Research Letter

Antiviral Potential of Selected Indian Medicinal (Ayurvedic) Plants Against Herpes Simplex Virus 1 and 2

Herpes simplex virus 1 and 2 (HSV-1 and HSV-2) belong to diverse family of *Herpesviridae*, causing oral herpes lesions (HSV-1), genital lesions (HSV-2), meningitis, and encephalitis.[1] Primary infection within the genital tract, followed by an established latency phase give rise to life-long infection in humans.[2] Treatment of herpes infection is thus cause of major concern owing to the difficulty in eliminating it from the ganglion, high cost of treatment, increasing drug resistance, and association with HIV-1.[3-7] We investigated the plants selected on the basis of their traditional usage by comparing the disease symptomatology and recommended use from the texts of *Ayurveda* and used them in the forms as used traditionally. Primary screening of 24 extracts of seven plants was done by cytopathic effect inhibition (CPE) assay followed by dose response, antiviral, and cytotoxicity assay conducted at eight concentrations from 3.125 to 400 µg/ml. Five extracts, *Punica granatum* aqueous extract (PGAE), peals powder (PGPP), freeze-dried powder (PGFP), *Ocimum sanctum* methanol extract (OSME), and *Azadirachta indica* leaves powder (AZLP) demonstrated favorable activity in primary screening. PGFP showed antiviral activity at 50, 100, and 200 µg/ml concentrations followed by PGAE, which showed highest selectivity index (SI) (14 and 12.5) followed by PGFP (7.6 and 12.9). Thus, aqueous extract and freeze-dried powder of *P. grantum* have potential to be further explored for its possible anti-HSV activity.

The plants selected, namely, *A. indica*, *Curcuma longa*, *Punica granatum*, *O. sanctum*, *Nyctanthes arbortristis*, *Carica papaya*, and *Holarrhena antidysentrica* were collected from Gujarat State of India. The plant species were taxonomically identified and confirmed using morphological and anatomical techniques. They were authenticated at the Botanical Survey of India, Pune and voucher specimens were deposited and standardized using High Performance Thin Layer Chromatography (HPTLC) method (data not provided here) [Figures 1 and 2].

We screened 24 extracts of 7 selected plants, of which 7 were water extracts, 3 were methanol extracts, and 14 were powders of various plant parts [Table 1]. The herb powders were prepared by drying and grinding the plant materials. Water and methanol extracts were prepared from 500 g of fresh raw material of plant parts in Soxhlet extraction vessel under reflux with stirrer for 3 cycles with methanol or water. After each cycle, solvent was added to replenish the remaining volume and dried in a rotatory evaporator at temperature not exceeding 35°C. The stock solution of 20 mg/ml was prepared in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, USA) and subsequent serial dilutions were carried out in Dulbecco's modified eagle medium (DMEM) (GIBCO Cat. No. 12430) supplemented with 2% fetal bovine serum (FBS) (GIBCO Cat. No. 16000-044). The final concentration of DMSO was <0.2%.[8]

All reagents and media for cell culture were purchased from GIBCO-BRL (Grand Island, NY, USA). Confluent monolayer of African Green Monkey kidney cell line (Vero) (ATCC CCL-81) was grown in T-25 flasks in a 5% carbon dioxide (CO_2) incubator at 37°C for 48 hours, in DMEM supplemented with 10% FBS, penicillin G sodium 200 U/ml, streptomycin sulfate 200 mg/L, and amphotericin B 0.5 mg/L. HSV-1 clinical strain and HSV-2 ATCC G strain virus stocks were propagated in Vero cells and used at a titer of 1:10,000 for HSV-1 and 1:1000 for HSV-2 in all *in-vitro* experiments.

Acyclovir (ACV) powder (Matrix Laboratories Limited, Hyderabad, India) was used as a standard antiviral drug at four different concentrations: 3.125, 6.25, 12.5, and 25 µg/ml for HSV-2 and 0.78, 1.56, 3.125, 6.25, and 12.5 µg/ml for HSV-1. It was dissolved with DMSO to give a stock concentration of 10 mg/ml and then diluted with DMEM-2% FBS (maintenance medium) before use. For all the assays, ACV control consisted of the cells, ACV at different concentrations: 3.125, 6.25, 12.5, and 25 µg/ml for HSV-2 and 0.78, 1.56, 3.125, 6.25, and 12.5 µg/ml for HSV-1 made by 1:2 serial dilutions and the virus suspension.

Primary screening of these extracts was conducted by CPE assay at 100 µg/ml concentration against HSV-2 in triplicates. Confluent Vero cells were grown in 96-well microtiter plates (2×10^4 cells/100 µl) for 24 hours. Two-fold serial dilutions of the samples were prepared in DMEM with 2% FBS. The cell control consisted of 200 µl maintenance medium; the virus control consisted of 100 µl of maintenance medium and 100 µl of the virus suspension. After 1 hour incubation of the cells with different sample concentrations, the virus (HSV-2) was added to the plates and the plates were again incubated

for 48 hours in 5% CO_2 at 37°C. The plates were then washed with saline and stained with crystal violet and incubated for 30 min at room temperature after which the plates were gently washed under running tap water and left overnight for drying and visually analyzed (qualitatively).[9]

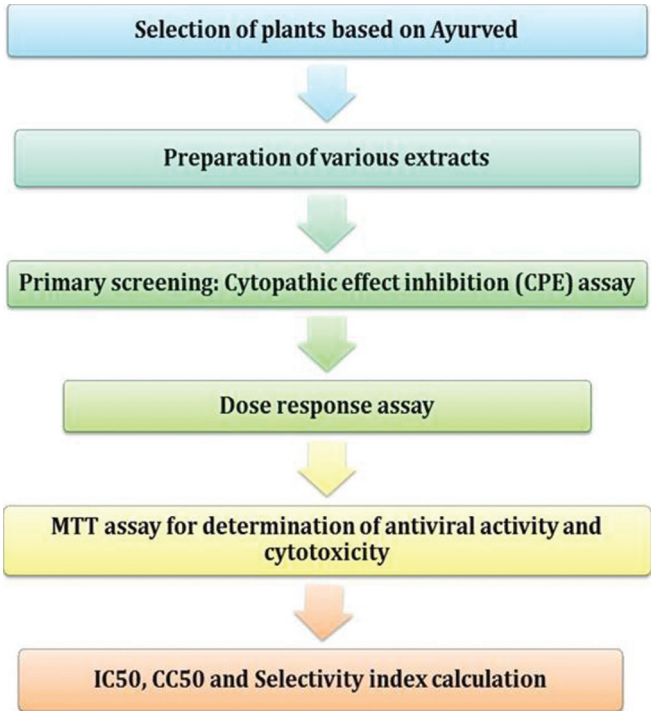

Figure 1: *In-vitro* screening of plant extracts against HSV-1 and HSV-2

Eight concentrations of extracts that exhibited activity in CPE assay were assayed from 3.125 µg/ml onwards higher in the ratio 1:2 till 400 µg/ml. After 1 hour incubation of test samples with the cells, virus was added and the plates were incubated for 48 hours before washing with saline and staining with crystal violet. After overnight drying, the plates were analyzed.[10,11]

The extracts that exhibited activity in CPE assay were analyzed at eight concentrations for antiviral assay. The assay plates contained virus and cell controls and the samples. The plates were then incubated for 48 hours after which the plates were washed with plain DMEM followed by the addition of tetrazolium compound MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent diluted 1:10 in 2% FBS-DMEM. After incubation of 4 hours, detergent was added to the wells and left overnight for incubation in a 5% CO_2 at 37°C. The absorbance was then read with the help of a plate reader at a wavelength of 570 nm. The data were analyzed by plotting cell number versus absorbance, allowing quantification of changes in cell proliferation.[12,13]

Table 1: Details of the plant and their extracts screened against herpes virus

Plant name	Family	Sanskrit/Local name	Parts used	Extracts prepared	Abbreviation
Azadiracta indica, A. Juss	Meliaceae	Nimb	Leaves and stem	Aqueous extract	AZAE
				Methanol extract	AZME
				Leaves powder	AZLP
Carica papaya, Linn.	Cariaceae	Papai	Raw and ripe fruits	Raw fruit powder	CRAF
				Ripe fruit powder	CRIF
				Raw fruit aqueous extract powder	CRAE
				Ripe fruit aqueous extract powder	CRIE
Curcuma longa, Linn.	Zingiberaceae	Haridra	Rhizomes	Aqueous extract	CLAE
				Methanol extract	CLME
				Yellow herb powder	CLYH
				Brown herb powder	CLBH
Holarrhena antidysentrica, Wall.	Apocynaceae	Kutaja	Stem bark	Stem bark powder	HABP
				Aqueous extract	HAAE
Ocimum sanctum, Linn.	Lamiaceae	Tulsi	Aerial parts	Herb powder	OSHP
				Aqueous extract	OSAE
				Methanol extract	OSME
Punica granatum, Linn.	Punicaceae	Dadima	Fruit juice, peel, seed and its oil	Freeze-dried juice powder	PGFP
				Aqueous extract powder	PGAE
				Seed oil	PGSO
				Seed herb powder	PGSP
				Peals powder	PGPP
				Juice powder	PGJP
Nyctanthes arbortristis, Linn.	Oleaceae	Parijata	Leaves and stem	Leaves powder	NALP
				Aqueous extract	NAAE

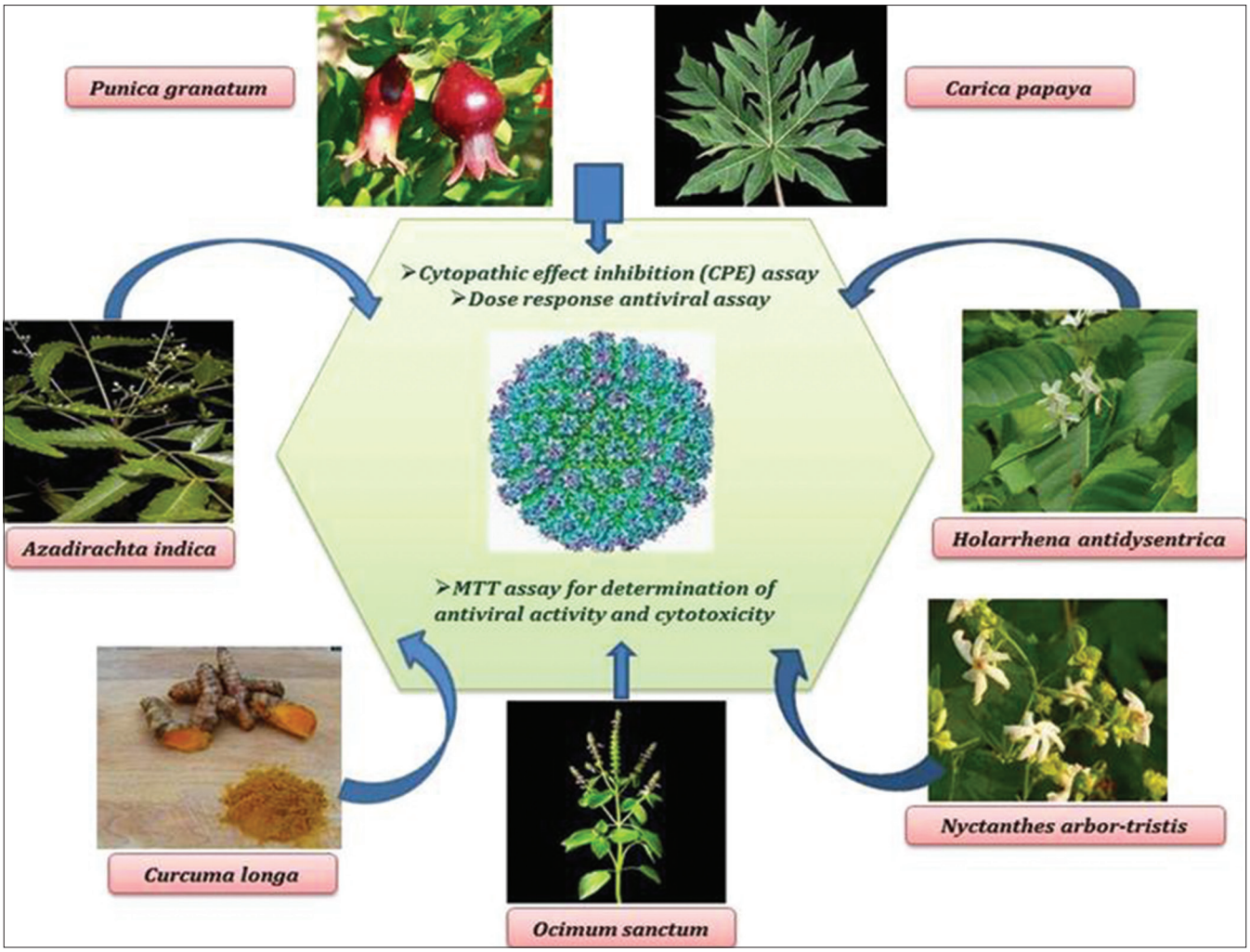

Figure 2: Twenty-four extracts of seven plants were screened for their antiviral activity *in-vitro*. The figure depicts the plants and the methodology used for screening

CPE assay and dose response assay

Extracts PGAE, PGPP, PGFP, OSME, and AZLP exhibited positive response in the CPE assay [Table 2] and were further screened at eight concentrations [Figure 3a]. All the three *Punica grantum* extracts showed a dose-dependent response against both HSV-1 and HSV-2 along with OSME. Based on the SI values, PGFP showed the most potent antiviral activity (CC_{50}: 233 µg/ml, IC_{50}: 30.6 and 18.14 µg/ml and SI: 14 and 12.5 for HSV-1 and HSV-2, respectively) at 50, 100, and 200 µg/ml concentrations followed by PGAE (CC_{50}: 220 µg/ml, IC_{50}: 15.8 and 17.6 µg/ml and SI: 7.6 and 12.9 for HSV-1 and HSV-2, respectively). OSME and PGPP showed identical antiviral activity. AZLP failed to exhibit any significant antiviral activity [Table 3].

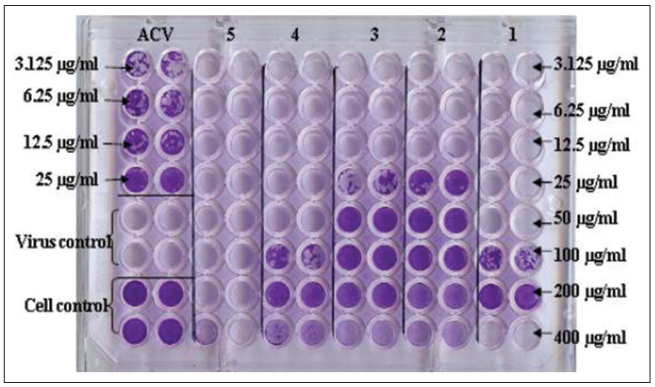

Figure 3a: Dose response analysis of samples 1 (OSME), 2 (PGAE), 3 (PFFP), 4 (PGPP), and 5 (AZLP) along with acyclovir (ACV), HSV-2 virus control and cell controls in a 96-well plate following CPE inhibition assay

Antiviral and cytotoxicity assay

To assess the antiviral potential and cytotoxicity of PGAE, PGPP, PGFP, and OSME, MTT was performed with and without the virus [Figure 3b]. Following formulae were used as described by Cheng, *et al*. (2004):[13]

$$\%\ \text{Viability} = \left[\frac{\text{O.D.}_{\text{TEST/SAMPLE}}}{\text{O.D.}_{\text{CELL CONTROL}}}\right] \times 100$$

$$\%\ \text{Toxicity} = 100 - \%\ \text{Viability}$$

$$\%\ \text{Antiviral activity} = \left[\frac{\text{O.D.}_{\text{TEST}} - \text{O.D.}_{\text{VIRUS CONTROL}}}{\text{O.D.}_{\text{CELL CONTROL}} - \text{O.D.}_{\text{VIRUS CONTROL}}}\right] \times 100$$

Table 4 illustrates the percentage (%) viability, % toxicity, and % antiviral activity of these four extracts. From this study, based on the CC_{50} values, it can be concluded that all the five samples showed no cytotoxicity at the potent antiviral concentrations. Based on the results, it was found that PGAE [Figure 4a] showed the highest SI (14 and 12.5); therefore, the promising antiviral activity against HSV-1 and HSV-2, is followed by PGFP (7.6 and 12.9) [Figure 4b], whereas PGPP and OSAE showed moderate SIs [Table 4].

Discussion

We attempted to generate the *in-vitro* data of activity for the plants and their extracts against herpes virus using similar drug formulations as used traditionally. The preliminary data of the selected plants and their extracts' antiviral activity based on *in-vitro* studies were not available to the extent of our knowledge. Five out of 24 extracts showed potential antiviral activity against HSV-2 at 100 µg/ml. Based on these results, further qualitative and subsequent quantitative analyses of these positive samples were carried out at a wider concentration range. In order to confirm that the inhibitory effect of the extracts seen was due only to

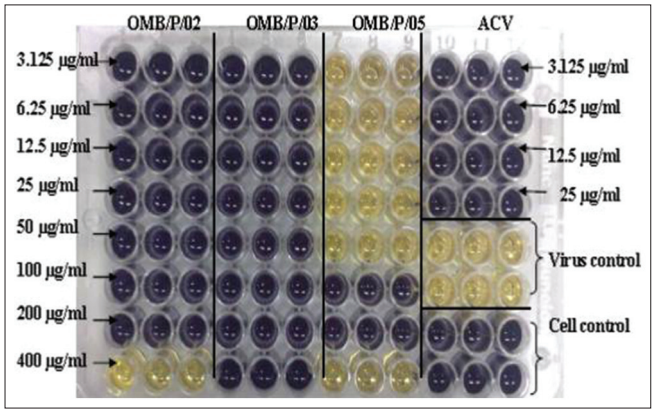

Figure 3b: MTT plate for samples: PGAE, PGFP, and PGPP assayed in a dose-dependent manner against HSV-2. Yellow indicates extremely low cell density due to plaque formation and deep purple indicates high cell density

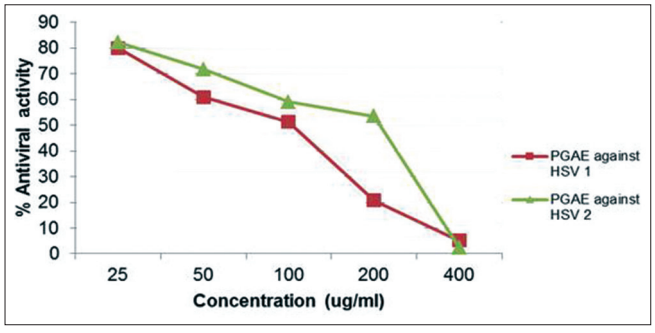

Figure 4a: Antiviral activity of PGAE against HSV-1 and HSV-2

Table 2: Primary screening results of the samples against HSV-2 at 100 µg/ml

Plant extract	Anti-HSV-2 activity % CPE inhibition
AZLP	+
OSME	+++
PGFP	++
PGAE	+
PGPP	+

-: 0-10%; +: 11-25% protection; ++: 26-50% protection; +++: 51-75% protection; ++++: 76-100% protection. Note: ACV exhibited 100% protection at 25 µg/ml and 11-25% inhibition at 50 µg/ml; ACV was not assessed at 100 µg/ml; HSV: Herpes simplex virus; CPE: Cytopathic effect; AZLP: *Azadirachta indica* leaves powder; OSME: *Ocimum sanctum* methanol extract; PGFP: *Punica granatum* freeze-dried juice powder; PGAE: *Punica granatum* aqueous extract; PGPP: *Punica granatum* peals powder

Table 3: Dose response assay outcome of selected extracts

Plant extract	Concentration (µg/ml)	Anti-HSV-1 activity % CPE inhibition	Anti-HSV-2 activity % CPE inhibition
PGAE	25	+	+++
	50	+++	+++
	100	+++	+++
	200	++	+
PGFP	50	+++	++++
	100	++++	+++
	200	+++	+++
PGPP	100	-	+
	200	+++	+++
	400	+	-
OSME	100	+	+
	200	-	+++
AZLP	400	+	-
ACV	50	+	+
	25	+++	++++
	12.5	++++	++++
	6.25	++++	++++
	3.125	++	+++

-: 0-10%; +: 11-25% protection; ++: 26-50% protection; +++: 51-75% protection; ++++: 76-100% protection; HSV: Herpes simplex virus; CPE: Cytopathic effect; AZLP: *Azadirachta indica* leaves powder; OSME: *Ocimum sanctum* methanol extract; PGFP: *Punica granatum* freeze-dried juice powder; PGAE: *Punica granatum* aqueous extract; PGPP: *Punica granatum* peals powder; ACV: Acyclovir

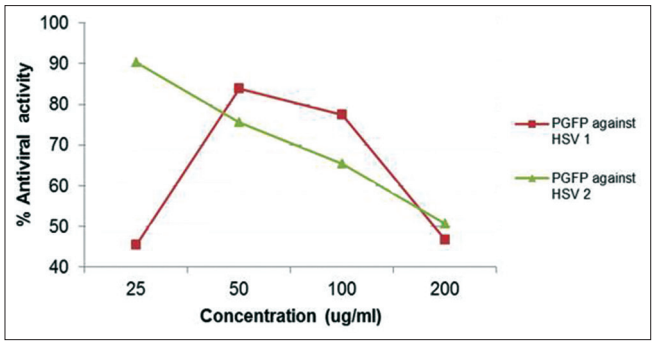

Figure 4b: Antiviral activity of against HSV-1 and HSV-2

Table 4: Antiviral activity and cytotoxicity effect of selected extracts

Extracts	Cytotoxicity CC_{50} [#] (µg/ml)	Antiviral activity			
		IC_{50} [§] (µg/ml)		Selectivity index	
		HSV-1	HSV-2	HSV-1	HSV-2
PGAE	220	15.8	17.6	14	12.5
PGFP	233.08	30.6	18.14	7.6	12.9
PGPP	381.4	250	185	1.52	2.06
OSME	278.5	-	112.6	-	2.5
ACV	230	2	2.5	115	92

[#]CC_{50}: The concentration that causes the reduction of viable cells by 50%; [§]IC_{50}: The concentration that protects 50% of the cells against destruction by viruses; -: No measurable effect

its antiherpes activity and not due to overlapping drug toxicity itself, the cytotoxicity assay was carried out to measure cell viability in presence of the drug alone.

A. indica A. Juss aqueous and methanol extract, *O. sanctum*, Linn. herb powder and aqueous extract, *Punica granatum*, Linn. seed oil, seed herb powder and juice powder and samples of *C. papaya*, Linn., *C. longa*, Linn., *H. antidysentrica*, Wall., and *Nyctanthes arbortristis*, Linn. failed to exhibit anti-HSV potential in primary screening at a concentration of 100 µg/ml against HSV-2.

There are no preliminary data on antiherpes studies on *C. papaya*, *H. antidysentrica*, and *N. arbortristis*. Although *H. antidysentrica* has demonstrated activity against various infective pathogens as antimalarial, antibacterial, anti-*Escherichia coli*, and anti-MRSA agent.[14-17] *N. arbortristis* is reported for its antileishmanial, antiinflammatory, and antiencephalitis causing virus activity.[18-22] *A. indica* Linn. bark aqueous extract demonstrated potent entry inhibitor activity against HSV-1 infection.[23] *A. indica* A. Juss aqueous extract and its pure compound Azadirachtin were studied against dengue cirus type-2. The crude aqueous extract revealed inhibition in a dose-dependent response *in-vitro* and *in-vivo*, whereas pure compound Azadirachtin failed to exhibit any activity against the virus.[24] Neem oil was assayed against HSV-1, which did not demonstrate antiviral activity.[9] Although no studies were found on *C. longa*, but the isolate curcumin exhibited potent antiviral potential against HSV-1 and HSV-2.[25-27] *O. basilicum* aqueous and ethanol extracts and isolate ursolic acid exhibited potent anti-HSV-1 activity *in-vitro*.[28] Ursolic acid is one of the important marker compounds of *O. sanctum*.[29] The aqueous extract and tannins from pericarp of *P. granatum* had shown anti-HSV-1 and HSV-2 activities *in-vitro*, respectively.[30,31]

Other plants mentioned in texts of *Ayurveda* were shown to have anti-HSV activity. Acetone, ethanol, and methanol extracts of *Phyllanthus urinaria* Linn. inhibited HSV-2 infection by disturbing the early stage of virus infection and by diminishing the virus infectivity.[32] Another Indian medicinal plant extract *Swertia chirata* inhibited HSV-1 viral dissemination.[33] Putranjivain A, isolate of *Euphorbia jolkini* Bioss, inhibited both the virus entry and late stage replication of HSV-2 *in-vitro*.[13] Kenyan plant *Carissa edulis* aqueous extract exhibited efficacy against both *in-vitro* and *in-vivo* models of HSV-1 infection.[34]

This study highlights the potential of these investigated plant extracts to be exploited for antiviral activity.[35] The aqueous extract and freeze-dried powder of *P. grantum* exhibited promising antiviral activity. The punicalagin present in extract and juice of *P. granatum* may be explored further for bioactivity guided fractionation studies.[36,37] The remaining extracts may have compounds of antiviral potential, which may be present at quantities insufficient to inactivate all infectious virus particles. It is possible that the elucidation of active constituents in these plants may provide important lead to the development of new and effective antiviral agents.

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Priyanka Jadhav, Natasha Kapoor[1], Becky Thomas[1], Hingorani Lal[2], Nilima Kshirsagar[3]

Department of Infectious Diseases, K.E.M. Hospital, and Interpathy Research and Technology, Maharashtra University of Health Sciences, Mumbai, [1]Department of Vaccine Development, Piramal Life Sciences Ltd., Mumbai, [2]Pharmanza Herbals Pvt. Ltd., Gujarat, [3]Office of National Chair (Clinical Pharmacology), Indian Council of Medical Research (ICMR), and ESI PGIMSR MGM Hospital, Government of India, Mumbai, India.
E-mail: priyanka.ing@gmail.com

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