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# A SIMPLIFIED PLAQUE REDUCTION ASSAY FOR ANTIVIRAL AGENTS FROM PLANTS. DEMONSTRATION OF FREQUENT OCCURRENCE OF ANTIVIRAL ACTIVITY IN HIGHER PLANTS

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ABSTRACT.—An improved plaque reduction assay for antiviral activity was developed and used to examine extracts from sixty-one higher plants. Substantial antiviral activity was observed in ca. 20%, which is higher than might be expected given the limited metabolic differences between viruses and their hosts. The frequent antiviral activity may represent phytoalexins elaborated to protect against plant viruses.

Most successful anti-infective agents act by inhibiting an essential step in a metabolic process required by the pathogen but absent in the host. For example, the majority of antibiotics inhibit either protein synthesis or cell wall synthesis processes that use different pathways in bacteria from those in mammals. In the case of viruses, these metabolic differences are not available, because viruses use the host's protein synthesis system and membranes, leaving only some aspects of nucleic acid synthesis and macromolecule processing distinct from the host metabolism. Consequently, discovery of antiviral agents from plants and other natural sources might be expected to be difficult.

Relatively few studies seeking antiviral agents from plants have been reported (1). The limited number of studies may reflect perceptions of a poor prognosis for reasons discussed above, but they may also reflect the technical challenges involved in conducting antiviral assays, which make them inherently more difficult than antibacterial assays. The two basic approaches used in assaying antiviral activity are plaque reduction assays and titer reduction assays. Plaque reduction assays use a constant number of viral particles and vary the concentration of test substance. Antiviral activity is expressed as a reduction in the size or number of viral plaques (areas of killed or morphologically altered cells) in a cell monolayer. Titer reduction assays use a constant amount of test substance and vary the number of viral particles. Antiviral activity is detected as a lower apparent virus titer, compared to the virus titer determined in the absence of test compound by serial dilution of the virus suspension to a concentration that gives a number of viral plaques small enough to be counted.

In the present study we have developed a simplified plaque reduction assay for activity against either a DNA virus (*Herpes simplex* type 1, HS-1) or an RNA virus (vesicular stomatitis virus, VSV). Plaque reduction assays typically use a monolayer of cultured host cells that are allowed to bind virus and then are overlaid with a layer of medium thickened with agar or another thickener that makes plaque formation possible by preventing mixing due to currents in the medium. Preparations to be tested for antiviral activity are either incorporated into the thickened layer or absorbed in a paper disc laid on the thickened layer. The thickened layer can cause several types of technical problems, including toxicity of the thickener to virus or host cells and absorption or other types of interference with unknown antiviral agents being tested. We have modified this approach to allow the production of acceptable HS-1 plaques without the use of a thickening agent. The assay size has been reduced to fit the wells (ca. 5 mm diameter) of 96-well microtiter trays. When these wells are $>\frac{2}{3}$ filled with medium (150–250 μl) and the cultures are not disturbed during incubation, the small size of the wells presumably limits convection currents, which play an important role in dispersing viral

particles in larger culture dishes. Presumably the large aggregate molecular weight of viral particles prevents significant dispersion by simple diffusion. The use of serial dilutions of extracts in parallel wells in a tray allows an estimation of end-point concentrations for antiviral agents. These modifications result in reduced sample size, reduced costs, and simplified processing, and they eliminate the potential for interference by thickening agents. The assay also allows for cytotoxicity estimations in extracts, which are reflected as loss of the cell monolayer in which the plaques are normally formed.

The modified assay has been used to examine extracts from 61 higher plants. These include 56 unselected plants of predominantly temperate origin and five plants (*Acorus gramineus* large and small varieties, *Kalanchoe pinnata*, *Perilla frutescens*, and *Polygonum cuspidatum*) used in Laotian (Hmong) folklore medicine to treat chicken pox (2). Identification of plant extracts as having antiviral activity was based on a selectivity index defined as the ratio of the maximum non-cytotoxic concentration giving 100% reduction in plaque number to the minimum concentration giving 100% reduction in plaque number. EtOH extracts that gave a selectivity index of 2 or more with either virus are given in Table 1. Saline extracts that exhibited a selectivity index of 2 or more are given in Table 2. Using this criterion for antiviral activity 11 out of 56 EtOH extracts (20%) and 10 out of 56 saline extracts (18%) exhibited activity among unselected plants. A modestly higher frequency (2 out of 5) was observed among plants selected for reported antiviral activity in folk medicine. Use of a less rigorous criterion for antiviral activity (e.g., 80% reduction in plaque number) would identify numerous additional extracts as antiviral in this study.

These results confirm previously reported (1, 3–5) observations of up to 30% incidence of antiviral activity in extracts of plants prepared and assayed by a variety of methods. The frequent occurrence of antiviral activity in higher plants contrasts with the anticipated difficulty in identifying antiviral agents. The reason for the high frequency is not known. One plausible explanation is that the antiviral agents detected in these studies are phytoalexins, substances produced by the plants to protect against plant viruses (6). Although much progress has been made in recent years in characterizing structures and mechanisms of action of phytoalexins, the full extent of types and actions is not known, particularly for plants that are not of major economic importance (6). Better understanding of plant antiviral agents and their roles in plants will require isolation and characterization of a number of these substances.

## EXPERIMENTAL

CULTURES.—*Herpes simplex* type 1 (HS-1) and vesicular stomatitis virus (VSV) were the gifts of Dr. R.G. Hughes, Roswell Park Memorial Institute, Buffalo, New York. Original cultures are available from American Type Culture Collection, Rockville, Maryland. Virus stocks were prepared as aliquots of culture medium from Vero cells infected at multiplicity of 1 virion per 10 cells and cultured 3 days. They were stored at −80°. Working stocks were prepared by titering virus stocks by serial dilution in culture medium and assayed in triplicate on Vero monolayers in the wells of microtiter tray. Virus working stock suspensions were stored at 4° until used. Vero African green monkey kidney cells were purchased from Viromed Laboratories, Minnetonka, Minnesota, and grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (HyClone Laboratories, Ogden, Utah), 60 μg/ml penicillin G, and 100 μg/ml streptomycin sulfate maintained at 37° in a humidified atmosphere containing about 15% (v/v) $CO_2$ in air. All medium components were obtained from Sigma Chemical Co., St. Louis, Missouri, unless otherwise indicated. Vero stocks were maintained at 34° in culture flasks filled with medium supplemented with 1% (v/v) calf serum. Subcultures for virus titration or antiviral screening were grown in the wells of microtiter trays (Falcon Microtest III 96-well trays, Becton Dickinson Labware, Lincoln Park, New Jersey) (7,8) by suspending Vero cells in medium following trypsin-EDTA treatment, counting the suspension with a hemocytometer, diluting in medium containing 10% calf serum to $2 \times 10^4$ cells per 200 μl culture, aliquoting into each well of a tray, and culturing until confluent.

PREPARATION OF EXTRACTS.—Green plant materials were obtained from the University of Min-

TABLE 1. Plants with Antiviral Activity in EtOH Extracts.[a]

| Plant | Organ tested[b] | *Herpes simplex* type 1 | | Vesicular stomatitis virus | | Cytotoxicity[c] (μg/ml) |
|---|---|---|---|---|---|---|
| | | MIC[c] (μg/ml) | Selectivity[d] | MIC[c] (μg/ml) | Selectivity[d] | |
| *Acorus gramineus* Soland (Araceae) | a | P | — | P–200 | 2.5 | 1000 |
| *Hippeastrum rhodophiala* K. Presl (Amaryllidaceae) | bu | 10 | 2.5 | 5–10 | 2.5–5 | 63 |
| *Anemone coronaria* L. (Ranunculaceae) | bu | P–100 | 2 | 100–200 | 1–2 | 500 |
| *Filipendula ulmaria* Maxim. (Rosaceae) | w | 100 | 2 | P–100 | 2 | 500 |
| *Fragaria virginiana* Duchesne (Rosaceae) | w | 250–500 | 1–2 | 250–500 | 1–2 | 1000 |
| *Fritillaria imperialis* (Lutea) L. (Liliaceae) | bu | 250–500 | 1–2 | P–250 | 2 | >1000 |
| *Fritillaria imperialis* (Rubra) L. (Liliaceae) | bu | 500 | 1 | P–250 | 2 | >1000 |
| *Geum triflorum* Pursh. (Rosaceae) | a | 250–500 | 1–2 | P–200 | 2.5 | 1000 |
| *Narcissus poeticus* L. (Amaryllidaceae) | bu | 10 | 1 | 5–10 | 1–2 | 25 |
| *Narcissus pseudonarcissus* L. (Amaryllidaceae) | bu | 10–25 | 1–2.5 | 10 | 2.5 | 63 |
| *Pachysandra terminalis* Sieb. & Zucc. (Buxaceae) | w | 200 | 2.5 | P–200 | 2.5 | >1000 |
| *Polygonum cuspidatum* Sieb. & Zucc. (Polygonaceae) | a | 250–500 | 1–2.5 | 500 | 1 | 750 |
| *Waldsteinia fragarioides* Tratt. (Rosaceae) | w | 25–100 | 5–20 | 100–200 | 2.5–5 | 1000 |

[a]The following plants did not exhibit antiviral activity by the criteria used for either EtOH or saline extracts of the indicated organ (a, aerial parts; bu, bulbs; f, flowers; l, leaves; r, roots; rh, rhizomes; s, stems; tu, tubers; w, whole plants): Apiaceae: *Zizia aurea* (L.) Koch f, r, l, s; Apocyanaceae: *Apocynum androsaemifolium* L., w; Araceae: *Caladium* sp., bu; Asteraceae: *Achillea filipendulina* Lam., w, *Dahlia* sp., bu; *Conyza canadensis* (L.) Crong., w, *Gaillardia aristata* Pursh, a, r, *Helenium autumnale* (L.), w, *Helianthus tuberosus* L., tu, *Erigeron annus* (L.) Pers., w; Boraginaceae: *Brunnera macrophylla* Johnston, w; Brassicaceae: *Descurainia richardsonii* (Sweet) Schultz, w; Campanulaceae: *Campanula glomerata* L., w; Crassulaceae: *Kalanchoe pinnata* Pers., a, *Sedum kamschaticum* Fisch., w, *Sedum rupestre* L., w, *Sedum spurium* Bieb., w; Fabaceae: *Coronilla varia* L., w, *Lotus corniculatus* L., w, *Melilotus officinalis* Lam., a, r; Geraniaceae: *Geranium ibericum* Cav., w, *Geranium sanguineum* L., w; Iridaceae: *Iris iberica* Siemss, rh, *Iris pseudacorus* L., rh, *Iris reticulata* Bieb, rh, *Iris virginica* L., rh, f, a, *Tigridia pavonia* Ker., bu; Laminaceae: *Perilla frustescens* Britt, f, l, s, *Nepeta grandiflora* Bieb., w; Liliaceae: *Allium ascalonicum* L., bu, *Allium giganteum* Regel, bu, *Allium sphaerocephalum* L., bu, *Ipheion uniflorum* Raf., bu, *Eucomis autumnalis* Mill., bu, *Hosta decorata* Bailey, w, *Hosta undulata* Bailey, w, *Hyacinthus orientalis* L., bu, *Lillium tigrinum* Ker., a, r; *Muscari armeniacum* Leicht., bu; Poaceae: *Arundo donax* L., w, *Deschampsia cespitosa* (L.) Beauv., w, Typhaceae: *Typha latifolia* L., a, l, r.

[b]Abbreviations: a, aerial parts; bu, bulbs; w, whole plants.

[c]MIC = minimum inhibitory concentration; i.e., the minimum concentration of plant extract which causes 100% inhibition of virus replication; P indicates that partial (i.e., <100%) inhibition of virus replication was observed at non-toxic concentrations. In the cases in which a range of MIC values is shown the higher value was obtained when cultures were preinfected with virus. Single value with absence of range indicates similar results with or without preinfection.

[d]Selectivity index, defined as the maximum non-toxic concentration which gives 100% inhibition of virus replication divided by the minimum concentration which causes 100% inhibition of virus replication.

[e]Approximate concentration of plant extract which causes 50% loss of cells from the substratum.

TABLE 2. Plants with Antiviral Activity in Saline Extracts.

| Plant | Organ tested[a] | *Herpes simplex* type 1 | | Vesicular stomatitis virus | |
|---|---|---|---|---|---|
| | | MIC[b] (fold dilution) | Selectivity[c] | MIC[b] | Selectivity[c] |
| *Comptonia peregrina* Coult. (Myriaceae) | w | 8 | 2 | P | — |
| *Coreopsis verticillata* L. (Asteraceae) | w | 13.2 | 3.3 | 8 | 2 |
| *Crepis tectorum* L. (Asteraceae) | w | 9 | 1.7 | 10.6 | 2 |
| *Filipendula ulmaria* Maxim (Rosaceae) | w | 8 | 2 | P | — |
| *Fragaria virginiana* Duchesne (Rosaceae) | w | 8 | 2 | P | — |
| *Geum triflorum* Pursh (Rosaceae) | r | 13.2 | 3.3 | P | — |
| *Geum triflorum* Pursh (Rosaceae) | a | 8 | 2 | P | — |
| *Heuchera sanguinea* Engelm. (Saxifragaceae) | w | 8 | 2 | P | — |
| *Oenothera missouriensis* Sims (Onagraceae) | a | 8 | 2 | 8 | 2 |
| *Waldsteinia fragarioides* Tratt. (Rosaceae) | w | 40 | 10 | 20 | 5 |

[a]Abbreviations: a, aerial parts; r, roots; w, whole plants.

[b]MIC = minimum concentration which exhibits 100% inhibition of virus proliferation expressed as the number of fold dilution of a saline extract; P indicates that partial (< 100%) inhibition of virus proliferation was observed at nontoxic concentrations.

[c]Maximum non-toxic concentration which gives 100% inhibition of virus replication divided by the minimum concentration which causes 100% inhibition of virus replication.

nesota Landscape Arboretum or commercial outlets. Five plants from Hmong folk medicine were submitted for antiviral testing by M. Spring.

*Alcoholic Extracts.*—Fresh plant material (50 g) was diced in 5 volumes of 95% EtOH, homogenized in a blender, and shaken for 2 h at room temperature. The mixture was filtered through Whatman No. 1 paper, and the filtrate was evaporated to dryness under reduced pressure at 40°. For assay, residues were dissolved in DMSO at 100 mg/ml and diluted 50-fold into sterile medium. If necessary, the pH was readjusted to about 7.2 with 0.1 N NaOH or 0.1 N HCl.

*Saline Extracts.*—Fresh plant material (20 g) was homogenized with 50 ml saline (0.15 M NaCl) and shaken for 2 h at room temperature. The mixture was filtered through Whatman No. 1 paper and sterilized by filtration through a type GS 0.22 μm membrane filter (Millipore Corp., Bedford, Massachusetts). Extracts were assayed as 1:1 dilutions in sterile medium.

ANTIVIRAL ASSAYS.—Microtiter trays with confluent monolayer cultures of Vero cells were inverted, and the medium was shaken out and replaced with serial dilutions of sterile extracts in triplicate in 100 μl medium followed by titered virus in 100 μl medium containing 10% calf serum in each well. For routine assays about 30 plaques per well of HS-1 were used or sufficient VSV (10 to 30 infectious particles) to almost eliminate the Vero monolayer. In each tray the last row of wells was reserved for controls that were not treated with extract or not treated with virus. The trays were cultured for 66 h (HS-1) or 36 h (VSV) with care being taken not to disturb the cultures during incubation. The trays were inverted onto a pad of paper towels, and the remaining cells were rinsed carefully with medium and fixed with 3.7% formaldehyde in saline for at least 20 min. The fixed cells were rinsed with $H_2O$, stained with 0.5% crystal violet in 20% aqueous EtOH for 30 min, rinsed with $H_2O$, and examined visually. Antiviral activity is identified as confluent, relatively unaltered monolayers of stained Vero cells treated with either virus. Cytotoxicity was identified as described previously (7,8) in HS-1 assays as loss of the monolayer in excess of plaques caused by HS-1.

Several minor variations in the assay procedure outlined above can be carried out to obtain additional information about the antiviral mechanisms of active substances. For example, EtOH extracts were also examined by an alternate approach in which the cultures were preinfected with virus by adding the virus suspension to each well in 200 μl of medium, incubating 90 min under normal culture conditions, and washing away unbound virus before adding extracts. This approach reduces the sensitivity of the assay for antiviral agents that act by inactivating viral particle infectivity. It is also possible to test for direct effects on viral particles by preincubating high concentrations of viral particles with test substances. Diluting ten thousandfold or more before assay minimizes potential effects on the host cells. In another variation, prolonged incubation (e.g., 1 week) without fixing and staining cells can be used to examine for 100% inhibition of virus multiplication. It is also possible to evaluate antiviral effects at higher virus multiplicity. For example, extracts of *Waldsteinia fragarioides* at noncytotoxic concentrations completely inhibit viral replication at up to 120,000 virions per well (i.e., about 2 virions per Vero cell in the monolayer).

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