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FURTHER INVESTIGATIONS ON THE ANTIVIRAL ACTIVITIES OF MEDICINAL PLANTS OF TOGO

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ABSTRACT

Further studies were done on the antiviral activities of 10 species of Togolese medicinal plants, previously shown to possess activity against herpes simpler virus (HSV). The dominant activity in all cases was virucidal (direct inactivation of virus particles), although Adansonia digitata *extracts also appeared to have intracellular antiviral activities as well, which could indicate the presence of multiple antiviral compounds, or a single compound with multiple actions. In the seven most active extracts, the anti-HSV activity was considerably enhanced by light, especially UVA (long wavelength UV), although they all showed "dark" antiviral activity as well. Thus, all the extracts contained antiviral photosensitizers. In all tests, the root-bark and leaf extracts of* A. digitata *were the most potent.*

INTRODUCTION

Most of the inhabitants of Togo (W. Africa) use traditional medicinal plants in the treatment of infectious diseases. Recently, we examined extracts of 19 of these plants for antiviral and antimicrobial activities (Anani et a1., 1999). Seventeen of the 19 species demonstrated antimicrobial activity, and 10 of them had activity against one or more viruses. The variation in the spectrum of susceptible organisms suggested that different bioactive phytochemicals were involved.

In this report we describe further investigations into the mechanisms of the antiviral activities. In our previous studies on medicinal flora from various parts of the world, we have frequently encountered bioactive photosensitizers (Hudson & Towers, 1991; Hudson et al., 1995; Towers et al., 1997). This observation and other factors have necessitated the modification of standard antiviral test procedures, in order to optimize the detection of potentially useful antiviral phytochemicals, which could be photosensitizers or non-photosensitizers (Vlietinck & Vanden Berghe, 1991; Hudson et al., 1994). We have therefore used these modified test protocols to study the active Togolese extracts in more detail.

Keyss-ords: Adansonia digitata, antiviral, herpes simplex *virus,* medicinal plants, photosensitizers, Togo.

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MATERIALS AND METHODS

Extracts

Methanol extracts of the active plants were prepared as described previously (Anani et al., 1999). The crude extracts were resuspended in methanol to a concentration of 50 mg/ml and stored in the dark at 4°C. For antiviral tests, each extract was diluted in Dulbecco's modified Eagle medium (MEM) with 0.1 % serum, to give 1 mg/ml final concentration of extract, and filtered through a sterile 0.2 W pore cellulose acetate filter.

Cells and Viruses

Vero cells (African green monkey kidney cell line - American Type Culture Collection) were cultivated as

monolayers in a 5% carbon dioxide atmosphere at 37°C in Dulbecco Modified Eagle Medium (MEM) with 5% fetal bovine serum (FBS) (Gibco Life Sciences, Ontario) plus 25 Wg/ml gentanticin sulfate. Herpes simplex virus type- 1(HSV) and poliovirus type 1 were clinical isolates that had undergone several passages in Vero cells in our laboratory. Sindbis virus (SINV) (originally from American Type Culture Collection) was also propagated in Vero cells (Marles et al., 1992).

Antiviral Tests

The standard procedure is a modification of the techniques described in Marles et al. (1992) and Anani et al. (1999). Cells were cultured in 96-well tissue culture trays, 0.1 ml per well, until confluent monolayers were obtained. In another set of empty trays, the extracts were prepared as a duplicate series of two-fold dilutions, in MEM with 0.1 % FBS (Hudson et al., 1994) to give a range of final concentrations from 500 to 4 l.Lg per ml. These solutions were transferred, by means of multipipetting devices, to the corresponding wells of a tray of cell cultures from which the media had been aspirated. After one hour at 37°C, 100 p.l of virus in MEM (plus 0.1% FBS), containing 100 pfu (plaque-forming units) were added to each well and the infected cultures were then exposed to lamps (a combination of fluorescent and long-wave UV, so as to cover the range of 320-600 nm wavelengths) on a shaker platform inside an environmental chamber at 37°C for 30 min. Total incident radiation, as measured by a photometer, was 4 Kjoules per m^2.

Control cultures were subjected to identical light exposure, and included cells without virus, and untreated virus. When the latter showed maximum cpe (virus-induced cytopathic effects, i.e., the characteristic pathological changes induced in the infected cells), 4 days after infection in the case of HSV; 3 days for SEW; 2 days for poliovirus; all cultures were examined microscopically and assessed for cpe. In those cases where no cpe were evident, the virus was assumed to be completely inactivated or inhibited. Cases of partial inactivation, i.e., a 50% decrease or more in cpe compared to untreated virus were also recorded, since these represent inactivation or inhibition of a fraction of the 100 pfu present in the standard virus dose. The minimum antiviral concentration was that dilution of extract that gave rise to complete or partial inactivation of the virus.

Modified Antiviral Tests

Several modified protocols were used, for the specific purposes explained in the Results section.

Determination of Site of Action

Three different modified protocols were compared.

i) In the "pre-infection" protocol, the diluted extracts were incubated with the cultures, in the incubator, for 4 to 24 h before virus infection. After this incubation, the extracts were aspirated and replaced with 100 pfu of virus in 0.1 ml as usual, and the cultures exposed to lamps in the usual way (but for 0 min instead of 30).

ii) "Virucidal"-protocol: extract dilutions were prepared in the empty trays as usual. The virus, 100 pfu in 0.1 ml was added to each well (except cell controls), and the trays were immediately exposed to light (60 min). After this, the virus-extract mixtures were transferred to a tray of aspirated cultures, which were then returned to the incubator.

iii) "Post-infection" protocol: in this modified protocol, the virus was added to the cells first, followed by 60 min incubation to allow the virus to adsorb to and penetrate the cells. Then the diluted extracts were transferred to the infected cultures and immediately exposed to light (60 min).

Light vs. Dark Protocol

The test procedure was similar to the virucidal one (ii, above) except that all 96-well test trays were duplicated and, immediately following mixing of extract and virus, half the trays were wrapped in aluminum foil to exclude light. These trays were otherwise treated identically except when the foil was periodically removed for microscopic examination of the cultures. In addition, light exposure was increased to 60 min (= 4 Kjoules/m^2).

UVA vs. Visible Light

In this modification of the virucidal protocol (ii, above), one third of the trays were exposed to the UVA lamps only (60 min), one third were exposed to fluorescent lamps only (60 min), and the others were covered with foil as above, within the environmental chamber. The doses of radiation were determined, by means of a photometer, to be approximately the same for the different kinds of lamp (4 Kjoules/m').

RESULTS AND DISCUSSION

Table 1 summarizes the data for the 11 plant extracts with antiviral activity (as determined previously by Anani et al., 1998), expressed as minimum antiviral

concentrations. The A. digitata and P. hirsuta extracts were evidently the most potent, and in fact they were found to be active at much lower concentrations than those indicated in the original tests shown in Table 1 (see below). Since all extracts were active against herpes simplex virus (but not the other viruses), this virus was used in the subsequent tests described here.

Sites of Antiviral Action

There are three ways in which an antiviral activity could manifest in virus-cell culture systems. The active phytochemical could protect cells from virus infection, by means of an interferon-like effect (although interferon itself may not necessarily be involved-, Andrei et al., 1988). Alternatively the activity could be virucidal (direct inactivation of the virus, a common finding among antiviral phytochemicals, Hudson, 1995). Or the phytochemical could inhibit some stage of the virus replication cycle in the infected cells (Vlietinck & Vanden Berghe,_1991). To distinguish between these possibilities we devised three test protocols (described in the "Materials and Methods" section), respectively referred to as "pre-infection", "virucidal", and "post infection' protocols. These tests were done with HSV, the most susceptible of the three viruses.

Table 2 shows the results. The predominant activity was virucidal, and this activity was sufficient to explain the previous results obtained from the standard procedure. Most of the pre- and post-infection activities were relatively low, and these could be due to

Table 1. Summary of species with antiviral activity.

Plant tested	Minimum Antiviral Activity (Ag/mb Herpes simplex	Sindbis	Polio
Asteraceae			
Conyza aegyptiaca (L.) Aiton	**500**	**250**	**500**
Bombacaceae			
Adansonia digirara L. (root-bark)	**125**	**250**	**250**
(leaves)	**<62.5**	**250**	
Commelinaceae			
Palisora hirsuta (Thunb.) K. Schum.	<62.5	**500**	**250**
Davalliaceae			
Davallia chaerophyUoides (Poir.) Steud.	**500**	–	–
Malvacae			
Sida acuta Burm. f.	**250**	–	–
Moraceae			
Ficus ovata Vahl	**125**	–	**250**
Rubiaceae			
Mitracarpus villosus (Sw.) DC.	**125**	–	**500**
Rutaceae			
Zanthorvlunt zanthoxvloides (Lam.) Zepernick & Tiniler	**500**	–	–
Simarubaceae			
Harrisonia abyssinica Oliv.	**250**		
Sapindaceae			
Paullinia ***pinnata*** L.	**125**		

-. no detectable activity at 500 Wg/ml

Table 2. Sites of antiviral action.

Plant tested	Minimum Antiviral Activity (Wglml) Virucidal	Pre-infection	Post-infection
Con vza aegyptiaca	125	500	500
Adansonia digitata (root-bark)	<62.5	250	250
(leaves)	<62.5	125	125
Palisora hirsuta	<62.5	250	250
Davallia chaerophi·lloides	500	–	–
Sida acuta	250	Soo	250
Ficus orata	<62.5	500	250
Mirracarpus villosus	125	–	500
Harrisonia abvssinica	250	500	500
Paullinia pinnata	125	500	500

residual amounts of virucidal compound which remained cell-associated and could then subsequently inactivate progeny virus from the first round of replication and thereby prevent development of visible cpe. In some cases, however, especially the A. *digitata* extracts, the pre- and post-infection activities were substantial. These activities could represent real multiple antiviral activities, due to several different mechanisms of action, or to multiple antiviral compounds with different mechanisms.

Tests for Antiviral Photosensitizers

To determine the presence of a photosensitizer, the virucidal test protocol was modified to include a duplicate set of test trays, which were covered in aluminum foil during and after light exposure to serve as "dark-reactions".

Table 3 shows the results for the seven most active extracts. Two conclusions can be made. Firstly, in all cases the antiviral activity was much greater in the presence of light, which indicates the presence of antiviral photosensitizers. Our experience with this test protocol for other unrelated phytochemicals/extracts has led us to expect a D/L ratio of approximately 1.0 (± 0.5) for non-photosensitizers (Taylor et al., 1996). This is illustrated in Table 3 by the data for two known algal extracts (Kim et al., 1997), tested in the same experiments. One of them, A. *japonica,* does not contain a photoactivated antiviral activity, and its D/L ratio has consistently been approximately 1.0, while the other, N. *luetkeana,* shows antiviral activity only in the presence of light (D/L ratio >62.5 in Table 3).

Secondly, all of the extracts had "dark" activity, indicating antiviral activity in the absence of photoactivation. Technically it is impossible to exclude ambient light completely during procedures that involve handling viruses in biosafety cabinets. Nevertheless, with the aid of photometers, we determined that ambient light irradiation of the test reactions was <2% of the deliberate light-irradiation dose. In addition we have often encountered extracts which gave rise to D/L ratios of 50 or more (and no detectable dark activity, e.g., the algal extract shown in Table 3), as well as some that showed no detectable activity at concentrations up to 2,500 lLg/ml. Therefore, we believe that the D/L values shown in Table 3 are significant. Consequently these extracts represent light-enhanced activities rather than an absolute requirement for light. This could be due in some or all cases to a single phytochemical with distinct photoactive and non-photoactive antiviral

Table 3. Test for antiviral photosensitizers.

Plant tested	Minimum antiviral concentration (Wg/ml) + light (L)	dark (D)	ratio D/L
Adansonia digitata (root-bark)	6.25	50	8.0
(leaf)	3.1	50	16.0
Palisota hirsuta	8.0	24	3.0
Davallia ehaerophylloides	46	125	2.7
Ficus ovara	8.0	20	2.5
Mitracarpus tzllosus	12	39	3.2
Paullinia pinnato	8.0	24	3.0
Analipus japonicusl Ralfsiaceae	4.0	4.0	1.0
Nereocvsris luetkeana (Mertens) Postels and Ruprecht. Lessoniaceae	8.0	- (>500)	>62.5

t control tests with methanol extracts of algae from British Columbia (Kim et al.. 1997)

Table 4. Light-mediated antiviral activities.

Plant tested	Minimum antiviral concentration (pg/ml) + UVA (UV)	+ visible (L)	dark (D)	DfUV	D/L
Adansonia digitata (root-bark)	6	24	40	6.7	1.7
(leaf)	5	24	125	25.0	5.2
Palisota hirsuta	47	125	125	2.7	1.0
Davollia chaerophvlloides	62	110	94	1.5	0.85
Ficus ovata	31	39	62	2.0	1.6
Mitracarpus villosus	24	28	47	2.0	1.7
Harrisonia abessinica	187	250	250	1.3	1.3
Paullinia pinnata	16	24	31	1.9	1.3

activities, or to the presence of two or more different antiviral compounds. However, in either case, the compounds are not likely to be common to all the extracts because the virus susceptibilities were not the same for all extracts (Table 1). Thus, it is more likely that a number of different antiviral compounds are involved.

The other three extracts were not shown in Table 3 because their minimum antiviral activities were close to the upper concentration tested and it was not possible to make definitive light-dark comparisons.

Comparison of UVA and Visible Light

In order to determine whether the antiviral photosensitizers were activated by UVA (long-wavelength ultraviolet), as is known for many furyl compounds, thiophenes, polyynes, and alkaloids (Hudson & Towers, 1991) or by visible light (e.g., complex quinones, Towers et al., 1997), the antiviral tests were conducted in three groups. One group of culture trays was exposed only to UVA lamps, a second group to fluorescent lamps only (same incident radiation dosage), and the third group was kept dark. Table 4 shows the results for eight extracts.

Once again, the data indicated dark activity in all cases, as noted in Table 3; but only the A. *digitata* extracts showed substantial light enhancement, and this was much more evident for UVA than visible light. Since there is a slight overlap between the emission spectra of the UVA and fluorescent lamps, in the 400 nm region, then the antiviral compound may be activated primarily by UVA with some contributions from higher wavelengths. *Palisota hirsuta* also showed a preference for UVA, but the others, including H. *abyssinica,* did not show a preference (Table 4). In these instances the presence of a photosensitizer was questionable, although the combination of data from Tables 3 and 4 consistently showed slightly greater antiviral potency in the presence of some kind of light, particularly UVA, which was invariably more effective than visible light (Table 4).

These results, taken together, indicate that all of the substantial antiviral activities were due to photosensitzers, although there may have been in addition some antiviral compounds that did not require light.

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