

# Chapter 3

## Antiviral Properties of Phytochemicals

Ai-Lin Liu and Guan-Hua Du

**Abstract** In recent years, significant progress has been achieved for the development of novel anti-viral drugs. These newly developed drugs belong to three groups of compounds, nucleoside analogues, thymidine kinase-dependent nucleotide analogues and specific viral enzyme inhibitors. It has been found that the natural products, like plant-derived compounds (phytochemicals) as well as traditional medicines, like traditional Chinese medicines (TCM), Ayurvedic medicines and so on, are the important sources for potential and novel anti-viral drugs. In this chapter, the history of natural products as antiviral drugs, the approaches to discover potential lead compounds, and the anti-viral properties of phytochemicals with different action mechanisms are discussed. The key conclusion is that natural products are most important sources for novel anti-viral drugs.

**Keywords** Phytochemicals • Polysaccharides • Flavonoids • Organic acids • Alkaloids • Saponins • Essential oils • Stilbenes • Antiviral activity assay

### Abbreviations

ADV	Adenovirus
AIDS	Acquired immunodeficiency syndrome
BHV	Bovine herpes virus
CB4	Coxsackie B4
CMV	Cytomegalovirus
Cox B3	Coxsackie B3
CPE	Cytopathic effect

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DENV-2	Dengue virus type-2
DHBV	Duck hepatitis B virus
EBV	Epstein-barr viurs
EC <sub>50</sub>	50% effective concentration
ELISA	Enzyme linked immunosorbent assay
EV71	Enterovirus 71
Flu A	Influenza A
HA	Haemoglutinin
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HRV2	Human rhinovirus 2
HRV3	Human rhinovirus 3
HSV	Herpes simplex virus
HSV-1	Herpesvirus type 1
HSV-2	Herpesvirus type 2
HTS	High throughput screening
IC <sub>50</sub>	50% inhibiting concentration
IVA	Influenza virus A
LD <sub>50</sub>	Half lethal dose
MES	2-N-Morpholino-ethanesulfonic acid
MDCK	Madin-Darby canine kidney
MOI	Multiplicity of infection
MoMLV	Moloney murine leukemia virus
MUNANA	2'-(4-methylumbelliferyl)- $\alpha$ -D-acetylneuraminic acid
MTT	3-[4,5-Dimethyl-thiazol-2-yl] -2,5-diphenyl tetrazolium bromide
NA	Neuraminidase
PAGE	Polyacrylamide gel electrophoresis
PI-3	Para influenza 3
PRRS virus	Porcine reproductive and respiratory syndrome virus
RBC	Red blood cells
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
RT-PCR	Real time polymerase chain reaction
SAR	Structure-activity relationship
SARS	Severe acute respiratory syndrome
SARS-CoV	Severe Acute Respiratory Syndrome associated coronavirus
SI	Selective index
SV40	Simian vacuolating virus 40 or Simian virus 40
TCM	Traditional Chinese medicine
VHSV	Viral haemorrhagic septicaemia virus
VSV	Vesicular stomatitis virus
VZV	Varicella-zoster virus

### 3.1 Introduction

A virus is a small, infectious agent, which contains little more than bundles of gene strands of either RNA or DNA, and may be surrounded by a shell of protein. Unlike bacterial cells, which are free-living entities, viruses utilize the host cell environment to propagate new viruses. The newly made viruses then leave the host cell, sometimes killing it in the process, and proceed to infect other cells within the host. Virus infection can cause a mild illness as a cold or a deadly illness as the bloody African fever. The viruses that cause Lassa fever and Ebola fever and the retrovirus that causes acquired immunodeficiency syndrome (AIDS) are examples spreading easily, threatening people's life and health sometimes swiftly, and for which there is no cure or vaccine (Jassim and Naji 2003). Viral infection has become one of the main causes of morbidity and mortality worldwide (Golean et al. 2009).

Since viruses have plenty of invasion strategies. Each strain of virus has its unique configuration of surface molecules, which work like keys in locks, enabling viruses enter into hosts by accurately fitting the molecules on their surfaces of target cells (Jassim and Naji 2003). Since viruses and hosts are intimately connected, the designing of effective anti-virals that will inhibit the viral enzymes or its replication without affecting the host cells has proved to be difficult (Golean et al. 2009; Chattopadhyay et al. 1999). The public health measures and prophylactic vaccines are still the important means, by which society controls the spread of viral infections.

With the advances in molecular biology and reverse genetics in the past two decades, many viruses have been known to have unique features in their structures or in their replication cycles, which are their potential targets. Since viral enzymes are crucial for disease progression and virus replication, inhibitors against viral enzymes have been the most desirable strategies. Most of the well-studied inhibitors against HIV, herpes simplex virus (HSV) or the influenza viruses target the host cell binding (T-20, betulinic acid, etc.), uncoating of capsid (amantadine derivatives, pleconaril), replication (reverse transcriptase inhibitors like zidovudine or abacavir, nevirapine, etc.), integrase inhibitors, DNA or RNA polymerase inhibitors (acyclovir, cidofovir, ribavirin, etc.), proteinase involved in viral polyprotein precursors and assembly/maturation inhibitors (indinavir, ritonavir and rimantadine, etc.). Based on this strategy, numerous compounds have been tested on different viruses in the past decades, but there are still less than 40 licensed anti-virals in the market (Chattopadhyay et al. 2009).

The development of viral resistance towards current antiviral agents enhances the need for new effective compounds against viral infections. Some new anti-viral compounds are currently undergoing either preclinical or clinical evaluation, and perspectives for finding new interesting antiviral drugs are promising (Chattopadhyay et al. 2009). Among these antiviral substances are several natural compounds isolated from plants used in traditional medicines.

According to statistics, natural products have become one of the main resources for new drug research and development in the past two decades, especially for the treatment of infectious diseases. Approximately 44% of the antiviral drugs approved between 1981 and 2006 were natural products, semi-synthetic natural product analogues or

synthetic compounds based on natural-product pharmacophores (Newman and Cragg 2007). Drug screening of plant extracts has led to the evaluation of their *in vitro* antiviral activities (Jassim and Naji 2003; Mukhtar et al. 2008). In addition, the application of new technologies and methods, such as high throughput screening (HTS) and molecular biology, has greatly increased the probability of finding valuable new bioactive plant extract(s) or compound(s) (Li and Vederas 2009).

In this chapter, (1) the history of natural products as antiviral drugs, (2) the main *in vitro* models used to discover and evaluate potential “lead” compounds as “antiviral substances”, and (3) the anti-viral properties of phytochemicals with special action mechanisms have been reviewed.

## 3.2 History of Natural Products as Antiviral Drugs

The history of medicinal plants can date back to the origin of human civilization on earth (Mukhtar et al. 2008). In the early times, natural products were directly used for the treatment of diseases, including viral infection diseases. Natural products have made great contributions to human health, many active constituents from natural products have been determined and their mechanisms of action have been elucidated.

The inhibitory effects of medicinal plants extracts on the replication of several viruses were reported in the past six decades. In 1952, 288 plants for anti-influenza activity were evaluated by the Boots drug company (Nottingham, England); 12 out of these plants were found to be effective against influenza viruses in embryonated eggs (Chantrill et al. 1952). In the 1970s, the antiviral activities of extracts from grape, apple, strawberry and other fruit juices against HSV, poliovirus type 1, coxsackievirus B5 and echovirus 7 were reported (Konowalchuk and Speirs 1976, 1978a, b). In 1995, 100 British Colombian medicinal plants were also reported to have antiviral activities, 12 out of which showed significant antiviral effect against corona viruses, respiratory syncytial virus (RSV), para-influenza virus type 3 (PI3), herpesvirus type 1 (HSV-1) and retavirus (McCutcheon et al. 1995). In 1998, more than 800 common Chinese herbal medicines were detected for antiviral activities against HIV and more than 100 plants exhibited anti-HIV activities (Lou 1998). In 2005, more than 200 Chinese herbal medicines were screened for antiviral activities against Severe Acute Respiratory Syndrome associated coronavirus (SARS-CoV), 4 out of which showed potent antiviral activities (Li et al. 2005a).

In addition, herpes simplex virus type 2 (HSV-2) (Debiaggi et al. 1988), HIV (Asres and Bucar 2005; Vermani and Garg 2002), hepatitis B virus (HBV) (Huang et al. 2003, 2006; Kwon et al. 2005), dengue virus type-2 (DEN-2) (Parida et al. 2002; Reis et al. 2008) and emerging viral infections associated with poxvirus and severe acute respiratory syndrome (SARS) virus (Kotwal et al. 2005) were strongly inhibited by various plants extracts. Most of these studies utilized either water soluble or alcoholic extracts of medicinal plants, and limited efforts have been directed toward the identification of active natural ingredients exhibiting antiviral effects. Some typical examples of traditional plant medicines and their antiviral activities are shown in Table 3.1.

**Table 3.1** Partial viruses inhibited by medicinal plants

Virus	Medicinal plant used	Antiviral effect	Reference
Hepatitis C virus (HCV)	<i>Acacia nilotica</i> L.	Exhibited significant activity against HCV <i>in vitro</i>	Rehman et al. (2011)
Hepatitis B virus (HBV)	<i>Solanum nigrum</i> L.	Displayed potential antiviral activity against HCV	Javed et al. (2011)
	<i>Ophioglossum pedunculatum</i> Desv.	Exhibit modest activity of blocking HBsAg secretion <i>in vitro</i>	Wan et al. (2011)
	<i>Boehmeria nivea</i> L.	Reduced HBV production in an <i>in vitro</i> and <i>in vivo</i> model	Huang et al. (2006)
	<i>Polygonum cuspidatum</i> Sieb. and Zucc.	Inhibited hepatitis B virus in a stable HBV-producing cell line	Chang et al. (2005)
Herpes simplex virus (HSV)	<i>Carissa edulis</i> Vahl.	Exhibited strong anti-HSV 1 and 2 activities both <i>in vitro</i> and <i>in vivo</i>	Tolo et al. (2006)
	<i>Geranium sanguineum</i> L.	Significantly inhibited the replication of HSV-1 and HSV-2 <i>in vitro</i>	Serkedjieva and Ivancheva (1999)
Influenza virus	<i>Geranium sanguineum</i> L.	Reduced the infectivity of various influenza virus strains <i>in vitro</i> and <i>in vivo</i>	Pantev et al. (2006) and Serkedjieva (1997)
	Elderberry	Showed an efficient, safe and cost-effective treatment for influenza <i>in vitro</i>	Zakay-Rones et al. (2004)
Poliovirus	<i>Guazuma ulmifolia</i> Lam.	Inhibited poliovirus replication, as well as, blocked the synthesis of viral antigens in infected cell cultures	Felipe et al. (2006)
Viral haemorrhagic septicaemia virus (VHSV)	<i>Olea europaea</i> L.	Inhibited viral replication <i>in vitro</i>	Micol et al. (2005)

(continued)

Table 3.1 (continued)

Virus	Medicinal plant used	Antiviral effect	Reference
Human immunodeficiency virus (HIV)	<i>Phyllanthus amarus</i> Schum. and Thonn Olive leaf	Inhibited HIV replication both <i>in vitro</i> and <i>in vivo</i>	Notka et al. (2004)
Vesicular stomatitis virus (VSV)	<i>Trichilia glabra</i> L.	Inhibited acute infection and cell-to-cell transmission of HIV-1	Lee-Huang et al. (2003)
Human adenovirus type 1	Black soybean	Inhibited VSV <i>in vitro</i>	Cella et al. (2004)
Coxsackievirus B1		Inhibited human adenovirus type 1 and coxsackievirus B1 <i>in vitro</i>	Yamai et al. (2003)
Dengue virus type-2 (DENV-2)	<i>Azadirachta indica</i> Juss.(Neem)	Inhibited DENV-2 both <i>in vitro</i> and <i>in vivo</i>	Parida et al. (2002)
	<i>Uncaria tomentosa</i> (Willd.) DC.	Displayed <i>in vitro</i> antiviral activity against DENV-2	Reis et al. (2008)
Severe Acute Respiratory Syndrome associated coronavirus (SARS-CoV)	<i>Lycoris radiata</i> , <i>Artemisia annua</i>	Showed potent antiviral activities against SARS-CoV <i>in vitro</i>	Li et al. (2005a)

Moreover, recent studies showed that plant extracts possess antiviral potential of against viral strains resistant to conventional antiviral agents (Serkedjieva 2003; Tolo et al. 2006), which have challenged the modern drug discovery practices. Thus, the further exploration of natural antiviral components of medicinal plants is necessary.

Although medicinal plants have been used throughout the world, however, their wide usage has been limited to China, India, Japan, Pakistan, Sri Lanka, Thailand and a number of African countries. However, developed countries are gradually accepting the usage of plant-based natural medicinal products in their healthcare systems. In England, because of the outstanding performance of TCMs on some stubborn diseases, such as skin eczema, currently the British government appears to support the research and the usage of TCMs, and therefore the international drug market for TCMs has been rapidly expanded, especially in England (Jiang 2005).

Besides, the Natural Health Product Regulations of Canada promulgated in 2004 and the performance of phase III clinical trial of Chinese medicine Danshen dripping pills in America in 2010 are also very important steps toward modernization of plant-based product usage in healthcare. These advances encourage the usage of modern technology and evidence-based scientific support toward promoting medicinal plants and the associated products (Siow et al. 2005).

### 3.3 Common Models for Antiviral Activity Assay

The antiviral activities of natural products, including ingredients, fractions and extracts, need to be evaluated by various anti-viral models, including *in vitro* and *in vivo* models. Besides, the analysis of assay results of natural products is also very important. In the present section, the models to evaluate the *in vitro* anti-viral activities of potential natural products have been summarized, and the evaluation methods of several representative models have been introduced. The antiviral models include molecular level models and cellular level models (Table 3.2; Chattopadhyay et al. 2009).

#### 3.3.1 Molecular Level Models

Molecular level models are usually the first step in screening a large number of substances, including compounds or extracts, for their inhibition and action mechanisms on viral enzymes or function proteins, and therefore provide useful activity information for further strategy for drug discovery, including the related molecular level models and/or cellular level models.

##### 3.3.1.1 Reverse Transcriptase (RT) Activity Assay

RT activity can be assayed by adding lysates to a 96-well microtiter plate which has a poly (A) nucleotide chain covalently bound to the base of each well. A mix of 5'

**Table 3.2** Molecular level, and cellular level for testing anti-viral agents against common viruses

Virus	Disease	Molecular level model	Cellular level model
HSV	Herpes	DNA polymerase assay	Plaque assay (Vero, MRC-5, HFF, BHK, HEp-2 cell lines)
Influenza	Flu	NA, HA, RNA polymerase assays	Plaque assay, HA, HAI, RT-PCR (MDCK, A549 cell lines)
Hepatitis C virus (HCV)	Liver cirrhosis	Protease, RNA polymerase assay	RT-PCR, no cell line facility
HBV	Liver cirrhosis	Reverse transcriptase assay, interferons	RT-PCR (HepG2 2.2.15)
Enterovirus	Diarrhoea		RT-PCR, PAGE
RSV	Common cold	RNA polymerase assay	Plaque assay, MTT assay, RT-PCR Cell-ELISA, HEp-2
HIV	AIDS	Reverse transcriptase and integrase activity assays	Primary macrophages, HFF cells
Rabies virus	Hydrophobia		McCoy cells
Polio virus	Paralysis, Aseptic meningitis	RNA polymerase assay	Plaque assay, RT-PCR (Hela cell line)

bromodeoxyuridine triphosphate (BrdUTP) is added, and the plate is incubated at 33°C for 48 h (Sivapalasingam et al. 2005).

The plate is washed to terminate the reaction and a BrdU-binding antibody conjugated to alkaline phosphatase (AP) is added and incubated at 33°C for 90 min.

Following a second wash, the AP substrate p-nitrophenyl phosphate is added and the resulting yellow color change is measured to quantify the amount of incorporated BrdUTP. Following addition of the AP substrate each plate is read at three time points: 10 min, 2–3 h, and 5–6 h or overnight at 15–24 h. The results are expressed in femtograms HIV-1 RT activity/ml plasma. The lower limit of detection is <1 fg/ml. Results are calculated using ExaVir Load Analyzer software that automatically determines the amount of RT activity in samples using a standard curve generated from an 11-point dilution of a known amount of recombinant HIV-1 RT. The results are also given in units of HIV-1 RNA equivalent copies/ml using a conversion factor derived from a Swedish cohort comprised mainly of subtype B virus. Assay precision is determined on duplicates of five samples, and assay reproducibility is determined on seven samples run on different days.

### 3.3.1.2 DNA Polymerase Activity Assay

Although different DNA polymerases have distinct functions and substrate affinities, their general mechanism of action is similar. Thus, they can all be studied using the same technical principle, the primer extension assay employing radioactive tags (Lope et al. 2007).



To explore the use of fluorescent primers in assaying DNA polymerase activity with *in vitro* primer extension, we can use the following 40 mer oligodeoxynucleotides as templates: an oligo dT (5' TTTTTTTTTTTTTTTTTTTTTTTT GTCGTG ACTGGGAAAAC) and a (dC.dA)<sub>n</sub> template (5' CACACACACACACACACA CACGTCGTGACTGGGAAAAC), where the underlined sequences are the 17-mer M13 (–40) universal primer sequence. Fluorescein-labeled M13 primer (0.5 μM; Alpha DNA, Montreal, Canada) is annealed to the two templates (0.8 μM each) in the presence of 50 mM Tris-HCl, pH 7.5, and 100 mM NaCl by heating to 90°C for 5 min, followed by cooling to 25°C over several hours, to obtain our substrate.

Since the Klenow fragment of DNA polymerase I is very well characterized, it is used as a model in the experiments. Maintaining the same conditions as in radioactive labeling primer experiments (Tuske et al. 2000; Lestienne et al. 2003), it could be assessed in different settings for Klenow activity. Briefly, DNA polymerase I (Klenow fragment) is incubated with a plant constituent, 5'-fluorescein-end-labeled primer-template substrate (50 nM), 200 μM deoxynucleotide, 10 mM Tris-HCl, pH 5, 6, 7 or 8, 5 mM dithiothreitol, 100 μg/ml bovine serum albumin, 0, 10, 100 or 200 mM MgCl<sub>2</sub>, and 0, 50, 100 or 200 mM NaCl. Reaction mixtures are incubated at 37°C for 30 min. The reaction is quenched by the addition of stop solution (10 μl) (80% deionized formamide, 10 mM EDTA, pH 8, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue) heated to 90°C for 2 min and placed on ice. Products are resolved on denaturing polyacrylamide sequencing gels in an automated ALF DNA sequencer (GE Healthcare). Analyses of the deoxynucleotide incorporation are carried out with Allelelinks software, version 1.0 (GE Healthcare).

### 3.3.1.3 Neuraminidase Inhibition Assay

A standard fluorimetric assay is used to measure influenza virus NA activity. The substrate MUNANA (2'-(methylumbelliferyl)-α-D-acetylneuraminic acid) is cleaved by NA to yield a fluorescent product, which can be quantified. The reaction mixture containing the test compounds/extracts and NA enzyme or a virus suspension in 32.5 mM MES buffer with 4 mM CaCl<sub>2</sub> (pH 6.5) is incubated for 40 min at 37°C. After incubation, the reaction is terminated by the addition of 34 mM NaOH. The fluorescence is quantified at an excitation wavelength of 360 nm and emission wavelength of 450 nm. The 50% inhibitory concentration (IC<sub>50</sub>) is defined as the concentration of NA inhibitor necessary to reduce the NA activity by 50% relative to that in a reaction mixture containing virus but no inhibitor (Liu et al. 2008a).

### 3.3.1.4 Hemagglutination Inhibition Assay

The hemagglutination assay (HA) is a quantitation of hemagglutination protein of viruses. Some viral families have surface or envelope proteins, which are able to agglutinate (stick to) human or animal red blood cells (RBC) and bind to its N-acetylneuraminic acid. The RBC will form a type of lattice in this case.

In contrast to plaque assay or  $LD_{50}$ , HA does not give any measure of viral infectivity, because no virus replication is required in this assay. It is an easy, simple and rapid method and can be applied to large amounts of samples. The detailed conditions depend on the type of virus. Some viruses bind RBCs only at certain pH values, others at certain ionic strengths.

If the test compounds inhibit virus replication and thus viral titre, the HA value will be reduced. HA inhibition assay is employed to test the effect of the test compounds in virus adsorption to target cells. Compound solutions (25  $\mu$ l) with twofold serial dilution with PBS are mixed with equal volume of influenza virus solution (200HAU/25  $\mu$ l). After incubation for 30 min at room temperature, 50  $\mu$ l of the solution is mixed with equal volume of 1% chicken erythrocyte suspension and incubated for 30 min at room temperature (Song et al. 2005).

### 3.3.2 Cellular Level Models

Cellular level models usually exploit the virus's ability to infect and replicate in specific cell lines in cell culture systems. The cell culture system provides a rapid method to grow viruses at higher titres to test the antiviral activity of compounds/extracts. It is based on the observation that virus infection and multiplication results in cytopathic effect (CPE) due to either release of virus or induction of apoptosis as a result of host immune responses. Inhibition of CPE in presence of test compound could be due to inhibition of virus replication. The assay results combined with molecular level assay results will guide the later study in animal models.

#### 3.3.2.1 Cytotoxicity in MDCK Cells

Cell viability is determined with the MTT (3-(4,5)-dimethylthiazolium (-z-y1)-3, 5-di-phenyltetrazolium bromide) method (Liu et al. 2008a). Consecutive threefold serial dilutions (100  $\mu$ l) of compounds/extracts and reference compounds are added to cell monolayers in replicates. Blank medium is used as a control. After 3 days of incubation at 37°C, 12  $\mu$ l of MTT solution (5 mg/ml in phosphate-buffered saline) is added to each well. The plate is further incubated at 37°C for 3 h to allow the formation of the formazan product. After removal of the medium, 100  $\mu$ l of dimethyl sulfoxide (DMSO) is added to dissolve the formazan crystals. After 15 min, the contents of the wells are homogenized on a microplate shaker. The optical densities are then measured with a microplate spectrophotometer at a wavelength of 540 nm. The median cytotoxic concentration ( $CC_{50}$ ) is calculated as the concentration of the tested sample that reduced the number of viable cells to 50% of the untreated control. The maximal non-cytotoxic concentration (MNCC) is defined as the maximal concentration of the sample that does not exert a cytotoxic effect and resulted in more than 90% viable cells.

### 3.3.2.2 CPE Reduction Assay

The antiviral activities of the test compounds/extracts are measured with the CPE inhibition assay (Liu et al. 2008b). Viral suspension (200 TCID<sub>50</sub>/ml, 100 µl) is added to each well of a 96-well plate containing a confluent cell monolayer. After incubation at 37°C for 2 h, the virus solution is removed, and 100 µl of consecutive threefold serial dilutions of the test compounds/extracts and reference compounds are added to each well, using the MNCC as the highest concentration. An infection control without samples is also included. The plates are incubated at 37°C in a humidified CO<sub>2</sub> atmosphere (5% CO<sub>2</sub>) for 24 h, after which the CPE is assessed. The virus-induced CPE is scored as follows: 0 = no CPE, 1 = 0–25% CPE, 2 = 25–50% CPE, 3 = 50–75% CPE, and 4 = 75–100% CPE. The reduction in virus multiplication is calculated as a percentage of the virus control ( $\% \text{ virus control} = \text{CPE}_{\text{exp}} / \text{CPE}_{\text{virus control}} \times 100$ ). The IC<sub>50</sub> of the CPE with respect to the virus control is estimated using the Reed–Muench method. The selective index (SI) is calculated as the ratio  $\text{CC}_{50} / \text{IC}_{50}$ .

### 3.3.2.3 Plaque Inhibition Assay

For plaque inhibition assays, confluent monolayer MDCK cells cultured in a 6-well tissue culture plate ( $1 \times 10^5$  cells/cm<sup>2</sup>) are infected with a mixture of approximately 500 PFU/ml of virus. After 60 min for virus adsorption, the solution is removed and the cells are washed twice with pre-warmed MEM medium, and replaced with overlay medium (DMEM containing 10 µg/ml trypsin, 1% low melting agarose, without serum), containing test compounds/extracts at different concentration. After incubating cultures for 2–3 days at 37°C with 5% CO<sub>2</sub>, monolayers are fixed with 4% formaldehyde solution for 30 min and the agarose is then removed by flowing water and stained with 1% (w/v) crystal violet solution. The plaques are counted by visual examination and percentage of plaque inhibition is calculated as relative to the control without samples. A required concentration to reduce the 50% plaque number (EC<sub>50</sub>), is calculated by regression analysis of the dose–response curves generated from these data (Song et al. 2005).

### 3.3.2.4 Influenza Virus Quantitative RT-PCR Analysis

MDCK cells are grown at about 90% confluence and infected with influenza virus at 0.1 MOI and cultured in the presence of compounds/extracts at various concentrations. At 16 h post-infection, cells are scraped off and collected by centrifugation (500 g for 5 min). Cell pellets are washed with PBS twice. Total cellular and viral RNAs are isolated from pellets using the RNeasy mini kit (QIAGEN) following the manufacture's protocol. First-strand cDNA is synthesized from 1 µg of total RNA with Omniscript RT kit (QIAGEN) using specific primers. PCR reactions are performed with 50 µl of reaction buffer [5 µl of cDNA template, 50 pmols of

primers, 0.1 mM dNTPs, and 0.5U of EX-Taq polymerase (Takara)]. The amplification conditions are as follows: 94°C for 5 min (1 cycle), 94°C for 1 min, 55°C 40 s and 72°C 1 min 40 s (18 cycles, respectively). NP RNA is chosen for detection and the primer sequences used for the detection of viral RNA are 5'-TGC TGG ATT CTC GTT CGG TC (sense) and 5'-CCCT TTA TGA CAA AGA AGA AATAAGGCG (antisense). The  $\beta$ -actin is used as internal control of cellular RNAs, with primer sequences of 5'-TCA CCC GAG TCC ATC ACG AT (sense) and 5'-GAA GTA CCC CAT TGA GCA CGG (antisense). The reverse transcription and PCR products are resolved on 1.0% agarose gels and stained with ethidium bromide (Song et al. 2005).

### 3.3.2.5 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA assays allow the detection of viral antigen or antibody, using a solid-phase assay system. Although qualitative, it offers a rapid, sensitive, and specific method for detection and gross quantitation of virus. Absolute quantitation is possible if a series of pre-determined viral titres are used to get elisa readings and matched with that unknown samples can be calculated to estimate the quantity of the virus samples (untreated or drug-treated). The method cannot differentiate between infectious and non-infectious virus particles as a result of which the drug affecting at the earlier or latter stage of viral replication or maturation may not be understood (Chattopadhyay et al. 2009).

Briefly, the untreated or drug-treated cells are infected with a known concentration of virus, adsorbed for 1 h, washed and incubated for 2–4 days depending on virus-induced CPE. Virus is harvested after freeze-thawing, centrifuged, and supernatant is diluted with the sample diluent and used for ELISA experiments. To each of the 96 well strip-plate coated with virus specific antibody 100  $\mu$ l control or test sample is added followed by 1 h incubation at room temperature with 100  $\mu$ l conjugate containing enzyme labeled virus-specific antibody. After washing five times 100  $\mu$ l of substrate is added and incubated in dark for 10 min at room temperature. Most of the assays employ horseradish peroxidase, alkaline phosphatase, or  $\beta$ -D-galactosidase. Reaction is stopped with stop solution in kit (usually 5%  $H_2SO_4$ ) and absorbance reading is taken photometrically at  $OD_{450}$ . Alternatively, the drug treatment and virus infection can be performed in 96-well formats, instead of preparing virus supernatants. For this, quadruplicate monolayers in 96-well microtitre plates are overlaid with log10 dilution of test compounds followed by the infection with virus. After 16–20 h incubation at 37°C, monolayers are fixed with 0.05% glutaraldehyde in PBS and assayed for protein specific for the virus on the cell surface. ELISA is performed with monoclonal antibodies (MAb) to specific protein of the corresponding virus strains and protein A horseradish peroxidase conjugate (Bio Rad, Hercules, CA). The optical densities ( $OD_{450}$ ) are measured and expressed as a percentage of nondrug-treated virus-infected cells (virus control). The concentration causing 50% reduction in optical density values ( $EC_{50}$ ) was evaluated from graphic plots. The selectivity index (SI) was determined from the ratio of  $IC_{50} \cdot EC_{50}$ .

### 3.4 Antiviral Activities of Different Phytochemicals

The ever increasing resistance of human pathogens to current anti-infective agents is a serious medical problem, leading to the need to develop novel antibiotic prototype molecules. In the case of viruses, the search for antiviral agents involves additional difficulties, particularly due to the nature of the infectious viruses. Thus, many compounds that may cause the death of viruses are also very likely to injure the host cell that harbours them. Natural products are increasingly appreciated as leads for drug discovery and development. Screening studies have been carried out in order to find antiviral agents from natural sources, and the extracts of traditional medicinal plants, marine organisms and fungi often show antiviral activities. The evidence indicates that there may be numerous potentially useful antiviral phytochemicals in nature, waiting to be evaluated and exploited. In addition, other plants, not previously utilized medicinally, may also reveal antivirals. Compounds from medicinal plants are of interest as possible source to control viral infection, which include flavonoids, alkaloids, polysaccharides, organic acids, essential oils, and others. Natural substances offer interesting pharmacological perspectives for antiviral drug development in regard to broad-spectrum antiviral properties and novel mechanisms of action.

#### 3.4.1 Polysaccharides

Among natural antiviral agents, recent investigations have reconsidered the interest of phyto-polysaccharides, which act as potent inhibitors of different viruses (Martinez et al. 2005). This section will illustrate a variety of antiviral polysaccharides from natural sources since 1990, with the aim of making this matter more accessible to drug development, some typical examples of such medicines and their antiviral activities are shown in Table 3.3.

The inhibitory effects of polyanionic substances on the replication of HSV and other viruses were reported almost five decades ago. Shortly after the identification of HIV as the causative agent of AIDS in 1984, heparin and other sulfated polysaccharides were found to be potent and selective inhibitors of HIV-1 replication in cell culture. Since 1988, the activity spectrum of the sulfated polysaccharides has been shown to extend to various enveloped viruses, including HSV *in vitro* (Karmakar et al. 2010; Herold et al. 1995), human cytomegalovirus (HCMV) (Baba et al. 1988), respiratory syncytial virus, influenza A and B virus (Damonte et al. 1994), DENV-2 and DENV-3 in the human hepatoma HepG2 (Talarico et al. 2005).

As potential anti-HIV drug candidates, sulfated polysaccharides offer a number of promising features. Some polysulfates show a differential inhibitory activity against different HIV strains, suggesting that marked differences exist in the target molecules with which polysulfates interact. They not only inhibit the cytopathic effect of HIV, but also prevent HIV-induced syncytium (giant cell) formation. Furthermore, experiments carried out with sulfate samples of increasing molecular weight and with sulfated cyclodextrins of different degrees of sulfation have shown that antiviral

**Table 3.3** Summary of the most active polysaccharides from medicinal plants

Compounds	Anti-viral effect	Examples of plant source	Reference
Sulfated polysaccharide fraction	Exerted activity against HSV-2, HCMV, RSV, influenza A and B virus	<i>Nothogenia fastigiata</i>	Damonte et al. (1994)
A polysaccharide	Exhibited activities against HSV-1	<i>Sclerotium glaucanicum</i>	Marchetti et al. (1996)
A polysaccharide	Exhibited activities against HSV	<i>Prunella vulgaris</i>	Xu et al. (1999)
A polysaccharide	Exhibited activities against HIV	<i>Rhizophora mucronata</i>	Premanathan et al. (1999b)
A polysaccharide	Exhibited activities against HSV	<i>Achyrocline flaccida</i>	Garcia et al. (1999)
Sulfated galactans	Exhibited antiviral activity against HSV-1 and HSV-2	<i>Bosrychia montagnei</i>	Duarte et al. (2001)
An acidic polysaccharides fraction (APS)	Exhibited activities against the replication of HSV-2 and VSV	<i>Cedrela tubiflora</i>	Craig et al. (2001)
Sulfated polysaccharides	Exhibited activities against DENV-2 and DENV-3	<i>Gymnogongrus griffithsiae</i> and <i>Cryptomenia crenulata</i>	Talarico et al. (2005)
Protein bound polysaccharides	Exhibited potent HSV-1 and HSV-2 antiviral activity	<i>Ganoderma lucidum</i>	Eo et al. (2000) and Lju et al. (2004)
Sulfated polysaccharide	Exhibited antiviral activity against HSV-1 and HSV-2 both <i>in vitro</i> and <i>in vivo</i>	<i>Porphyridium</i> sp.	Huheibel et al. (2002)
Polysaccharide fractions	Exhibited antiviral activity against HSV-1 and HIV-1	<i>Arthrospira platensis</i>	Rechter et al. (2006)
Polysaccharide fractions	Exhibited antiviral activity against HSV	<i>Grateloupia indica</i>	Chattopadhyay et al. (2007)
Polysaccharide extract	Displayed activity against WSN influenza A <i>in vivo</i>	<i>Echinacea purpurea</i>	Fusco et al. (2010)

activity increases with increasing molecular weight and degree of sulfation (Duarte et al. 2001; Wang et al. 2010; Karmakar et al. 2010). A sugar backbone is not strictly needed for the anti-HIV activity of polysulfates because sulfated polymers composed of a carbon–carbon backbone have also proved to be highly efficient anti-HIV agents *in vitro*. Other, yet to be defined, structural features may also play an important role. From studies on their mechanism of action, polysulfates appear to exert their anti-HIV activity by shielding off the positively charged sites in the V3 loop of the viral envelope glycoprotein (gp120), which is necessary for virus attachment to cell surface heparan sulfate, a primary binding site, before more specific binding occurs to the CD4 receptor of CD4<sup>+</sup> cells (Huheihel et al. 2002). This general mechanism also explains the broad antiviral activity of polysulfates against enveloped viruses. Variations in the viral envelope glycoprotein region may result in differences in the susceptibility of different enveloped viruses to compounds that interact with their envelope glycoproteins. The efficacy of sulfated polysaccharide against herpes simplex virus types 1 and 2 (HSV-1 and -2) were demonstrated *in vivo* (rats and rabbits) (Huheihel et al. 2002).

The proteoglycan seems to be a potential candidate for anti-HSV agents. A proteoglycan (GLPG), extracted and purified from the mycelia of *Ganoderma lucidum* by ethanol (EtOH) precipitation and DEAE-cellulose column chromatography, displayed antiviral activities against HSV-1 and type 2 (HSV-2). The study on its action mechanism suggested that GLPG inhibits viral replication by interfering with the early events of viral adsorption and entry into target cells (Liu et al. 2004).

The *in vivo* anti-viral activity has partially been demonstrated (Fusco et al. 2010). The *in vivo* experiment of polysaccharide extract from *Echinacea purpurea*, a widely consumed botanical product, indicated that mice infected with WSN influenza A and treated with *E. purpurea* polysaccharide extract had less weight loss than untreated mice but similar pulmonary viral titers. *Echinacea*-treated mice had lower systemic and pulmonary KC and IL-10 levels and lower systemic IFN- $\gamma$  levels following influenza infection. These suggest that *E. purpurea* alters the clinical course of influenza infection in mice through modulation of cytokines and not direct antiviral activity.

### 3.4.2 Flavonoids

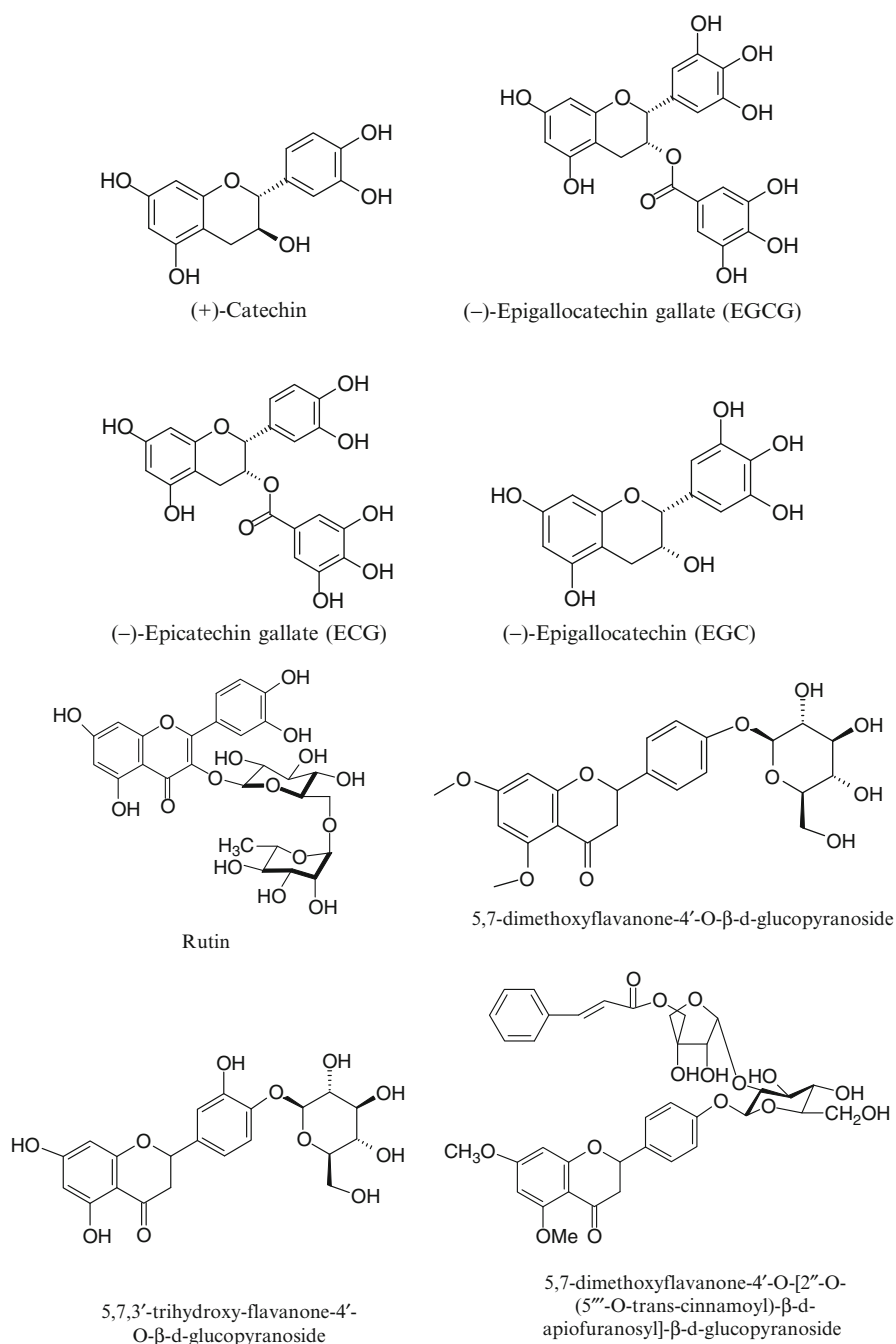
Flavonoids are very common natural products widely existing in plant kingdom. Flavonoids, including genistein, catechins and so on, have been shown to reduce the infectivity of a variety of viruses affecting humans and animals, including adenovirus, HSV, HIV, porcine reproductive and respiratory syndrome virus, and rotavirus (Andres et al. 2009). Current results about the mechanisms of action underlying their antiviral properties suggest a combination of effects on both the virus and the host cell. Flavonoids have been reported to affect virus binding, entry, replication, viral protein translation, formation of certain virus envelope glycoprotein complexes and virus release (Andres et al. 2009). They also affect a variety of host cell signaling

**Table 3.4** Viruses inhibited by flavonoids

Flavonoids	Inhibited viruses	Model	Reference
Catechins	Adenovirus	Hep2 cells	Webster et al. (2006)
	Influenza virus	MDCK cells	Song et al. (2005)
	HIV-1	T cells	Williamson et al. (2006)
	HSV	Vero and CV1 cells	Isaacs et al. (2008)
	HPV	Human condyloma	Reuter et al. (2010)
Genistein	Adenovirus	SW480 cell	Li et al. (2000b)
	Arenaviruses	Vero cells	Vela et al. (2008)
	BHV-1	MDBK cells	Akula et al. (2002)
	Bovine viral diarrhea virus	MDBK cells	Lecot et al. (2005)
	EBV	Ramos cells	Fukuda and Longnecker (2005)
	HSV-1, HSV-2	Vero cells	Yura et al. (1993) and Lyu et al. (2005)
	Human CMV	HEL 299 cells	Evers et al. (2005)
	HHV-8	HFF cells	Sharma-Walia et al. (2004)
	HIV	Primary macrophages	Stantchev et al. (2007)
	MoMLV	XC cells	Kubo et al. (2003)
	RSV	Vero cells	Rixon et al. (2005)
	Rotavirus	MA-104 cells	Andres et al. (2007)
	SV40	CV-1 cells	Dangoria et al. (1996) and Pelkmans et al. (2002)
	Quercetin	Adenovirus	BCC-1/KMC cells
Coronavirus		Vero cells	Yi et al. (2004)
Luteolin	Coronavirus	Vero cells	Yi et al. (2004)
	Influenza virus	MDCK cells	Liu et al. (2008a)
Apigenin	Influenza virus	MDCK cells	Liu et al. (2008a)
	Influenza virus	MDCK cells	Liu et al. (2009)
3-Deoxysappanchalcone	Influenza virus	MDCK cells	Liu et al. (2009)
Sappanchalcone	Influenza virus	MDCK cells	Liu et al. (2009)
Isoflavones mix	PRRS virus	Pigs ( <i>in vivo</i> )	Greiner et al. (2001)
Gorvanol A	HSV-1	Vero cells	Arthan et al. (2002)
Kaempferol	HSV-1	Vero cells	Amoros et al. (1992)
5,6,7-Trimethoxyflavone	HSV-1, Human CMV, Poliovirus	Vero and MRC-5 cells	Hayashi et al. (1997)
Irisolidone	JC virus	Primary astrocytes	Kim et al. (2006)
	Poliovirus	Vero cells	Robin et al. (2001)
3-Methylkaempferol	Poliovirus	HeLa R19 cells	Salvati et al. (2004)
3(2H)-isoflavene	Poliovirus	HeLa R19 cells	Salvati et al. (2004)
Pedunculumosides A and G	Human B Virus (HBV)	HepG2 2.2.15 cells	Wan et al. (2011)

processes, including induction of gene transcription factors and secretion of cytokines (Andres et al. 2009). Although enormous promising results were from *in vitro* experiments, a few *in vivo* results can partly confirm their *in vivo* efficacy (Miki et al. 2007). Flavonoids possess antiviral properties against a wide range of viruses under both *in vitro* and *in vivo* conditions (see Table 3.4 and Fig. 3.1).





**Fig. 3.1** The chemical structures of the active flavonoids from medicinal plants

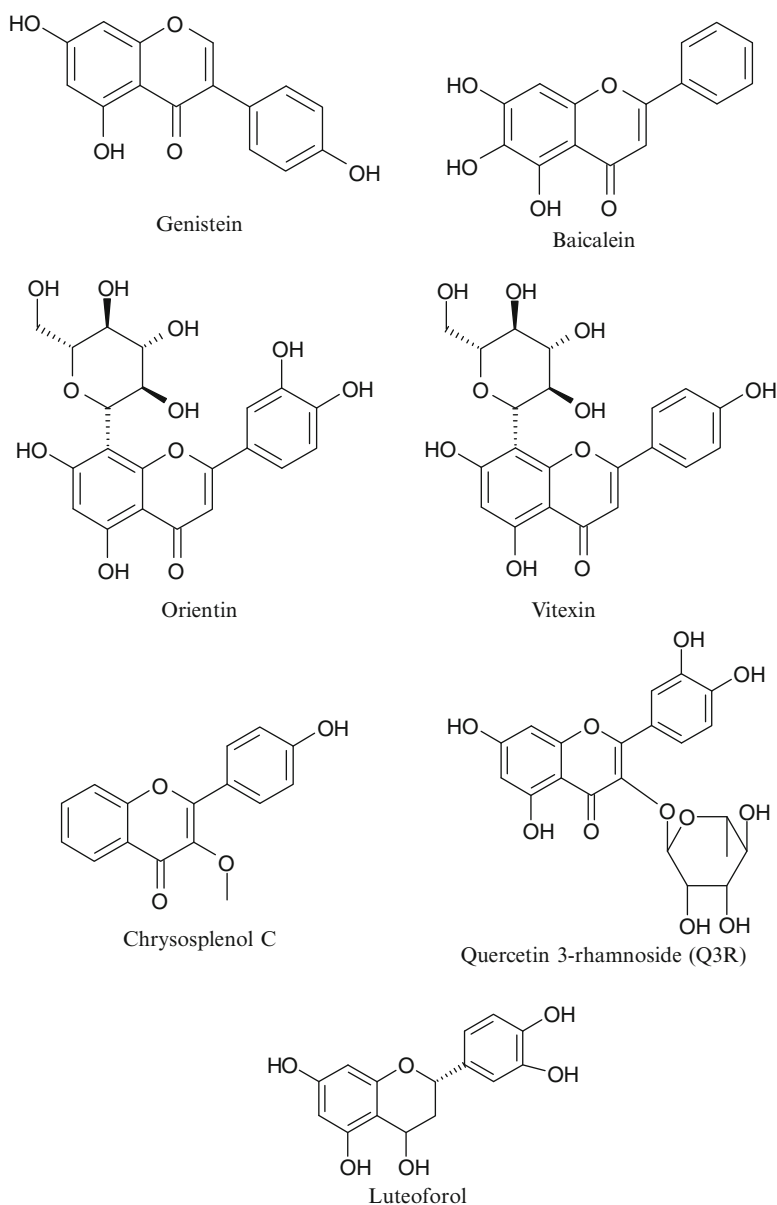


Fig. 3.1 (continued)

Tea polyphenols from green tea mainly consist of catechins ((-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin (EGC)). Their ability to inhibit several different viruses was evaluated. Among the catechins, the EGCG and ECG were found to be potent inhibitors of influenza virus replication

in MDCK cell culture and this effect was observed in all influenza virus subtypes tested, including A/H1N1, A/H3N2 and B virus (Demeule et al. 2002). However, the sensitivity in hemagglutination inhibition was widely different among three different subtypes of influenza viruses tested. Quantitative RT-PCR analysis revealed that, at high concentration, EGCG and ECG also suppressed viral RNA synthesis in MDCK cells whereas EGC failed to show similar effect (Song et al. 2005). Similarly, EGCG and ECG inhibited the neuraminidase activity more effectively than the EGC (Song et al. 2005). The results show that the 3-galloyl group of catechin skeleton plays an important role on the observed antiviral activity, whereas the 5'-OH at the trihydroxy benzyl moiety at 2-position plays a minor role (Song et al. 2005). In addition, EGCG has not only potential use as adjunctive therapy in HIV-1 infection (Williamson et al. 2006), but has greater anti-HSV activity than other green tea catechins and inactivates multiple clinical isolates of HSV-1 and HSV-2 by binding to gB, gD, or another envelope glycoprotein, it appears to be a promising candidate for use in a microbicide to reduce HSV transmission (Isaacs et al. 2008). Among green tea catechins, EGCG was the most effective when added to the cells during the transition from the early to the late phase of adenovirus infection, suggesting that EGCG inhibits one or more late steps in virus infection (Webster et al. 2006). It is worth to mention that Veregen (80% catechins) as a prescription drug for condyloma treatment was approved by US FDA in 2006, suggesting that medicinal plants will open up new prospects for the pharmaceutical industry (Reuter et al. 2010).

The flavonoids apigenin and luteolin displayed significant *in vitro* anti-influenza viral activity, which were isolated from the traditional Chinese medicinal plant *Elsholtzia rugulosa* Hemsl. (Liu et al. 2008a). The structure-activity relationship (SAR) of flavonoids as influenza virus neuraminidase inhibitors revealed that for good inhibitory effect, the 4'-OH, 7-OH, C4=O, and C2=C3 functionalities were essential, and the presence of a glycosylation group greatly reduced NA inhibition (Liu et al. 2008b). The ability of flavonoids to inhibit the activity of NA is one of the pathways to inhibit the replication of influenza virus. These findings provide important information for the exploitation and utilization of flavonoids as NA inhibitors for influenza treatment.

It was reported that rutin, 5,7-dimethoxyflavanone-4'-O- $\beta$ -d-glucopyranoside and 5,7,3'-trihydroxy-flavanone- 4'-O- $\beta$ -d-glucopyranoside were active against PI-3 (Parainfluenza-3 virus), while 5,7-dimethoxyflavanone-4'-O-[2''-O- (5'''-O-trans-cinnamoyl)- $\beta$ -d-apiofuranosyl]- $\beta$ -d- glucopyranoside inhibited potently HSV-1 (Orhan et al. 2009).

While baicalein and genistein can block human cytomegalovirus (HCMV) replication at concentrations that were significantly lower than those producing cytotoxicity against growing or stationary phase host cells. The study of their mechanisms of action suggested that the primary mechanism of action for baicalein may be to block HCMV infection at entry while the primary mechanism of action for genistein may be to block HCMV immediate-early protein functioning (Evers et al. 2005). Flavonoids orientin and vitexin found in the flower of Chinese folk medicine *Trollius chinensis* Bunge exhibited potent or moderate antiviral activity against PI-3 (Li et al. 2002).

The flavonoid chrysofenol C yielded from the green aerial parts of the Australian plant *Pterocaulon sphacelatum* (Labill.) Benth. and Hook. f. ex F. Muell., which is a 4'-hydroxy-3-methoxyflavone, has the ability to inhibit the replication of rhinoviruses (Semple et al. 1999). While the antiviral activity of quercetin 3-rhamnoside (Q3R) from traditional Chinese medicine *Houttuynia cordata* was evaluated using a cytopathic effect (CPE) reduction method, the assay results demonstrated that Q3R possessed strong anti-influenza A/WS/33 virus reducing the formation of a visible CPE. In addition, Q3R inhibited virus replication in the initial stage of virus infection by indirect interaction with virus particles. Therefore, these findings provide important information for the utilization of Q3R for influenza treatment (Choi et al. 2009b).

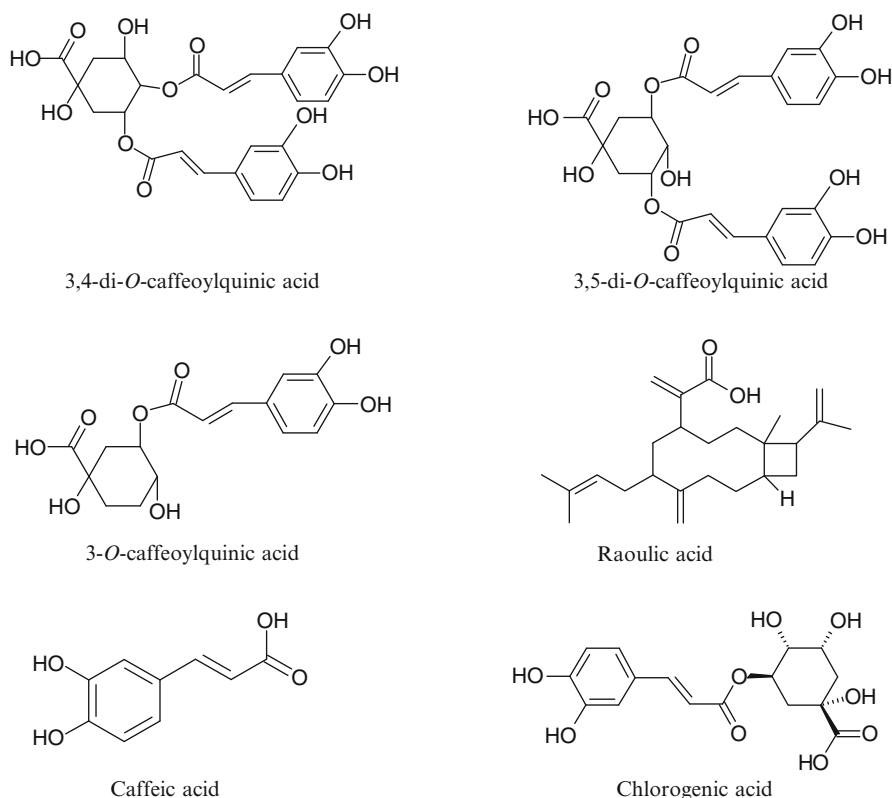
Flavonoid luteoforol isolated from *Hypericum connatum* (Guttiferae), which is used in southern Brazil in the treatment of lesions in the mouth and often related to acute herpetic gingivo-stomatitis, displayed antiviral activity against HSV-1 DNA viral strains KOS and VR733 (ATCC) (Fritz et al. 2007). Moreover, flavones are inhibitors of HIV-1 proteinase (Brinkworth et al. 1992).

Therefore, flavonoids and their structurally similar compounds possess antiviral properties against a wide range of viruses under both *in vitro* and *in vivo* conditions. These findings will provide important information for new drug design and for their exploitation and utilization for the treatment of viral infection.

### 3.4.3 Organic Acids

Besides, some natural organic acids also possess antiviral activities. Three caffeoylquinic acid derivatives, namely 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 3-O-caffeoylquinic acid isolated from traditional Chinese medicinal plant *Schefflera heptaphylla* were investigated for their antiviral activity against RSV (see Fig. 3.2; Li et al. 2005b). 3,4-Di-O-caffeoylquinic acid and 3,5-di-O-caffeoylquinic acid exhibited potent anti-RSV activity with  $IC_{50}$  values of 2.33  $\mu$ M (1.2  $\mu$ g/ml) and 1.16  $\mu$ M (0.6  $\mu$ g/ml), respectively, in a plaque reduction assay. The antiviral action of 3,4-di-O-caffeoylquinic acid and 3,5-di-O-caffeoylquinic acid was specific against RSV, as they had no obvious antiviral activity against influenza A (Flu A), Coxsackie B3 (Cox B3), and Herpes simplex type one (HSV-1) viruses (Li et al. 2005b). Studies indicated that the dicaffeoylquinic acids could inhibit RSV directly, extracellularly, but only at much higher concentrations than seen in standard assays. Moreover, they could not inhibit RSV attachment to host cells, and could not protect HEp-2 cells from RSV infection at lower concentrations. The data suggest that the compounds exert their anti-RSV effects via the inhibition of virus-cell fusion in the early stage, and the inhibition of cell-cell fusion at the end of the RSV replication cycle (Li et al. 2005b).

Raoulic acid is a principal ingredient of the plant *Raoulia australis* Hook. F (Fig. 3.2; Choi et al. 2009a). Antiviral assay using cytopathic effect (CPE) reduction method showed that raoulic acid possessed strong antiviral activity against



**Fig. 3.2** The chemical structures of the active organic acids from medicinal plants

human rhinovirus 2 (HRV2) with  $IC_{50}$  value of less than 0.1  $\mu\text{g/ml}$ , human rhinovirus 3 (HRV3) with a  $IC_{50}$  value of 0.19  $\mu\text{g/ml}$ , coxsackie B3 (CB3) virus with  $IC_{50}$  values of 0.33  $\mu\text{g/ml}$ , coxsackie B4 (CB4) virus with  $IC_{50}$  values of 0.40  $\mu\text{g/ml}$ , and enterovirus 71 (EV71) virus with  $IC_{50}$  values of less than 0.1  $\mu\text{g/ml}$ . However, the compound did not possess antiviral activity against influenza A (Flu A/PR, Flu A/WS, H1N1) and B viruses.

Moreover, the antiviral activity of aqueous extract and pure compounds of *Plantago major* L., a popular traditional Chinese medicine, were studied on a series of viruses, namely herpesviruses (HSV-1, HSV-2) and adenoviruses (ADV-3, ADV-8, ADV-11). The results showed that aqueous extract of *P. major* possessed only a slight anti-herpes virus activity (Wang et al. 2009). While certain pure compounds exhibited potent antiviral activity. Among them, caffeic acid exhibited the strongest activity against HSV-1 ( $EC_{50}$  = 15.3  $\mu\text{g/ml}$ , SI = 671), HSV-2 ( $EC_{50}$  = 87.3  $\mu\text{g/ml}$ , SI = 118) and ADV-3 ( $EC_{50}$  = 14.2  $\mu\text{g/ml}$ , SI = 727), whereas chlorogenic acid possessed the strongest anti-ADV-11 ( $EC_{50}$  = 13.3  $\mu\text{g/ml}$ , SI = 301) activity (Wang et al. 2009). The study concludes that pure compounds of *P. major*, which possess antiviral activities are mainly derived from the phenolic compounds, especially

caffeic acid. Its mode of action against HSV-2 and ADV-3 was found to be at multiplication stages (postinfection of HSV-1: 0–12 h; ADV-3: 0–2 h) (Chiang et al. 2002a). In addition, chlorogenic acid and caffeic acid also showed an inhibitory effect on HBV (hepatitis B virus) replication (Fig. 3.2; Wang et al. 2009).

#### 3.4.4 Alkaloids

There are some alkaloids which exhibit antiviral activities both *in vitro* and *in vivo*. The naphthoindozidine alkaloid, together with 7-demethoxytylophorine and 7-demethoxytylophorine N-oxide isolated from the aerial parts of *Cynanchum komarovii*, had antiviral activities against tobacco mosaic virus (An et al. 2001). While the sophoridine is one of the active constituents extracted from Chinese medicinal herb, *Sophora flavescens*, exhibited significantly antiviral activity against coxsackievirus B3 (CVB3) *in vitro* (primarily cultured myocardial cells) and *in vivo* (BALB/c mice) by regulating cytokine expression, and it is likely that sophoridine itself, not its metabolites, is mainly responsible for the antiviral activities (Zhang et al. 2006). While another alkaloid lycorine, isolated from *Lycoris radiate* possesses anti-SARS-CoV activity (Fig. 3.3; Li et al. 2005a).

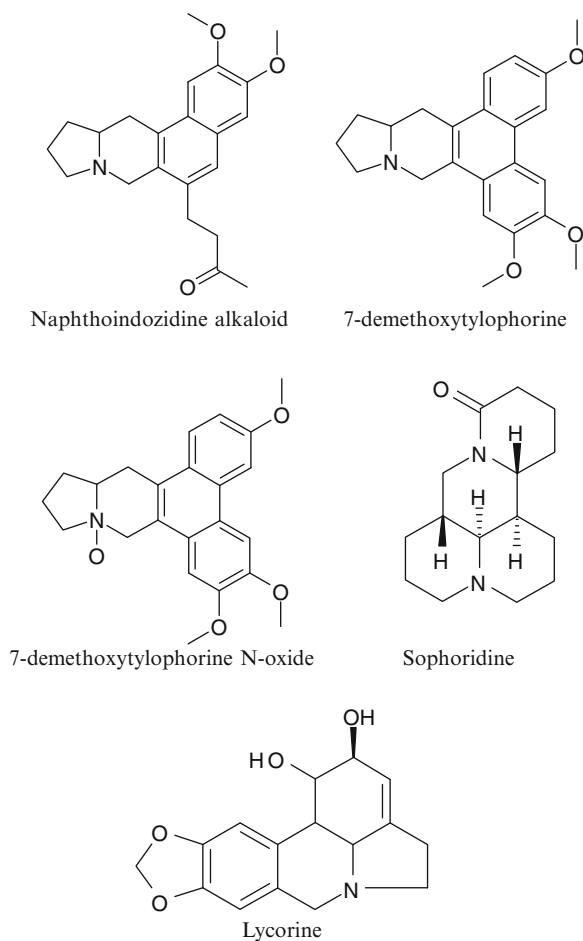
#### 3.4.5 Saponins

Plant saponins are a group of naturally occurring triterpene or steroid glycosides which include a large number of biologically and pharmacologically active compounds. Saponins have been shown in both *in vitro* and *in vivo* experimental test systems during the last decade to possess a broad spectrum of biological and pharmacological activities. This section will summarize some of the recent advances concerning antiviral activities of saponins.

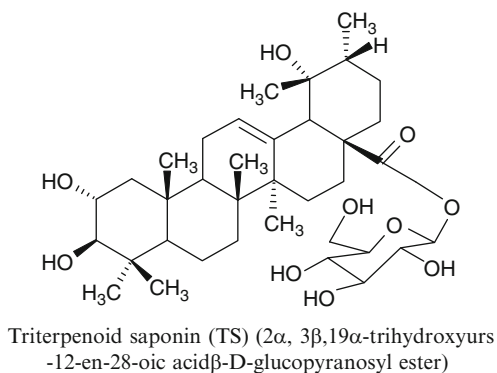
The antiviral activity of a triterpene saponin isolated from *angallis arvensis*, was studied *in vitro* against several viruses including HSV-1, adenovirus type 6, vaccinia, vesicular stomatitis and poliovirus (Amoros et al. 1987). The drug was found to inhibit the replication of HSV-1 and poliovirus type 2 as shown by inhibition of cytopathic effect and reduction of virus production. The action was not due to a virucidal effect but might involve inhibition of virus-host cell attachment. Single cycle experiments indicated that saponins interfered with both early and late events of herpes virus replication (Amoros et al. 1987).

The Tibetan herb *Potentilla anserina* L. has been widely used in China for many thousands of years to treat hepatitis-B. Bioassay-guided fractionation of the ethanol extract of the rhizomes led to the isolation of a triterpenoid saponin (TS) that was determined to be 2 $\alpha$ ,3 $\beta$ ,19 $\alpha$ -trihydroxyurs-12-en-28-oic acid  $\beta$ -d-glucopyranosyl ester (Fig. 3.4; Zhao et al. 2008). Using models of HBV infection, this compound was evaluated for its effect on HBV antigene expression in the 2.2.15 cell line

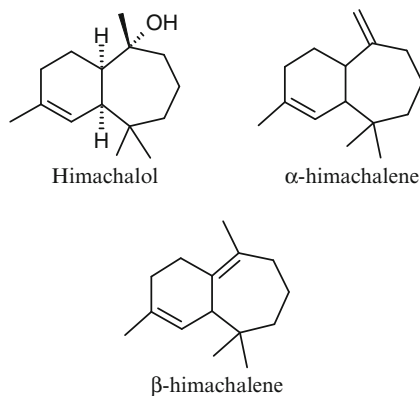
**Fig. 3.3** The chemical structures of the active alkaloids from medicinal plants



**Fig. 3.4** The chemical structure of the most active saponin from *Potentilla anserina* L.



**Fig. 3.5** The chemical structures of the active essential oils from medicinal plants



*in vitro* and anti-hepatitis B virus (HBV) activities in Peking ducklings *in vivo* (Zhao et al. 2008). Results showed that it could decrease the expression levels of HBsAg, HBeAg and HBVDNA in the 2.2.15 cell culture. The antiviral study *in vivo* on Peking ducklings also demonstrated that this compound inhibits duck hepatitis B virus (DHBV) DNA replication (Zhao et al. 2008).

### 3.4.6 Essential Oils

The application of essential oils as possible therapeutic agents against diseases is gaining attention due to various factors. Growing consumer preferences for inexpensive, natural and traditional medicines that are also effective on resistant bacterial or viral strains have stimulated investigations into the bioactive properties of essential oils. This section will summarize some of the recent advances concerning antiviral activities of essential oils.

The *Artemisia arborescens* L. essential oil showed significant antiviral activity against Herpes simplex virus type 1 (HSV-1) (Sinico et al. 2005). Peppermint oil, the essential oil of *Mentha piperita*, exhibited high levels of virucidal activity against HSV-1 and HSV-2, and it is also active against an acyclovir resistant strain of HSV-1 (HSV-1-ACV<sup>res</sup>) (Schuhmacher et al. 2003).

The essential oil from *Santolina insularis* was also inhibited virus replication against HSV-1 and HSV-2 *in vitro* (Schnitzler et al. 2008). Its antiviral activity was principally due to direct virucidal effects (De Logu et al. 2000). Lemon balm oil, the essential oil of *Melissa officinalis*, is capable of exerting a direct antiviral effect on HSV-1 and HSV-2. Considering the lipophilic nature of essential oils, which enable it to penetrate the skin, and a high selectivity index, essential oils might be suitable for topical treatment of herpetic infections (Schnitzler et al. 2008).

The wood essential oil from *Cedrus libani* widely used as traditional medicine in Lebanon for treatment of different infection diseases are mainly consist of himachalol (22.50%), β-himachalene (21.90%), and α-himachalene (10.50%) was identified for their *in vitro* antiviral activities against HSV-1 (Fig. 3.5; Loizzo et al. 2008).



All essential oils from anise, hyssop, thyme, ginger, camomile and sandalwood possess virucidal activity against HSV-2 mainly before adsorption probably by interacting with the viral envelope (Koch et al. 2008). Camomile oil exhibited a high selectivity index and seems to be a promising candidate for topical therapeutic application as virucidal agents for treatment of herpes genitalis (Koch et al. 2008).

### 3.4.7 Stilbenes

In recent years, stilbenes, which widely exist in natural kingdom, have attracted much attention for their various biological activities, including antimicrobial, anti-cancer, anti-inflammation, hepatoprotective and hepatotoxic and so on (Liu et al. 2010).

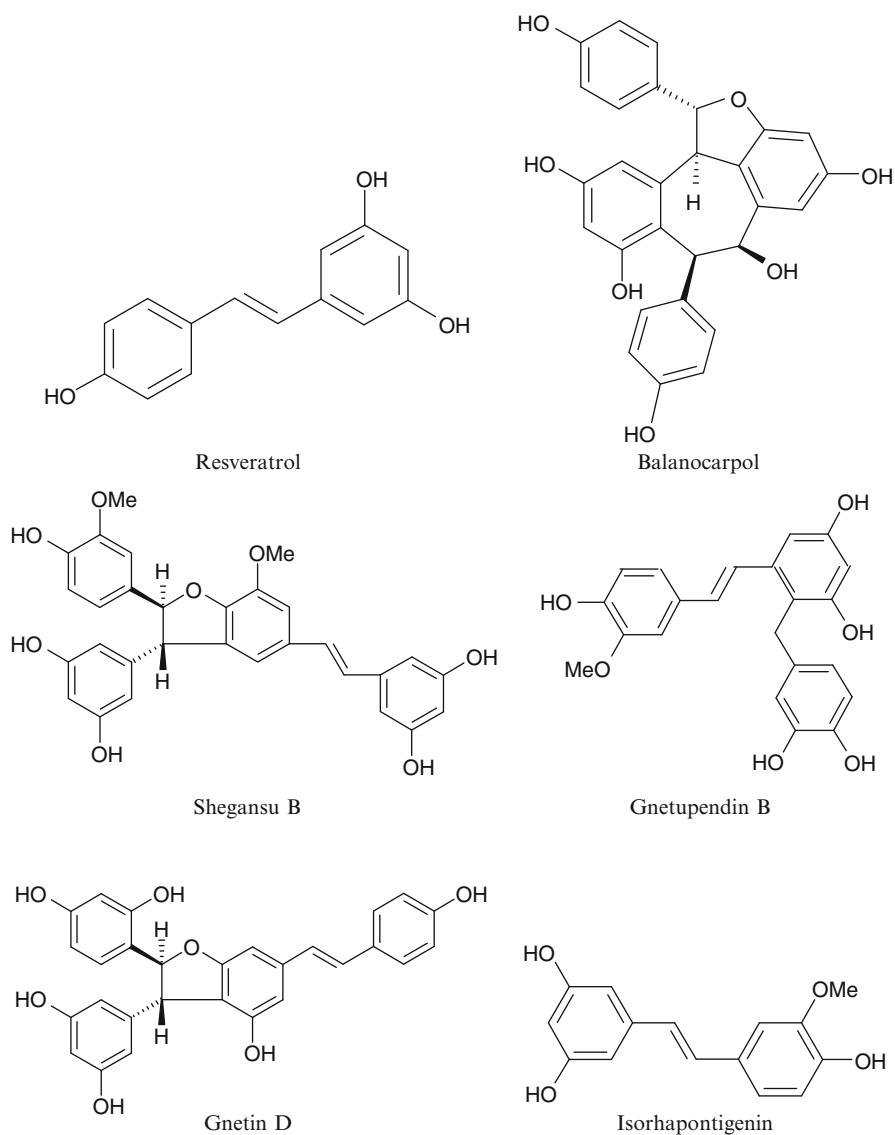
Resveratrol (3,5,4'-trihydroxystilbene) is a natural component of certain foods, such as grapes, has been shown to have anti-HSV activities *in vitro* and *in vivo* (Fig. 3.6; Docherty et al. 2004, 2005). Moreover, resveratrol was found to inhibit varicella-zoster virus (VZV) replication in a dose-dependent and reversible manner (Docherty et al. 2006). Two oligostilbenes dibalanocarpol and balanocarpol isolated from the leaves of *Hopea malibato* exhibited very modest HIV-inhibitory activity *in vitro* (Dai et al. 1998).

Besides, oligostilbenes also possess antiviral effect. Four stilbenoids, including isorhapontigenin, shegansu B, gnetupendin B and gnetin D from the lianas of *Gnetum pendulum* (Gnetaceae), exhibited significant *in vitro* anti-influenza viral activities in MDCK cells with  $IC_{50}$  values from 0.67 to 11.99  $\mu\text{g/ml}$ , their action mechanism is mainly through influenza neuraminidase (NA) inhibitory effects (see Fig. 3.6; Liu et al. 2010).

### 3.4.8 Others

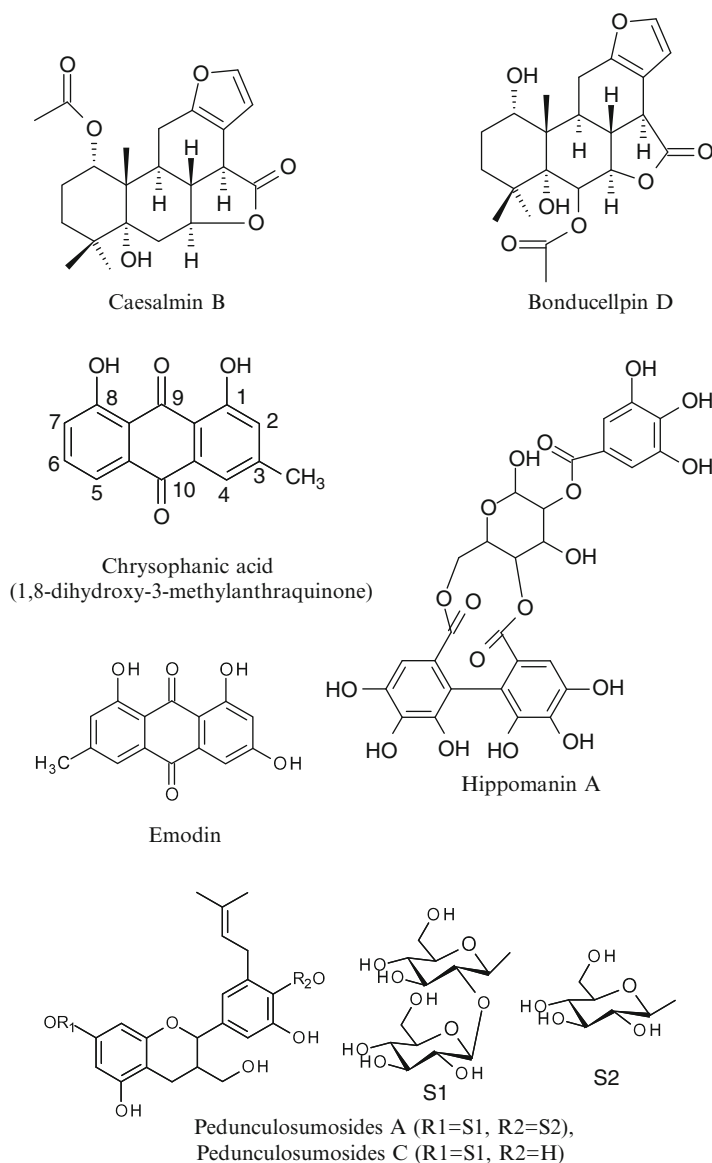
In addition, the antiviral activity of several other kinds of natural products was also reported. Terpenoids caesalmin B and bonducellpin D from the seeds of traditional Chinese medicinal plant *Caesalpinia minax* exhibited inhibitory activities on the PI-3 virus *in vitro* by cytopathogenic effects (CPE) reduction assay (Jiang et al. 2002). Hippomanin A from *Phyllanthus urinaria* Linnaea (Euphorbiaceae), which is a commonly used traditional medicinal plant in oriental countries, exhibited antiviral activity against HSV-2 but not HSV-1 infection (Fig. 3.7; Yang et al. 2007).

Chrysophanic acid (1,8-dihydroxy-3-methylanthraquinone), isolated from the Australian Aboriginal medicinal plant *Dianella longifolia*, has been found to inhibit the replication of poliovirus types 2 and 3 (*Picornaviridae*) *in vitro* (Fig. 3.7; Semple et al. 2001). The compound inhibited an early stage in the viral replication cycle, but did not have an irreversible virucidal effect on poliovirus particles. Chrysophanic acid did not have significant antiviral activity against five other viruses which were tested: Coxsackievirus types A21 and B4, human rhinovirus type 2 (*Picornaviridae*), and the enveloped viruses Ross River virus (*Togaviridae*) and HSV-1 (*Herpesviridae*).



**Fig. 3.6** The chemical structures of the active stilbenes from medicinal plants

Four structurally-related anthraquinones – rhein, 1,8-dihydroxyanthraquinone, emodin (3-methyl-1,6,8-trihydroxyanthraquinone) and aloe-emodin were also tested for activity against poliovirus type 3 (Semple et al. 2001). None of the four compounds was as active as chrysophanic acid against the virus. The results suggested that two hydrophobic positions on the chrysophanic acid molecule (C-6 and the methyl group attached to C-3) were important for the compound's activity against poliovirus (Semple et al. 2001).



**Fig. 3.7** The chemical structures of the other kinds of constituents from medicinal plants

Recently, it was reported that emodin isolated from the roots of *Rheum tanguticum* possess remarkable antiviral activity against HSV infection *in vitro* and *in vivo* (Xiong et al. 2011), and thus emodin is a promising agent in the clinical therapy of HSV infection. Besides, seven new homoflavonoid glucosides, pedunculosumosides A–G were isolated from the whole plant of *Ophioglossum pedunculosum*,

pedunculumosides A and C exhibit modest activity of blocking HBsAg secretion with  $IC_{50}$  values of 238.0 and 70.5  $\mu$ M, respectively (Wan et al. 2011).

With increasing of interest in the drug discovery from natural products, more and more antiviral activities of phytochemicals will be reported, which will greatly promote the new drug discovery and development for the treatment of viral infection disease.

### 3.5 Future Perspective

Not only the common viral diseases are still fatal, but new viral infections have emerged in worldwide in recent years. The currently available antivirals though effective are too expensive for most developing countries. Thus, the development of safe, effective and inexpensive antiviral drugs is among the top global priorities since many viruses are not yet curable and mortality rates are still high for example with HIV and hepatitis. Because of the tendency of viral mutation to drug-resistant forms, it is essential to continue the search for useful and novel natural antiviral agents, which can be expected to prolong the efficacy of drug therapy. Recently a lot of attention has been given to screening of various species of medicinal plants especially with antiviral activity (Asres et al. 2001; Jassim and Naji 2003; Chattopadhyay et al. 2006, 2009).

Since an obvious number of plant extracts have showed positive results, it seems reasonable to conclude that there are probably many potential antiviral agents. Further characterization of the active ingredients will reveal useful compounds. Some of these compounds belong to a wide range of different structural classes, e.g. coumarins, flavonoids, tannins, alkaloids, lignans, terpenes, naphtho- and anthraquinones, polysaccharides, proteins and peptides. There may also be novel phytochemicals. Although large numbers of new compounds have been isolated from medicinal plants, only some have been marketed as pharmaceutical products. Several compounds have been or are undergoing various phases of clinical trials. The traditional use of some of the medicinal plants for the treatment of infectious diseases of viral origin, therefore, is justified. In conclusion, active phytochemicals will provide important information for the development of new medicinal plant products in controlling the threats posed by some pathogenic viruses.

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