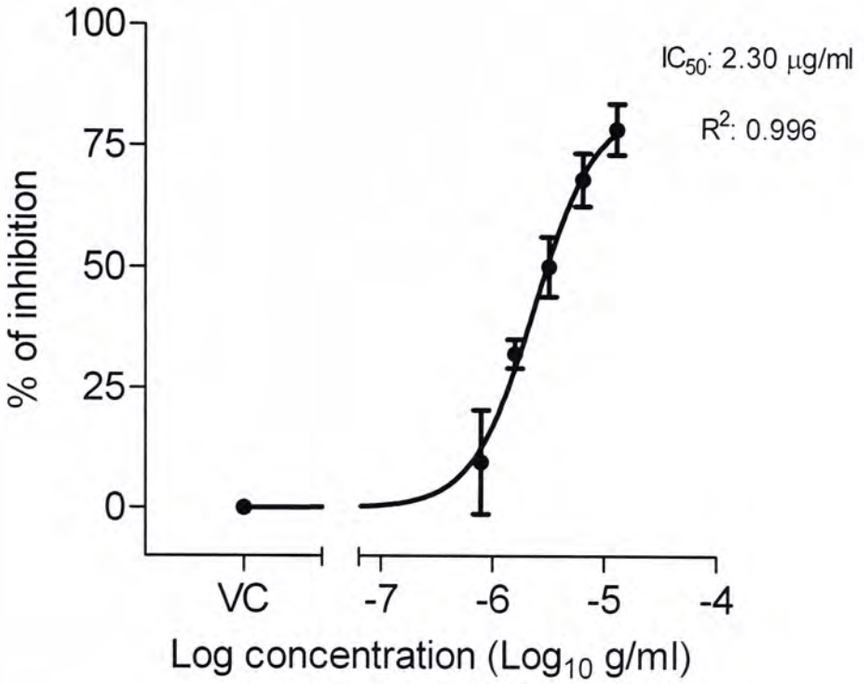


Figure 4.8 Inhibition of RSV plaque formation by NTL.

Percentage of RSV-plaque inhibition after addition of different concentrations of NTL to infected HEp-2 cells were calculated and dose-response curve was generated by GraphPad Prism 4.0 software. Data were obtained from three independent experiments and expressed as mean ± S.D.

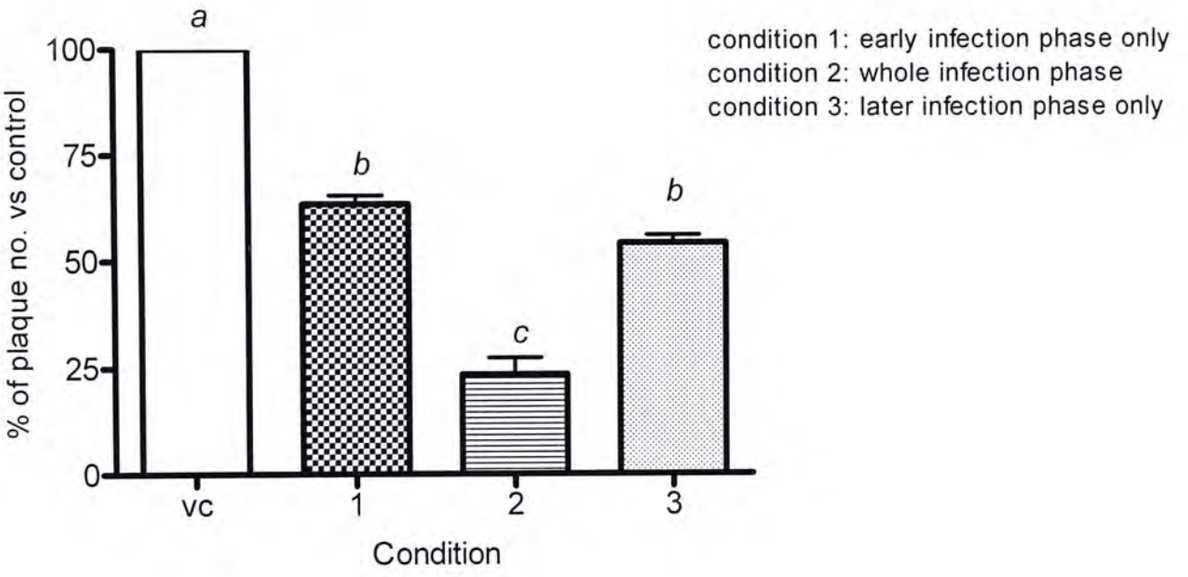


Figure 4.9 Mode of anti-RSV action of NTL by plaque reduction assay.
HEp-2 cells were infected by RSV at about 80 pfu and 10 μg/ml NTL were added during the early infection phase only (2 h post-infection only), during the whole infection phase, or during later infection phase only (after 2 h post-infection). The percentage of number of plaques formed under different conditions compared to the virus control (VC) were calculated and analyzed by one-way ANOVA followed by Tukey's multiple comparison post-test, $P<0.001$. The bars marked by the same letter have no significant difference. Results were expressed as mean ± S.D. obtained from three independent experiments with duplicate wells for each condition.

4.4 Discussion

Protein is considered as one of the major antiviral phytocompounds. Antiviral protein researches mainly focus on two major groups of proteins — RIPs and lectins. For lectins, interest has been mainly focused on anti-HIV activities and many monocot mannose-specific lectins, such as *Cymbidium* agglutinin (CA) from *Cymbidium* hybrid, *Listera ovata* agglutinin (LOA) from *L. ovata* and *Epipactis helleborine* agglutinin (EHA) from *E. helleborine*, have been proven to possess *in vitro* antiviral activities against HIV as well as other enveloped viruses (Balzarini *et al.*, 1992). A mannose-binding lectin *Narcissus pseudonarcissus* agglutinin (NPA) from *N. pseudonarcissus* has been isolated (Kaku *et al.*, 1990) and shows marked *in vitro* inhibition to HIV and CMV (Balzarini *et al.*, 1991). Therefore, it is possible that some other monocot lectins or proteins possess antiviral, including anti-RSV, activity.

In this study, the proteins from the daffodils, including *N. tazetta* cultivar and *N. tazetta* var. *chinensis*, were examined for their anti-RSV activity. From the bulbs of *N. tazetta* cultivar, two mannose-binding proteins NTM2 and NTM3 were isolated after affinity and size-exclusion chromatography. The two proteins have different molecular mass as analyzed by SDS-PAGE. The first 20 N-terminal amino acid sequence of NTM2 shared a 45 % homology with that of NTL from *N. tazetta*

var. *chinensis* as analyzed by N-terminal amino acid sequencing. Both NTM2 and NTM3 are mannose-binding proteins, and whether they are lectins remain to be elucidated by testing their carbohydrate specificity, haemagglutinating activity, and study of their complete amino acid sequences. When tested for their ability on RSV-induced CPE inhibition, NTM2 and NTM3 only showed mild inhibitory effect. Besides, they were rather cytotoxic to HEp-2 cells. Therefore, they were not considered as potent anti-RSV agents.

NTL from the bulbs of *N. tazetta* var. *chinensis* has been isolated, characterized and showed inhibition on bovine immunodeficiency virus (Ooi *et al.*, 2000). In the present study, NTL inhibited RSV-induced plaque formation (IC_{50} = 2.30 μg/ml), and its cytotoxicity to HEp-2 cells was not high (CC_{50} = 325 μg/ml). Its high SI value of 141 indicated that it possesses potent anti-RSV activity. Further investigation on its mode of action revealed that significant reduction on number of plaques was observed when the lectin was either only present during the early infection phase or only present in the later infection phase, although the extent of inhibition under these two conditions were not as much as when the lectin was present during the whole infection cycle. As the early infection events were inhibited, NTL may be virucidal or, like some other antiviral lectins, interact with glycans on glycoproteins present on RSV envelope that are crucial for attachment or fusion and

prevented their normal functions. It has been showed that *Urtica dioica* agglutinin (UDA) from *U. dioica* strongly binds to the glycans on the HIV-1 attachment protein gp120, and prevents HIV entry and transmission when being co-cultivated with persistently HIV-infected cells and uninfected cells in the lower μg/ml range (Balzarini, 2006). Some other lectins were also able to inhibit viral entry. *Galanthus nivalis* agglutinin (GNA), HHA, CA and UDA were found able to inhibit entry of HCV and HIV-1 to their target cells (Bertaux *et al.,* 2007). *Hippeastrum* hybrid agglutinin (HHA) from *Hippeastrum* hybrid inhibits SARS-CoV replication when added at the moment of infection, and the lectin also able to inhibit extracellular viral load when added up to 5 hours post-infection (a time near the end of the virus replication cycle), thus HHA interacts both at virus entry and at virus release (Keyaerts *et al.,* 2007). To inhibit RSV infection, the possible targets that NTL can bind are the glycoproteins present on RSV envelope. Three envelope glycoproteins are present on RSV, including the G protein, the F protein and the SH protein. The G protein of RSV Long strain contains 8 *N*-glycosylation sites and up to 70 potential *O*-glycosylation acceptor sites (Rawling & Melero, 2007). The F protein contains six potential *N*-glycosylation sites (Zimmer *et al.,* 2001). The SH protein contains polylactosaminoglycan (Feldman *et al.,* 2001). The properties of viral glycoproteins are heavily influenced by the nature, position, and extent of glycosylation. Both the

G and F proteins may be involved in the initial attachment of RSV to host cell, and the F protein is largely responsible for the subsequent fusion process, though highest level of fusion was observed when all three envelope glycoproteins are present (Feldman *et al.*, 2001). Besides, although the cellular receptor for RSV still remains to be identified, interactions between G or F protein with cellular glycosaminoglycans (GAGs), including heparan sulfate, have been shown to be important for virus infectivity (Feldman *et al.*, 2000; Hallak *et al.*, 2000). If NTL binds to G or F protein, the interaction of the two glycoproteins with GAGs may be interfered and the attachment/fusion process may be hindered. This may explain the observed significant reduction on plaque formation when NTL was added during the infection phase only. Moreover, RSV glycoproteins expressed on infected cells at a later stage of the viral replication cycle may interact with GAGs expressed on neighboring uninfected cells, which may be responsible for the cell-to-cell fusion and syncytium formation (Feldman *et al.*, 2001). This interaction may be blocked if the RSV glycoproteins were bound by NTL, and the spreading of RSV would be affected. In the present study, NTL was also found to reduce plaque formation when added only in the later infection phase. Whether NTL interfered with RSV spreading by binding to viral glycoproteins or inhibited other events during the viral replication or assembly processes remains to be elucidated.

Chapter 5

General Discussion and Conclusions

Respiratory syncytial virus infection remains a serious global health concern. Presently there is no licensed vaccine or effective drug available for the disease. The search for new therapy to the disease is urgently needed. Besides synthesis of novel antiviral compounds, natural products also serve as a major source of anti-RSV agents. In order to identify more naturally occurring anti-RSV agents, in the first part of the present project, two purified plant compounds, seven commercially available phytochemicals, as well as the water and ethanol extracts of nine medicinal plants were screened for their anti-RSV activities by observing their inhibition on RSV-induced cytopathic effect and their cytotoxicity were examined by MTT assay. A purified compound from *W. indica*, designated as daphnoretin, a purified lectin from *Narcissus tazetta* var. *chinensis*, designated as NTL, the ethanol extract of *Adina pilulifera*, as well as the ethyl acetate fraction of *W. indica* exhibited potent anti-RSV activities. Therefore, in the second part of the present study, the ethanol extract of *A. pilulifera* was further fractionated using petroleum ether, ethyl acetate and butanol. Among them, the ethyl acetate fraction was found as

the most potent anti-RSV fraction. Besides, as daphnoretin was isolated from the ethyl acetate fraction of *W. indica*, it may partly explain the anti-RSV activity of the fraction. Thus, the IC_{50} of daphnoretin and the ethyl acetate fraction of *A. pilulifera* were further studied by plaque assay, and their SI were found to be 28.2 and 86.2, respectively, that are comparable to or higher than that of the positive control drug ribavirin (SI = 30.0). The modes of action of the samples were studied by the addition of compounds at different times during the virus infection cycle, so the samples were present during the early infection phase only (i.e. the first 2 h of infection only), during the whole infection cycle (i.e. throughout the whole experiment), or during the later infection phase only (i.e. after 2 h of infection). The antiviral activity of the *A. pilulifera* ethyl acetate fraction was found unlikely through inhibition of RSV attachment or penetration, but by inhibition of the later events of the virus replication cycle or by modulation of host cell's biological events. On the other hand, daphnoretin may be able to slightly inhibits viral attachment and/or fusion, and mainly inhibits later viral development and spreading. Daphnoretin was reported as a PKC activator (Ko *et al.*, 1993; Wang *et al.*, 1995; Chen *et al.*, 1996) that leads to activation and translocation of intracellular PKC to the plasma membrane. At the same time, activation and translocation of PKCα to cell membrane was observed upon RSV contact and facilitates fusion

(San-Juan-Vergara *et al.*, 2004). It is speculated that daphnoretin could compete with RSV for the intracellular PKC pool and affects RSV fusion.

Many plant proteins have been reported for their antiviral activities against human viruses. Lectin is considered as one of the major groups of antiviral plant proteins. Different lectins have been shown for their antiviral activities against HIV, HCMV, HCV, influenza virus, poxvirus, HSV, RSV and SARS-CoV (Ooi *et al.*, 2004; Bertaux *et al.*, 2007; Favacho *et al.*, 2007; Kaur *et al.*, 2007; Keyaerts *et al.*, 2007; Luo *et al.*, 2007), and some of these studies focused on monocot mannose-binding lectins. Thus there is a potential for finding more antiviral, especially anti-RSV, lectins from monocots. Therefore, in the third part of the study, two proteins (NTM2, 15.2 kDa and NTM3, 6.70 kDa) were isolated from *N. tazetta* cultivar by ammonium sulfate precipitation, affinity chromatography on mannose-agarose column and size exclusion chromatography on Superose 12 FPLC column. N-terminal amino acid sequence of the two proteins were analyzed and compared to that of NTL, which was found to possess anti-RSV activity in the first part of the present study. The sequence of the first 20 N-terminal amino acids of NTM2 showed a 45 % homology with that of NTL, while the sequence of NTM3 obtained was too short for comparison. Thus, NTM2 may, like NTL, belongs to the monocot mannose-binding lectin superfamily. *In vitro* antiviral study showed that

NTM2 and NTM3 only have mild anti-RSV effect and they are rather cytotoxic to HEp-2 cells. On the contrary, NTL potently inhibited RSV and has low cytotoxicity. The IC_{50} of NTL is 2.30 μg/ml and its SI is 141.36, which is much higher than that of the positive control drug ribavirin. Further investigation on its mode of action revealed that significant reduction on number of plaques was observed when NTL was either only present during the early infection phase or only present in the later infection phase. NTL may, similar to some other anti-HIV lectins, able to recognize and bind to RSV envelope glycoproteins on the virus and on host cells, thereby interfere with the normal function of the glycoproteins and affect RSV attachment, fusion, syncytium formation and spreading.

Further studies could be directed to the isolation and characterization of pure antiviral compound(s) from the *A. pilulifera* ethyl acetate fraction and more in-depth investigations on the antiviral mechanisms of the pure compound, daphnoretin, and NTL could be carried out. More than one method should be employed for both anti-RSV and cytotoxicity assays so as to confirm the results. Moreover, more *in vitro* cytotoxicity tests using a panel of both primary and continuous cell lines as well as understanding of any known toxicity or adverse effects of the compounds are needed before proceeding to *in vivo* or pre-clinical testing. Besides, as NTL is a protein, it is also important to test for the occurrence of

any unfavorable protein properties such as allergenicity, haemagglutination and unwanted interaction of the lectin with cellular glycoproteins.

In conclusion, the ethyl acetate fraction of *A. pilulifera*, daphnoretin and NTL were shown for the first time that they possess anti-RSV activity. The three candidates exhibited different modes of action. This study provided some scientific information for the future development of the three candidates as potential anti-RSV drugs. Further *in vivo* studies and in-depth investigation on their antiviral mechanisms could be implemented to provide more information on their efficacy and safety.

References

Adah SA, Bayly SF, Cramer H, Silverman RH, Torrence PF. 2001. Chemistry and biochemistry of 2',5'-oligoadenylate-based antisense strategy. *Current Medicinal Chemistry* **8**: 1189-1212.

Ahmad A, Davies J, Randall S, Skinner GRB. 1996. Antiviral properties of extract of *Opuntia streptacantha. Antiviral Research* **30**: 75-85.

Ahmadian G, Chambers P, Easton AJ. 1999. Detection and characterization of proteins encoded by the second ORF of the M2 gene of pneumoviruses. *Journal of General Virology* **80**: 2011-2016.

Alché LE, Barquero AA, Sanjuan NA, Coto CE. 2002. An antiviral principle present in a purified fraction from *Melia azedarach* L. leaf aqueous extract restrains herpes simplex virus type 1 propagation. *Phytotherapy Research* **16**: 348-352.

Bae G, Yu JR, Lee J, Chang J, Seo EK. 2007. Identification of nyasol and structurally related compounds as the active principles from *Anemarrhena asphodeloides* against respiratory syncytial virus (RSV). *Chemistry & Biodiversity* **4**: 2231-2235.

Balzarini J. 2006. Inhibition of HIV entry by carbohydrate-binding proteins. *Antiviral Research* **71**: 237-247.

Balzarini J. 2007a. Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy. *Nature Reviews Microbiology* **5**: 583-597.

Balzarini J. 2007b. Carbohydrate-binding agents: a potential future cornerstone for the chemotherapy of enveloped viruses? *Antiviral Chemistry & Chemotherapy* **18**: 1-11.

Balzarini J, Neyts J, Schols D, Hosoya M, Van Damme EJM, Peumans W, De Clercq E. 1992. The mannose-specific plant lectins from *Cymbidium* hybrid and *Epipactis helleborine* and the (N-acetylglucosamine)n-specific plant lectin from *Urtica dioica* are potent and selective inhibitors of human immunodeficiency virus and cytomegalovirus replication *in vitro*. *Antiviral Research* **18**: 191-207.

Balzarini J, Schols D, Neyts J, Van Damme EJM, Peumans W, De Clercq E. 1991. α-(1-3)- and α-(1-6)-D-mannose-specific plant lectins are markedly inhibitory to human immunodeficiency virus and cytomegalovirus infections *in vitro*. *Antimicrobial Agents and Chemotherapy* **35**: 410-416.

Barnard DL, Hill CL, Gage T, Matheson JE, Huffman JH, Sidwell RW, Otto MI, Schinazi RF. 1997. Potent inhibition of respiratory syncytial virus by polyoxometalates of several structural classes. *Antiviral Research* **34**: 27-37.

Barnard DL, Huffman JH, Meyerson LR, Sidwell RW. 1993. Mode of inhibition of respiratory syncytial virus by a plant flavonoid, SP-303. *Chemotherapy* **39**: 212-217.

Bennett CR Jr., Hamre D. 1962. Growth and serological characteristics of respiratory syncytial virus. *The Journal of Infectious Diseases* **110**: 8-16.

Bertaux C, Daelemans D, Meertens L, Cormier EG, Reinus JF, Peumans WJ, Van Damme EJM, Igarashi Y, Oki T, Schols D, Dragic T, Balzarini J. 2007. Entry of hepatitis C virus and human immunodeficiency virus is selectively inhibited by carbohydrate-binding agents but not by polyanions. *Virology* **366**: 40-50.

Bhendrich A. 2006. Flavonoid-membrane interactions: possible consequences for biological effects of some polyphenolic compounds. *Acta Pharmacologica Sinica* **27**: 27-40.

Bies C, Lehr CM, Woodley JF. 2004. Lectin-mediated drug targeting: history and applications. *Advanced Drug Delivery Reviews* **56**: 425-435.

Bitko V, Barik S. 1998. Persistent activation of RelA by respiratory syncytial virus involves Protein Kinase C, underphosphorylated IκBβ, and sequestration of Protein Phosphatase 2A by the viral phosphoprotein. *Journal of Virology* **72**: 5610-5618.

Bitko V, Barik S. 2001. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiology* **1**: 34.

Bitko V, Musiyenko A, Shulyayeva O, Barik S. 2005. Inhibition of respiratory viruses by nasally administered siRNA. *Nature Medicine* **11**: 50-55.

Black CP. 2003. Systematic review of the biology and medical management of Respiratory Syncytial Virus infection. *Respiratory Care* **48**: 209-231.

Bonfanti JF, Doublet F, Fortin J, Lacrampe J, Guillemont J, Muller P, Queguiner L, Arnoult E, Gevers T, Janssens P, Szel H, Willebrords R, Timmerman P, Wuyts K, Janssens F, Sommen C, Wigerinck P, Andries K. 2007. Selection of a respiratory syncytial virus fusion inhibitor clinical candidate, part 1: improving the pharmacokinetic profile using the structure-property relationship. *Journal of Medicinal Chemistry* **50**: 4572-4584.

Bosch ME, Sánchez AJR, Rojas FS, Ojeda CB. 2007. Ribavirin: Analytical determinations since the origin until today. *Journal of Pharmaceutical and Biomedical Analysis* **45**: 185-193.

Bourgeois C, Bour JB, Lidholt K, Gauthray C, Pothier P. 1998. Heparin-like structures on respiratory syncytial virus are involved in its infectivity *in vitro*. *Journal of Virology* **72**: 7221-7227.

Bourinbaiar AS, Lee-Huang S. 1996. The activity of plant-derived antiretroviral proteins MAP30 and GAP31 against herpes simplex virus infection *in vitro*. *Biochemical and Biophysical Research Communications* **219**: 923-929.

Broekaert WF, Van Parijs J, Leyns F, Joos H, Peumans WJ. 1989. A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. *Science* **245**: 1100-1102.

Brown G, Aitken J, Rixon HWM, Sugrue RJ. 2002. Caveolin-1 is incorporated into mature respiratory syncytial virus particles during virus assembly on the surface of virus-infected cells. *Journal of General Virology* **83**: 611-621.

Budge PJ, Li YQ, Beeler JA, Graham BS. 2004. RhoA-derived peptide dimers share mechanistic properties with other polyanionic inhibitors of respiratory syncytial virus (RSV), including disruption of viral attachment and dependence on RSV G.. *Journal of Virology* **78**: 5015-5022.

Cane PA. 2001. Molecular epidemiology of respiratory syncytial virus. *Reviews in Medical Virology* **11**: 103-116.

Cella M, Riva DA, Coulombié FC, Mersich SE. 2004. Virucidal activity presence in *Trichilia glabra* leaves. *Revista Argentina de Microbiología* **36**: 136-138.

Chanock RM, Finberg L. 1957. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). II. Epidemiologic aspects of infection in infants and young children. *The American Journal of Hygiene* **66**: 291-300.

Chanock RM, Roizman B, Myers R. 1957. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization. *The American Journal of Hygiene* **66**: 281-290.

Chantrill BH, Coulthard CE, Dickinson L, Inkley GW, Morris W, Pyle AH. 1952. The action of plant extracts on a bacteriophage of *Pseudomonas pyocyanea* and on influenza A virus. *Journal of General Microbiology* **6**: 74-84.

Chattopadhyay D, Naik TN. 2007. Antivirals of ethnomedicinal origin: structure-activity relationship and scope. *Mini-Reviews in Medicinal Chemistry* **7**: 275-307.

Chen HC, Chou CK, Kuo YH, Yeh SF. 1996. Identification of a Protein Kinase C (PKC) activator, daphnoretin, that suppresses hepatitis B virus gene expression in human hepatoma cells. *Biochemical Pharmacology* **52**: 1025-1032.

Chen CC, Lin YC, Chen YP, Hsu HY. 1981. A study on the constituents of *Wikstroemia indica* C.A. Mey. *Journal of the Taiwan Pharmaceutical Association* **33**: 28-29.

Chen JL, Blanc P, Stoddart CA, Bogan M, Rozhon EJ, Parkinson N, Ye ZJ, Cooper R, Balick M, Nanakorn W, Kernan MR. 1998. New iridoids from the medicinal plant *Barleria prionitis* with potent activity against respiratory syncytial virus. *Journal of Natural Products* **61**: 1295-1297.

Chen JL, Kernan MR, Jolad SD, Stoddart CA, Bogan M, Cooper R. 2007. Dysoxylins A-D, tetranortriterpenoids with potent anti-RSV activity from *Dysoxylum gaudichaudianum*. *Journal of Natural Products* **70**: 312-315.

Chen WY, Yu ZY, Li SL. 1999. Effects of the water-soluble extracts from the single herb of ganduqing against hepatitis B virus *in vitro*. *Zhong Yao Cai* **22**: 463-465.

Choi SB, Park S. 2002. The effects of water extract of *Polygonatum Odoratum* (Mill) Druce on insulin resistance in 90% pancreatectomized rats. *Journal of Food Science* **67**: 2375-2379.

Cianci C, Meanwell N, Krystal M. 2005. Antiviral activity and molecular mechanism of an orally active respiratory syncytial virus fusion inhibitor. *Journal of Antimicrobial Chemotherapy* **55**: 289-292.

Cos P, Vlietinck AJ, Berghe DV, Maes L. 2006. Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. *Journal of Ethnopharmacology* **106**: 290-302.

Crim RL, Audet SA, Feldman SA, Mostowski HS, Beeler JA. 2007. Identification of linear heparin-binding peptides derived from human respiratory syncytial virus fusion glycoprotein that inhibit infectivity. *Journal of Virology* **81**: 261-271.

Crotty S, Andino R. 2002. Implications of high RNA virus mutation rates: lethal mutagenesis and the antiviral drug ribavirin. *Microbes and Infection* **4**: 1301-1307.

Domachowske JB, Dyer KD, Bonville CA, Rosenberg HF. 1998. Recombinant human eosinophil-derived neurotoxin/RNase 2 functions as an effective antiviral agent against respiratory syncytial virus. *The Journal of Infectious Diseases* **177**: 1458-1464.

Domingo E, Holland JJ. 1997. RNA virus mutations and fitness for survival. *Annual Review of Microbiology* **51**: 151-178.

Douglas JL, Panis ML, Ho E, Lin KY, Krawczyk SH, Grant DM, Cai R, Swaminathan S, Cihlar T. 2003. Inhibition of respiratory syncytial virus fusion by the small molecule VP-14637 via specific interactions with F protein. *Journal of Virology* **77**: 5054-5064.

Douglas JL, Panis ML, Ho E, Lin KY, Krawczyk SH, Grant DM, Cai R, Swaminathan S, Chen XW, Cihlar T. 2005. Small molecules VP-14637 and JNJ-2408068 inhibit respiratory syncytial virus fusion by similar mechanisms. *Antimicrobial Agents and Chemotherapy* **49**: 2460-2466.

Easton AJ, Domachowske JB, Rosenberg HF. 2004. Animal Pneumoviruses: molecular genetics and pathogenesis. *Clinical Microbiology Reviews* **17**: 390-412.

Elhassan NO, Sorbero MES, Hall CB, Stevens TP, Dick AW. 2006. Cost-effectiveness analysis of palivizumab in premature infants without chronic lung disease. *Archives of Pediatrics and Adolescent Medicine* **160**: 1070-1076.

Ennaciri J, Ahmad R, Menezes J. 2007. Interaction of monocytic cells with respiratory syncytial virus results in activation of NF-κB and PKC-α/β leading to up-regulation of IL-15 gene expression. *Journal of Leukocyte Biology* **81**: 625-631.

Fabricant DS, Farnsworth NR. 2001. The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives* **109**: 69-75.

Fang SY, He ZS, Gao JH, Wang P. 1995. Triterpenoid glycosides from *Adina rubella*. *Phytochemistry* **39**: 1241-1243.

Favacho ARM, Cintra EA, Coelho LCBB, Linhares MIS. 2007. *In vitro* activity evaluation of *Parkia pendula* seed lectin against human cytomegalovirus and herpes virus 6. *Biologicals* **35**: 189-194.

Fearns R, Collins PL. 1999. Role of the M2-1 transcription antitermination protein of respiratory syncytial virus in sequential transcription. *Journal of Virology* **73**: 5852-5864.

Feldman SA, Audet S, Beeler JA. 2000. The fusion glycoprotein of human respiratory syncytial virus facilitates virus attachment and infectivity via an interaction with cellular heparan sulfate. *Journal of Virology* **74**: 6442-6447.

Feldman SA, Crim RL, Audet SA, Beeler JA. 2001. Human respiratory syncytial virus surface glycoproteins F, G and SH form an oligomeric complex. *Archives of Virology* **146**: 2369-2383.

Felipe AMM, Rincão VP, Benati FJ, Linhares REC, Galina KJ, de Toledo CEM, Lopes GC, de Mello JCP, Nozawa C. 2006. Antiviral effect of *Guazuma ulmifolia* and *Stryphnodendron adstringens* on Poliovirus and Bovine Herpesvirus. *Biological & Pharmaceutical Bulletin* **29**: 1092-1095.

Garcia-Barreno B, Palomo C, Penas C, Delgado T, Perez-Brena P, Melero JA. 1989. Marked differences in the antigenic structure of human respiratory syncytial virus F and G glycoproteins. *Journal of Virology* **63**: 925-932.

Gentil M, Pereira JV, Silva-Sousa YTC, Pietro R, Sousa Neto MD, Vansan LP, de Castro França S. 2006. *In vitro* evaluation of the antibacterial activity of *Arctium lappa* as a phytotherapeutic agent used in intracanal dressings. *Phytotherapy Research* **20**: 184-186.

Gower TL, Pastey MK, Peeples ME, Collins PL, McCurdy LH, Hart TK, Guth A, Johnson TR, Graham BS. 2005. RhoA signaling is required for respiratory syncytial virus-induced syncytium formation and filamentous virion morphology. *Journal of Virology* **79**: 5326-5336.

Hacking D, Hull J. 2002. Respiratory syncytial virus — viral biology and the host responses. *Journal of Infection* **45**: 18-24.

Hall CB. 2001. Respiratory syncytial virus and parainfluenza virus. *The New England Journal of Medicine* **344**: 1917-1928.

Hall IH, Tagahara K, Lee KH. 1982. Antitumor agents LIII: The effects of daphnoretin on nucleic acid and protein synthesis of Ehrlich ascites tumor cells. *Journal of Pharmaceutical Sciences* **71**: 741-744.

Hallak LK, Collins PL, Knudson W, Peeples ME. 2000. Iduronic acid-containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection. *Virology* **271**: 264-275.

Haynes LM, Jones LP, Barskey A, Anderson LJ, Tripp RA. 2003. Enhanced disease and pulmonary eosinophilia associated with formalin-inactivated respiratory syncytial virus vaccination are linked to G glycoprotein CX3C-CX3CR1 interaction and expression of substance P. *Journal of Virology* **77**: 9831-9844.

Heinrich EL, Welty LAY, Banner LR, Oppenheimer SB. 2005. Direct targeting of cancer cells: a multiparameter approach. *Acta Histochemica* **107**: 335-344.

Hemming VG, Rodriguez W, Kim HW, Brandt CD, Parrott RH, Burch B, Prince GA, Baron PA, Fink RJ, Reaman G.. 1987. Intravenous immunoglobulin treatment of respiratory syncytial virus infections in infants and young children. *Antimicrobial Agents and Chemotherapy* **31**: 1882-1886.

Hogervorst PAM, Ferry N, Gatehouse AMR, Wäckers FL, Romeis J. 2006. Direct effects of snowdrop lectin (GNA) on larvae of three aphid predators and fate of GNA after ingestion. *Journal of Insect Physiology* **52**: 614-624.

Holt PG, Sly PD. 2002. Interactions between RSV infection, asthma, and atopy: unraveling the complexities. *The Journal of Experimental Medicine* **196**: 1271-1275.

Hosoya M, Balzarini J, Shigeta S, De Clercq E. 1991. Differential inhibitory effects of sulfated polysaccharides and polymers on the replication of various myxoviruses and retroviruses, depending on the composition of the target amino acid sequences of the viral envelope glycoproteins. *Antimicrobial Agents and Chemotherapy* **35**: 2515-2520.

Hruska JF, Bernstein JM, Douglas RG Jr., Hall CB. 1980. Effects of ribavirin on respiratory syncytial virus *in vitro*. *Antimicrobial Agents and Chemotherapy* **17**: 770-775.

Hu K, Kobayashi H, Dong AJ, Iwasaki S, Yao XS. 2000. Antifungal, antimitotic and anti-HIV-1 agents from the roots of *Wikstroemia indica*. *Planta Medica* **66**: 564-567.

Huang KL, Lai YK, Lin CC, Chang JM. 2006. Inhibition of hepatitis B virus production by *Boehmeria nivea* root extract in HepG2 2.2.15 cells. *World Journal of Gastroenterology* **12**: 5721-5725.

Huntley CC, Weiss WJ, Gazumyan A, Buklan A, Feld B, Hu W, Jones TR, Murphy T, Nikitenko AA, O'Hara B, Prince G, Quartuccio S, Raifeld YE, Wyde P, O'Connell JF. 2002. RFI-641, a potent respiratory syncytial virus inhibitor. *Antimicrobial Agents and Chemotherapy* **46**: 841-847.

Jairath S, Vargas PB, Hamlin HA, Field AK, Kilkuskie RE. 1997. Inhibition of respiratory syncytial virus replication by antisense oligodeoxyribonucleotides. *Antiviral Research* **33**: 201-213.

Jassim SAA, Naji MA. 2003. Novel antiviral agents: a medicinal plant perspective. *Journal of Applied Microbiology* **95**: 412-427.

Jiratchariyakul W, Wiwat C, Vongsakul M, Somanabandhu A, Leelamanit W, Fujii I, Suwannaroj N, Ebizuka Y. 2001. HIV inhibitor from Thai bitter gourd. *Planta Medica* **67**: 350-353.

Jordan WS Jr. 1962. Growth characteristics of respiratory syncytial virus. *Journal of Immunology* **88**: 581-590.

Kaku H, Van Damme EJM, Peumans WJ, Goldstein IJ. 1990. Carbohydrate-binding specificity of the daffodil (*Narcissus pseudonarcissus*) and amaryllis (*Hippeastrum* hybr.) bulb lectins. *Archives of Biochemistry and Biophysics* **279**: 298-304.

Kato A, Hashimoto Y, Kidokoro M. 1979. (+)-Nortrachelogenin, a new pharmacologically active lignan from *Wikstroemia indica*. *Journal of Natural Products* **42**: 159-162.

Kaul TN, Middleton E Jr, Ogra PL. 1985. Antiviral effect of flavonoids on human viruses. *Journal of Medical Virology* **15**: 71-79.

Kaur A, Kamboj SS, Singh J, Singh R, Abrahams M, Kotwal GJ, Saxena AK. 2007. Purification of 3 monomeric monocot mannose-binding lectins and their evaluation for antipoxviral activity: potential applications in multiple viral diseases caused by enveloped viruses. *Biochemistry and Cell Biology* **85**: 88-95.

Kernan MR, Amarquaye A, Chen JL, Chan J, Sesin DF, Parkinson N, Ye ZJ, Barrett M, Bales C, Stoddart CA, Sloan B, Blanc P, Limbach C, Mrisho S, Rozhon EJ. 1998. Antiviral phenylpropanoid glycosides from the medicinal plant *Markhamia lutea*. *Journal of Natural Products* **61**: 564-570.

Keyaerts E, Vijgen L, Pannecouque C, Van Damme EJM, Peumans W, Egberink H, Balzarini J, Van Ranst M. 2007. Plant lectins are potent inhibitors of coronaviruses by interfering with two targets in the viral replication cycle. *Antiviral Research* **75**: 179-187.

Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *American Journal of Epidemiology* **89**: 422-434.

Kisch AK, Johnson KM, Chanock RM. 1962. Immunofluorescence with respiratory syncytial virus. *Virology* **16**: 177-189.

Ko FN, Chang YL, Kuo YH, Lin YL, Teng CM. 1993. Daphnoretin, a new protein kinase C activator isolated from *Wikstroemia indica* C.A. Mey. *The Biochemical Journal* **295**:321-327.

Komath SS, Kavitha M, Swamy MJ. 2006. Beyond carbohydrate binding: new directions in plant lectin research. *Organic & Biomolecular Chemistry* **4**: 973-988.

Kotelkin A, Prikhod'ko EA, Cohen JI, Collins PL, Bukreyev A. 2003. Respiratory syncytial virus infection sensitizes cells to apoptosis mediated by tumor necrosis factor-related apoptosis-inducing ligand. *Journal of Virology* **77**: 9156-9172.

Kott V, Barbini L, Cruañes M, Muñoz JdeD, Vivot E, Cruañes J, Martino V, Ferraro G, Cavallaro L, Campos R. 1999. Antiviral activity in Argentine medicinal plants. *Journal of Ethnopharmacology* **64**: 79-84.

Krusat T, Streckert HJ. 1997. Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. *Archives of Virology* **142**: 1247-1254.

Lam SK, Ng TB. 2002. A xylanase from roots of sanchi ginseng (*Panax notoginseng*) with inhibitory effects on human immunodeficiency virus-1 reverse transcriptase. *Life Sciences* **70**: 3049-3058.

Lambert DM, Barney S, Lambert AL, Guthrie K, Medinas R, Bucy T, Erickson J, Merutka G, Petteway SR Jr. 1996. Peptides from conserved regions of paramyxovirus fusion (F) proteins are potent inhibitors of viral fusion. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 2186-2191.

Leaman DW, Longano FJ, Okicki JR, Soike KF, Torrence PF, Silverman RH, Cramer H. 2002. Targeted therapy of respiratory syncytial virus in African green monkeys by intranasally administered 2-5A antisense. *Virology* **292**: 70-77.

Lee KH, Tagahara K, Suzuki H, Wu RY, Haruna M, Hall IH, Huang HC, Ito K, Iida T, Lai JS. 1981. Antitumor agents. 49. Tricin, kaempferol-3-*O*-β-D-glucopyranoside and (+)-nortrachelogenin, antileukemic principles from *Wikstroemia indica*. *Journal of Natural Products* **44**: 530-535.

Lee-Huang S, Huang PL, Huang PL, Bourinbaiar AS, Chen HC, Kung HF. 1995. Inhibition of the integrase of human immunodeficiency virus (HIV) type 1 by anti-HIV plant proteins MAP30 and GAP31. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 8818-8822.

Levine S, Hamilton R. 1969. Kinetics of the respiratory syncytial virus growth cycle in HeLa cells. *Archives of Virology* **28**: 122-132.

Levine S, Klaiber-Franco R, Paradiso PR. 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. *Journal of General Virology* **68**: 2521-2524.

Leyssen P, De Clercq E, Neyts J. 2008. Molecular strategies to inhibit the replication of RNA viruses. *Antiviral Research* **78**: 9-25.

Li H, Wang QJ, Zhu DN, Yang Y. 2008. Reinioside C, a triterpene saponin of *Polygala aureocauda* Dunn, exerts hypolipidemic effect on hyperlipidemic mice. *Phytotherapy Research* **22**: 159-164.

Li SY, Chen C, Zhang HQ, Guo HY, Wang H, Wang L, Zhang X, Hua SN, Yu J, Xiao PG, Li RS, Tan XH. 2005a. Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antiviral Research* **67**: 18-23.

Li YL, But PPH, Ooi VEC. 2005b. Antiviral activity and mode of action of caffeoylquinic acids from *Schefflera heptaphylla* (L.) Frodin. *Antiviral Research* **68**: 1-9.

Li YL, Jiang RW, Ooi LSM, But PPH, Ooi VEC. 2007. Antiviral triterpenoids from the medicinal plant *Schefflera heptaphylla*. *Phytotherapy Research* **21**: 466-470.

Li YL, Leung KT, Yao FH, Ooi LSM, Ooi VEC. 2006. Antiviral flavans from the leaves of *Pithecellobium clypearia*. *Journal of Natural Products* **69**: 833-835.

Li YL, Ooi LSM, Wang H, But PPH, Ooi VEC. 2004. Antiviral activities of medicinal herbs traditionally used in southern mainland China. *Phytotherapy Research* **18**: 718-722.

Lin YM, Flavin MT, Schure R, Chen FC, Sidwell R, Barnard DL, Huffman JH, Kern ER. 1999. Antiviral activities of biflavonoids. *Planta Medica* **65**: 120-125.

Lis H, Sharon N. 1998. Lectins: carbohydrate-specific proteins that mediate cellular recognition. *Chemical Reviews* **98**: 637-674.

Liu BL, Li SJ, Hu J. 2004. Technological advances in high-throughput screening. *American Journal of Pharmacogenomics* **4**: 263-276.

Liu J, Li Y, Ren W, Hu WX. 2006. Apoptosis of HL-60 cells induced by extracts from *Narcissus tazetta* var. *chinensis*. *Cancer Letters* **242**: 133-140.

Liuzzi M, Mason SW, Cartier M, Lawetz C, McCollum RS, Dansereau N, Bolger G, Lapeyre N, Gaudette Y, Lagacé L, Massariol MJ, Dô F, Whitehead P, Lamarre L, Scouten E, Bordeleau J, Landry S, Rancourt J, Fazal G, Simoneau B. 2005. Inhibitors of respiratory syncytial virus replication target cotranscriptional mRNA guanylylation by viral RNA-dependent RNA polymerase. *Journal of Virology* **79**: 13105-13115.

Luo YT, Xu XC, Liu JW, Li J, Sun YS, Liu Z, Liu JZ, Van Damme EJM, Balzarini J, Bao JK. 2007. A novel mannose-binding tuber lectin from *Typhonium divaricatum* (L.) Decne (family Araceae) with antiviral activity against HSV-II and anti-proliferative effect on human cancer cell lines. *Journal of Biochemistry and Molecular Biology* **40**: 358-367.

Ma SC, Du J, But PPH, Deng XL, Zhang YW, Ooi VEC, Xu HX, Lee SHS, Lee SF. 2002. Antiviral Chinese medicinal herbs against respiratory syncytial virus. *Journal of Ethnopharmacology* **79**: 205-211.

Maggon K, Barik S. 2004. New drugs and treatment for respiratory syncytial virus. *Reviews in Medical Virology* **14:** 149-168.

Martinez FD. 2003. Respiratory syncytial virus bronchiolitis and the pathogenesis of childhood asthma. *The Pediatric Infectious Disease Journal* **22**: S76-S82.

Mason SW, Lawetz C, Gaudette Y, Dô F, Scouten E, Lagacé L, Simoneau B, Liuzzi M. 2004. Polyadenylation-dependent screening assay for respiratory syncytial virus RNA transcriptase activity and identification of an inhibitor. *Nucleic Acids Research* **32**: 4758-4767.

McConnochie KM, Hall CB, Walsh EE, Roghmann KJ. 1990. Variation in severity of respiratory syncytial virus infections with subtype. *The Journal of Pediatrics* **117**: 52-62.

McKimm-Breschkin J. 2000. VP-14637 Viropharma. *Current Opinion in Investigational Drugs* **1**: 425-427.

Monick MM, Cameron K, Staber J, Powers LS, Yarovinsky TO, Koland JG, Hunninghake GW. 2005. Activation of the epidermal growth factor receptor by respiratory syncytial virus results in increased inflammation and delayed apoptosis. *The Journal of Biological Chemistry* **280**: 2147-2158.

Monick MM, Staber JM, Thomas KW, Hunninghake GW. 2001. Respiratory syncytial virus infection results in activation of multiple Protein Kinase C isoforms leading to activation of mitogen-activated protein kinase. *The Journal of Immunology* **166**: 2681-2687.

Morris JA, Blount RE, Savage RE. 1956. Recovery of cytopathic agent from chimpanzees with coryza. *Proceedings of the Society for Experimental Biology and Medicine* **92**: 544-549.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *Journal of Immunological Methods* **65**: 55-63.

Mukhtar M, Arshad M, Ahmad M, Pomerantz RJ, Wigdahl B, Parveen Z. 2008. Antiviral potentials of medicinal plants. *Virus Research* **131**: 111-120.

Murphy BR, Collins PL. 2002. Live attenuated virus vaccines for respiratory syncytial and parainfluenza viruses: applications of reverse genetics. *The Journal of Clinical Investigation* **110**: 21-27.

Nikitenko AA, Raifeld YE, Wang TZ. 2001. The discovery of RFI-641 as a potent and selective inhibitor of the respiratory syncytial virus. *Bioorganic & Medicinal Chemistry Letters* **11**: 1041-1044.

Notka F, Meier G, Wagner R. 2004. Concerted inhibitory activities of *Phyllanthus amarus* on HIV replication *in vitro* and *ex vivo*. *Antiviral Research* **64**: 93-102.

Nuijten MJC, Wittenberg W, Lebmeier M. 2007. Cost effectiveness of palivizumab for respiratory syncytial virus prophylaxis in high-risk children: a UK analysis. *Pharmacoeconomics* **25**: 55-71.

Ojwang JO, Wang YH, Wyde PR, Fischer NH, Schuehly W, Appleman JR, Hinds S, Shimasaki CD. 2005. A novel inhibitor of respiratory syncytial virus isolated from ethnobotanicals. *Antiviral Research* **68**: 163-172.

Ooi LSM, Ng TB, Geng YQ, Ooi VEC. 2000. Lectins from bulbs of the Chinese daffodil *Narcissus tazetta* (family Amaryllidaceae). *Biochemistry and Cell Biology* **78**: 463-468.

Ooi LSM, Sun SSM, Wang H, Ooi VEC. 2004. New mannose-binding lectin isolated from the rhizome of sarsaparilla *Smilax glabra* Roxb. (Liliaceae). *Journal of Agricultural and Food Chemistry* **52**: 6091-6095.

Ooi LSM, Wang H, Ng TB, Ooi VEC. 1998. Isolation and characterization of a mannose-binding lectin from leaves of the Chinese daffodil *Narcissus tazetta*. *Biochemistry and Cell Biology* **76**: 601-608.

Openshaw PJM. 2005. Antiviral immune responses and lung inflammation after respiratory syncytial virus infection. *Proceedings of the American Thoracic Society* **2**: 121-125.

Openshaw PJM, Culley FJ, Olszewska W. 2002. Immunopathogenesis of vaccine-enhanced RSV disease. *Vaccine* **20**: S27-S31.

Parida MM, Upadhyay C, Pandya G, Jana AM. 2002. Inhibitory potential of neem (*Azadirachta indica* Juss) leaves on dengue virus type-2 replication. *Journal of Ethnopharmacology* **79**: 273-278.

Parikh BA, Tumer NE. 2004. Antiviral activity of ribosome inactivating proteins in medicine. *Mini-Reviews in Medicinal Chemistry* **4**: 523-543.

Pastey MK, Gower TL, Spearman PW, Crowe JE Jr., Graham BS. 2000. A RhoA-derived peptide inhibits syncytium formation induced by respiratory syncytial virus and parainfluenza virus type 3. *Nature Medicine* **6**: 35-40.

Peumans WJ, Van Damme EJM. 1995. Lectins as Plant Defence Proteins. In *Lectins: biomedical perspectives*, Pusztai A, Bardocz S, editors. London: Taylor & Francis; 1-22.

Peungvicha P, Temsiririrkkul R, Prasain JK, Tezuka Y, Kadota S, Thirawarapan SS, Watanabe H. 1998. 4-Hydroxybenzoic acid: a hypoglycemic constituent of aqueous extract of *Pandanus odorus* root. *Journal of Ethnopharmacology* **62**: 79-84.

Polack FP, Karron RA. 2004. The future of respiratory syncytial virus vaccine development. *The Pediatric Infectious Disease Journal* **23** (Supplement 1): S65-S73.

Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H, Lirman DD, Rabold R, Hoffman SJ, Karp CL, Kleeberger SR, Wills-Karp M, Karron RA. 2002. A role for immune complexes in enhanced respiratory syncytial virus disease. *The Journal of Experimental Medicine* **196**: 859-865.

Prince GA, Curtis SJ, Yim KC, Porter DD. 2001. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. *The Journal of General Virology* **82**: 2881-2888.

Reed JL, Muench H. 1938. A simple method of estimating fifty percent endpoints. *The American Journal of Hygiene* **27**: 493-497.

Rodriguez WJ, Gruber WC, Groothuis JR, Simoes EA, Rosas AJ, Lepow M, Kramer A, Hemming V. 1997. Respiratory syncytial virus immune globulin treatment of RSV lower respiratory tract infection in previously healthy children. *Pediatrics* **100**: 937-942.

Romero JR. 2003. Palivizumab prophylaxis of respiratory syncytial virus disease from 1998 to 2002: results from four years of palivizumab usage. *The Pediatric Infectious Disease Journal* **22**: S46-S54.

Rüdiger H, Gabius HJ. 2001. Plant lectins: occurrence, biochemistry, functions and applications. *Glycoconjugate Journal* **18**: 589-613.

San-Juan-Vergara H, Peeples ME, Lockey RF, Mohapatra SS. 2004. Protein Kinase C-α activity is required for respiratory syncytial virus fusion to human bronchial epithelial cells. *Journal of Virology* **78**: 13717-13726.

Seifert G, Jesse P, Laengler A, Reindl T, Lüth M, Lobitz S, Henze G, Prokop A, Lode HN. 2008. Molecular mechanisms of mistletoe plant extract-induced apoptosis in acute lymphoblastic leukemia *in vivo* and *in vitro*. *Cancer Letters* **264**: 218-228.

Sharon N, Lis H, editors. 2003. Lectins. 2nd ed. The Netherlands: Kluwer Academic Publishers.

Shi HM, Min ZD. 2003. Two new triterpenoid saponins from *Adina pilulifera*. *Journal of Asian Natural Products Research* **5**: 11-16.

Shigeta S, Mori S, Kodama E, Kodama J, Takahashi K, Yamase T. 2003. Broad spectrum anti-RNA virus activities of titanium and vanadium substituted polyoxotungstates. *Antiviral Research* **58**: 265-271.

Shigeta S, Mori S, Yamase T, Yamamoto N, Yamamoto N. 2006. Anti-RNA virus activity of polyoxometalates. *Biomedicine & Pharmacotherapy* **60**: 211–219.

Shirai Y, Saito N. 2002. Activation mechanisms of Protein Kinase C: maturation, catalytic activation, and targeting. *Journal of Biochemistry* **132**: 663-668.

Simoes EAF, Sondheimer HM, Top FH Jr, Meissner HC, Welliver RC, Kramer AA, Groothuis JR. 1998. Respiratory syncytial virus immune globulin for prophylaxis against respiratory syncytial virus disease in infants and children with congenital heart disease. The Cardiac Study Group. *The Journal of Pediatrics* **133**: 492-499.

Soike KF, Zhang JY, Meyerson LR. 1992. Reduction of respiratory syncytial virus (RSV) shedding in african green monkeys treated with SP-303. *Antiviral Research* **17** (supplement 1): 91.

Stevens TP, Hall CB. 2004. Controversies in Palivizumab Use. *The Pediatric Infectious Disease Journal* **23**: 1051-1052.

Stott EJ, Taylor G. 1985. Respiratory syncytial virus. Brief review. *Archives of Virology* **84**: 1-52.

Su MX, Li YL, Leung KT, Cen YZ, Li T, Chen RZ, Ooi VEC. 2006. Antiviral activity and constituent of *Ardisia chinensis* benth against coxsackie B3 virus. *Phytotherapy Research* **20**: 634-639.

Sudo K, Konno K, Watanabe W, Shigeta S, Yokota T. 2001. Mechanism of selective inhibition of respiratory syncytial virus by a benzodithiin compound (RD3-0028). *Microbiology and Immunology* **45**: 531-537.

Sudo K, Miyazaki Y, Kojima N, Kobayashi M, Suzuki H, Shintani M, Shimizu Y. 2005. YM-53403, a unique anti-respiratory syncytial virus agent with a novel mechanism of action. *Antiviral Research* **65**: 125-131.

Sudo K, Yoshida K, Konno K, Ninomiya S, Hibino M, Shigeta S, Yokota T. 2002. Pharmacokinetics of a benzodithiin (RD3-0028) following aerosol treatment in rat. *Xenobiotica* **32**: 19-27.

Sullender WM. 2000. Respiratory syncytial virus genetic and antigenic diversity. *Clinical Microbiology Reviews* **13**: 1-15.

Takayama H, Ichikawa T, Kitajima M, Nonato MG, Aimi N. 2002. Isolation and structure elucidation of two new alkaloids, pandamarilactonine-C and -D, from *Pandanus amaryllifolius* and revision of relative stereochemistry of pandamarilactonine-A and -B by total synthesis. *Chemical & Pharmaceutical Bulletin* **50**: 1303-1304.

Teng MN, Collins PL. 1998. Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles. *Journal of Virology* **72**: 5707-5716.

Teng MN, Whitehead SS, Collins PL. 2001. Contribution of the respiratory syncytial virus G glycoprotein and its secreted and membrane-bound forms to virus replication *in vitro* and *in vivo*. *Virology* **289**: 283-296.

The IMpact-RSV Study Group. 1998. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. *Pediatrics* **102**: 531-537.

Torrence PF. 1999. 2-5A-antisense chimeras: inhibitors of respiratory syncytial virus infection. *Current Opinion in Molecular Therapeutics* **1**:307-315.

Valentiner U, Fabian S, Schumacher U, Leathem AJ. 2003. The influence of dietary lectins on the cell proliferation of human breast cancer cell lines *in vitro*. *Anticancer Research* **23**: 1197-1206.

van der Meer FJUM, de Haan CAM, Schuurman NMP, Haijema BJ, Verheije MH, Bosch BJ, Balzarini J, Egberink HF. 2007. The carbohydrate-binding plant lectins and the non-peptidic antibiotic pradimicin A target the glycans of the coronavirus envelope glycoproteins. *Journal of Antimicrobial Chemotherapy* **60**: 741-749.

Van Parijs J, Joosen HM, Peumans WJ, Genus JM, Van Laere AJ. 1992. Effect of the lectin UDA (*Urtica dioica* agglutinin) on germination and cell wall formation of *Phycomyces blakesleeanus* Burgeff. *Archives of Microbiology* **158**: 9-25.

Vasconcelos IM, Oliveira JTA. 2004. Antinutritional properties of plant lectins. *Toxicon* **44**: 385-403.

Verpoorte R. 2000. Pharmacognosy in the new millennium: leadfinding and biotechnology. *Journal of Pharmacy and Pharmacology* **52**: 253-262.

Walsh EE, McConnochie KM, Long CE, Hall CB. 1997. Severity of respiratory syncytial virus infection is related to virus strain. *The Journal of Infectious Diseases* **175**: 814-820.

Wang JP, Raung SL, Kuo YH, Teng CM. 1995. Daphnoretin-induced respiratory burst in rat neutrophils is, probably, mainly through Protein Kinase C activation. *European Journal of Pharmacology* **288**: 341-348.

Weiss WJ, Murphy T, Lynch ME, Frye J, Buklan A, Gray B, Lenoy E, Mitelman S, O'Connell J, Quartuccio S, Huntley C. 2003. Inhalation efficacy of RFI-641 in an African green monkey model of RSV infection. *Journal of Medical Primatology* **32**: 82-88.

Wilson SD, Roberts K, Hammond K, Ayres JG, Cane PA. 2000. Estimation of incidence of respiratory syncytial virus infection in school-children using salivary antibodies. *Journal of Medical Virology* **61**: 81-84.

World Health Organization. Initiative for Vaccine Research: respiratory syncytial virus. http://www.who.int/vaccine_research/diseases/ari/en/index3.html. Accessed 2008 Feb 20.

Wu H, Pfarr DS, Losonsky GA, Kiener PA. 2008. Immunoprophylaxis of RSV infection: advancing from RSV-IGIV to palivizumab and motavizumab. *Current Topics in Microbiology and Immunology* **317**: 103-123.

Wyde PR, Ambrose MW, Meyerson LR, Gilbert BE. 1993. The antiviral activity of SP-303, a natural polyphenolic polymer, against respiratory syncytial and parainfluenza type 3 viruses in cotton rats. *Antiviral Research* **20**: 145-154.

Wyde PR, Chetty, SN, Timmerman, P, Gilbert, BE, Andries, K. 2003. Short duration aerosols of JNJ 2408068 (R170591) administered prophylactically or therapeutically protect cotton rats from experimental respiratory syncytial virus infection. *Antiviral Research* **60**: 221-231.

Wyde PR, Laquerre S, Chetty SN, Gilbert BE, Nitz TJ, Pevear DC. 2005. Antiviral efficacy of VP14637 against respiratory syncytial virus *in vitro* and in cotton rats following delivery by small droplet aerosol. *Antiviral Research* **68**: 18-26.

Wyde PR, Moore-Poveda DK, O'Hara B, Ding WD, Mitsner B, Gilbert BE. 1998. CL387626 exhibits marked and unusual antiviral activity against respiratory syncytial virus in tissue culture and in cotton rats. *Antiviral Research* **38**: 31-42.

Wyde PR, Moore-Poveda DK, Pimentel DM, Blough HA. 1995. Evaluation of the antiviral activity of *N*-(phosphonoacetyl)-$_{L}$-aspartate against paramyxoviruses in tissue culture and against respiratory syncytial virus in cotton rats. *Antiviral Research* **27**: 59-69.

Wyde PR, Moore-Poveda DK, Pimentel DM, Gilbert BE, Nimrod R, Panet A. 1996. Recombinant superoxide dismutase (SOD) administered by aerosol inhibits respiratory syncytial virus infection in cotton rats. *Antiviral Research* **31**: 173-184.

Xu Z, Kuang M, Okicki JR, Cramer H, Chaudhary N. 2004. Potent inhibition of respiratory syncytial virus by combination treatment with 2-5A antisense and ribavirin. *Antiviral Research* **61**: 195-206.

Xue JY. 2007. Studies on the cytotoxic and antiviral constituents of *Wikstroemia indica* (L.) C. A. Mey [thesis]. Guangzhou, China: Jinan University. p.19.

Xue JY, Li YL, Fan ZY, Su MX, Cen YZ. 2007. Studies on the chemical constituents of *Adina pilulifera*. *Zhong Yao Cai* **30**: 1084-1086.

Yamai M, Tsumura K, Kimura M, Fukuda S, Murakami T, Kimura Y. 2003. Antiviral activity of a hot water extract of black soybean against a human respiratory illness virus. *Bioscience, Biotechnology, and Biochemistry* **67**: 1071-1079.

Yang CM, Cheng HY, Lin TC, Chiang LC, Lin CC. 2007. The *in vitro* activity of geraniin and 1,3,4,6-tetra-*O*-galloyl-β-$_{D}$-glucose isolated from *Phyllanthus urinaria* against herpes simplex virus type 1 and type 2 infection. *Journal of Ethnopharmacology* **110**: 555-558.

Zakay-Rones Z, Thom E, Wollan T, Wadstein J. 2004. Randomized study of the efficacy and safety of oral elderberry extract in the treatment of influenza A and B virus infections. *The Journal of International Medical Research* **32**: 132-140.

Zhang Q, Sun LR. 2006. Studies on chemical constituents of *Serissa serissoides* roots. *Zhong Yao Cai* **29**: 786-788.

Zhang WD, Yang H, Kong XY, Mohapatra S, San Juan-Vergara H, Hellermann G, Behera S, Singam R, Lockey RF, Mohapatra SS. 2004. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nature Medicine* **11**: 56-62.

Zheng YT, Chan WL, Chan P, Huang H, Tam SC. 2001. Enhancement of the anti-herpetic effect of trichosanthin by acyclovir and interferon. *FEBS Letters* **496**: 139-142.

Zhu HX, Min ZD. 2005. Study on identification of *Adina pilulifera* (Lam.) Franch. ex Drake. *Chinese Wild Plant Resources* **6**: 68-69.

Zhu W, Chiu LCM, Ooi VEC, Chan PKS, Ang PO Jr. 2006. Antiviral property and mechanisms of a sulphated polysaccharide from the brown alga *Sargassum patens* against Herpes simplex virus type 1. *Phytomedicine* **13**: 695-701.

Zimmer G, Trotz I, Herrler G. 2001. *N*-glycans of F protein differentially affect fusion activity of human respiratory syncytial virus. *Journal of Virology* **75**: 4744-4751.