The Inhibition of Infection by Cucumber Mosaic Virus and Influenza Virus by Extracts from *Phytolacca americana*

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SUMMARY

Extracts of the leaves of *Phytolacca americana* and partially purified preparations of such extracts caused marked inhibition of the infection of *Chenopodium quinoa* by cucumber mosaic virus (CMV) and of the infection of monkey kidney cells and embryonated hen eggs by the A2/Hong Kong/1/68 (H3N2) strain of influenza virus.

Both viruses became non-infective when mixed with preparations of the extracts but infectivity was regained when the viruses were separated from them by centrifuging.

INTRODUCTION

It has been known for many years that certain plants contain substances which, when mixed with plant viruses, can inhibit the mechanical transmission of the viruses to host plants. These substances can inhibit the infection of numerous plants by many different viruses and are thus non-specific in their action (Gendron & Kassanis, 1954). They cannot inhibit the infection of the plants which contain them, however, and they appear to be effective only when mixed with the infecting virus before the inoculation takes place. Their inhibitory effects can be greatly reduced by dilution and the infectivity of the virus is restored if it is separated from the inhibitor.

*Phytolacca americana* was the first plant species shown to contain an inhibitor (Duggar & Armstrong, 1925). Although substances in the sap of other species such as *Beta vulgaris*, *Spinacia oleracea*, *Dianthus caryophyllus* and *Chenopodium amaranticolor* will also inhibit the transmission of plant viruses, the inhibitor in *Phytolacca* sap is probably the most potent of those so far studied.

In view of the wide range of effects of the inhibitors in plants on plant virus infection, experiments were made with preparations of *Phytolacca* leaves to determine whether they also inhibited infection of animal tissue by an animal virus. These involved comparative tests with cucumber mosaic virus (CMV) and the A2/Hong Kong/1/68 (H3N2) strain of influenza virus.

METHODS

Phytolacca extracts. These were made from the leaves of *Phytolacca americana* plants grown in the glasshouse from seed kindly supplied by Dr R. J. Shepherd, University of California, Davis. Leaves were harvested and stored frozen until used.

Crude extracts were made by homogenizing leaves (1 g/2 ml) in 0.05 M-potassium
phosphate buffer (pH 7.5). The homogenate was filtered through muslin and centrifuged at 9000g for 30 min in a No. 30 rotor of a Beckman ultracentrifuge. The supernatant fluid was separated and stored at −12 °C. For use in tests, samples were thawed and clarified by centrifuging at 350g for 20 min and the deposit discarded. Crude tobacco leaf extracts were made in the same way.

The extracts were further purified by a method similar to that described by Wyatt & Shepherd (1969). A lyophilized preparation (60 to 70 mg) obtained by differential ethanol precipitation of an acidified crude extract was dissolved in 10 ml of 0.05 M-phosphate buffer, pH 6.0, and loaded on to a column (30 × 1.5 cm) of CM-50 Sephadex (Pharmacia, Uppsala, Sweden) previously equilibrated with the same buffer. The column was then washed with more buffer to remove unadsorbed or weakly adsorbed components. The effluent was continuously monitored at 280 nm through a 5 mm flow cell of an Isco UA2 u.v. analyser and 5 ml fractions collected with an Isco 326 fraction collector. Subsequently, the inhibitor was displaced with 0.05 M-phosphate buffer, pH 6.0, containing 0.5 M-NaCl and further 5 ml fractions collected. The u.v. extinction profile of the effluent fractions is shown in Fig. 1.

Tests of the various fractions of the effluent for their inhibition of CMV (see below) showed that this was confined almost exclusively to the five fractions corresponding to the second peak. These fractions were pooled, dialysed against water for 24 h and stored at −12 °C. They contained 1.1 mg/ml of the purified extract.

**Cucumber mosaic virus**

*Virus.* The W strain (Tomlinson *et al.* 1970) of CMV was sap-transmitted to and maintained in tobacco (*Nicotiana tabacum* c.v. White Burley). Purified virus preparations were made from infected tobacco leaves by the method of Tomlinson *et al.* (1973). They were
Virus inhibition with Phytolacca extracts

Virus inhibition with Phytolacca extracts

kept in 0.005 M-sodium tetraborate + 0.005 M-EDTA, pH 9.0 (borate/EDTA buffer) and stored in liquid nitrogen.

Assay of inhibitory effects of Phytolacca preparations. Samples of purified CMV (dilution end-point 10^-6) in borate/EDTA buffer, pH 9.0, were added either to equal vol. of the buffer or to equal vol. of serial dilutions of the Phytolacca preparations in the same buffer. The infectivity of each mixture was estimated by counting the number of local lesions produced in eight leaves of Chenopodium quinoa inoculated by rubbing the 'Celite' dusted leaf surfaces with a muslin pad soaked in the inoculum after which the leaves were air-dried (Yarwood, 1963).

Reversible effect of Phytolacca extract on infectivity. One ml purified CMV and 1 ml purified Phytolacca extract were mixed for 1 h at 20 °C, centrifuged at 12,000 g for 2 h and the pellet resuspended in borate/EDTA buffer, pH 9.0, equal to the original vol. of the mixture. The infectivity of the preparation was compared with that of other preparations mixed with equal vol. of buffer and with non-centrifuged preparations.

Influenza and other viruses

Virus strains. Influenza A/PR8 (H1N1), A/DSP (H1N1), A2/Hong Kong/1/68 (H3N2), adenovirus types 3 and 10, measles virus, ECHO type 11, and vaccinia virus were used.

The influenza strains were propagated by allantoic inoculation of 10- to 11-day-old embryonated eggs. Indicator suspensions of the strains of influenza virus for use in haemagglutination-inhibition tests were prepared by diluting infected allantoic fluid 1/5 in borate-citrate buffer, pH 8.4 (Clarke & Lubs, 1917), and heating for 30 min at 56 °C.

Virus haemagglutinin. Adenovirus types 3 and 10, measles virus, ECHO type 11 and vaccinia virus were grown in the appropriate tissue cultures and haemagglutinin was produced by standard methods. For measles virus and adenovirus, infected cells were frozen and thawed three times, treated for 30 s in a Headland H 62 ultrasonic bath and the suspension clarified by centrifuging at 1000 g for 5 min. ECHO 11 infected cultures were frozen and thawed once, treated in a sonifier, but not centrifuged and vaccinia virus was clarified by sedimentation only. When necessary, the haemagglutinin was concentrated by dialysis against polyethylene glycol (Kohn, 1959).

Phytolacca and tobacco-leaf extracts. All leaf extracts and partially purified preparations were sterilized by passage through a collodion membrane filter.

Haemagglutinin-inhibition tests. Two series of tests were made, one with four and one with eight minimum haemagglutinating doses of virus. Twofold serial dilutions of crude Phytolacca extract were made in 0.15 ml of veronal-buffered saline (pH 7.2) and either four or eight minimum haemagglutinating doses of virus in 0.25 ml were added to each dilution of the extract.

The samples containing influenza and adenovirus were kept at room temperature for 30 min and 1 h, respectively; those with measles virus and vaccinia virus were incubated for 1 h at 37 °C and ECHO 11 for 1 h at 4 °C.

After absorption, 0.25 ml of washed blood cells were added to each dilution using 1 % fowl cells for the influenza strains, 0.75 % rat cells previously adsorbed by an estimated vol. of normal rabbit serum for adenovirus 10, 0.75 % rhesus monkey cells for measles virus, 0.75 % human group O cells for ECHO 11 and 0.5 % fowl, 'vaccinia sensitive' cells for vaccinia virus. Tests were read after 1 h at room temperature for the influenza strains, 1 h at 37 °C for adenovirus types 3 and 10, measles virus and vaccinia virus, and 1 h at 4 °C for ECHO 11.
**Haemadsorption-plaque test.** The method used was adapted from the plaque assay test for influenza A2 viruses described by Bentley & Wickham (1971).

Five cm plastic Petri dishes were seeded with 2 to 3 x 10⁶ primary monkey kidney (M/K) cells in 5 ml Eagle's MEM growth medium, containing 0.02 M-glutamine, 10% foetal calf serum, 2% of a 4.4% solution of sodium bicarbonate and per ml: penicillin 100 units and streptomycin 100 µg, amphotericin B 5 µg, 'fungizone' 5 µg, and neomycin 35 µg. Cultures were incubated in sealed plastic boxes at 37 °C in an atmosphere containing 5% CO₂ and the medium changed after 2 and 7 days. A confluent cell monolayer formed in 5 to 6 days. Monolayers were kept in a maintenance medium which was the same as the growth medium described above but which contained only 2% foetal calf serum.

Dilutions of virus were mixed either in the maintenance medium (pH 7.4) or in dilutions of the *Phytolacca* or tobacco-leaf preparations in the maintenance medium (pH 7.4). Before infection with virus, the growth medium was removed, the monolayers were washed with phosphate-buffered saline (pH 7.3) and 0.3 ml of virus mixture was introduced to give a conveniently countable number (about 100) of plaques per plate. After adsorption for 1 h at room temperature (22 °C) during which time the cultures were rocked frequently, 5 ml of overlay was added.

The overlay consisted of equal parts of 1.2% (w/v) agarose (L'Industrie Biologique Française) and double strength MEM (Flow Laboratories Ltd) containing 0.04 M-glutamine, phenol red as in Eagle's MEM (Eagle, 1959) and twice normal strength antibiotics, penicillin, streptomycin, amphotericin B and neomycin buffered with Hepes.

Cultures were incubated at 37 °C for 3 days in a humid atmosphere. The overlay was then removed and the cell sheet washed with phosphate-buffered saline. Two ml of 0.1% guinea pig red blood cells were added to each dish and left 30 min at 4 °C. Cultures were then washed with cold phosphate-buffered saline and examined under a low-power inverted binocular microscope to count areas of haemadsorption.

Virus was titrated to determine the dilution required to produce about 100 plaques per plate and stored in phials at -70 °C.

**Inhibition of influenza virus growth in embryonated eggs.** Dilutions of 10⁻⁵ and 10⁻⁶ of virus in allantoic fluid were made in partially purified samples of the *Phytolacca* extract containing 1 mg/ml.

After standing 1 h at room temperature 0.3 ml vol. were inoculated allantoically in 10- to 11-day-old embryonated eggs, which were then incubated for 48 h at 37 °C. Harvested allantoic fluid was titrated for haemagglutinin and infectivity.

**Electron microscopy.** Equal vol. of undiluted virus and partially purified *Phytolacca* extract were mixed and left for 3 h (1 h at room temperature and 2 h at 4 °C); 4 drops of the mixture and 1 drop of 2% potassium phosphotungstate were mixed and mounted on electron microscope grids.

**Reversible effect of Phytolacca extract on infectivity.** One ml undiluted virus with 1 ml partially purified *Phytolacca* extract were left 1 h at 20 °C then centrifuged at 20000 g for 1 h. The pellet was resuspended in 0.1% bovine serum albumen in Earle's medium containing 2% Hepes and centrifuged at 20000 g for 30 min. The resuspended pellet was treated in a sonifier for 30 s and assayed for plaque formation by the haemadsorption technique.

**RESULTS**

In the first experiment crude *Phytolacca* extract was mixed with an equal vol. of purified CMV or influenza virus and the infectivity of the virus/extract mixtures compared with that of the viruses mixed with buffer only. CMV infectivity was determined by inoculating the
**Virus inhibition with Phytolacca extracts**

Table 1. *Effect of crude Phytolacca extract on the infectivity of cucumber mosaic virus*

<table>
<thead>
<tr>
<th>Extract tested*</th>
<th>Mean no. lesions†</th>
<th>Lesions as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted Phytolacca</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Phytolacca 1/10</td>
<td>58</td>
<td>43</td>
</tr>
<tr>
<td>Phytolacca 1/100</td>
<td>149</td>
<td>111</td>
</tr>
<tr>
<td>Undiluted tobacco</td>
<td>138</td>
<td>103</td>
</tr>
<tr>
<td>Buffer (control)</td>
<td>134</td>
<td>100</td>
</tr>
</tbody>
</table>

* Extract or buffer (0.05 M-phosphate, pH 7.5) mixed with virus in equal vol.
† Mean no. lesions in eight *Chenopodium quinoa* leaves.

Table 2. *Effect of crude Phytolacca extract on the infectivity of influenza virus*

<table>
<thead>
<tr>
<th>Dilution of Phytolacca extract tested*</th>
<th>No. plaques per plate†</th>
<th>Plaques as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1/10</td>
<td>36</td>
<td>12</td>
</tr>
<tr>
<td>1/20</td>
<td>93</td>
<td>47</td>
</tr>
<tr>
<td>1/40</td>
<td>146</td>
<td>74</td>
</tr>
<tr>
<td>Virus only (control)</td>
<td>197</td>
<td>100</td>
</tr>
</tbody>
</table>

* Extract mixed with an equal vol. of virus.
† Infectivity estimated by the haemadsorption plaque count technique.

leaves of *Chenopodium quinoa* and counting the local lesions subsequently formed. Influenza virus infectivity was tested with M/K cells and infection estimated by the haemadsorption plaque count technique. The results (Tables 1 and 2) showed that crude *Phytolacca* extract was strongly inhibitory to both viruses. In cytotoxicity tests, when the extract (diluted 1/10 and 1/20) was incorporated in the overlay, the appearances of treated and untreated cells were indistinguishable after 3 days.

Inhibition of infection of M/K cells by influenza virus occurred only when the *Phytolacca* extract was mixed with the virus before the cells were inoculated. It did not occur if the crude extract was applied to the cells for 1 to 3 h and then washed off before application of the virus. Crude tobacco leaf extracts prepared in the same way as the crude *Phytolacca* extract had no effect on the infectivity of the influenza virus. As the haemadsorption technique was used to assay the presence of the virus in M/K cells, tests were made which showed that the purified *Phytolacca* preparation contained no haemagglutinin. Furthermore, the preparation did not contain a component which inhibited the agglutination of red blood cells by influenza, measles, ECHO, adenovirus or vaccinia virus.

The effects of partially purified *Phytolacca* extract were then tested (Table 3) and it also was shown to be strongly inhibitory to the infectivity both of CMV to *Chenopodium quinoa* and of influenza virus to M/K cells. In another experiment cell monolayers, 1 h after inoculation, received overlay with and without partially purified *Phytolacca* extract diluted 1/10. After 3 days the mean numbers of plaques were 42 and 41, respectively, indicating that virus production was unaffected and that the extract had no detectable cytotoxic effect on the cells.

Tests were also made in embryonated eggs inoculated with influenza virus mixed either with buffer or with the partially purified *Phytolacca* extract. After inoculation, eggs were incubated for 48 h at 37 °C and the allantoic fluid subsequently harvested and tested for haemagglutination. The titres obtained (Table 4) showed that the yield of virus in eggs was considerably reduced if the inoculated virus was mixed with the extract prior to inoculation. The results were similar to those obtained using M/K cells.
Table 3. Effect of partially purified *Phytolacca* extract on the infectivity of cucumber mosaic virus and influenza virus

<table>
<thead>
<tr>
<th>Dilution of <em>Phytolacca</em> extract*</th>
<th>Cucumber mosaic virus</th>
<th>Influenza virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. lesions ‡</td>
<td>Lesions as % of control</td>
</tr>
<tr>
<td>Undiluted †</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1/10</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>1/100</td>
<td>51</td>
<td>128</td>
</tr>
<tr>
<td>Buffer control</td>
<td>40</td>
<td>100</td>
</tr>
</tbody>
</table>

* Extract (1 mg/ml) and virus (dilution end-point $10^{-x}$) mixed in equal vol.
† *Phytolacca* extract concentration approx. 1.0 mg/ml.
‡ Mean no. lesions in eight *Chenopodium quinoa* leaves.
§ Mean no. plaques of plates of monkey kidney cells estimated by the haemadsorption plaque count technique.

Table 4. Haemagglutination titre of allantoic fluid of embryonated hen eggs inoculated with influenza virus mixed with partially purified *Phytolacca* extract

<table>
<thead>
<tr>
<th>Reciprocal haemagglutination titre of allantoic fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test no.</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

* Virus (A2/Hong Kong/1/68) mixed with an equal vol. of partially purified extract (1 mg/ml) and stored for 1 h at 20 °C before allantoic inoculation.

Table 5. Infectivity of viruses after sedimentation from buffer or partially purified *Phytolacca* extract

<table>
<thead>
<tr>
<th>Dilution of preparation</th>
<th>Buffer</th>
<th>Buffer and centrifuge</th>
<th>Extract</th>
<th>Extract and centrifuge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cucumber mosaic virus*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>&gt; 200</td>
<td>&gt; 200†</td>
<td>16</td>
<td>154</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>&gt; 200</td>
<td>153</td>
<td>19</td>
<td>176</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>159</td>
<td>18</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza virus†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>&gt; 500</td>
<td>&gt; 500§</td>
<td>5</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td></td>
<td>&gt; 500</td>
<td></td>
<td>&gt; 500</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td></td>
<td>68</td>
<td></td>
<td>260</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td></td>
<td>9</td>
<td></td>
<td>38</td>
</tr>
</tbody>
</table>

* Mean no. local lesions in eight *Chenopodium quinoa* leaves.
† Mean no. plaques per plate.
‡ Purified CMV + equal vol. borate/EDTA buffer (pH 9.0) centrifuged for 2 h at 112000 g.
§ Purified influenza virus + equal vol. phosphate-buffered saline, centrifuged 1 h at 20000 g.
—, Not tested.
Virus inhibition with Phytolacca extracts

To determine if the Phytolacca extract permanently inactivated CMV or influenza virus, the viruses were first mixed with partially purified extract (or buffer) as described, separated from the mixtures by centrifuging and their infectivity determined after resuspension in buffer containing no inhibitor. The results obtained (Table 5) showed that all the infectivity of each virus was regained and that treatment with the extract had no permanent effect.

No aggregation or agglutination of influenza or CMV particles was detected by electron microscopy of mixtures of virus and inhibitor.

DISCUSSION

The present studies provide evidence that the infection of animal cells by influenza virus can be inhibited by an extract from Phytolacca which also inhibits the infection of plant cells by CMV.

Attempts were made to purify the Phytolacca extract to where it contained only one compound which could thus have been conclusively implicated in the inhibition of both viruses. No proof was obtained that the extract was entirely homogeneous, however, and the possibility remains that the purified extract contained more than one substance. Wyatt & Shepherd (1969) demonstrated that purified Phytolacca extracts, as made in the present study, contained three components all inhibitory to southern bean mosaic virus.

The possibility was considered that the inhibition of infection by influenza virus was an artefact caused by the presence of a haemagglutinin in the Phytolacca extract (Reisfeld et al. 1967) or by the inhibition of virus haemagglutination of red blood cells. Tests showed, however, that neither was so and it was further shown that the inhibitory effect was removed when CMV and influenza virus were separated from the inhibitor. In this respect, therefore, the action of the extract was the same on both viruses.

The evidence obtained of the extension of the effects of the Phytolacca extract to animal cell/virus interactions renews the question of the mode of action of the inhibitor. Conceivably, this might be an effect on the host, the virus or the reaction of the virus with the host.

Direct effects on host cells seem unlikely because inhibition of infection did not occur when the extract was applied to the cells separately. Evidence of cytotoxicity, such as that reported by Fantes & O’Neill (1964), when avian fibroblasts were treated with Dianthus caryophyllus extracts, was not obtained. It was not due to a mitogen; our colleague Dr R. A. Thompson kindly examined the purified extract for mitogenic activity on human lymphocytes, and found none. It is also apparent, from the reversibility of the reaction, that the inhibitor had no direct or permanent effect on the virus particles as infectivity was restored when the inhibitor was removed.

It seems most likely, therefore, that in the presence of the inhibitor some essential process of infection is prevented. One essential process is the attachment of the virus to the host cell. Studies by some workers (Burger & Stahmann, 1951) suggested that this may be prevented by an alteration of the charge on the virus particle caused by polybasic substances in plant extract. Such effects could be produced by the lysine amino groups of the basic proteins which have been identified with the inhibitors from Dianthus caryophyllus (Ragetli & Weintraub, 1962), Phytolacca americana (Wyatt & Shepherd, 1969) and various Chenopodiaceae (Smookler, 1971). It has also been shown that synthetically prepared polylysine reversibly inhibited infection by tobacco mosaic virus (Stahmann et al. 1951), an effect attributed to the formation of ionic bonds between NH\_3 groups of polylysine and the acidic groups of the virus.

Although inhibitors may prevent the attachment of the virus particle to infectible cell
sites, another mechanism might be in causing the failure of a protein uncoating mechanism, thus preventing virus RNA replication. In recent tests, however, with TMV-RNA (J. A. Tomlinson, unpublished observations) mixed with the *Phytolacca* inhibitor, all infectivity was inhibited, suggesting that the failure of an uncoating mechanism cannot provide a full explanation.

Whatever the cause of the inhibition of infection, it is apparent that it applies to a very wide range of host/virus interactions and further studies on its mechanism are now in progress.

We thank Dr R. A. Thompson for suggesting, and testing for, mitogenic activity in the partially purified *Phytolacca* extract.

**REFERENCES**


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