Chapter 16

# Antiviral Evaluation of Herbal Drugs

## Chapter Outline



## 16.1 ANTIVIRAL POTENTIALS OF HERBS

Although vaccines have been very successful in controlling many viral diseases, some diseases are likely to be controlled only by antiviral chemotherapy. The concept of antiviral drugs has only been accepted slowly, partly because of the toxicity of many of the earlier antiviral agents. In contrast to the development of antibiotics, attempts to develop antiviral drugs have indeed met a variety of problems. Being strictly dependent on cellular metabolic processes, viruses possess only limited intrinsic enzyme systems and building blocks that may serve as specific targets for a drug. Moreover, contrary to a bacteriostatic compound, an effective antiviral drug should not only display considerable specificity in its antiviral action, but should also irreversibly block viral synthesis in order to stop cell suicide due to the viral infection and restore normal cell synthesis (Vanden Berghe et al., 1986). In addition to this inhibition, the antiviral agent must have a broad spectrum of activity, favorable pharmacodynamic properties, and not be immunosuppressive. In the ideal situation, the antiviral drug checks the infection while the immune system prepares to destroy the last virus particles (Munro et al., 1987). This point is critical for those immune-compromised by illness (AIDS, cancer) or drug therapy (transplants, cancer). A frequent cause of death in these instances is from viral infections, so that adjuvant antiviral chemotherapy is vital in these circumstances (Shannon and Schabel, 1980).

Many viral infectious diseases still cause high mortality. Although antiviral chemotherapy has shown outstanding progress, antiviral agents are still required. The emergence of drug-resistant viruses during treatment raises a potential problem for effective therapy. Furthermore, new viral pathogens may be discovered. Biologically active substances of plant origin have long been known as viral inhibitors. These antiviral compounds may be extracted from sources, such as higher plants, which have, for various reasons, been explored considerably less than the traditional ones.

The first clinically useful antiviral drug was methylisatin–thiosemicarbazone (Methisazone), which was active against pox viruses, especially smallpox virus. Methisazone (25 mg/kg) was found to inhibit Variola virus in mice and was later

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used successfully to treat cases of eczema vaccinatum and to treat and prevent smallpox. Kaufman et al. (1962), an ophthalmologist, successfully treated a herpes eye infection with an antineoplastic drug, Idoxuridine. At the same time, a group of chemists at the Du Pont chemical company in the United States investigated the antiviral activity of a molecule called Amantadine. It was active against influenza virus type A. In rapid succession, further nucleoside analogs, cytosine arabinoside, trifluorothymidine, and adenine arabinoside, were found to inhibit herpes virus. In the 1970s, the antiherpes activity of a new compound, 9-(2-hydroxyethoxymethyl) guanine (Acyclovir), was detected by J. Bauer at the Wellcome Research Labs. Within a decade, the same group was to discover azidothymidine (Zidovudine), the first effective inhibitor of the newly emerged HIV. The alkaloid from the Australian horse chestnut (Castanospermine australe), isolated in the 1980s, was also found to be active against HIV.

A research program to detect and isolate antiviral compounds from higher plants is best carried out by a multidisciplinary team, consisting of at least a pharmacognosist and a virologist. The antiviral screening system should meet all requirements of any good assay, including validity, lack of ambiguity, accuracy, reproducibility, simplicity, and reasonable cost. Moreover, because we are dealing with plant extracts, the antiviral screen should be highly selective, specific, and sensitive; it is advisable to discriminate a true antiviral activity from a viricidal one at this stage. Because most of the aforementioned requirements are better met by in vitro testing, we not only prefer in vitro screening of the plant extracts, but also the use of the same bioassay to guide the isolation of the antivirally active compounds from the plant extracts. The antiviral activity of the pure compounds then has to be confirmed in a later stage by in vivo assays (Leven et al., 1982; Vanden Berghe et al., 1986).

One of the possible strategies for finding new antiinfective drugs may involve the search for compounds with chemotherapeutic activities supplementary to, and structures widely different from, those in current use. These compounds could be extracted from sources that have, for various reasons, been explored considerably less than the traditional microorganisms, including, among others, higher plants (Mitscher and Rao, 1984). Considering the enormous number and the amazing structural diversity of the currently available antimicrobially and antivirally active plant constituents, one might hope that promising systemic and/or locally acting antiinfective agents might be discovered in the plant kingdom (Vanden Berghe et al., 1986; Vlientinck et al., 1988).

The increase of drug-resistant viruses in treatment raises an important issue for effective treatment. Moreover, new viral pathogens might be found. Along these lines, there is a strong requirement for promptly accessible antiviral medications at moderate costs with minimal side effects. Henceforth, traditional medicines ought to be investigated as novel antiviral agents, as many of these ancient medicaments, containing different plant metabolites, have potent antiviral activities (Chattopadhyay and Khan, 2008).

The design of new antiviral drugs potentially focuses on the structural dynamics and replication cycles of the various infections causing viruses. A suitable example is the invention of acyclovir, which hinders certain proteins of herpes infections responsible for the triggering of disease. Ethnomedicines are a vast repository of structural diversities and extensive bioactivities that can serve as a huge source of potential antiviral drugs. A significant number of medicinal plants from Ayurveda and the traditional Chinese system of medicine serve as potential remedies to decrease the severity of illness caused by viruses (Chattopadhyay et al., 2009; Khan et al., 2005; Jadhav et al., 2012).

Research on the antiviral potentials of plants was first started in 1952, and 12 out of 288 plants were found to be effective against influenza (Chattopadhyay and Naik, 2007). Numerous screening studies have been conducted in the last few years to determine the antiviral efficacy of medicinal plants using in vitro and in vivo assays. A few out of a 100 British Colombian medicinal plants showed antiviral efficacy against respiratory syncytial virus (RSV), corona virus, influenza virus, and herpes simplex virus (HSV) (McCutcheon et al., 1995), while the marine algae *Spirulina* showed antimutagenic, immunomodulatory, and antiviral activities (Chamorro et al., 1996). Interestingly, Cyanovirin N, an 11-kDa protein of blue green algae, inactivates HIV-1 by binding with its glycoprotein120 (Clercq De, 2000), while sulfated polysaccharides of seaweeds and algae showed anti-HIV and anti-HSV activities (Schaeffer and Krylov, 2000).

The plant kingdom is highly diverse and ranges from unicellular microscopic plants to long lived, huge trees. To screen each and every plant or their individual parts for the identification of antiviral components is a huge task. Several examples of plants having antiviral properties and newly identified active compounds from them are reported in various journals. One of the examples that can be cited here is cyanovirin N (CV-N), an 11-kDa protein isolated from the cyanobacterium *Nostoc ellipsosporum*, which exhibits the property of inhibiting HIV-1 infection and also possesses broad-spectrum activity. The phytochemical, Podophyllotoxin, isolated from the aqueous extract of *Podophyllum peltatum* L., inhibited HSV type 1 (HSV-1) (Bedows and Hatfield, 1982). The acetone extract of another plant, *Phyllanthus urinaria*, also suppressed HSV-2 and HSV-1 (Yang et al., 2007). Bessong et al. (2006) reported a comparison of various plants and their individual parts (stem, leaves, roots, and so forth.) in repressing viral reverse transcriptase (RT) and integrase, the two basic enzymes in HIV disease. After comparing all the extracts and fractions of the various plants, it was found that the *n*-butanol fraction of *Bridelia micrantha* (Hochst) exhibited the highest anti-RT activity. It has also been reported that the aqueous extract from the

roots of *Carissa edulis* (Forssk.) Vahl, a plant grown in Kenya, displayed noteworthy activity against HSV for both wild type and resistant strains (Tolo et al., 2006). Polyphenol-rich extract from the medicinal plant *Geranium sanguineum* L. has been reported to show a strong antiinfluenza virus activity, as well as antioxidant and radical scavenging capacities (Sokmen et al., 2005)

Hepatitis A, B, C, D, and E viruses are the leading causes for the prevalence of viral hepatitis and liver inflammation. Despite the fact that presentation to any of these infections prompts intense disease, in any case, types B, C, and D are unique in causing chronic infection. Plants belonging to the genus *Phyllanthus* of the Euphorbiaceae family were extensively used as a traditional remedy against these infections. Clinical investigations were additionally intended to look at the inhibitory effects of different species of *Phyllanthus*, that is, *P. amarus* (L.), *P. niruri* (L.), and *P. urinaria* (L.) (Wang et al., 1995). The screening of 56 different Chinese medicinal herbs led to the identification of two potent plant extracts against Duck hepatitis B virus, namely, *Ardisia chinensis* and *Pithecellobium clypearia* (Leung et al., 2006). Similarly, this also led to the identification of *Oenanthe javanica* (Blume) DC flavones (OjF). They acted as a strong inhibitor of HBsAg and HBeAg secretion (involved in viral pathogenesis) in 2.2.15 cells and also reduced DHBV-DNA levels in the HBV-infected duck model (Wang et al., 2005). Because of the strong prevalence of HCV infection in poor countries, screening for the identification of anti-HCV potentials from medicinal plants are still ongoing. According to Hussein et al. (2000) various plant extracts, such as methanol extracts *Acacia nilotica* L. Willd ex Delile, *Boswellia carterii*, *Embelia schimperi*, *Quercus infectoria*, *Trachyspermum ammi* L., and aqueous extracts of *Piper cubeba* L., *Q. infectoria*, and *Syzygium aromaticum* L., were found to possess significant inhibitory activity against HSV.

Combination therapy for treating diseases is an age-old practice of traditional medicine in which several plants are mixed together to develop an effective formulation for a particular disease. Such combination therapies have also been tried for the inhibition of viral hepatitis. As an example, a Chinese herbal medicine, prepared by liquid fermentation broth of *Ganoderma lucidum* supplemented with an aqueous extract of *Radix Sophorae flavescentis*, was potent against hepatitis B virus activity in vitro and in vivo. Viral infections are a matter of great concern for this planet. Plants having broad-spectrum activity against many viruses could be evaluated for isolation and identification of molecules for treating viral infections. As an example, glycyrrhizin, a bioactive component of licorice (*Glycyrrhiza uralensis* Fisch), and lycorine, isolated from *Lycoris radiata* L., showed strong anti-SARS-CoV activity, and was initially used for treating other indications (Li et al., 2005a, b).

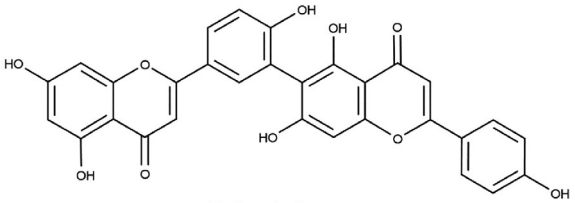
A variety of herbal preparations have shown potentials for inhibiting viruses that cause serious infections among humans, such as measles viruses (Olila et al., 2002), human rotaviruses (HRV) (Husson et al., 1994; Takahashi et al., 2001), respiratory syncytial virus (RSV), human rhinoviruses (Glatthaar-Saalmuller et al., 2001), the coxsackie group of viruses (Evstropov et al., 2004; Su et al., 2006), neurotropic Sindbis virus (NSV) (Paredes et al., 2001), and various strains of polio virus (Andrighetti-Frohner et al., 2005; Melo et al., 2008). One such illustration is the atomic investigation of the heated water concentrates of *Stevia rebaudiana* L., which blocked a section of different irresistible serotypes of HRV into permissive cells by an anionic polysaccharide having a molecular weight of 9800 with uronic acid as a noteworthy sugar constituent (Takahashi et al., 2001). Thus, an alkaloid concentrate of *Haemanthus albiflos* globules repressed RNA amalgamation of HRV spread in MA-104 cells (Husson et al., 1994).

## 16.2 PHYTOCONSTITUENTS HAVING ANTIVIRAL POTENTIALS

In contrast to the many publications on antibacterial and antifungal screening of plant extracts that have appeared in the last decades, far fewer antiviral screening studies of plant extracts have been reported. This is due chiefly to the complexity of the different techniques involved in such research, which consequently requires the know-how and dedication of a multidisciplinary team. Nevertheless, many antiviral agents have been isolated from natural sources and partly or completely characterized. From these studies, several substances have emerged as true antivirals having a good chemotherapeutic index based on the viability of infected cells and on in vivo tests. Thus, different 3-methoxy flavones and synthetic derivatives have shown to be promising leads for the development of antirhinovirus drugs (Van Hoof et al., 1984; Vlientinck et al., 1988), whereas the spanonin, glycyrrhizic acid, was found to be highly active in vitro against herpes simplex (Pompei et al., 1979), varizella-zoster (Baba and Shijeta, 1987), and human immunodeficiency viruses (Ito et al., 1987). Whether these compounds have any clinical potential, that is, in the therapy of the corresponding viral diseases, remains to be determined. There, in vivo bioavailability and other pharmacokinetic parameters are subjects of future study.

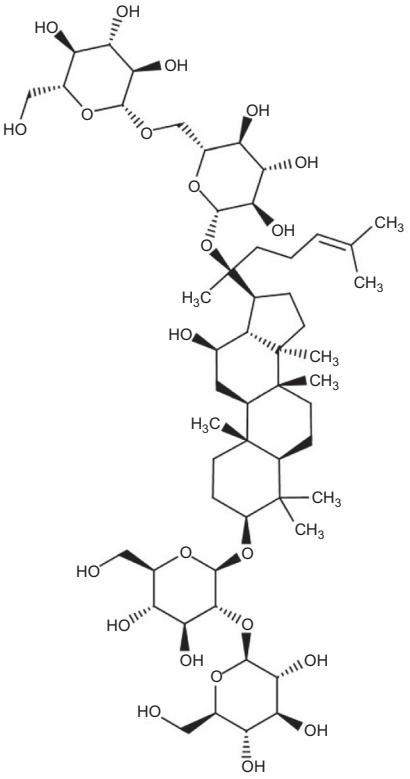
Because of the problems of drug resistance stated earlier and of side effects, the pharmaceutical industry is looking forward toward natural products (mainly medicinal plant extracts) as a source of possible antiviral agents. Approximately 2500 medicinal plant species have been recorded globally to treat a myriad of inflictions and diseases. Polyphenols, alkaloids, flavonoids, saponins, quinones, terpenes, proanthocyanidins, lignins, tannins, polysaccharides, steroids, thiosulfonates, and coumarins are prominent bioactive phytochemicals that have been observed to combat viral infections, as they are harmless to the systems of the human body. Some selected anthraquinones and anthraquinone derivatives are noted for their

antiviral and viricidal activities against viruses representing several taxonomic groups. One of these compounds, hypericin, has activity against vesicular stomatitis virus, HSV types 1 and 2, parainfluenza virus, and vaccinia virus (Andersen et al., 1991). The mechanism of inhibition of HIV-1 infection by purified extracts of *Prunella vulgaris* in the lymphoid cell line MT-4, in the monocytoid cell line U-937, and in peripheral blood mononuclear cells is that it antagonizes HIV-1 infection by preventing HIV-1 viral attachment to the CD-4 receptor (Yao et al., 1992). The mannose-specific lectins from the orchid species *Cymbidium hybrid*, *Epipactis helleborine*, and *Listera ovata* were highly inhibitory to HIV-1 and HIV-2 in MT-4 and showed a marked anti-HCMV, antirespiratory syncytial virus and antiinfluenza A virus activity in HEL, HeLa, and MDCK cells, respectively (Balzarini et al., 1992). Five Australian *Phyllanthus* species have been found to have an antiviral effect on the duck hepatitis B virus endogenous DNA polymerase (Shead et al., 1992). Hudson and Towers (1995) noted the influence of reaction parameters on antiviral assays of phytochemicals.

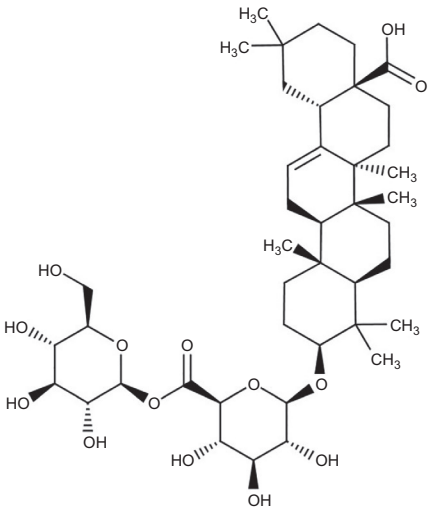


**Robustaflavone**

Dammarane saponins, ginsenoside Rb1 (GRb1), and Chikusetsusaponin (Chi-III) have been found to possess antiviral activity against HSV-I using an in vitro plaque elimination assay. Chi-III prevented plaque formation at half the concentration of GRb1 (Fukushima et al., 1995).



**Ginsenoside Rb1**



**Chikusetsusaponin**

Hudson and his coworkers have assayed 31 species of medicinal plants used in the treatment of diseases of viral origin in the Yunnan province of China for the inhibition of Sindbis virus and Murine Cytomegalovirus in mammalian cell cultures in which 16 species displayed antiviral activity (Hudson and Towers, 1995). The inhibitory effect of ferulic acid and isoferulic acid on murine interleukin-8 production in response to influenza virus infections in vitro has been reported (Hirabayashi et al., 1995) and the effect of isoferulic acid was found to be greater than that of ferulic acid. Hayashi et al. (1995)

found a direct inhibitory activity of the steam distillate prepared from fresh plants of *Houttuynia cordata* (Saururaceae) against HSV-1, influenza virus, and HIV-1, without showing cytotoxicity, but not against Poliovirus and Coxsackie virus. Montanha et al. (1995a) evaluated the action of a series of 19 Aporphine alkaloids against HSV-1 in cell cultures. On the basis of viral titer reduction, six alkaloids were found to be active. Ellagitannins isolated from *Phyllanthus myrtifolius* and *P. urinaria* (Euphorbiaceae) showed activity against Epstein–Barr virus DNA polymerase. Flavonoidal constituents, such as biflavonoids and robustaflavones, exhibited strong inhibitory effects against influenza A and B viruses. The antiviral potential of the flavonoids of *Chamaesyce thymifolia* has been reported; they showed high cytotoxicity on HEp-2 cells and moderate inhibitory activity against HSV-1 and bovine viral diarrhea virus (Amaral et al., 1999). Sotanaphun et al. (1999) isolated Sclerocarpic acid from the stem bark of *Glyptopetalum sclerocarpum*, which exhibited antiviral activity against Herpes simplex virus types 1 and 2. Rhamnan sulfate, a natural sulfated polysaccharide isolated from *Monostroma latissimum*, showed potent inhibitory effects on the virus replication of HSV-1, HCMV, and HIV-1 in vitro (Lee et al., 1999). Matsuse et al. (1999) tested aqueous and methanolic extracts of 39 Panamanian medicinal plants for anti-HIV effects. Seven of these were found to be moderate inhibitors of HIV-protease enzyme. Serkedjieva and Ivancheva (1999) investigated the inhibitory effect of five extracts from the Bulgarian medicinal plant *G. sanguineum* on Herpes simplex virus types 1 and 2. Yoosook et al. (1999) evaluated the anti-HSV-2 activities of *Barleria lupulina* and *Clinacanthus nutans*. The results suggested a therapeutic potential against HSV-2 for *B. lupulina*, but not for *C. nutans*. The antiviral activities of various water and methanol soluble substances isolated from *G. lucidum* against HSV types 1 and 2, influenza A virus, and vesicular stomatitis virus were studied using cytopathic effect inhibition assay and plaque reduction assay (Eo et al., 1999). Kudi and Myint (1999) investigated the antiviral activity of medicinal plant extracts against Poliovirus, Astrovirus, HSV, and Parvovirus. Most of the extracts showed activity against more than one virus at a dose rate of between 100 and 400 µg/100 µL (Eo et al., 1999). Bourne et al. (1999) assessed 19 plant products in vitro by plaque reduction assay to determine their activity against a commonly transmitted pathogen, Herpes Simplex virus type 2. Docherty et al. (1999) found that Resveratrol, a phytoalexin, inhibited HSV-1 and HSV-2 replication in a dose-dependent reversible manner.

Anani et al. (2000) prepared methanol extracts from 19 medicinal plants of Togo and analyzed them for antiviral and antibiotic activities. Ten of the 19 showed significant antiviral activity against one or another of the test viruses (Herpes Simplex, Sindbis, and Poliovirus). Hudson et al. (2000a, b) evaluated ethanolic extracts of 11 plants, endemic to Madagascar, in order to determine the potential of Malagasy plants as sources of antiviral activities. Nine of the extracts had significant activity against HSV, whereas only four were active against the Sindbis virus. A bioactive flavonoid, "Baicalein," isolated from the Chinese medicinal plant *Scutellaria baicalensis* Georgi showed antiviral properties using the high-speed countercurrent chromatography (HSCCC) technique (Li and Chen, 2005).

Many other substances, including flavonoids, phenolics, tannins, triterpenes, and alkaloids, interfere with host cell replication at their antivirally active concentrations or only exhibit extracellular viricidal activities. Some of these viricides, including flavonoids and tannins present in foodstuffs, might be important, because they can inhibit virus replication of picorna-, rota-, and arena viruses in the gastrointestinal tract of humans and animals.

*Artemisia capillaris* was found to possess an active 6,7-dimethylesculetine having potent cytotoxic potential. In the fruits of *Schisandra chinensis* (schizandraceae) used in oriental medicine, 22 lignans were identified, some of which, such as Wuweizisu C and Gomisine N, are very active. The same method threw light on the mechanism of the antihepatotoxic action of such well-known compounds as glycyrrhizin and its intestinal metabolites, which are protective against the first stages preceding hepatic lesions. Other tests of this type are used to track down active substances. From *Taxus baccata*, Potier's group isolated new analogs of taxol, a terpenic compound displaying very good antileukemic and antitumor activities. Taxol promotes the polymerization of tubulin into microtubules and inhibits their depolymerization. The choice among various fractions obtained by extraction was guided by the antitubulin activity in an in vitro test. Many substances that are present in only trace quantities and are difficult to purify have been studied chemically; for example, the demonstration of xylose-bearing derivatives is new in this series of compounds. Regarding structure activity relationships, in vitro cytotoxicity tests have shown that the activity is mainly related to the presence of a complex ester function in the compound. A list of plant extracts and their phytoconstituents that have antiviral potentials are listed in Table 16.1.

In recent years, a lot of development has taken place regarding the identification of antiviral molecules from plant sources and the molecular mechanistic approach. Compounds, such as Spiroketalenol ether derivatives isolated from rhizome extract of *Tanacetum vulgare*, have been reported to block virus entry and also arrest the synthesis of HSV-1 gC and HSV-2 gG glycoproteins (Fernandes et al., 2012). Samarangenin B from the roots of *Limonium sinense* exhibited inhibition of HSV-1α gene expression (Kuo et al., 2002), whereas Oxyresveratrol from *Artocarpus lakoocha* plant was found to inhibit the early and late phases of viral replication of HSV-1 and HSV-2, respectively (Chuanasa et al., 2008). Also, Pterocarnin A compound isolated from *Pterocarya stenoptera* inhibited HSV-2 from binding and penetrating to the host cells (Cos et al., 2003). The structures of some of the potential phytoconstituents having significant antiviral activity are depicted in Fig. 16.1.

**TABLE 16.1 Several Viruses Inhibited by Plants and Their Active Constituent(s)**

| Plant Species | Family | Active Constituent | Activity | Reference |
|---|---|---|---|---|
| *HIV virus* | | | | |
| *Buchenavia capitata* | Combretaceae | *O*-demethyl-buchenavianine | Produces partial protection against the cytopathic effect of HIV in cultured human lymphoblastoid cells | Vlietinck et al. (1997) |
| *Ancistrocladus korupensis* D. Thomas and Gereau | Ancistrocladaceae | Michellamines D and Michellamines F | Exhibited in vitro HIV-inhibitory activity | Halloch et al. (1997) |
| *Schumanniophyton magnificum* | Rubiaceae | Schumannificine | Activity against HIV and anti-anti-HSV | Vlietinck et al. (1997) |
| *Berberis vulgaris* | Berberidaceae | Berberine | Antiviral effects against HIV-1 | Manske and Brossi (1990) |
| *Glycyrrhiza glabra* | Leguminosae | Licopyranocoumarin | Inhibit giant cell formation in HIV-infected cell cultures without any observable cytotoxicity | Vlietinck et al. (1997) |
| *Syzygium claviflorum* | Myrtaceae | Betulinic acid | Potent against HIV | Fujioka et al. (1994) and Cichewicz and Kouzi (2004) |
| *Curcuma longa* | Zingiberaceae | Curcumin | Active against HIV | Mathew and Hsu (2018) |
| *Rheum palmatum* | Polygonaceae | Sennoside A | Active against HIV-1 | Esposito et al. (2016) |
| *Securigera securidaca* | Fabaceae | Kaempferol | Active against HIV-1 | Behbahani et al. (2014) |
| *Olea europaea* | Oleaceae | Maslinic acid | Anti-HIV | Qian et al. (2011) |
| *Artemisia annua* | Asteraceae | Artemisinin | Anti-HIV activity | Lubbe et al. (2012) |
| *HSV virus* | | | | |
| *Actinodaphne hookeri* | Lauraceae | Actinophnine | Active against HSV-1 | Montanha et al. (1995a) |
| *Peganum harmala* | Zygophyllaceae | Harmaline and harmine | Have antiviral effect against the DNA-containing herpes virus type 1 (HSV-1) | Rashan (1990) |
| *Pedilanthus tithymaloides* | Euphorbiaceae | Luteolin | Active against HSV-2 | Ojha et al. (2015) |
| *Mallotus peltatus* | Euphorbiaceae | Ursolic acid | Active against HSV-1 and HSV-2 | Bag et al. (2012) |
| *Achyranthes aspera* | Amaranthaceae | Oleanolic acid | Active against HSV | Mukherjee et al. (2013) |
| *Terminalia chebula* | Combretaceae | Chebulagic acid and punicalagin | Active against HSV-1 | Lin et al. (2011) |
| *Artocarpus lakoocha* | Moraceae | Oxyresveratrol | Inhibit viral replication | Chuanasa et al. (2008) |
| *Influenza virus* | | | | |
| *Syzygium aromaticum* | Myrtaceae | Eugenol | Inhibit the activation of extracellular signal-regulated kinase, p38-mitogen-activated protein kinase, IkB kinase (IKK)/ NF-kB signal pathways | Dai et al. (2013) |

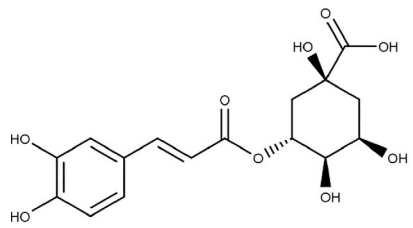
**TABLE 16.1 Several Viruses Inhibited by Plants and Their Active Constituent(s)—cont'd**

| Plant Species | Family | Active Constituent | Activity | Reference |
|---|---|---|---|---|
| *Houttuynia cordata* | Saururaceae | Quercetin 3 rhamnoside | Inhibit replication in the initial stage of antiinfluenza A virus infection by indirect interaction with virus particles | Choi et al. (2009) |
| *Caesalpinia sappan* | Fabaceae | 3-Deoxysappanchalcone | Antiinfluenza, apoptosis, and antiinflammation | Yang et al. (2012) |
| *Angelica keiskei* | Apiaceae | Xanthokeistal A | Neuraminidase inhibitor | Park et al. (2011) |
| *Rubus coreanus* | Rosaceae | Gallic acid | Influenza virus | Lee et al. (2016) |
| *Lonicera japonica Thunb* | Caprifoliaceae | Chlorogenic acid | Influenza A (H1N1/H3N2) virus | Ding et al. (2017) |
| *Melaleuca alternifolia* | Myrtaceae | Terpinen-4-ol, terpinolene | Anti-A/PR/8 virus (H1N1) | Garozzo et al. (2011) |
| *Hepatitis B virus (HBV)* | | | | |
| *Liriope platyphylla* | Asparagaceae | LPRP-Et-97543 | Inhibit viral gene expression and replication. Inhibit viral promoter activity | Huanga et al. (2014) |
| *Piper longum* Linn. | Piperaceae | Piperine | Inhibit the secretion of HBV surface antigen | Jiang et al. (2013) |
| *Swertia macrosperma* | Gentianaceae | Swermacrolactones and luteolin | Inhibit secretion of HBV surface antigen | Wang et al. (2013) |
| *Phyllanthus niruri* L. | Phyllanthaceae | Nirtetralin A | Anti-HBV activities | Wei et al. (2012) |
| *Phyllanthus urinaria* | Phyllanthaceae | Ellagic acid | Effective against hepatitis B | Kang et al. (2006) |
| *Hepatitis C virus (HCV)* | | | | |
| *Bupleurum kaoi* | Apiaceae | Saikosaponin b2 | Inhibiting early HCV entry, including neutralization of virus particles, preventing viral attachment | Lin et al. (2015) |
| *Ruta angustifolia* | Rutaceae | Chalepin and pseudane IX | Inhibited HCV at the postentry step and decreased the levels of HCV RNA replication and viral protein synthesis | Wahyuni et al. (2014) |
| *Syncephalastrum racemosum* | Syncephalastraceae | Ursolic acid | Anti-HCV activity | Fu et al. (2013) |
| *Embelia ribes* | Primulaceae | Quercetin | Active against HCV | Bachmetov et al. (2012) |
| *Vaccinium virgatum* Aiton | Ericaceae | Proanthocyanidin | Inhibit HCV replication | Takeshita et al. (2009) |
| *Respiratory syncytial virus (RSV)* | | | | |
| *Schefflera heptaphylla* | Araliaceae | Dicaffeoylquinic acids | Inhibition of virus–cell fusion in the early stage and the inhibition of cell–cell fusion at the end of the RSV replication cycle | Li et al. (2005a, b) |

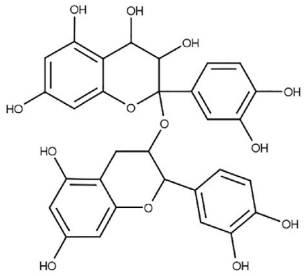
*Continued*

**TABLE 16.1 Several Viruses Inhibited by Plants and Their Active Constituent(s)—cont'd**

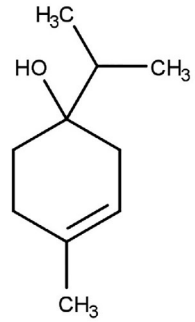
| Plant Species | Family | Active Constituent | Activity | Reference |
|---|---|---|---|---|
| *Citrus reticulata* | Rutaceae | Tangeretin and nobiletin | Affected the intracellular replication of RSV. Tangeretin down regulated the expression of RSV phosphoprotein (P protein) | Xu et al. (2014) |
| *Rosmarinus officinalis* | Lamiaceae | Carnosic acid | Inhibit replication of RSV | Shin et al. (2013) |
| *Cimicifuga foetida* L. | Ranunculaceae | Cimicifugin | Inhibit viral attachment and internalization | Wang et al. (2012) |
| *Vesicular stomatitis virus (VSV)* | | | | |
| *Melia azedarach* L. | Meliaceae | 1-Cinnamoyl-3,11-dihydroxymeliacarpin (CDM) | CDM blocks VSV entry and the intracellular transport of VSV-G protein and confined it only to the Golgi apparatus | Barquero et al. (2006) |
| *G. glabra* | Fabaceae | Glycyrrhizin | Inhibit phosphorylation enzymes and latency of VSV | Fiore et al. (2008) |
| *Calendula arvensis* | Asteraceae | Oleanolic acid | Inhibit VSV multiplication | De Tommasi et al. (1991) |
| *Dengue virus (DEN)* | | | | |
| *Magnolia grandiflora* | Magnoliaceae | Honokiol | Inhibits dengue virus type 2 infection | Fang et al. (2015) |
| *Scutellaria baicalensis* | Lamiaceae | Baicalein | Viricidal against DEN-2 | Zandi et al. (2012) |
| *Epstein–Barr virus (EBV)* | | | | |
| *Glycyrrhiza radix* | Leguminosae | Glycyrrhizic acid | Interferes with an early step of EBV replication cycle | Lin (2003) |
| *Saururus chinensis* | Saururaceae | Manassantin B | Inhibitory effects toward EBV lytic replication | Cui et al. (2014) |
| *Polio virus (PV)* | | | | |
| *Baccharis gaudichaudiana* | Compositae | Apigenin | Anti-PV type 2 | Visintini et al. (2013) |
| *Dianella longifolia* | Xanthorrhoeacea | Chrysophanic acid | Inhibit PV 2 and PV 3 replication | Semple et al. (2001) |
| *Pterocaulon sphacelatum* | Asteraceae | Chrysosplenol C | Inhibit PV | Semple et al. (1999) |
| *SARS-corona virus (SARS-CoV)* | | | | |
| *G. glabra* | Fabaceae | Glycyrrhizin | Anti-SARS-CoV | Fiore et al. (2008) |
| *Lycoris radiata* | Amaryllidaceae | Lycorine | Anti-SARS-CoV | Li et al. (2005a, b) |
| *Ebola virus* | | | | |
| *Aglaia foveolata* | Meliaceae | Silvestrol | Active against Ebola virus | Biedenkopf et al. (2016) |
| *C. longa* | Zingiberaceae | Curcumin | Active against Ebola virus | Mathew and Hsu (2018) |



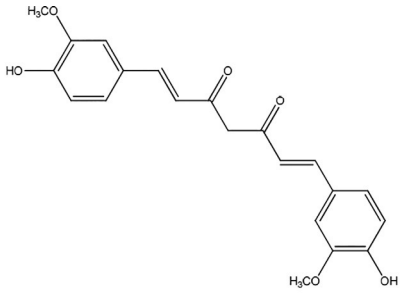
**Chlorogenic acid**



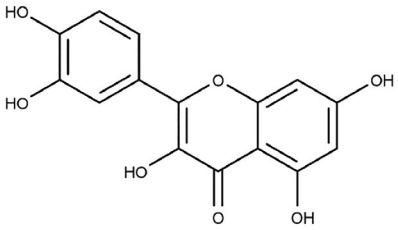
**Curcumin**



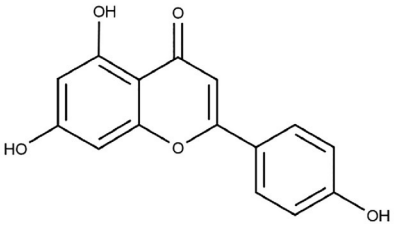
**Terpinen-4-ol**



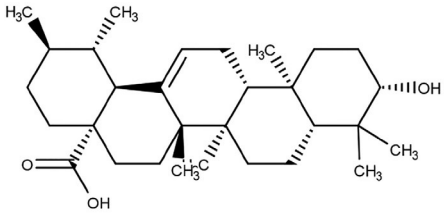
**Procyanidin**



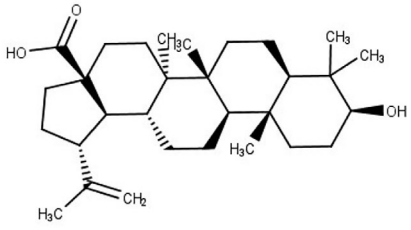
**Quercetin**



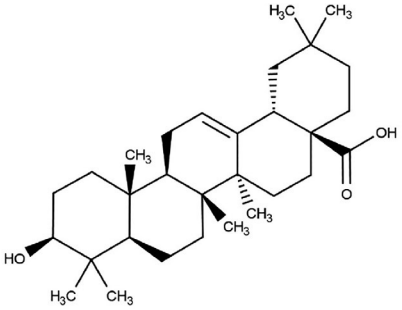
**Apigenin**



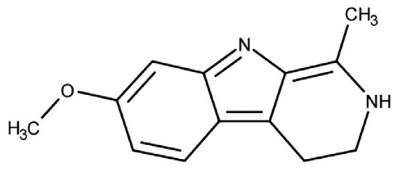
**Ursolic acid**



**Betulinic acid**



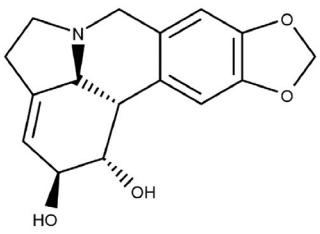
**Oleanolic acid**



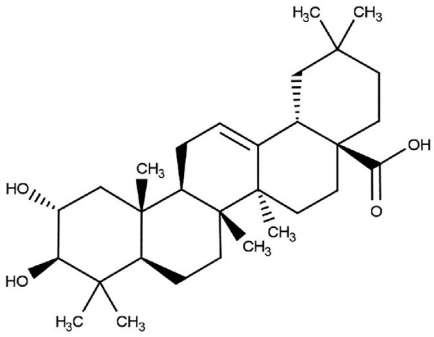
**Harmaline**

**FIG. 16.1** Several important antiviral compounds from plant sources.

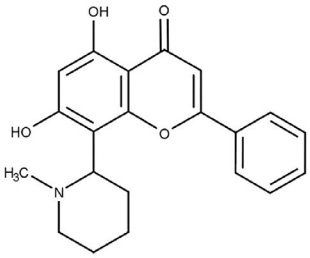
*Continued*



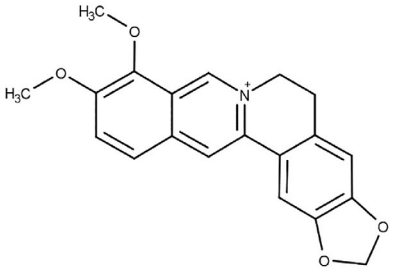

**Lycorine**



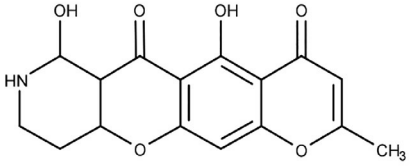

**Maslinic acid**



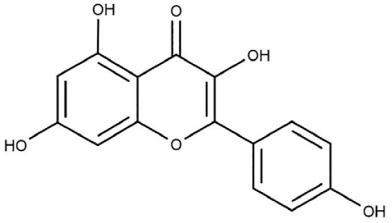

***O*-Demethylbuchevianine**



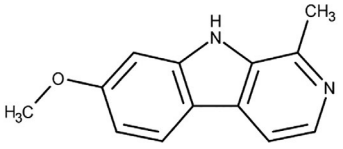

**Berberine**



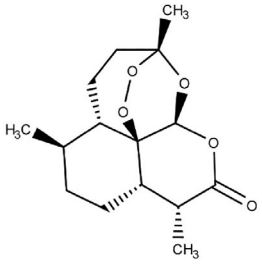

**Schumannificine**




**Kaempferol**

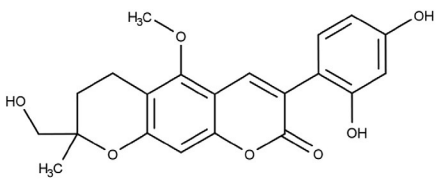



**Harmine**

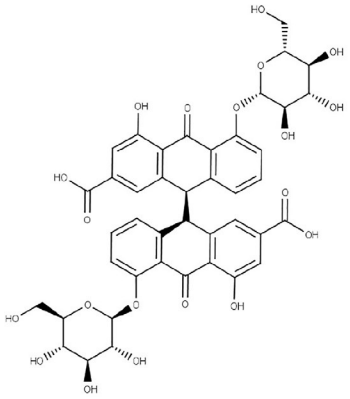



**Artemisinin**

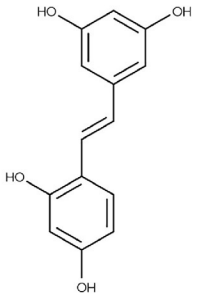
**FIG. 16.1** Cont'd



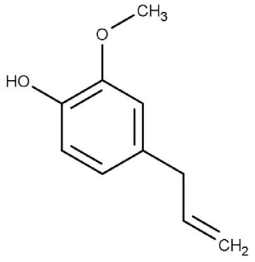
Licopyranocoumarin



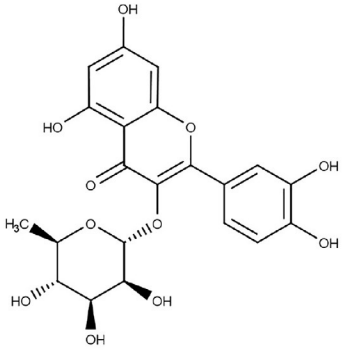
Sennoside A



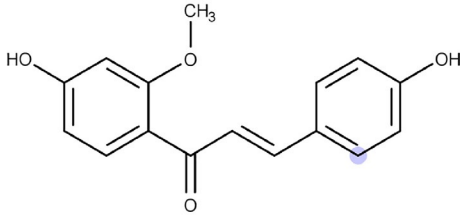
Oxyresveratrol



Eugenol



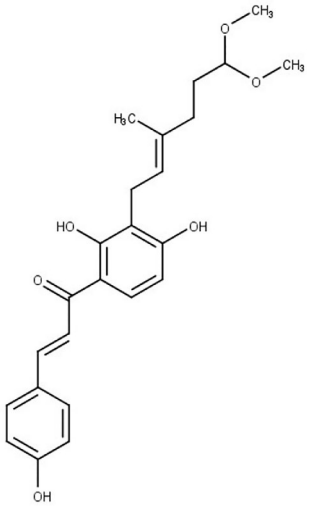
Quercetin 3 rhamnoside



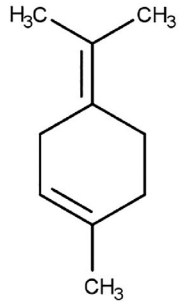
3-Deoxysappanchalcone

**FIG. 16.1** Cont'd

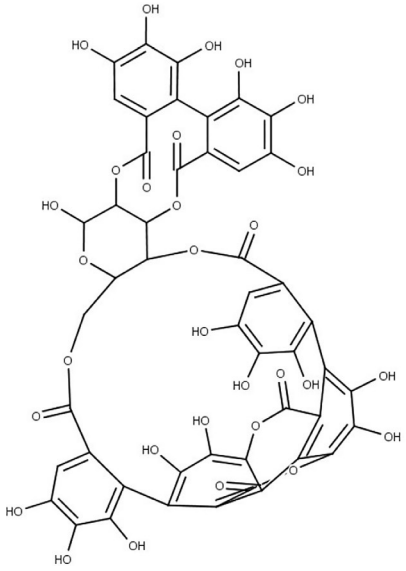
*(Continued)*



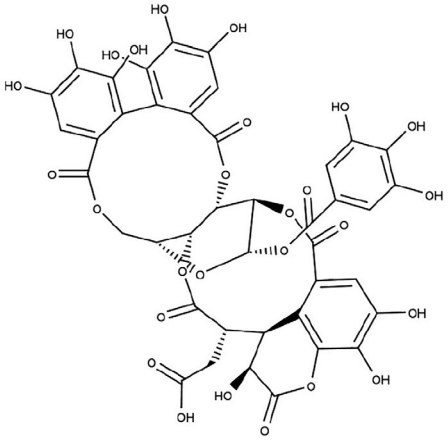
Xanthokeistal A



Terpinolene

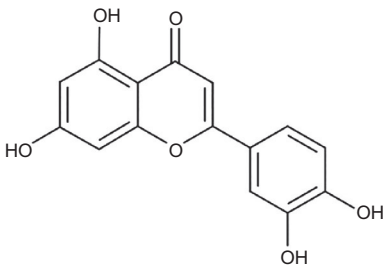


Luteolin

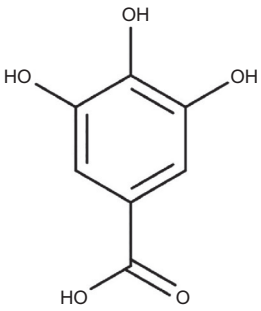


Chebulagic acid

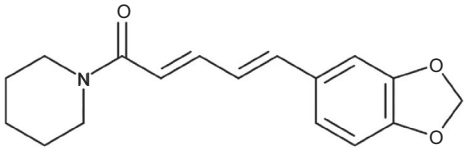
**FIG. 16.1** Cont'd



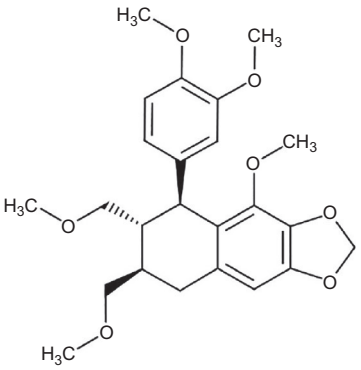
**Punicalagin**



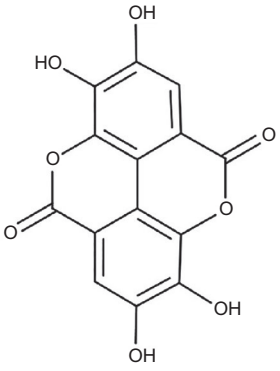
**Gallic acid**



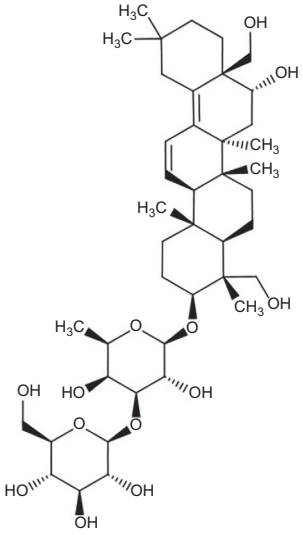
**Piperine**



**Nirtetralin**



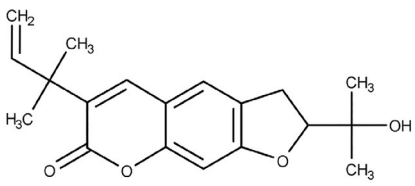
**Ellagic acid**



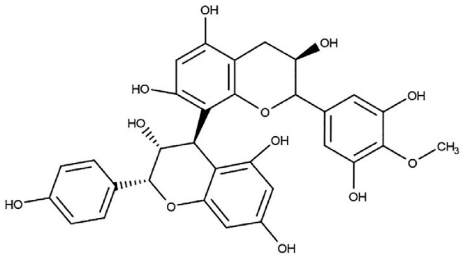
**Saikosaponin B2**

**FIG. 16.1** Cont'd

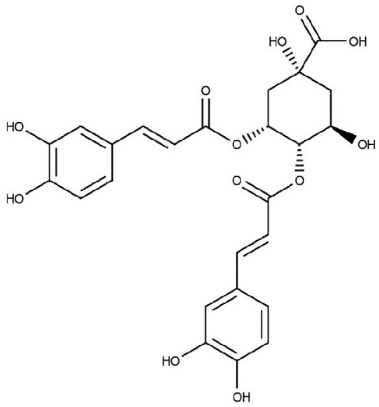
*(Continued)*



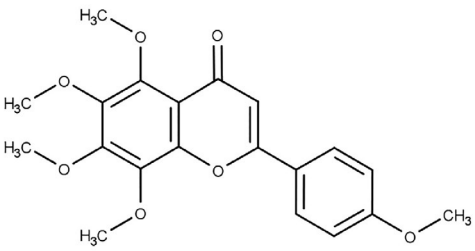
Chalepin



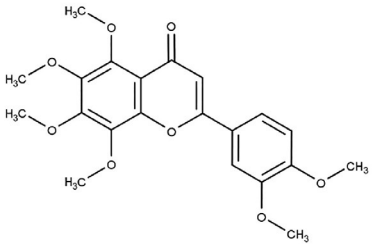
Proanthocyanidin



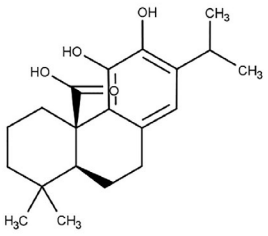
Dicaffeoylquinic acid



Tangeretin

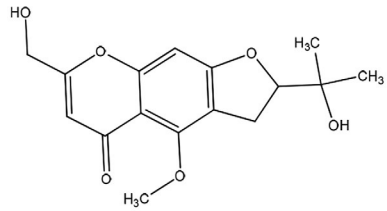


Nobiletin

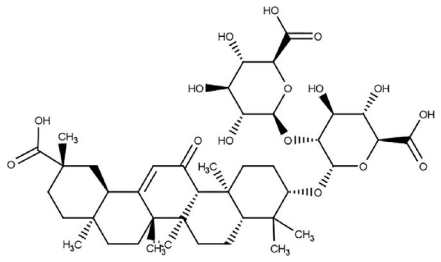


Carnosic acid

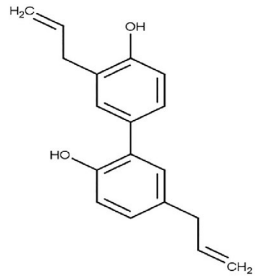
**FIG. 16.1** Cont'd



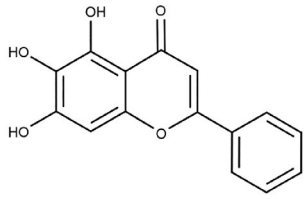
**Cimicifugin**



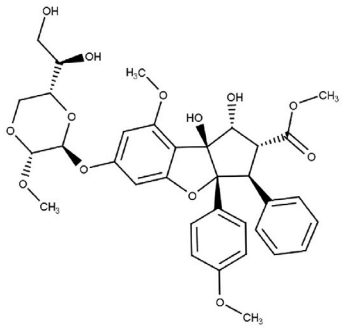
**Glycyrrhizin**



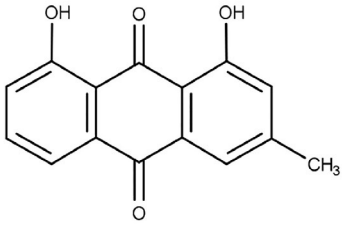
**Honokiol**



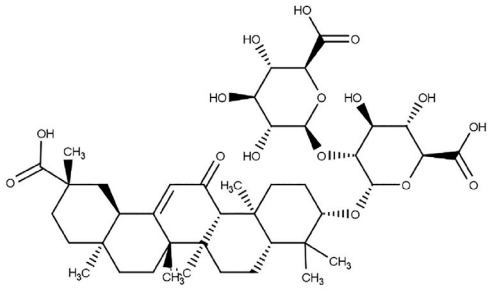
**Baicalein**



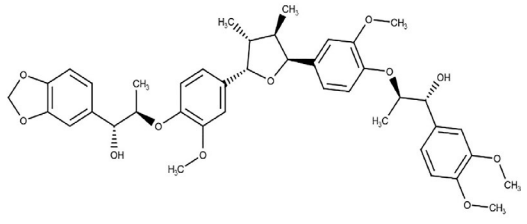
**Silvestrol**



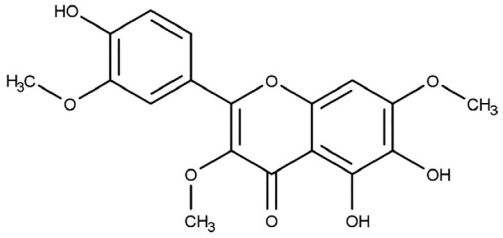
**Chrysophanic acid**



**Glycyrrhizic acid**



**Manassantin B**



**Chrysosplenol C**

**FIG. 16.1** Cont'd

## 16.3 IN VITRO PERSPECTIVES FOR ANTIVIRAL EVALUATION

Many of the antiviral drugs now known have been discovered by random search in the laboratory. Most labs use a biological test system in which new molecules are added to tissue culture cells at a range of concentrations (e.g., 100–1000 µg/mL); the drug-treated cells (and untreated cells as control) are then infected with a known multiplicity of infective virus particles. Thousands of compounds can inhibit viral replication in a cell culture. In general, the more complex the regulatory mechanisms of a virus, the easier it is to find molecules that inhibit it. A broad estimate of the ratios of the activity of antiviral compounds in a cell culture, animal models, and humans is 1000:10:1.

During the evaluation of antiviral agents, many test conditions, such as the cell culture system, virus strain, virus challenge dose, virus input multiplicity of infection, and time of harvesting, can affect or even alter the test results. To test the inhibitory activity of a new antiviral agent, it is first necessary to select the host cell system(s) in which the virus replication can be measured. Viruses vary considerably in their ability to replicate in cultured cells. Many viruses can cause CPE while some of them can form plaques. Others can produce some specialized functions, such as hemagglutination, hemadsorption, or cell transformation. Virus replication in cell cultures can also be detected by the presence of viral products, namely, viral DNA, RNA, or polypeptides.

The *antiviral tests selected may be based on:*

**(a)** Inhibition of the virus-induced cytopathic effect (CPE) in which the 50% effective dose ($ED_{50}$) of the antiviral agent is expressed as the concentration that inhibits CPE in half of the quadruplicate cultures.

**(b)** Plaque reduction assay in which the dose of the drug required to reduce the plaque number by 50%, that is, $ED_{50}$ is calculated.

**(c)** Virus yield reduction assay in which the drug concentration required to reduce 90% (1 $\log_{10}$ reduction), or 99% (2 $\log_{10}$ reduction) of the virus yield as compared with the virus control (infected cultures without drug) are determined from the dose–response curves and are expressed as $ED_{90}$ or $ED_{99}$ of the antiviral agent.

**(d)** Assay systems based on the measurement of specialized functions and viral products; a number of viruses do not produce plaques nor do they cause CPE readily, but they may be quantified by certain specialized functions based on their unique properties, for example, hemagglutination and hemadsorption tests used to study the antiviral activity against myxoviruses and ELISA, used to determine the extent of virus replication and, thus, obtain a measure of the inhibitory effect of various antiviral agents on virus replication, etc. (Hu and Hsiung, 1989).

### 16.3.1 MTT and Neutral Red (NR) Assays

Colorimetric assays quantify cell viability through enzyme-mediated biochemical reactions owing to ingress of certain dyes inside living cells. Mosmann, Borenfreund, and Puerner first advocated the application of tetrazolium (MTT) and Neutral Red (NR) assay, respectively, to quantitate cell viability and the cytotoxicity to cells in vitro. Parida et al. (1999) compared the efficacy of two colorimetric assays (MTT and Neutral Red) to determine viral infectivity in microculture virus titration employing Polio virus type-3 and Dengue virus type-4. MTT assay, also known as tetrazolium assay, has been exploited extensively to reveal the protective efficacy of therapeutic agents and plant extracts against cancer, HIV-1, HSV, Polio virus type 3, DEN-4 virus, and to the determination of neutralizing antibody levels to HIV and Respiratory syncytial virus. MTT assay using microculture virus titration (MCVT) was applied for the determination of infectivity titers of Influenza viruses and was found to be compatible with the well-established procedure of egg infectivity assay.

Unlike MTT, neutral red dye uptake assay has not been substantially exploited in virologic research. NR dye assay was earlier performed for the study of the antiviral efficacy of basil leaves extract against Polio virus type 3. MTT, a tetrazolium salt, is a yellow-colored dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] which gets cleared by mitochondrial succinate dehydrogenase enzyme into a blue-colored formazan in active cells. This product does not form crystals when it interacts with isopropyl alcohol and thus can be accurately measured. There is no need to harvest the viable cells, wherein the cell viability can be directly measured by a spectrophotometer (Parida et al., 1999). The assay procedure has been discussed in Section 16.6.7.

### 16.3.2 WST-1 Cell Proliferating Reagent Assay

A colorimetric assay based on the cleavage of the tetrazolium salt WST-1 has been developed for human cytomegalovirus (HCMV) antiviral susceptibility testing and adapted to a microtiter plate format. Bedard et al. (1999) developed a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 for human cytomegalovirus antiviral susceptibility testing.

## 16.4 FACTORS INFLUENCING ANTIVIRAL EVALUATION

**(i)** *Host cell system*

The response of different cell cultures to a given antiviral agent, including the drug metabolism and toxicity, among other factors, may vary greatly. To perform antiviral testing against a particular virus infection, it is necessary to obtain a suitable host cell system for that virus infection. The same antiviral agent may behave very differently in different cell culture systems, although the same virus strain is used.

**(ii)** *Virus strain and passage history*

The variability of sensitivity to a given antiviral agent has been noted among different strains of HSV and CMV. Drug-resistant strains have emerged to some antiviral agents, especially in the herpes virus group. The passage history of a virus strain may also affect its sensitivity to some antiviral agents.

**(iii)** *Virus input multiplicity of infection (MOI)*

MOI can influence substantially the evaluation of antiviral activity by the plaque reduction method or the virus yield reduction assay. High MOI will decrease the sensitivity of the virus to an antiviral agent.

**(iv)** *Virus yield harvesting time*

This may also contribute to the disparity among antiviral evaluation results, even when the virus MOI is kept constant (Hu and Hsiung, 1989).

**(v)** *The influence of reaction parameters on antiviral assays of phytochemicals*

The activities of several known antiviral phytochemicals are profoundly affected by the presence of serum components. For example, the terthiophene, α-terthienyl (α-T) was inhibited in a concentration-dependent manner by serum. In the case of the carboxylic acid derivative of α-T, the compound appeared to have no antiviral activity at all in the presence of serum, yet in its absence this compound was as effective as α-T. In contrast, the complex anthraquinone hypericin required a small amount of serum for maximal antiviral activity. The reactions were also strongly affected by the order of incubation of the components: virus, compound, serum, and light (Hudson and Towers, 1995).

## 16.5 HOST CELLS, ORGANISMS, AND CULTURE MEDIA

The viruses to be selected for initial evaluation of plant extracts are obviously of major importance. They must be chosen to represent the different groups of viruses according to their morphology and various multiplication mechanisms and a range of virus diseases for which chemical control would be useful. Besides the need for control, also the prevalence of the viral diseases and the resulting projection of the market potential, which are determined by the antigenic abundance of the causative viruses and the problems this represents for vaccine control, are important selection criteria (Grunert, 1979).

In vitro methods are therefore more appropriate, because they allow simultaneous screening of a battery of viruses. In vivo screening of extracts against a broad array of viruses, in contrast, is not only very expensive but also extremely laborious. In vitro antiviral bioassays utilize thinly confluent monolayers of tissue culture cells with sufficient susceptibility to the infecting viruses that a visibly cytopathogenic effect (CPE) occurs, for example, rounding up. Shrinking or detaching of cells from the monolayer can be produced and readily observed microscopically. A monolayer of cells consists of animal or human cells, such as chick embryo, rabbit, or green monkey kidney cells (Vero cells), or human skin fibroblasts and carcinoma cells (HeLa cells), grown in a culture medium. Such continuous cell lines used in virology are mostly "transformed" cells that can be maintained for an indefinite number of generations.

The host cells require an appropriate tissue culture medium in which they can survive for at least 1 week without having to renew the medium. Renewal of the medium causes changes in intra- and extracellular products and alters the virus concentration. The medium must have a stable pH during the whole incubation time and may contain only a small amount of serum, because blood products tend to absorb many compounds. Mostly, a defined synthetic medium, supplemented by some type of serum (such as fetal bovine, calf, or horse), a buffer on sometimes bacterial and fungal inhibitory antibiotics, is used. According to experience, Vero cells, which allow the growth of many human viruses with visible CPE, grown in the medium described by Hronovosky et al. (1975), and slightly modified by lowering the pyruvate concentration, are most suitable for antiviral screening of plant extracts (Van den Berghe et al., 1978).

Many combinations of test viruses are possible, but a battery of six viruses seems to be quite acceptable. Virus types and strains may vary in sensitivity, but have to be selected as a function of their ability to multiply in the same tissue culture when cell culture models are used as screening systems. In this way, an objective comparison of antiviral activities is possible, whereas toxicity tests are minimized. Moreover, virus multiplication must cause a visible CPE within a reasonable period of time, preferably within a week after infection.

## 16.6 METHODS FOR ANTIVIRAL EVALUATION OF HERBAL DRUGS

An in vitro screening bioassay involves the detection of inhibition of the visible CPE on monolayer tissue culture cells after inoculation of the cells with a pretitered virus suspension and incubation in a maintenance medium containing the plant extract or test component. Virus suspension is characterized by their "virus titers," which are expressed as the number of infectious units per volume. An infectious unit is defined as the smallest amount of virus capable of producing a reaction after virus inoculation and can be carried out by two generally applied methods, namely, the plaque test (PT) and the 50% endpoint titration technique (EPTT).

In the plaque test, monolayers of cells grown in plastic or glass Petri dishes are inoculated with dilutions of a viral suspension. After adsorption of infectious virus particles to the host cell, the monolayers are overlaid with an agarose-containing medium so that the newly formed virus particles are localized by the solid agar over layer. These newly formed infectious particles spread from the initially infected cell to the adjacent cells and develop well-circumscribed foci of cellular degeneration. These areas of dead cells are called "plaques" and are visualized by staining the cell monolayer with a vital dye, such as Neutral Red. The plaques may also be detected microscopically by determining the multinucleated cells (e.g., measles) or by immunofluorescence. The concentrations of viral suspensions measured by counting the plaques are expressed as plaque-forming units per mL (PFU $mL^{-1}$). In the endpoint titration technique, the concentration of infectivity is measured by determining the highest dilution of the suspension, which produces CPE in 50% of the cell cultures inoculated. This dilution is called the 50% tissue culture dose endpoint ($TCD_{50}$). Dilutions are therefore made in a maintenance medium and a certain volume of each of them (0.05–0.1 mL) is added to four or more tube cultures. The proportion of positive cultures is registered for each dilution and the precise dilution at which 50% of the inoculated tube cultures are infected is calculated. At this dilution, the inoculum contains, on average, one $TCD_{50}$ or one tissue culture (infectious) dose for 50% of the tissue culture tubes.

The influence of a plant extract on virus multiplication can be determined as a viricidal or an antiviral activity. The viricidal activity is measured by titration of the residual infectious virus after incubation of the plant extract with a virus suspension of at least $10^6$ $TCD_{50}$ $mL^{-1}$. On the other hand, the antiviral activity is determined by comparing the virus titers of infected cells, which have been cultured with a maintenance medium containing plant extracts or test substances and a maintenance medium without test material (Colegate and Molyneux, 1993).

### 16.6.1 Preparation of Samples for Antiviral Testing

In contrast with antibacterial screening, no solvents, other than physiological buffer solutions, should ideally be used in the in vitro antiviral screening of plant extracts because the samples have to be added to tissue culture cells. It has been observed that many nonpolar plant extracts, prepared and evaporated, are reasonably soluble in dimethyl sulfoxide (DMSO), especially if little or no water is present in the sample and the dissolving sample is heated on a water bath. Viricidal and antiviral determinations may then be carried out on test solutions containing not more than 10% and 1% DMSO, respectively. Therefore, dissolved samples of nonpolar plant extracts in DMSO are added dropwise to the maintenance medium in a ratio of 1:10 or 1:100 under stirring. As already mentioned, the maintenance medium may contain antibiotics, such as penicillin G (20 µg $mL^{-1}$), neomycin (1 µg $mL^{-1}$), and amphotericin B (1 µg $mL^{-1}$), in order to avoid sterilization of the test solutions. Any contamination by bacteria or fungi would indeed ruin the in vitro antiviral bioassay (Colegate and Molyneux, 1993).

There are various methods for validation of antiviral activity. The major techniques have been highlighted in the preceding sections and in Fig. 16.2.

### 16.6.2 Extracellular Viricidal Evaluation Procedure

Most currently used antiseptics and disinfectants kill pathogenic bacteria and fungi at 25°C within 5 min when present in a concentration of about 0.5% (3-log titer reduction). Because it has been noticed that most of these preparations fail to kill all pathogenic viruses under these circumstances, a method has been developed for testing the in vitro viricidal effect of plant extracts, as will be described in the following section.

Thoroughly mix the preincubated (25°C) plant extracts, dissolved in a physiological buffer, or their twofold dilutions (e.g., 1/2 to 1/16), with the same volume of a preincubated (25°C) virus suspension of, for example, $10^6$ PFU $mL^{-1}$ or $TCD_{50}$ $mL^{-1}$ in physiological buffer. Incubate the mixture at 25°C for 5 min. Stop the incubation by the addition of a 10-fold volume of ice-cold maintenance medium and filter the mixture immediately through a 0.22 µm filter to eliminate all possible precipitate. Then, filter the ice-cold filtrate through a 0.01 µm filter to concentrate residual virus on the filter and separate the virus from possibly cytotoxic plant components, which pass the filter. Remove the residual virus from the filter

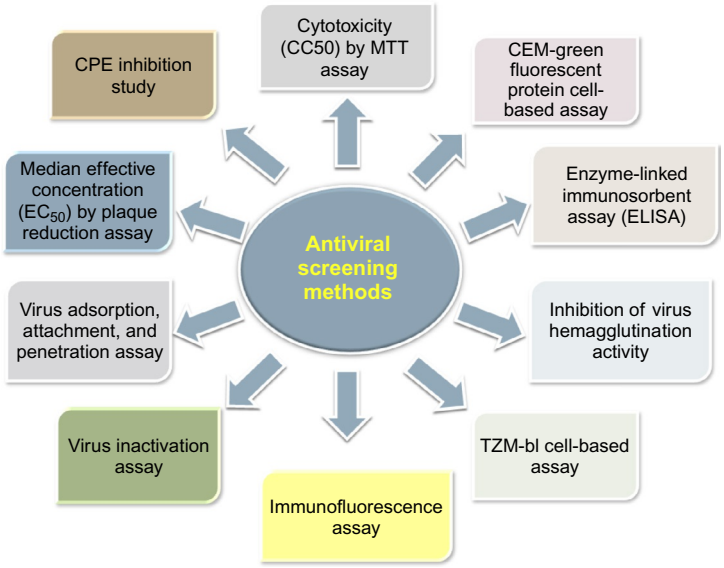



**FIG. 16.2** Antiviral screening methods.

with maintenance medium, supplement with 5% serum (1 mL), sonicate in an ice-bath for 30 s, and titrate in 10-fold dilutions at 37°C by plaque formation or in microtiter plates according to the EPTT. Carry out a virus control in a physiological buffer containing no plant extract simultaneously.

An essential step of this methodology is the separation of all cytotoxic plant components from the residual virus, which has to be measured at 37°C. Cytotoxic substances have a greater influence on the activity of an extracellular virus at 37°C than at 25°C. This step, however, can be omitted when the plant extracts to be tested are not toxic to the host cells under the conditions of the evaluation procedure (Colegate and Molyneux, 1993).

### 16.6.3 End Point Titration Technique (EPTT)

The EPTT technique is performed on preemptied confluent monolayers of Vero or other cells, grown in the holes of microtiter plates, which are infected with serial 10-fold dilutions of a virus suspension (100 µL). Starting with monolayers containing $10^4$ cells per hole and a virus suspension of, for example, $10^7$ $TCID_{50}$ $mL^{-1}$ or PFU $mL^{-1}$, infect the first monolayers of cells with a multiplicity of infection (MOI). The antiviral activity is expressed as the virus titer reduction at the maximal nontoxic dose (MNTD) of the test substance, that is, the highest concentration (µg $mL^{-1}$) that does not affect the monolayers under the antiviral test condition. Viral titer reduction factors, that is, the ratio of the viral titer reduction in the absence (virus control) and presence of the MNTD of the test sample of $1 \times 10^3$ to $1 \times 10^4$ indicate a pronounced antiviral activity and are suitable as selection criteria for further investigation of plant extracts (Colegate and Molyneux, 1993). The EPTT is more suitable for testing complex samples, such as plant extracts, for many reasons.

**(i)** First, the concentration of many compounds in the extract remains constant, and consequently the proportion of toxic versus active compounds does not change.

**(ii)** Second, the exact duration of the antiviral action can be determined by using the EPTT, because the action starts from the moment the extract is added to the cells.

**(iii)** Third, the EPTT using serial diluted extracts deals with a dynamic process, because the cells are subsequently infected with different MOI.

**(iv)** This system allows the correlation of all possible MOI values in the same microtiter plate with decreasing amounts of plant extracts, so that the noncytotoxic concentration of plant extracts can be determined. At the same time, a correlation between extract toxicity and antiviral activity according to the corresponding MOI can be determined in the same microtiter plate.

**(v)** It can be stated as a general rule that the detected antiviral activity should be stable in at least two subsequent dilutions of nontoxic concentration of the extract; otherwise the activity is directly correlated with the toxicity of the extract or is only viricidal. Moreover, a true antiviral product has to protect the cells, which have been infected with low virus dilutions (starting from 0.1 PFU per cell onward).

**(vi)** Finally, it should be stressed that all possible steps of virus manipulation are to be completed before the plant extracts are added. This means that an antiviral product $TCD_{50}\ mL^{-1}$), under nontoxic conditions, must act on virus replication steps after uncoating. When the cells are completely protected only in the lower MOI ($0.1\ TCD_{50}\ mL^{-1}$), replication processes before uncoating may be involved.

### 16.6.4 Effect of Test Compounds on Virus CPE Inhibition

An important aspect of the inhibition of viral cytopathic effect (CPE) is the determination of $TCID_{50}$ (50% tissue culture infective dose). After harvesting, dilute the virus stock 10-fold. Add 0.1 mL of each dilution in 10 wells each of a 96-well microtiter plate. Add 0.1 mL of cell suspension of 10,000 cells/well, incubate at 37°C with 5% $CO_2$ atmosphere, and observe for vial CPE on alternate days. Take a final reading on the fifth day and calculate $TCID_{50}$ as per the method of Reed and Muench (1938), from which $TCID_{50}$ can be further calculated from the log value.

$$\mathrm{TCID}_{50} = \left( \frac{(\%\text{CPE at dilution next above } 50\%) - 50}{(\%\text{CPE at dilution next above } 50\%) - (\%\text{CPE at dilution next below } 50\%)} \right)$$

An antiviral drug will inhibit the CPE of a virus. Therefore, for detecting an antiviral agent, the amount of inhibition of CPE of a virus can be observed microscopically (Kenny et al., 1985). For this purpose, trypsinise the monolayer and allow to seed in 96-well microtiter plates. After a 24-h incubation, wash the monolayer in each well and add different test drug dilutions and incubate. After 24 h, wash the cell culture and inoculate with 0.1 mL of 10 $TCID_{50}$, 50 $TCID_{50}$, and 100 $TCID_{50}$ of the virus suspensions in different wells. Incubate them for 1 h at 37°C in an incubator for the adsorption of the virus onto the cells. After incubation, remove excess virus suspension by washing with RPMI. Add 0.1 mL of selected concentration of the test compound and keep both virus and cell control wells with 0.1 mL of RPMI containing 2% sheep serum. Observe the plates under a microscope every 24 h until the virus control shows 100% CPE and tabulate the results (Hu and Hsiung, 1989).

### 16.6.5 Virus Yield Assay

Trypsinize the cell monolayer, allow to seed in a 96-well microtiter plate and incubate for 24 h at 37°C with 5% $CO_2$ atmosphere. Remove the medium, wash the cell monolayer with RPMI without serum, and add 0.1 mL of different virus suspensions in different wells containing the cell layer and incubate for 1 h for virus adsorption; wash off the excess virus suspension after adsorption. To each well, add the selected concentration of the test drug diluted in RPMI containing 2% sheep serum. To the virus control and cell control, RPMI is added (2% serum) and incubated for 24 h. Freeze the plates at −70°C and thaw at room temperature a couple of times to liberate the associated virus. Determine the virus titer by the end point dilution method and express as $TCID_{50}$ (Cinatl et al., 1997).

### 16.6.6 MTT Assay

Trypsinize the monolayer culture and allow to seed in a 96-well microtiter plate. After a 24-h incubation, wash the monolayer in each well and add different test drug dilutions and incubate. After 24 h, wash the cell culture and inoculate with 0.1 mL of 10 $TCID_{50}$, 50 $TCID_{50}$, and 100 $TCID_{50}$ of the virus suspensions in different wells. Incubate for 1 h at 37°C in a $CO_2$ incubator for adsorption of the virus onto the cell. After incubation, remove excess virus suspension by washing with RPMI without serum. Add 0.1 mL of the selected concentrations of the test compound and keep both the virus and cell control wells with 0.1 mL of RPMI containing 2% sheep serum. Incubate the plates at 37°C for 72 h. After 72 h, discard the old media from the cell cultures and add 50 µL of 2 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to each well and incubate for 3 h. After 3 h of incubation, discard the MTT and add 100 µL of isopropyl alcohol to each culture and set aside for 10 min. Record the absorbance using an Elisa plate reader at 540 nm. In this experiment, the effect of the test drug on mitochondrial synthesis due to viral infection is studied.

### 16.6.7 Plaque Reduction Assay

The "plaque" is a confined region of contaminated cells formed by multiplying virus particles. The plaques are revealed either as regions of dead/decimated cells recognized by cell stains or as zones of polluted cells by immunostaining. The blended cell monolayer is infected with a log10 dilution of viral plaque-forming unit (PFU) in the absence or presence of the test drug and permitted to adsorb (1 h at 37°C in 5% $CO_2$); afterward, the cells should be washed twice with prewarmed

minimum essential medium (MEM). The overlaid drug dilutions are arranged in the overlay medium on the contaminated culture, without the test drug. Then, 45 mL of carboxy methyl cellulose is added to 9 mL of the medium; the plates are incubated for 3–5 days, then settled with 10% formalin or 4% formaldehyde for 30 min. The cells are stained with methylene blue (1 mL/well) or 1% gem violet (w/v), washed, and dried to check the plaques (dark areas) by low-power amplification of a binocular microscope.

The antiviral impact should be measured as the percentage inhibition of plaque formation:

$$\left[\left(\text{mean number of plaques in control}\right)-\left(\text{mean number of plaques in sample}\right)\right]\times 100 \,/\, \text{Mean number of plaque in control.}$$

The concentration of the test drug required to exert 50% of virus inhibition ($IC_{50}$ or $EC_{50}$), as compared with the infection control, is evaluated from the graphical plot as dose–response curves by regression analysis (Chattopadhyay et al., 2015).

### 16.6.8 Inhibition of Virus Hemagglutination Activity (HA)

Viruses, for example, influenza, can agglutinate RBCs due to surface HA proteins, which can be analyzed visually by blending viral dilutions with RBC. This can be utilized to inspect the inhibitory impact of any medication on HA. The 10-overlay serially diluted (1–1000X) test drug is used alongside the diluted viral stocks (1:4 to 1:128). This dilution (50 mL/well) is added to drug-containing wells. It should be preincubated for 45 min and mixed with RBC (1/20 in PBS) sample solution. Here, up to a specific dilution, the viral particles possibly lose their capacity to agglutinate RBCs, which shows a linkage of the drug with the viral HA.

### 16.6.9 Immunofluorescence Assay

Known quantities of virus (MOI 5–10) are used for infecting both untreated or drug-treated cells and allowed to adsorb for 45–60 min. The unabsorbed virus particles are washed and fresh media is added to incubate for 24–36 h. Then, cells are washed with PBS, fixed with paraformaldehyde (3%–4%), and permeabilized with acetone or triton X-100. The cells are again washed and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. Then, the cells are incubated with mouse or rabbit antibody against a specific viral protein for 1–4 h at 37°C. After that, the cells are subjected to repeated washing and incubated with a fluorescent-tagged secondary antibody for 1 h and washed again. After washing, the cells are visualized under a fluorescent microscope and compared with the fluorescence of untreated and drug-treated cells. On the other hand, for quantitation, the cells are trypsinized after treatment and fixed with 4% paraformaldehyde. The cells are then washed, permeabilized, and labeled with a fluorescent-tagged antibody, followed by propidium iodide (PI: 50 mg/mL in PBS). The cells are then counted in a fluorescent-activated cell counter to quantitate the fluorescence percentage.

### 16.6.10 Enzyme-Linked Immunosorbent Assay

Known quantities of virus (MOI 5–10) are used for infecting both untreated and drug-treated cells, adsorbed for 1 h, washed, and incubated (2–4 days) for evaluation of the inhibition of the virus-induced cytopathic effect (CPE). The virus stock is centrifuged after freeze thawing and diluted for ELISA. Each well of a plate coated with a virus-specific antibody is mixed with 100 mL of controls or test drug and incubated at room temperature (1 h) with Horseradish peroxidase conjugate, alkaline phosphatase, or b-D-galactosidase-labeled virus-specific antibody. The wells are washed five times and the substrate (100 mL) is added and incubated in the dark for 10 min. The reaction is stopped by adding 5% $H_2SO_4$ solution and the absorbance is read at 450 nm. The 96-well plates are seeded with a quadruplicate cell monolayer having a log 10 dilution of the test drug followed by infection with the virus. After 16–20 h of incubation at 37°C, the monolayers are fixed with 0.05% glutaraldehyde and examined for virus-specific protein(s) on the cell surface. The ELISA should be performed with a monoclonal antibody to the specific protein of the corresponding virus and protein and the OD (optical density) is measured at 450 nm. The concentration is calculated by 50% reduction in OD values ($EC_{50}$) from extrapolating graphical plots followed by the determination of SI (ratio of $CC_{50}$:$EC_{50}$) in which the results are expressed as a percentage of virus-infected cells (virus control) (Chattopadhyay et al., 2015).

### 16.6.11 Virus Inactivation Assay

The test drug and the virus ($10^4$ PFU/mL) are mixed to incubate for 1 h at 37°C. Then, immediately dilute the virus-drug mixture to 100-fold (final inoculums 50 PFU/well) with media containing 2% FBS to get a subtherapeutic concentration of

the test drug. Following that, mix the monolayer, with the virus inoculums seeded using a 12-well plate. Alternately, add the virus-test drug mix diluted to 100-fold, with no incubation period, with the respective cells for infection. Allow to adsorb for 1 h at 37°C, discard the diluted inoculums, and wash the cells with PBS. Pour an overlay medium (with 2% FBS), and incubate at 37°C for 72 h, followed by plaque reduction assay. Count the viral plaque numbers obtained from infections set in the presence of the test drug and compare it with the control.

### 16.6.12 Attachment Assay

Viral attachment to the cell surface is carried out at 4°C, as it permits binding, but stops viral entry, by ELISA. Briefly, incubate susceptible cells ($2 \times 10^4$ cells/well) in 96-well plates and allow growth overnight. The cell monolayers are cooled at 4°C. The cells are infected with the virus (MOI 5) using heparin in presence of the test drug for 3 h at 4°C as a control. After washing the wells with ice-cold PBS, fix with prechilled 4% paraformaldehyde in PBS for 1 h on ice, blocked with 5% BSA at 4°C. The samples are incubated at 37°C for 1 h with a primary antibody in PBST PBS with 0.05% Tween 20 along with 5% BSA. The wells are washed twice with PBST, 5% BSA, and again only with PBST twice, at 5-min intervals on a shaker. This is mixed with secondary antibody in PBST with 5% BSA and incubated at 37°C for 1 h. The reaction is developed with a 3,3′,5,5′-tetramethyl-benzidine two component microwell peroxidase substrate for 20 min; the reaction is stopped with 1 M phosphoric acid. The absorbance is measured at 450 nm, and the values are expressed as the fold change of absorbance relative to the mock infection control (Lin et al., 2011).

### 16.6.13 Penetration Assay

The cell monolayers are cooled and grown in 12-well plates at 4°C for 1 h. Subsequently, infect the prechilled cells with HSV-1 (100 PFU/well) and incubate for 3 h at 4°C to allow for viral adsorption. Incubate the infected cell monolayers in the presence of test drug or heparin (100 mg/mL) for an additional 20 min at 37°C to facilitate HSV-1 penetration. The extracellular virus is inactivated by citrate buffer (pH 3.0) for 1 min, and the cells are washed with PBS followed by overlay with DMEM containing 2% FBS. After 48 h of incubation at 37°C, the viral plaques are stained and counted (Lin et al., 2011).

### 16.6.14 Virus Adsorption Assay

Add the plated cells ($0.8 \times 10^5$ cells/well for a 12-well plate) grown overnight at 30% confluence (300 mL) with virus dilution and DEAE dextran at a final concentration of 20 mg/mL. After adsorption (2 h at 37°C in $CO_2$ incubator), place the plates in a rocker to prevent the cells from drying and add fresh medium (1–2 mL) containing the test drug to each well and incubate for 40–48 h in 5% $CO_2$ at 37°C for subconfluent growth. After removing the media, add fixing solution (1–2 mL) to each well and incubate for 5 min at room temperature (β-galactosidase activity decreases dramatically if the fixing solution is left for >10 min). Then, discard the fixing solution, wash the cells twice with PBS, stain, and incubate at 37°C for 50 min. Finally, stain the plates to count the number of blue syncytia and express the titration values as the number of stained cells multiplied by the viral dilution (Chattopadhyay et al., 2015).

## 16.7 VIRUS-SPECIFIC ANTIVIRAL ASSAYS

### 16.7.1 Human Immunodeficiency Virus (HIV)

**(a)** TZM-bl cell-based assay

This assay is applicable for HIV-1, simian immunodeficiency virus (SIV), and simian-HIV and is carried out in TZM-bl cells as it reveal the reduction in Tat-induced luciferase (Luc) reporter gene expression after a single round of virus infection. Place the TZM-bl cells ($4 \times 10^4$/well) in a 24-well plate and incubate overnight. In a separate vial, mix the HIV-1NL4.3 (MOI 0.05) virion with the test drug or vehicle for 1 h at 37°C, then add to TZM-bl cells and incubate for 4 h. Wash the cells (with cold PBS), and add fresh media with the test drug to culture for 48 h, using untreated HIV-1-infected cells (negative) and azidothymidine (AZT)-treated cells (positive) as controls. Wash the cells twice with PBS, lyse with 1X lysis buffer, and add the supernatant with the substrate, which should then be analyzed for luciferase activity in an optiplate using a fluorimeter. The results are expressed as percentage inhibition:

$$\text{luminescence in the experimental group}\left(\text{test drug or AZT}\right) / \text{luminescence of infected cells without the drug} \times 100$$

and the percent inhibition is calculated by subtracting the above value from 100 (Wan et al., 2012).

**(b)** CEM-green fluorescent protein (GPT) cell-based assay

CEM-GFP is a stable T-cell line-containing a plasmid encoding GFP and is suitable for HIV-1NL4.3 (MOI 0.05) culture. For postinfection, incubate the cells ($2.0 \times 10^5$/well) with the test drug up to 8 days, using AZT and solvents (used to prepare the test drug) as control(s). Lyse the virus-infected cells with 1X Promega cell lysis buffer (150 mL), and transfer to a culture plate to read the absorbance at 485 nm (excitation) and 520 nm (emission) by a fluorimeter. The results can be expressed as percentage inhibition:

$$\text{GFP fluorescence in the experimental group / fluorescence in infected cells without the test drug} \times 100$$

with the he percent inhibition calculated by subtracting the above value from 100 (Chattopadhyay et al., 2015).

### 16.7.2 Hepatitis B Virus

**(a)** Hep AD38 assay

Place Hep AD38 cell suspension ($6 \times 10^5$ viable cells/mL of Hep AD38 seeding medium) in a 96-well microtiter plate, and incubate at 37°C for 3 days. Discard the medium and wash the cell monolayers with warm (37°C) DPBS. To the proper wells, add 100 µL of HepAD38 assay medium that contains either test or control compounds at the desired concentrations. Also include wells with Hep AD38 assay medium alone as "virus only" controls. Incubate at 37°C for 3 days. On day 3, wash the cells once with warm DPBS and add fresh medium containing the appropriate compound to the wells. After 24 h, transfer the supernatants to v-bottomed 96-well plates and remove cellular debris by centrifugation (15 min, 2500 rpm at 4°C). Transfer 90 µL of the clarified supernatants to new v-bottomed plates and store at −70°C for quantification of HBV DNA.

Thaw the supernatants that were collected and add 90 µL of 2X denaturation solution to each well and mix. Incubate at 37°C for 20 min. Cut the nylon membrane to size and prepare it for blotting by wetting it first with distilled water and then 20X hybridization buffer, SSC (Saline sodium-citrate). Dot-blot the denatured supernatants on to the nylon membrane as directed by the manufacturer. Wash the blot with 200 µL of neutralization solution followed by 200 µL of 20X SSC. Remove the blot, rinse it briefly in 2X SSC, and then crosslink the DNA to the nylon filter by UV irradiation.

Prehybridize the blot at 42°C for 1 h in 20 µL of hybridization solution. Prepare a 32P-labeled probe by random priming using a portion of the HBV genome as a template. Purify the probe through a Clontech Chroma Spin column. Denature the probe by boiling for 5 min and add it immediately to the hybridization solution. Hybridize the nylon filter overnight at 42°C. Wash the nylon filters twice with 50 mL of 2X SSC, 0.1% SDS (sodium dodecyl sulfate) at room temperature for 20 min and twice with 50 mL of 0.2X SSC, 0.1% SDS at 65°C for 20 min. Expose the nylon filters to a molecular imager screen for 4 h and scan on a phosphor imager to obtain the data. To determine the percent inhibition of HBV replication, subtract the background value (counts of radiation detected from the nylon filter itself) from all control and experimental values. Divide the average values of the experimental wells (cells treated with test compounds) by the average value for the "virus only" control (cells not treated with compound or tetracycline during the experiment) and multiply this number by 100 (King and Ladner, 2000). The above mentioned in vitro studies are listed in Table 16.2.

## 16.8 CYTOTOXICITY STUDIES

Drug development programs include preclinical screening of immense quantities of chemicals for specific and nonspecific cytotoxicity against numerous sorts of cells, which is imperative to show the potential therapeutic target and safety evaluation. The screening of plant extracts or pure compounds for investigating their antiviral properties can be more significant with cytotoxicity measures (Meyer et al., 1982).

It is essential for an investigational item to establish the antiviral activity at concentrations that can be accomplished in vivo without inducing toxicity to cells. Moreover, in a cell culture display, antiviral activity of an investigational item can be the aftereffect of host cell death after exposure to the item. Cytotoxicity tests utilize a series of increasing concentrations of the antiviral product to determine what concentration results in the death of 50% of the host cells. This value is referred to as the median cellular cytotoxicity concentration (CC50 or CTC50 or CCIC50). The relative effectiveness of the investigational product in inhibiting viral replication compared with inducing cell death is defined as the therapeutic or selectivity index (CC50 value/$EC_{50}$ value). It is desirable to have a high therapeutic index giving maximum antiviral activity with minimal cell toxicity. According to US FDA guidelines, it is recommended to determine CC50 values in both stationary and dividing cells from multiple relevant human cell types and tissues to establish the potential for cell cycle, species, or tissue-specific toxicities. Studies determining cytotoxicity and therapeutic indexes should be conducted before the initiation of phase 1 clinical studies. There are a number of advantages for in vitro testing using cell cultures, which include

**TABLE 16.2 In Vitro Evaluation for Antiviral Potentials: Determination of the Viral Infectivity in Cultured Cells During Virus Multiplication**

| Virus Assay | Specificity and Application |
|---|---|
| End point titration technique (EPTT) | • Determination of virus titer reduction in the presence of twofold dilutions of test compound(s) |
| Virus-induced cytopathic effect inhibition (CPE) | • For viruses that induce CPE but do not readily form plaques<br>• Determination of virus-induced CPE in monolayers, cultured in liquid medium, infected with a limited dose of virus, and treated with a nontoxic dose of the test substance(s) |
| Virus yield reduction assay | • Determination of the virus yield infected with a given amount of virus and treated with a nontoxic dose of the test substances(s)<br>• Virus titration is carried out after virus multiplication by the plaque test or the 50% tissue culture dose end point test ($TCD_{50}$) |
| MTT assay | • Determination of inhibition of virus infection treated with a nontoxic dose of the test substances(s) by MTT reagent<br>• Inhibition is determined by calculating optical density (OD) in an ELISA plate reader |
| Plaque reduction assay | • Only for viruses that form plaques<br>• Titration of residual virus infectivity after extracellular action of test substance(s)<br>• Cytotoxicity should be eliminated, for example, by dilution, filtration, etc., before the titration |
| Assays on specialized functions and viral products | • Determination of virus-specific parameters, e.g., hemagglutination and hemadsorption tests (myxovirus), inhibition of cell transformation (Epstein–Barr virus, EBV), immunological tests detecting antiviral antigens in cell cultures (EBV, HIV, HSV, and CMV), TZM-bl cell-based assay (HIV), CEM-green fluorescent protein cell-based assay (HIV), Hep AD38 assay (HBV), immunofluorescence assay, enzyme-linked immunosorbent assay (ELISA)<br>• Reduction or inhibition of virus-specific polypeptides synthesis in infected cell cultures, e.g., viral nucleic acids, determination of the uptake of radioactive isotope labeled precursors or viral genome copy numbers |
| Other assays for validation of antiviral activity | • Virus inactivation assay, virus adsorption assay, virus attachment, and penetration assay |

analysis of species specificity, feasibility of using only small amounts of test substances, and facility to do mechanistic studies (Guidance for Industry, 2006).

After confirming the cytotoxic concentration, the drug concentrations are selected for antiviral studies based on the percentage viability of cells and are used to study the antiviral activity by CPE inhibition assay, virus yield assay, followed by MTT assay.

### 16.8.1 Cytotoxic Study by Trypan Blue Dye Exclusion Technique

Any compound that is cytotoxic to cells inhibits the cell proliferation and kills the cells. Trypan blue is a dye that is capable of penetrating dead cells; therefore, the dead cells take up the blue stain whereas the viable cells do not. This method gives an exact number of dead and viable cells (Strober, 2001).

### 16.8.2 Determination of Cell Metabolic Function by Protein Estimation

Protein content is widely used for estimating total cellular material and can be used in growth experiments. The colorimetric method of estimating protein is more sensitive. The cell pellets are treated with 11% cold trichloroacetic acid to remove amino acid pools and dissolved in alkaline cupric sulfate and folin ciocalteau phenolic reagent. Folin's reagent and cupric sulfate together react with amino acid to give a blue color and this color intensity is proportional to the protein concentration, which can be measured colorimetrically (Maya et al., 1995).

To proceed with the same technique, the cells from the wells were trypsinized using 100 µL trypsin and transferred into Eppendorf tubes and centrifuged at 5000 rpm for 10 min to obtain pellets. The cell pellets are dissolved in NaOH and diluted

to 0.1 N. The test drug is to be added to 200 µL of protein sample, mixed, and left for 10 min. To this, 100 µL of test reagent is added with constant mixing and left for 40 min in incubator. The absorbance was read at 655 nm using an Elisa reader (Bio-Rad). The color development was correlated with the cell number as follows:

$$\%\text{Growth inhibition} = 100 - \frac{\text{OD of the sample}}{\text{OD of control}} \times 100$$

The cytotoxic concentration found by dye exclusion techniques gives superficial data. The selected concentrations from a trypan blue dye exclusion study are used further for estimating proteins.

## 16.8.3 Determination of Mitochondrial Synthesis by MTT Assay

MTT (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide) in live cells enters the cells and enzyme succinate dehydrogenase present in mitochondria reduces it to formazan blue product. The color intensity is directly proportional to the number of live cells.

To perform this process, the plates were seeded with HEp-2 cells at 10,000 cells/well. They are incubated for 24 h. After 24 h, the medium is discarded and drug concentrations were added and incubated for 72 h. Then, 50 µL of 2 mg/mL of MTT is to be added and incubated for 3 h and then 100 µL of Isopropyl alcohol is added and absorbance is read at 540 nm in an ELISA plate reader (Bio-Rad). The results are tabulated and percentage growth inhibition is calculated using the following formula:

$$\%\text{Growth inhibition} = 100 - \frac{\text{Mean OD of the individual test group}}{\text{Mean OD of control group}} \times 100$$

The concentrations of the test drug used in the previous experiments can be further confirmed by studying the mitochondrial synthesis by MTT assay. The formazan blue color formation is directly proportional to the number of viable cells and therefore the absorbance is to be read at 540 nm.

## 16.8.4 Brine Shrimp Bioassay

In this test, brine shrimp (*Artemia salina*) eggs are hatched in artificial sea water (38 g/L of sea salt). The Brine Shrimp test (BST) bioassay experiment is performed according to the procedure described by Meyer et al. (1982). Generally, samples of the test drugs for the experiment are prepared in methanol solution, which acts as control vehicle. After 48 h of incubation, 10 brine shrimps are transferred to each sample vial using a Pasteur pipette and artificial sea water is added to make 5 mL. Sample vials are previously prepared by dissolving specific concentrations of test drugs with different dilutions. The solvent is then evaporated overnight. Survivors are counted after 24 h and the $LC_{50}$ values, with 95% confidence intervals are determined using probit analysis, as described by Finney (1971). Control vials are prepared using methanol only. Three replicates are prepared for each concentration of the test drugs.

Control disks are prepared using only methanol. Replicates are prepared for each dose level. To begin the bioassay, brine shrimp eggs are hatched in a shallow rectangular dish ($22 \times 32\,cm^2$) under the same conditions described in the literature except that natural instead of artificial seawater is used. Ten shrimps are selected and transferred into each sample vial by means of a 23-cm disposable Pasteur pipette and the final volume in each vial is adjusted to 5 mL using natural seawater. A drop of dry yeast suspension (3 mg in 5 mL seawater) is added as food to each vial. The vials are maintained under illumination. Survivors are counted with the aid of a stereomicroscope, after 6, 24, and 48 h, and the deaths at each dose level and control are determined. No deaths are usually observed to occur in the control after 48 h.

The brine shrimp test (BST) represents a rapid, inexpensive, and simple bioassay for testing plant extract lethality, which, in most cases, correlates reasonably well with cytotoxic properties (McLaughlin, 1991). Following the procedure of Meyer et al. (1982), the lethality of the test drugs/plant extracts to brine shrimp is determined.

### *16.8.4.1 Lethal Concentration Determination*

The lethal concentrations of test drugs/plant extract resulting in 50% mortality of the brine shrimp ($LC_{50}$) and 95% confidence intervals are determined from the 24 and 48-h counts and the dose–response data are transformed into a straight line by means of a trend line fit linear regression analysis; the $LC_{50}$ is derived from the best-fit line obtained. Caffeine ($LC_{50} = 306$ µg/mL) (Meyer et al., 1982) is used as a positive control and methanol (500 µL) as a solvent and a negative control in the bioassay experiments.

The current scenario of viral diseases is lethal and there is an upsurge in new viral diseases and resistance to existing viral infections worldwide. The currently accessible antivirals, however effective, are exorbitant and past the reach of a majority of individuals. Along these lines, the advancement of safe, effective, and low-cost antiviral medications, for example, RT inhibitors, is among the top priorities, as many viral infections are not yet treatable and have high death rates. For the past few years, substantial work has been carried out regarding the effectiveness of medicinal plants on HIV infection (Premanathan et al., 1999; Calabrese et al., 2000; Asres et al., 2001) and an increasing popularity of over-the-counter plant products containing orthodox drugs has been observed. The main focus is to lower the adverse effects associated with viral infections and an inclination toward synergistic interactions of multiple molecules present in plant extracts. Be that as it may, because mostly pharmacological mechanisms of the combinations are not studied, antagonistic impacts or remedial disappointments have been seen (Chan et al., 2000). A prerequisite that should be considered significant for medicinal plants is to identify and standardize the method of extract preparation, the suitable season for collecting plant material, and the details of its administration (Chattopadhyay et al., 2006). As a lot of plant extracts and subsequent formulations have shown significant outcomes, it seems to be rational to endorse the idea of the study of medicinal plants as a quest to find potential antivirals.

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