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Inactivation of Enveloped Viruses by Anthraquinones Extracted from Plants

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To determine the extent of antiviral activity present in a number of plant extracts, hot glycerin extracts were prepared from *Rheum officinale*, *Aloe barbadensis*, *Rhamnus frangula*, *Rhamnus purshianus*, and *Cassia angustifolia* and their virucidal effects were tested against herpes simplex virus type 1. All the plant extracts inactivated the virus. The active components in these plants were separated by thin-layer chromatography and identified as anthraquinones. A purified sample of aloe emodin was prepared from aloin, and its effects on the infectivity of herpes simplex virus type 1 and type 2, varicella-zoster virus, pseudorabies virus, influenza virus, adenovirus, and rhinovirus were tested by mixing virus with dilutions of aloe emodin for 15 min at 37°C, immediately diluting the sample, and assaying the amount of infectious virus remaining in the sample. The results showed that aloe emodin inactivated all of the viruses tested except adenovirus and rhinovirus. Electron microscopic examination of anthraquinone-treated herpes simplex virus demonstrated that the envelopes were partially disrupted. These results show that anthraquinones extracted from a variety of plants are directly virucidal to enveloped viruses.

Many kinds of benzoquinones, naphthaquinones, and anthraquinones occur in plants, but until recently none of them has been tested for their effects on viruses. Vanden Berghe et al. (13) have shown that the naphthaquinone juglone was active against herpes simplex virus (HSV) but inactive against poliovirus, Simliki forest virus, coxsackievirus, and measlesvirus. Hypericin and pseudohypericine, naphthodianthrones found in *Hypericum* species, have been shown to have activity against animal retroviruses (4, 6). A recent report by Schinazi et al. (7) demonstrated that polyphenolic- and/or polysulfonate-substituted anthraquinones as well as hypericin inhibited human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. Kemp and coworkers (2) also showed that the glycoside acemannan inhibited replication and pathogenesis of HIV-1, HSV, and Newcastle disease virus. McDaniel and coworkers (3) reported its effectiveness in patients with AIDS. All of these studies demonstrated inhibition of the replication of certain viruses, but none examined the presence of direct virucidal effects.

MATERIALS AND METHODS

Plant sources and glycerin extraction procedure. The following plant materials were used for extraction: the rhizome of Chinese rhubarb (*Rheum officinale*), the leaves of aloe (*Aloe barbadensis*), a sample of aloe emodin prepared from aloe leaves, the bark of frangula (*Rhamnus frangula*), the bark of cascara sagrada (*Rhamnus purshianus*), and the leaves of senna (*Cassia angustifolia*). The extracts were prepared by mixing 0.3 g of ground, dried plant material with 3 ml of glycerin, heating at 105°C for 20 min, and filtering the extracted material. The extract was then diluted to a final starting concentration of 1.0 mg/ml in 50% glycerin for antiviral activity testing. In preliminary studies, the 50% glycerin control was processed exactly the same as the plant extractions, i.e., the glycerin was heated at 105°C for 20 min and then filtered and diluted with phosphate-buffered saline (PBS).

Identification of anthraquinones. Aloe emodin (1,8-dihydroxy-3-hydroxymethylanthraquinone) was semisynthetically prepared by oxidative hydrolysis of aloin. Its purity was verified by radial thin-layer chromatography (5), and its chemical structure was confirmed by nuclear magnetic resonance spectrometry.

Aloe emodin was isolated in form of orange needles and gave the following ^{1}H nuclear magnetic resonance data (instrument, General Electrics QE-300): ($CDCl_3$) 1.98 ppm (1H, t, J = 5.1 Hz, 3-alcoholic OH), 4.84 ppm (2H, d, J = 5.1 Hz, 3-methylene), 7.31 ppm (1H, dd, J = 8.1, 1.2 Hz, 7-H), 7.36 ppm (1H, br s, 2-H), 7.69 ppm (1H, dd, center signals coalesced into one, 6-H), 7.81 ppm (1H, br s, 4-H), 7.85 ppm (1H, dd, J = 7.5, 1.2 Hz, 5-H), 12.09 ppm (1H, s, 1-OH), 12.10 ppm (1H, s, 8-OH).

Aloe emodin and other emodins were also found in the hot glycerin extracts from frangula, rhubarb, cascara, and senna. Chromatographically, these extracts showed to be mixtures which were subjected to antiviral testing in toto. In all of these plant extracts the bulk of the phytoconstituents are anthraquinone glycosides which are inactive as antiviral agents, and the extraction with hot glycerol proved to be very useful in recovering the sum of the active aglycones.

The other emodins found in the glycerin extracts were compared with commercially available material. The purity of these was established by nuclear magnetic resonance and thin-layer chromatography. Radial thin-layer chromatography was performed on BAKER Si250 plates with the solvent system toluene-ethylacetate, 3:1. The spots were observed under long-wave UV light (360 nm) and in visible light after exposure to ammonia vapors.

Viruses and cells. The strain of HSV type 1 (HSV-1) used in this study (HSV-1 KOS) was originally isolated from a lip lesion by Smith (8) and was obtained from Fred Rapp, University of Pennsylvania Medical School, Hershey, Pa. The HSV-2 strain (HSV-2 G strain) (ATCC VR-734) and (ATCC VR) pseudorabies (PSV) virus were obtained from

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the American Type Culture Collection (ATCC), Rockville, Md. These viruses were propagated in Vero cells and quantitated by a plaque assay in Vero cells. Varicella-zoster virus (VZV) was obtained from ATCC (ATCC VR-916), propagated in WI-38 cells, and quantitated by an infectivity or cytopathic effect assay in WI-38 cells. A laboratory reference strain of influenza virus A (INF) was obtained from Jagdish Patel, State of Maryland Health Laboratories Virology Section (Baltimore, Md.), propagated in Vero cells, and quantitated by an infectivity assay using Vero cells. Rhinovirus (RH) strain 2060 and adenovirus (AD) type 37 were obtained from ATCC (ATCC VR-1100 and ATCC VR-929, respectively) and were propagated in WI-38 cells and quantitated by an infectivity assay in WI-38 cells.

African green monkey kidney cells (Vero) and WI-38 cells were obtained from Flow Laboratories (McLean, Va.). They were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and penicillin, streptomycin, and fungizone (100 U/ml, 100 μg/ml, and 2.5 μg/ml, respectively). Overlay medium for the plaque assays of HSV and PSV consisted of MEM plus 2% FCS containing antibiotics as described above and 1.5% methylcellulose.

PRA. All samples were tested for antiviral activity against HSV-1, HSV-2, and PSV by a plaque reduction assay (PRA) with monolayer cultures of Vero cells grown in MEM. Virus stock samples were diluted to give a final starting concentration of approximately 10^5 PFU per 0.1 ml. A 0.1-ml volume of this virus concentration was inoculated into 1.0 ml of each test sample dilution or PBS placed in a 37°C water bath for 15 min and then diluted in PBS to give a final virus concentration of approximately 100 PFU/ml in the control samples. Samples were titrated by inoculation of 1 ml of the appropriate dilution onto duplicate Vero cell monolayers in 60-mm dishes. After adsorption, the inoculum was removed, a methylcellulose-MEM overlay medium was added, and after 3 days of incubation the number of plaques per dish was counted and averaged. The results of duplicate experiments were normalized by setting the PFU obtained in the 15-min control virus dishes at 100% and calculating the average reduction of plaques in the test dishes as a percentage of the control. The inhibitory doses (IDs) were calculated as the concentrations of sample in milligrams per milliliter that reduced plaque formation by 50% (ID_{50}) or 90% (ID_{90}).

Infectivity assays. All samples were tested for antiviral activities against VZV, INF, AD, and RH by an infectivity assay with the same 15-min incubation conditions as described for the PRA. After incubation of the mixtures, samples were diluted in 10-fold steps, and 2.0 ml of each dilution was inoculated into triplicate screw-cap culture tubes containing monolayers of Vero cells (for INF) or WI-38 cells (for VZV, AD, and RH). The tubes were incubated at 37°C and examined on day 8 postinfection and scored as positive or negative. A positive tube was any tube which exhibited any area(s) of virus-specific cytopathic effect. The infectivity endpoints were calculated by graphing the datum points, and the results of duplicate experiments were averaged. The ID_{50} and ID_{90} were then calculated from these data, and the concentrations were given in milligrams per milliliter.

Cytotoxicity assays. Cell cytotoxicity of plant materials were tested in vitro by two methods. In the first method, cell medium alone or medium containing 10-fold dilutions of senna extract or purified aloe emodin, beginning with the highest concentrations attainable (0.1 mg/ml in 5% glycerin-PBS), were inoculated onto monolayer cultures of Vero cells and WI-38 cells in 24-well tissue culture plates. The cells were observed daily for 6 days for any signs of cell cytotoxicity compared with the controls. In the second method, a cell viability assay previously reported by Dumsha and Sydiskis (1) which measures the cell replication was used. Vero cells (1.2×10^6) and WI-38 cells (30×10^6) were inoculated into triplicate 75-cm^2 flasks (Falcon) with growth medium or medium containing either senna extract or aloe emodin to give final concentrations of 0.1 mg/ml. The cells were incubated at 37°C for 3 days until confluent and were observed for any signs of cytotoxic effects. The cells were removed from the flasks, and total numbers of viable cells were counted and averaged by using the trypan-blue dye exclusion procedure.

Statistical testing was performed with a Hewlett-Packard computer and computer program entitled ONEWAY SPSS, which compares the mean of all groups within each experiment with one another by the use of analysis of variance.

Electron microscopic studies of anthraquinone-treated herpesvirus. A large pool of HSV-1 was prepared by infecting six 150-cm^2 flasks of Vero cells with HSV-1 at a multiplicity of infection of 3. After 24 h of incubation at 37°C, the cells exhibiting 100% cytopathic effect were scraped from the flasks and centrifuged at 250 × *g* for 20 min. Next, 300 ml of supernatant was collected and centrifuged at 100,000 × *g* in a Sorval rotor in a Sorval Ultracentrifuge (DuPont, Newton, Conn.). The resultant pellets from the six tubes were resuspended in PBS and pooled, and one-half was treated with purified aloe emodin dissolved in 100% glycerin to give a final concentration of 50% glycerin. The other half of the virus pellet was treated with glycerin to a final concentration of 50%. Samples were incubated at 37°C for 15 min, diluted to 13-ml volumes in PBS, and centrifuged at 100,000 × *g* in an SW41 rotor for 1 h. The pellets were resuspended in a 0.5-ml volume of 3% paraformaldehyde. The samples were layered onto continuous 10 to 50% potassium tartrate gradients and centrifuged at 100,000 × *g* for 2 h. The visible bands of virus from each sample were removed by direct aspiration from the side of the polycarbonate tubes, and samples were placed on grids. Small samples were also saved for virus quantitation. Viral suspensions were placed on Formvar-carbon-coated grids and negatively stained with 1% sodium phosphotungstate, pH 7.0. Grids were examined in a JEOL 1200 EX II transmission electron microscope (JEOL, Peabody, Mass.) operating at 60 kV. Grids were examined independently by three blinded researchers. Fifty randomly selected grid sections were examined, and the number and type of virus particles present (whole or disrupted) were counted and averaged. At the viewing magnification used each grid section contained 2 to 5 virus particles for a total of 175. The experiment was repeated twice, and the results were averaged.

RESULTS

Effect of glycerin extracts on HSV-1. In preliminary studies, HSV-1 was used to screen the effect of various extracts obtained first from *Aloe barbadensis* and later from other plant extracts (Table 1). All the plant extracts, which are rich in natural anthraquinones or anthraquinonelike compounds, were completely virucidal to HSV-1 during the 15-min incubation period. Chinese rhubarb appeared to be the most active. The 50% glycerin-PBS control was identical to the hot glycerin control and to the PBS control showing that the glycerin had no effect on the inactivation of the virus.

Cytotoxicity assay. Purified aloe emodin- or senna extract-

TABLE 1. IDs of various plant extracts for HSV-1

Plant extract	ID (μg/ml)[a] ± SD	
	50%	90%
Chinese rhubarb	0.005 ± 0.0004	0.008 ± 0.0007
Cascara	0.01 ± 0.002	0.075 ± 0.006
Frangula	0.35 ± 0.02	0.75 ± 0.06
Aloe	1.8 ± 0.09	7.0 ± 0.56
Senna	1.8 ± 0.01	7.0 ± 0.60

[a] IDs were calculated from the average of two experiments as the concentration of plant extract that reduced plaque formation of HSV-1 by 50 or 90% compared with the controls.

treated Vero or WI-38 cells did not exhibit altered morphologies indicative of cytotoxic effects compared with the controls. In the cell growth assay, the total numbers of viable cells recovered after 3 days of incubation of the cells with extract-containing medium showed no statistically significant differences compared with those from the medium controls (data not shown). These results indicate that neither the senna nor the aloe emodin were cytotoxic to cells or inhibited their replication at the concentration was used. This concentration was 90-fold higher than that having an ID_{50} effect on HSV-1 (Table 1).

Effect of aloe emodin on different viruses. Since the active component from all the plants tested proved to be anthraquinones, one of them, aloe emodin, was tested against a variety of viruses (Table 2) at a starting concentration of 0.1 mg/ml in 50% glycerin. The compound was active against HSV-1, HSV-2, VZV, PSV, and INF. However, even at the highest concentration of the anthraquinone compound tested, AD and RH were not affected compared with the appropriate controls.

Mechanism of aloe emodin effect. The effect of aloe extract on HSV-1 inactivation was concentration dependent and occurred more rapidly at 37 than 4°C (Fig. 1). At the higher concentration (0.01 mg/ml), the antiviral effect of the extract was evident even in the zero time sample.

Samples of anthraquinone-treated and control HSV-1 were collected from potassium tartrate gradients and examined by electron microscopy. One band was seen in both samples, and both banded at the same density in the two gradients. Ninety-eight percent of the untreated control HSV-1 samples examined on the 50 grid sections as previously described contained whole undisrupted HSV-1. The virus particles were seen as containing completely intact envelopes with their glycoprotein spikes evident on the periphery of the particles typical of completely enveloped viruses. In the anthraquinone-treated samples, 95% of the virus particles observed on the 50 grid sections contained viruses which were partially disrupted, allowing the phosphotungstic acid stain to penetrate into the envelope to elucidate the inner nucleocapsid of the virus.

TABLE 2. IDs of aloe emodin on a variety of viruses

Virus	ID (μg/ml)[a]	
	50%	90%
HSV-1	1.6 ± 0.10	7.2 ± 0.80
HSV-2	1.5 ± 0.11	7.0 ± 0.65
PSV	5.0 ± 0.4	10.0 ± 1.1
VZV	6.0 ± 0.5	15.0 ± 1.4
INF	4.5 ± 0.5	8.5 ± 0.7
RH	0	0
AD	0	0

[a] IDs were calculated from the average of two experiments as the concentration of aloe emodin that reduced virus plaque formation or infectivity by 50 or 90% compared with the appropriate controls.

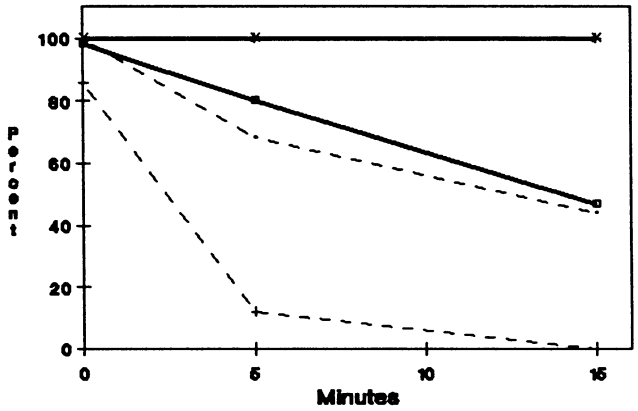

FIG. 1. Direct inactivation of two different concentrations of aloe extract on HSV-1 at various times and temperatures. HSV-1 was mixed with aloe extract to give final concentrations of 0.01 dashed lines or 0.001 (solid lines) mg of aloe extract per ml and incubated at various times from 0 to 15 min as described for a PRA. Temperatures of incubation were either 4 (asterisk and dot) or 37°C (square and plus). Results are presented as mean percentage of control of two separate experiments.

DISCUSSION

The original intent of our study was to determine if the gel from the aloe plant had virucidal activity. During the course of the study it was found that the virucidal activity was due to the anthraquinones extracted from the inner leaf of aloe and the roots, bark, or leaves of a number of other anthraquinone-containing plants. One of the emodins, aloe emodin (1,8-dihydroxy-3-hydroxymethyl anthraquinone) was isolated and semisynthetically prepared from its C-glycoside, aloin. These anthraquinones have been shown to be extractable with a number of organic solvents including acetone, ethyl acetate, and methanol (12). However, the best extractions can be achieved with hot glycerin as described here.

The results of the toxicity study demonstrate that the aloe emodin and senna extract were not toxic to cells nor did they prevent or reduce the rate of replication of either Vero cells or WI-38 cells. The concentration tested was the maximum soluble concentration achievable with the glycerin extraction and still 90-fold higher than the ID_{50} needed to produce a virucidal effect. Because of this limited solubility, a therapeutic index could not be calculated, since a concentration that would have produced a 50% cellular inhibition could not be achieved.

In the initial virus studies, glycerin extracts of plants were used and the amounts adjusted from weight per volume input. The glycerin procedure enriched for anthraquinones present in the plants. The results shown in Table 1 reflect the differences in anthraquinone concentrations present in the extracts as measured by the differences in virucidal effects of the extracts against HSV-1. The aloe extract sample was also used to determine the effects of dose, time, and temperature on the inactivation of HSV-1 (Fig. 1). Once anthraquinones were identified as being the active component responsible for these results, a chemically purified sample of aloe

emodin was prepared as previously described and used to test its effect on a variety of enveloped and unenveloped viruses (Table 2). The results indicated that aloe emodin directly affected both DNA- and RNA-containing enveloped viruses but had no effect on naked (unenveloped) viruses.

These results were confirmed in the electron microscopic study. The virus sample was prepared from extracellular fluids which have been shown to contain enveloped HSV only if the multiplicity of infection and time of preparation are carefully controlled (11). The intact envelope of herpesviruses presents an amorphic sphere with little detail except for spikes visible around the edges. Once the integrity of the envelope is disrupted, the negative stain can penetrate and reveal details of the internal capsid and individual capsomeres (11). The envelope of herpesviruses can be preserved intact during banding in the heavy-salt gradients by fixation in formaldehyde or paraformaldehyde (9, 10). Therefore, damage to the envelope of these viruses prior to fixation in this study was most probably a function of the preincubation of the virus with the anthraquinone and not an artifact of sample preparation.

On the basis of these results, we conclude that, under the conditions tested, the anthraquinones acted directly on the envelope of the anthraquinone-sensitive viruses, resulting in the prevention of virus adsorption and subsequent replication.

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