Antiviral properties of the seed extract of an Indian medicinal plant, *Pongamia pinnata*, Linn., against herpes simplex viruses: in-vitro studies on Vero cells

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Summary. *Pongamia pinnata*, Linn., an Indian medicinal plant used in the Ayurvedha and Siddha traditional medicine systems, for treatment of clinical lesions of skin and genitalia, was evaluated for antiviral properties against herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2) by in-vitro studies in Vero cells. A crude aqueous seed extract of *P. pinnata* completely inhibited the growth of HSV-1 and HSV-2 at concentrations of 1 and 20 mg/ml (w/v), respectively, as shown by complete absence of cytopathic effect.

Introduction

Acute and recurrent herpes simplex virus (HSV) infections are worldwide in distribution and several antiviral compounds have been introduced into therapeutic use during the past two decades.^{1,2} Their conflicting efficacy in recurrent infection and in immunodeficient patients^{2,3} as well as the problem of prohibitive costs in developing countries, has necessitated the search for alternative drugs. Two traditional Indian medicine systems, Ayurvedha and Siddha, mention the usefulness of the Indian medicinal plant *Pongamia pinnata*, in clinical lesions of skin and genitalia.^{4,5} However, the validity of its medicinal properties has not been evaluated scientifically. In this pilot study, the antiviral effect of an extract of *P. pinnata* seeds against herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2) was evaluated in Vero cells.

Materials and methods

Cell culture

Vero cells obtained from the National Facility for Animal Tissue and Cell Culture, Department of Biotechnology, University of Poona Campus, Pune, India, were grown in Eagle's Minimum Essential Medium (EMEM) containing Earle's salts, L-glutamine, non-essential amino acids, sodium bicarbonate, goat serum (heat inactivated at 56°C for 30 min) 5%

and antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml). The maintenance minimum essential medium (MMEM) was prepared similarly with goat serum 2%. The cells were cultivated according to the standard procedure of Grist *et al.*⁶

Virus strains and inhibition assays

HSV-1 (AC strain) and HSV-2 (HV-219 strain) were propagated in Vero cells and used at a concentration of 10⁶ pfu/ml in the experiments. The sensitivity of HSV strains to *P. pinnata* seed extract was tested by quantitative CPE reduction assays⁷ and the percentage inhibition of the virus growth was calculated according to the formula of Wachsmann and Coto.⁸

Preparation of aqueous *P. pinnata* seed extract

P. pinnata is a terrestrial tree belonging to the family Papilionaceae. Because Ayurvedha and Siddha literatures stress the use of the seeds, they were used in this study. Seeds were collected from dried fruits, surface-sterilised in ethyl alcohol 70% and powdered after weighing. Aqueous extracts (1 g/ml) were prepared in sterile double-distilled water. This was filtered through a sterile gauze cloth and then through a seitz filter. The concentration of the filtered extract was calculated by comparing the weight difference between similar quantities of plain double-distilled water and plant extract. No preservative was used and the sterility of the preparations was checked by standard procedures. The sterile extract was distributed in small volumes and stored at –20°C until required.

In-vitro antiviral studies of *P. pinnata* seed extract

Qualitative analysis. Two groups of six tubes containing Vero cell cultures were inoculated with HSV-1 (10^6 pfu). Seed extract (0.1 ml of 1 mg/ml w/v extract) was added to one of the groups. Control groups were inoculated with phosphate buffered saline (PBS). MEM (2 ml) was added to each culture after incubation for 90 min at room temperature. The tubes were then incubated at 37°C for 2 weeks. They were observed with a Nikon inverted microscope each day for evidence of cytopathic effect (CPE). Similar experiments were done for HSV-2.

Dose-response analysis

Dose response was estimated by a CPE reduction assay on Vero cell monolayers and the percentage virus inhibition was calculated according to the formula described by Wachsmann and Coto.⁸

Virus inhibition (%) =

$$100 - \frac{\text{Number of plaques in the tubes inoculated with virus + plant extract}}{\text{Number of plaques in the tubes inoculated with virus alone}} \times 100$$

For HSV-1, 36 tubes containing Vero cell cultures were used in six groups: A, B, C, D (test groups), E (positive virus control) and F (PBS control). In groups A, B, C and D, different concentrations of the seed extract were used. A similar experiment was done for HSV-2, in which 43 Vero cell cultures were used in

seven groups: A, B, C, D, E (test groups), F (virus positive control) and G (PBS control).

The dose-response pattern was assessed from the percentage inhibition of CPE at each concentration of the extract at 24, 48, 72, 96, 120, 144 and 168 h. Statistical evaluation was by Student's *t* test.

Results*Qualitative analysis*

Qualitative analysis of HSV-1 gave the following results. The virus control showed extensive CPE after incubation for 72 h, whereas the group treated with seed extract showed no CPE even after a week. The uninfected cells in the extract control tubes were unaffected. The seed extract completely inhibited the multiplication of HSV-1 at a concentration of 1 mg/ml w/v. Qualitative analysis of HSV-2 showed that an extract concentration of 1 mg/ml w/v could not bring about complete inhibition and only a concentration of 20 mg/ml completely inhibited replication by HSV-2.

Dose-response analysis

Table I shows the dose-response pattern of HSV-1 to *P. pinnata* seed extract, confirming the ability of a concentration of 1 mg/ml to completely inhibit growth of HSV-1; the lower concentrations were less effective. The mean percentage inhibition of each concentration of the extract was statistically significant. However, the tubes with lower concentrations of the extract

Table I. Dose-response pattern of HSV-1 to *P. pinnata*

Concentration of extract (mg/ml)	Mean percentage inhibition (SD)* at						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
0.25 ^a	62.5 (1.7)	4.3 (0.4)	0				
0.50 ^b	85.6 (1.5)	70.3 (1.5)	15.0 (3.2)	0			
0.75 ^c	98.5 (0.5)	86.0 (1.6)	73.0 (1.2)	18.0 (1.6)	0		
1.0 ^d	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)

All values were statistically significant at 24 h: a versus b, $p < 0.05$; a versus c and c versus d, $p < 0.001$.

* Expressed as a percentage of the input virus dose (10^6 pfu).

Table II. Dose-response pattern of HSV-2 to *P. pinnata*

Concentration of extract (mg/ml)	Mean percentage inhibition (SD)* at						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
1 ^a	61.3 (0.8)	3.3 (0.8)	0				
5 ^b	73.8 (1.5)	63.3 (1.5)	4.8 (3.9)	0			
10 ^c	83.5 (1.1)	73.3 (0.8)	62.8 (1.5)	10.3 (3.6)	0		
15 ^d	98.3 (0.4)	83.5 (1.1)	75.5 (0.5)	66.3 (1.3)	16.5 (1.1)	0	
20	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)

Statistical comparisons: a versus b at 24 h not significant but at 48 h statistically significant ($p < 0.05$); b versus c and c versus d at 24 h highly significant ($p < 0.001$).

* Expressed as a percentage of the input virus dose (10^6 pfu).

showed viral proliferation (appearance of CPE) on further incubation. In addition, the dose-response study of HSV-2 (table II), revealed that an extract concentration of 20 mg/ml was required to bring about 100% inhibition of viral growth. The mean percentage inhibition at each concentration of the extract was statistically significant. As with HSV-1, growth of HSV-2 was observed after further incubation of the tubes with the lower concentrations of the extract.

Discussion

Viral inhibition studies with the extract of *P. pinnata* seeds against HSV-1 and HSV-2 were evaluated *in vitro*. The most striking observation was the total inhibition of growth of HSV-1 and HIV-2 at concentrations of 1 mg/ml and 20 mg/ml w/v respectively, whereas even at the highest concentrations the extract was not toxic for Vero cells. Acute and chronic toxicological studies conducted in Swiss albino rats showed the safety of the *P. pinnata* seed extract (unpublished observations). When these observations are compared with those of Fiala *et al.*,⁹ the higher

MIC required for *P. pinnata* seed extract against HSV-1 and HSV-2 may be due to the crude nature of the extract. The difference in MIC *P. pinnata* extract for HSV-1 and HSV-2 is similar to their observation with adenine arabinoside, cytosine arabinoside and idoxuridine. Purification of the active principle(s) isolated from *P. pinnata* might resolve its real anti-HSV potential. Even though the above observations could be considered as most encouraging, it must be remembered that they are preliminary, and further chemical characterisation of the active principle(s) and re-analysis of their antiviral properties must be undertaken.

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