



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



# Recent advancements for the evaluation of anti-viral activities of natural products

Debprasad Chattopadhyay<sup>1</sup>, Mamta Chawla- Sarkar<sup>2</sup>, Tapan Chatterjee<sup>3</sup>, Rakhi Sharma Dey<sup>2</sup>, Paromita Bag<sup>1</sup>, Sekhar Chakraborti<sup>2</sup> and Mahmud Tareq Hassan Khan<sup>4</sup>

<sup>1</sup>ICMR Virus Unit, I.D. & B.G. Hospital, GB-4, First Floor, 57 Dr Suresh C Banerjee Road, Beliaghata, Kolkata 700010, India

<sup>2</sup>Division of Virology, National Institute of Cholera & Enteric Diseases, P-33, CIT Road, Scheme XM, Beliaghata, Kolkata 700010, India

<sup>3</sup>Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India

<sup>4</sup>Department of Pharmacology, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway

Significant progress has been achieved for the development of novel anti-viral drugs in the recent years. Large numbers of these newly developed drugs belong to three groups of compounds, nucleoside analogues, thymidine kinase-dependent nucleotide analogues and specific viral enzyme inhibitors. It has been found that the natural products, like plant extract, plant-derived compounds (phytochemicals) and so on, as well as traditional medicines, like Ayurvedic, traditional Chinese medicine (TCM), Chakma medicines and so on, are the potential sources for potential and novel anti-viral drugs based on different *in vitro* and *in vivo* approaches. In this chapter some of these important approaches utilised in the drug discovery process of potential candidate(s) for anti-viral agents are being discussed. The key conclusion is that natural products are one of the most important sources of novel anti-viral agents.

## Contents

Introduction . . . . .	348
Developments of effective anti-viral drugs . . . . .	349
Overview of medicinal plants worldwide as an effective source of anti-microbial drugs . . . . .	349
Common methods for assay of anti-viral activity . . . . .	349
In vitro assay . . . . .	349
Indirect assays . . . . .	349
Direct assays . . . . .	352
HBV DNA copy number determination and quantitative PCR . . . . .	357
Studies on the mechanism of action . . . . .	358
<i>In vivo</i> assays . . . . .	358
Established assays for <i>in vivo</i> testing of anti-virals . . . . .	358
Herpes viruses . . . . .	358
Skin irritation test . . . . .	358

Corresponding author. Present address at: Department of Pharmacology, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway. Khan, M.T.H. (mahmud.khan@fagmed.uit.no), (mthkhan2002@yahoo.com)

Cutaneous lesion assay . . . . .	358
Reduction in viral vaginitis in mice or cotton rats . . . . .	358
Model for ocular herpes virus study . . . . .	360
Latency and reactivation in rabbits . . . . .	360
Murine models for latency and reactivation . . . . .	360
The footpad/dorsal root ganglia model . . . . .	360
The mouse eye/trigeminal ganglia model . . . . .	360
Vaginal inoculation of HSV in guinea pigs . . . . .	360
Respiratory viruses . . . . .	361
Experimental animals, RSV infection, and treatment with NOS inhibitors . . . . .	361
Inoculation of virus in nasal cavity . . . . .	361
Harvesting of lungs and respiratory tract . . . . .	362
Mouse versus ferrets model . . . . .	362
Influenza virus . . . . .	362
Treatment of Balb/c mice with extract . . . . .	362
Mouse monitoring . . . . .	362
Histology and immunohistology . . . . .	363
Rabies virus . . . . .	363
Mouse inoculation test . . . . .	363
Cell culture for Rabies virus . . . . .	363
Human immunodeficiency virus . . . . .	363
Transgenic rat model . . . . .	364
Hepatitis B virus . . . . .	364
Anti-HBV study with ducklings . . . . .	365
Measurement of duck liver DHBV DNA by Southern blot analysis . . . . .	365
Histopathological examination of duck liver . . . . .	365
Endpoint measurement of reduction of virus load . . . . .	365
Future prospects and directions . . . . .	365
References . . . . .	365

## Introduction

Viruses are ultramicroscopic, acellular, metabolically inert nucleoprotein particles containing bundles of gene strands of either RNA or DNA, with or without surrounded by a lipid-containing envelope [1]. Unlike free-living bacteria, viruses are obligate intracellular parasites, utilise the host cell machinery to propagate new viruses and can cause ailments as benign as a common wart, as irritating as a cold, or as deadly as the bloody African fever. Hence, the viruses can be termed as the '*acellular parasites of cellular hosts*' [1]. Viruses have numerous invasion strategies and each strain has its own unique configuration of surface molecules [2,3], enabling them to enter into host cells by precisely fitting their surface molecules with the molecules of target cell. For example, the viruses that cause Lassa and Ebola fever and AIDS spread easily, kill swiftly and have no cure or vaccine. Each strain of virus is unique in its surface antigenic structure, its receptors on host cells and its life cycle. As a consequence of genetic variation, variety in mode of transmission, efficient replication and the ability to persist within the host, viruses have adapted to all forms of life and have occupied numerous ecological niches resulting in widespread diseases in humans, livestock and plants [2,4].

The discovery of novel anti-viral drugs deserves great efforts. There are several reviews been published in recent years stating discovery of new drugs against different viral infections from

natural resources [5–7]. The role of traditional medicine for the development of anti-viral compounds is also been discussed in different reviews. Interestingly, it was found that traditional medicines, like Ayurvedic, traditional Chinese medicine (TCM), Chakma medicines, are good sources for potential anti-viral drugs [5]. A wide variety of active phytochemicals, including the flavonoids, terpenoids, organosulfur compounds, limonoids, lignans, sulfides, polyphenolics, coumarins, saponins, chlorophyllins, furyl compounds, alkaloids, polyines, thiophenes, proteins and peptides have been found to have therapeutic applications against different genetically and functionally diverse viruses [3–7]. The anti-viral mechanism of these agents may be explained on basis of their antioxidant activities, scavenging capacities, inhibiting DNA, RNA synthesis, inhibition of the viral entry, or inhibiting the viral reproduction and so on [4,7,8]. Large numbers of candidate substances, such as phytochemicals and their synthetic derivatives have been identified by a combination of *in vitro* and *in vivo* studies in different biological assays [7,8].

In this chapter, we review different approaches or methods that are being used to discover potential 'lead' molecules as 'anti-viral substances' from natural sources, including both extracts and pure compounds from herbal medicines. Our major conclusion is that phytochemicals from medicinal plant extracts are very important source of anti-viral agents.

## Developments of effective anti-viral drugs

Despite continuous advances made in anti-viral therapy, viral diseases become the leading cause of death globally. Being obligate intracellular parasites, viruses are dependent on the metabolic pathways of the host cell for their replication. Since viruses and hosts are intimately connected, the designing of effective anti-virals that will attack the viral enzymes or its replication, without affecting the host cell has proved to be difficult [1,2]. With the advances in molecular biology and reverse genetics in the past two decades, we have come to know more about the biochemistry of virus replication leading to a more rational approach to the search of anti-viral agents targeting the virus binding or fusion, replication intermediates, DNA/RNA polymerases or viral assembly proteins. Since viral enzymes are crucial for disease progression and virus replication, inhibitors against viral enzymes have been the most desirable strategy. Most of the well-studied inhibitors against HIV, HSV or the Influenza viruses target the host cell binding (T-20, betulinic acid, etc.), uncoating of capsid (amantadine derivatives, pleconaril), replication (reverse transcriptase inhibitors like zidovudine or Abacavir, nevirapine, etc.), integrase inhibitors, DNA or RNA polymerase inhibitors (acyclovir, cidofovir, ribavirin, etc.), proteinases involved in viral polyprotein precursors and assembly/maturation inhibitors (indinavir, ritonavir and rimantadine, etc.). On the basis of this strategy numerous compounds have been tested on different viruses in the past decade, but still only 37 licensed anti-virals are in the market.

Rapid advances in viral genomics, gene function and regulation, as well as in rational drug design, have led to the development of gene-based drugs that can induce protective anti-viral immunity, interfere with viral replication, suppress viral gene expression or cleave viral mRNAs. Several such drug candidates have been developed in recent years against various viruses including HIV. Although gene-based agents show promise as anti-viral agents their therapeutic efficacy may be restricted by limited delivery to intracellular sites of viral replication and *in vivo* nuclease degradation [8].

## Overview of medicinal plants worldwide as an effective source of anti-microbial drugs

All ancient cultures in world like China, India, Persia and so on have relied historically and continue to rely on medicinal plants for primary health care. In recent years, owing to the fear of side effects of common drugs over the counter medicines, there has been huge upsurge in people preferring to use more and more natural plant product for preventing and treating serious ailments. However since no scientific background is provided, most of the over the counter herbal products do not have quality control or provide consistent results. Natural plant products have traditionally provided 30–40% of modern anti-microbial or anti-cancer drugs to pharmaceutical industry, but we still have barely analysed the tip of iceberg in our efforts to design more anti-microbial drugs from plants. Thus, it is imperative to search for more useful phytochemicals before we lose our herbal resources with the upward march of industrialisation (Table 1). Fortunately, many viruses have unique features in their structure or replication cycles that can be the potential targets as evident with nucleoside analogue acycloguanosine (acyclovir) that specifically blocks certain viral enzymes of herpes viruses [1,9] that play the key role in triggering disease. Owing to the amazing structural diversity and broad range of

bioactivities natural products can be explored as a source of complementary anti-virals, as many of them are reported to inhibit several steps of replication cycle and certain cellular factors of many DNA and/or RNA viruses. Many traditional medicinal plants used in Indian (Ayurveda) or TCM have been reported to have strong anti-viral activity and are used to reduce the disease severity [4,6,10–12]. Research interests for anti-viral agents started after Second World War in Europe, and in 1952 12 out of 288 plants were found to be effective against influenza viruses in embryonated eggs [13]. During the past 20 years, there have been numerous broad based screening programmes initiated in different parts of the world for screening of anti-viral activity by *in vitro* and *in vivo* assays. One hundred British Colombian medicinal plants were screened for anti-viral activity against seven viruses [14], and anti-viral activities against corona viruses, RSV, para influenza, HSV and so on were observed in few. The marine algae such as blue green algae Spirulina has been studied for its immunomodulatory, anti-mutagenic and anti-viral properties [15,16]. Cynovirin N, an 11 kDa protein from blue green algae has been shown to interact with gp120 and inactivate HIV [17]. The presence of various sulfated polysaccharide groups extracted from seaweeds and alga have shown anti-HIV and anti-HSV activity [18].

Several other herbal medicinal products are potential sources of functional foods and have various bioactivities like immunomodulatory and antimicrobial functions. We will summarise here the diverse methodologies and ways to evaluate the potential natural compounds having anti-viral activities, mainly against the common viral diseases.

## Common methods for assay of anti-viral activity

*In vitro* assays usually exploit the virus's ability to infect and replicate in specific cell lines in cell culture systems. The cell culture system provides a rapid and less cumbersome method to grow viruses at higher titres, vaccine strain cultures, reverse genetics and testing of anti-viral compounds. An example of cell lines characterised and routinely used for common viruses is provided in Table 2.

### *In vitro* assay

#### **Indirect assays**

Indirect assays are usually the first step in screening large number of compounds for their anti-viral activity. It is based on the observation that virus infection and multiplication results in cytopathic effects due to either release of virus or induction of apoptosis as a result of host immune responses. Inhibition of CPE in presence of test compound could be due to inhibition of virus replication. Various indirect tests have been developed and modified for individual viruses.

#### **Visual observation: cytopathic effect (CPE)**

For visual observation of CPE with the help of a microscope, in 96 or 48 well plate cells infected with virus in presence or absence of drugs are run in duplicate. Broadly the method of testing is that the test compounds are initially evaluated in a 2-concentration test (with the 10-fold difference for example 1 and 10 ng/ml or  $\mu\text{g/ml}$ ). The standard is to use an 18 h monolayer (80–100% confluent) of the appropriate cells, medium is drained and each of the concentrations of test compound or placebo is added, immediately after virus infection. In case effect of drug needs to be tested as

TABLE 1

## Partial list of viruses inhibited by medicinal plants [110].

Virus	Medicinal plant	Antiviral effect	References
HSV	<i>Carissa edulis</i> Vahl.	Exhibit strong anti-HSV- 1, and 2 activities <i>in vitro</i> and <i>in vivo</i>	Tolo <i>et al.</i> [111]
	<i>Phyllanthus urinaria</i> L.	Geraniin and 1346TOGDG isolated from <i>P. urinaria</i> inhibit HSV-1 and HSV-2, respectively	Yang <i>et al.</i> [112]
Influenza virus	<i>Geranium sanguineum</i> L.	Reduce the infectivity of various influenza virus strains <i>in vitro</i> and <i>in vivo</i>	Serkedjieva <i>et al.</i> [113]; Pantev <i>et al.</i> [114] and Serkedjieva [115]
	Elderberry ( <i>Sambucus</i> Sp.) extract	Randomized, double-blinded placebo-controlled study revealed an efficient, safe and cost-effective treatment for influenza	Zakay-Rones <i>et al.</i> [116]
HBV	<i>Boehmeria nivea</i> L.	Root extract reduced HBV production <i>in vitro</i> and <i>in vivo</i> model	Huang <i>et al.</i> [117]
	<i>Polygonum cuspidatum</i> Sieb & Zucc.	Inhibits hepatitis B virus in a stable HBV-producing cell line	Chang <i>et al.</i> [118]
HCV	<i>Saxifraga melanocentra</i> Engl. & Irmsch.	1,2,3,4,6-penta-O-galloyl-beta-D-glucoside from <i>S. melanocentra</i> inhibit HCV	Zuo <i>et al.</i> [119]
Poliovirus	<i>Guazuma ulmifolia</i> Lam.	Extract inhibit poliovirus replication, and blocked the synthesis of viral antigens in infected cell cultures	Felipe <i>et al.</i> [120]
Viral haemorrhagic septicaemia virus (VHSV)	<i>Olea europaea</i> L.	Leaf extract inhibited replication of VHSV	Micol <i>et al.</i> [121]
SARS-CoV	<i>Lycoris radiata</i>	Lycorine, isolated from <i>Lycoris radiata</i> possesses anti-SARS-CoV	Li <i>et al.</i> [122]
HIV	<i>Phyllanthus amarus</i> Schum. & Thonn.	Inhibits HIV replication both <i>in vitro</i> and <i>in vivo</i>	Notka <i>et al.</i> [123]
	Olive ( <i>Olea europaea</i> ) leaf extract	Inhibits acute infection and cell-to-cell transmission of HIV-1	Lee-Huang [124]
Vesicular stomatitis virus (VSV)	<i>Trichilia glabra</i> L.	Leaf extract of <i>T. glabra</i> inhibits VSV	Cella <i>et al.</i> [125]
Human adenovirus type 1	Black soybean ( <i>Glycine max</i> ) extract	Inhibit human ADV-1 and coxsackievirus B1 in a dose-dependent manner	Yamai <i>et al.</i> [126]
Dengue virus type-2 (DEN-2)	<i>Azadirachta indica</i> Juss. (Neem)	Aqueous leaf extract inhibited DEN-2 both <i>in vitro</i> and <i>in vivo</i>	Parida <i>et al.</i> [127]

pretreatment, cells can be treated with test compound 15 min to 6 h before virus infection. The plate is sealed and incubated for standard time period required to induce near maximal viral CPE. After every 24 h until the end of experiment, the plate is visualised microscopically for any changes in cell appearance compared with normal control cells. During the course of experiment, only test compound but no virus treatment will give an idea of maximum dose of drug causing minimum cell toxicity. These changes may be enlargement, granularity, rounding, detachment from plate and so on. These changes are given a designation of T (100% toxic), PVH (partially toxic – very heavy – 80%), PH (partially toxic – heavy – 60%), P (partially toxic—40%), PS (partially toxic – slight – 20%), or 0 (no toxicity—0%), conforming to the degree of cytotoxicity seen. A 50% cell inhibitory (cytotoxic) concentration (IC<sub>50</sub>) and 50% endpoint of virus inhibitory concentration (EC<sub>50</sub>) can be determined. General selectivity index is calculated as SI = (IC<sub>50</sub>)/(EC<sub>50</sub>), SI of more than three indicates potential anti-viral activity of test compound and should be further evaluated in increasing concentrations.

### Neutral red dye uptake

If a compound shows SI of more than three, four–five log<sub>10</sub> dilutions of each test compound (e.g. 1, 10, 100, 1000, and 10,000 ng/ml, etc.) should be used in triplicate in a 96-well plate.

The monolayer of cells is treated with drug for 30 min to 1 h followed by virus adsorption (pretreated) or the drug is added after virus adsorption (postinfection treatment). The plate is sealed and incubated at 37 °C and CPE observed microscopically when untreated infected controls develop a 3 to 4+ CPE (approximately 72–120 h). A known positive control drug should be evaluated in parallel to validate the test, for example ribavirin is used in case of dengue, influenza, measles, RSV and so on, and cidofovir for adenoviruses, interferon for SARS and so on. After the CPE has been read under microscope, neutral red is added to the medium, so that live and healthy cells take up more dye. The plate is read in a microplate reader at dual wavelengths of 405 and 540 nm. The difference in reading is used to eliminate background. EC<sub>50</sub> (concentration of drug at which 50% inhibition of virus is observed [19]).

#### Brief protocol: neutral red uptake cytotoxicity assay

- Plate containing mammalian cell lines in culture medium in the 96-well plate, at concentration of  $1 \times 10^5$  cells/ml.
- The cells are removed from the incubator after  $24 \pm 2$  h of plating and the culture medium is replaced. The freshly prepared culture medium, 100  $\mu$ l each well, is added to the blank control group and the negative control group, while the increasing concentration of test substance, and the virus infection is done in the experimental well.

TABLE 2

***In vitro* and *in vivo* models for Testing anti-viral agents against common viruses**

Name of virus	Disease	<i>In vitro</i> model	<i>In vivo</i> model
HSV	Herpes	Plaque Assay (Vero, MRC-5, HFF, BHK, HEp-2)	Mice, Rat, Rabbit Guinea pig
Influenza	Flu	Plaque Assay, HA, HAI, RT-PCR, Real Time PCR (MDCK, A549 cell lines)	Mice, Ferrets, Chicken
Polio virus	Paralysis, Aseptic meningitis	Plaque Assay, RT-PCR (Hela cell)	Mice, Rat
HCV	Liver cirrhosis	RT-PCR, Real time, No cell line facility	Chimpanzee
<b>HBV</b>			
Enterovirus	Diarrhoea	RT-PCR, PAGE	Mice, Rabbit
RSV	Common cold	Plaque assay, MTT assay, RT-PCR Cell-ELISA, HEp-2	Mice, Rabbit
Rabies	Hydrophobia	McCoy cells	Mice
HIV	AIDS		Transgenic mice

(iii) After required period of inoculation (24–120 h), the culture medium is removed and the plate is rinsed three times with 200  $\mu$ l PBS, each well, and then washed. The 200  $\mu$ l medium containing neutral red (50  $\mu$ g/ml) is added to each well. After 3 h of culture, the medium is removed and the plate is rinsed two times with 200  $\mu$ l PBS, each well. The desorbing solution, 100  $\mu$ l each well, containing 1% glacial acetic acid, 50% ethanol and 49% H<sub>2</sub>O, is added and shaken for 15 min with a micromixer in a dark place. The absorbance of coloured solution is measured at both 405 nm and 540 nm with a microplate reader. The concentration producing 50% inhibition for neutral red uptake (NR 50  $\mu$ g/ml) can be calculated by putting untreated versus test compound treated cells.

**MTT assay**

Another rapid and sensitive *in vitro* procedure of evaluating anti-viral agents is based on spectrophotometrical assessment for viability of virus-infected and mock-infected cells via *in situ* reduction of a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and is proved to have similar sensitivity like plaque reduction assay [20]. Mitochondrial enzymes of viable cells convert yellow water soluble dye MTS or MTT to a soluble, purple coloured insoluble formazan. The quantitation of the amount of the formazan product present in each well of the microtitre plate is then determined spectrophotometrically at 490/650 nm. While the toxicity of the test compounds to host cells is measured concurrently in the same microtitre plate. Data can be analysed by a statistical software programme along with determinations of the efficacy (IC<sub>50</sub>), toxicity (TC<sub>50</sub>) and selectivity (therapeutic index, TI) of the compounds. It not only significantly simplifies the assay procedures but also allows the evaluation of larger numbers of compounds at a time.

**Brief protocol**

- (i) Count and plate 100–200  $\mu$ l cells in central 10 columns of (column 2–11) a flat-bottomed 96-well plate (initial density approximately  $2 \times 10^4$  cells/ml) to get 70% confluency. On all side rows, just add media to avoid edge effect.
- (ii) After 24 h, prepare a serial twofold dilution of test compound to give eight concentrations in serum-free medium.
- (iii) Remove medium from the plate and add virus (1–5 moi depending on virus type) in 100  $\mu$ l medium in six wells of

Columns 3–11 (rows 3–8). Add only media in Column 2 (uninfected control) and rows 1–2 of Columns 3–11. After adsorption of 45 min at 37 °C, remove remaining virus. Wash cells gently with 200  $\mu$ l media.

- (iv) Add media (0, highest concentration of drug) to all wells of Columns 3–11. Add only media to Column 2 with no virus.
- (v) Seal the plate and keep at 37 °C humidified CO<sub>2</sub> incubator for 24–48 h.
- (vi) After the drug treatment is over, remove the media and drug. Add 200  $\mu$ l fresh media and 50  $\mu$ l of MTT solution (5 mg/ml in PBS) to all wells in Columns 1–11. Wrap the plate in aluminium foil and incubate for 3–4 h at 37 °C.
- (vii) Remove the media and MTT from wells. Add 200  $\mu$ l of DMSO to dissolve the formazan crystals in Columns 1–11. After 15 min, add 25  $\mu$ l of glycine buffer to all wells containing DMSO to change pH to 10.5.
- (viii) Read absorbance at 570 nm, with wells in Column 1 containing media and MTT but no cells as blank.
- (ix) Plot the absorbance (Y-axis) with drug concentration (X-axis). The Column 2 with no virus acts as positive cell viability control and wells in Column 3 with virus but no drug as maximum virus induced loss of cell viability.
- (x) The rows 1–2 of Columns 3–11 with increasing concentration of drug but no virus provides IC<sub>50</sub> value of drug (50% cell inhibitory concentration).
- (xi) The EC<sub>50</sub> or EC<sub>90</sub> can be deduced by plotting absorbance value of virus-infected wells (Column 3, rows 3–8) and that of increasing concentration of drug. Concentration of drug that shows increase in absorbance by 50% or 90% over virus alone can be deduced from the plot [20].

**MTT assay for screening anti-HSV compounds**

The method has been used successfully as a high throughput screening 96-well assay for anti-HSV-1 or HSV-2 drugs in Vero cells. The ability of the compounds to inhibit HSV-induced cell killing is measured five days postinfection using the tetrazolium dye MTS or MTT following the methods described by [21,22]. Briefly, Cells ( $2.5 \times 10^4$  cells/ml) were seeded on 96-well tissue culture plates. After a 24 h period of incubation, the medium was removed and replenished with 200  $\mu$ l of medium containing increasing concentrations of the compounds (serially diluted twofold). As cell control, 200  $\mu$ l of only medium is added. After three to five days of incubation, the medium is removed and 50  $\mu$ l of MTT solution

(1 mg/ml) is added to each well for 4 h at 37 °C. Then, 100 µl of DMSO is added to each well in order to dissolve the formazan crystals. After shaking gently the plates for 10 min to dissolve the crystals, the colour reaction is measured in an automated microplate reader at 562 nm. The untreated control was arbitrarily set as 100%. For each compound, the percentage of cytotoxicity can be calculated as  $[(A - B)/A \times 100]$ , where *A* and *B* correspond to the absorbances of control and treated cells, respectively. The  $CC_{50}$  value is defined as the concentration of each compound that reduced the absorbance of treated cells by 50% when compared with cell control [21].

### Decrease in virus yield assay

Compounds considered active by CPE inhibition and by NR dye uptake should be re-tested for, effect on reduction of virus yield. The CPE inhibition should be repeated as per Neutral dye uptake assay with freshly prepared test compounds showing potential anti-viral activity in initial experiments. After the test period of three to five days as described previously, the plates are freeze-thawed to lyse cells and elute virus in supernatant. After centrifugation at  $10,000 \times g$ , supernatants are collected from each column representing increasing concentration of drug. The titre of infectious virus from drug-treated wells can be evaluated by measuring CPE induced by these viruses in fresh untreated cells. Briefly cells are plated in fresh 96 well plates with susceptible cell lines. After 24 h, add serially diluted virus preparations from drug-treated cells. Development of CPE in these cells is the indication of presence of infectious virus. The 90% effective concentration ( $EC_{90}$ ), which is that test drug concentration that inhibits virus yield by one  $\log_{10}$ , is determined from these data.

### Data analysis and secondary testing of potential anti-viral test compounds

Each test compound's anti-viral activity is expressed as a selectivity index (SI), which is the  $IC_{50}$  divided by the  $EC_{50}$ . In general, an SI of 10 or greater is indicative of positive anti-viral activity, although other factors, such as a low SI for the positive control, are also taken into consideration. Compounds having confirmed SI values of 10 or greater may, be evaluated against additional strains of the original virus inhibited in order to more fully determined the spectrum of anti-viral activity of the compound.

The potential anti-viral test compound is further screened by direct assays measuring viral replication or inhibition of titre.

### Direct assays

Direct assays are a measure of >50% reduction in viral titre in presence of test compound, compared with the untreated cells. The inhibition of cytopathic effect on cell monolayer infected with virus can be measured by endpoint titration method [23], which is also helpful to determine virucidal activity after preincubations of test compound and virus [24,25]. The 50% endpoint titration is performed on confluent monolayers of cells ( $10^4$  cells per well) infected with serial 10-fold dilutions ( $10^7$  TCD<sub>50</sub>/ml) of virus suspension. The virus is allowed to absorb for 1 h at 37 °C, and then serial twofold dilutions of extract or test compound (in maintenance medium, supplemented with 2% foetal bovine serum and antibiotics) are added. The plates are incubated

(37 °C) and viral CPE is recorded by light microscopy for four to five days. It is important to run cytotoxicity control (uninfected but treated cells), and cell control (uninfected untreated cell) at each treatment concentration, and the virus control (infected but untreated). Toxic doses (CT) of the test extract or compound are considered to be dilutions that cause destruction of monolayer of cells so that no virus titre can be determined. The anti-viral activity is expressed as the virus titre reduction at the maximum non-toxic dose (MNTD) of the test extract or substance, that is the highest concentration that does not affect the monolayers under test conditions. In this method, virus titre reduction factors (RF, the ratio of the virus titre reduction in the absence and presence of the MNTD of the test sample) of  $10^3$ – $10^4$  indicate a pronounced anti-viral activity and are suitable as selection criteria for further investigation of the said extract or compound. It is important that the anti-viral activity should be present in at least two subsequent dilutions of the test substance, otherwise the activity is likely to be due to its toxicity, or the activity is only virucidal. The extracellular virucidal activity can also be determined by titration method of the residual infectious virus at room temperature after incubation of the test compound with virus suspension ( $10^6$  TCD<sub>50</sub>/ml) during 1 h at 37 °C (Figure 1).

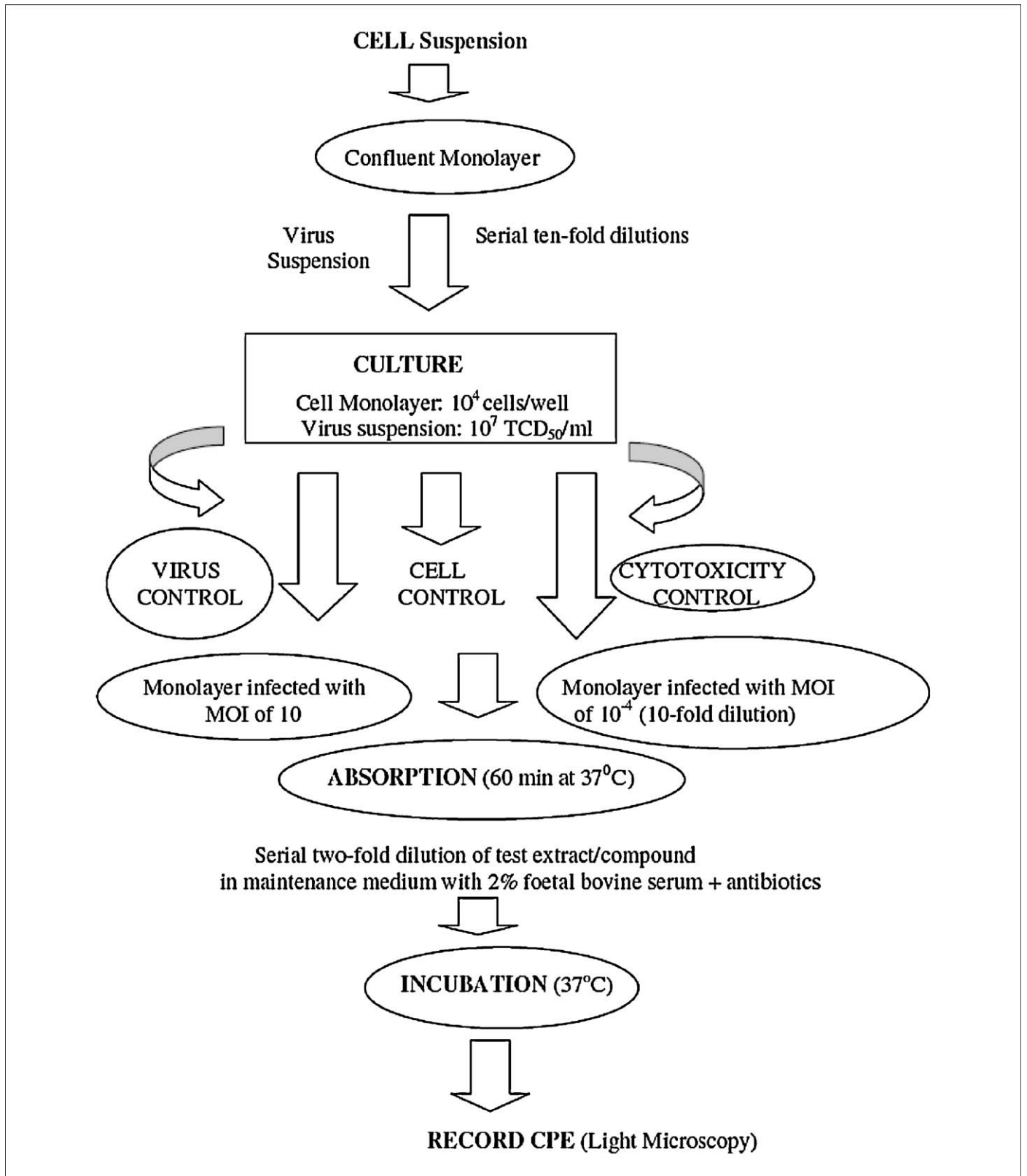
### Immunofluorescence assay: microscope or FACS analysis

This assay can be performed only for viruses against which antibodies are available either commercially or in-house. It can provide the estimate of quantity of virus though it cannot differentiate between infectious and non-infectious viral particles. Thus, drugs affecting later stages such as virus maturation or budding may not give good estimation of viral inhibition.

Briefly, the untreated or drug-treated cells are infected with a known concentration of virus (moi 5–10). After virus adsorption of 45 min, virus is removed, and cells are washed once with media. Fresh media is added and cells are incubated for 24–36 h (tentatively 50% of time required to achieve 3+ CPE). The media is removed and cells are washed with PBS. The cells are fixed with 3–4% paraformaldehyde (in PBS) at room temperature. After washing with PBS, cells are permeabilised with either acetone or 0.5% triton X-100, washed twice with PBS. Cells are blocked with 1% 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 several steps of washing, cells are incubated with a fluorescent tagged secondary antibody in 1% BSA for 1 h. Cells are washed three to four times with PBS and can be visualised under fluorescent microscope and fluorescence is compared between untreated and drug-treated cells. Alternatively for quantitation, the cells are trypsinised after treatment, fixed with 4% paraformaldehyde. The cells are washed with PBS, permeabilised and labeled with fluorescent-tagged antibody, followed by propidium iodide (50 µg/ml PI) in PBS. The cells are counted in the fluorescent activated cell counter and fluorescence positive percentage can be quantitated. The measure of PI will indirectly measure cytotoxicity caused by drug or virus infection.

### Enzyme linked immunosorbent assay (ELISA)

ELISA assays allow the detection of viral antigen or antibody, using a solid-phase assay system. Although qualitative, it offers a rapid, sensitive, and specific method for detection and gross quantitation

**FIGURE 1**

The *in vitro* anti-viral testing protocol.

of virus. Absolute quantitation is possible if a series of pre-determined viral titres are used to get elisa readings and matched with that unknown samples can be calculated to estimate the quantity of the virus samples (untreated or drug-treated). The method

cannot differentiate between infectious and non-infectious virus particles as a result of which the drug affecting at the earlier or latter stage of viral replication or maturation may not be understood (Figure 2).



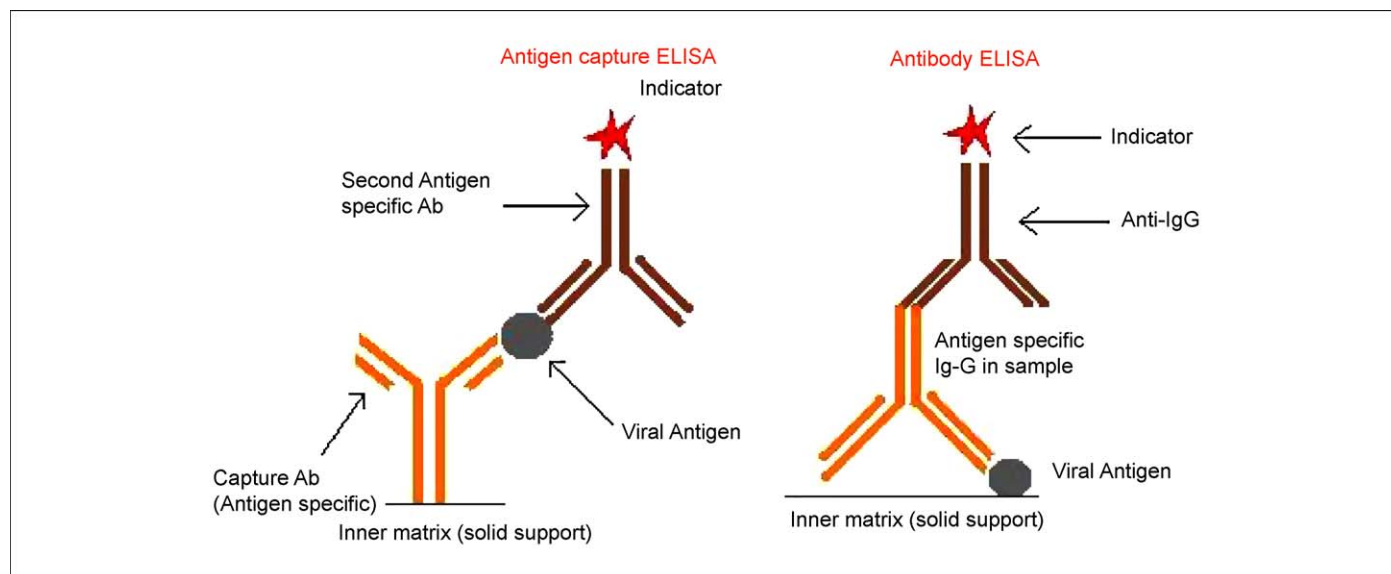


FIGURE 2

Schematic representation of two principles based on either antigen or antibody detection in clinical samples by ELISA (modified from [109]).

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

### Plaque reduction assay

Plaque assay is one of the most reliable and oldest methods of titrating infectious virus particles in samples. The effectiveness of drug in reducing the plaque-forming units (pfu) of virus compared with controls is an indicator of anti-viral activity. There are various

protocols standardised for different viruses though the principle is same (Figure 3).

1. Corresponding mammalian cell lines ( $1 \times 10^5$  cells/cm<sup>2</sup>) are cultured in a 6-well tissue culture plate.
2. 80–90% confluent cells should be infected with a five concentrations of  $\log_{10}$  dilution of plaque-forming units of virus either in absence of test drug or in presence of drug.
3. Briefly the virus is allowed to adsorb for 1 h at 37 °C in 5% CO<sub>2</sub> in air. The solution is removed and the cells are washed twice with prewarm MEM medium. While virus is adsorbing, drug dilutions are prepared in the overlay medium.
4. The infected cells are overlaid with agarose medium without test drug used as control.  
Overlay Medium Type I: (MEM or DMEM containing 10  $\mu$ g/ml trypsin, 1% low melting agarose, without serum and various concentration of test drug).
5. Overlay Medium Type II: 100 ml 10X MEM, supplemented with 10 ml glutamine, 10 ml antibiotics, approximately 40 ml bicarbonate and 20 ml foetal bovine serum. 45 ml of CMC is added to 9 ml of the concentrated medium.
6. Plates are incubated at 37 °C in 5% CO<sub>2</sub> for three to five days before fixing with 10% formalin or 4% formaldehyde solution for 30 min.
7. Cells are either stained with 1 ml per well of methylene blue or 1% crystal violet solution (w/v).
8. Stain is removed and rinsed gently three times with tap water and allowed to dry inverted overnight and plaques (dark areas) are counted using low power magnification on a binocular microscope.
9. Calculation of anti-viral effects: The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control) – (mean number of plaques in sample)]  $\times$  100/mean number of plaque in control. The value of  $IC_{50}$  or  $EC_{50}$ , which is the concentration of test sample required to inhibit up to 50% of virus growth as compared with

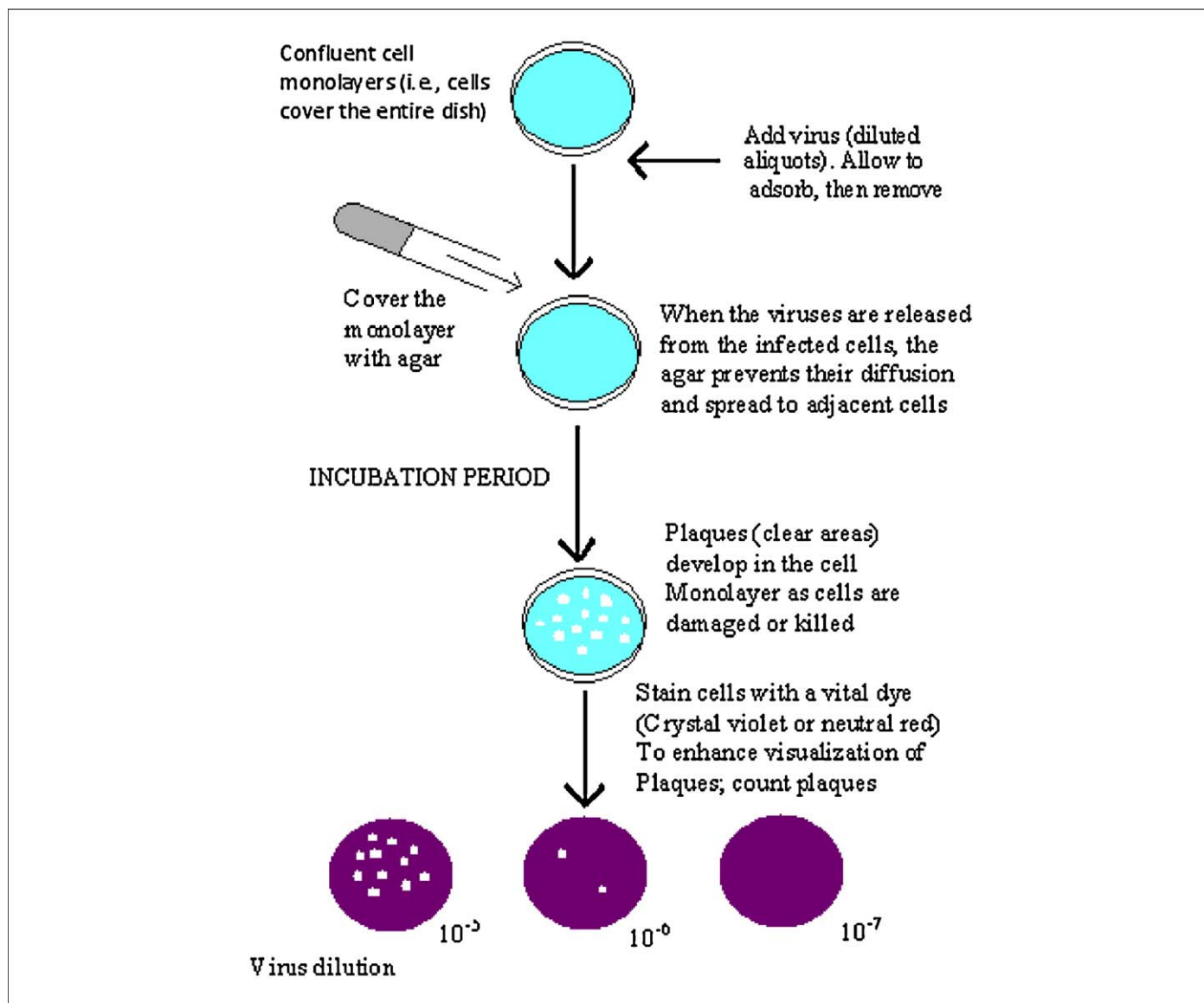


FIGURE 3

Schematic representation of the viral plaque assay (modified from [109]).

the virus control group, are estimated from the graphical plot of the data.

### Example of plaque reduction assay utilised for screening of anti-HSV compounds

The Hep2 or Vero cells were grown in suitable cell culture media with incubation at 37 °C for 24 h. Confluent cell monolayers are then infected with 100–200 plaque-forming units (PFU) of the virus. After 1 h incubation (to allow viral adsorption), the cells are washed with phosphate buffer saline (PBS) and overlaid with agar in cell culture medium containing twofold dilutions of the extracts or test compounds, and recultured at 37 °C, until plaques appeared. Finally the monolayer cells are fixed with formalin, stained, dried and the number of plaques are microscopically counted. The percentage inhibition of plaque formation [(mean number of plaques in control – mean number of plaques in test)/(mean number of plaques in control) × 100], or the effective concentration for 50% plaque reduction [EC<sub>50</sub>; the lowest extract

concentration that reduced plaque number by 50% in the treated cultures compared with untreated ones], or the 50% inhibitory concentration [IC<sub>50</sub>; the extract concentration required to reduce the virus plaque number by 50%] is calculated. A recent study with different herbal preparations (cold aqueous, hot aqueous, ethanolic, acid ethanolic, and methanolic) by plaque reduction assay, it was showed that the aqueous extract of *P. suffruticosa* and ethanolic extract of *Melia toosendan* inhibit the attachment and replication of HSV-1 and HSV-2 [26], indicating that these herbs can be the potential source for new anti-HSV compounds.

### Hemagglutination assay: measurement of viral titre

The hemagglutination assay (HA) is a quantitation of hemagglutination protein of viruses. Some viral families have surface or envelope proteins, which are able to agglutinate (stick to) human or animal Red blood cells (RBC) and bind to its *N*-acetylneuraminic acid. The RBC will form a type of lattice in this case. In contrast to plaque assay or LD<sub>50</sub>, HA does not give any measure of viral

infectivity, because no virus replication is required in this assay. It is an easy, simple and rapid method and can be applied to large amounts of samples. The detailed conditions depend on the type of virus. Some viruses bind RBCs only at certain pH values, others at certain ionic strengths (Figure 4).

If the test compound inhibits virus replication and thus viral titre, the HA value will be reduced. Briefly the virus dilution (e.g. twofold from 1:4 to 1: 512) will be applied to a RBC dilution (e.g. 0.1% to 0.7% in steps of 0.2%) for approximately 30 min, often at 4 °C, because the viruses with neuraminidase activity will detach the virus from the RBCs. Then the lattice forming parts will be counted and the titre calculated as per

$$\frac{\text{Virus concentration}}{\text{ml}} = 10^7 \times \text{HA titre}$$

#### Preparation of red blood cells (RBCs)

1. Blood from chicken or guinea pigs is collected in Alsever's solution (20.5 g Dextrose, 8.0 g Sodium citrate-dihydrate, 4.2 g sodium chloride and 0.55 g citric acid per litre, pH 6.1). RBCs are kept overnight at 4 °C. Alsever's solution is removed and cells are washed with PBS 2X.
2. The quantity of cell pack (generally 2.0 ml original blood yields 0.5–1.0 ml pack) is measured. 10% suspension in PBS is prepared. Resuspend 0.75 ml 10% Guinea Pig RBCs in 10 ml PBS to prepare 0.75% erythrocyte solution.

#### Hemagglutination assay

1. A U-bottom 96-well microtitre plate is taken.
2. Using a micropipette, 50 µl of PBS (pH 7.2) is added to all wells except first well of each row.
3. 50 µl of antigen (tissue culture fluid with virus from drug treated and no drug controls of infected plate) is added to the first two wells of each row.
4. Twofold dilution is made by transferring 50 µl from the second well of each column A2-K2 to A3-JK3 by using a multi-channel micropipette. Proceed till the ninth column and the remaining 50 µl is discarded after the ninth column.
5. 50 µl of 0.75% RBCs is added to all wells and mixed by manually agitating the plates thoroughly.
6. The plate is incubated for 60 min at room temperature. Cell control is checked for complete settling of the RBCs.
7. Results are recorded in HA sheet.
8. RBCs will form a button or a ring at the bottom of the wells and recorded as 'O'. If hemagglutination occurs, that is RBCs remain in suspension, it is recorded using a '+' symbol. The highest dilution of virus that causes complete hemagglutination was considered the endpoint in HA titration. The HA titre is the reciprocal of the dilution of virus in the last well with complete hemagglutination (Figure 4).

### Inhibition of hemagglutination activity of viruses:

#### Drug-viral HA interactions

Many viruses such as influenza viruses have surface HA proteins that are characterised by their ability to agglutinate erythrocytes. This hemagglutinating activity can be visualised upon mixing virus dilutions with chicken or guinea pig erythrocytes in microtitre plates. It can thus be used to investigate the inhibitory effect of any drug onto the hemagglutinating activity. Untreated ery-

throcytes precipitate to the bottom of the plate, while upon preincubation with virus the blood cells show an even and diffuse distribution. Briefly, the drug is 10-fold serially diluted (1–1000X) as described previously. From virus stocks (1:4 to 1:128) dilutions were made, and 50 µl/well of this virus dilution was added to drug containing wells as indicated. After preincubation of 45 min, chicken erythrocytes (1/20 in PBS) were mixed with the solution. In the samples where viruses are preincubated with drug, up to a certain dilution the viral particles may lose ability to agglutinate erythrocytes, indicating an interaction of drug with the viral HA (Figure 4).

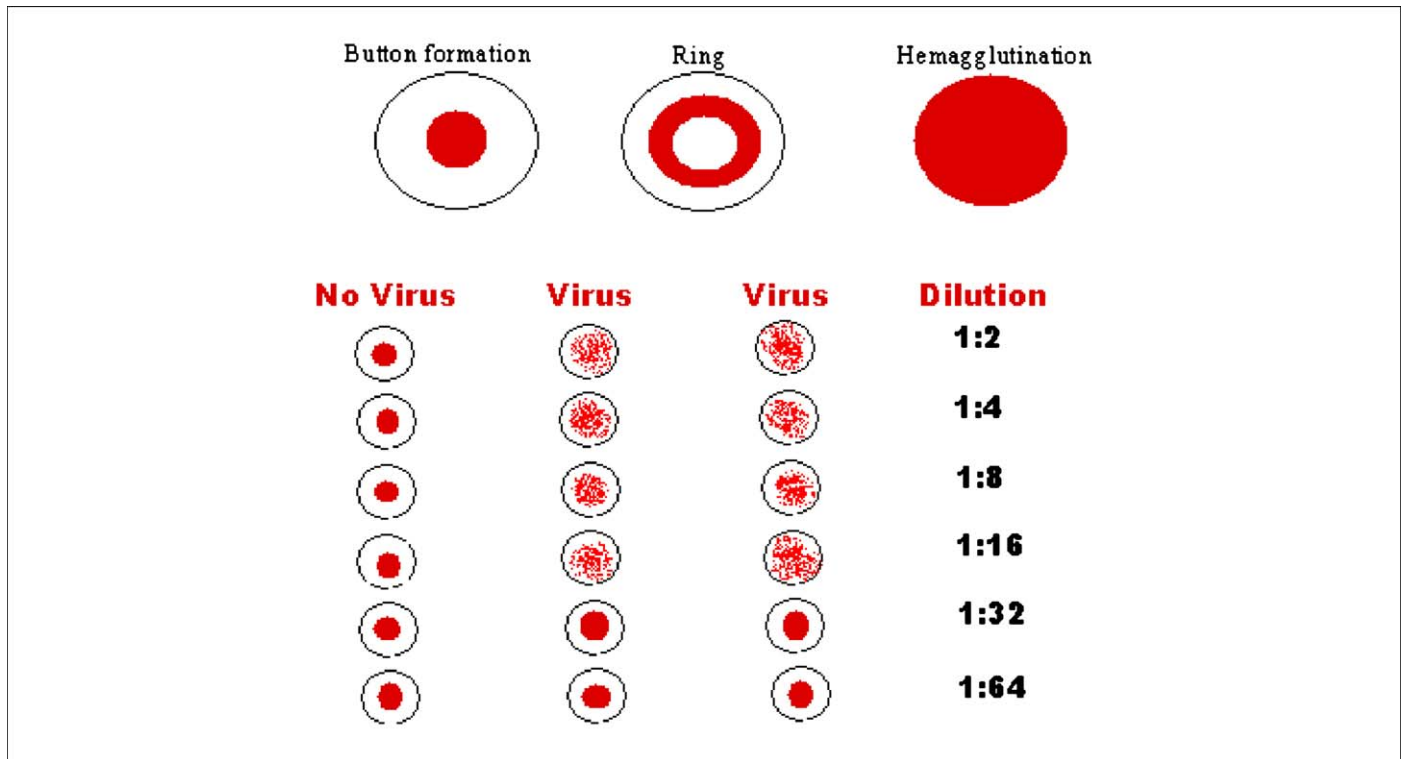
#### Quantitative real time PCR

Real time PCR has engendered wider acceptance of the PCR for the study of viral load due to its improved rapidity, low inter-assay and intra-assay variability, sensitivity, reproducibility and reduced risk of carry-over contamination. The method involves direct measurement of the amount of PCR product produced while the reaction is in progress. Mathematical analysis of the data, and comparison to control reactions containing known amounts of template, allows one to calculate the amount of input DNA in the initial reaction. The severity of some diseases has been shown to correlate with the viral load, making real-time PCR quantitation useful to study, not simply the presence of a virus but the role of viral reactivation or persistence in the progression of the disease [27–29]. This property has been successfully adapted to screen potential anti-viral test compounds *in vitro*. The fold decrease in viral genetic material in presence of drug is a measure of its anti-viral activity.

#### RTQ PCR procedure

Briefly, for RNA viruses, initially cDNA is prepared using standard Reverse transcriptase kits (Applied Biosystems, Invitrogen or Ambion). Quantitative real-time polymerase chain reaction (PCR) are carried out with 5 µl cDNA in a volume of 25 µl with TaqMan1 PCR Core Reagent Kit (Applied Biosystems, USA) or any other established manufacturer. Primers and probe are used at its optimum concentration. For all PCR amplifications positive and negative controls are included. Positive controls consist of RNA/DNA of known virus sample. Negative controls are carried out with water instead of RNA. PCR runs are carried out as per manufacturer's instructions. Amplification of target DNA and detection of PCR products are performed with a GeneAmp1 5700 Sequence Detection System (Applied Biosystems, USA). Amplification of the target sequence is detected by an increase of fluorescence above a baseline with no or little change in fluorescence. In order to analyse data, the reporter (FAM) fluorescence is automatically normalised to a passive reference to avoid the measurement of non-PCR-related fluorescence [30]. A threshold is set above the baseline and a threshold cycle value (Ct) is defined as the cycle number at which the fluorescence passes the fixed threshold and a statistically significant increase in fluorescence is first detected. The qPCR amplifications standard curve for test samples is designed from Ct values plotted versus the log of standard concentrations [31–33].

*Calculation and interpretation of anti-viral effect:* A higher Ct value corresponding to the drug-treated sample compared with the untreated sample will prove the anti-viral effect of the drug when

**FIGURE 4**

Representative diagram of hemagglutination assay plate showing calculation of Viral HA units. The virus in lanes 2 and 3 has 1:16 HA units (modified from [109]).

viral load ( $\times 10^6$  particles or fold decrease) is calculated from the standard plot of Ct values and is found to be less in case of drug-treated sample rather than untreated one. From these data  $IC_{50}$  or  $IC_{90}$  may also be calculated getting 50% or 90% reduction of viral particles or of fold change.

**Cell culture and experimental design for HBV:** The human HBV-transfected cell line HepG2.2.15 [34] were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% (v/v) foetal bovine serum (Gibco, USA), 100 unit/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM l-glutamine at 37 °C in a humidified incubator at 5%  $CO_2$ . Cells were seeded in 24-well tissue culture plates at approximately  $5 \times 10^5$ /well and maintained for 48 h before the extract treatment (at different concentrations) to allow the HBV DNA levels to stabilise. The cell medium and HepG<sub>2</sub> cells were collected right before the first dose (day 0) and after 3, 6, 9, 12 days of treatment (in duplicate), and stored at -70 °C for analysis of Hepatitis B surface antigen (HBsAg), Hepatitis B e antigen (HBeAg) and HBV DNA level [35].

**Cell toxicity:** The cytotoxic effect of extract towards HepG<sub>2</sub> 2.2.15 was evaluated using the MTT assay [35,36] to determine the drug concentrations that did not affect the cell viability. These drug concentrations were used in subsequent assays.

**Determination of HBsAg and HBeAg:** After incubation with various concentrations of extract at 37 °C in 5%  $CO_2$  for 3, 6, 9, 12 days, the culture medium was collected. The concentration of HBsAg or HBeAg was detected by an enzyme-linked immunosorbent assay (ELISA) kit (KeHua Inc., China) following the manufacturer's protocol [35] and [37]. Inhibition Ratio % =  $OD$  (Control) -  $OD$  (Sample)/ $OD$  Control  $\times$  100%.

**Analysis of HBV DNA:** For the intracellular HBV DNA analysis, cells in 24-well plates were lysed with 0.5 ml/well lysis buffer (4 M guanidine isothiocyanate, 7% 2-mercaptoethanol, 2% sarkocyl) and cells in 10 cm dishes were lysed in 6 ml lysis buffer. The cellular DNA was prepared as previously described [36]. The DNAs (10  $\mu$ g each) were then digested with EcoRI, electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose membrane. A purified 3.2 kb EcoRI HBV DNA fragment labeled with digoxigenin was used for the detection following the manufacturer's protocol (Roche Inc., USA) [37].

#### HBV DNA copy number determination and quantitative PCR

PCR reactions were performed in Biorad iCycler (Biorad, USA) following the manufacturer's manual. The oligonucleotide sequences of the primers were as follows: HBV upstream 5'-CCTCTTCATCCTGCTGCT-3' downstream 5'-AACTGAAAGC-CAAACAGTG-3'. The PCR was carried out in a 25  $\mu$ l reaction volume containing 5.0  $\mu$ l  $5 \times$  R-PCR Buffer, 0.3  $\mu$ l 250 mM  $Mg^{2+}$ , 1.0  $\mu$ l 10 mM dNTP, 1.0  $\mu$ l 10  $\mu$ M primers, 1.0  $\mu$ l  $25 \times$  SYBR SYBR Green I, 1.0  $\mu$ l  $10^{-3} \times$  Calibration, 0.25  $\mu$ l 5 U/ $\mu$ l Taq-E (TaKaRa BIO, Japan), 13.7  $\mu$ l ddH<sub>2</sub>O and 2.0  $\mu$ l HBV DNA. After initial incubation at 95 °C for 90 s to activate the Taq polymerase, 40 cycles of amplification were conducted using the following programme: 95 °C for 5 s, 60 °C for 30 s and 72 °C for 20 s. The PCR products were finally held at 4 °C before analysis [32,37]. The GAPDH gene was used as the DNA standard. Primers for GAPDH were as follows: 5'-AAGGTCCGAGTCAACGGATT-3' and 5'-CTGGAAGATGGTGATGATGGGATT-3'. The RT-PCR standard was generated using serial dilutions of a known DNA. The HBV DNA copy number was normalised by GAPDH and calculated by

the iCycler iQ™ Real-Time System software (Version 3.0 for windows, BIO-RAD, USA) according to the standard curve [32]. HBV DNA inhibition rate (%) = (copy number of the control–copy number of the study sample)/copy number of the control × 100%.

### Studies on the mechanism of action

Compounds can also be evaluated for activity when challenged with different amounts of virus like HSV-1, HSV-2, HCMV and VZV ranging from very low to very high multiplicity of infection.

**Time of addition and time of removal:** Compounds are added or removed from cultures at various times preinfection and postinfection. By comparison with other known viral inhibitors, this allows to determine the relative point in the virus life cycle that is being inhibited (immediate early, early, late functions, DNA polymerisation, etc.). This standard technique typically used early during the process of determining mechanism of action as it allows one to narrow in on a smaller target window of activity for further experimentation. It also allows for an easy way to determine if a compound is acting by a unique or novel mechanism compared with other known inhibitors. Furthermore, time of removal studies allow one to determine the reversibility of a compounds activity.

**Analysis of viral DNA:** The effect of compounds on the production of viral DNA can be evaluated using various hybridisation techniques, PCR or TaqMan PCR.

**Analysis of viral proteins:** The effect of compounds on the production of immediate early, early and late viral proteins can be evaluated using Western blots and/or Flow cytometry.

**Selection and characterisation of drug-resistant virus isolates:** Resistant virus isolates are selected in tissue culture by serial passage of the virus in the presence of gradually increasing concentrations of the compound. Resistance evaluations can be performed in any of the available cell lines with a variety of virus isolates. In addition, resistance selection can be evaluated using combinations of anti-viral agents to evaluate the relative ability of the virus to become resistant to multiple agents that might be used in the clinic.

### In vivo assays

On the basis of the target site of infection and disease presentation various animal models can be used for different viruses namely mouse, guinea pigs, ferrets, rabbit, primates and so on.

#### Established assays for *in vivo* testing of anti-virals

##### Herpes viruses

**Animal models for studying HSV:** The broad host range of HSV has allowed the use of different animal models for the study of these viruses. An *ideal* animal model would be able to recreate all aspects of the human disease, but, obviously, this is not attainable. Still, in terms of the ability of HSV to establish a localised initial infection followed by neuronal spread and establishment of latency, a number of very useful models exist for understanding of the disease in humans. The most appropriate model for latency must allow virus reactivation similar to humans. Both rabbit and the guinea pig, approximate this ideal situation, although both suffer from limitations, and expense. A third model, the mouse (the most reasonable in cost), is being used extensively, but suffers from the lack of efficient *in vivo* reactivation. These three animal systems are exten-

sively used for generating the vast majority of data now available on HSV latency and reactivation, but other models also exist.

As the HSV infections in mice provide a good model for human disease, we will describe here the different mouse models as well as with other animals that provide receptors for HSV entry and expression of viral glycoproteins that influence disease and pathogenicity in man. To test the *in vivo* toxicity and efficacy of the herbal products in animal several models have been developed. The most commonly used method of *in vivo* toxicity determination is dermal toxicity testing of the extract or substance, usually done by skin irritation test; while the efficacy of any extract or compound is measured by cutaneous lesion development in guinea pigs (Figure 5).

##### Skin irritation test

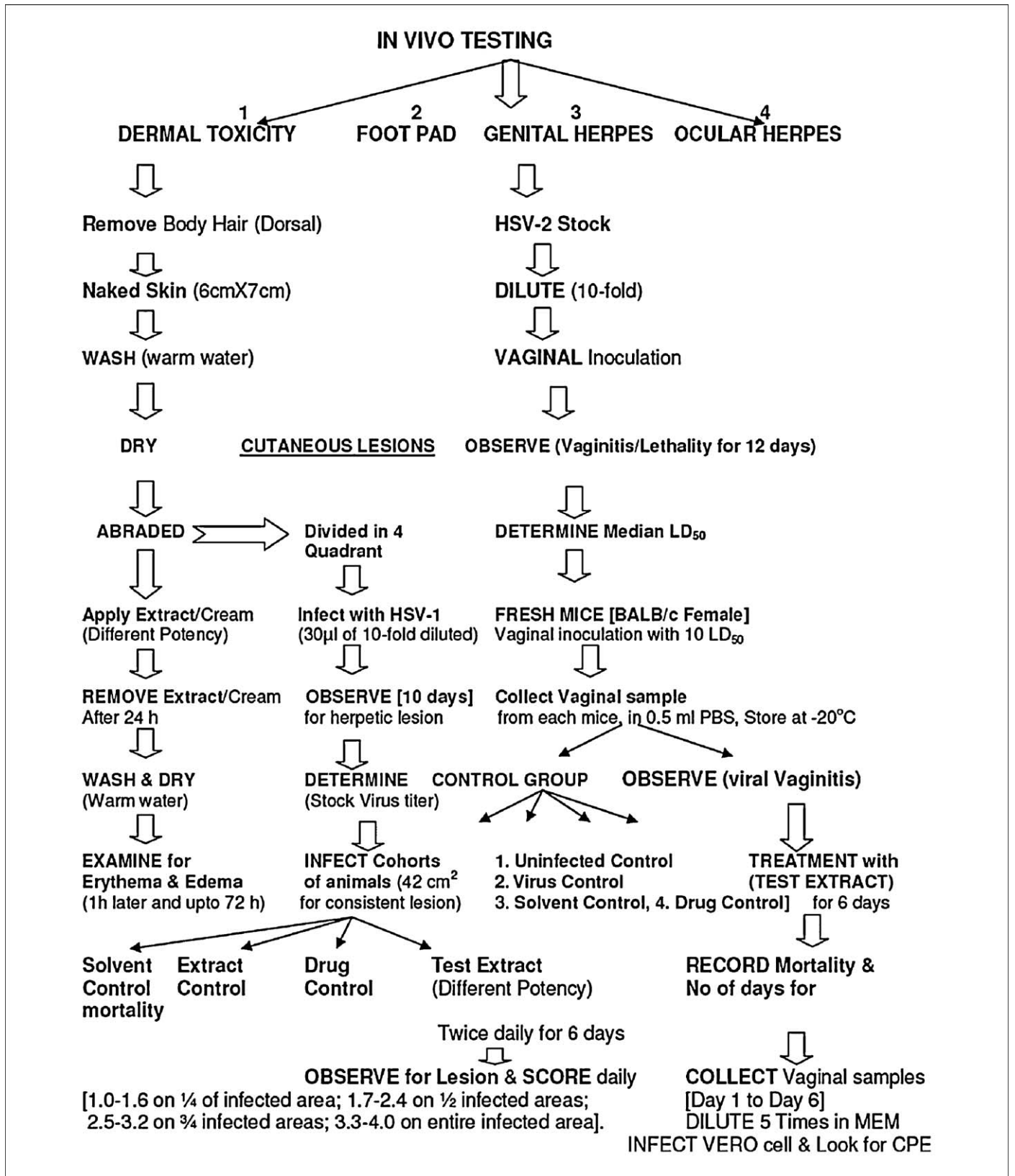
The dermal toxicity of the herbal extract or extract-based creams can be tested in Guinea pigs of either sex (200–250 g). After removing the body hair from the dorsal side of the animal with 8% BaS, the naked skin (6 × 7 cm) is washed with warm water, dried and abraded with dermal (Seven-Star) needles. The extract or extract-based creams of different potency (like 30, 15, and 7.5%) is then being applied to the abraded area of cohorts of animals ( $n = 5$ ) at the rate of 2 g per animal. After 24 h, the creams will be removed with warm water and the animals will be examined for erythema and oedema 1 h later, up to the next 72 h.

##### Cutaneous lesion assay

To study the extract potency on HSV-1 induced cutaneous lesion, the dorsal skin of Guinea pigs can be prepared and abraded as above. The abraded area should be divided into four quadrants and each of the quadrants will be infected with 30  $\mu$ l of 10-fold diluted HSV-1. The animals ( $n = 5$ ) will be observed for 10 days for typical herpes lesion development. On the basis of initial result, amount of stock virus will be determined (usually 150  $\mu$ l of  $10^8$  PFU) to infect 42 cm<sup>2</sup> and to obtain consistent lesion development. Once the lesion developed, the Cohorts of animals ( $n = 15$ ) will be infected as above and treated with the (i) extract or extract-based cream, (ii) 3% (wt/wt) acyclovir cream, or (iii) base cream (1.5 g per dose) to the infected area with sterile cotton swabs twice daily for a 6-day period (Figure 5). The extent of lesion will be scored daily as follows: 1.0–1.6, lesions on 1/4 of infected area; 1.7–2.4, lesions on 1/2 of infected area; 2.5–3.2, lesions on 3/4 of infected area; and 3.3–4.0, lesions on the entire infected area [38].

##### Reduction in viral vaginitis in mice or cotton rats

To test the *in vivo* efficacy of an extract or formulation against HSV-2, the Genital herpes model was developed in random breed BALB/c female mice or in female *Sigmodon hispidus* (cotton) rats [39] by intravaginal inoculation of HSV-2 in anaesthetised inbred 6-week-old mice or rats. After one-week acclimatisation at room temperature ( $23 \pm 3$  °C) the animals (10 animals for each dilution) are inoculated with HSV-2 (30  $\mu$ l of  $10^{-3}$  virus stock) to the vagina by a size 12 needles and observed for 12 days to develop vaginitis or lethality to determine the median lethal dose (LD<sub>50</sub>). To test the efficacy of the extract or formulation, fresh batches of animals are infected with 10 LD<sub>50</sub> dose of the virus ( $10^5$  PFU) as described above. Following inoculation, a vaginal cotton swab



**FIGURE 5**

*In vivo* testing protocols for anti-HSV extract/agents.

sample is collected from each animal, transferred to 0.5 ml of PBS and stored at -20 °C. The animals are divided into test groups (different potency), positive control group (acyclovir), negative control group (solvent or base cream), and one no treatment

(virus control) group as well as an additional group of uninfected control. Symptoms of viral vaginitis (topical oedema of the vaginal tract with turbid secretions) will be observed on the third day of infection. Treatment began on day 3 postinfection, by applying

the extract or formulations to the vaginal tract with cotton swabs, at a dose of 2 mg per mouse twice daily for a 6-day period [38]. Mortality and the number of days for mortality to occur are recorded. From day one following the completion of the treatment, as well as from the deceased animals immediately following their death vaginal swab samples should be collected (Figure 5). The vaginal samples are then diluted five times in MEM and used to infect Vero cells. Samples that gave positive CPE are considered positive for HSV-2 [38]. A polysaccharide lignin-carbohydrate complex from *Prunella vulgaris* (PPS-2b), when tested by the plaque reduction assay showed strong activities against HSV-1 and HSV-2, as the complex block HSV binding and penetration; while a cream with semi-purified fraction of *P. vulgaris* showed a significant reduction ( $P < 0.01$ ) in skin lesions and animal ( $P < 0.01$ ) mortality in a HSV-1 skin lesion guinea pigs model and HSV-2 genital infection model in BALB/c mice, indicating that this complex had potent anti-HSV activity [38].

#### Model for ocular herpes virus study

A fast, simple reactivation model to study the ocular herpesvirus infection and latency was successfully established by Gordon *et al.* [40] in New Zealand female rabbits (1.5–2.0 kg). Following topical anaesthesia with 0.5% proparacaine HC1 eye drops, each unscarified rabbit eye was inoculated with a suspension of thymidine-kinase-positive HSV-1W strain ( $5 \times 10^4$  pfu/eye) into the lower fornix, following a topical anaesthesia with eye drops. The HSV-1W establishes latency and reactivates in a manner similar to mouse pathogenic strain HSV-1 McKrae [41]. Successful inoculation (100%) of eyes was found on day 7 with typical herpetic dendritic ulcers and significant HSV-1 titre ( $10^4$  pfu/ml) and viral shedding can be determined by neutralisation test. After satisfactory anaesthesia the globe is proptosed with a wooden cotton applicator and an operating microscope is used to facilitate all surgical manipulations. The intrastromal injection (by a no. 30 short bevel needle attached to a 0.25-ml tuberculin syringe) is injected into the central corneal stroma. One group will receive deionised sterile endotoxin-free water; another group will get 100  $\mu$ l air while the third group will receive no injection, and for all three groups, the needle should be carefully withdrawn, and the proptosed globe gently returned to the orbit by gentle digital pressure. The anterior chamber injection with deionised sterile water is made at the limbus, inserted into the anterior chamber parallel to the iris plane. Needle is carefully withdrawn, and the insertion site will be pressed with a cotton swab for 30 s, to avoid aqueous loss. This pressure also returned the proptosed globe to its proper place in the orbit. For topical administration of 100 ml deionised sterile water will be made onto the cornea of the proptosed globe by a pipette and the globe will return to the orbit by gentle digital pressure. Viral Shedding (detection of latent HSV-1 after reactivation and induced shedding into the tear film) is determined by swabbing the eyes two days before treatment and for 7 consecutive days after treatment. Each eye swab is mixed with 0.3 ml MEM (modified 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 period, an additional 1.5 ml media is added to the well, and the plate can be examined daily for seven days for the progressive CPE characteristic of

HSV-1. Random HSV-1 isolates can be confirmed by neutralisation [40,42].

#### 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 only following explant and co-cultivation with indicator cells. In addition, virus can be sporadically recovered from the eye following periods of latency. Of particular use is the fact that the reactivation can be efficiently induced by the iontophoresis of epinephrine into the eye. This model has been very important in establishing the requirement for LAT expression for efficient reactivation.

#### Murine models for latency and reactivation

##### The footpad/dorsal root ganglia model

Direct demonstration of the ability of HSV to establish and maintain a latent infection in neuronal cells in mouse footpad infection followed by latent infection of spinal ganglia. This model system is roughly analogous to genital infection of HSV in humans, and has been central to describing many of the parameters of HSV latent infection including the identification of the neuron as the site of latent infection, axonal transport of virus through the sciatic nerve, ability of non-replicating virus to establish latent infections, and the characterisation of restricted transcription of the latency specific transcription unit during the latent phase of infection. Further, the model is useful with HSV-2, despite this virus' greater neuropathology. Following infection of the footpad, local pathology is observed with clear evidence of involvement of the central nervous system (CNS), the mice that recover are evidently physiologically normal. When dorsal root ganglia are dissected and cultured on feeder cells, HSV-induced cytopathology can be detected within 4–12 days. This explant recovery of HSV from such latently infected spinal ganglia has been an extremely useful and relatively inexpensive means of assaying the presence of viral genomes within the tissue.

##### The mouse eye/trigeminal ganglia model

A second murine model for HSV-1 and HSV-2 latency involves the infection of the cornea followed by virus latency in the trigeminal ganglia. As in the footpad model, latent HSV genomes express LAT (the latency associated transcript) in a portion of those neurons maintaining them, and virus can be recovered by co-cultivation of explanted ganglia. An interesting variation on this method that comes closer 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 immunohistochemical methods or genetic engineering (if recombinant virus with an expressible marker in the genome is used).

##### Vaginal inoculation of HSV in guinea pigs

Vaginal inoculation of female Guinea pigs with HSV-1 or HSV-2 results in obvious primary infection with some mortality. 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 fact that HSV-2 spontaneously reactivates with much higher frequency than HSV-1 makes this

a very attractive system for comparative analysis of the influence of viral genes on reactivation and HSV-1 and HSV-2 recombinant viruses are being investigated with an eye towards attempting to identify features important in this difference. The value of guinea pigs in studying drug or vaccine efficiency and other aspects of experimental pathogenesis makes this an extremely valuable and promising system.

### Respiratory viruses

**Respiratory syncytial virus:** Respiratory syncytial virus (RSV) is a member of the family Paramyxoviridae, subfamily Pneumovirinae, with a nonsegmented negative stranded genome of 15,222 nucleotides (strain A2; [43]), and a nucleocapsid within a lipid envelope with irregular size and shape. The virion contains two major surface glycoproteins (fusion protein and attachment protein), along with a minor hydrophobic protein. The virus has two major antigenic groups (A and B) with dissimilarities in the attachment protein (1%–7% relatedness). The related viruses are bovine, ovine, and caprine RSV, mice pneumonia virus, and turkey rhinotracheitis virus. RSV is structurally and functionally similar to parainfluenza viruses, with very little antigenic or sequence relationship, and is distantly related to Orthomyxoviridae (influenza virus). More than 50% children is infected during the first year of life and by 24 months of age all children had been infected at least once [44], and 50% had two infections, with an incubation period of four to five days, and transmission probably occurs via fomites not aerosols. There is no viremia, but pulmonary involvement occurs in 25%–40% of initial which is uncommon in subsequent infections, except in bone marrow transplant recipients or elderly patients. Virus may be shed as long as 20 days. The protective immune response of infected individuals lasts for a few months. The development of vaccine (formalin inactivated) and subsequent vaccination of children ended with disastrous results [45], and till date not successful.

Several animal models of RSV infection have been developed using primates, cotton rats, mice, calves, guinea pigs, ferrets, and hamsters, with an aim to know the mechanisms of disease and for testing efficacy and safety of drugs and candidate vaccine (prophylactic and therapeutic). For RSV multiple animal models is needed as in humans RSV is a multifaceted disease whose clinical manifestations and sequelae depend upon age, genetic makeup, immunologic status. There is no single human subpopulation in which all forms of RSV disease manifest, nor is there a single animal model that duplicates all forms of RSV disease. Hence, the choice of animal model will be governed by the specific manifestation of disease to be studied and thus no single animal model can duplicate all aspects of primary RSV disease in man. The cost, availability, and opposition from animal rights activists will restrict the study of RSV in primate models, particularly the chimpanzee. The primary advantage of the models are: the calf is the natural host of an RSV species; the cotton rat have high permissiveness and reliability as a predictor of prophylactic and therapeutic strategies; the mouse had wide availability of specialised strains and reagents; the guinea pig is best for parallels in reactive airway disease; and the ferret produce age-dependent pulmonary infection. Utilisation of the calf, cotton rat, mouse, guinea pig, and ferret models probably will increase as the relative strengths of each model become well defined.

### Experimental animals, RSV infection, and treatment with NOS inhibitors

Female BALB/c mice, after being sedated with ketamine and xylazine, are infected by intranasal inoculation with  $5 \times 10^5$  pfu of RSV A2 [46,47]. RSV infection is confirmed by measuring titres in the lungs. To evaluate the effects of inhibition of NOS activity in *in vivo* model, RSV infection is performed in the absence or presence of AMT (2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; Sigma Chemical) or L-NAME (N-nitro-L-arginine methyl ester; Sigma Chemical), a specific inhibitor of iNOS and a nonspecific inhibitor of NOS isoforms, respectively [48]. These agents are given i.p. daily for four days, beginning 1 h before RSV infection, at the following doses: 4 mg/kg for AMT and 100 mg/kg for L-NAME. As shown by other investigators [48] and preliminary studies, this experimental protocol resulted in inhibition of production of NO in mouse system. Control mice received normal saline intraperitoneally in a similar manner and schedule. On the basis of preliminary dose-response and time-response studies, the experiments using NOS inhibitors are performed on day 4 after RSV infection, at the peak of viral replication.

### Inoculation of virus in nasal cavity

Primary RSV infection was initially described in nasal tissues of only few Chimpanzees [49,50]. The genetic heterogeneity among outbred animals, the statistical insignificance of data and verification of published observations by other laboratories are not possible owing to limited number of animals, and thus raise questions about the scientific validity of these experiments. Experimental RSV infection has also been described in the owl monkey [51–53], rhesus monkey [49], African green monkey [54], cebus monkey [51], squirrel monkey [49], bonnet monkey [55], and baboon [56] but both purchase and maintenance costs of these species are high and handling is cumbersome, though unlike chimpanzees, these species can be used in terminal experimentation, thus allowing more detailed virological and histological studies of pulmonary RSV disease. Lack of inbreeding in these species limits other studies, and none develop clinical or radiological signs of pulmonary RSV disease, except owl monkeys that develop mild rhinorrhea. The most useful of these is the African green monkey because the pulmonary disease it develops is histologically similar to humans. However, the lack of sufficient number of animals, lack of inbreeding, and the scarcity of immunologic reagents suggest that it will be a secondary model.

The cotton rats are uniformly susceptible to pulmonary infection, thus become a useful model for long-term studies [57] and is 100-fold more permissive (per input dose of virus) and more responsive (develop serum antibody titres 10-fold or higher [46,52,53], than mouse, and is parallel to humans. A recent work studying combined anti-viral/anti-inflammatory approach [58] suggests that modulation of lung inflammation, in addition to clearance of virus, will be required for rapid reversal of clinical disease and clinical trials are planned to test this approach. Although inbred cotton rats are commercially available but lack of reagents, other plasma proteins, antigens, cytokines and congenic, transgenic or knockout strains limit its use.

The diseases caused by RSV and bovine RSV have several common characteristics, suggesting that the calf may be a useful model of human RSV disease. For example, both viruses cause acute



disease in the respiratory tract, induce an incomplete immune response permitting repeated life long infections, cause epidemic during winter, are attenuated by high levels of maternally derived antibody, and cause severe pulmonary disease in neonates [59]. The calf model however, has many drawbacks like cost of purchasing and maintaining animals, lack of inbred strains, and lack of specific reagents. Additionally two characteristics of clinical disease caused by bovine RSV in calves raise concerns about its usefulness. First, bovine RSV commonly causes fever, but human RSV does not [60] and it is unknown whether this is due to differences in the viruses or in host responses. Second, bacterial (pasteurella, haemophilus [61] and mycoplasmal [62] coinfections are common complications of bovine RSV infection, whereas such coinfections with human RSV are rare.

#### Harvesting of lungs and respiratory tract

The only animal on which quantitative virological studies have been reported is the Syrian hamster [63]. Although RSV replicated in both the lungs and nose, the relative permissiveness of the hamster was equivalent to that of the mouse and was 100-fold less than that of the cotton rat.

#### Mouse versus ferrets model

Coates and Chanock [64] examined several species, including four strains of inbred mice (DBA/2, BALB/c, AKR, and C3H), to determine permissive for pulmonary viral infection and found that none of those four mice developed CF antibody, but only one strain (AKR) developed neutralising antibody. Prince *et al.* [52], Prince *et al.* [53] found that each of 20 inbred strains, including the four tested earlier, as permissive for RSV infection in the lungs and nose. Levels of viral replication varied by two orders of magnitude from the least permissive (CBA/CaHN) to the most permissive (DBA/2N) strains, and the most permissive strain was about 100-fold less sensitive than the cotton rat [57]. The mouse model has several advantages over other species, like availability of inbred, congenic, transgenic, and knockout strains; specific reagents allowing identification and quantitation of cell types, immunoglobulins, cytokines, and relatively low purchase and maintenance costs. Although no prophylactic or therapeutic formulations have yet been licensed on the basis of these studies, but a lot of information on RSV immunology have emerged (for reviews see [65,66]).

RSV replicates in high titres in the nasal tissues of ferrets of all ages [64], but the virus replicates in the lungs only of infant animals [67]. Although the rapidly decreasing permissiveness of the lung severely limits the utility of ferrets, it provides an intriguing model for the age dependence severe RSV disease in humans. Despite the fact that the ferret model has disadvantages similar to those of the guinea pig and primate models (lack of inbred strains and immunologic reagents), it is the only model in which age-dependent pulmonary infection has been described and thus offers a potential tool for dissecting the mechanisms of age-dependent disease in humans.

#### Influenza virus

Inbred female Balb/c and C57Bl/6 mice of 6–8 weeks old are used. The animals are anaesthetised by intraperitoneal injection of 150  $\mu$ l of a ketamine rompun-solution (equal amounts of a 2%-

rompun solution and a 10%-ketamin solution are mixed at the rate of 1:10 with PBS) and infected intranasally (i.n.) with  $1 \times 10^2$  pfu/50  $\mu$ l of Influenza A virus. For infection of mice with Influenza A virus pre-incubated with specific drug, either 102/25  $\mu$ l, 103/25 or 104/25  $\mu$ l virus is incubated either with 25  $\mu$ l specific drugs (1 mg/ml) or with 25  $\mu$ l PBS for 30 min at room temperature [68].

#### Treatment of Balb/c mice with extract

Five mice are treated at the same time in an inhalation chamber ( $2.1 \times 10^{-2}$  m<sup>3</sup>) or single mice are treated in an inhalation tube. Five tubes are connected to a central cylinder with an overall volume of  $8.1 \times 10^{-4}$  m<sup>3</sup>. A PARI<sup>®</sup> nebuliser (Aerosol Nebulizer; Art. No. 73-1963) is connected to either the inhalation chamber or the central tube cylinder. Specific extract (10 mg/ml) or buffer solution with a pressure of 1.5 bar is given for 10 min (roughly 2 ml) to the chambers. The extract was dissolved in sterile ddH<sub>2</sub>O (stock solution: 10 mg extract/ml distillate water) and incubated for 1 h at 100 °C in a water bath. Balb/c mice are placed in the tube-cylinder and exposed to 2 ml of aerosolised extract for 10 min three times a day at 9:00 a.m., 12:00 a.m. and 3:00 p.m. The treatment is performed for five days. Ten minutes after the first treatment mice are infected with Influenza A. Balb/c controls are treated with the same amount of sterile ddH<sub>2</sub>O. After virus infection, the general health status of the animals is controlled twice a day. Furthermore, the animals are weighted every day. The mice are sacrificed after they had lost 25% of their initial weight. All animals are monitored for 15 days after infection.

#### Mouse monitoring

The monitoring of body temperature and gross motor activity of the animals is performed with the VitalView<sup>®</sup> software and hardware system (Mini Mitter, USA) that allows data acquisition of physiological parameters. The hardware includes a transmitter (E-Mitter)/receiver system. The E-Mitter collects data on temperature and gross motor activity for the lifetime of the animal. The temperature sensitive devices alter their pulse rate in response to temperature changes. The Vital View system records the average rate and converts this to temperature using temperature calibration values specific to each unit. The vitality or gross motor activity measurement provides a basic index of the movement of mice with implanted E-Mitter. As the mouse moves, movement of the implanted E-Mitter results in subtle changes in the transmitted signal. These small changes are detected by a receiver via telemetry and registered by a connected computer as activity counts. In our experiments the Vital View software recorded an index of movement every 5 min to produce a longitudinal record of the activity. For implantation of the E-Mitters the mice are anaesthetised with intraperitoneal injection of 150  $\mu$ l ketamine/rompun. The ventral surface of the abdomen was shaved and a midline abdominal skin incision is made 0.5–1 cm below the diaphragm with no more than 2 cm in length. The abdomen is opened with a 2 cm incision along the *linea alba* and the E-Mitter is positioned in the abdominal cavity. After the incision was closed with two to three 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. Health status is controlled for seven days before infection.

### Histology and immunohistology

Mice are treated with nebulised extract (10 mg/ml) three times at 9:00 a.m., 12:00 a.m. and 3:00 p.m. for 10 min or H<sub>2</sub>O (as control). Immediately after the treatment the mice are killed and lungs are obtained and fixed in buffered 4% paraformaldehyde. The lungs are stained with haematoxylin and 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 was performed with an ABC-kit (Vector) for 30 min at RT. Substrate reaction was performed with DAB kit (Vector Laboratories).

### Rabies virus

Rabies is caused by a neurotropic virus of the genus *Lyssavirus* of the family Rhabdoviridae, and is transmissible to all mammals. As it is transmissible to humans by inoculation or inhalation of infectious virus, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organisation [69]. Seven distinct genetic lineages can be distinguished within the genus *Lyssavirus* by cross-protection tests and molecular biological analysis [70–72], namely the classical rabies virus itself (RABV, genotype 1, serotype 1), Lagos bat virus (LBV, genotype 2, serotype 2), Mokola virus (MOKV, genotype 3, serotype 3), and Duvenhage virus (DUUV, genotype 4, serotype 4). The European bat lyssaviruses (EBLV), subdivided into two biotypes (EBLV1, genotype 5 and EBLV2, genotype 6) and the Australian bat lyssavirus (ABLV, genotype 7), recently isolated in Australia (24), are also members of the *Lyssavirus* genus, but are not yet classified into serotypes. Viruses of serotypes 2–4, EBLV and ABLV are known as rabies-related viruses. An experimental model of rabies was established in the fruit-eating bat *Artibeus jamaicensis*, infecting with CVS-N2c and CVS-B2c, which are both stable variants of CVS-24 in the right masseter muscle. CVS-N2c produced neurologic signs of rabies with paresis, ataxia, and inability to fly, while CVS-B2c did not produce neurologic signs. Bats were sacrificed and the distribution of rabies virus antigen was assessed in tissue sections with immunoperoxidase staining. Both viruses spread to the brain stem and bilaterally to the trigeminal ganglia by days 2 to 3. CVS-N2c had disseminated widely in the central nervous system (CNS) by day 4 and had involved the spinal cord, thalamus, cerebellum, and cerebral cortex. CVS-B2c had infected neurons in the spinal cord on day 5 and in the cerebellum, thalamus, and cerebral cortex on day 6. Infected pyramidal neurons of the hippocampus were observed on day 5 in CVS-N2c infection, but infected neurons were never noted in the hippocampus in CVS-B2c infection. CVS-N2c infected many more neurons and more prominently involved neuronal processes than CVS-B2c. CVS-N2c spread more efficiently in the CNS than CVS-B2c. Morphologic changes of apoptosis or biochemical evidence of DNA fragmentation were not observed in neurons with either virus after this route of inoculation. The different neurovirulent properties of these CVS variants in this model were not related to their *in vivo* ability to induce apoptosis [73].

These tests detect the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result or when the FAT is negative in the case of known human exposure.

### Mouse inoculation test

Five-to-ten mice, 3–4 weeks old (12–14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally. It is recommended, though not strictly essential, to use specific pathogen-free (SPF) mice. The inoculum is the clarified supernatant of a 20% (w/v) homogenate of brain material (cortex, Ammon's horn, cerebellum, medulla oblongata) in an isotonic buffered solution containing antibiotics. To reduce animal pain, mice should be anaesthetised when inoculated. The young adult mice are observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For street fox rabies strains, deaths due to rabies generally begin nine days postinoculation. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9 and 11 postinoculation. This *in vivo* test is quite expensive, particularly if SPF mice are used, and should be avoided where possible. It does not give rapid results (compared with *in vitro* inoculation tests), but when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes. Another advantage of this low-tech test is that it can be easily and practicably be applied in situations where skills and facilities for other tests (e.g. cell culture) are not available.

### Cell culture for Rabies virus

Neuroblastoma cell lines (CCL-131, ATCC: American Type Culture Collection, 10801), is used for routine diagnosis of rabies. The cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% foetal calf serum, incubated at 36 °C with 5% CO<sub>2</sub>. Its sensitivity has been compared with that of baby hamster kidney (BHK-21) cells [74]. This cell line is sensitive to street isolates without any adaptation step, but should be checked for susceptibility to locally predominant virus variants before use. Presence of rabies virus in the cells is revealed by the FAT. The result of the test is obtained after at least 18 h (one replication cycle of virus in the cells); generally incubation continues for 48 h [75] or in some laboratories up to four days. This test is as sensitive as the mouse inoculation test. Once a cell culture unit exists in the laboratory, this test should replace the mouse inoculation test as it avoids the use of live animals, is less expensive and gives more rapid results. It is often advisable to carry out more than one type of test on each sample, at least when there has been human exposure [76].

### Human immunodeficiency virus

The current testing of anti-HIV drugs is hampered owing to the lack of a small animal that is readily available, easy to handle, infected systemically with HIV-1, harbours the major HIV-1 target cells in a physiological frequency, organ distribution, and activation state and is established as a pharmacological model. The potential of outbred Sprague–Dawley rats that transgenically express the HIV-1 receptor complex on CD4 T cells and macrophages was explored by Goffinet *et al.* [77] as a model for the preclinical evaluation of inhibitors targeting virus entry or reverse transcription (RT). It was observed that the concentrations of enfuvirtide (peptidic fusion inhibitor) or efavirenz (nonnucleoside RT inhibitor) required to inhibit HIV-1 infection of cultured primary CD4 T cells and macrophages from human CD4 and CCR5-transgenic rats differed by threefold from those required for human reference cultures [77]. Prophylactic treatment of dou-

ble-transgenic rats with a weight-adapted paediatric dose for either enfuvirtide (s.c., twice-daily) or efavirenz (oral, once-daily) achieved 92.5% or 98.8% reduction, respectively, of the HIV-1 cDNA load in the spleen four days after i.v. HIV-1 challenge. While a once-daily dose for enfuvirtide resulted in fivefold weaker inhibition of infection. The study provides proof that HIV-susceptible transgenic rats can allow a rapid and predictive preclinical evaluation of the inhibitory potency and of the pharmacokinetic properties of anti-viral compounds targeting early steps in the HIV replication cycle.

### Transgenic rat model

The generation and initial characterisation of hCD4/hCCR5-transgenic rats has been reported [78] and Cultures of primary T lymphocytes and macrophages from transgenic rats and random human donors were generated by many workers [79–81]. For virus stock the HIV-1<sub>YU-2</sub> proviral DNA was cloned directly from brain tissue of a patient who died of AIDS dementia complex [82], and the generation of replication-competent HIV-1<sub>YU-2</sub> stocks for *in vivo* infection was studied [78]. Virus stocks were characterised for p24 concentration and for infectious titre (TZM-BL IU) by Keppler *et al.* [81]. The molecular clones HIV-1<sub>NL4-3</sub> E<sup>-</sup> EGFP, which carries an *egfp* gene within the *nef* locus [83], HIV-2<sub>ROD-A</sub> E<sup>-</sup> EGFP [84], or HIV-1<sub>NL4-3</sub> RT<sub>mut</sub>(K20R, K32R, V35L, K65R, L100I, K103N, L214F, P272A, I293 V) E<sup>-</sup> EGFP were pseudotyped with VSV-G [79]. HIV-1<sub>YU-2</sub> virions containing BlaM-Vpr were produced by triple-transfection of 293T cells by calcium phosphate DNA precipitation [85]. Two days posttransfection, the supernatant was concentrated by Centricon Plus-70 spin columns (Millipore, USA), and then virus particles were purified through a 20% sucrose cushion (27,000 × g, 4 °C, 60 min). The virion-enriched pellet was resuspended in medium and stored at -80 °C. The drug enfuvirtide (Roche, USA) was dissolved (9 mg/ml) and further diluted in distilled water; while efavirenz (Bristol-Myers Squibb, USA) was used as a drinking solution at 30 mg/ml and diluted in PBS.

The flow cytometry based *ex vivo* HIV-1 virion-fusion assay was conducted by standard methods [85,86]. Briefly, primary T cells and macrophages were pretreated with the indicated concentrations of enfuvirtide for 30 min. Subsequently, cells were challenged with HIV-1<sub>YU-2</sub> BlaM-Vpr virions (50 ng per 2 × 10<sup>6</sup> T cells or per ≈2 × 10<sup>5</sup> macrophages) for 3–4 h, washed, and then loaded with CCF2/AM dye overnight. Fusion was monitored with a three-laser BD FACSAria Cell Sorting System. While for *ex vivo* efficacy testing of drug Efavirenz, macrophages were pretreated overnight and T cells for 1 h with efavirenz at the indicated concentrations, and then they were subsequently challenged with single-round VSV-G HIV-1<sub>NL4-3</sub> EGFP reporter virions (200–500 ng of p24 per 3 × 10<sup>6</sup> T cells or per ≈2 × 10<sup>5</sup> macrophages). On day 3 postinfection, the percentage of EGFP-positive T cells and macrophages was scored by flow cytometry on a FACS-Calibur by using BD CellQuest Pro 4.0.2 software. For *in vivo* efficacy testing two independent studies with a similar design were performed (animal age 8 weeks; weight 140–306 g). For the NNRTI study, groups consisting of six hCD4/hCCR5-transgenic rats were treated with either efavirenz at 25 mg/kg per day or PBS by once-daily oral gavage for three days. On day 3, rats from both experimental groups were anaesthetised and challenged by tail vein injection through a plastic catheter with HIV-1<sub>YU-2</sub> (6.7 × 10<sup>6</sup> TZM-BL IU;

5000 ng of p24 per rat; [78]). For the fusion inhibitor study, groups consisting of five or six hCD4/hCCR5-transgenic rats were treated with enfuvirtide at 4 mg/kg per day, applied by either twice-daily or once-daily s.c. injection. The control group was injected twice daily with an equivalent volume of distilled water. On day 3, all rats were challenged i.v. with HIV-1<sub>YU-2</sub> (6.7 × 10<sup>5</sup> TZM-BL IU; 500 ng of p24 per rat). In both efficacy studies, one HIV-nonsusceptible, hCD4-single transgenic rat was challenged with the identical virus inoculum. Dosing was continued for 4 more days postchallenge, and then all animals were killed. Total DNA was prepared from single-cell suspensions of splenocytes by using DNeasy tissue kits (Qiagen, Valencia, CA), and it was analysed by quantitative duplex PCR.

**Quantitation of HIV-1 DNA species:** The amount of total HIV-1 cDNA and HIV-1 2-LTR circles in splenocyte extracts was analysed by quantitative duplex PCR (ABI 7500 sequence detection system, Applied Biosystems, USA) and amplified by a primer pair specific for *LTR U5* and *gag* and the probe 5'-[fluorescein (FAM)]-CAGTGGCGCCCGAACAGGGA-[rhodamine (TAMRA)]-3' [76,77]. For quantitation of 2-LTR circle junctions a forward primer annealing at *U5*, a reverse primer annealing at *U3* and the probe 5'-(FAM)-TCCACACTGACTAAAA GGGTCTGGGGATCTCT-(TAMRA)-3' were used [87,88]. For standard curves, dilutions of pHIV-1<sub>NL4-3</sub> E<sup>-</sup> EGFP and pU3U5 covering five logs was used, supplemented with DNA from uninfected cells. Results obtained for HIV-1 cDNA species were normalised to the amount of cellular DNA, quantified in the same reaction by amplification of the rat GAPDH gene DNA. For the latter, dilutions of genomic DNA extracted from primary rat T cells were used for standard curves. The cycling programme was: 2 min at 50 °C; 10 min at 95 °C; 40 cycles of 15 s at 95 °C then 1 min at 60 °C. All samples were run in duplicate, and the data were analysed by 7500 system software. The lowest detection limit ranged from 0.02 to 0.03 copies per ng of DNA and from 0.05 copies per ng of DNA for total HIV-1 cDNA and for 2-LTR circles, respectively.

### Hepatitis B virus

Hepatitis B remains a major public health problem worldwide, despite the available effective vaccines. Hepatitis B virus (HBV), a member of the hepadnaviruses (hepatotropic DNA viruses), causes acute and chronic infection of the liver [89]. Approximately 80% of HBV carriers have different levels of hepatocyte destruction, which could develop into liver cirrhosis and hepatocellular carcinoma (HCC) or liver cancer [90]. There are about 400 million people with chronic HBV infection, who are at a life-long high risk of developing cirrhosis and/or liver cancer. Up to 30% of the chronic carriers will die of complications of chronic liver diseases [91–93]. Several anti-viral drugs have been approved for the treatment of hepatitis B, including interferon-γ and nucleoside analogues. However, unresolved significant issues such as moderate to low efficacy, dose-dependent side effects, and drug resistance remain with current drugs [94]. Therefore, there exists a significant unmet medical need for safe and efficacious new anti-HBV drugs. On the contrary, natural products provide a large reservoir of potentially active agents for safer and more efficacious anti-HBV agents.

The recent study with polyphenolic extract of *Geranium carolinianum* L on HBV replication both *in vitro* and *in vivo* in human HBV-transfected liver cell line HepG<sub>2</sub> 2.2.15, showed that it effec-

tively suppressed the secretion of the HBV antigens in a dose-dependent manner (IC<sub>50</sub> 46.85 µg/ml for HBsAg and 65.60 µg/ml for HBeAg at day 9). Consistent with the HBV antigen reduction, the extract (100 µg/ml) reduced HBV DNA level by 35.9%. In duck hepatitis B virus (DHBV) infected ducks (intra-gastrically (i.g.) once a day for 10 days), the plasma DHBV DNA level was reduced (ED<sub>50</sub> 47.54 mg/kg). In addition, Southern blot analysis confirmed the *in vivo* anti-HBV effect of the extract in ducks and extract also reduced the plasma and the liver DHBV DNA level in a dose-dependent manner. Furthermore, significant improvement of the liver was observed after extract treatment, as evaluated by the histopathological analysis. Another study with HBV-transfected liver cell line revealed that wogonin, a potent anti-inflammatory and anti-cancer drug, isolated from the traditional Chinese medicine *Scutellaria radix* (used for the treatment of inflammatory conditions including hepatitis) reduces the levels of the HBV antigens and DNA and inhibits HBV DNA polymerase with much higher potencies [91] and more importantly, demonstrated the anti-HBV activity *in vivo* in Duck hepatitis B virus (DHBV)-infected ducks and in human HBV-transgenic mice. In the human HBV-transfected liver cell line HepG2.2.15, wogonin effectively suppressed the secretion of HBV antigens (both HBsAg and HBeAg) with an IC<sub>50</sub> of 4 µg/ml at day 9, and reduced HBV DNA level in a dose-dependent manner. DHBV DNA polymerase was dramatically inhibited by wogonin with an IC<sub>50</sub> of 0.57 µg/ml, and in DHBV-infected ducks wogonin (once a day for 10 days) reduced plasma DHBV DNA level with an ED<sub>50</sub> of 5 mg/kg. The *in vivo* anti-HBV effect of wogonin in ducks was confirmed by Southern blotting of duck viral DNA in the liver. Additionally in human HBV-transgenic mice, wogonin (i.v. once a day for 10 days) significantly reduced plasma HBsAg level [95].

#### Anti-HBV study with ducklings

DHBV-positive (from vertical transmission) female ducks (12 months old, 900–1000 g) were maintained under normal daylight and fed with a standard commercial diet and water *ad libitum*. Human HBV-transgenic mice (5 weeks old with positive plasma HBsAg) from lineage 1.3.32 [96] and maintained under a 12/12 h light/dark cycle with a standard commercial diet and water *ad libitum*. Ducklings at one day of age were intravenously infected with a  $5.7 \times 10^6$  viral genome equivalent (VGE, 1 VGE =  $3.3 \times 10^6$  pg) of DHBV [97]. Seven days later, extract, solubilised in isotonic saline solution, was administered (112, 56, 28 mg/kg) once daily orally in a liquid diet for 10 days. The isotonic saline liquid diet was also administered to the animals as negative control. Lamivudine (200 mg/kg) was used as the positive control. DHBV DNA levels were measured at 0, 5, 10 days, and 3 after cessation of treatment at 10 days (day p3) by dot blot analysis.

#### Measurement of duck liver DHBV DNA by Southern blot analysis

Four grams of duck liver tissues were ground in 4 ml of a buffer containing 10 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 1.27 mM

EDTA, 20 mg/ml SDS, 5 µg/ml salmon sperm DNA, and 0.5 mg/ml proteinase K at 50 °C for 3 h, followed by centrifugation at  $13,000 \times g$  for 10 min. The supernatant was extracted with phenol/chloroform before the DNA precipitation in two volumes of ethanol and 1/10 volume of acetic acid. The DNA was then dissolved in 800 µl of TE buffer. Finally, the purified DNA was separated on a 0.8% agarose gel and analysed by Southern blot analysis using a DHBV DNA probe as described previously [98,99].

#### Histopathological examination of duck liver

The DHBV-positive ducks were treated with PPGC and 3 TC (i.g.) once daily for 10 days. The animals were sacrificed and the liver tissues were removed, fixed in formalin and embedded in paraffin. The tissue samples were then sliced at 5 µm of each section, stained with haematoxylin and eosin, and examined by light microscopy.

#### Endpoint measurement of reduction of virus load

The virus-infected organs are harvested after sacrificing the animal. The viruses are purified by either mincing the tissue, or treating with collagenase and freeze-thawing in media. Titres are measured by any of the *in vitro* tests described previously such as immunofluorescence assay on tissue sections, plaque reduction assay and quantitative PCR [100,101]. Indirect measure of inhibition of viral infection can also be done by monitoring the fever, activation of immune response, inflammatory cytokines and mortality post virus infection.

#### Future prospects and directions

Not only the common viral diseases are still fatal but there has been also upsurge in new viral infections worldwide. The currently available anti-virals though effective are beyond means of most developing countries. Thus, the development of safe, effective and inexpensive anti-viral drugs like RT inhibitors is among the top global priorities as many viruses are not yet curable and have high mortality rates. Recently considerable attention has been given to screening of various species of medicinal plants especially with focus on anti-HIV activity [102–106]. There has been considerable rise in using over the counter medicinal plant products containing orthodox medicinal 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 well studied, adverse effects or therapeutic failures have been observed [107]. The most important consideration involving medicinal plants is to identify and standardise the exact method of preparation of an extract, appropriate season of collecting plant material and details of administration [10,108].

Since significant number of plant extracts have yielded positive results it seems reasonable to conclude there are probably many potential anti-viral agents. Further characterisation of active ingredients will reveal useful compounds.

#### References

- 1 Chattopadhyay, D. *et al.* (1999) Viruses, the acellular parasites of cellular hosts: biology and pathology with special reference to HIV. *Ind. J. STD AIDS* 20, 54–60
- 2 Wagner, E.K. and Hewlett MJ, (1999) *Basic Virology* (1st ed.), Blackwell Science, Inc. 1-4051-0346-9
- 3 Chattopadhyay, D. and Bhattacharya, S.K. (2008) Ethnopharmacology: a new search engine for the development of antivirals from naturaceuticals. In *Handbook of Ethnopharmacology* (Eddouks, M., ed.), pp. 129–197, Research Signpost Publication

- 4 Chattopadhyay, D. and Naik, T.N. (2007) Antivirals of ethnomedicinal origin: structure–activity relationship and scope. *Mini Rev. Med. Chem.* 7, 275–301  
Review
- 5 Khan, M.T.H. *et al.* (2005) Extracts and molecules from medicinal plants against herpes simplex viruses. *Antivir. Res.* 67, 107–119 and references cited therein
- 6 Chattopadhyay, D. (2006) Ethnomedicinal antivirals: scope and opportunity. In *Modern Phytomedicine: Turning Medicinal Plants into Drugs* (Ahmad, I., Aquil, F., Owais, M., eds), pp. 313–338, Wiley-VCH
- 7 Naithani, R. *et al.* (2008) Antiviral activity of phytochemicals: a comprehensive review. *Mini Rev. Med. Chem.* 8, 1106–1133
- 8 Christopher, M.E. and Wong, J.P. (2006) Recent developments in delivery of nucleic acid-based antiviral agents. *Curr. Pharm. Des.* 12, 1995–2006
- 9 Chattopadhyay, D. and Khan, M.T.H. (2008) Ethnomedicines and ethnomedicinal phytophores against herpesviruses. *Biotechnol. Annu. Rev.* 14, 297–348
- 10 Hudson, J.B. (1990) *Antiviral Compounds from Plants*. CRC Press pp. 119–131
- 11 Venkateswaran, P.S. *et al.* (1987) Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: *in vitro* and *in vivo* studies. *Proc. Natl. Acad. Sci. U.S.A.* 84, 274–278
- 12 Thyagarajan, S.P. *et al.* (1990) *Phyllanthus amarus* and hepatitis B. *Lancet* 336 (8720), 949–950;  
Thyagarajan, S.P. *et al.* (1990) *Phyllanthus amarus* and hepatitis B. *Lancet* 335 (8705), 1600–1601
- 13 Chantrill, B.H. *et al.* (1952) The action of plant extracts on a bacteriophage of *Pseudomonas pyocyanea* and on influenza A virus. *J. Gen. Microbiol.* 6, 74–84
- 14 McCutcheon, A.R. *et al.* (1995) Antiviral screening of British Columbian medicinal plants. *J. Ethnopharmacol.* 49, 101–110
- 15 Chamorro, C. *et al.* (1996) Comparative study of propofol versus midazolam in the sedation of critically ill patients: results of a prospective, randomized, multicenter trial. *Crit. Care Med.* 24, 932–939
- 16 Chamorro, G. *et al.* (1996) Pharmacology and toxicology of *Spirulina* alga. *Rev. Invest. Clin.* 48, 389–399
- 17 De Clercq, E. (2000) Novel compounds in preclinical/early clinical development for the treatment of HIV infections. *Rev. Med. Virol.* 10, 255–277
- 18 Schaeffer, D.J. and Krylov, V.S. (2000) Anti-HIV activity of extracts and compounds from algae and cyanobacteria. *Ecotoxicol. Environ. Safety* 45, 208–227
- 19 McManus, N.H. (1976) Microtiter assay for interferon: microspectrophotometric quantitation of cytopathic effect. *Appl. Environ. Microbiol.* 31, 35–38
- 20 Sudo, K. *et al.* (1994) A sensitive assay system screening antiviral compounds against herpes simplex virus type 1 and type 2. *J. Virol. Methods* 49, 169–178
- 21 Takeuchi, H. *et al.* (1991) An application of tetrazolium (MTT) colorimetric assay for the screening of anti-herpes simplex virus compounds. *J. Virol. Methods* 33, 61–71
- 22 Sieuwerts, A.M. *et al.* (1995) The MTT tetrazolium salt assay scrutinized: how to use this assay reliably to measure metabolic activity of cell cultures *in vitro* for the assessment of growth characteristics, IC<sub>50</sub>-values and cell survival. *Eur. J. Clin. Chem. Clin. Biochem.* 33, 813–823
- 23 Vanden Berghe, D.A.R. *et al.* (1993) Antiviral agents from higher plants and an example of structure–activity relationship of 3-methoxyflavones. In *Bioactive Natural Products: Detection, Isolation, and Structural Determination* (Colegate, S.M. and Milyneux, R.J., eds), pp. 405–440, CRC Press
- 24 Vlietinck, A.J. *et al.* (1997) Plant substances as antiviral agents. *Curr. Org. Chem.* 1, 307–344
- 25 Apers, S. *et al.* (2002) Antiviral activity of simalikalactone D, a quassinoid from *Quassia africana*. *Planta Med.* 68, 20–24
- 26 Hsiang, C.Y. *et al.* (2001) Inhibitory effect of anti-pyretic and anti-inflammatory herbs on herpes simplex virus replication. *Am. J. Chin. Med.* 29, 459–467
- 27 Kimura, H. *et al.* (1999) Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. *J. Clin. Microbiol.* 37, 132–136
- 28 Laue, T. *et al.* (1999) Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. *J. Clin. Microbiol.* 37, 2543–2547
- 29 Tanaka, N. *et al.* (2000) Quantitative analysis of cytomegalovirus load using a real-time PCR assay. *J. Med. Virol.* 60, 455–462
- 30 Josefsson, A. *et al.* (1999) Detection and quantification of human papillomavirus by using the fluorescent 59 exonuclease assay. *J. Clin. Microbiol.* 37, 490–496
- 31 Locatelli, G. *et al.* (2000) Real-time quantitative PCR for human herpesvirus 6 DNA. *J. Clin. Microbiol.* 38, 4042–4048
- 32 Loeb, K. *et al.* (2000) High-throughput quantitative analysis of hepatitis B virus DNA in serum using the TaqMan fluorogenic detection system. *Hepatology* 32, 626–629
- 33 Schutten, M. *et al.* (2000) Development of a real-time quantitative RT-PCR for the detection of HIV-2 RNA in plasma. *J. Virol. Methods* 88, 81–87
- 34 Sells, M.A. *et al.* (1987) Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc. Natl. Acad. Sci. U.S.A.* 84, 1005–1009
- 35 Zhou, Z. *et al.* (2007) Prodocutechnic aldehyde inhibits hepatitis B virus replication both *in vitro* and *in vivo*. *Antivir. Res.* 74, 59–64
- 36 Korba, B.E. *et al.* (1989) Hepatocellular carcinoma in woodchuck hepatitis virus-infected woodchucks: presence of viral DNA in tumor tissue from chronic carriers and animals serologically recovered from acute infections. *Hepatology* 9, 461–470
- 37 Shin, M.S. *et al.* (2005) A flavonoid from medicinal plants blocks hepatitis B virus-antigen secretion in HBV-infected hepatocytes. *Antivir. Res.* 67, 163–168
- 38 Zhang, Y. *et al.* (2007) Chemical properties, mode of action, and *in vivo* anti-herpes activities of a lignin–carbohydrate complex from *Prunella vulgaris*. *Antivir. Res.* 75, 242–249
- 39 Yim, K.C. *et al.* (2005) The cotton rat provides a novel model to study genital herpes infection and to evaluate preventive strategies. *J. Virol.* 79, 14632–14639
- 40 Gordon, Y.J. *et al.* (1990) A fast, simple reactivation method for the study of HSV-1 latency in the rabbit ocular model. *Investig. Ophthalmol. Vis. Sci.* 31, 921–924
- 41 Gordon, Y.J. *et al.* (1986) The development of an improved reactivation model for the study of HSV-1 latency. *Invest. Ophthalmol. Vis. Sci.* 27, 1230
- 42 Gordon, Y.J. *et al.* (1983) The role of herpesvirus type 1 thymidine kinase in experimental ocular infections. *Am. J. Ophthalmol.* 95, 175
- 43 Collins, P.L. *et al.* (1996) Respiratory syncytial virus. In *Fields Virology* (3rd ed.) (Fields, B.N., Knipe, D.M., Howley, P.M., eds), pp. 1313–1351, Lippincott-Raven Publishers
- 44 Glezen, W.P. *et al.* (1986) Risk of primary infection and reinfection with respiratory syncytial virus. *Am. J. Dis. Child.* 140, 543–546
- 45 Kim, H.W. *et al.* (1969) Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am. J. Epidemiol.* 89, 422–434
- 46 Graham, B.S. *et al.* (1988) Primary respiratory syncytial virus infection in mice. *J. Med. Virol.* 26, 153–162
- 47 Kao, Y.J. *et al.* (2001) Induction and regulation of nitric oxide synthase in airway epithelial cells by respiratory syncytial virus. *Am. J. Respir. Crit. Care Med.* 163, 532–539
- 48 Trifilieff, A. *et al.* (2000) Inducible nitric oxide synthase inhibitors suppress airway inflammation in mice through down-regulation of chemokine expression. *J. Immunol.* 165, 1526–1533
- 49 Belshe, R.B. *et al.* (1977) Experimental respiratory syncytial virus infection of four species of primates. *J. Med. Virol.* 1, 157–162
- 50 Crowe, J.E., Jr *et al.* (1996) Live subgroup B respiratory syncytial virus vaccines that are attenuated, genetically stable, and immunogenic in rodents and nonhuman primates. *J. Infect. Dis.* 173, 829–839
- 51 Richardson, L.S. *et al.* (1978) Evaluation of five temperature-sensitive mutants of respiratory syncytial virus in primates: I. Viral shedding, immunologic response, and associated illness. *J. Med. Virol.* 3, 91–100
- 52 Prince, G.A. *et al.* (1979) Respiratory syncytial virus infection in inbred mice. *Infect. Immun.* 26, 764–766
- 53 Prince, G.A. *et al.* (1979) Respiratory syncytial virus infection in owl monkeys: viral shedding, immunological response, and associated illness caused by wild-type virus and two temperature-sensitive mutants. *Infect. Immun.* 26, 1009–1013
- 54 Kakuk, T.J. *et al.* (1997; 1993) A human respiratory syncytial virus (RSV) primate model of enhanced pulmonary pathology induced with 1368 Byrd and Prince CID; 25 (December) a formalin-inactivated RSV vaccine but not a recombinant FG subunit vaccine. *J. Infect. Dis.* 167, 553–561
- 55 Simoes, E.A. (1995) *Natural and Vaccine Induced Immunity to RSV in Primates*. NIH Publication No R01 AI37271-02. National Institutes of Health
- 56 Hildreth, S.W. *et al.* (1989) A parenterally administered subunit RSV vaccine: safety studies in animals and adult humans. *Pediatr. Res.* 25, 180A
- 57 Prince, G.A. *et al.* (1978) The pathogenesis of respiratory syncytial virus infection in cotton rats. *Am. J. Pathol.* 93, 771–791
- 58 Prince, G.A. and Porter, D.D. (1996) Treatment of parainfluenza virus type 3 bronchiolitis and pneumonia in a cotton rat model using topical antibody and glucocorticosteroid. *J. Infect. Dis.* 173, 598–608
- 59 Van der Poel, W.H.M. *et al.* (1994) Respiratory syncytial virus infections in human beings and in cattle. *J. Infect.* 29, 215–228
- 60 Belknap, E.B. *et al.* (1995) Experimental respiratory syncytial virus infection in calves and lambs. *J. Vet. Diagn. Invest.* 7, 285–298

- 61 Baker, J.C. (1993) The characteristics of respiratory syncytial viruses. *Vet. Med. (Praha)* 88, 1190–1195
- 62 Viuff, B. *et al.* (1996) Sites of replication of bovine respiratory syncytial virus in naturally infected calves as determined by *in situ* hybridisation. *Vet. Pathol.* 383–390
- 63 Wright, P.F. *et al.* (1970) Temperature-sensitive mutants of respiratory syncytial virus: *in vivo* studies in hamsters. *J. Infect. Dis.* 122, 501–512
- 64 Coates, H.V. and Chanock, R.M. (1962) Experimental infection with respiratory syncytial virus in several species of animals. *Am. J. Hyg.* 76, 302–312
- 65 Openshaw, P.J.M. (1995) Immunopathological mechanisms in respiratory syncytial virus disease. *Springer Semin. Immunopathol.* 17, 187–201
- 66 Graham, B.S. (1996) Immunological determinants of disease caused by respiratory syncytial virus. *Trends Microbiol.* 4, 290–294
- 67 Prince, G.A. and Porter, D.D. (1976) The pathogenesis of respiratory syncytial virus infection in infant ferrets. *Am. J. Pathol.* 82, 339–352
- 68 Droebner, K. *et al.* (2007) CYSTUS052, a polyphenol rich plant extract, exerts anti influenza virus activity in mice. *Antivir. Res.* 76, 1–10
- 69 World Health Organisation, (1996) *Laboratory Techniques in Rabies* (. In *Laboratory Techniques in Rabies* 4th ed. (Meslin, F-X., . In *Laboratory Techniques in Rabies* 4th ed. (Kaplan, M.M., Koprowski, H., eds), WHO
- 70 Baer, G.M. (1991) *The Natural History of Rabies* (2nd ed.), CRC Press p. 620
- 71 Bourhy, H. and Kissi Band Tordo, N. (1993) Molecular diversity of the lyssavirus genus. *Virology* 194, 70–81
- 72 Fekadu, M. *et al.* (1988) Efficacy of rabies vaccines against Duvenhage virus isolated from European house bats (*Eptesicus serotinus*), classic rabies virus and rabies-related viruses. *Vaccine* 6, 533–539
- 73 Reid, J.E. and Jackson, A.C. (2001) Experimental rabies virus infection in *Artibeus jamaicensis* bats with CVS-24 variants. *J. NeuroVirol.* 7, 511–517
- 74 Rudd, R.J. and Trimachi, C.V. (1987) Comparison of sensitivity of BHK-21 and murine neuroblastoma cells in the isolation of a street strain rabies virus. *J. Clin. Microbiol.* 25, 145–168
- 75 Barrat, J. *et al.* (1986) Diagnostic de la rage sur culture cellulaire, comparaison des résultats de l'inoculation au neuroblastome murin et de l'inoculation à la souris. *Comp. Immunol. Microbiol. Infect. Dis.* 11, 207–214
- 76 Chávez, J.H. *et al.* (2006) Evaluation of antiviral activity of phenolic compounds and derivatives against rabies virus. *Vet. Microbiol.* 116, 53–59
- 77 Goffinet, C. *et al.* (2007) HIV-susceptible transgenic rats allow rapid preclinical testing of antiviral compounds targeting virus entry or reverse transcription. *PNAS* 104, 1015–1020
- 78 Keppler, O.T. *et al.* (2002) Progress toward a human CD4/CCR5 transgenic rat model for *de novo* infection by human immunodeficiency virus type 1. *J. Exp. Med.* 195, 719–736
- 79 Keppler, O.T. *et al.* (2001) Susceptibility of rat-derived cells to replication by human immunodeficiency virus type 1. *J. Virol.* 75, 8063–8073
- 80 Goffinet, C. and Keppler, O.T. (2006) Efficient non-viral gene delivery into primary lymphocytes from mice and rats. *FASEB J.* 20, 500–502
- 81 Keppler, O.T. *et al.* (2005) Rodent cells support key functions of the human immunodeficiency virus type 1 pathogenicity factor Nef. *J. Virol.* 79, 1655–1665
- 82 Li, Y. *et al.* (1991) Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. *J. Virol.* 65, 3973–3985
- 83 He, J. *et al.* (1997) CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* 385, 645–649
- 84 Reuter, S. *et al.* (2005) Role of HIV-2 envelope in Lv2-mediated restriction. *Virology* 332, 347–358
- 85 Venzke, S. *et al.* (2006) Expression of Nef proteins downregulates CXCR4, the major HIV-1 coreceptor, from the cell surface and thereby enhances resistance to superinfection. *J. Virol.* 80, 11141–11152
- 86 Cavois, M. *et al.* (2002) *Nat. Biotechnol.* 20, 1151–1154
- 87 Van Maele, B. *et al.* (2003) Impact of the central polyurine tract on the kinetics of human immunodeficiency virus type 1 vector transduction. *J. Virol.* 77, 4685–4694
- 88 Brussel, A. *et al.* (2003) Longitudinal monitoring of 2 long terminal repeat circles in peripheral blood mononuclear cells from patients with chronic HIV-1 infection. *AIDS* 17, 645–652
- 89 Gitilin, N. (1997) Hepatitis B: diagnosis, prevention and treatment. *Clin. Chem.* 43, 1500–1506
- 90 Park, N.H. *et al.* (2006) Chronic hepatitis B in hepatocarcinogenesis. *Postgrad. Med.* 82, 507–515
- 91 Huang, R.L. *et al.* (2000) Anti-hepatitis B virus effects of wogonin isolated from *Scutellaria baicalensis*. *Planta Med.* 66, 694–698
- 92 Gish, R.G. (2005) Current treatment and future directions in the management of chronic hepatitis B viral infection. *Clin. Liver Dis.* 9, 541–565
- 93 McMahon, B.J. (2005) Epidemiology and natural history of hepatitis B. *Semin. Liver Dis.* 25 (Suppl. 1), 3–8
- 94 Perrillo, R.P. (2005) Current treatment of chronic hepatitis B: benefits and limitations. *Semin. Liver Dis.* 25 (Suppl. 1), 20–28
- 95 Guo, Q.L. *et al.* (2007) Anti-hepatitis B virus activity of wogonin *in vitro* and *in vivo*. *Antivir. Res.* 74, 16–24
- 96 Guidotti, L.G. *et al.* (1995) High-level hepatitis B virus replication in transgenic mice. *J. Virol.* 69, 6158–6169
- 97 Jibert, A.R. *et al.* (1992) Rapid resolution of duck hepatitis B virus infections occurs after massive hepatocellular. *J. Virol.* 66, 1377–1388
- 98 Yao, Y. *et al.* (2001) Study on the replication of hepatitis B virus compared with that of duck hepatitis B virus in primary duck hepatocytes. *Zhonghua Yi Xue Za Zhi* 81, 1157–1161
- 99 Freiman, J.S. *et al.* (1988) Experimental duck hepatitis B virus infection: pathology and evolution of hepatic and extrahepatic infection. *Hepatology* 8, 507–513
- 100 Guiver, M. *et al.* (2001) Evaluation of CMV viral load using TaqMan CMV quantitative PCR and comparison with CMV antigenemia in heart and lung transplant recipients. *Transplantation* 71, 1609–1615
- 101 Lallemand, F. *et al.* (2000) Quantitative analysis of human herpesvirus 8 viral load using a real-time PCR assay. *J. Clin. Microbiol.* 38, 1404–1408
- 102 Hussein, G. *et al.* (1999) Inhibitory effects of Sudanese plant extracts on HIV-1 replication and HIV-1 protease. *Phytother. Res.* 13, 31–36
- 103 Premanathan, M. *et al.* (1999) Antiviral properties of a mangrove plant, *Rhizophora apiculata* Blume, against human immunodeficiency virus. *Antivir. Res.* 44, 113–122
- 104 Premanathan, M. *et al.* (1999) *In vitro* anti-human immunodeficiency virus activity of polysaccharide from *Rhizophora mucronata* Poir. *Biosci. Biotechnol. Biochem.* 63, 1187–1191
- 105 Calabrese, C. *et al.* (2000) A phase I trial of andrographolide in HIV positive patients and normal volunteers. *Phytother. Res.* 14, 333–338 PMID: 10925397
- 106 Asres, K. *et al.* (2001) Antiviral activity against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) of ethnobotanically selected Ethiopian medicinal plants. *Phytother. Res.* 15, 62–69
- 107 Chan, P.K. *et al.* (2000) Survey for the presence and distribution of human herpesvirus 8 in healthy brain. *J. Clin. Microbiol.* 38, 2772–2773 109
- 108 Chattopadhyay, D. *et al.* (2006) Dose dependent therapeutic anti-infectives from ethnomedicines of Bay Islands. *Chemotherapy* 52, 151–157
- 109 Flint, S.J. *et al.* (2004) *Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses* (2nd ed.), ASM Press
- 110 Mukhtar, M. *et al.* (2008) Antiviral potentials of medicinal plants. *Virus Research* 131 (2), 111–120
- 111 Tolo, F.M. *et al.* (2006) Anti-viral activity of the extracts of a Kenyan medicinal plant *Carissa edulis* against herpes simplex virus. *J. Ethnopharmacol.* 104, 92–99
- 112 Yang Chien-Min, *et al.* (2007) The *in vitro* activity of geraniin and 1,3,4,6-tetra-O-galloyl- $\beta$ -D-glucose isolated from *Phyllanthus urinaria* against herpes simplex virus type 1 and type 2 infection. *J. Ethnopharmacol.* 110, 555–558
- 113 Serkedjieva, J. *et al.* (2008) Protective efficacy of an aerosol preparation, obtained from *Geranium sanguineum* L., in experimental influenza infection. *Pharmazie* 63, 160–163
- 114 Pantev, A. *et al.* (2006) Biologically active constituents of a polyphenol extract from *Geranium sanguineum* L. with anti-influenza activity. *Z. Naturforsch. [C]* 61, 508–516
- 115 Serkedjieva, J. (1997) Anti-infective activity of a plant preparation from *Geranium sanguineum* L.. *Pharmazie* 52, 799–802
- 116 Zakay-Rones, Z. *et al.* (2004) Randomized study of the efficacy and safety of oral elderberry extract in the treatment of influenza A and B virus infections. *J. Int. Med. Res.* 32, 132–140
- 117 Huang, K.L. *et al.* (2006) Inhibition of hepatitis B virus production by *Boehmeria nivea* root extract in HepG2 2.2.15 cells. *World J. Gastroenterol.* 12 (35), 5721–5725
- 118 Chang, J.S. *et al.* (2005) Ethanol extract of *Polygonum cuspidatum* inhibits hepatitis B virus in a stable HBV-producing cell line. *Antivir. Res.* 66, 29–34
- 119 Zuo, G.Y. *et al.* (2005) *In vitro* anti-HCV activities of *Saxifraga melanocentra* and its related polyphenolic compounds. *Antivir. Chem. Chemother.* 16, 393–398
- 120 DeFelipe, A.M. *et al.* (2006) Antiviral effect of guazuma ulmifolia and strychnodendron adstringens on poliovirus and bovine herpesvirus. *Biol. Pharm. Bull.* 29, 1092–1095
- 121 Micol, V. *et al.* (2005) The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). *Antivir. Res.* 66, 129–136

- 122 Li, S.Y. *et al.* (2005) Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antivir. Res.* 67, 18–23
- 123 Notka, F. *et al.* (2004) Concerted inhibitory activities of *Phyllanthus amarus* on HIV replication *in vitro* and *ex vivo*. *Antivir. Res.* 64, 93–102
- 124 Lee-Huang, S. *et al.* (2003) Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. *Biochem. Biophys. Res. Commun.* 307, 1029–1037
- 125 Cella, M. *et al.* (2004) Virucidal activity presence in *Trichilia glabra* leaves. *Rev. Argent. Microbiol.* 36, 136–138
- 126 Yamai, M. *et al.* (2003) Antiviral activity of a hot water extract of black soybean against a human respiratory illness virus. *Biosci. Biotechnol. Biochem.* 67, 1071–1079
- 127 Parida, M.M. *et al.* (2002) Inhibitory potential of neem (*Azadirachta indica* Juss.) leaves on dengue virus type-2 replication. *J. Ethnopharmacol.* 79, 273–278