CHAPTER

8

Validation of Antiviral Potential of Herbal Ethnomedicine

*Debprasad Chattopadhyay[1], Durbadal Ojha[1],
Supriya Mondal[1], Debayan Goswami[1,2]*

[1]ICMR Virus Unit, I.D. & B.G. Hospital, Beliaghata, Kolkata, India; [2]School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

8.1 INTRODUCTION

Viruses are ultramicroscopic, acellular, metabolically inert nucleoprotein particles of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), with or without a lipid envelop [1,2]. They are obligate intracellular parasites, utilize the host's cell machineries to propagate, and cause ailments as benign as a common wart, as irritating as a cold, or as deadly as the bloody African fever [2]. Entry of a virus into the specific host cell depends on the precise attachment or fitting of the viral surface molecules with the molecules of the host cell. Each viral strain is unique in its surface antigenic structure, host cell receptors, and life cycle, and thus adopts various invasion strategies to infect every form of life. Due to their genetic variation, mode of transmission, effective replication, and the ability to persist within the host, viruses can cause widespread diseases from bacteria to humans [3−6].

Thus, the development of antiviral drugs requires great efforts. Several reports on the discovery of new drugs from traditional medicines [7−12] indicated the use of medicinal plants as a potential source for antiviral drug development [1,7−9]. Several reports indicated that a wide variety of phytochemicals, including alkaloids, flavonoids, terpenoids, polyphenolics, coumarins, lignans, have therapeutic applications [4−12] due to their interferences on viral entry or replication, along with their antioxidative properties [6,9,13]. Different in vitro and in vivo bioassays on phytochemicals lead to the identification of potential antiviral molecules. In this chapter, we will summarize the scientific approaches used to validate potential "antiviral leads" against selected genetically and functionally diverse viral families, from crude extracts to pure compounds. In-depth studies on some common in vitro antiviral assays with testing of efficacies, modes, and molecular mechanisms of action will be described here with in vivo methods against a few selected viruses.

Viral infection control strategies include (1) public health measures to minimize the risk of infection, (2) vaccination to the exposed individuals, and (3) antiinfective drugs for infected individuals. Public health measures such as safe drinking water can prevent Polio and Rota virus infections, while pest control can prevent arthropod and rodent-borne yellow fever, dengue, encephalitis, and hanta and arena viruses. However, to provide these benefits to the people of underdeveloped and developing world is a challenge. A second challenge is to discover means to control airborne respiratory infections. As public health measures are never totally effective, vaccination is a second strategy. Although vaccines dramatically decreased the incidence of polio, yellow fever, rabies, measles, mumps, rubella, and hepatitis B, they are not yet effective in human immunodeficiency virus (HIV), herpes simplex virus (HSV), hepatitis C virus (HCV), influenza, including common cold, caused by 100$^+$ strains of Rhinoviruses. Developing a polyvalent vaccine against all these viruses is also a challenge. Moreover, Alpha and Filovirus (Ebola) cause devastating epidemics with sporadic outbreaks, and it is difficult to vaccinate the entire population to eradicate them. Thus, infection can and will occur despite the best effort of humankind. Hence, for antiviral drug development, scientists are looking into the features of an ideal antiviral drug that (1) effectively inhibits some essential viral processes, (2) prevents the development of drug-resistant viruses, (3) has broad activity (single drug against any of the 100$^+$ common cold viruses), and (4) has minimum or no effect on the host system. The first feature is obvious while the remaining depend on the viral process being targeted.

8.2 RATIONALE FOR ANTIVIRAL DRUG DEVELOPMENT

Till 2014, only 37 antivirals are approved by the US Food and Drug Administration, although many viral diseases have no effective drug(s). Moreover, several viral diseases require long-term treatment that usually leads to the development of drug-resistant viruses. The existing antiviral *nucleoside analogs* act as antimetabolites in DNA synthesis and prevent viral replication in infected cells, and the related *nucleotide analogs* are used against hepatitis B virus (HBV), HCV, HSV, and HIV. In infected cells, these analogs are incorporated into the replicating DNA strand and phosphorylated as a chain terminator to stop viral DNA polymerase activity, and affect the mitochondrial DNA, leading to adverse effects such as bone marrow suppression. Another family of synthetic nucleoside analog reverse-transcriptase (RT) inhibitors help to design drugs with preferential activity, while some nucleoside analogs can act as nucleoside analog reverse-transcriptase inhibitors and polymerase inhibitors for HBV. Thus, antiviral *nucleotides analogs* repress viral reproduction by interfering with viral nucleic acid replication, compete with natural deoxynucleotide triphosphates (dNTP)/NTP substrates, and incorporate into the nascent viral nucleic acid leading to chain termination or mutagenesis (Table 8.1).

Extensive long-term clinical use of antivirals, such as acyclovir, the reference treatment for herpes viruses [14], results in the emergence of drug-resistant strains [15], due to mutations in viral *thymidine kinase* and/or DNA *polymerase* [16], along with renal impairment [17,18]. As antiviral drugs are virus specific and target oriented, they require appropriate diagnosis before therapeutic intervention. Another factor is the availability of suitable vaccines and their degree of effectiveness against a particular virus, as vaccination is the most effective approach for the prevention of a disease. However, in

TABLE 8.1 Some Nucleoside and Nucleotide Analogs and Their Antiviral Properties

Nucleotide	Analog	Incorporated to viral	Mechanism of viral inhibition
Vidarabine triphosphate	ATP	DNA polymerase	Unknown
Ribavirin triphosphate	GTP	RNA polymerase	Mutagenesis of genome replication
Acyclovir triphosphate	GTP	DNA polymerase	Chain termination of DNA synthesis
Zidoduvine triphosphate	TTP	Reverse transcriptase	CT of cDNA synthesis
Stavudine triphosphate	TTP	Reverse transcriptase	Do
Lamivudine triphosphate	CTP	Reverse transcriptase	Do
Nucleoside	**Analog**	**Incorporated to viral**	**Mechanism of inhibition**
Didanosine	Deoxyadenosine	HIV-RT	CT of viral cDNA synthesis
Abacavir, Entecavir	Deoxyguanosine	HIV-RT, HBV	Do
Telbivudine, zidovudine	Deoxythymidine	HBV, HIV-RT	Do
Emtrici- and Zalci-tabine	Deoxycytidine	HIV-RT and HBV	Do
Idoxuridine Trifluridine	Deoxyuridine	HIV-RT	Do

ATP, Adenosine triphosphate; GTP, Guanosine triphosphate; TTP, Thymidine triphosphate; CTP, Cytidine triphosphate; CT, Chain termination, (Dai L, Huang Q, Boeke JD. (2011). Effect of reverse transcriptase inhibitors on Line-1 and Ty1 reverse transcriptase activities and on LINE-1 retrotransposition. *BMC Biochemistry* **12**:18−28).

diseases such as hepatitis B, infected patients provide a large market for chemotherapy. Therefore, there is an unmatched need for readily available antiviral drugs at affordable prices with minimal side effects. Hence, traditional medicines are explored as novel antiviral agents, as many of these ancient medicaments, containing diverse plant metabolites, have potent antiviral activities [10−12,19,20].

8.3 DEVELOPMENT OF EFFECTIVE ANTIVIRAL DRUGS

Despite continuous advancement in medical science, viral diseases are a major cause of death around the globe. The development of an antiviral drug depends on the unique features of host−virus interactions, as viruses are acellular and host- and route-specific, and infect only by their nucleic acid(s) that regulate the host cell DNA for multiplication. Being an obligate intracellular parasite, viral replication depends on the metabolic pathways of the host. Thus, designing an effective antiviral drug to target virus-specific enzymes or replication steps without affecting the host cell is very difficult [20]. Advancement in molecular biology and genetics helps to design a rational approach for developing antiviral agents by targeting specific sites/steps of the viral life cycle, including (1) inactivation of the extracellular virus particles, (2) prevention of viral attachment or fusion, (3) entry or penetration, (4) replication of viral genome, (5) synthesis of viral-specific proteins or intermediates, and (6) assembly or release of new virions. However, the most desirable target is the

inhibition of major viral enzymes essential for replication and spread of the disease. Most of the well-studied inhibitors of HIV, HSV, or Influenza viruses target the host cell binding (T-20, betulinic acid), uncoating of capsid (amantadine, pleconaril), viral replication (RT inhibitors: zidovudine, abacavir, nevirapine), viral enzymes integrase or DNA/RNA polymerase (acyclovir, cidofovir, ribavirin), proteinases of polyprotein precursors, and assembly or maturation (indinavir, ritonavir, rimantadine). On the basis of these strategies, several compounds were tested on different viruses, but only 40 licensed antivirals are approved till 2014 [1,5]. Moreover, studies on viral genomics, gene functions, and regulations led to the rational designing of gene-based drugs that induce protective antiviral immunity, interference with viral replication, gene expression, or viral messenger RNAs with limited delivery to the sites of replication and nuclease degradation [13].

8.4 MEDICINAL PLANTS AS A SOURCE OF ANTIVIRAL DRUGS: AN OVERVIEW

The history of human civilization revealed that almost all ancient cultures rely upon medicinal plants for treating ailments of the body and mind, and about 80% of the world population uses traditional medicine for primary health care [4]. The discovery of synthetic drugs with better efficacy and quick recovery in many diseases leads to several adverse effects during long-term clinical use, particularly in chronic or "difficult-to-treat" diseases. Thus, there has been a huge upsurge in the use of natural products for preventing and

treating serious viral diseases. However, most herbal products have no quality control, are unable to provide consistent results, and even produce undesirable effects. About 40% of the modern-day anticancer and antimicrobial drugs have their roots in traditional medicine [4,5,20], but till 2014, we have barely analyzed the tip of iceberg to design more drugs from plants. Hence, it is of utmost importance to search for phytochemicals with antiviral potential from unexplored plants used in ethnomedicine, before we lose our herbal resources due to rapid anthropogenic activities including industrialization and urbanization [1,8].

Many viruses have unique features in their structure or replication cycle that may act as the potential target for designing new antivirals, as evident with acyclovir (acycloguanosine), which blocks certain enzymes of herpes viruses responsible for triggering disease. Due to the structural diversity and broad range of bioactivities, ethnomedicines can serve as a source of complementary antivirals by inhibiting some specific enzymes or steps of viral replication or cellular factors (Table 8.2) of many DNA/RNA viruses [5].

Ayurveda and traditional Chinese medicine, in particular, use several medicinal plants to reduce disease severity, and many of them have antiviral activity

TABLE 8.2 The Viruses and Their Viral and Cellular Targets for Antiviral Drug Development

Virus	Viral target	Cellular target
Parvo, Polyoma, Papiloma viruses	DNA polymerase	—
Adenovirus	DNA polymerase	Cellular factors
Herpesviruses	DNA pol, kinase, helicase	—
Poxvirus	DNA and RNA polymerase	4 Enzymes*
Hepadna virus	DNA polymerase (RT)	—
Picornavirus	Capsid RNA polymerase	—
Flavivirus	RNA polymerase	—
Arenavirus	RNA polymerase	4 Enzymes*
Bunya, Toga, Rhabdo Filovirus	RNA polymerase	4 Enzymes*
Hepacivirus	-Do-, helicase, protease	—
Orthomyxovirus	Matrix neuraminidase	—
Paramuxovirus	Fusion polypeptides	4 Enzymes*
Coronavirus	RNA poly, helicase, protease	—
Rheovirus	—	4 Enzymes*
Retrovirus	Protease, integrase, TAT	Integration-transcription factors

* Inosine 5′ monophosphate dehydrogenase, S-adenosylomocysteine hydrolase, Oritidine 5′-phosphate decarboxylase, Cytosine 5′-triphosphate synthetase.

[1,5,7,8,21]. Research on the antiviral potentials of plants was first started in 1952, and 12 out of 288 plants were found to be effective against influenza [4,5]. During the past 40 years, numerous broad-based screening programs for the antiviral activity of medicinal plants have been initiated using in vitro and in vivo assays. Out of 100 British Colombian medicinal plants, only a few showed activities against corona viruses, respiratory syncytial virus (RSV), para influenza, and HSV [22], while the marine algae *Spirulina* showed antimutagenic, immunomodulatory, and antiviral activities [23,24]. Interestingly, Cyanovirin N, an 11-kDa protein of blue green algae, inactivates HIV-1 by binding with its glycoprotein120 [25], while sulfated polysaccharides of seaweeds and alga showed anti-HIV and anti-HSV activities [26]. A list of selected medicinal plants and their compounds having antiviral activities against common viral diseases is presented in Table 8.3, and the structures of some important compounds are depicted in Figure 8.1.

8.5 METHODS FOR THE VALIDATION OF ANTIVIRAL ACTIVITY OF PLANTS

In vitro assays are the preliminary step toward the identification of antiviral activity of a plant extract or phytocompounds. These assays measure the ability of a virus to infect and replicate in specific cell lines and the response of a particular extract toward the relevant virus infection. The cell culture system provides a rapid and less cumbersome method for the growth of viruses at higher titers, testing of cytotoxicity and antiviral efficacy, maintenance of cultures, and genetic manipulations. The cell lines characterized and routinely used for common viruses along with their respective methods are given in Table 8.4.

8.5.1 In vitro Assay

8.5.1.1 Indirect Assays

Indirect assays are the first step for screening a large number of extracts or phytocompounds at a time, as virus infection and multiplication result in a cytopathic effect (CPE) due to the release of infectious virion or induction of apoptosis. Inhibition of CPE in the presence of a test extract or phytocompound may be due to the inhibition of viral replication. Till 2014, various indirect assays have been developed, validated, and modified for individual viruses.

8.5.1.2 Visual Observation of CPE

Visual observation of CPE under an inverted microscope is usually carried out by infecting a semiconfluent cell monolayer with a susceptible virus in

TABLE 8.3 Partial List of Viruses Inhibited by Plants Used in Traditional Medicines

Virus	Medicinal plant	Family name	Isolated molecule	Antiviral effect	References
HSV	*Tanacetum vulgare*	Asteraceae	Parthenolide	Anti-HSV-1 and -HSV-2	[27]
	Ophiorrhiza nicobarica	Rubiaceae	Harmaline	Inhibit IE gene synthesis	[10,11]
	Rhus javanica	Anacardiaceae	Moronic acid	Anti-HSV-1 and -HSV-2	[28]
	Achyranthes aspera	Amaranthaceae	Oleanolic acid	Anti-HSV-1 and -HSV-2	[12]
	Odina wodier	Anacardiaceae	Chlorogenic acid	Prevent attachment	[29]
	Mallotus peltatus	Euphorbiaceae	Ursolic acid	HSV replication	[30]
	Terminalia chebula	Combretaceae	Chebulagic acid, punicalagin	Inhibit entry and cell spread	[31]
	Ilex asprella	Aquifoliaceae	Asprellanoside A, Oblonganoside H	Anti-HSV-1	[32]
	Azadirachta indica	Meliaceae	Sulfonoquinovosyldiacylglyceride	Anti-HSV-1 and -HSV-2	[33]
	Ficus benjamina	Moraceae	Quercetin 3-O-rutinoside, Kaempferol 3-O-robinobioside	Anti-HSV-1 and -HSV-2	[34]
	Artocarpus lakoocha	Moraceae	Oxyresveratrol	Inhibit viral replication	[35]
HIV	*Mimusops elengi*	Sapotaceae	Gallocatechin, Epigallocatechin	HIV-1 integrase activity	[36]
	Schisandra sphenanthera	Schisandraceae	Schisphendilactones A, B	Anti-HIV-1 activity	[37]
	Olea europaea	Oleaceae	Maslinic acid	Anti-HIV	[38]
	Stellera chamaejasme	Thymelaeaceae	Stelleralides A	Anti-HIV	[39]
	Artemisia annua	Asteraceae	Artemisinin	Anti-HIV activity	[40]
	Sargassum fusiforme	Sargassaceae	Palmitic acid	Inhibits HIV entry	[41]
	Melochia odorata	Sterculiaceae	Waltherione A	Inhibition of HIV P24	[42]
	Emblica officinalis	Phyllanthaceae	Curcumin	Anti-HIV activity	[43]
	Pelargonium sidoides	Geraniaceae	—	Blocks attachment of HIV-1, and prevent entry	[44]
Influenza virus	*Syzygium aromaticum*	Myrtaceae	Eugenol	Inhibit the activation of extracellular signal-regulated kinase, p38-mitogen-activated protein kinase, IκB kinase (IKK)/NF-κB signal pathways	[45]
	Silybum marianum L.	Asteraceae	23-(S)-2-Amino-3-phenyl-propanoyl-silybin	activate ERK/p38 MAPK and IKK pathways	[46]
	Ribes nigrum folium	Saxifragaceae	—	Antiinfluenza A	[47]
	Taraxacum officinale	Asteraceae	—	Antiinfluenza (H1N1)	[48]
	Caesalpinia sappan	Fabaceae	3-Deoxysappanchalcone	Antiinfluenza, apoptosis, and antiinflammation	[49]

Continued

TABLE 8.3 Partial List of Viruses Inhibited by Plants Used in Traditional Medicines—cont'd

Virus	Medicinal plant	Family name	Isolated molecule	Antiviral effect	References
	Vaccinium angustifolium, *Vitis vinifera* L, *Cinnamomum cassia*	Ericaceae, Vitaceae, Lauraceae	Procyanidin	Antiinfluenza A by inhibiting the formation of Atg5-Atg12/Atg16 heterotrimer	[50]
	Adenium obesum	Apocynaceae	Oleandrigenin-β-D-glucosyl l (1→4)-β-D-digitalose	Antiinfluenza A/PR/8/34 (H1N1)	[51]
	Angelica keiskei	Apiaceae	Xanthokeistal A	Neuraminidase inhibitor	[52]
	Melaleuca alternifolia	Myrtaceae	Terpinen-4-ol, terpinolene	Anti-A/PR/8 virus (H1N1)	[53]
Human RSV	*Rosmarinus officinalis*	Lamiaceae	Carnosic acid	Inhibit replication of RSV	[54]
	Gentiana lutea	Gentianaceae	—	Anti-RSV	[55]
	Cimicifuga foetida L.	Ranunculaceae	Cimicifugin	Inhibit viral attachment and internalization	[56]
HBV	*Artemisia capillaris*	Asteraceae	8-(Z)-Decene-4, 6-diyne-1, 3, 10-triol (1), 1, 3S, 8S-trihydro xydec-9-en-4, 6-yne (2)	Inhibit HBV DNA replication	[57]
	Aster tataricus L.	Asteraceae	Astataricusones, astataricusol A	Inhibit HBV DNA replication	[58]
	Swertia macrosperma	Gentianaceae	Swermacrolactones, luteolin	Inhibit secretion of HBV surface antigen	[59]
	Piper longum Linn.	Piperaceae	Piperine	Inhibit the secretion of HBV surface antigen	[60]
	Phyllanthus niruri L.	Phyllanthaceae	Nirtetralin A	Anti-HBV activities	[61]
HCV	*Morinda citrifolia*	Rubiaceae	Pyropheophorbide, pheophorbide	Inhibit entry and postentry steps of HCV	[62]
	Syncephalastrum racemosum	Syncephalastraceae	Ursolic acid	Anti-HCV activity	[63]
	Marrubium peregrinum L	Lamiaceae	Ladanein	HCV entry inhibitor	[64]
	Acacia nilotica	Fabaceae	—	Anti-HCV activity	[65]
	Vaccinium virgatum Aiton	Ericaceae	Proanthocyanidin	Inhibit HCV replication	[66]

PV	*Coffea arabica*	Rubiaceae	N-methyl-pyridinium formate	Anti-PV	[67]
	Baccharis gaudichaudiana	Asteraceae/ Compositae	Apigenin	Anti-PV type 2	[68]
	Heteropteris aphrodisiaca	Malpighiaceae	Aliphatic nitro compound	Inhibit PV type 1	[69]
	Dianella longifolia	Xanthorrhoeaceae	Chrysophanic acid	Inhibit PV 2 and PV 3 replication	[70]
	Pterocaulon sphacelatum	Asteraceae	Chrysosplenol C	Inhibit PV	[71]
VHSV	*Rhus verniciflua*	Anacardiaceae	Fisetin	Anti-VHSV	[72]
	Olea europaea L.	Oleaceae	Oleuropein	Inhibit VHSV replication	[73]
SARS-CoV	*Lycoris radiata*	Amaryllidaceae	Lycorine	Anti-SARS-CoV	[74]
	Glycyrrhiza glabra	Fabaceae	Glycyrrhizin	Anti-SARS-CoV	[75]
VSV	*Glycyrrhiza glabra*	Fabaceae	Glycyrrhizin	Inhibit phosphorylation enzymes and latency of VSV	[75]
	Calendula arvensis	Asteraceae	Oleanolic acid	Inhibit VSV multiplication	[76]
	Trichilia glabra L.	Meliaceae	–	Leaf extract inhibit VSV	[77]
Human ADV	*Glycine max*	Fabaceae	–	Inhibit ADV-1, Coxsackie B1	[78]
	Gentiana lutea	Gentianaceae	–	Against ADV-5	[55]
	Astragalus membranaceus	Fabaceae	Astragaloside IV	Inhibit ADV-3 replication	[79]
	Ficus carica	Moraceae	–	Inhibit replication	[80]
DEN	*Aedes aegypti*	Culicidae	Triptamine	Larvicidal	[81]
	Scutellaria baicalensis	Lamiaceae	Baicalein	Virucidal against DEN-2	[82]
	Azadirachta indica Juss.	Meliaceae	–	Leaf extract inhibit DEN-2	[83]

ADV, adenovirus; DEN, Dengue virus; PV, Poliovirus; SARS-CoV, severe acute respiratory syndrome coronavirus; VHSV, viral hemorrhagic septicemia virus; ERK, extracellular signal-regulated kinase, MAPK, p38-mitogen-activated protein kinase; IKK, IκB kinase.
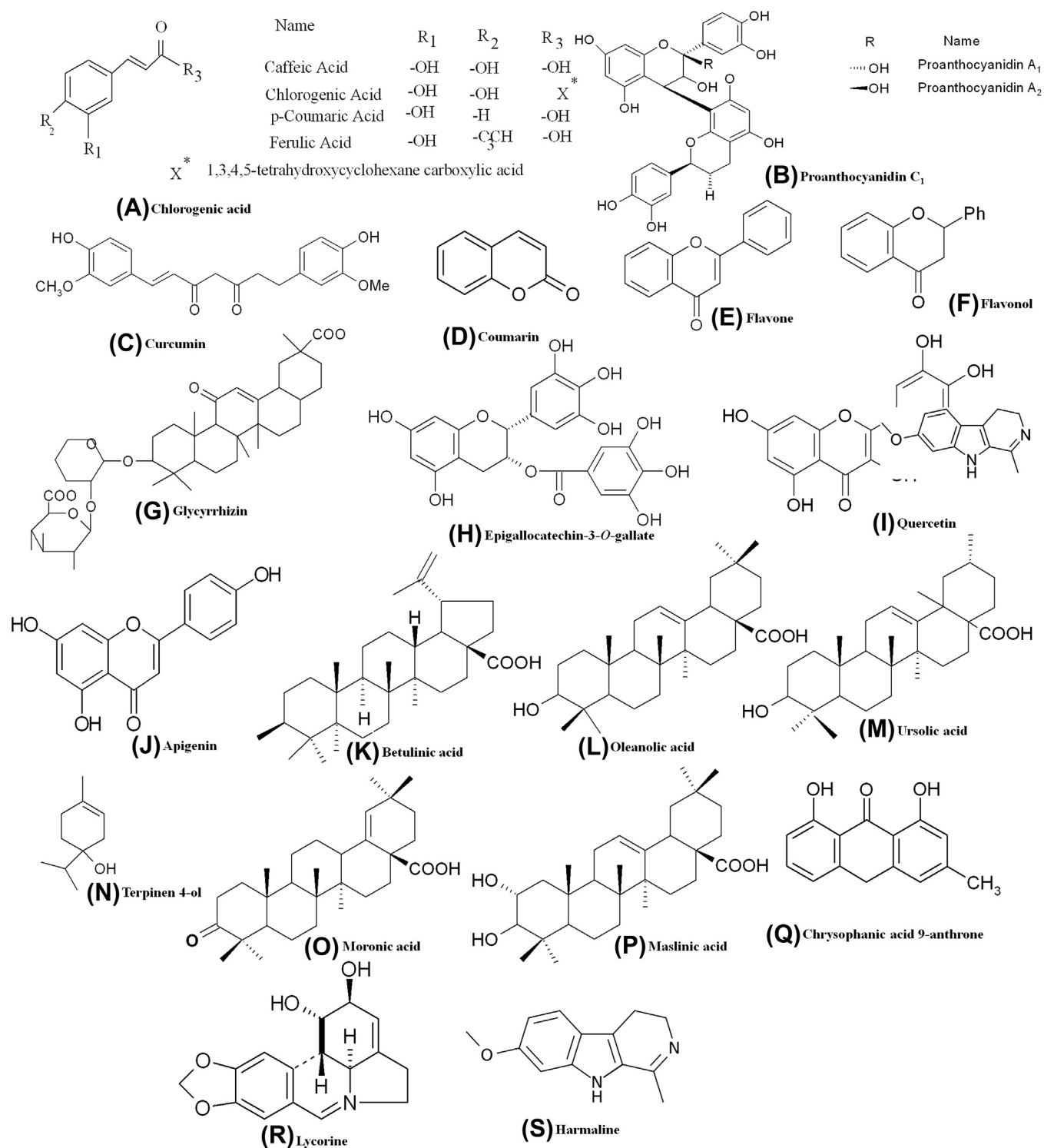
FIGURE 8.1   Structures of some important compounds.

the presence or absence of a test drug, in a microtiter plate (Figure 8.2).

This initial evaluation is usually done at a twofold concentration (with a 10-fold difference, e.g., 1 and 10 ng/ml or µg/ml). An 18 h culture (80–90% confluency) of an appropriate cell line should be added with the test drug or placebo, immediately after the virus infection. When the drug effect was tested as pre-treatment, cells need to be treated with the test drug for 15–180 min before virus infection, and incubated

Virus	Disease	In vitro assay (Cell line)	In vitro assay		In vivo model	In vivo methods
			Antiviral activity	Mechanistic study		
HSV	Herpes	Vero, MRC-5, HFF, BHK, HepG2cells	MTT assay, plaque reduction assay, CPE reduction, $TCID_{50}$ [10,11,29,30]	IFA, ELISA, quantitative RT-PCR, Western blot, electrophoretic mobility shift assay (EMSA), Attachment assay, penetration assay [10,11,29,30]	Mouse, rat, rabbit guinea pig	Skin irritation test, cutaneous lesion assay, viral vaginitis assay, ocular HSV study, latency and reactivation study, footpad/dorsal root ganglia model [10,11,29,30]
Influenza	Flu	MDCK, A549 cell lines	MTT, hemagglutination, and plaque assay [47]	Virus adsorption assay, Q-Rt PCR, Western blot, flow cytometry, IFA [47]	Mice, ferrets, Chicken	Treatment with extract/compound, mouse monitoring, histology and immunohistology [47]
Polio virus	Paralysis, aseptic meningitis	Hela, Vero, Human Rhabdomyosarcoma cells	MTT, plaque reduction And luciferase assay [125]	EMSA, Q-RT PCR, Western blot, siRNA transfection [125]	Mouse, rat	Prophylactic and therapeutic efficacy, infectivity titers in brain/spinal cord [111]
HBV	Liver inflammation, Jaundice	HepG2, HepG2.2.15, COS-7 cells	MTT, ELISA [126,127].	Q-Rt PCR, Western blot [126,127]	Mice, Ducklings	Infection and treatment, detection of duck hepatitis B virus-DNA, Histopathology of duck liver [126,127]
HCV	Liver cirrhosis	Huh7, Ava5 cells	Full-replication, replicon, and RdRp assay, $TCID_{50}$ [89,90]	Pseudoparticle infection assay, ELISA, Western blot [90].	Chimpanzee, Cynomolgus monkey	10 and 18-Day treatment schedule [90]
HIV	Acquired immunodeficiency syndrome	TZM-bl, MT-4 cells, Vk2/E6E7, lymphocytic reporter cell line	CPE reduction, TZM-bl and CEM-GFP cell-based assay [44, 85]	Quantitative RT-PCR, Virus infectivity assays, pulse-chase analysis, Western blot, siRNA transfection [85]	Transgenic mice, rabbits	Transgenic rat model, vaginal irritation study [112]
Rabies	Hydrophobia	McCoy cells	MTT assay, CPE reduction assay [128].	Confocal fluorescence Microscopy, Q-Rt PCR, Western blot [129]	Mice	Protection assay [113], determination of mortality rate [114]
DENV-2	Dengue fever	C6/36 mosquito and Vero cells	Foci forming unit reduction and prophylactic activity assay [130,131]	Quantitative RT-PCR, extracellular virucidal activity [130,131]	Mice	Virus inhibition assay [83]
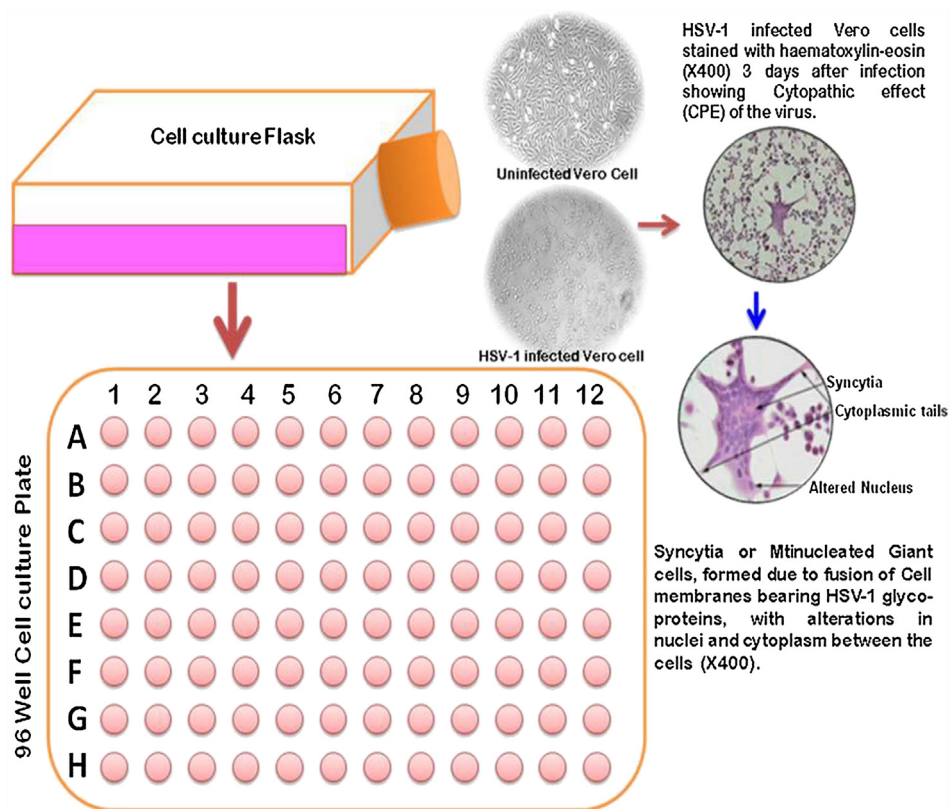
FIGURE 8.2 HSV-1 infected Vero (African green monkey kidney) cells showing CPE as the formation of Syncytia (multinucleated giant cell). This assay can be used to detect the antiviral activity of the test drug and cytopathicity can be expressed microscopically as $0 =$ No CPE; $1 = 0-25\%$; $2 = 25-50\%$; $3 = 50-75\%$; $4 = 75-100\%$.

for a standard time to induce viral CPE. After every 24 h until the end of the experiment, the plate needs to be visualized microscopically for changes in cell morphology, compared to that for the control cells. Here, the uninfected cell monolayer treated with the test drug will provide an idea of the maximum dose of drug causing minimum cell toxicity, as enlargement, granularity, rounding off, detachment, etc., and the degree of cytotoxicity as T (100% toxic), PVH (partially very heavy toxic, 80%), PH (partially heavy toxic, 60%), P (partial toxic 40%), PS (partial slightly toxic, 20%), or 0 (nontoxic, 0%). A 50% cell inhibitory or cytotoxic concentration (CC_{50}) and 50% end point of virus inhibitory or effective concentration (EC_{50}) can be determined from a graph plotted with the concentration versus cellular effect due to virus infection, while the selectivity or therapeutic index (SI or TI) is calculated as CC_{50}/EC_{50}.

8.5.1.3 MTT or MTS Assay

Another rapid and sensitive in vitro assay for the evaluation of antiviral agents is based on the spectrophotometric assessment of the viability of virus-infected or mock-infected cells, via the in situ reduction of a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS); this has an equal sensitivity to that of the plaque reduction assay [10,11,84]. The mechanism is based on the conversion of a yellow water-soluble dye MTT or MTS to a purple colored insoluble formazan crystal by the mitochondrial enzymes of the viable cells. The quantitation of the amount of formazan produced in each well is then determined spectrophotometrically at 490 nm, and the same is subtracted from the background absorption of the plate at 650 nm. The cytotoxicity of the test drug needs to be evaluated in the same plate, and for data analysis, a statistical software program is used to determine the efficacy (EC_{50}), cell toxicity (CC_{50}), and SI or TI of the test drug. This method allowed the screening of a larger number of extract/compounds at the same time with a simplified procedure.

Protocol: Cell Monolayer (100−200 μl) cultured in 10 Columns (C2−11) of a 96-well flat-bottom plate ($\sim 2 \times 10^4$ cells/ml) needs to be added at a twofold dilution of the test drug, after 24 h (eight concentrations in serum-free medium), the medium is removed and

added with the virus (0.5—5 multiplicity of infection (MOI)) in six wells of C3—11 (Rows 3—8). Then, C-2 (uninfected Control) and Rows 1—2 of C3—11 are added with the media and after adsorption (45—60 min) at 37 °C, unabsorbed viruses are removed by washing with fresh media. The infected cells should then be mixed with the test drug (0—highest concentration) in all wells of C3—11, and incubated (37 °C in a CO_2 incubator) for 24—48 h. After removing the drug-containing media, 50 μl of MTT solution (5 mg/ml in phosphate buffered saline (PBS)) should be added with the fresh media (200 μl) to all wells in C1—11, and incubated for 3—4 h at 37 °C. Finally, the medium should be replaced with dimethyl sulfoxide (DMSO; 200 μl) to dissolve the formazan in C1—11, and after 15 min, glycine buffer (25 μl) should be mixed in all wells (pH 10.5) and the absorbance should be read (570 nm). Usually, the results are calculated by plotting a graph with absorbance (Y-axis) versus drug concentration (X-axis). Here, Column 2 with no virus serves as the cell viability control and wells in Column 3 with virus without the drug serve as the virus-induced loss of cell viability. Rows 1—2 of Columns 3—11 with an increasing concentration of the drug without virus provide IC_{50} (50% cell inhibitory concentration) of the drug, while EC_{50} or EC_{90} can be deduced by plotting absorbance of virus-infected wells (Column 3, rows 3—8) versus concentration of the drug showing an increased absorbance by 50 or 90% over the virus alone [29].

8.5.1.4 TZM-bl Cell-Based Assay

This neutralizing antibody assay, used for HIV-1, simian immunodeficiency virus (SIV), and simian-HIV, is done in TZM-bl cells as it reflects the reduction in Tat-induced luciferase (Luc) reporter gene expression after a single round of virus infection. TZM-bl cells are HeLa cell clones, engineered to express cluster of differentiation 4 and C—C chemokine receptor type 5, and contain integrated reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under the control of an HIV-1 long terminal repeat. Thus, they are highly sensitive and prone to infection by immunodeficiency viruses including primary HIV-1 isolates and cloned Env-pseudotyped viruses. Here, diethylaminoethyl (DEAE) dextran is used to enhance infectivity during neutralization. Soon after infection, reporter gene expression is induced by a viral transactivator protein *Tat*. Luciferase activity can be quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inoculum [85]. This high-throughput assay requires a 96-well plate, and clonal cells provide enhanced precision and uniformity. This has been validated for a single-round infection with either uncloned viruses grown in human lymphocytes or molecularly cloned Env-pseudotyped viruses produced by transfection in 293T/17 cells [85].

Protocol: TZM-bl cells (4×10^4/well) are usually seeded in a 24-well plate and incubated overnight. In a separate vial, HIV-1NL4.3 (MOI 0.05) virion is mixed with the test drug or vehicle for 1 h at 37 °C, and then added to TZM-bl cells, and incubated for 4 h. After washing the cells (with cold PBS), fresh media with the test drug should be added and cultured for 48 h, using untreated HIV-1 infected cells (negative) and azidothymidine (AZT)-treated cells (positive) as a control. The cells then need to be washed twice with PBS, lysed with 1X lysis buffer, and the supernatant added with the substrate can be analyzed for luciferase activity in an optiplate using a fluorimeter. The results are expressed as percentage inhibition as luminescence in the experimental group (test drug or AZT)/luminescence of infected cells without the drug x 100; and percent inhibition can be calculated by subtracting the above value from 100 [44,85].

8.5.1.5 CEM-Green Fluorescent Protein Cell-Based Assay

CEM-green fluorescent protein (GFP) is a stable T-cell line-containing a plasmid encoding GFP and is suitable for HIV-1NL4.3 (MOI 0.05) culture. For postinfection, the cells (2.0×10^5/well) should be incubated with the test drug up to 8 days, using AZT and solvents (used to prepare the test drug) as control(s). The virus-infected cells are then lysed with 1x Promega cell lysis buffer (150 μl), and transferred to culture plate to read the absorbance at 485 nm (excitation) and 520 nm (emission) by means of a fluorimeter. The results can be expressed as percentage inhibition: GFP fluorescence in the experimental group/fluorescence in infected cells without the test drug X 100. Percent inhibition should be calculated by subtracting the above value from 100 [85].

8.5.1.6 Virus Yield Reduction Assay

This is generally used to confirm the results of the CPE reduction or inhibition assay with the freshly prepared test drug that showed activity in the initial experiments. After 3—5 days of the test, the cells are lysed by a freeze—thaw cycle, to elute the virion, centrifuged ($10,000 \times g$), and the resulting supernatants with increasing drug concentration are titrated for infectious virion and subjected to a fresh CPE inhibition test. Briefly, cells plated in a 96-well plate with susceptible cell lines need to be incubated for 24 h, and then added with the serially diluted virus preparations from drug-treated cells. Development of CPE indicates the presence of infectious virus, and 90% effective concentration (EC_{90}) of the test drug is determined as the concentration that inhibits virus yield by one $\log_{10}$. Infectious virus yield assay, pretreated or posttreated with the test

drug (reduced virus infectivity by 90% or $TCID_{50}/ml$) can be calculated from this assay.

Secondary testing of potential antiviral compounds: The antiviral activity of the test compound is expressed as a TI or SI, determined by dividing CC_{50} by EC_{50}. In general, an SI of ≥ 10 is considered as a potential antiviral agent, although a low SI for the positive control needs to be considered. Compounds having SI values of ≥ 10 need to be evaluated against additional virus strains to establish the full spectrum of activity, and the potential antiviral drug needs to be screened further by direct assays to measure viral replication or titer inhibition [1].

8.5.1.7 Direct Assays

Direct assays can measure $\geq 50\%$ reduction in the viral titer in the presence of the test drug, compared to the untreated cells. CPE inhibition can be determined by end point titration [86], which evaluates the virucidal activity after preincubation with the virus plus test compound [87,88]. Fifty percent end point titration is done on confluent monolayers (10^4 cells/well) infected with serial 10-fold dilutions (10^7 $TCID_{50}/ml$) of the virus suspension in 96-well plate. After adsorption for 1 h at $37\,°C$, the test drug (serial twofold dilutions) is added (to the maintenance medium with 2% fetal bovine serum (FBS) plus antibiotics), and incubated at $37\,°C$ to record the viral CPE under a light microscope after 4−5 days with virus control, uninfected drug-treated and untreated cell controls. Cytotoxicity of the test agent is the concentration that killed cell monolayers when no virus titer can be determined, while the antiviral activity is the inhibition of the virus titer at the maximum nontoxic dose of the highest concentration of the test drug without affecting cell morphology. Antiviral activity needs to be present in at least two subsequent dilutions of the test agent; otherwise, the activity is only virucidal or is due to the toxicity of the test drug. Extracellular virucidal activity can also be determined by titration of residual infectious virus particles after incubation of the test drug with virus suspension (10^6 $TCID_{50}/ml$) for 1 h at $37\,°C$.

8.5.1.8 Immunofluorescence Assay

This assay is used for the quantitative estimation of viruses against which antibodies are available, either commercially or in-house, but it is unable to differentiate between infectious and noninfectious viral particles. Briefly, the untreated or drug-treated cells are infected with a known amount of virus (MOI 5−10). After adsorption (45−60 min), the unabsorbed virus particle is removed by washing, and then added with the fresh media to incubate for 24−36 h (50% of time required to achieve 3^+ CPE). Then, the cells need to be rewashed with PBS, fixed with 3−4% paraformaldehyde, washed, and permeabilized with acetone or 0.5% triton X-100. After washing, the cells should be blocked with 1% bovine serum albumin (BSA) in PBS for 30 min followed by incubation with mouse or rabbit antibody against a specific viral protein for 1−4 h at $37\,°C$. After repeated washing, the cells need to be incubated with a fluorescent-tagged secondary antibody for 1 h, washed, and visualized under a fluorescent microscope compared to the fluorescence of untreated and drug-treated cells. Alternatively, for quantitation, the cells are trypsinized after treatment, fixed with 4% paraformaldehyde, washed, permeabilized, and then labeled with a fluorescent-tagged antibody, followed by propidium iodide (PI: 50 µg/ml in PBS). The counting of cells is done in a fluorescent-activated cell counter to quantitate the fluorescence percentage. Here, the measure of PI will indirectly measure cytotoxicity caused by drug treatment or virus infection [10,11,30].

8.5.1.9 Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) is a solid-phase rapid, sensitive, and specific assay used for the qualitative detection of the viral antigen or antibody and gross quantitation of the virus. Absolute quantitation is done by a series of predetermined viral titers in ELISA matched with unknown samples that estimate the quantity of the virus (untreated or drug-treated). As this method cannot differentiate between infectious and noninfectious virus particles, the drug affecting at the earlier or later stage of viral replication or maturation will not be differentiated. Briefly, the untreated or drug-treated cells are infected with a known amount of virus, adsorbed for 1 h, washed and incubated (2−4 days) for CPE. The virus stock harvested after freeze thawing needs to be centrifuged, and diluted for ELISA. Each well of a plate coated with virus-specific antibody needs to be mixed with 100 µl of controls or test drug and incubated (1 h, at room temperature) with Horseradish peroxidase conjugate (100 µl), alkaline phosphatase, or β-D-galactosidase-labeled virus-specific antibody. After washing five times, the substrate (100 µl) is added and reincubated in the dark for 10 min. The reaction is then stopped with a stop solution (5% H_2SO_4) and the absorbance is read (optical density $(OD)_{450}$). Alternatively, a quadruplicate cell monolayer in 96-well plates is overlaid with a $\log_{10}$ dilution of the test drug followed by infection with the virus. After 16−20 h of incubation at $37\,°C$, monolayers are fixed with 0.05% glutaraldehyde and assayed for virus-specific protein(s) on the cell surface. An ELISA is performed with monoclonal antibody to the specific protein of the corresponding virus and protein-A horseradish peroxidase conjugate, and the OD is measured at 450 nm. The results are expressed as a percentage of virus-infected cells (virus control), and the concentration causing 50% reduction in OD
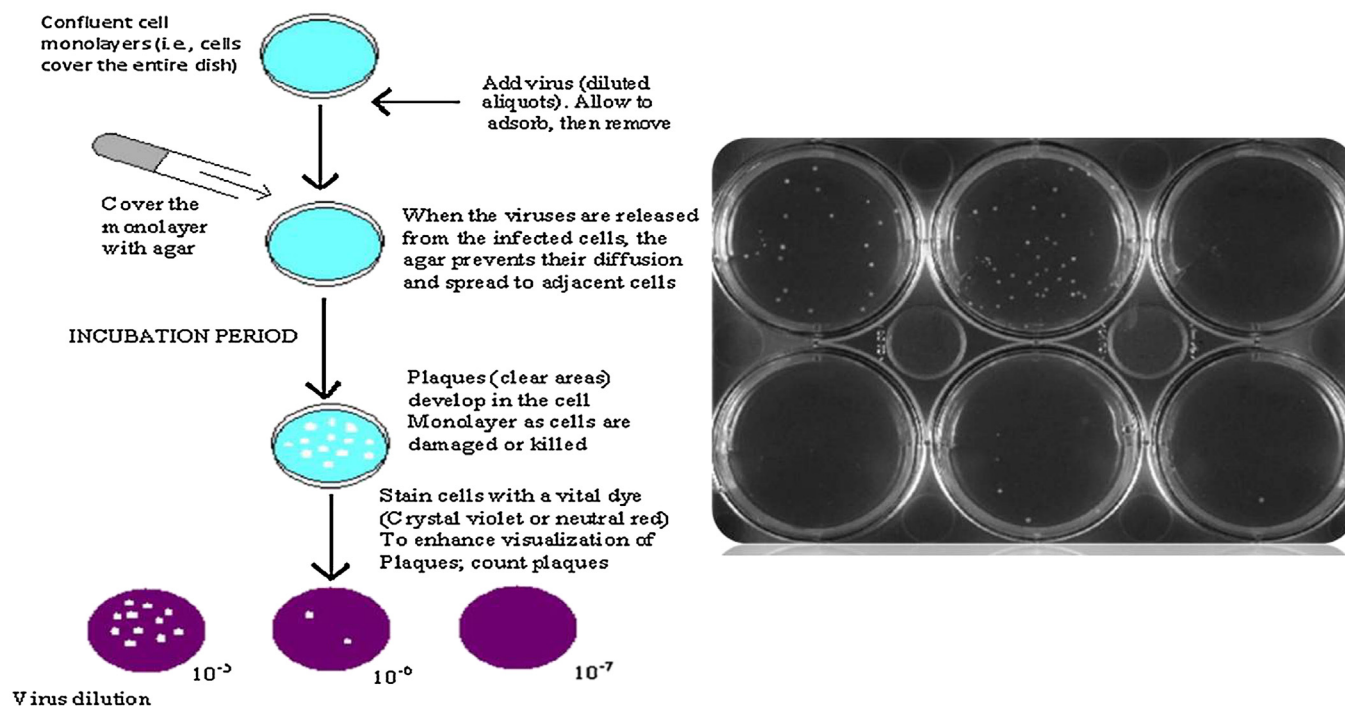
FIGURE 8.3 Schematic representation and pictures of viral plaque assay.

values (EC_{50}) should be calculated from graphic plots, by the determination of SI (ratio of CC_{50}:EC_{50}) [1,10,29].
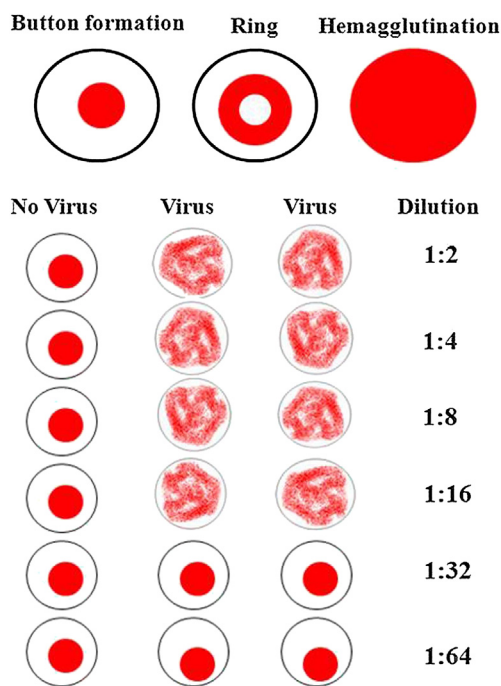
8.5.1.10 Plaque Reduction Assay

The plaque reduction assay, a phenotypic assay, can be used to determine the IC_{50} values of drugs and to determine inhibitor sensitivity (e.g., neuraminidase). In this assay, each infectious virus particle multiplies to form a localized area of infected cells or "plaque." The plaques are revealed either as areas of dead/destroyed cells detected by cellular stains or as areas of infected cells by immunostaining (Figure 8.3).

Protocol: Here, the confluent (80−90%) cell monolayer (1×10^5 cells/cm^2) is infected with a $\log_{10}$ dilution of viral plaque-forming unit (PFU) in the presence or absence of the test drug, allowed to adsorb (1 h at 37 °C in 5% CO_2), and then the cells are washed twice with prewarm minimum essential medium (MEM). Drug dilutions prepared in the overlay medium are then overlaid on the infected culture, without the test drug. Overlay medium I comprises MEM/Dulbecco's modified Eagle's medium (DMEM) with trypsin (10 μg/ml), 1% low melting agarose, without serum and the test drug. Overlay medium II comprises: 100 ml 10x MEM, supplemented with glutamine (10 ml), antibiotics (10 ml), bicarbonate (40 ml), and FBS (20 ml). Usually, 45 ml of carboxy methyl cellulose is added to 9 ml of the medium, and the plates are incubated (37 °C in 5% CO_2) for 3−5 days and then fixed with 10% formalin or 4% formaldehyde for 30 min.

The cells are then stained with methylene blue (1 ml/well) or 1% crystal violet (w/v), and rinsed with tap water, allowed to dry overnight, and the plaques (dark areas) are counted by low power magnification of a binocular microscope. The antiviral effect is usually measured as the percentage inhibition of plaque formation: [(mean number of plaques in control)−(mean number of plaques in sample)] × 100/Mean number of plaque in control. The concentration of the test drug required to inhibit up to 50% of virus growth (IC_{50} or EC_{50}), as compared to the virus control, is estimated from the graphical plot as dose−response curves by regression analysis.

8.5.1.11 Hemagglutination Assay to Measure Viral Titer

The hemagglutination activation (HA) assay is a quantitation of the viral surface or envelop hemagglutination protein of some viral families that can agglutinate (stick to) human or animal red blood cells (RBCs) and bind to its N-acetylneuraminic acid to form a lattice. In contrast to the plaque assay, HA cannot measure of viral infectivity, because no virus replication is required in this assay. It is an easy, simple, and rapid method for large samples, and the conditions depend on the type of virus, as some viruses bind RBCs only at certain pH values, others are at certain ionic strengths. If a test drug inhibits virus replication, it will also affect the viral titer and will thus reduce the HA value. Briefly, the virus dilution (1:4−1:512) is applied to an RBC dilution (0.1−0.7% for

Button formation   **Ring**   **Hemagglutination**

No Virus	Virus	Virus	Dilution
			1:2
			1:4
			1:8
			1:16
			1:32
			1:64

FIGURE 8.4   HA showing viral HA units. Lanes 2−3 has 1: 16 HA units.

30 min at 4 °C, because the viruses with neuraminidase activity will detach from the RBCs, and then the lattice-forming parts will be counted to calculate the titer as Virus concentration/ml $= 10^7$ X HA titer.

Protocol: The RBCs separated from blood is collected in Alseiver's solution (20.5 g dextrose, 8.0 g sodium citrate dihydrate, 4.2 g sodium chloride, and 0.55 g citric acid per liter, pH 6.1) and kept overnight at 4 °C. The cells are washed with PBS (2X), and the quantity of the cell pack (2.0 ml blood yields 0.5−1.0 ml pack) is measured in 10% suspension with PBS. The RBC solution (0.75%) in PBS (0.75 ml 10% RBCs in 10 ml PBS, pH 7.2) is added to each well of a 96-well plate, except for the first well of each row, along with the antigen (culture fluid containing virus from drug-treated and untreated controls) to the first two wells of each row. A twofold dilution (transferring 50 μl from the second well of each column A2-K2 to A3-K3) is mixed with 0.75% RBCs (50 ml) in all wells, and incubated for 60 min at room temperature. The cell control needs to be checked for the complete settling of the RBCs, and the results are recorded in the HA sheet. The RBCs form a button or ring at the bottom of the wells that is recorded as "O," while hemagglutination (RBCs remain in suspension) is recorded as a + symbol. The highest dilution of virus that causes complete hemagglutination is considered as the end point. The HA titer is the reciprocal of the dilution of the virus in the last well with complete hemagglutination (Figure 8.4).

Inhibition of virus hemagglutination activity (HA): Viruses having surface HA proteins, such as influenza, are able to agglutinate RBCs, which can be visualized by mixing virus dilutions with RBC, and can thus, be used to investigate the inhibitory effect of any drug onto the HA. Briefly, the 10-fold serially diluted (1−1000X) test drug, along with the diluted virus stocks (1:4 to 1:128), is used. The virus dilution (50 μl/well) is added to drug-containing wells, preincubated for 45 min, and need to be mixed with RBC (1/20 in PBS) solution. Here, up to a certain dilution, the viral particles may lose their ability to agglutinate RBCs, which indicates an interaction of the drug with the viral HA.

8.5.1.12 Virus Inactivation Assay

The virus (10^4 PFU/ml)−test drug mix is usually incubated for 1 h at 37 °C, and then diluted 100-fold (100 PFU/well) with media containing 2% FBS to get a subtherapeutic concentration of the test drug. Then, the monolayer, seeded in the 12-well plate, is mixed with the virus inoculums. For comparison, virus−test drug mix diluted 100-fold (no incubation period), are added with the respective cells for infection. The 100-fold dilution helps to titrate the test agent below its effective doses and prevent meaningful interactions with the host cell surface. After adsorption for 1 h at 37 °C, the diluted inoculums are discarded, and the cells washed with PBS, should be added with an overlay medium (with 2% FBS), and incubated at 37 °C for 72 h before being subjected to the plaque assay, and the viral plaque numbers obtained from infections set in the presence of the test drug are compared with the control [29].

8.5.1.13 Attachment Assay

Viral attachment to the host cell surface can be assayed at 4 °C, as it allows binding but prevents viral entry, by ELISA [31]. Briefly, 96-well plates seeded with susceptible cells (2×10^4 cells/well) are grown overnight, and the cell monolayers chilled at 4 °C for 1 h, are challenged with the virus (MOI 5) in the presence of the test drug using heparin as the control, for 3 h at 4 °C. The wells are then washed with ice-cold PBS, fixed with prechilled 4% paraformaldehyde in PBS for 1 h on ice, and blocked with 5% BSA at 4 °C. To detect bound virus, the samples are incubated at 37 °C for 1 h with a primary antibody in PBST (PBS with 0.05% Tween 20) plus 5% BSA. The wells are washed twice with PBST plus 5% BSA and twice with PBST only, each at 5-min interval on a shaker, and mixed with secondary antibody in PBST with 5% BSA. After incubation (37 °C for 1 h), the wells are washed and developed with a 3,3′,5,5′-tetramethyl-benzidine two-component microwell peroxidase substrate for 20 min and the reaction is stopped with 1 M phosphoric acid. The absorbance is read immediately at 450 nm, and the

values are expressed as the fold change of absorbance relative to the mock infection control [11,31].

8.5.1.14 Penetration Assay

Cell monolayers grown in 12-well plates are chilled at 4 °C for 1 h and incubated with the virus (100 PFU/well) for 3 h at 4 °C. The infected cell monolayer is then reincubated with the test drug, or heparin (100 µg/ml), for 20 min at 37 °C to facilitate penetration. Then, the extracellular virus is inactivated by citrate buffer (pH 3.0) for 1 min, and washed with PBS before being overlaid with DMEM containing 2% FBS. After 48 h of incubation at 37 °C, viral plaques are stained and counted [31].

8.5.1.15 Virus Adsorption Assay

The plated cells (0.8×10^5 cells/well for a 12-well plate) grown overnight at 30% confluence are added (300 µl) with virus dilution, and DEAE dextran at a final concentration of 20 µg/ml. After adsorption (2 h at 37 °C in CO_2 incubator), the plates are placed in a rocker, to prevent the cells from drying, and fresh medium (1−2 ml) containing the test drug is added to each well and incubated for 40−48 h in 5% CO_2 at 37 °C, for subconfluent growth. After removing the media, fixing solution (1−2 ml) is added to each well and incubated for 5 min at room temperature (β-galactosidase activity decreases dramatically if the fixing solution is left for >10 min). Then on discarding the fixing solution, the cells are washed twice with PBS, stained, and incubated at 37 °C for 50 min. Finally, the plates are stained to count the number of blue syncytia, and the titration values are expressed as the number of stained cells multiplied by the viral dilution.

8.5.1.16 Replicon Assay

Cells harboring viral subgenomic replicon are usually maintained with 0.25 mg/mL G418, and the viral replicon cells should be seeded in a 96-well plate and incubated at 37 °C in 5% CO_2. After 24 h, the culture medium needs to be replaced with a medium containing the serially diluted test drug with 2% FBS and 1% DMSO and incubated for 72 h. Then, the total RNAs are extracted, and the RNA levels are quantified by a quantitative real-time polymerase chain reaction (qRTPCR), using a real-time PCR system with specific primers, and glyceraldehyde-3-phosphate dehydrogenase gene as control. Antiviral activity can be determined by RNA levels in drug-treated cells as compared to that in mock-treated cells [89].

8.5.1.17 Pseudoparticle Infection Assay

This assay requires HIV-1 based pseudoparticles containing HCV E1E2 or vesicular stomatitis virus (VSV) glycoprotein G. Pseudotyped viruses added to Huh7.5.1 cells are incubated for 3 h at 37 °C, and mixed with the test agent 15 min prior to virus addition. After removing the supernatant, the cells are incubated at 37 °C for 72 h and measured for luciferase activity [90].

8.5.2 Studies on the Mechanism of Action

The mechanism of action of a test drug can be predicted from the studies of addition and removal of the drug along with inhibition of preinfection, coinfection, and postinfection, as depicted in Figure 8.5.

Based on the time when the test drug yielded maximum inhibition, a mechanistic study can be done by targeting each and every step (surface glycoproteins for fusion and entry, different proteins, transcriptional and translational events for gene expression) separately, using several modern methods.

8.5.2.1 Confocal Fluorescence Microscopy

Cells adsorbed on a microscopy-adapted slide for 6 days will be infected with the virus in the presence or absence of the test drug for 3 h. The cells are then washed and incubated with or without polyclonal anti-gp160 (50 µg/ml) antibodies at 4 °C for 30 min, rewashed with PBS, 0.01% azide, 0.5% BSA, and labeled with polyclonal mouse antihuman immunoglobulin G−fluorescein isothiocyanate (FITC), and then fixed with 1% paraformaldehyde. Cover slides need to be mounted in Mowiol to be observed by sequential acquisition using a microscope with planapochromat ×63, 1.4 numerical aperture oil immersion objectives, and optical sections at an image resolution of 512×512 pixels [85].

8.5.2.2 Quantitative Real-Time PCR

Real-time PCR is widely accepted for viral load determination due to its rapidity, low interassay and intraassay variabilities, sensitivity, reproducibility, and reduced risk of carryover contamination. The method involves the direct measurement of the amount of PCR product obtained, even during the reaction. Mathematical analysis of the data, and comparison to control reactions containing known amounts of template, allows the calculation of the input DNA amount in the initial reaction. The severity of some diseases can be correlated well with the viral load, making real-time PCR quantitation useful, not only the presence of a virus but also the viral reactivation or persistence in disease progression [91−93]. This property is successfully adapted to screen potential antiviral compounds in vitro, where the fold decrease in viral genetic material in the presence of drug will measure its antiviral activity.

8.5.2.3 Quantitative Reverse Transcription-PCR

Briefly, cDNA prepared from the respective RNA virus by an RT kit is subjected to quantitative real-time
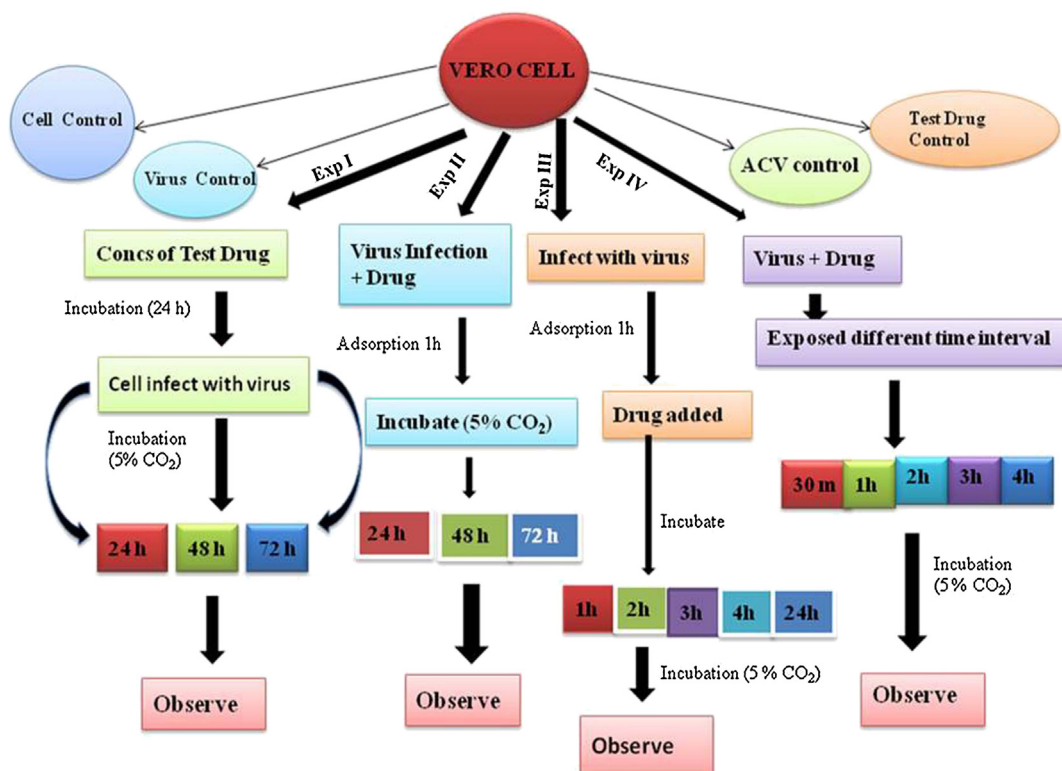
FIGURE 8.5 Schematic protocol of time of addition and removal along with preinfection, coinfection, and postinfection assays of drugs against viruses grown in Vero cells (e.g., Herpes simplex virus, Dengue).

PCR using cDNA (5 μl) in a volume of 25 μl with a Taq-Man1 PCR Core Reagent Kit. Primers and probe are used at the optimum concentration. For PCR amplifications, carried out as per the manufacturer's instructions, RNA/DNA of the known virus serves as the positive control, and water serves as the negative control. Amplification of target DNA and detection of PCR products are performed with a GeneAmp1 5700 Sequence Detection System. Amplification of the target sequence is detected by an increase in fluorescence above a baseline with no or little change in fluorescence. To analyze the data, the reporter fluorescence is automatically normalized to a passive reference to avoid the measurement of non-PCR-related fluorescence [94]. A threshold is set above the baseline to get a threshold cycle value (Ct), which is the cycle number at which the fluorescence passes the fixed threshold with a statistically significant increase in fluorescence. The qPCR amplification standard curve for the test agent is designed from Ct values versus the log of standard concentrations [95–97].

Calculation and interpretation of antiviral effect: A higher Ct value corresponding to the drug-treated sample, compared with the untreated one, is the antiviral effect of the drug when the viral load ($\times 10^6$ particles or fold decrease) calculated from the standard plot of Ct values is less in the drug-treated sample than in the untreated

one. From these data, IC_{50} or IC_{90} (50 or 90% reduction) of viral particles or of fold change may also be calculated.

8.5.2.4 Western Blot Analysis

The corresponding cell lines (1×10^6 cells/well) at an 80–90% confluence are infected with a five concentrations of $\log_{10}$ dilution of PFU of the respective virus, in the absence or presence of a test drug. The infected cells were incubated for 24 h at 37 °C in 5% CO_2, and equal amounts of protein (40 μg/sample) extract from whole cells are harvested in buffer (200 μl/well) containing 20 mM Tris (pH 7 ± 0.5), 50 mM NaCl, 5% nonyl phenoxypolyethoxylethanol-40 (NP-40%), and 0.05% deoxycholate (DOC), added with 2X sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1:1) and heated to 100 °C for 5 min. The gel washed with SDS-PAGE running buffer is then loaded with the above sample and run at 100 V through the stacking part at 200 V, after the proteins move through the stack and migrate through the resolving gel (until the blue dye front reaches the end of the glass plates). After soaking a PVDF membrane in methanol (30 second) and distilled water the membrane will be placed in between two fiber pads and four Whatmann

papers (precut) in a shallow tray, fitted with a Transfer buffer for a few minutes. After cutting off the stacking gel and soaking in the transfer buffer (few min), the gel is transferred in gel cassettes to prepare the transfer sandwich, cover it, and insert the gel cassette into the electrode module and the bioice cooling unit into the buffer chamber (filled with the transfer buffer), and allow 1—2 h at 4 °C at 100 V. The gel is then stained with 1X Pongee S for 1 min, destained with ddH_2O, rinsed with PBS, and the membrane is incubated with blocking buffer on a shaker for 1—2 h at 37° or at 4 °C overnight. The membrane is then incubated with the diluted primary antibody (in dilution buffer) on a shaker for 1 h at 37 °C or overnight at 4 °C, washed four times with washing buffer (5—10 min). The membrane is then incubated with the diluted secondary antibody (in blocking buffer) on a shaker for 1 h at 37 °C or overnight at 4 °C, washed four times with washing buffer for 5—10 min, and visualized using the commercial enhanced chemiluminescence Western blot detection kit.

8.5.2.5 Pulse-Chase Analysis

This examines a time-dependent cellular process by successive exposure of the cells to a labeled compound (pulse) and then in an unlabeled form (chase), by radioactivity. Corresponding cell lines (1×10^6 cells/well) cultured in a six-well plate at an 80—90% confluency is infected with five concentrations of $\log_{10}$ dilution of viral PFUs, either in the absence or presence of a test drug. The infected cells are incubated for 24 h at 37 °C in 5% CO_2, and then equal amounts of protein (40 µg/sample) extract from whole cells are harvested in buffer (200 µl/well) containing 20 mM Tris (pH 7 ± 0.5), 50 mM NaCl, 5% NP-40%, and 0.05% DOC. Synthesized protein labeled with Click-iT Metabolic Labeling Reagents and 293T cells transfected with pNL4-3 are used. At 3 h posttransfection, the cell supernatant is replaced with fresh medium with or without 10 µM fangchinoline. After 24 h, the cells need to be labeled with azidohomoalanine (AHA; 50 µM) for 1 h in methionine-free medium with or without fangchinoline. The supernatant is then replaced with complete medium in the presence or absence of fangchinoline, and the cells should be chased for the indicated time, after being lysed in lysis buffer (50 mM Tris HCl pH 8.0, 1% SDS, protease inhibitor). The AHA-incorporated protein is then biotinylated by the Click-instant Protein reaction buffer Kit and Biotin Alkyne. After precipitation and dissolution, the biotinylated protein needs to be collected with Dynabeads MyOne Streptavidin T1, and the purified nascent protein will be eluted into the SDS-PAGE buffer by boiling for Western blot analysis [85].

8.5.2.6 Flow Cytometry

The cells (10^6/well) were first dissociated by a nonenzymatic cell dissociation buffer, and then infected with the virus (MOI 1) in the presence or absence of the test drug for 1 h at 37 °C, using DMSO (0.1%) as control. The cells are washed twice with ice-cold fluorescence-activated cell sorting (FACS) buffer (1X PBS, 2% fetal calf serum (FCS), and 0.1% sodium azide), need to be blocked with 5% FCS for 30 min on ice, and then stained with an FITC-conjugated antibody, washed with FACS buffer, and then fixed with 1% paraformaldehyde before being subjected to standard flow cytometry analysis. Normal rabbit serum can serve as the isotype control, while the data acquisition and flow Cytometry can be performed on a Cyan flow cytometer [31].

8.5.2.7 Electrophoretic Mobility Shift Assay

The mobility shift electrophoresis is a gel or band shift retardation assay, which uses the common affinity electrophoresis technique for studying protein—DNA or protein—RNA interactions to determine whether a protein or mixture of proteins is capable of binding to a given DNA or RNA sequence, and to indicate whether more than one protein molecule is involved in the binding complex. Gel shift assays are often performed to study transcription initiation, DNA replication, DNA repair, or RNA processing and maturation. Here, an oligonucleotide sequence (5′-GCATGCTAATGA-TATTCTTTG-3′) of the promoter gene of a virus (e.g., ICP0 of HSV) is biotinylated by a Biotin 3′ end DNA labeling kit. The nuclear extracts of virus-infected cells treated with the test drug for the indicated time are mixed with reaction mixtures (20 µl) containing 3 µg of nuclear extracts, 20 fmol of Biotin 3′ end-labeled probe, 50 ng/µl of poly (dI-dC), 2.5% glycerol, 0.05% NP-40 (1%), 5 mM $MgCl_2$, and 1X binding buffer. After incubation for 20 min at room temperature, reaction mixtures are applied to 4% polyacrylamide gels in 0.5X Tris-borate-ethylenediaminetetraacetic acid buffer at 4 °C, and the gels are transferred to Nylon membranes using a Semi Dry Transfer Cell (Bio-Rad, USA). The transferred oligos are then immobilized by ultraviolet crosslinking for 10 min. For detection of bound oligos, membranes are blocked with blocking buffer, followed by the addition of Streptavidin—Horseradish Peroxidase conjugate and developed according to the manufacturer's instructions. For supershift assays, nuclear extracts are preincubated with host cell factor-1 polyclonal antibodies for 30 min on ice [9,10].

8.5.2.8 Small Interfering RNA Transfection

Small interfering RNA (siRNA or silencing RNA) is a short (21—23 bp), double-stranded RNA nucleotide, and its transfection is "transference," involved in the

silencing of genes. The siRNA is extremely valuable in silencing gene expression and studying gene functions. Usually, the corresponding cells are treated with siRNA, specific for specific viral gene, and grown in 6/12-well plates at a 70−80% confluency, and the medium is removed. The cells are washed twice with PBS, and the transfection mixture [71.5 pmol of duplex siRNA (siRNA[specific gene] or siRNA[lamin A/C]) and 0.25% Lipofectamine 2000 in OptiMEM] is incubated for 30 min at room temperature before adding to each well. The cells are then incubated with the transfection mixture for 4−5 h at 37 °C in 5% CO_2. The transfection medium is then replaced with fresh MEM with 10% FBS, repeated at 15 h posttransfection, and the transfected cells are infected with specific viruses at 36 hour posttransfection (hpt) to perform the immunofluorescence assays as mentioned above.

8.5.3　In vivo Assays

The in vitro antiviral activity of the test drug can be validated by in vivo testing in specific models. On the basis of the target site of infection and disease presentation, various animal models, namely, mouse, guinea pigs, ferrets, rabbit, and primates, can be used for different viruses.

8.5.3.1　Herpes Simplex Virus

HSV is an enveloped double-stranded DNA virus, classified into two types HSV-1 and HSV-2. Usually, HSV-1 is associated with orolabialis, keratitis, and encephalitis, whereas HSV-2 is associated with genital herpes [98], and both are transmitted through close personal contact [99]. HSV can hide from the immune attack of the host by latency and reactivate frequently to cause recurrent episodes [100]. Moreover, HSV-2 is a high risk factor for acquisition of HIV infection [101,102]. A recent report showed that HSV-suppressive therapy can greatly reduce HIV-1 load in persons coinfected with HSV [103]. Moreover, the determinants of effective immunity against HSV infection are not yet identified [104,105], and therapeutic vaccines failed to provide protection from infection or recurrences [104]. Hence, broad spectrum antiviral(s) of natural origin is an important strategy for the management of HSV infection. Thus, any agent that showed anti-HSV activity is usually tested for toxicity along with a skin irritation test to determine its acceptability for topical application, while in vivo efficacy studies are performed by developing cutaneous, vaginal, ocular (Figure 8.6), and latency associated infection models, when required.

8.5.3.1.1　Skin Irritation Test

The dermal hypersensitivity and related allergic manifestation of any anti-HSV agent alone or in ointment dosage form can be tested in batches (3) of mice. Specific doses of test drug/ointment (0.5% w/w) or ointment base are applied on the shaved and cleaned dorsal area (100- to 150-mm² area) of each animal. After 4 h, the residual ointment is removed, washed, and blotted dry to observe for any signs of inflammation, redness, flash, flare, and wheel corresponding to hypersensitivity. For further confirmation of dermal toxicity, the dorsal hair of female mice is shaved (with hair remover cream, cleaned with luke warm water, and dried) and the naked skin (100- to 150-mm² area) is abraded with a dermal (Seven-Star) needle to apply 0.1 g of ointment (desired %) to the abraded area of cohorts of animals. After 24 h, the ointment is removed, washed with warm water, and the animals are examined for erythema and edema within another 1 h. The animals are also observed up to the next 72 h for additional confirmation of toxicity.

8.5.3.1.2　Cutaneous Lesion Assay

To study the drug potency on HSV-1-induced cutaneous lesion, the dorsal skin of mice/Guinea pigs can be prepared and abraded (as above), and the abraded area is divided into four quadrants, and each is infected with HSV-1 (30 μl of 10-fold dilution), and observed up to 10 days for herpes lesion. On the basis of the initial result, stock virus (usually 150 μl of 10^8 PFU) is used to infect a 42-cm² area for consistent lesion development. Once the lesion develops, animal cohorts (n = 15) are infected and treated with the (1) test drug or drug-based ointment, (2) 3−5% (wt/wt) acyclovir, or (3) ointment base (1.5 g per dose) on the infected area with sterile cotton swabs, twice daily for 6 days (Figures 8.7 and 8.8).

The extent of the lesion needs to be scored daily as 1.0−1.6, lesions on one-fourth of the infected area; 1.7−2.4, lesions on one-half of the infected area; 2.5−3.2, lesions on three-fourths of the infected area; and 3.3−4.0, lesions on the entire infected area [106].

8.5.3.1.3　Reduction in Viral Vaginitis in Mice or Cotton Rats

To test the in vivo efficacy of test drug/formulation against HSV-2, the genital herpes model with random breed BALB/c female mice (Figure 8.9) or *Sigmodon hispidus* (cotton) rats [107] is developed by intravaginal inoculation of the virus in anesthetized 6-week-old animals.

After one-week of acclimatization (23 ± 3 °C), the animals (n = 10 for each dilution) will be inoculated with the virus (30 μl of 10^{-3} stock) into the vagina by a needle (size 12) and observed for 12 days for vaginitis or lethality (LD_{50}). To test the efficacy, fresh batches of animals are infected with 10 LD_{50} of the virus (10^5 PFU). Following inoculation, a vaginal cotton swab sample is collected from each animal, transferred to 0.5 ml of PBS, and stored at −20 °C. The animals are divided

DERMAL TOXICITY

Remove Dorsal Hair, Clean

Naked Skin (6cm X 7cm)

WASH (warm water)

DRY

CUTANEOUS LESION

ABRADED ⟹ Divided in 4 Quadrant

Apply Compound (Different Potency)

Infect with HSV-1 (30μl of 10-fold diluted)

REMOVE Compound After 24 h

OBSERVE [10 days] for herpetic lesion

WASH and DRY (Warm water)

DETERMINE Virus titer

EXAMINE for Erythema/Edema (1h later to 72 h)

INFECT Cohorts of animals

Solvent Control   Drug Control   Virus Control   Test Compound (Different Potency) Twice daily for 7 days

OBSERVE Lesion and SCORE (0-10) daily
[1.0-1.6 on ¼ of infected area; 1.7-2.4 on ½ infected areas; 2.5-3.2 on ¾ infected areas; 3.3-4.0 on entire infected area].

GENITAL HERPES

HSV-2 Stock

DILUTE (10-fold)

VAGINAL Inoculation

OBSERVE (Vaginitis/Lethality)

DETERMINE Median LD_{50}

FRESH Animal [Female] Vaginal inoculation with 10 MLD

Collect Vaginal sample in 0.5 ml PBS, Store at -20°C

CONTROL GROUP   Experiment (Vaginitis)

1. Uninfected Control
2. Virus Control
3. Solvent Control
4. Drug Control]

TREATMENT with (Compound) for 6 days

RECORD Mortality & No of days

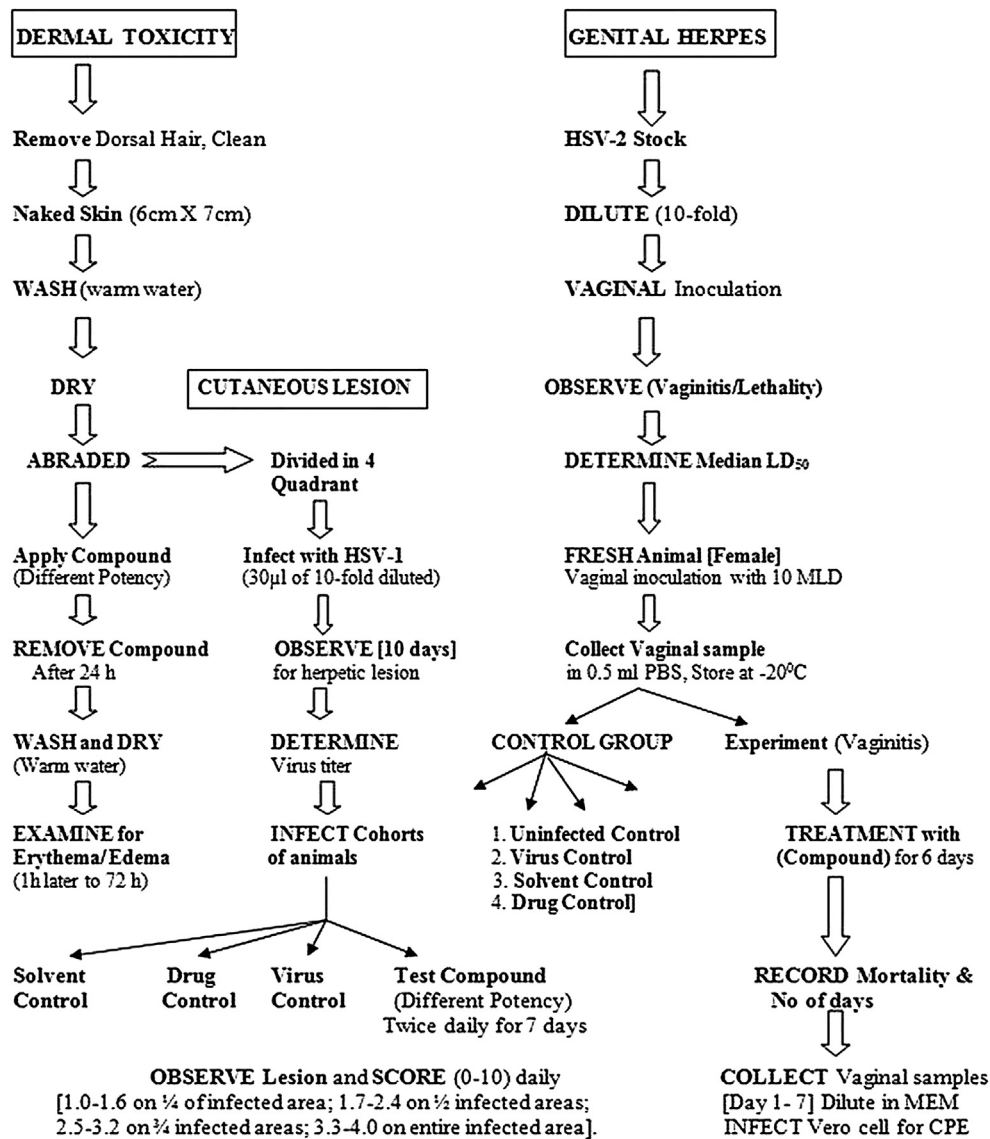COLLECT Vaginal samples [Day 1- 7] Dilute in MEM INFECT Vero cell for CPE

FIGURE 8.6   Dermal toxicity, cutaneous and vaginal infection model flowchart for herpes simplex virus.

Group 1(n=5)   Group 2 (n=5)   Group 3 (n=5)

6X7 cm²

Test Drug   Acyclovir control   Control (infected without treatment)

Dorsal skin abraded (4 quadrants)

Infected (HSV-1,10^8 pfu)

Observed (10 days for Herpes lesion)

Animal Infected (n=15)

Treatment (Test Drug/acyclovir by sterile cotton swabs twice daily, 6-days)

Lesion Score (Daily)
1.0-1.6= lesions on 1/4 of infected area; 1.7-2.4 = on 1/2 of infected area; 2.5-3.2 = on 3/4 of infected area; 3.3-4.0= on entire infected area (Zhang et al., 2007).
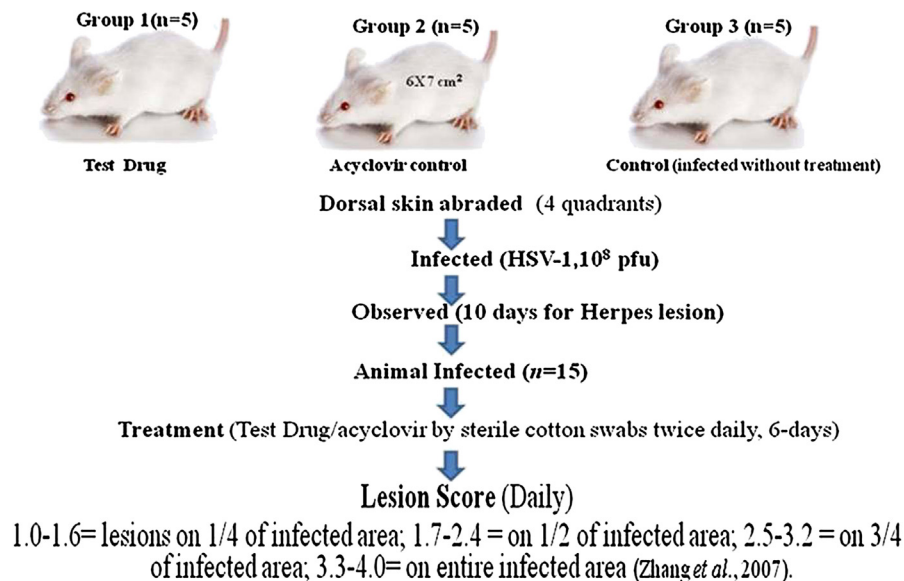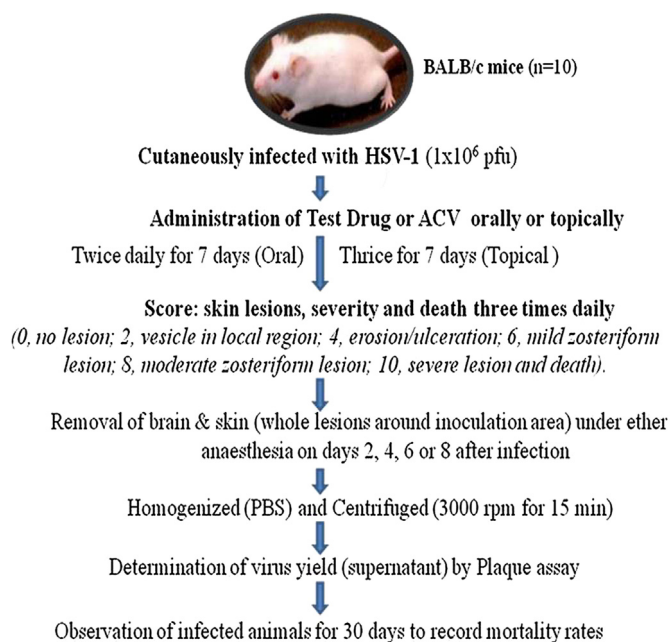
FIGURE 8.7   Cutaneous model development (outline).

FIGURE 8.8 Efficacy test protocol on cutaneously infected mice (schematic).

into test groups (different potency), positive control (acyclovir), negative control (solvent or ointment base), and no treatment (virus control) groups along with an additional uninfected control group. Symptoms of viral vaginitis (topical edema of the vaginal tract with turbid secretions) will be observed on the third day of infection. Treatment begins on day 3 postinfection, by applying the test agent or formulations to the vaginal tract with cotton swabs (2 mg per mouse) twice daily for 6 days [106]. Mortality and the number of days for mortality to occur are recorded. The vaginal swab samples are
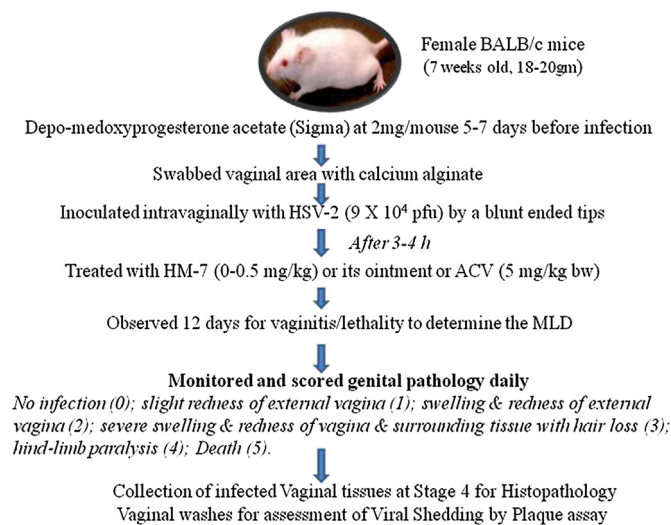
FIGURE 8.9 Viral virginities model with HSV-2.

collected from day 1 following the completion of treatment, as well as from the deceased animals immediately following their death (Figure 8.5). The vaginal samples are then diluted five times in MEM and used to infect Vero cells. Samples that gave a positive CPE are considered positive for HSV-2 [106].

8.5.3.1.4 Vaginal Inoculation of HSV in Guinea Pigs

Vaginal inoculation of female Guinea pigs with HSV-1 or HSV-2 results in obvious primary infection. Following recovery, survivors of primary infection periodically display vesicular recrudescence in the vaginal area from which infectious virus and/or viral DNA can be recovered. Although reactivation cannot be reliably induced, the HSV-2 spontaneously reactivates with a much higher frequency than HSV-1, making it a very attractive model for comparative analysis of the influence of viral genes on reactivation and HSV recombinant viruses. This model helps to investigate and identify important features in this difference. The value of guinea pigs in studying drug or vaccine efficiency and experimental pathogenesis makes this an extremely valuable and promising system.

8.5.3.1.5 Ocular Herpes Virus Model

A fast, simple reactivation model to study ocular herpes virus infection and latency is developed by Gordon et al. [108] in New Zealand female rabbits (1.5−2.0 kg). Following topical anesthesia (0.5% proparacaine HC1 eye drops), each unscarified rabbit eye will be inoculated with a thymidine kinase-positive HSV-1W (5×10^4 pfu/eye) into the lower fornix, following topical anesthesia with eye drops. The virus establishes latency and reactivates [109]. Successful inoculation (100%) of eyes on day 7 will produce herpetic dendritic ulcers with significant HSV-1 titer (10^4 pfu/ml) and viral shedding, determined by a neutralization test. After satisfactory anesthesia, the globe is proptosed with a wooden cotton applicator and an operating microscope for all surgical manipulations. The intrastromal injection (0.25-ml tuberculin syringe with a no. 30 short bevel needle) is given into the central corneal stroma. The first group is given deionized sterile endotoxin-free water; the second group is given 100 μl air, while the 3rd group is not given any injection. For all three groups, the needle should be carefully withdrawn with gentle pressure so that the proptosed globe gently returns to the orbit. The anterior chamber, injected with deionized sterile water, is made at the limbus, inserted into the anterior chamber parallel to the iris plane. The needle is carefully withdrawn, and the insertion site is pressed with a cotton swab for 30 s, to avoid aqueous loss, and return of the proptosed globe to its proper place in the orbit. For topical administration, 100 ml deionized sterile water is administered onto the cornea of the proptosed globe

by means of a pipette, and the globe returns to the orbit by gentle digital pressure. Viral Shedding (latent HSV-1 after reactivation and induced shedding into the tear film) is determined by swabbing the eyes 2 days before treatment and for seven consecutive days after the treatment. Each eye swab is mixed with 0.3 ml MEM (Eagle's medium with Earle's salt, 10% newborn calf serum, 1% penicillin–streptomycin, 1% Fungizone), vortexed, and the eluant is plated onto a Vero cell monolayer. After a 1-h adsorption, 1.5 ml of medium is added to the well, and the plate is examined daily for 7 days for the CPE of HSV-1. Random HSV-1 isolates can be confirmed by neutralization [108,110].

8.5.3.1.6 Latency and Reactivation in Rabbits

Infection of rabbit eyes leads to a latent infection in which virus can be recovered from the trigeminal nerve ganglia following explantation and cocultivation with indicator cells. In addition, virus can be sporadically recovered from the eye following latency. In fact, reactivation can be efficiently induced by the iontophoresis of epinephrine into the eye, and this model can help to establish the latency associated transcript (LAT) expression for efficient reactivation.

8.5.3.1.6 The Mouse Eye or Trigeminal Ganglia Model

A second murine model for HSV-1 and HSV-2 latency involves the infection of the cornea followed by latency in the trigeminal ganglia. As in the footpad model, latent HSV genomes express LAT in a portion of those neurons maintaining them, and the virus can be recovered by cocultivation of the explanted ganglia. Interestingly, this method is similar to an in vivo method. Here, latently infected mice are transiently exposed to hyperthermia, and then trigeminal ganglia are excised, sectioned, and assayed for the presence of observable virus by immunohistochemistry or genetic engineering (when recombinant virus with an expressible marker in the genome is used).

8.5.3.2 *Influenza Virus*

Inbred female Balb/c or C57Bl/6 mice of six to eight weeks old are anesthetized by an intraperitoneal injection [150 µl of ketamine rompun-solution (2%-rompun and 10% ketamine solution are mixed at 1:10 with PBS)] and infected intranasally (i.n.) with 1×10^2 pfu/ 50 µl of Influenza A virus. For infection of Influenza A virus, preincubated with the specific drug, $102/25$ µl, $103/25$, or $104/25$ µl of virus is incubated with 25 µl drug (1 mg/ml) or with 25 µl PBS for 30 min at room temperature [68].

8.5.3.2.1 Treatment of Balb/C Mice with the Test Drug

Eight-week-old BALB/c mice are anesthetized using an intraperitoneal (i.p.) injection of ketamine (200 µl of 10% ketamine solution) and xylazin (2% xylazin solution) at 1:10 with PBS. Mice are treated with the test drug using the COAALA Mouse Aerosol Application System. BALB/c mice are placed in the tube cylinder and exposed to 1.5 ml (per mouse), 2 bars of an aerosolized test agent for 10 min twice a day for 3 (lung titer) or 5 days (body weight), along with a control group (H_2O). Mice are infected 10 min after the first exposure via the intranasal route with the virus A/FPV/ Bratislava/79 (H7N7) (10^3 pfu) or the recombinant A/ Puerto Rico/8/34 (H1N1rec) (4×10^2 pfu) in a 50 µl volume to determine the viral replication in the lung and monitor the body weight. The health status of the animals is controlled twice a day, and their body weight is measured every day. The animals are sacrificed upon a body weight loss of 20%. For the determination of lung virus titer, mouse lungs are collected in PBS (3 ml) on day 3 post-infection (p.i.) and homogenized using a FastPrep24 homogenizator with Lysing Matrix D, centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatants are subjected to the plaque assay.

8.5.3.2.2 Mouse Monitoring

Body temperature and gross motor activity of the animals are monitored with suitable software (Vital View®) and hardware (Mini Mitter, USA) for data acquisition on physiological parameters. The hardware includes a transmitter (E-Mitter)/receiver system that collects data on temperature and motor activity. The vitality or gross motor activity provides a basic index of the movement of mice with implanted E-Mitter. As the mouse moves, the movement of the implanted E-Mitter results in subtle changes in the transmitted signal detected by a receiver and registered by a computer. The Vital View software recorded an index of movement every 5 min to produce a longitudinal record of the activity, and for implantation of E-Mitters the mice are anesthetized with an i.p. injection of 150 µl ketamine/rompun. The shaved ventral surface of the abdomen with a midline abdominal skin incision 0.5–1 cm below the diaphragm with a 2-cm length. The abdomen is opened with a 2-cm incision along the linea alba to position the E-Mitter in the abdominal cavity. Then the closure of the incision is achieved with wound clips (autoclip 9 mm; Becton & Dickinson, Germany). The animals are placed into the cage, and successful implantation of the E-Mitter is controlled by Vital View software. Their health status is controlled for seven days before infection.

8.5.3.2.3 Histology and Immunohistology

Mice are treated with the nebulized extract (10 mg/ml) three times at 9 am, 12 am, and 3 pm for 10 min or with H_2O. Immediately after the treatment, the mice are killed and the collected lungs are fixed in buffered 4%

paraformaldehyde, and stained with hematoxylin—eosin. Lectin staining of lung sections is performed with *Sambucus nigra* agglutinin (SNA; Vector Laboratories) for sialic acid 2, 6 linked to galactose and *Maackia amurensis* agglutinin (MAL II; Vector Laboratories) for sialic acid 2, 3 linked to galactose. Secondary staining is done with the ABC-kit for 30 min at room temperature, and substrate reaction with the Diaminiobenzidine (DAB) kit.

8.5.3.3 Dengue Virus

The dengue virus (DENV) is a mosquito-borne *Flavivirus* (*Flaviviridae*) virus with a positive sense single-stranded RNA [115,116] of about 11,000 bases, which codes for capsid protein C; membrane protein M; envelop protein E; seven nonstructural NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5; and short noncoding regions on $5'$ and $3'$ ends [115,117]. Further classification of each serotype (1—5) into genotypes often relates to the region where particular strains are found.

8.5.3.3.1 Virus Inhibition Assay

The inhibitory potential of a test drug can be evaluated in suckling mice [118]. Briefly, the serial twofold dilutions of test agent are mixed with 100 LD_{50} of virus in equal proportions and incubated for 1 h at room temperature. After incubation, 0.02 ml of the mixture (virus + drug) per mouse pup should be inoculated intracerebrally along with the virus and mice control, and observed daily for signs of Dengue, that is, weight loss, slow gait, inability to suck mother's milk, and flaccid paralysis followed by death. On postinoculation days 5—6, the mouse pups are collected, killed (chloroform inhalation), and stored at $-20\,°C$ for RNA extraction. In addition to the preincubation inhibition assay, pretreatment and posttreatment of the test drug are attempted as per the same protocol, except that the time of drug addition is varied.

RT-PCR: In order to demonstrate the presence or absence of viral RNA in infected C6/36 cells and mouse brain, RT-PCR can be performed [118], employing group specific primers. Briefly, RNA is extracted and eluted and then subjected to RT-PCR after initial reverse transcription with Moloney murine leukemia virus reverse-transcriptase (MMLV-RT) to synthesize the cDNA. The cDNA synthesis is usually carried out in a 10-µl volume with RT-mix (5 × -RT buffer, 10 mM dNTPs, 5U RNasin, 5U MMLV-RT) at $37\,°C$ for 1 h using a downstream consensus primer (MP4). The amplification of cDNA is carried out by PCR (50 µl volume) congaing PCR mix (10 × -buffer, 1.5 mM MgCl2, 10 mM dNTPs, 2.5 U Taq-DNA Polymerase) using an upstream consensus primer (MP3) in a DNA thermal cycler. The thermal profile of the PCR reaction includes initial denaturation at $95\,°C$ for 2 min followed by 30 cycles of denaturation at $94\,°C$ 30 s, annealing at $54\,°C$, 2 min, and extension at $72\,°C$, 2 min, and final extension at $72\,°C$, 10 min. The amplified product can be analyzed on a 2% agarose gel for the presence of 511 bp virus-specific amplicon.

8.6 FUTURE PROSPECTS AND DIRECTIONS

Not only are the existing viral diseases fatal but also there is an upsurge of new viral infections worldwide. The currently available antivirals, though effective, are costly and beyond the reach of a vast majority of people. Thus, the development of safe, effective, and low cost antiviral drugs such as RT inhibitors is among the top priorities, as many viruses are not yet curable and have high mortality rates. Recently, considerable work has been done on medicinal plants focusing on anti-HIV activity [119—122], and there has been a considerable rise in the use of over-the-counter plant products containing orthodox drugs. The rationale is to reduce the side effects and to produce synergistic effects. But since in most cases pharmacological mechanisms of the combinations are not studied, adverse effects or therapeutic failures have been observed [123]. The most important consideration involving medicinal plants is to identify and standardize the method of preparation of extract, appropriate season of collecting plant material, and details of its administration [20,124]. Since a significant number of plant extracts has yielded positive results, it seems reasonable to conclude that there are probably many potential antiviral agents, and further characterization of active ingredients of those potential plants will reveal useful compounds.

References

[1] Chattopadhyay D, Sarkar MC, Chatterjee T, Sharma DR, Bag P, Chakraborti S, et al. Recent advancements for the evaluation of anti-viral activities of natural products. N Biotechnol 2009;25: 347—68.

[2] Chattopadhyay D, Chakraborty MS, Saha GC. Viruses, the acellular parasites of cellular hosts: biology and pathology with special reference to HIV. Ind J STD AIDS 1999;20:54—60.

[3] Wagner EK, Hewlett MJ. Basic virology. 1st ed. Blackwell Science, Inc; 1999. ISBN 1-4051-0346-9.

[4] Chattopadhyay D, Bhattacharya SK. ethnopharmacology: a new search engine for the development of antivirals from naturaceuticals. In: Eddouks M, editor. Handbook of ethnopharmacology. Trivandrum: Research Signpost Publication; 2008. p. 129—97.

[5] Chattopadhyay D, Naik TN. Antivirals of ethnomedicinal origin: structure—activity relationship and scope. Mini Rev Med Chem 2007;7:275—301.

[6] Tombacz K, Patterson R, Grierson SS, Werling D. Lack of genetic diversity in newly sequenced porcine circovirus type 1 strains isolated 20 years Apart. Genome Announc 2014;2:e00156—14.

[7] Khan MT, Ather A, Thompson KD, Gambari R. Extracts and molecules from medicinal plants against herpes simplex viruses. Antiviral Research 2005;67:107—19.

[8] Chattopadhyay D. Ethnomedicinal antivirals: scope and opportunity. In: Ahmad I, Aquil F, Owais M, editors. Modern phytomedicine: turning medicinal plants into drugs. Wiley-VCH; 2006. p. 313—38.

[9] Naithani R, Huma LC, Holland LE, Shukla D, McCormick DL, Mehta RG, et al. Antiviral activity of phytochemicals: a comprehensive review. Mini Rev Med Chem 2008;8:1106—33.

[10] Bag P, Ojha D, Mukherjee H, Halder UC, Mondal S, Chandra NS, et al. An indole alkaloid from a tribal folklore inhibits immediate early event in HSV-2 infected cells with therapeutic efficacy in vaginally infected mice. PLoS One 2013;8:e77937.

[11] Bag P, Ojha D, Mukherjee H, Halder UC, Mondal S, Biswas A, et al. A dihydro-pyrido-indole potently inhibits HSV-1 infection by interfering the viral immediate early transcriptional events. Antiviral Res 2014;105:126—34.

[12] Mukherjee H, Ojha D, Bag P, Chandel HS, Bhattacharyya S, Chatterjee TK, et al. Anti-herpes virus activities of *Achyranthes aspera*: an Indian ethnomedicine, and its triterpene acid. Microbiol Res 2013;168:238—44.

[13] Christopher ME, Wong JP. Recent developments in delivery of nucleic acid-based antiviral agents. Curr Pharm Des 2006;12:1995—2006.

[14] Kleymann G. New antiviral drugs that target herpesvirus helicase primase enzymes. Herpes 2003;10:46—52.

[15] Miserocchi E, Modorati G, Galli L, Rama P. Efficacy of valacyclovir vs acyclovir for the prevention of recurrent herpes simplex virus eye disease: a pilot study. Am J Ophthalmol 2007;144:547—51.

[16] Sweetman S. The complete drug reference. 34th ed. London: Pharmaceutical Press; 2004.

[17] Narayana K. A purine nucleoside analogue-acyclovir [9-(2-hydroxyethoxymethyl)-9h-guanine] reversibly impairs testicular functions in mouse. J Toxicol Sci 2008;33:61—70.

[18] Sawyer MH, Webb DE, Balow JE, Straus SE. Acyclovir-induced renal failure, clinical course and histology. Am J Med 1988;84:1067—71.

[19] Greco M, Silva AP, Merchán-Hamann E, Jeronymo ML, Andrade JC, Greco DB. Differences in HIV-risk behaviour of bisexual men in their relationships with men and women. Revista de Saúde Pública 2007;41:109—17.

[20] Chattopadhyay D, Khan MTH. Ethnomedicines and ethnomedicinal phytophores against herpes viruses. Biotechnology Annual Review 2008;14:297—348.

[21] Jadhav P, Kapoor N, Thomas B, Lal H, Kshirsagar N. Antiviral potential of selected Indian medicinal (Ayurvedic) plants against herpes simplex virus 1 and 2. N Am J Med Sci 2012;4:641—7.

[22] McCutcheon AR, Roberts TE, Gibbons E, Ellis SM, Babiuk LA, Hancock RE, et al. Antiviral screening of British Columbian medicinal plants. J Ethnopharmacol 1995;49:101—10.

[23] Chamorro C, de Latorre FJ, Montero A, Sánchez-Izquierdo JA, Jareño A, Moreno JA, et al. Comparative study of propofol versus midazolam in the sedation of critically ill patients: results of a prospective, randomized, multicenter trial. Crit Care Med 1996;24:932—9.

[24] Chamorro G, Salazar M, Favila L, Bourges H. Pharmacology and toxicology of *Spirulina* alga. Rev invest clin 1996;48:389—99.

[25] Clercq De E. Novel compounds in preclinical/early clinical development for the treatment of HIV infections. Rev Med Virol 2000;10:255—77.

[26] Schaeffer DJ, Krylov VS. Anti-HIV activity of extracts and compounds from algae and cyanobacteria. Ecotoxicol Environ Saf 2000;45:208—27.

[27] Alvarez AL, Habtemariam S, Juan-Badaturuge M, Jackson C, Parra F. In vitro anti HSV-1 and HSV-2 activity of *Tanacetum vulgare* extracts and isolated compounds: an approach to their mechanisms of action. Phytother Res 2011;25:296—301.

[28] Kurokawa M, Basnet P, Ohsugi M, Hozumi T, Kadota S, Namba T, et al. Anti-herpes simplex virus activity of moronic acid purified from *Rhus javanica* in vitro and in vivo. J Pharmacol Exp Ther 1999;289:72—8.

[29] Ojha D, Mukherjee H, Ghosh S, Bag P, Mondal S, Chandra NS, et al. Evaluation of anti-infective potential of a tribal folklore *Odina wodier* Roxb against some selected microbes and herpes simplex virus associated with skin infection. J Appl Microbiol 2013;115:1317—28.

[30] Bag P, Chattopadhyay D, Mukherjee H, Ojha D, Mandal N, Sarkar MC, et al. Anti-herpes virus activities of bioactive fraction and isolated pure constituent of *Mallotus peltatus*: an ethnomedicine from andaman islands. Virol J 2012;9:98.

[31] Lin LT, Chen TY, Chung CY, Noyce RS, Grindley TB, McCormick C, et al. Hydrolyzable tannins (chebulagic acid and punicalagin) target viral glycoprotein—glycosaminoglycan interactions to inhibit herpes simplex virus 1 entry and cell-to-cell spread. J Virol 2011;85:4386—98.

[32] Zhou M, Xu M, Ma XX, Zheng K, Yang K, Yang CR, et al. Antiviral triterpenoid saponins from the roots of *Ilex asprella*. Planta Med 2012;78:1702—5.

[33] Bharitkar YP, Bathini S, Ojha D, Ghosh S, Mukherjee H, Kuotsu K, et al. Antibacterial and antiviral evaluation of sulfonoquinovosyldiacylglyceride: a glycolipid isolated from *Azadirachta indica* leaves. Lett Appl Microbiol 2014;58:184—9.

[34] Yarmolinsky L, Huleihel M, Zaccai M, Ben-Shabat S. Potent antiviral flavone glycosides from Ficus benjamina leaves. Fitoterapia 2012;83:362—7.

[35] Chuanasa T, Phromjai J, Lipipun V, Likhitwitayawuid K, Suzuki M, Pramyothin P, et al. Anti-herpes simplex virus (HSV-1) activity of oxyresveratrol derived from Thai medicinal plant: mechanism of action and therapeutic efficacy on cutaneous HSV-1 infection in mice. Antiviral Res 2008;80:62—70.

[36] Suedee A, Tewtrakul S, Panichayupakaranant P. Anti-HIV-1 integrase activity of *Mimusops elengi* leaf extracts. Pharm Biol 2014;52:58—61.

[37] Liang CQ, Luo RH, Yan JM, Li Y, Li XN, Shi YM, et al. Structure and bioactivity of triterpenoids from the stems of *Schisandra sphenanthera*. Arch Pharmacal Res 2014;37:168—74.

[38] Qian Y, Guan T, Tang X, Huang L, Huang M, Li Y, et al. Maslinic acid, a natural triterpenoid compound from *Olea europaea*, protects cortical neurons against oxygen—glucose deprivation-induced injury. Eur J Pharmacol 2011;670:148—53.

[39] Asada Y, Sukemori A, Watanabe T, Malla KJ, Yoshikawa T, Li W, et al. Stelleralides A-C, novel potent anti-HIV daphnane-type diterpenoids from *Stellera chamaejasme* L. Organic Letter 2011;13:2904—7.

[40] Lubbe A, Seibert I, Klimkait T, Van der Kooy F. Ethnopharmacology in overdrive: the remarkable anti-HIV activity of *Artemisia annua*. J Ethnopharmacol 2012;141:854—9.

[41] Paskaleva EE, Arra M, Liu Y, Guo H, Swartz G, Kennedy JS, et al. Evaluation of potential genotoxicity of HIV entry inhibitors derived from natural sources. PLoS One 2014;9:e93108.

[42] Jadulco RC, Pond CD, Van Wagoner RM, Koch M, Gideon OG, Matainaho TK, et al. 4-Quinolone alkaloids from *Melochia odorata*. J Nat Prod 2014;77:183—7.

[43] Talwar GP, Dar SA, Rai MK, Reddy KV, Mitra D, Kulkarni SV, et al. A novel polyherbal microbicide with inhibitory effect on bacterial, fungal and viral genital pathogens. Int J Antimicrob Agents 2008;32:180—5.

[44] Helfer M, Koppensteiner H, Schneider M, Rebensburg S, Forcisi S, Müller C, et al. The root extract of the medicinal plant *Pelargonium sidoides* is a potent HIV-1 attachment inhibitor. PLoS One 2014;9:e87487.

[45] Dai JP, Zhao XF, Zeng J, Wan QY, Yang JC, Li WZ, et al. Drug screening for autophagy inhibitors based on the dissociation of

Beclin1-Bcl2 complex using BiFC technique and mechanism of eugenol on anti-influenza A virus activity. PLoS One 2013a;8: e61026.

[46] Dai JP, Wu LQ, Li R, Zhao XF, Wan QY, Chen XX, et al. Identification of 23-(s)-2-amino-3-phenylpropanoyl-silybin as an antiviral agent for influenza A virus infection in vitro and in vivo. Antimicrob Agents Chemother 2013b;57:4433−43.

[47] Ehrhardt C, Dudek SE, Holzberg M, Urban S, Hrincius ER, Haasbach E, et al. A plant extract of Ribes nigrum folium possesses anti-influenza virus activity in vitro and in vivo by preventing virus entry to host cells. PLoS One 2013;8:e63657.

[48] He W, Han H, Wang W, Gao B. Anti-influenza virus effect of aqueous extracts from dandelion. Virol J 2011;8:538.

[49] Yang F, Zhou WL, Liu AL, Qin HL, Lee SM, Wang YT, et al. The protective effect of 3-deoxysappanchalcone on in vitro influenza virus-induced apoptosis and inflammation. Planta Med 2012;78: 968−73.

[50] Dai J, Wang G, Li W, Zhang L, Yang J, Zhao X, et al. High-throughput screening for anti-influenza A virus drugs and study of the mechanism of procyanidin on influenza A virus-induced autophagy. J Biomol Screen 2012;17:605−17.

[51] Kiyohara H, Ichino C, Kawamura Y, Nagai T, Sato N, Yamada H, et al. In vitro anti-influenza virus activity of a cardiotonic glycoside from Adenium obesum (Forssk). Phytomedicine 2012;19: 111−4.

[52] Park JY, Jeong HJ, Kim YM, Park SJ, Rho MC, Park KH, et al. Characteristic of alkylated chalcones from Angelica keiskei on influenza virus neuraminidase inhibition. Bioorg Med Chem Lett 2011;21:5602−4.

[53] Garozzo A, Timpanaro R, Stivala A, Bisignano G, Castro A. Activity of Melaleuca alternifolia (tea tree) oil on Influenza virus A/PR/8: study on the mechanism of action. Antiviral Res 2011; 89:83−8.

[54] Shin HB, Choi MS, Ryu B, Lee NR, Kim HI, Choi HE, et al. Antiviral activity of carnosic acid against respiratory syncytial virus. Virol J 2013;10:303.

[55] Glatthaar-Saalmüller B, Rauchhaus U, Rode S, Haunschild J, Saalmüller A. Antiviral activity in vitro of two preparations of the herbal medicinal product Sinupret against viruses causing respiratory infections. Phytomedicine 2011;19:1−7.

[56] Wang KC, Chang JS, Lin LT, Chiang LC, Lin CC. Antiviral effect of cimicifugin from Cimicifuga foetida against human respiratory syncytial virus. Am J Chin Med 2012;40:1033−45.

[57] Zhao Y, Geng CA, Sun CL, Ma YB, Huang XY, Cao TW. Polyacetylenes and anti-hepatitis B virus active constituents from Artemisia capillaris. Fitoterapia 2014;95:187−93.

[58] Zhou WB, Zeng GZ, Xu HM, He WJ, Tan NH. Astataricusones A-D and astataricusol A, five new anti-HBV shionane-type triterpenes from Aster tataricus L. f. Molecules 2013;18:14585−96.

[59] Wang HL, Geng CA, Ma YB, Zhang XM, Chen JJ. Three new secoiridoids, swermacrolactones A-C and anti-hepatitis B virus activity from Swertia macrosperma. Fitoterapia 2013;89:183−7.

[60] Jiang ZY, Liu WF, Zhang XM, Luo J, Ma YB, Chen JJ. Anti-HBV active constituents from Piper longum. Bioorg Med Chem Lett 2013;23:2123−7.

[61] Wei W, Li X, Wang K, Zheng Z, Zhou M. Lignans with anti-hepatitis B virus activities from Phyllanthus niruri L. Phytother Res 2012;26:964−8.

[62] Ratnoglik SL, Aoki C, Sudarmono P, Komoto M, Deng L, Shoji I, et al. Antiviral activity of extracts from Morinda citrifolia leaves and chlorophyll catabolites, pheophorbide and pyropheophorbide a, against hepatitis C virus. Microbiol Immunol 2014;58: 188−94.

[63] Fu SB, Yang JS, Cui JL, Sun DA. Biotransformation of ursolic acid by Syncephalastrum racemosum CGMCC 3.2500 and anti-HCV activity. Fitoterapia 2013;86:123−8.

[64] Haid S, Novodomská A, Gentzsch J, Grethe C, Geuenich S, Bankwitz D, et al. A plant-derived flavonoid inhibits entry of HCV genotypes into human hepatocytes. Gastroenterology 2012;143:213−22.

[65] Rehman S, Ashfaq UA, Riaz S, Javed T, Riazuddin S. Antiviral activity of Acacia nilotica against Hepatitis C Virus in liver infected cells. Virol J 2011;8:220.

[66] Takeshita M, Ishida Y, Akamatsu E, Ohmori Y, Sudoh M, Uto H, et al. Proanthocyanidin from blueberry leaves suppresses expression of subgenomic hepatitis C virus RNA. J Biol Chem 2009;284: 21165−76.

[67] Tsujimoto K, Sakuma C, Uozaki M, Yamasaki H, Utsunomiya H, Oka K, et al. Antiviral effect of pyridinium formate, a novel component of coffee extracts. Int J Mol Med 2010;25:459−63.

[68] Visintini Jaime MF, Redko F, Muschietti LV, Campos RH, Martino VS, Cavallaro LV. In vitro antiviral activity of plant extracts from Asteraceae medicinal plants. Virol J 2013;10:245.

[69] Melo FL, Benati FJ, Roman Jr WA, de Mello JC, Nozawa C, Linhares RE. The in vitro antiviral activity of an aliphatic nitro compound from Heteropteris aphrodisiaca. Microbiol Res 2008; 163:36−9.

[70] Semple SJ, Pyke SM, Reynolds GD, Flower RL. In vitro antiviral activity of the anthraquinone chrysophanic acid against poliovirus. Antiviral Res 2001;49:169−78.

[71] Semple SJ, Nobbs SF, Pyke SM, Reynolds GD, Flower RL. Antiviral flavonoid from Pterocaulon sphacelatum, an Australian aboriginal medicine. J Ethnopharmacol 1999;68:283−8.

[72] Kang SY, Kang JY, Oh MJ. Antiviral activities of flavonoids isolated from the bark of Rhus verniciflua stokes against fish pathogenic viruses in Vitro. J Microbiol 2012;50:293−300.

[73] Micol V, Caturla N, Pérez-Fons L, Más V, Pérez L, Estepa A. The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). Antiviral Res 2005;66: 129−36.

[74] Li SY, Chen C, Zhang HQ, Guo HY, Wang H, Wang L, et al. Identification of natural compounds with antiviral activities against SARS-associated coronavirus. Antiviral Res 2005;67:18−23.

[75] Fiore C, Eisenhut M, Krausse R, Ragazzi E, Pellati D, Armanini D, et al. Antiviral effects of Glycyrrhiza species. Phytother Res 2008;22:141−8.

[76] De Tommasi N, Conti C, Stein ML, Pizza C. Structure and in vitro antiviral activity of triterpenoid saponins from Calendula arvensis. Planta Med 1991;57:250−3.

[77] Cella M, Riva DA, Coulombié FC, Mersich SE. Virucidal activity presence in Trichilia glabra leaves. Rev Argent Microbiol 2004;36: 136−8.

[78] Yamai M, Tsumura K, Kimura M, Fukuda S, Murakami T, Kimura Y. Antiviral activity of a hot water extract of black soybean against a human respiratory illness virus. Biosci Biotechnol Biochem 2003;67:1071−9.

[79] Shang L, Qu Z, Sun L, Wang Y, Liu F, Wang S, et al. Astragaloside IV inhibits adenovirus replication and apoptosis in A549 cells in vitro. J Pharm Pharmacol 2011;63:688−94.

[80] Lazreg Aref H, Gaaliche B, Fekih A, Mars M, Aouni M, Pierre Chaumon J, et al. In vitro cytotoxic and antiviral activities of Ficus carica latex extracts. Nat Prod Res 2011;25:310−9.

[81] Oliveira GL, Cardoso SK, Lara CR, Vieira Jr TM, Guimarães EF, Figueiredo LS, et al. Chemical study and larvicidal activity against Aedes aegypti of essential oil of Piper aduncum L. (Piperaceae). An Acad Bras Ciênc 2013;85:1227−34.

[82] Zandi K, Teoh BT, Sam SS, Wong PF, Mustafa MR, Abubakar S. Novel antiviral activity of baicalein against dengue virus. BMC Complement Alternat Med 2012;12:214.

[83] Parida MM, Upadhyay C, Pandya G, Jana AM. Inhibitory potential of neem (Azadirachta indica Juss.) leaves on dengue virus type-2 replication. J Ethnopharmacol 2002;79:273−8.

[84] Sudo K, Konno K, Yokota T, Shigeta S. A sensitive assay system screening antiviral compounds against herpes simplex virus type 1 and type 2. J Virol Methods 1994;49:169–78.

[85] Wan Z, Lu Y, Liao Q, Wu Y, Chen X. Fangchinoline inhibits human immunodeficiency virus type 1 replication by interfering with gp160 proteolytic processing. PLoS One 2012;7: e39225.

[86] Berghe Vanden DA, Haemers A, Vlietinck AJ. Antiviral agents from higher plants and an example of structure–activity relationship of 3-methoxyflavones. In: Colegate SM, Milyneux RJ, editors. Bioactive natural products: detection, isolation, and structural determination. Florida: CRC Press; 1993. p. 405–40.

[87] Vlietinck AJ, De Bruyne T, Vanden Berghe D. Plant substances as antiviral agents. Curr Org Chem 1997;1:307–44.

[88] Apers S, Cimanga K, Vanden Berghe D, Van Meenen E, Longanga AO, Foriers A, et al. Antiviral activity of simalikalactone D, a quassinoid from *Quassia Africana*. Planta Med 2002; 68:20–4.

[89] Kim JW, Park SJ, Lim JH, Yang JW, Shin JC, Lee SW, et al. Triterpenoid saponins isolated from *Platycodon grandiflorum* inhibit hepatitis C virus replication. Evid-Based Complement Alternat Med 2013;2013:560417.

[90] Takebe Y, Saucedo CJ, Lund G, Uenishi R, Hase S, Tsuchiura T, et al. Antiviral lectins from red and blue–green algae show potent in vitro and in vivo activity against hepatitis C virus. PLoS One 2013;8:e64449.

[91] Kimura H, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, et al. Quantitative analysis of Epstein–Barr virus load by using a real-time PCR assay. J Clin Microbiol 1999;37:132–6.

[92] Laue T, Emmerich P, Schmitz H. Detection of dengue virus RNA inpatients after primary or secondary dengue infection by using the TaqMan automated amplification system. J Clin Microbiol 1999;37:2543–7.

[93] Tanaka N, Kimura H, Iida K, Saito Y, Tsuge I, Yoshimi A, et al. Quantitative analysis of cytomegalovirus load using a real-time PCR assay. J Med Virol 2000;60:455–62.

[94] Josefsson A, Livak K, Gyllensten U. Detection and quantification of human papillomavirus by using the fluorescent 59 exonuclease assay. J Clin Microbiol 1999;37:490–6.

[95] Locatelli G, Santoro F, Veglia F, Gobbi A, Lusso P, Malnati MS, et al. Real-time quantitative PCR for human herpesvirus 6 DNA. J Clin Microbiol 2000;38:4042–8.

[96] Loeb KR, Jerome KR, Goddard J, Huang M, Cent A, Corey L. High-throughput quantitative analysis of hepatitis B virus DNA in serum using the TaqMan fluorogenic detection system. Hepatology 2000;32:626–9.

[97] Schutten M, van den Hoogen B, van der Ende ME, Gruters RA, Osterhaus AD, Niesters HG. Development of a real-time quantitative RT-PCR for the detection of HIV-2 RNA in plasma. J Virol Method 2000;88:81–7.

[98] Smith JS, Robinson NJ. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. J Infect Dis 2002;186:S3–28.

[99] Hardin TC. Sexually transmitted diseases. In: Herfindal ET, Gourley DR, editors. Textbook of therapeutics-drug and disease management. Baltimore: Williams & Wilkins; 1996. p. 1389–404.

[100] Tyring SK. Advances in the treatment of herpesvirus infection: the role of famciclovir. Clin Ther 1998;20:661–70.

[101] Cowan FM, French RS, Mayaud P, Gopal R, Robinson NJ, de Oliveira SA, et al. Seroepidemiological study of herpes simplex virus types 1 and 2 in Brazil, Estonia, India, Morocco, and Sri Lanka. Sex Transm Infect 2003;79:286–90.

[102] White MK, Gorrill TS, Khalili K. Reciprocal transactivation between HIV-1 and other human viruses. Virology 2006;352: 1–13.

[103] Nagot N, Ouédraogo A, Foulongne V, Konaté I, Weiss HA, Vergne L, et al. Reduction of HIV-1 RNA levels with therapy to suppress herpes simplex virus. N Engl J Med 2007;356:790–9.

[104] Stanberry LR. Clinical trials of prophylactic and therapeutic herpes simplex virus vaccines. Herpes 2004;11:161A–9A.

[105] Koelle DM, Huang J, Hensel MT, McClurkan CL. Innate immune responses to herpes simplex virus type 2 influence skin homing molecule expression by memory CD4+ lymphocytes. J Virol 2006;80:2863.

[106] Zhang Y, But PP, Ooi VE, Xu HX, Delaney GD, Lee SH, et al. Chemical properties, mode of action, and *in vivo* anti-herpes activities of a lignin–carbohydrate complex from *Prunella vulgaris*. Antiviral Res 2007;75:242–9.

[107] Yim KC, Carroll CJ, Tuyama A, Cheshenko N, Carlucci MJ, Porter DD, et al. The cotton rat provides a novel model to study genital herpes infection and to evaluate preventive strategies. J Virol 2005;79:14632–9.

[108] Gordon YJ, Romanowski E, Araullo-Cruz T. A fast, simple reactivation method for the study of HSV-1 latency in the rabbit ocular model. Invest Ophthalmol Vis Sci 1990;31:921–4.

[109] Gordon YJ, Araullo-Cruz TP, Romanowski E, Ruziczka L, Balouris C, Oren J, et al. The development of an improved reactivation model for the study of HSV-1 latency. Invest Ophthalmol Vis Sci 1986;27:1230.

[110] Gordon YJ, Armstrong JA, Brown SI, Becker Y. The role of herpesvirus type 1 thymidine kinase in experimental ocular infections. Am J Ophthalmol 1983;95:175.

[111] McKinlay MA, and Steinberg BA. Oral efficacy of WIN 51711 in mice infected with human poliovirus. Antimicrobial Agents and Chemotherapy 29:30–2.

[112] Lodmell DL, Esposito JJ, Ewalt LC. Rabies virus anti-nucleoprotein antibody protects against rabies virus challenge in vivo and inhibits rabies virus replication in vitro. J Virol 1993;67(10):6080–6.

[113] Finke S, Conzelmann KK. Replication strategies of rabies virus. Virus Res 2005;111:120–31.

[114] Admasu P, Deressa A, Mengistu A, Gebrewold G, Feyera T. In vivo antirabies activity evaluation of hydroethanolic extract of roots and leaves of *Phytolacca dodecandra*. Global Veterinaria 2014;12:12–8.

[115] Rodenhuis-Zybert IA, Wilschut J, Smit JM. Dengue virus life cycle: viral and host factors modulating infectivity. Cell Mol Life Sci 2010;67:2773–86.

[116] WHO. Dengue guidelines for diagnosis, treatment, prevention and control. World Health Organization; 2009. ISBN 92-4-154787-1.

[117] Hanley KA, Weaver SC. Frontiers in dengue virus research. Caister Academic Press; 2010. ISBN 978-1-904455-50-9.

[118] Parida MM, Upadhyay C, Pandya G, Jana AM. Inhibitory potential of neem (*Azadirachta indica* Juss) leaves on Dengue virus type-2 replication. J Ethnopharmacol 2002;79:273–8.

[119] Hussein G, Miyashiro H, Nakamura N, Hattori M, Kawahata T, Otake T, et al. Inhibitory effects of Sudanese plant extracts on HIV-1 replication and HIV-1 protease. Phytother Res 1999;13: 31–6.

[120] Premanathan M, Kathiresan K, Yamamoto N, Nakashima H. *In vitro* anti-human immunodeficiency virus activity of polysaccharide from *Rhizophora mucronata* Poir. Biosci Biotechnol Biochem 1999;63:1187–91.

[121] Calabrese C, Berman SH, Babish JG, Ma X, Shinto L, Dorr M, et al. A phase I trial of andrographolide in HIV positive patients and normal volunteers. Phytother Res 2000;14:333–8.

[122] Asres K, Bucar F, Kartnig T, Witvrouw M, Pannecouque C, De Clercq E. Antiviral activity against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) of ethnobotanically selected Ethiopian medicinal plants. Phytother Res 2001;15:62–9.

[123] Chan PK, Ng HK, Cheung JL, Cheng AF. Survey for the presence and distribution of human herpesvirus 8 in healthy brain. J Clin Microbiol 2000;38:2772–3.

[124] Chattopadhyay D, Arunachalam G, Mandal AB, Bhattacharya SK. Dose dependent therapeutic antiinfectives from ethnomedicines of Bay Islands. Chemotherapy 2006;52:151–7.

[125] Bufalo MC, Figueiredo AS, de Sousa JP, Candeias JM, Bastos JK, Sforcin JM. Anti-poliovirus activity of *Baccharis dracunculifolia* and propolis by cell viability determination and real-time PCR. J Appl Microbiol 2009;107:1669–80.

[126] Li J, Huang H, Feng M, Zhou W, Shi X, Zhou P. In vitro and in vivo anti-hepatitis B virus activities of a plant extract from *Geranium carolinianum* L. Antiviral Res 2008;79:114–20.

[127] Hana Y-Q, Huang Z-M, Yang X-B, Liud H-Z, Wua G-X. *In vivo* and *in vitro* anti-hepatitis B virus activity of total phenolics from *Oenanthe javanica*. J Ethnopharmacol 2008;118:148–53.

[128] Chavez JH, Leal PC, Yunes RA, Nunes RJ, Barardi CR, Pinto AR, et al. Evaluation of antiviral activity of phenolic compounds and derivatives against rabies virus. Vet Microbiol 2006;116:53–9.

[129] Lee JH, Park DY, Lee KJ, Kim YK, So YK, Ryu JS, et al. Intracellular reprogramming of expression, glycosylation, and function of a plant-derived antiviral therapeutic monoclonal antibody. PLoS One 2013;8:e68772.

[130] Zandi K, Teoh BT, Sam SS, Wong PF, Mustafa MR, Abubakar S. Antiviral activity of four types of bioflavonoid against dengue virus type-2. Virol J 2011;8:560.

[131] Zandi K, Lim TH, Rahim NA, Shu MH, Teoh BT, Sam SS, et al. Extract of *Scutellaria baicalensis* inhibits dengue virus replication. BMC Complement Alternat Med 2013;13:91.

LIST OF ABBREVIATIONS

AHA Azidohomoalanine
AIDS Acquired immunodeficiency syndrome
AZT Azidothymidine
BSA Bovine serum albumin
CC$_{50}$ 50% cytotoxic concentration
CCR5 C–C chemokine receptor type 5
CD4 Cluster of differentiation 4
CNS Central nervous system
CO$_2$ Carbon dioxide
CPE Cytopathic effects
DAB kit Diaminiobenzidine (DAB) as substrate used by Vectastain ABC kit (Vector Labs, USA)
DEAE Diethylaminoethyl
DHBV Duck hepatitis B virus
DMEM Dulbecco's modified Eagle's medium
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
dNTP Deoxynucleotide triphosphates
DOC Deoxycholate
EC$_{50}$ Concentration of compound producing 50% inhibition of virus-induced cytopathic effect
ECL Enhanced chemiluminescence
EDTA Ethylenediaminetetraacetic acid
FACS Fluorescence-activated cell sorting
FDA Food and Drug Administration
FITC Fluorescein isothiocyanate
GAPDH Glyceraldehyde-3-phosphate dehydrogenase gene

GFP Green fluorescent protein
H$_2$SO$_4$ Sulfuric acid
HA Hemagglutination activation
HBsAg HBV antigens
HBV Hepatitis B virus
HCC Hepatocellular carcinoma
HCF Host cell factor
HCl Hydrochloric acid
HCV Hepatitis C virus
HIV Human immunodeficiency virus
HSV Herpes simplex virus
IC$_{50}$ half minimal (50%) inhibitory concentration.
ICP0 Infected cell protein 0
IgG Immunoglobulin G
LAT Latency associated transcript
LD$_{50}$ Median lethal dose
LTR Long terminal repeat
MAb Monoclonal antibodies
MEM Minimum Essential Medium
MgCl$_2$ Magnesium chloride
MMLV-RT Moloney Murine Leukemia Virus Reverse Transcriptase
MNTD Maximum Nontoxic Dose
MOI Multiplicity of infection
mRNA messenger RNA
MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl Sodium chloride
NP-40 Nonyl phenoxypolyethoxylethanol-40
NRTIs Nucleoside analog reverse-transcriptase inhibitors
OD Optical density
OptiMEM Reduced Serum Media is a modification of MEM
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
PBST PBS solution with 0.1% Tween 20
PCR Polymerase chain reaction
PFA Paraformaldehyde
PFU Plaque-forming uni*t*
PI Propidium iodide
PVDF Polyvinylidene difluoride
qRT-PCR quantitative real-time polymerase chain reaction
RBCs Red blood cells
RF Reduction factors
RNA Ribonucleic acid
RSV Respiratory syncytial virus
RT Reverse transcriptase
RTQ-PCR Quantitative reverse transcription PCR
SARS Severe acute respiratory syndrome
SDS Sodium dodecyl sulfate
SHIV Simian-HIV
SI selectivity index
siRNA Small interfering RNA
SIV Simian immunodeficiency virus
TBE Tris-borate-EDTA
TCID$_{50}$ Titration expressed in 50% tissue culture infectious doses
TCM Traditional Chinese Medicine
TI Therapeutic index
TMB 3,3′,5,5′-tetramethyl-benzidine
UV Ultraviolet
VGE Viral genome equivalent
VHSV Viral hemorrhagic septicemia.
VSV Vesicular stomatitis virus