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# In vitro antiviral activity of a saponin from *Anagallis arvensis*, Primulaceae, against herpes simplex virus and poliovirus

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## Summary

The antiviral activity of a triterpene saponin isolated from *Anagallis arvensis*, Primulaceae, was studied in vitro against several viruses including herpes simplex type 1, adenovirus type 6, vaccinia, vesicular stomatitis and poliovirus. The drug was found to inhibit the replication of herpes simplex virus type 1 and poliovirus type 2 as shown by inhibition of cytopathic effect and reduction of virus production. The action was not due to a virucidal effect but might involve inhibition of virus-host cell attachment. Single cycle experiments indicated that saponin interfered with both early and late events of herpes virus replication.

Triterpene saponin; *Anagallis arvensis*; Primulaceae; Herpes simplex virus; Poliovirus

## Introduction

In the course of a study on the antiviral properties of plants from Britanny, we reported the in vitro antiviral activity against herpes simplex virus type 1 and poliovirus type 2 of some higher plants [4,21] and in particular of *Anagallis arvensis*, Primulaceae [5] and *Matricaria inodora*, Composaceae [22]. The antiviral compounds isolated from *Anagallis arvensis* were found to be saponins of which the

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isolation and chemical structure have been described [6]. In this paper, we report the in vitro efficacy of an isolated triterpene saponin against poliovirus and herpes simplex virus, in comparison with 5-iodo-2′-deoxyuridine, and suggest a possible mechanism of action of the compound.

## Materials and Methods

### *Cells*

The Vero monkey kidney cell line utilized in this study was obtained from Flow Laboratories (Scotland) and was routinely grown in Eagle's minimum essential medium (MEM) supplemented with 10% newborn calf serum (Flow Laboratories), 400 U of penicillin and 80 μg of gentamycin per ml.

### *Viruses*

The deoxyribonucleic acid (DNA) viruses used were: type 1 herpes simplex virus (HSV-1), vaccinia virus (VV) and type 6 adenovirus (AV). The ribonucleic acid (RNA) viruses used were vesicular stomatitis virus (VSV) and type 2 poliovirus (PV). Herpes simplex virus and adenovirus were provided by the Virology Department, Pontchaillou Hospital, Rennes. Vaccinia and poliovirus were vaccinal strains propagated in our laboratory by serial passages on Vero cells. Vesicular stomatitis virus was supplied by the Virology Department where it is currently used for interferon titrations.

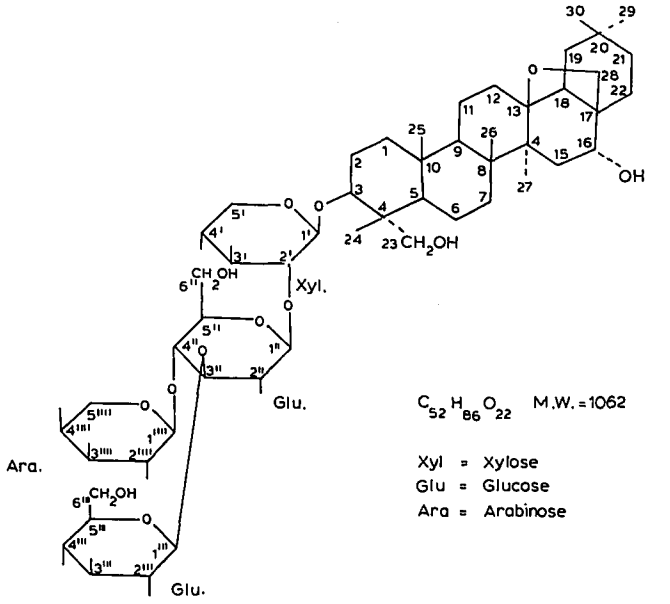
Virus stocks were prepared by inoculating Vero monolayers at low multiplicity and incubating at 37°C. Two days after infection, the cultures were frozen and thawed and stocks were maintained at −70°C. Virus titrations were performed by the Reed and Muench dilution method [18], using six wells of a 96-cell Nunclon microplate per dilution. The virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infectious doses per milliliter ($TCID_{50}$).

### *Antiviral compound*

The antiviral compound was a natural saponin isolated from *Anagallis arvensis*, Primulaceae. The structure of the compound was: 3-O-glucose-(1 → 3 or 4)-[arabinose (1 → 4 or 3)]-glucose (1 → 2)-xyloside of 23-hydroxyprotoprimulagenin A, as shown in Fig. 1. Saponin was dissolved in sterile, double-distilled water and sterilized by filtration with 0.2 μm Millex filter in order to obtain a concentration of 10 mg/ml. Then, suitable dilutions were made in the cell culture medium.

### *Drug cytotoxicity*

The effect of saponin on Vero cells was assessed in two ways: (i) cytopathic effect in drug-exposed, uninfected monolayers; (ii) inhibition of cell growth. Sus-




3-O-glucose(1 → 3 or 4)-[arabinose(1 → 4 or 3)]-glucose(1 → 2)-xyloside of 23-hydroxyprotoprimulagenin A.

Fig. 1. Structure of the antiviral saponin from *Anagallis arvensis*.

pension of $2 \times 10^5$ Vero cells in 5 ml portions of growth medium containing appropriate drug dilutions or no drug were placed in 25 $cm^2$ culture flasks. After incubation for 24 h or 96 h, supernatant fluids were discarded, adherent monolayers cells were washed with phosphate buffered saline, removed by trypsination and then harvested and counted using an hemocytometer. The protein content in various preparations was estimated by Lowry's method [12] and DNA was extracted and assayed by the method of Munro [14].

*Determination of antiviral activity*

For determination of antiviral activity two techniques were employed. Inhibition of cytopathic effect with estimation of $MIC_{50}$ [8,10] and virus rating [9,13,19]. To evaluate the cytopathic effect, confluent monolayers were prepared by seeding $4 \times 10^4$ cells into each well of microtiter tissue culture plates. The cultures were incubated at 37°C in a humidified $CO_2$ (5%) atmosphere, and the monolayers were confluent within 24 h. Monolayers were infected with viruses at low multiplicities of infection: $10^{-3}$, $10^{-2}$, $10^{-1}$ $TCID_{50}$/cell, in presence of a range of concentrations of saponin: 0.1, 0.25, 0.5, 1.0, 2.5 μg/ml (six wells/concentration/inoculum). Each panel included toxicity controls, virus controls and cell controls. Each experiment was made in quadruplate. After 72 h for poliovirus and VSV, 96 h for HSV-1 and 110 h for adeno and vaccinia, the cytopathic effect was examined under an in-

verted microscope. Viral CPE was graded on a progressive scale of 0 (normal cells) to 4 (complete destruction of the cell layer).

The concentration at which the viral cytopathic effect was inhibited by 50% in comparison to the control was designated as 50% minimal inhibitory concentration ($MIC_{50}$).

Sidwell [19] described the modified virus rating (VR) method as follows: the sum of the values assigned to the CPE of each cup of the treated, infected cells (T) at each drug level was substracted from the sum value of the CPE in an equal number of virus control (C) cups. The C – T total of all drug levels was then divided by 10 times the number of test cups used per drug level.

*Assay of virus titer.* Vero cells grown in test tubes in MEM containing 0.1 – 4 μg/ml of saponin were inoculated with viruses at a MOI of 0.001. After 96 h incubation at 37°C, the cultures were submitted to three cycles of freezing and thawing, then clarified by low-speed centrifugation (6000 rpm). Thereafter, the 50% infectivity end-point of the supernates was determined in microtissue culture plates. Cultures treated with 5-iodo-2′-deoxyuridine and infected with HSV-1 were carried out and assayed under similar experimental conditions.

*Experimental approach to the possible mechanisms of the antiviral action*

*Virus inactivation.* To test possible virucidal activity, equal volumes (1 ml) of virus stocks ($10^6$ $TCID_{50}$/ml) and MEM containing 0 – 1000 μg of saponin were mixed and incubated for 2 h at 37°C. Thereafter, the virus suspensions were diluted 10-fold serially and assayed by the method of Reed and Muench [18].

*Determination of prophylactic activity.* Prophylactic activity was determined by incubating the saponin with the cell monolayers for 24 h at 37°C. The drug solution was discarded and the cells were washed three times, then covered again with maintenance medium and inoculated at a MOI of 0.001. The remainder of the procedure was identical to that described above for the assay of virus titer.

*Yield reduction.* Confluent monolayer cultures of Vero cells were infected at a multiplicity of infection of about 1. After 60 min at 37°C, the excess virus was removed and the monolayers washed twice with MEM. Then MEM containing 0 – 10 μg/ml of saponin was added. Single cycle conditions were achieved by incubating the cultures for 18 h (HSV-1) or 8 h (poliovirus). The cultures were frozen at −70°C and extracts were prepared by freezing and thawing three times and sonicating the resulting suspension for 30 s. The cell debris was removed by low-speed centrifugation and the supernatants were titrated as usually.

*Effect of time of addition.* Monolayers of Vero cells in test tubes were inoculated with the viruses at a MOI of 1. At various times (0, 0.5, 1, 2, 3, 4, 5, 6 and 8 h post-infection) culture medium containing saponin at a concentration of 10 μg/ml was added and incubated with cells until the end of the experiment. After 18 h

(HSV-1) or 8 h (poliovirus), i.e. one step growth cycle, the cultures were frozen and virus yields were determined as above.

*Electron microscopy.* Vero cells grown in 25 $cm^2$ flasks were infected with HSV-1 at a MOI of 1. Cultures were incubated at 37°C for 1.5 h, the inoculum was discarded and the monolayers were washed twice with 5 ml of MEM. Fresh MEM supplemented with 10% calf serum and 7 μg/ml of saponin was added and incubation was continued at 37°C. Controls without drug or virus were included. Even after 18 h incubation, the virus controls did not show cytopathic effect. The cells were fixed in 2.5% glutaraldehyde, fixed in 10% osmium tetroxyde, dehydrated in a graded series of ethanols and embedded in an Epon-Araldite mixture. Thin sections of the embedded cells were stained with uranyl acetate and viewed with a JEOL 7-A electron microscope.

## Results

### *Drug cytotoxocity*

After 96 h of treatment, concentrations of saponin below 10 μg/ml did not inhibit either cell growth or protein or DNA synthesis, as shown in Fig. 2. Saponin concentrations higher than 10 μg/ml induced granularity, retraction and then lysis of Vero cells. Therefore, the maximum non-toxic dose was 10 μg/ml and a maxi-

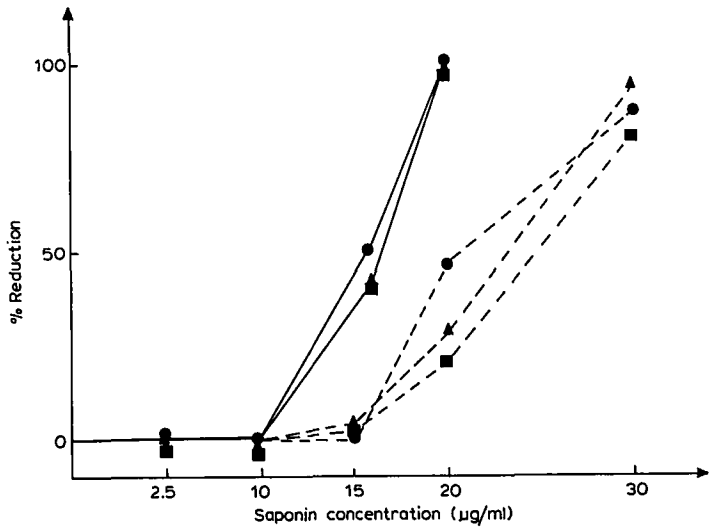



Fig. 2. Effect of saponin on the growth of uninfected Vero cells. Percentages of reduction in cell number (●), protein (■) and DNA synthesis (▲) are plotted in function of saponin concentration. Points represent the mean of three experiments. Solid lines = incubation for 96 h, broken lines = incubation for 24 h.

mum concentration of 4 μg/ml was used for the determination of antiviral activity.

When the time of incubation was reduced to 24 h, saponin concentration up to 15 μg/ml did not cause any decrease in cell number, DNA and protein synthesis. Consequently, tests with short incubation time (yield reduction effect of addition time, electron microscope) may allow higher concentrations of the drug (10 μg/ml).

*Antiviral spectrum*

The virus battery used in our study may be considered as representative of both the DNA and RNA virus groups.

Herpes: double-strand DNA virus, intranuclear maturation, enveloped.

Adeno: double-strand DNA virus, intranuclear maturation, non-enveloped.

Vaccinia: double-strand DNA virus, cytoplasmic maturation, enveloped.

Vesicular stomatitis: minus-strand RNA virus, cytoplasmic maturation, enveloped.

Polio: plus-strand RNA virus, cytoplasmic maturation, non-enveloped.

All these viruses could be propagated in the same cell line, the Vero cells which minimized toxicity tests and allowed an objective comparison of antiviral activity [23].

Under the same experimental conditions (saponin added to the maintenance medium at concentrations of 0.1 – 2.5 μg/ml, 24 h before and then throughout infection and incubation), the drug reduced the cytopathogenic effect caused by herpes simplex and poliovirus. But no activity was found against adeno, vaccinia and vesicular stomatitis virus.

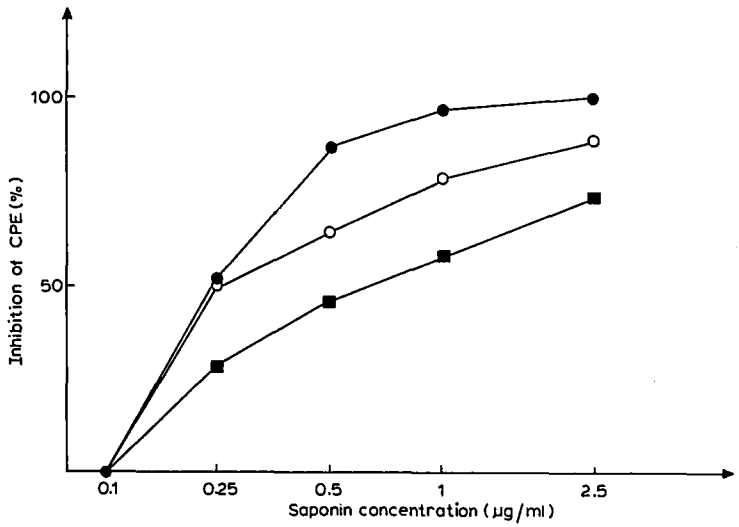



Fig. 3. Inhibition by saponin of cytopathic effect (CPE) of HSV-1 on Vero cells at three multiplicities of infection: ● = 0.001 $TCID_{50}$/cell; ○ = 0.01 $TCID_{50}$/cell; ■ = 0.1 $TCID_{50}$/cell.

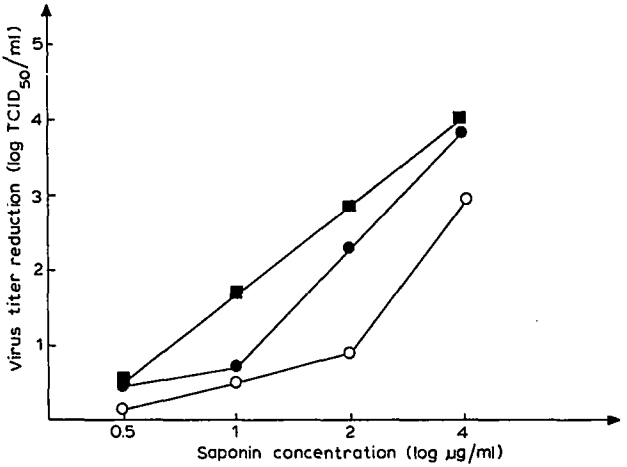



Fig. 4. Effect of varying saponin concentrations on the replication of HSV-1 and poliovirus in Vero cells. Cells cultured with saponin were infected at a MOI of 0.01. Three days later, the total virus content of these cultures was compared with controls which had been infected but not treated with saponin; ■ = Poliovirus-infected, treated with saponin; ● = HSV-1-infected, treated with saponin; ○ = HSV-1-infected, treated with IUdR.

*Activity of saponin extracted from Anagallis arvensis against HSV-1 and poliovirus*

Inhibition of the cytopathic effect of HSV-1 at three multiplicities of infection ($10^{-3}$, $10^{-2}$, $10^{-1}$ $TCID_{50}$/cell) in presence of various saponin concentrations is shown in Fig. 3. $MIC_{50}$ values were determined graphically. The concentrations required to inhibit HSV-1 CPE by 50% were as low as 0.24, 0.25 and 0.6 µg/ml depending on the MOI. At a MOI of $10^{-3}$ and $10^{-2}$ $TCID_{50}$/cell, the virus-rating was higher than 1 (1.3 and 1.2, respectively), which would indicate a definite antiviral activity [13,19]. But the antiviral indexes calculated by dividing the 50% maximum non-toxic dose (5 µg/ml) by the $MIC_{50}$ were low: 20 at a MOI of $10^{-3}$ and $10^{-2}$, and 8 at a MOI of $10^{-1}$.

Poliovirus was found slightly less susceptible. At a MOI of $10^{-3}$ $TCID_{50}$/cell, the $MIC_{50}$ was 0.8 µg/ml, indicating that the antiviral index was only about 6.

The activity of the saponin was also evaluated by determining inhibition of virus production in Vero cells in the presence and absence of drug. Viral titer reductions were plotted against the logarithm of the concentration to give dose-response lines, as shown in Fig. 4. The anti-HSV-1 activity was compared to that of an anti-herpes nucleoside agent, IUdR.

Treatment of infected Vero cells with saponin at concentrations of 0.1 – 4 µg/ml produced a dose-dependent decrease in virus titer. A concentration of 0.1 µg/ml was ineffective but from a concentration of 1 µg/ml, the reduction of infectivity was evident. At 2 µg/ml (1.88) µM) saponin produced a reduction in virus titer at 3 log (poliovirus) and more than 2 log (HSV-1) whereas the virus yield reductions at 4 µg/ml (3.7 µM) were 4 log for the both viruses. Treatment of HSV-1 infected

Vero cells with IUdR produced a less steep decrease in virus titer. At a concentration of 4 μg/ml, i.e. 11.8 μM, IUdR suppressed production of infectious virus by only 3 log. To obtain a viral titer reduction of two log, 1.47 μM of saponin and 8.5 μM of IUdR were required, i.e. concentrations respectively 5 times and 7 times lower than the maximum non-toxic dose. Thus, under the experimental conditions used, saponin was more active against HSV-1 than was IUdR, although the therapeutic index of the nucleoside was higher.

*Virus inactivation*

Exposure of poliovirus to saponin at concentrations of 0 – 1000 μg/ml resulted in no loss of infectivity. Virus suspension incubated with or without saponin (1000 μg) had the same titer ($10^{7.1}TCID_{50}$/ml).

At concentrations higher than 100 μg/ml, the drug inactivated herpes simplex virus and at 50 μg/ml the infectivity was reduced by 1 log; at lower concentrations the compound did not exert a virucidal effect on HSV-1. Thus, the antiviral activity could not be attributed to a direct virus inactivation since inhibitory effects on virus replication in culture were measured at a concentration of 4 μg/ml.

*Induction of an antiviral state by cell pretreatment*

When Vero cell monolayers were pretreated with saponin for 24 h, the virus production was strongly decreased, as shown in Table 1. For example, if the Vero cells were preincubated with 5 μg of saponin per ml, the inhibition reached 99.7% for both HSV-1 and poliovirus. A possible mechanism that could account for this result would be an alteration of the cell membrane which prevents virus adsorp-

TABLE 1

Inhibition of growth of herpes simplex virus type 1 and poliovirus by various concentrations of saponin from *Anagallis arvensis*

| Saponin concentration (μg/ml) | In Pretreated cultures[a] | | In Single cycle experiments[b] | |
|---|---|---|---|---|
| | HSV-1 | Poliovirus | HSV-1 | Poliovirus |
| 2.5 | 94.56 | 98.14 | 77.67 | 89.16 |
| 5 | 99.71 | 99.73 | 90.03 | 91.28 |
| 7.5 | – | 99.99 | 96.99 | 98 |
| 10 | 99.99 | 100 | 99.99 | 99.49 |

Inhibition is expressed as percentage of virus titer in infected, untreated controls.

[a] Vero cells were pretreated with various concentrations of saponin for 24 h. Washed cells were then infected at a MOI of $10^{-3}$ and harvested after incubation for three days at 37°C.

[b] Monolayers were inoculated with the viruses at a MOI of 1, and, after a 1 h absorption period, overlaid with maintenance medium containing varying concentrations of drug or no drug (controls). Cells were harvested after 18 h incubation at 37°C for HSV-1 and after 8 h incubation at 37°C for poliovirus.

Each experiment was carried out in triplicate and repeated three times.

tion on cell surface and subsequently inhibits virus penetration into the cell. Another possibility should be a competition with infectious virus for the virus-specific receptor sites [17].

*Yield reduction*

Under single cycle conditions, saponin concentrations higher than 5 μg/ml suppressed the viral replication by more than 90% (Table 1). For example, when infected cells were incubated in the presence of 7.5 μg of saponin per ml, the yield of HSV-1 was reduced by approximately 97% and poliovirus by 98%. Since the saponin was added to the cultures after infection, these results cannot be accounted for by a failure of the viruses to adsorb to the cell.

*Effect of time of saponin addition*

The effect of time of addition of saponin on the reduction of virus titer is shown in Fig. 5.

The drug was equally effective when added immediately before or after 1-h poliovirus adsorption. Saponin was somewhat less effective when it was added after 2 h or 3 h, but when added after 5 h it was remarkably less effective. Thus, at least one site of action of the saponin might be associated with a relatively early event in the virus replicative cycle.

Whereas untreated HSV-1 infected Vero cells yielded $10^5$ $TCID_{50}$ of virus within 18 h, cells treated with 10 μg/ml of saponin added at the time of infection or 0.5

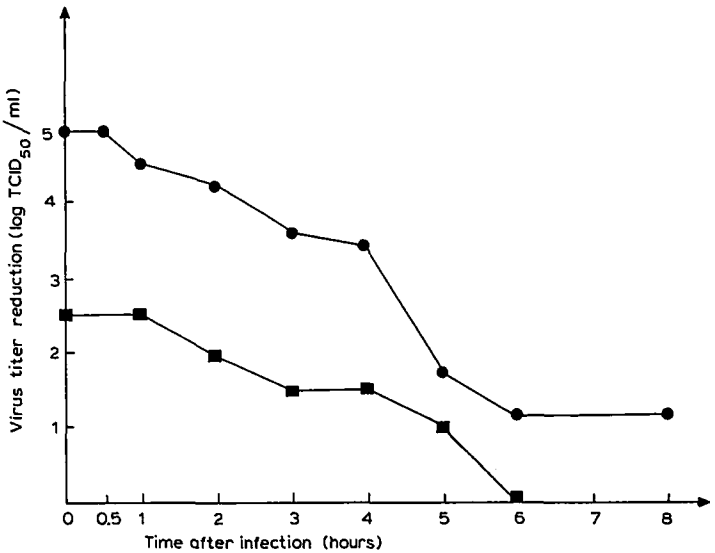



Fig. 5. Effect of adding saponin (10 μg/ml) at various times post-infection to infected Vero cells. ● = HSV-1 ■ = Poliovirus.

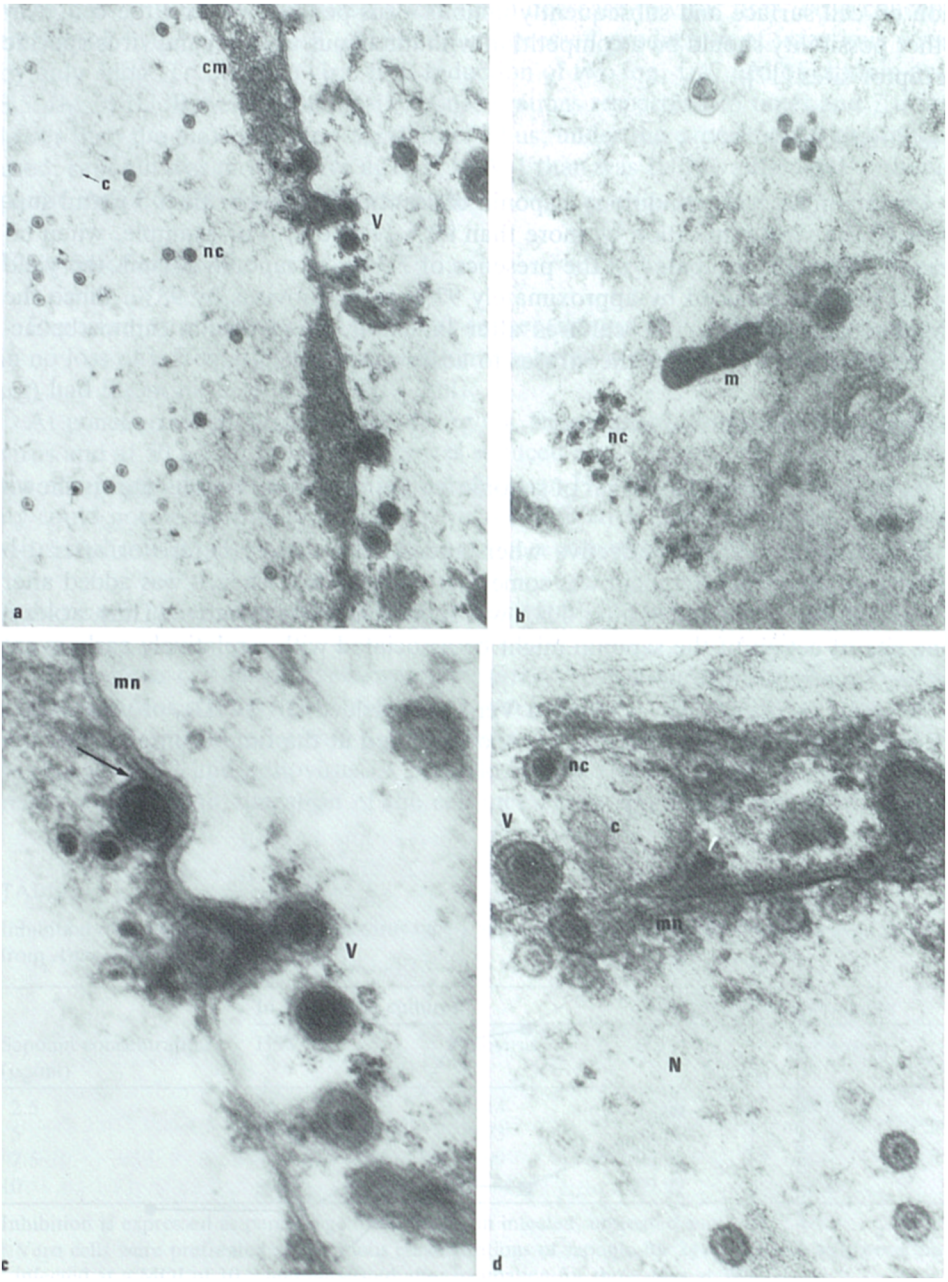



Fig. 6. Electron micrographs of infected cells treated with saponin (7 μg/ml) (b and d) or not treated (a and c).

h later produced only 10 $TCID_{50}$. When the saponin was added at 2 h after HSV-1 infection, the synthesis of infectious virus was reduced by more than 4 log and when added at 4 h post-infection saponin inhibited virus production by more than 3 log. When the drug was added later, its effect decreased. However, inhibition still reached 1 log if saponin was added as late as 8 h post-infection. These results suggest that saponin must interfere with an earlier event of the HSV-1 replication but also inhibits a later stage. To further assess this matter, electron micrographs of Vero cells infected with HSV-1, whether or not treated from 1 h post-infection, and incubated for 18 h at 37°C were carried out.

*Electron microscopy*

Electron micrographs are shown in Fig. 6. In untreated Vero cells infected with HSV-1, margination of chromatin was apparent (cm) and numerous non-enveloped nucleocapsids (nc) were present in the nucleus of the cell (panel a × 36000). Some viral particles acquired their outer layer by budding (arrow) through the nuclear membrane (mn) and enveloped virions (V) were apparent in the cytoplasm (panel c × 82000). On panel b (× 36000), several naked nucleocapsids were visible near a mitochondria (m) in the cytoplasm of an infected cell treated with 7 μg of saponin. The panel d (× 82000) also showed an infected treated cell. As in the controls, nucleocapsids were observed in the nucleus (N) but in the cytoplasm only one enveloped virion (V), some naked nucleocapsids (nc) and one empty capsid without nucleoid (c) could be observed.

The number of virus particles in nuclei of treated and untreated cells was not compared. However, it was apparent that saponin affects the structural integrity of the virus.

## Discussion

The above experiments indicate that 3-O-glucose-(1 → 3 or 4)-[arabinose (1 → 4 or 3)]-glucose (1 → 2)-xyloside of 23-hydroxyprotoprimulagenin A, isolated from *Anagallis arvensis*, exerts a significant inhibitory effect in vitro on herpes simplex type 1 and poliovirus type 2. This activity was demonstrated, by both inhibition of virus-induced cytopathogenicity and reduction of virus yield, at concentrations below the toxic dose. Therefore, as suggested by Vandenberghe et al. [23], the saponin might be considered as a rather selective antiviral product. However, we cannot explain why it was ineffective against adenovirus, vaccinia virus and vesicular stomatitis virus.

Antiviral saponins have also been extracted from some other plants [24]. For example, aescine from *Aesculus hippocastanum*, primula saponin from *Primula veris*, saikosaponin A from *Bupleurum falcatum*, theasaponin from *Thea sinensis* and gymnemic acid from *Gymnema sylvestre* showed activity against influenza A2 virus [17,20]. It would be interesting to test activity of saikosaponin A and primula saponin against HSV-1 and poliovirus since the structure of these saponins is close

to that of our compound. Another natural triterpene, glycyrrhizic acid, inhibits the growth of several DNA and RNA viruses [15,16], whereas the effect of glycyrrhizin on varicellazoster virus has just been reported [7].

In order to understand the mechanism of inhibition, the effect of saponin under different experimental conditions was studied. We showed that the antiviral effect could not be attributed to a direct inactivation of the viruses. The non-enveloped poliovirus was not affected and herpes simplex virus was inactivated at a concentration much higher than the minimum inhibitory dose for virus replication in cell culture.

Abe [1] has reported on an effect of biological membranes of saikosaponins having a bridged 13 β-28 epoxyoleanane system in the aglycone D-ring and a C-16 OH in α position. The structures of the aglycone moieties of the saikosaponins, which are extracted from *Bupleurum falcatum*, and of the saponin isolated from *Anagallis arvensis* are very close. The saikosaponins, like our compound, show a strong hemolytic activity [1]. Saikosaponins were also found to cause a significant decrease in the negative charge at the surface of Ehrlich ascites tumor cells, which is suggestive of an alteration on the negatively charged carbohydrate portions of the cell membrane [3]. Saikosaponins must also interact with the lipid layer of membrane since they have been found to cause a remarkable decrease in membrane fluidity [2]. Such mechanisms might prevent adsorption of the viruses to the cell surface and hence penetration and replication.

In studies of the single cycle replication, which implied a high multiplicity of infection and addition of the drug at 1 h post-infection, saponin was found to inhibit the growth of both HSV-1 and poliovirus. These results suggests that our compound exerts its antiviral effect not only via inhibition of attachment and penetration but also interferes with a further step of the virus replication cycle. This interpretation was strengthened by the study on the effect of time of addition of saponin. Addition of saponin at times between 0.5 h and 8 h post-infection resulted in a significant antiviral activity. This means that the compound might act at both an early and later event in the viral replicative cycle. Observations by thin-section electron microscopy of cells infected with HSV-1 suggested that this late event may be the maturation of virions. Changes in the morphology of HSV particles (lack of envelope) were observed as a result of the treatment with the saponin and it is well established that the outer layer is necessary for infectivity. Perhaps, the mechanism of action of saponin is comparable to that of arildone [11], although the chemical structure of the two compounds is totally different. However, the precise step(s) of viral replication which are blocked by saponin are yet unknown.

In summary, the triterpene saponin isolated from *Anagallis arvensis* appears to be a potent anti-herpes virus and anti-poliovirus drug. The compound is highly water soluble but relatively toxic. In vivo studies are being carried out to determine whether anti-herpes activity could be confirmed in a model of experimental keratitis in rabbits.

**Acknowledgement**

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